

DOCTORAL THESIS

Extracellular Vesicles:
Novel Approaches for
Characterization, Functional
Assessment, and Translation

Francesca Loria

TALLINN UNIVERSITY OF TECHNOLOGY

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Novel Approaches for Characterization,
Functional Assessment, and Translation**

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Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for doctoral or equivalent academic degree.

Francesca Loria

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**Ekstratsellulaarsed vesiikulid: uudsed
lähenemisviisid nende iseloomustamiseks,
funktsionaalseks hindamiseks ja kliiniliseks
rakendamiseks**

FRANCESCA LORIA



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List of Publications

The list of author's publications, on the basis of which the thesis has been prepared:

- I Fortunato, D., Mladenović, D., Criscuoli, M., **Loria, F.**, Veiman, K.-L., Zocco, D., Koort, K., & Zarovni, N. (2026). Correction: Fortunato et al. Opportunities and Pitfalls of Fluorescent Labelling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms. *Int. J. Mol. Sci.* 2021, 22, 10510. *International Journal of Molecular Sciences*, 27(7), 3199. <https://doi.org/10.3390/ijms27073199>.
- II Zuppone, S., Zarovni, N., Noguchi, K., **Loria, F.**, Morasso, C., Löhmus, A., Nakase, I., & Vago, R. (2024). Novel loading protocol combines highly efficient encapsulation of exogenous therapeutic toxin with preservation of extracellular vesicles properties, uptake and cargo activity. *Discover Nano*, 19(1). <https://doi.org/10.1186/s11671-024-04022-8>.
- III **Loria, F.**, Boysen, A. T., van Herwijnen, M. J. C., Kleijn, M., Zendrini, A., Candini, O., Tauro, M., Frabetti, S., Löhmus, A., Mladenović, D., Stulova, I., Kaleda, A., Paolini, L., Guazzi, P., Chiesi, A., Dominici, M., Nejsun, P., Bergese, P., Wauben, M. H. M., Korulu, S., Vilu, R., & Zarovni, N. Unravelling biochemical signatures in small extracellular vesicles: a comparative study across distinct taxa. In preparation.
- IV **Loria, F.**, Kattel, A., Junusova, M., Brucale, M., Valle, F., Bergese, P., Kobrin, E.-G., Chiesi, A., Guazzi, P., Korulu, S., Stulova, I., Zarovni, N., & Vilu, R. (2026). Investigating the Effect of Ginger-Derived Nanovesicles on the Growth and Metabolic Activity of *Bacteroides thetaiotaomicron*: An Isothermal Microcalorimetric Study. *Journal of Extracellular Biology*, 5(7), e70163. <https://doi.org/10.1002/jex2.70163>.
- V **Loria, F.**, Picciotto, S., Adamo, G., Zendrini, A., Raccosta, S., Manno, M., Bergese, P., Liguori, G. L., Bongiovanni, A., & Zarovni, N. (2024). A decision-making tool for navigating extracellular vesicle research and product development. *Journal of Extracellular Vesicles*, 13(12). <https://doi.org/10.1002/jev2.70021>.

Author's Contribution to the Publications

Contribution to the papers in this thesis are:

- I The author was involved in the investigation, data curation, formal analysis, and visualization of the results collected by nanoparticle tracking analysis.
- II The author contributed to the experimental work assessing the biochemical and biophysical integrity of extracellular vesicles, and participated in the curation of the resulting data.
- III The author participated in the conceptualization of the study, contributed to the provision of resources, managed work methodology, investigation, validation, data curation, formal analysis, and visualization, as well as drafted the original version of the manuscript.
- IV The author was responsible for the study conceptualization, contributed to the methodology, investigation, validation, formal analysis, data curation, and visualization, and prepared the original draft of the manuscript.
- V The author was involved in the conceptualization the study, actively contributed to the methodology, investigation, validation, data curation, formal analysis, visualization, and led the preparation of the original draft of the manuscript.

Introduction

Over the past few decades, extracellular vesicles (EVs) have gained significant momentum due to their potential to transform several domains of nanoscience, offering promising clinical and commercial solutions across diverse industrial sectors. Hopes and expectations stem from their unique nature as naturally occurring, nanoscale-to-microscale biological particles enclosed by a lipid bilayer and carrying a diverse repertoire of biological cargo, including proteins, lipids, carbohydrates, nucleic acids, and metabolites, either encapsulated within or associated with their membrane. By virtue of their physicochemical properties, EVs have been shown to promote cell-to-cell communication, ultimately shaping the functionality of any organism and regulating its response to the surrounding environment. Despite their remarkable promise, EVs exhibit a profound level of complexity and heterogeneity that remains largely unresolved, which continues to hinder rigorous research and effective exploitation.

EV-related uncertainty underlies critical methodological, biological, and translational gaps that are interdependent and need to be systematically tackled to unlock the full potential of EVs. At the methodological level, the primary bottlenecks arise from the absence of appropriate reference materials, standardized methodologies, and reliable data normalization approaches. Collectively, these challenges result in method-dependent EV recovery and purity outcomes, limited analytical resolution of EV subpopulations, substantial inter-study and inter-laboratory variability, as well as poor data comparability across distinct studies and sample types. Furthermore, current exogenous engineering approaches, designed to enhance the performance of EVs as nanodelivery systems through the incorporation of exogenous molecules, generally prioritize cargo loading efficiency over the maintenance of EV structural integrity, often resulting in either impaired or suboptimal downstream functional performance. From a biological perspective, an additional limitation is the conventional adoption of EV bioactivity assessment methods that provide end-point measurements rather than capturing the biological responses induced by EVs in their dynamic evolution, potentially leading to simplified and likely misleading mechanistic interpretations. At the innovation-to-application interface, the EV field lacks multidimensional study approaches that are also user-friendly and transferable, which can be strategically employed to minimize biased and context-insensitive decision-making, ultimately unlocking the effective deployment of EV-based products into meaningful, fit-for-purpose offerings.

The current thesis aims at addressing several of these constraints, focusing primarily on the following objectives: i) understanding how EV detection and the resolution of EV heterogeneity are influenced by different analytical capabilities and EV labelling strategies (**Publication I**); ii) investigating how to strengthen EV cargo loading efficiency without compromising EV integrity, cellular uptake, and biological activity (**Publication II**); iii) assessing the impact of different normalization approaches on the comparative evaluation of EVs from diverse biological sources, while identifying quality metrics that enable more robust comparisons and informed applications (**Publication III**); iv) implementing dynamic methodologies for EV bioactivity assessment to explore their potential for enhancing mechanistic interpretability of EV-mediated biological effects (**Publication IV**). The specific case study addressing the latter objective involved the application of isothermal microcalorimetry to evaluate the biological response of the human gut bacterium *Bacteroides thetaiotaomicron* following exposure to nanovesicles derived from ginger rhizome. Additionally, this thesis explores the application of multi-

criteria decision-making to enable systematic and sustainable decision-making in the selection and management of EV preparations and related processes (**Publication V**).

Overall, the research outputs generated within the framework of this thesis provide deeper insights into EV heterogeneity and advance the understanding of how methodological choices influence EV characterization, comparison, functional interpretation, and translational applicability. Furthermore, this work introduces novel methodological approaches that support more robust EV assessment and application, while enabling more informed, transparent, traceable, and responsible decision-making across research and translational settings.

Abbreviations

Abbreviation	Definition
AFM	Atomic force microscopy
ALIX	ALG-2-interacting protein X
APC	Allophycocyanin
APD	Avalanche photodiode
AS	<i>Ascaris suum</i>
BCA	Bicinchoninic acid
BM	Bovine milk
<i>Bt</i>	<i>Bacteroides thetaiotaomicron</i>
CCM	Cell conditioned media
CFSE	Carboxyfluorescein succinimidyl ester
ChoPL	Choline-containing phospholipid
CMG	CellMask™ Green
CMOS	Complementary metal-oxide-semiconductor
CMR	CellMask™ Red
CONAN	Colorimetric nanoplasmonic
CQA	Critical quality attribute
CTL	(Extracellular vesicle-depleted) control
CTR	CellTrace™ Red
CTRL	Control
DLS	Dynamic light scattering
DMT	Decision-making tool
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ENO	Enolase
EV	Extracellular Vesicle
EV-DMG	EV decision-making grid
FAA	Free amino acid
FACS	Fluorescence-activated cell sorting
FI	Fluorescence intensity
FITC	Fluorescein isothiocyanate
(F-)NTA	(Fluorescence) nanoparticle tracking analysis
FSC	Forward scatter
G	Ginger
<i>gDW</i>	<i>Grams dry weight</i>
HEK293	Human embryonic kidney 293
HGM	Human gut microbiota
HPLC	High-performance liquid chromatography
IMC	Isothermal microcalorimetry
ISEV	International Society for Extracellular Vesicles
LAP	Leucine aminopeptidase
LoD	Limit of detection
MCDM	Multi-criteria decision-making
MISEV	Minimal Information for Studies of Extracellular Vesicles
MSC	Mesenchymal stem/stromal cell
MW	Molecular weight

MWCO	Molecular weight cut-off
ND	Not detected
nd	Not determined
NV	Nanovesicle
nFCM	Nanoscale flow cytometry
NQ	Not quantified
NTA	Nanoparticle tracking analysis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDV	Plant-derived vesicle
PE	Phycoerythrin
PLS-DA	Partial least squares discriminant analysis
POD	Peroxidase
QbD	Quality-by-Design
qPCR	Quantitative polymerase chain reaction
R&D	Research and development
R&I	Research and innovation
RR&I	Responsible research and innovation
RT	Room temperature
SAP	Saporin
SCFA	Short-chain fatty acid
SD	Standard deviation
SEC	Size exclusion chromatography
SEM	Standard error of the mean
SNR	Signal-to-noise ratio
SP-IRIS	Single-particle interferometric reflectance imaging sensor analysis
SSC	Side scatter
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TSG101	Tumor susceptibility gene 101
UC	Ultracentrifugation
UF	Ultrafiltration
UPLC	Ultra-performance liquid chromatography
W	Importance weight
WB	Western blot
WSM	Weighted sum model
XO	Xanthine oxidase
ZP	Zeta potential

1 Literature Review

1.1 A brief history of Extracellular Vesicles (EVs)

The earliest evidence of the existence of biological structures and phenomena that, in light of current knowledge, may have reflected or involved the activity of what are now recognized as extracellular vesicles (EVs) can be traced back to the mid-1600s (Couch et al., 2021; Hargett & Bauer, 2013; Quick, 1966). Nevertheless, only studies on blood coagulation conducted in the 1940s by Chargaff and West paved the way for their formal identification and definition in the 1980s and 1990s. These investigations demonstrated that a sediment derived from blood plasma centrifuged at high speed (*i.e.*, 31,000 g) had the property of accelerating blood clotting. This induced the authors to hypothesize that the procoagulant activity shown by the sediment, which was named “particulate fraction”, could be attributed to the presence of a “thromboplastic agent” coexisting with “a variety of minute breakdown products of blood corpuscles”. In 1967, Wolf published electron microscopy (EM) images revealing an enrichment of platelet-derived “material in minute particulate form” in a high-speed centrifugation sediment, described as “platelet dust”, which could be distinguished from intact platelets. Similarly, in 1971, using EM, Crawford was able to detect “microparticles” in platelet-free plasma, which were shown to contain lipids, adenosine triphosphate (ATP), and contractile proteins, thus suggesting bioactivity. Around the same period, Anderson (1969) and Bonucci (1967) identified membrane-bound “matrix vesicles” within hypertrophic cartilage, which were assumed to play a functional role in bone mineralization. The first observation of multivesicular bodies (MVBs) harbouring small vesicles (1-10 nm) in animal cells, specifically in the bat thyroid gland during hibernation, can be attributed to Nunez and Gershon (1974) (Figure 1). Later studies on non-animal sources, including algae (Aaronson, 1971), yeast (Chigaleichik 1977), and bacteria (Käppeli & Finnerty 1979) suggested that vesicle production was ubiquitous across organisms, rather than exclusive to mammals, foreshadowing communication functions. Over the same decade, misidentified EVs, defined as “virus-like particles”, were observed in biofluids of both cancer patients and control groups, which were later recognized as vesicular artefacts (Dalton, 1975).

From the 1980s onward, EV research underwent a breakthrough, as studies demonstrated that vesicle secretion reflected specific sorting and trafficking mechanisms, rather than being artificially induced. Complementary publications by Johnstone (Pan & Johnstone 1983) and Stahl (Harding et al. 1983) demonstrated that the transferrin receptor was secreted by maturing reticulocytes via vesicle release (Figure 1). EM images reported by Harding et al. (1983) further proved that such vesicles were contained within MVBs, hence their definition as “intraluminal vesicles” (ILVs), and secreted into the extracellular milieu upon MVB fusion with the plasma membrane. In 1981, Trams et al. coined the term “exosomes” to describe the EVs released after MVB fusion with the plasma membrane. Six years later, Johnstone et al. used the same name to specifically refer to the ILVs released from MVBs following fusion with the plasma membrane. Since then, exosomes have been demonstrated to act as cargo carriers, specifically associated with lipid and protein diffusion (Gawrisch 1986), also expressing flippases (Vidal 1989), guanosine triphosphatases (GTPases), such as Rab and ARF (Vidal & Stahl 1993), and tetraspanins (Escola 1998). In 1989, Johnstone et al. demonstrated that exosomes were enzymatically active, thereby promoting a paradigm shift, from

considering them mere artificial fragments to functional structures. When bacterial vesicles were shown to interact with mammalian cells (Kay, 1990), a putative role of exosomes in mediating the communication between distinct organisms was unveiled. One year later, however, Johnstone's group attributed exosome secretion to stress-induced "waste disposal" of "obsolete membrane proteins", such as transferrin receptor and nucleoside transporter, a view that persisted in the field for several years after that, thereby marking the beginning of the "dust era".

Despite this, the interest in exosomes triggered by earlier studies continued to grow, unlocking associations of elevated particle levels with multiple disease conditions, such as ischemia (Lee 1993), angina (Singh 1995), and Crohn's (Powell 1996). In 1996, Raposo et al. discovered that exosomes derived from B lymphocytes were able to present antigens, catalyzing a massive interest in the EV immunomodulatory functions for the following decades (Figure 1). The field's expansion within the immunological area culminated with the study of dendritic cell-derived EVs engineered with tumor peptides as tumor vaccines (Zitvogel 1998), eventually contributing their entry into clinical trials a few years later (Escudier 2005). Proving the putative exploitable potential of EVs as either functional cargo carriers, to be used as biomarkers, or prophylactic/therapeutic platforms boosted the field's momentum in the early 21st century. After 2000, the EV research and development arena experienced an intensive and dynamic activity in both academic and industrial settings, as highlighted by the exponentially growing number of scientific publications in peer-reviewed journals, the increase in public funding and patent filing, as well as the globally higher investment trend by industry or venture capital funds (Couch et al., 2021; Zarovni et al., 2021).

In addition to collecting increasing evidence of the immunomodulatory potential of EVs, researchers started exploring EV composition via high-throughput proteomic and lipidomic analyses (Bard et al., 2004; Théry et al., 2001; Wubbolts et al., 2003; Subra et al., 2007). A key scientific advancement in the field was achieved between 2006 and 2008, when Ratajczak et al. (2006), Skog et al. (2008), and Valadi et al. (2007) uncovered the EV-mediated functional transfer of nucleic acids, ultimately revolutionizing our understanding of EVs and suggesting an active role in cell-to-cell communication (Figure 1). EV functionality was concurrently proved *in vivo* by Colino and Snapper (2007), demonstrating that EVs exerted a protective role in animal models against disease onset and progression. In 2009, the therapeutic potential of EVs gathered particular momentum, as highlighted by the publication of several studies focused on EVs derived from mesenchymal stem cells (Couch et al., 2021).

In the 2010s, particular attention was devoted to EV community building, standardization efforts, and methodological advances. Although the first international meeting for EVs was held in Montreal in 2005, the International Society for Extracellular Vesicles (ISEV) was formed in 2011, arranging annual meetings on EVs from that year onwards and paving the way for the establishment of national EV societies worldwide. The 2010s were also characterized by the launch of the Journal of Extracellular Vesicles (JEV) in 2012, along with the publication of several position papers, especially the Minimal Information for Studies of EVs (MISEV) guidelines (Lötvall et al., 2014; Théry et al., 2018), and EV-related databases (Kalra et al., 2012; Simpson et al., 2012). EV data reporting initiatives [e.g., Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research (EV-TRACK)] (Van Deun et al., 2017), survey outputs, and meeting reports contributed to reinforcing the EV community's efforts in harmonizing methodologies, data reporting, and nomenclature. The methodological advancements

experienced over the last decade, including the introduction of novel platforms and approaches for EV sample preparation and analysis, enabled researchers to minimize technical challenges related to EV manufacturing, engineering, and characterization, thereby attempting to unlock the full potential of EVs. More recently, the field has been emphasizing the need for addressing issues related to EV production scalability and the definition of specific quality metrics tailored to EV-based products and intended use (Figure 1).

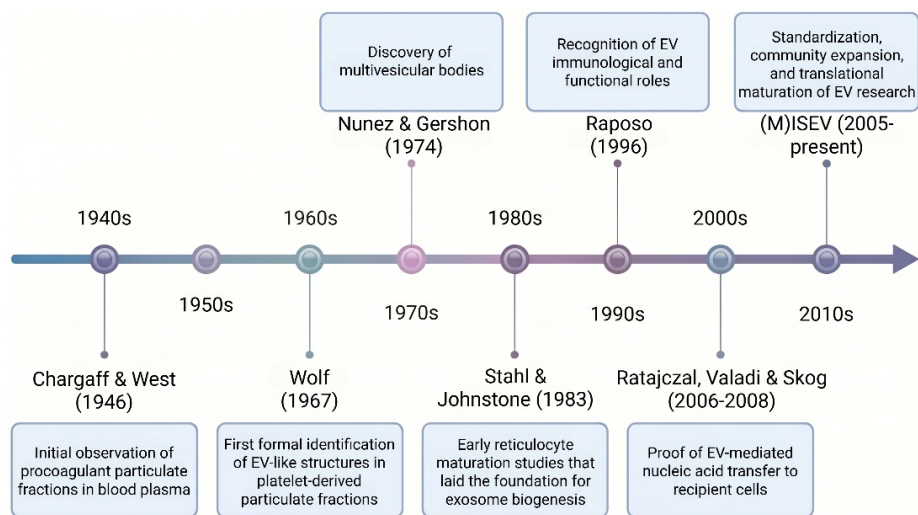


Figure 1. Timeline of major milestones in the EV field. The figure was adapted from Figure 2 in Couch, Y., Buzàs, E. I., Di Vizio, D., Gho, Y. S., Harrison, P., Hill, A. F., Lötvall, J., Raposo, G., Stahl, P. D., Théry, C., Witwer, K. W., & Carter, D. R. F. (2021). A brief history of nearly EV-erything – The rise and rise of extracellular vesicles. *Journal of Extracellular Vesicles*, 10(14), e12144. <https://doi.org/10.1002/jev2.12144>. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Modifications were made. Abbreviations: EV = extracellular vesicle; ISEV = International Society for Extracellular Vesicles; MISEV = Minimal Information for Studies of Extracellular Vesicles. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/5cqam2f>.

1.2 Overview of EV biology and applications

1.2.1 Biological fundamentals and state-of-the-art classification of EVs

Over time, cells have been shown to constantly exchange biochemical messages to finely coordinate functions in multicellular organisms and between single-celled organisms. Only in recent decades, these evolutionarily conserved biological mechanisms have been attributed to the cell secretion and internalization of nano-sized and micro-sized membranous particles, collectively designated as EVs (Gill et al., 2019; Lawson et al., 2017; Yáñez-Mó et al., 2015).

EVs are universally delimited by a lipid bilayer and carry a plethora of biomolecules, including proteins, lipids, carbohydrates, nucleic acids, and metabolites, with a composition that can reflect the identity and the status of their parental cells. They exhibit a density ranging from 1.08 to 1.21 g/mL and a predominantly negative surface charge, as reflected by a zeta potential (ZP) spanning -50 to -10 mV (Hendrix et al., 2023). By promoting direct cargo transport from donor cells to recipient cells, EVs have been

demonstrated to actively regulate the functional state of target cells and their interactions with the surrounding environment (Buzas, 2023; Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015). Besides the molecules sorted into their membrane and lumen during biogenesis, EVs can co-express unique biomolecular features adsorbed when exposed to the surrounding extracellular space, ultimately leading to the formation of a protein-enriched surface corona, which may have an impact on their functionality (Dietz et al., 2023; Liam-Or et al., 2024; Tóth et al., 2021) (Figure 2).

Historically, EVs were classified into three major categories based on differences in biogenesis, release pathways, and size: i) exosomes, of endosomal origin, released via MVB fusion with the plasma membrane, and typically in the range of 30-150 nm in diameter; ii) microvesicles, also named ectosomes or microparticles, formed through outward budding of the plasma membrane and with diameter ranging from 100-1,000 nm; iii) apoptotic bodies, secreted by plasma membrane blebbing during programmed cell death and constituted by a size range of 50-5,000 nm (Gurunathan et al., 2019). Furthermore, Di Vizio and colleagues identified a distinct subset of larger EVs, ranging from 1 to 10 μ m in diameter, which are highly associated with cancer biology. Termed large oncosomes, these vesicles originate from highly migratory and aggressive tumor cells, as a result of the outward budding and detachment of plasma membrane blebs (Di Vizio et al., 2012; Minciacchi et al., 2015).

Recent advances in EV detection and analytical assessment have highlighted multiple limitations associated with the traditional EV classification system, as the lack of molecular markers specific to individual EV subtypes, along with their substantial overlap in size ranges, makes their discrimination challenging using currently available study methods. Accordingly, the ISEV has encouraged researchers to categorize EVs using operational terms based on the following characteristics: (a) EV physical properties, such as size (*i.e.*, <100 nm or <200 nm for small EVs and >200 nm for medium/large EVs) and density (*e.g.*, low, intermediate, and high); (b) biochemical composition, highlighted by the expression of specific markers (*e.g.*, tetraspanins CD63 and CD81) or labelling with specific macromolecules (*e.g.*, Annexin A5); (c) cell of origin or pathophysiological conditions (*e.g.*, hypoxia-associated EVs, large oncosomes, apoptotic bodies) (Buzas, 2023; Théry et al., 2018; Welsh et al., 2024).

1.2.2 EVs across taxa

The ubiquity of EVs across all domains of life has been highlighted over the past half century. To date, the great biological significance and translational potential of EVs have been mostly disclosed via studies conducted on samples of mammalian origin (*e.g.*, human and bovine). Nevertheless, the earliest observations on EV production can be traced back to late 1950s and mid 1970s in less investigated non-mammalian sources, including algae, bacteria, plants, fungi, and parasitic helminths, which have increasingly gained attention for their contribution to the evolutionary footprint of EVs within and across distinct taxa (Couch et al., 2021; Gill et al., 2019; Woith et al., 2019). Furthermore, they have been shown to offer a putative competitive advantage over mammalian EV sources for the promotion of more sustainable and faster-paced translation into clinical and commercial settings (Zarovni et al., 2021).

EVs derived from organisms belonging to different taxonomic groups exhibit biomolecular (*e.g.*, proteins, lipids, nucleic acids, glycosylation) and biophysical (*e.g.*, size, morphology, curvature, rigidity) homologies that suggest a common ancestral origin. Concomitantly, the evolutionary processes shaping organismal diversity in the tree of life

underlies the biochemical heterogeneity that characterize EVs of different origin (Gill et al., 2019; Lawson et al., 2017; Yáñez-Mó et al., 2015; Woith et al., 2019). This can be exemplified by the numerous universally conserved and taxa-specific macromolecular profiles that have been highlighted in both eukaryotic and prokaryotic EVs, revealing overlapping but not identical compositions, thereby diversifying EV functional implications according to the source (Woith et al., 2019). To date, most compositional profiling studies have focused on disclosing the EV proteome, lipidome, and transcriptome (Ghadami & Dellinger, 2023; Greening et al., 2024; Joyce & Doran, 2023; Lischmig et al., 2022; Miceli et al., 2024; Skotland et al., 2020; T. Wang et al., 2023). Recent research efforts have also unveiled the potential role of glycans in mediating EV biogenesis and functionality, pioneering EV glycomics and carbohydrate analysis, especially on mammals and parasitic helminths (Whitehead et al., 2020; Williams et al., 2018). Given the primary EV molecular constituents and biological sources explored in this thesis, the following subsections provide a more detailed overview of protein, lipid, and carbohydrate composition across EVs derived from animal (*i.e.*, mammalian and parasitic helminth) and plant sources (Figure 2).

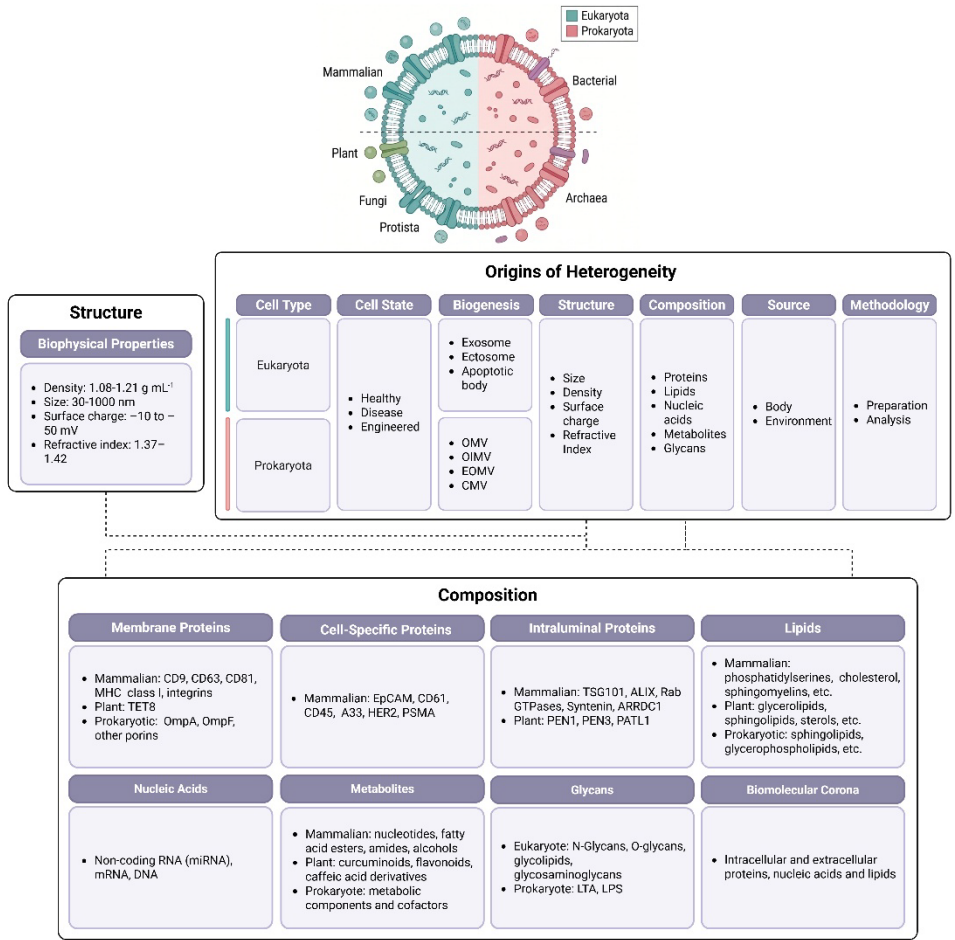


Figure 2. Structure, composition, and source of heterogeneity of EVs. The figure was adapted from Figure 1 in Hendrix, A., Lippens, L., Pinheiro, C., Théry, C., Martin-Jaular, L., Lötvall, J., ... & Witwer, K. W. (2023). *Extracellular vesicle analysis. Nature Reviews Methods Primers*, 3(1), 56.

<https://doi.org/10.1038/s43586-023-00240-z>, with permission from Springer Nature. Modifications were made. Abbreviations: ALIX = ALG-2-interacting protein X; ARRDC1 = arrestin domain-containing protein 1; CMV = cytoplasmic membrane vesicles; EOMV = explosive outer membrane vesicles; EpCAM = epithelial cell adhesion molecule; GTPase = guanosine triphosphatase; HER2 = human epidermal growth factor receptor 2; LPS = lipopolysaccharide; LTA = lipoteichoic acid; MHC = major histocompatibility complex; miRNA = micro ribonucleic acid; mRNA = messenger ribonucleic acid; OIMV = outer inner membrane vesicles; OmpA/F = outer membrane protein A/F; OMV = outer membrane vesicles; PEN1/3 = penetration 1/3; PSMA = prostate-specific membrane antigen; TET8 = tetraspanin 8; TSG101 = Tumor susceptibility gene 101. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/8wq4o7t>.

1.2.2.1 Proteins

Proteins represent the most extensively characterized molecular constituents of EVs, playing major roles in EV membrane structure and biogenesis, cargo sorting and trafficking, intercellular communication, environmental sensing and adaptation (Gill et al., 2019; Théry et al., 2018; Yáñez-Mó et al., 2015). A representative, non-exhaustive overview of the major protein categories and illustrative examples is presented below. EV membrane proteins may be categorized according to whether they are conserved across biological sources belonging to the same taxonomic group or exhibit cell-/tissue-/species-specificity. Taxa-conserved proteins include both transmembrane proteins (e.g., tetraspanins CD9, CD63, and CD81 in animal EVs; tetraspanins 8 and 9 in plant EVs) and membrane-associated proteins [e.g., flotillins and glycosylphosphatidylinositol (GPI)-anchored proteins CD55, CD59, and CD73 in animal EVs; PEN1, aquaporins, and ATPase in plant EVs]. Cell-/tissue-/species-specific proteins comprise major histocompatibility complex (MHC) class I and II, integrins, and lysosome-associated membrane protein (LAMP)1/2 in animal EVs and proteins mediating environmental sensing, host recognition, defence responses, or inter-organism interactions in EVs derived from other eukaryotic and prokaryotic sources. Among the cytosolic proteins, the most well-established categories include heat shock proteins (HSP)/chaperone proteins (e.g., HSP70 and HSP90 in animal and plant EVs), structural proteins (e.g., actin and tubulin in animal EVs), metabolic enzymes [e.g., glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, ATPase in animal and plant EVs], ribonucleic acid (RNA)/deoxyribonucleic acid (DNA)-binding proteins [e.g., Argonaute (AGO) proteins in animal and plant EVs], defence or virulence cargo (e.g., plant defence enzymes, bacterial toxins, fungal hydrolytic enzymes, and archaeal anti-microbial proteins), and endosomal sorting complexes required for transport (ESCRT)-associated proteins [e.g., ALG-2-interacting protein X (ALIX) and tumor susceptibility gene 101 (TSG101) in animal and plant EVs].

Although no universal marker has yet been accepted for EV identification across taxa, certain of these protein classes, especially tetraspanins, are routinely employed as representative EV markers. Furthermore, membrane proteins may also serve as antigens for selective EV capture and purification (Di Naro et al., 2025; Gill et al., 2019; Lian et al., 2022; Théry et al., 2018; Urzì et al., 2022; Woith et al., 2019).

1.2.2.2 Lipids

Several studies have shown that EVs derived from different cell types, even belonging to the same organism, are characterized by distinct lipid profiles contributing to important EV source-specific functional roles. EV enrichment with lipid classes that commonly characterize plasma membranes, especially phospholipids and sterols, has been reported

(Ghadami & Dellinger, 2023; Skotland et al., 2020). Among phospholipids, glycerophospholipids provide the structural backbone to EV membranes across all domains of life, with specific composition and relative abundance varying according to the EV source. In mammalian EVs, phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylethanolamine (PE) represent the predominant species and play a supporting role in EV biogenesis, membrane organization and stability, cellular interaction, and signalling functions. Conversely, plant EVs, in addition to PC, are enriched in phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylglycerol (PG), which are actively involved in defense signalling and stress responses. Beyond PC, PE, and PS, parasitic helminth EVs exhibit elevated content of plasmalogens and lysophospholipids, contributing to membrane resilience, oxidative protection, and host-pathogen immunomodulation. Diversity of sphingolipid and sterol profiles in EVs of different biological origin have been shown to reflect EV source-dependent membrane structural and functional specialization. Mammalian EV membranes display enrichment in sphingomyelin, ceramides, glycosphingolipids, and cholesterol, which are key lipid raft components collectively contributing to reinforcing EV rigidity and stability, as well as supporting biogenesis and signalling pathways. Instead, in parasitic helminth EVs, characterized by reduced levels of sphingolipids and cholesterol, membrane rigidity and stabilization are primarily conferred by plasmalogens. Unlike EVs of animal origin, plant EVs are associated with higher levels of glycosylceramides, ceramides, and phytosterols, such as sitosterol, campesterol, and stigmasterol, which are involved not only in membrane structural enhancement, but also in environmental resilience and defence-related signalling (Di Naro et al., 2025; Ghadami & Dellinger, 2023; Lian et al., 2022; Skotland et al., 2020; Whitehead et al., 2020; Woith et al., 2019).

1.2.2.3 Glycans

Glycoproteins and glycolipids are critical structural and functional components of biological membranes. These biomolecules exert a fundamental role in regulating multiple cellular processes, including cell adhesion, communication, signal transduction, and membrane trafficking (Seeberger et al., 2024). Diverse glycan moieties have been recently identified in association with EVs derived from multiple sources, highlighting their potential involvement in EV biogenesis, uptake, and EV source-specific biological effects (Williams et al., 2018). To date, glycan characterization has been mostly explored in mammalian EVs, whose membrane has been shown to express a diverse array of complex N-linked glycans [e.g., high-mannose glycans, complex bi-, tri, and tetra-antennary N-glycans, polylactosamine extensions, fucosylated glycans, bisecting N-acetylglucosamine (GlcNAc)-modified glycans, and terminal sialylated glycans, especially with α 2,6-linked sialic acid motifs] and O-linked glycans (e.g., truncated mucin-type structures), with either selective enrichment or exclusion (e.g., ABO blood group antigens; Batista et al., 2011) of certain glycotypes, compared with parental cells. Altered patterns have been demonstrated in correlation with disease states, such as cancer. Furthermore, surface enrichment in heparan sulfate proteoglycans has been also reported. Glycan profiling of parasitic helminth EVs remains limited and has been thus far addressed mostly through indirect approaches, such as assays based on the use of lectins – proteins that bind to carbohydrate complexes associated with glycoproteins and glycolipids (Kk. Kumar et al., 2012) – or glycosidase treatments (Cwiklinski et al., 2015; Huang et al., 2025; Torre-Escudero et al., 2019; Whitehead et al., 2020; Woith et al., 2021; Q. Zhang et al., 2025). Available evidence from *Fasciola hepatica* and *Schistosoma*

mansoni parasitic helminths have highlighted distinct glycan composition between EVs and parasite tegumental surface, supporting the predominant EV enrichment with N-linked glycan structures, especially high- and oligo-mannose residues, alongside a general absence of sialic acid motif (Kuipers et al., 2023; Torre-Escudero et al., 2019; Whitehead et al., 2020). Current knowledge about plant EV glycobiology is limited and detailed mapping of N-glycan or O-glycan structures in plant EVs is still lacking. However, recent lipidomic studies have reported plant EV membranes to be enriched in glycosphingolipids, especially glycosyl inositol phosphoryl ceramides (GIPCs), and galactolipids, such as mono- and di-galactosyldiacylglycerols (Huang et al., 2025; N.-J. Liu et al., 2020; Q. Zhang et al., 2025).

1.2.3 Biological functions and applications of EVs

As intercellular cargo carriers with intrinsic effector and delivery properties, EVs have been demonstrated to possess a wide spectrum of pleiotropic functions across, virtually, every major biological process, ultimately showcasing their broad market potential (Zarovni et al., 2021). The physiological conditions in which EV functions have been most extensively characterized comprise, and are not limited to, coagulation and haemostasis, erythrocyte maturation, angiogenesis and vascular repair, tissue repair and regeneration, innate and adaptive immunity, bone calcification and mineralization, nervous system communication, liver homeostasis and drug metabolism, reproduction and embryonic development. Likewise, extensive research has highlighted the critical implication of EVs in numerous pathological mechanisms, which primarily encompass cancer, neurodegenerative disorders, cardiovascular diseases, infectious diseases, metabolic disorders, and autoimmune and inflammatory diseases (Kalluri & LeBleu, 2020; M. A. Kumar et al., 2024; Li et al., 2026; Yáñez-Mó et al., 2015). As a result, over the years, EVs of both eukaryotic and prokaryotic origin have increasingly gathered visibility for their potential to deliver innovative, fit-for-purpose solutions across numerous market niches: from the more consolidated and less profitable EV life science, research products & tools and diagnostics segments to the less mature yet more remunerative EV therapeutics, drug delivery and regenerative medicine. Great promise for accelerating EV-based product development, market validation, and adoption lies within the lucrative personal care and food industries, in which EV-based innovations have already begun early commercialization attempts in the form of cosmetic and cosmeceutical products, nutraceuticals, as well as pesticides and seed priming agents. State-of-the-art methodologies for EV study and limitations (Zarovni et al., 2021).

1.3 State-of-the-art methodologies for EV study and limitations

1.3.1 Enrichment, separation, and fractionation of EVs

It is well recognized that, in addition to arising from the intrinsic biological diversity of EV populations, EV heterogeneity is fundamentally shaped by the extrinsic technical variability introduced during EV recovery due to the adoption of non-standardized methodologies, in the absence of a universal, “one-size-fits-all” solution. As EVs are highly complex structures comprising multiple biological constituents, methods for separating EVs from their original source are selected based on the complexity (e.g., type of macromolecular contaminants, presence of excipients) and the properties (e.g., volume, viscosity) of the source, the target EV populations to examine, the specific study

context, and the intended downstream EV applications. In turn, these dictate specific yield, purity, and integrity requirements.

EV preparation typically comprises most, if not all, of the following stages: i) EV source pre-processing or pre-clearing, to remove cells and large cellular debris; ii) EV source concentration, to facilitate storage and make downstream steps more sustainable; iii) EV enrichment, to retain EVs based on distinct physicochemical properties (e.g., size, density, surface charge, surface antigen composition), while decreasing, but not fully depleting, contaminating materials; iv) EV separation, involving two or more consecutive enrichment steps, often targeting distinct EV physicochemical characteristics, to increase EV purification and specificity; v) EV fractionation, to further resolve bulk EVs into different EV subpopulations with distinct physicochemical profiles (Hendrix et al., 2023) (Figure 3). It is noteworthy that, depending on the EV source complexity and the specific study objectives, EV enrichment and separation can often be integrated into a streamlined, single-step workflow, involving, for instance, tangential flow filtration (TFF) (Busatto et al., 2018).

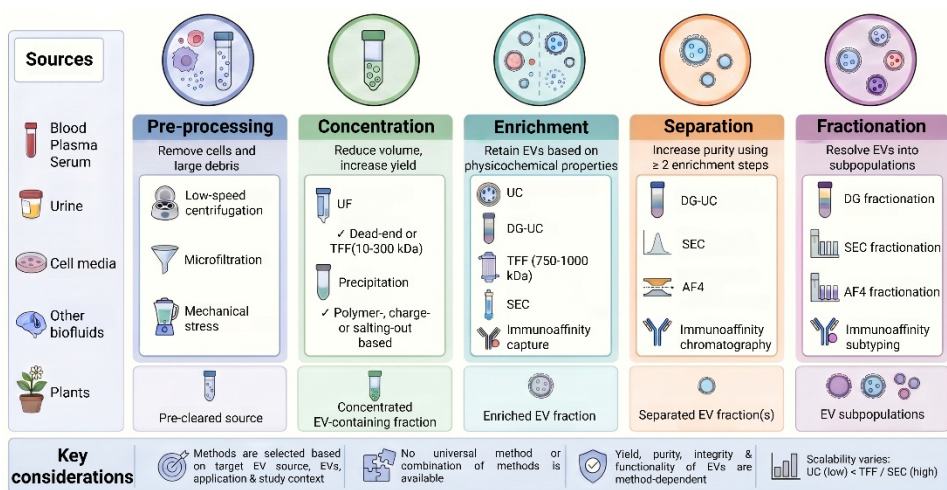


Figure 3. Overview of EV preparation workflow. Abbreviations: AF4 = asymmetric flow-field-flow fractionation; DG = density gradient; EV = extracellular vesicle; IAC = immunoaffinity chromatography; SEC = size exclusion chromatography; TFF = tangential flow filtration; UC = ultracentrifugation; UF = ultrafiltration. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/rct7xc5>.

EV source pre-processing traditionally relies on either differential low-speed centrifugations or microfiltration (Hendrix et al., 2023). Application of mechanical stress (e.g., grinding using mixers/blenders) can be also introduced when handling particularly stiff sources, such as plant roots or specific types of plant tissues (Lian et al., 2022). However, this procedure is often associated with the introduction of artificial nanoparticles into the purified EV preparations. Subsequent concentration of the pre-cleared EV source can be traditionally performed by either ultrafiltration (UF), using filter units with molecular weight (MW) cut-offs ranging from 10 to 300 kDa, or precipitation combined with low-speed centrifugation to promote EV sedimentation after mixing with the precipitating agents. The most commonly used UF systems rely on either dead-end filtration, traditionally leading to particle aggregation and membrane pore blockage, or TFF, which minimizes this issue by directing the fluid flow tangentially across the

membrane surface (Hendrix et al., 2023). Regardless of their nature [e.g., hydrophilic polymer-based (e.g., polyethylene glycol), protamine-induced charge-based, or acetate-based salting-out], precipitants are known to affect the composition of the final EV product, causing EV co-sedimentation with other contaminants and residual precipitating agents, thereby often requiring additional enrichment and separation steps (Hendrix et al., 2023) (Figure 3).

A wide range of methodologies for EV enrichment, separation, and fractionation, each featured by certain limitations and a specific level of scalability, are currently available in the market. Among those recovering EVs based on size or mass, ultracentrifugation (UC), which relies on high-speed centrifugation to pellet EVs, has been used as a gold standard for more than a decade. Nevertheless, depending on the specific settings used (e.g. centrifugation speeds, durations, and rotors), UC is known to potentially affect EV preparation efficiency and specificity, as some EVs may either skip sedimentation or co-sediment with non-EV components. Furthermore, it is well recognized that UC can also compromise EV surface properties, potentially leading to aggregation. Integrating a density gradient into the workflow, a procedure commonly referred to as density gradient UC (DG-UC), promotes EV separation from similarly sized particles with different density profiles, thereby facilitating improved purification from contaminants, such as soluble proteins, cell-free nucleic acids, and lipoproteins (Hendrix et al., 2023). Despite this, recent economic evaluations have ranked UC's scalability as low to very limited, especially for clinical-grade or commercial-grade manufacturing (Ng et al., 2019). Currently, this limitation can be overcome by TFF and size exclusion chromatography (SEC), which represent the primary commercially available systems that support industrial-scale EV processing (e.g., tens-to-thousands of liters per batch), depending on unit size and architecture, and whether parallelization is implemented (AxiChromTM 300 to 1600 Mm Process-Scale Chromatography Columns, n.d.; Ng et al., 2019; Sartoflow[®] 5000 SU, n.d.). For faster EV enrichment with simultaneous depletion of smaller contaminants, TFF cartridges with MW cut-offs ranging from 750 to 1000 kDa are traditionally used. TFF enables further fractionation when employing filters with varying MW cut-offs in sequence. By gravity or via an automated liquid chromatography system, SEC operates by separating EVs and EV subpopulations as they pass through a stationary phase (i.e., a porous gel polymer or resin beads packed in a column). These elute earlier than small contaminants accessing the pores, including soluble proteins, and eluting later than larger particles, which are excluded from the pores and travel more rapidly through the interstitial spaces between the beads. Although SEC is recognized to yield a high level of purification from protein contaminants, it dilutes the sample, thus requiring possibly further concentration steps (Hendrix et al., 2023). Furthermore, its performance has been shown to correlate with reduced EV functionality, compared with certain alternative methodologies, potentially due to depletion of active protein corona constituents (Wolf et al., 2022). When size-based and density-based methods do not achieve the envisaged performance and low scalability is acceptable, EVs, being negatively charged, may be separated from less negatively or positively charged components, using surface charge-based methods, such as ion exchange (IEX) chromatography and electrophoresis-based approaches, combined with modulation of buffer ionic strength or pH to release EVs. Alternatively, affinity capture using different molecules, such as antibodies, aptamers, or peptides, immobilized on distinct platforms (e.g., magnetic or latex beads, affinity columns, filter units, or chips) can be employed to target specific EV subpopulations based on the composition of their surface antigens

(e.g., cell-type specific tetraspanins and other molecules). However, the procedure followed to elute captured EVs can affect their integrity, potentially compromising their downstream uses (Hendrix et al., 2023) (Figure 3).

Depending on the specific EV characteristics required for an intended research objective or use, each of these methodological approaches is characterized by its own set of benefits and limitations. This makes the entire process of EV preparation fundamentally hindered by the need to achieve a trade-off between efficiency and specificity of EV recovery, as enhancing one automatically lowers the other, and vice versa (Hendrix et al., 2023). Certain EV applications, such as drug delivery and engineering, may demand either high purity levels or enrichment of specific EV subtypes to manipulation, targeting specificity, dosing, and ultimate functionality. Others, relying on EVs in their native, intrinsically bioactive state, including EV therapeutics, may benefit from higher production yields and the presence of co-isolated non-EV components that contribute to therapeutic efficacy. The paradigm “the process is the product” embodies these principles, emphasizing the fundamental interdependence of EV manufacturing processes and resulting structure, composition, functionality, and suitability for translational uses (Loria et al., 2024; Poupardin et al., 2024; Rohde et al., 2019).

1.3.2 Physicochemical characterization of EVs

A major challenge to EV research and development (R&D) remains our inability to achieve a comprehensive understanding of EV structural and functional properties due to the lack of “all-in-one” methodological solutions, capturing the intrinsic complexity and heterogeneity of EV populations with sufficient analytical resolution (Fortunato et al., 2021; Théry et al., 2018; Zarovni et al., 2021). In the attempt to address this gap and meet the requirements for robust EV characterization, the MISEV guidelines have consistently emphasized the need to rely on orthogonal and complementary analytical approaches, integrating bulk measurements with single-particle analyses, each offering advantages and suffering from technical constraints. As a substantial repertoire of analytical tools is currently available for EV quantitative and qualitative assessment, their selection is often driven by the study context, the specific research objectives to address, and the resource availability (Théry et al., 2018; Welsh et al., 2024) (Figure 4).

1.3.2.1 Bulk EV characterization methods

Bulk analyses are techniques that provide average readouts of specific measurable properties that are relative to a collective sample, rather than individual sample components (Donovan, 2018; Kolodziejczyk et al., 2015; Welsh et al., 2024). The major bulk methods that have been historically used for EV characterization can be categorized as follows: i) methods for biomolecular quantification and characterization [e.g., quantification assays, Western blot (WB), chromatography, capillary electrophoresis, polymerase chain reaction (PCR), omics technologies, such as mass spectrometry (MS) and RNA-sequencing]; ii) particle sizing methods [e.g., dynamic light scattering (DLS)]; iii) imaging-based techniques [e.g., transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic TEM (cryo-TEM), atomic force microscopy (AFM)]; iv) spectroscopic analyses [e.g., Raman, Fourier-transform infrared spectroscopy (FTIR)]. Although informative, all these methods reflect the existence of a critical gap between what a bulk analytical method is assumed to measure and what it truly measures (Welsh et al., 2024). Such discrepancy often leads to misinterpretation of EV sample characteristics and mostly arises from three fundamental issues: i) inability to uncover

EV subpopulations; ii) inadequacy in discriminating EVs from non-EV particles; iii) limited ability to detect EV particles beyond specific thresholds imposed by each individual technology (Coumans et al., 2017; Gandham et al., 2020; Welsh et al., 2024).

Among the methods used for bulk biomolecular quantification, the conventional quantification of total protein content as a surrogate measure of EV concentration exemplifies the matter. When EVs are co-isolated with non-EV protein contaminants, especially when processing complex biofluids or adopting purification methods that are not optimal, EV quantification based on protein measurement necessarily leads to EV number overestimation. Alternatively, qualitative and (semi-)quantitative analysis of specific molecular markers [*e.g.*, tetraspanins (CD9, CD63, CD81, TET8), ALIX, TSG101, *etc.*], whose performance is generally recommended for supporting EV identification and purity assessment, are unable to resolve EV heterogeneity, as no universal molecular markers – present in all EVs, regardless of their biological origin, subtype classification, and study context – have yet been identified. Furthermore, considering an analytical technique, such as DLS, that estimates particle hydrodynamic diameter based on the light scattering collected from all particles at once, particle size overestimation may be expected, compared with imaging technologies enabling direct visualization and accurate structural assessment of the same particles. Nevertheless, most of the latter ones do not provide reliable estimations of particle concentrations and produce images that are unlikely to be representative of the whole particle population in solution, given that larger EVs tend to be statistically underestimated, compared with smaller EVs. On the other hand, spectroscopic methods share comparable uncertainty in detection efficiency, which can vary depending on sample molecular composition and arise from either instrumental sensitivity constraints - as in the case of FTIR – or the intrinsic intensity weakness of the specific signal to be detected – as in the case of Raman spectroscopy (Welsh et al., 2024) (Figure 4).

1.3.2.2 Single-particle EV characterization methods

Over the last decade, multiple single-particle analytical approaches, including nanoparticle tracking analysis (NTA) and nanoscale flow cytometry (nFCM), have been developed to address EV heterogeneity with higher sensitivity and resolution, thereby overcoming some technical limitations associated with bulk EV assessment. In contrast to bulk methods, single-particle platforms enable the detection and phenotyping of individual particles in suspension without collapsing collective data into possibly misleading averages. Depending on the intrinsic capabilities and operational settings of the instrument in use, each technology exploits either light scattering, interference, diffusion, or electrical resistance to provide estimates of multiple physical properties attributable to the assessed particles, which may be either concentration within specific size ranges, size distribution, ZP, or refractive index. Despite these advancements, single-particle measurements that rely solely on light scattering are ineffective in discriminating EVs from non-EV components, such as soluble proteins in aggregated form and cell media components, which can be identified as particles with comparable physical characteristics (Coumans et al., 2017; Fortunato et al., 2021; Kwon & Park, 2022; Welsh et al., 2024). To address this, along with characterizing relevant EV subpopulations, the EV community has increasingly adopted multiple fluorescent labelling strategies, enhancing single-particle analysis with biochemical evaluation of either EV membrane surface markers (*e.g.*, tetraspanins CD9, CD63, CD81) or internal components (*e.g.*, cytoplasmic proteins, nucleic acids) (Théry et al., 2018; Welsh et al., 2024).

Most single-particle technologies that are currently used for EV analysis share comparable technical constraints associated with detection, quantification, sizing, and discrimination from contaminants. As providing indirect measurements based on optical and transport phenomena, their capability mainly relies on inferring the physical features of the detected particles, thus potentially leading to analytical artefacts (*e.g.*, false positives, incorrect size assignments, misclassifications). In terms of particle detectability, comparisons between different platforms are challenged by differences in limit of detection (LoD), which is difficult to define, especially for smaller size and lower concentration thresholds. Particle quantification and sizing, especially in complex fluids, is also prone to introduce bias dependent on the specific algorithm and software in use, refractive index-related assumptions, and fluorescent labelling effects. Moreover, different instruments may require different pre-analytical steps to make samples suitable for assessment, which can selectively alter final sample composition, ultimately contributing to data misinterpretation. Overall, this implies that rigorous calibration procedures, appropriate reference materials, transparent reporting, and orthogonal validation must be applied to ensure reliable, robust, and reproducible measurements (Welsh et al., 2024) (Figure 4).

1.3.2.2.1 Nanoparticle Tracking Analysis *versus* Nanoscale Flow Cytometry

NTA tracks the Brownian motion of individual particles over time within a sample cell. Using laser illumination, specific optical filters, and a complementary metal-oxide-semiconductor (CMOS) camera sensor, either forward-scattered light (FSC) or fluorescence is recorded using a single detection channel or multiple detection channels, as implemented in more advanced instruments (*e.g.*, ZetaView® Evolution by Particle Metrix). The acquired particle trajectories and speed of motion are then analyzed to calculate particle hydrodynamic diameter via the Stokes–Einstein equation (McComiskey & Tajber, 2018; Mladenović et al., 2025). Conversely, nFCM is capable of simultaneously detecting particle side-scattered light (SSC) and fluorescence by means of laser illumination and more sensitive single-photon counting module (SPCM) avalanche photodiode (APD) detectors across multiple channels. In contrast to NTA, particle size is inferred based on calibrated SSC intensity derived from silica nanoparticle standards with refractive index that is similar yet not equivalent to EVs. Furthermore, the two instruments make different estimations of particle concentration measurements: NTA counts particles across multiple positions within the same sample cell (*e.g.*, ZetaView® by Particle Metrix) or under controlled flow conditions (*e.g.*, NanoSight® by Malvern Panalytical), whereas nFCM detects particles in flow, using calibration beads of known concentration and fixed detection thresholds (Fortunato et al., 2021; Mladenović et al., 2025) (Figure 4).

1.3.2.3 Fluorescent labelling strategies of EVs

Depending on the molecular target, fluorescent dyes may be subdivided into the following categories: i) lipid-binding dyes; ii) antibody-conjugated dyes; iii) protein-reactive dyes; iv) nucleic acid-binding dyes (Figure 4).

Lipid-binding dyes are typically designed to target lipid membranes. Most commercially available options are constitutively fluorescent in aqueous solution (*e.g.*, CellMask™ Plasma Membrane Stain, BODIPY™, PKH®, *etc.*) and are used for their brightness and localized diffusion. Others have been developed as environment-sensitive, non-fluorescent molecules, which emit fluorescence only upon insertion into

lipid membranes (e.g., Di-8-ANEPPS) (Adamo et al., 2021; Barreca et al., 2023; Deghani et al., 2020; Fortunato et al., 2021; Th  ry et al., 2018; Welsh et al., 2024).

Fluorescently labelled antibodies [e.g., Alexa Fluor 488 (AF488)-conjugated, phycoerythrin (PE)-conjugated] targeting epitopes on EV membrane surface proteins (e.g., tetraspanins) offer the advantage of targeting specific surface membrane protein antigens, thereby enabling the discrimination of EVs of different sources, the identification of EV subpopulations, and the assessment of EV purity by detection of contaminating markers. Fluorophore-conjugation makes them suitable for multiple analytical platforms and their multiplex capabilities allow for comprehensive EV phenotyping. Upon proper calibration, they can contribute to measuring epitope abundance and epitope density on individual EVs. Analogously to lipophilic dyes, they can also bind to non-EV biocomponents, such as free proteins and non-vesicular extracellular particles, and require rigorous implementation of procedural controls and blanks. Moreover, they can suffer from intrinsic epitope accessibility issues, which would impose the introduction of additional steps to the labelling procedure, such as EV membrane permeabilization for internal cargo and enzymatic treatments (e.g., deglycosylation) (Fortunato et al., 2021; Mladenovi   et al., 2025; Nie et al., 2025; Th  ry et al., 2018; Welsh et al., 2024).

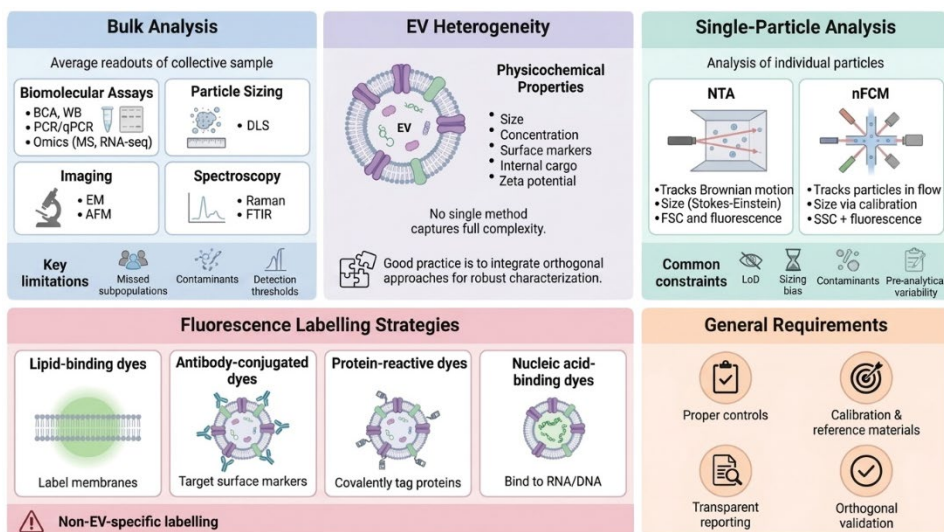


Figure 4. Overview of EV analytical assessment strategies. Abbreviations: AFM = atomic force microscopy; BCA = bicinchoninic acid; DLS = dynamic light scattering; DNA = deoxyribonucleic acid; EM = electron microscopy; EV = extracellular vesicle; FSC = forward scatter; FTIR = Fourier Transform Infrared; LoD = limit of detection; MS = mass spectrometry; NTA = nanoparticle tracking analysis; nFCM = nanoscale flow cytometry; (q)PCR = (quantitative) polymerase chain reaction; RNA = ribonucleic acid; RNA-seq = RNA sequencing; SSC = side scatter; WB = Western blot. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/377qgxq>.

Protein-reactive dyes are molecules that function upon covalent binding to proteins. Depending on the chemical group they react with, they can be categorized into multiple classes, including the following: i) amine-reactive [e.g., CellTrace[®] carboxyfluorescein succinimidyl ester (CFSE)]; ii) thiol-reactive, combining common fluorescent tags, such as AF488, with maleimide; iii) carboxyl-reactive; iv) tyrosine-reactive; v) histidine-reactive; vi) photo-activated protein-reactive (Hermanson, 2008). Among these, amine-reactive

probes have been the most extensively employed in EV detection studies (Bao et al., 2023; Ender et al., 2019; Fortunato et al., 2021; Morales-Kastresana et al., 2017). Except for those that are initially administered as membrane-permeable precursors, such as carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (*i.e.*, the non-fluorescent, esterified form of CFSE), most of the others are not permeable to the lipid bilayer, when the latter is intact (Ender et al., 2019; Fortunato et al., 2021; Perfetto et al., 2010). As the primary chemical reaction driving the labelling is not epitope-dependent and can potentially occur in any protein-containing particle, these dyes have the potential to reveal particles that can escape antibody-based detection in the case antigen epitopes are lacking or inaccessible (Fortunato et al., 2021; Morales-Kastresana et al., 2017).

Being designed as lipid bilayer-permeable, nucleic acid-binding dyes (*e.g.*, SYTO™ RNASelect™) do not require the disruption of EV membrane integrity for internalization and can enable the selective identification of EV subpopulations based on the presence and abundance of RNA or DNA molecules. Although commonly exploited in research settings, fluorescent single-particle measurements are acknowledged to share various technical limitations, which impose complementary validation with orthogonal methods and assessment of the labelled samples of interest in parallel with proper procedural controls (Cordero-Rivera et al., 2026; de Voogt et al., 2021; Dlugolecka & Czystowska-Kuzmicz, 2024; Fortunato et al., 2021; Morales-Kastresana et al., 2017; Saftics et al., 2025; Théry et al., 2018; Welsh et al., 2024). These include: i) non-EV-specificity, as other potentially co-isolated particles, such as lipoproteins, and soluble proteins can be stained; ii) variable dye affinity for or accessibility to EV membrane-associated and internal cargo according to EV composition; iii) dye tendency to self-aggregate, especially at high concentrations; iv) vulnerability to background noise derived from excess of unbound dye; v) dye requirement for resistance to photobleaching (Dlugolecka & Czystowska-Kuzmicz, 2024; Fortunato et al., 2021; Morales-Kastresana et al., 2017).

1.4 Impact of EV structure on EV function

The molecular cargo of EVs was originally considered dominant in determining final EV functional outcomes (Kalluri & LeBleu, 2020; Raposo & Stoorvogel, 2013; Valadi et al., 2007). Nevertheless, growing evidence suggests that EV membrane integrity and surface composition play a pivotal role in influencing EV-cell interaction, EV uptake mechanisms, and the activation of downstream cellular pathways (Hallal et al., 2022; Russell et al., 2019). Despite this, most adopted EV engineering strategies persist in focusing on EV cargo loading efficiency, often overlooking whether and how induced structural perturbations impact EV functionality (Somiya & Kuroda, 2021; Théry et al., 2018).

1.4.1 Mechanisms of EV uptake

Upon contact with host cells, EVs have been demonstrated to trigger distinct, energy-dependent membrane reorganization mechanisms, activating specific uptake pathways according to the target cell type and the specific physicochemical properties of the EVs that are involved. To date, accumulated evidence has identified the endocytic pathway as the primary route for EV internalization. Nevertheless, a substantial body of research has also demonstrated the existence of a minor pathway involving direct membrane fusion. As particularly enhanced under acidic pH conditions, this internalization process has been suggested as the primary mechanism driving the EV uptake within endosomes (Gandek et al., 2023; Joshi et al., 2020; Mulcahy et al., 2014; Ribovski et al., 2023).

1.4.1.1 Endocytosis

Endocytosis is an umbrella term referring to a wide range of molecular and particle uptake processes that are commonly classified into two categories: clathrin-dependent and clathrin-independent pathways.

During clathrin-dependent endocytosis, also known as receptor-mediated endocytosis, clathrin and adaptor proteins [i.e., adaptor protein complex 2 (AP-2) associated with EPS15 (epidermal growth factor receptor pathway substrate clone 15)] are recruited to the plasma membrane around the receptor-ligand complexes to be internalized. Their sequential assembly progressively deforms the membrane up to the formation of a pit. Upon maturation, the clathrin-coated pit evolves into a vesicular bud that is cleaved at its neck by the action of the GTPase dynamin 2, operating as both a membrane-binding and a fission protein. This is followed by the release of a clathrin-coated vesicle, which undergoes uncoating, before fusing with an endosome to unload its content (Mulcahy et al., 2014).

The endocytic pathways that are not mediated by clathrin are broadly subdivided into four categories: caveolin-dependent endocytosis, lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis. Caveolin-dependent endocytosis involves the formation of small invaginations of the plasma membrane, known as caveolae, in subdomains of glycolipid rafts enriched in cholesterol, sphingolipids, caveolins, and integral membrane proteins residing on the cytoplasmic leaflet. Upon oligomerization, caveolin-1 promotes the inward budding of the bilayer, leading to the formation of a vesicle (or caveola), which is ultimately pinched off the plasma membrane by dynamin 2. Unlike caveolin-dependent endocytosis, lipid raft-mediated endocytosis occurs in planar lipid rafts marked by the presence of flotillins. By direct binding, these mediate cargo internalization with the assistance of the other structural components enriched in these regions, which promote the assembly of signalling molecules, affect membrane fluidity, and mediate membrane protein trafficking. Differently, macropinocytosis is characterized by the development of long membrane ruffles that protrude outward from the cell surface. Mainly driven by actin, cholesterol, and the GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1), a critical regulator of cytoskeleton reorganization, this process triggers the enclosure of a wide area of extracellular fluid, which is then internalized within large vesicles, known as macropinosomes, after the ruffles fold back on themselves and fuse with the plasma membrane. Although promoting the occurrence of events that have multiple mechanistic similarities with the macropinocytotic pathway, phagocytosis is not Rac1-dependent and requires receptor-ligand interactions for direct contact with the material to be internalized. The latter is commonly represented by opsonized particulate matter of larger size, including bacteria and fragments of apoptotic cells, which makes this uptake route largely restricted to specialized cells, such as macrophages (Mulcahy et al., 2014; Feng et al., 2010; French et al., 2017; Lin et al., 2020).

1.4.1.2 Membrane fusion

As occurring during caveolin-dependent endocytosis, EV uptake by direct fusion with the plasma membrane or the endosomal membrane after primary endocytic internalization involves structural changes in the membrane curvature, which are mainly mediated by the (v- and t-) soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNARE) proteins. When the two fusogenic membranes are brought into close proximity, the water at the polar head groups is displaced, leading to the fusion of the outer leaflets, a phenomenon defined as hemifusion. This is followed by complete

membrane fusion, which triggers the formation of a pore and cargo release upon pore expansion (Lehninger et al., 2013; Mulcahy et al., 2014).

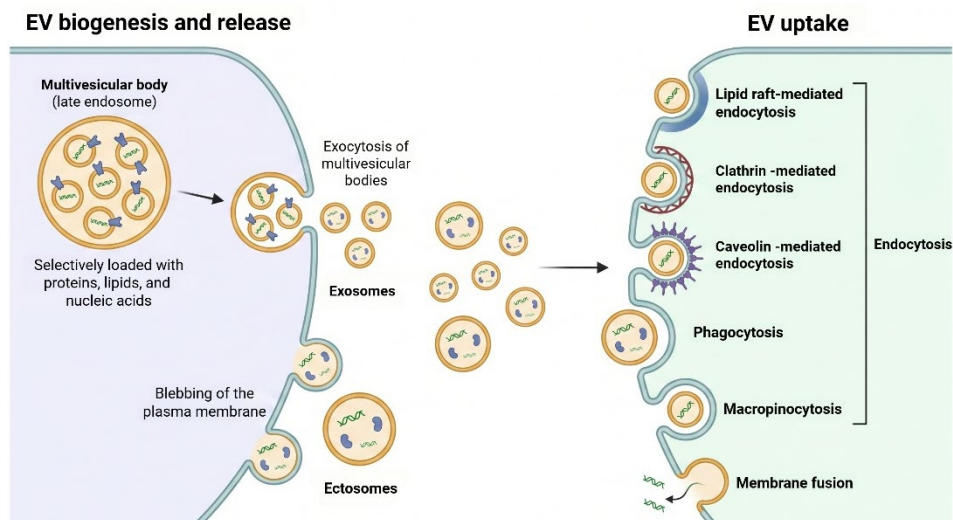


Figure 5. Overview of EV biogenesis, release, and uptake. The figure was adapted from Figure 1 in Thompson, W., & Papoutsakis, E. T. (2023). The role of biomechanical stress in extracellular vesicle formation, composition and activity. *Biotechnology Advances*, 66, 108158. <https://doi.org/10.1016/j.biotechadv.2023.108158>, with permission from Elsevier. Modifications were made. Abbreviations: EV = extracellular vesicle. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/8y0gp8j>.

1.4.2 Structural determinants of EV interaction and uptake

The EV membrane is a sophisticated, multifaceted interface, whose composition and integrity govern the pillar aspects of EV-mediated cargo delivery to recipient cells: from tissue homing and targeting to cellular uptake or signal transduction. Because of their small size, EVs possess a high surface area-to-volume ratio, which makes the membrane itself a functional, interactive platform capable of establishing extensive connections with cells and extracellular molecules within the extracellular microenvironment. Lipids, proteins, and carbohydrates provide structural support to the vesicular scaffold, overall contributing to EV morphology, stiffness, integrity, and stability. They may also play critical functional roles, acting either as i) physical binding platforms for receptors, ligands, or extracellular matrix (ECM) components, ii) effector molecules, triggering specific cellular signalling pathways and biological responses, or iii) recognition signals, driving selective EV-cell interactions (Buzás et al., 2018).

Phospholipids and sterols represent the main lipid constituents of EV lipid bilayers. From a structural standpoint, their composition and relative abundance, which are highly dependent on the EV source, critically affect EV membrane stiffness, curvature, and resistance to mechanical stress, thereby influencing EV stability in circulation and adhesion to target cells. Functionally, among the most enriched lipid species, those that are mainly concentrated within lipid rafts, such as sterols (e.g., cholesterol and phytosterols) and sphingolipids, are known to regulate, at the cellular level, various functions relevant for maintaining cellular homeostasis, including signal transduction, molecular and vesicular trafficking, cell adhesion, and uptake. Furthermore, in specific EV subtypes (e.g., plasma membrane-derived medium-to-large EVs, apoptotic bodies,

hypoxia-induced EVs, and certain tumor-derived EVs), PS is an example of an anionic glycerophospholipid that can act as a recognition signal depending on its location within the membrane. PS is predominantly concentrated in the inner membrane leaflet. However, when externalized to the outer membrane leaflet and bound to macrophage scavenger receptors or PS-binding proteins (e.g., Annexin V), it is involved in determining EV fate by promoting selective uptake pathways (Buzás et al., 2018; Skotland et al., 2020).

Owing to their complex three-dimensional structure, EV membrane proteins, compared with lipids, are characterized by greater recognition and binding capabilities, which underlie their prominent role in determining EV cell-/tissue-/organ-specificity and interaction modalities. Depending on their conformation, charge, and localization within the lipid bilayer, they can enhance EV docking through the following processes: i) the creation of binding pockets for adhesion molecules, strengthening receptor-ligand interactions, as in the case of tetraspanins (e.g., CD9, CD63, CD81); ii) recognition of specific ECM proteins, leading to organ-/tissue-specific biodistribution and accumulation, as observed for integrins (e.g., $\alpha 6 \beta 4$, $\alpha 6 \beta 1$, $\alpha v \beta 5$ integrins); iii) cell-type-specific EV targeting through receptor-ligand interactions, as occurring via adhesion proteins [e.g., intercellular adhesion molecule 1 (ICAM-1)], proteoglycans (e.g., CD44) – proteins with covalently attached carbohydrate chains (e.g., glycosaminoglycans) projecting out of the membrane – and lectins (e.g., galectin 3) – proteins that preferentially bind to carbohydrate residues conjugated to either proteins (i.e., glycoproteins, proteoglycans) or lipids (i.e., glycolipids) (Andreu & Yáñez-Mó, 2014; Bänfer et al., 2018; French et al., 2017; Hoshino et al., 2015; Lehninger et al., 2013; Segura et al., 2005; Szatanek & Baj-Krzyworzeka, 2021; Théry et al., 2018).

When released into the surrounding environment, especially when circulating in complex biological fluids, the EV membrane has been demonstrated to rapidly adsorb non-intrinsic extracellular biomolecules, which form a surface corona that ultimately shapes EV identity and activity (Dietz et al., 2023; Musicò et al., 2025; Tóth et al., 2021; Wolf et al., 2022). The thermodynamic instability of bare EVs, which is linked to the elevated free energy of their surfaces, tends to be naturally minimized upon formation of complexes with other secreted molecules and particles (Monopoli et al., 2012; Tóth et al., 2021). As the interactions between EVs and target cells are governed by the structural characteristics of both the EV and recipient cell membranes, the EV biomolecular corona acts as a dynamic interface determining EV homing, targeting, and uptake routes by recipient cells (Dietz et al., 2023; Monopoli et al., 2012). By modifying the physicochemical properties of EVs, such as optical and hydrodynamic diameters, surface charge, and aggregation status, it can directly affect adhesion and binding to cell membranes, internalization efficiency, and systemic biodistribution (Monopoli et al., 2012; Tenzer et al., 2013; Tóth et al., 2021). Instead, alteration of the biochemical composition of EV surfaces with the acquisition of a variety of molecules, such as immunoglobulins, enzymes, DNA, cytokines, coagulation factors, and apolipoproteins, can either introduce or mask ligands and receptors, thereby leading to distinct outcomes of biological interactions and systemic responses (e.g., modulation of immune recognition and clearance) (Caracciolo et al., 2017; Dietz et al., 2023; Tóth et al., 2021).

Substantial evidence has been accumulated about the putative compromise of EV structural and functional integrity when subjected to mechanical damage, freeze-thaw cycles, detergents, or oxidative stress (Ahmadian et al., 2024; Chiaradia et al., 2021; Jeyaram & Jay, 2017; Osteikoetxea et al., 2015; Sivanantham & Jin, 2022). The resulting

alteration of EV membrane fluidity, permeability, surface charge, and exposure patterns of surface components can cause cargo leakage and modification of interaction capabilities, ultimately impairing their interaction specificity, route of internalization, internalization efficiency, and functional delivery of bioactive cargo (Mathieu et al., 2019; Midekessa et al., 2020; Mulcahy et al., 2014; Skotland et al., 2020). On this basis, it is generally recommended to implement EV handling and storage procedures that ensure long-term maintenance of EV stability after rigorous validation of their non-detrimental impact on EV qualitative and quantitative characteristics (Théry et al., 2018; Welsh et al., 2024).

1.4.3 EV engineering strategies

Despite being natural carriers of effector and regulatory biomolecules, the successful translation of EVs into clinical practice and beyond remains limited by several challenges, including heterogeneous cargo composition, limited bioavailability and targeting specificity, variable loading capacity, and incomplete understanding of their mechanisms of action. As a result, to maximize their translational potential across various fields of application, EV engineering has emerged as a technological approach to generate EVs with more predictable biological properties, improve their performance as nanodelivery systems with enhanced bioavailability and targeting properties, and enable clearer mechanistic interpretation of their biological effects (Bader et al., 2025; Pacciani et al., 2026; X. Wang et al., 2025).

A wide range of EV engineering strategies have been explored to either increase the payload capacity or improve the delivery properties of EVs. Depending on the vesicular site in which the manipulation occurs, engineering approaches can be broadly divided into two complementary categories: i) EV surface modification methods, aimed at enhancing targeting specificity and cellular internalization; ii) EV content modification methods, designed to augment biological activity. According to the stage at which manipulation is performed, a second classification system can be applied, which discriminates modifications occurring at the endogenous (or donor cell-based) level and at the exogenous (or EV-based) level (Bader et al., 2025; Danilushkina et al., 2023; (Ma et al., 2025)Pacciani et al., 2026; X. Wang et al., 2025) (Figure 6).

Endogenous EV loading entails the genetic or molecular engineering of donor cells to produce EVs with desired structural and functional properties. Active manipulation of donor cells is commonly conducted by transfecting or transducing cells with vectors (e.g., plasmids, lentiviruses) encoding the desired cargo molecule at high expression levels and/or as a ligand fused with proteins enriched in EVs [e.g., EV surface-enriched proteins, including tetraspanins (CD63, CD9, CD81), Lamp2b, or GPI-anchored proteins] (Danilushkina et al., 2023). To boost EV yield, secretion rate, or alter their molecular composition by inducing non-targeted genetic manipulation, further approaches can be employed. These include: i) challenging the surrounding environment of the parental cells (e.g., nutrient deprivation, hypoxia, pH adjustment); ii) inducing physical stimulation of the parental cells through application of external forces, such as an electric field (e.g., by electroporation), ultrasound waves (e.g., by sonication), and fluid shear stress (e.g., by microfluidics); iii) promoting biochemical stimulation of the parental cells via biochemical compounds, such as chemical inducers (e.g., growth factors, cytokines) and pharmacological agents (e.g., chemotherapeutic drugs) (Danilushkina et al., 2023; (Ma et al., 2025) (Figure 6).

Exogenous EV loading involves the direct manipulation of EVs after their preparation. This can be promoted by either i) direct surface functionalization, ii) cargo encapsulation, or iii) hybridization, each offering distinct advantages and limitations in terms of encapsulation efficiency, cargo type compatibility, and scalability. Direct EV surface functionalization exploits the intrinsic properties of the EV membrane (e.g., hydrophobicity, endogenous biomolecules, functional groups) to promote the conjugation of exogenous moieties through either covalent (e.g., via click chemistry) or non-covalent (e.g., through electrostatic interactions and protein-ligand recognition) coupling, thereby enhancing targeting properties, cellular uptake, or cargo-loading capacity. Molecular encapsulation approaches involve the internalization of the target payload either passively or actively. Passive cargo loading can occur via simple diffusion or endocytosis (e.g., incubation), alongside transient destabilization of the EV membrane to augment loading efficiency (e.g., freeze-thaw cycles, extrusion, use of surfactants). In contrast, active cargo loading relies on the adoption of physicochemical methods (e.g., electroporation, sonication) that cause pronounced perturbation or disruption of the EV lipid bilayer to promote more efficient cargo internalization. Finally, to optimize EV payload capacity, tissue penetration properties, and immune evasion capability, as well as minimize cargo leakage during circulation, hybrid-based nanoplatfroms have recently gained increasing attention as a means of integrating the biological advantages of EVs with the versatility and scalability of synthetic nanoparticles. Specifically, this strategy combines natural EVs with synthetic materials, either carrying or grafting specific cargoes, through multiple mechanisms: i) EV membrane fusion (e.g., with lipid nanoparticles); ii) physical perturbation of the EV membrane, such as via extrusion, sonication, and microfluidics (e.g., for the incorporation of metal and inorganic nanoparticles); iii) the insertion of lipophilic monomers (e.g., cholesterol and derivatives) into the EV membrane (Ma et al., 2025).

Although cell genetic manipulation enables selective cargo sorting into EVs upon natural biogenesis, thereby exerting no or minimal disrupting effects on EV integrity, the endogenous EV loading approach faces multiple challenges, ranging from technical constraints (e.g., low EV yield, inefficient and non-standardized EV production procedures, applicability restricted to EVs derived from cell cultures) to economic (e.g., insufficient scalability, high production and analytical costs) and regulatory hurdles [e.g., quality control (QC), stability, and safety concerns] (Danilushkina et al., 2023). Furthermore, excessive cellular stimulation through external forces or environmental conditions can induce the release of EVs with unwanted molecular profiles, potentially compromising their efficacy and safety. Conversely, exogenous engineering approaches are generally more user-friendly and accessible for implementation. By bypassing the time-consuming and cost-intensive procedures required for creating and maintaining genetically modified cell lines, they offer cheaper engineering solutions, which do not carry any risk of oncotransformation from viral vectors. Moreover, they are suitable for most cargoes (e.g., small molecules, proteins, nucleic acids) and applicable to EVs recovered from any source. However, as applying physicochemical stress on EVs, most of these strategies share comparable implementation challenges related to the maintenance of EV integrity (Ma et al., 2025)

Disruption of EV integrity can involve physical damage to the membrane structure and alteration of the surface or cargo composition by loss or introduction of procedural artifacts (e.g., aggregation, contaminants), overall affecting biophysical properties and biological function. Freeze-thaw cycles, electroporation, sonication, and

extrusion are among the main exogenous engineering methods that have been reported to induce physical perturbation and rupture of the EV membrane. Specifically, by causing the temporary formation of ice crystals, freeze-thaw cycles have been demonstrated to promote lateral phase separation of the membrane components and membrane fusion, ultimately leading to irreversible structural changes and putative aggregation processes. Depending on the level of exposure to ultrasounds during sonication, the EV bilayer can be subjected to either temporary permeabilization or permanent structural impairment, leading to alterations in membrane architecture and microviscosity, potential adherence of exogenous molecules to the outer membrane layer, as well as loss of intrinsic molecules. When performed excessively or at high intensity, EV extrusion through membranes with pore sizes of 100-400 nm, as well as the application of electrical impulses to form transient pores in the EV lipid bilayer, have been linked to altered surface ZP and irreversible membrane deformation. Furthermore, the use of chemical agents [e.g., saponin, copper-catalyzed click chemistry reactions] can introduce contaminating artifacts causing oxidative stress or cytotoxic effects, if not coupled with thorough purification steps post-treatment (Auqui re et al., 2025; Ciferri et al., 2024; Danilushkina et al., 2023).

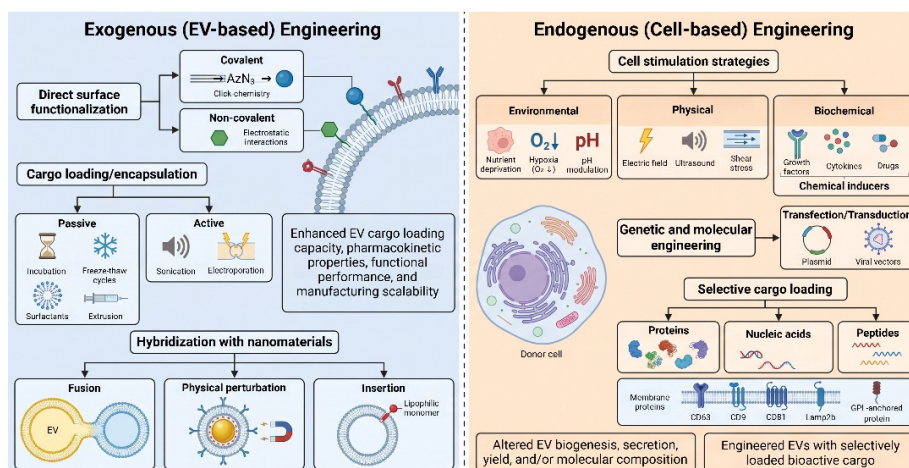


Figure 6. Overview of EV engineering strategies. The figure was adapted from Figure 2 in Ma, Y., Dong, S., Grippin, A. J., Teng, L., Lee, A. S., Kim, B. Y. S., & Jiang, W. (2025). Engineering therapeutical extracellular vesicles for clinical translation. *Trends in Biotechnology*, 43(1), 61–82. <https://doi.org/10.1016/j.tibtech.2024.08.007>, with permission from Elsevier. Modifications were made. Abbreviations: EV = extracellular vesicle; GPI = glycosylphosphatidylinositol; Lamp = lysosome-associated membrane protein. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/954j4ad>.

There is mounting evidence that EV structural perturbations induced by harsh engineering methods can compromise EV stability (e.g., resistance to aggressive environmental conditions), pharmacokinetic behavior (e.g., biodistribution, circulation time, and clearance rate), and cellular cargo delivery at the cellular level (e.g., uptake, intracellular transport, and payload release), while contributing to inter-batch and inter-study variability of functional outcomes (Ni et al., 2025; Peruzzi et al., 2024). Moreover, orthogonal analysis of cargo loading efficiency has shown inconsistencies across multiple analytical platforms (Lowe et al., 2024). Overall, this suggests that EV cargo encapsulation efficiency and increased surface ligand density, frequently reported as preferred metrics

for successful engineering, may represent misleading indicators of biological performance and targeting capability.

1.5 Comparative analysis and normalization approaches in EV research

EV heterogeneity, arising from both their biological origin and the specific methodologies used for their production, poses a significant methodological challenge for quantitative and qualitative comparisons of EVs across different sources. As a result, datasets are required to be normalized to ensure that the observed analytical outputs reveal true similarities or differences among EV preparations (Hartjes et al., 2019; Royo et al., 2020). Data normalization is a mathematical approach that uses quantitative parameters as reference values to adjust raw data for fair and consistent comparisons (Evans et al., 2017). Its application is especially relevant when conducting omics studies, involving the analysis of large and complex data pools, and meta-analyses, where data from independent studies are systematically combined to increase statistical power, resolve inconsistencies, and formulate more reliable conclusions (Ten-Doménech et al., 2024). In EV analysis, it is essential for reducing technical bias caused by batch effects, differences in sample size, and variability in analytical settings, while also addressing biological variance relative to EV abundance by emphasizing relative compositional changes (Li et al., 2026; Pinheiro et al., 2024; Ten-Doménech et al., 2024; Théry et al., 2018). Despite this, correcting data has been demonstrated to affect EV studies by varying the interpretation of experimental outcomes depending on the normalization approach adopted. Moreover, the lack of standardized normalization procedures challenges data consistency and harmonization across independent studies (Li et al., 2026; Ten-Doménech et al., 2024).

1.5.1 Normalization strategies and their interpretative impact

Normalization of EV datasets has historically relied upon the use of metrics reflecting either EV physicochemical properties or the study context. As directly relating to the inherent nature of EVs as biological particles, particle number and cargo content (*e.g.*, proteins, lipids, and nucleic acids) are among the most widely used normalization factors in the field and often combined in ratios to facilitate EV analysis (*e.g.*, total particle number: total protein amount, traditionally used as an indirect indicator of EV purity) (Hill et al., 2013; Pinheiro et al., 2024; Royo et al., 2020; Ten-Doménech et al., 2024; Théry et al., 2018; Zickler et al., 2025). Nonetheless, they face several limitations, which mainly consist in: i) sensitivity to co-isolated contaminants, such as aggregated proteins and lipoproteins, which can be detected as particles, thereby affecting EV-specific particle counts; ii) quantification bias arising from the specific settings, detection limits, and manual handling of the analytical platforms in use; iii) biased biological interpretations and study conclusions depending on the specific property selected for normalization (Buschmann et al., 2018; Hartjes et al., 2019; Li et al., 2026; Royo et al., 2020; Simonsen, 2017; Ten-Doménech et al., 2024; Théry et al., 2018; Welsh et al., 2024; Zarovni et al., 2025). On the other hand, as EV preparation methods have been shown to substantially affect EV particle recovery, molecular composition, and purity, further reference parameters reflecting the EV source or experimental setting adopted are often used and comprise: i) starting tissue mass or producing cell number, which is typical in studies involving EVs from cell cultures; ii) volume or endogenous molecule level of input sample, particularly when studying EVs derived from biofluids, including plasma, serum, and

urine; iii) spike-in controls, such as synthetic RNAs, mainly used to monitor technical variations upon nucleic acid extraction and analysis.

By accounting for the technical inconsistency arising from both bioprocessing conditions (i.e., pre-analytical variability) and analytical settings (i.e., analytical and post-analytical variability), these normalization approaches contribute to associating EV-related measurements with environmental parameters that directly affect EV detection and characterization. However, the variations highlighted by their use can lead to a misleading understanding of EV biology, as potentially correlating with inherent fluctuations of the parameter selected for normalization, rather than with changes related to EV abundance and compositional traits (Blijdorp et al., 2021; Royo et al., 2020; Srinivasan et al., 2019; Théry et al., 2018). For instance, the normalization of EVs based on input biofluid volume (e.g., per mL of urine or plasma) is unable to reveal whether the level of the measured EV property is dependent on the original EV abundance in the assessed biofluid or cell secretion rate; a high measurement could indicate either a regular cell secretion rate with a greater concentration of the biofluid (e.g., due to dehydration) or a more pronounced cell secretion rate with a regular biofluid hydration status. In this regard, normalization to endogenous molecules in biofluids, such as creatinine in urine, may be implemented to remove variations due to differences in biofluid dilution (Auber & Svenningsen, 2022; Blijdorp et al., 2021; Erdbrügger et al., 2021; Puhka et al., 2017; Vago et al., 2022; Welsh et al., 2024). From this perspective, the combination of context-dependent normalization strategies with approaches based on EV-associated metrics is generally recommended to mitigate individual limitations and improve EV data assessment.

1.5.2 Role of control samples in EV processing

Most methodologies used to isolate EVs from their original matrices are ineffective in achieving full separation of EVs from non-vesicular contaminants (e.g., protein aggregates, non-conditioned media components, lipoproteins, RNA-protein complexes, and other extracellular particles) (Simonsen, 2017; Sódar et al., 2016; Théry et al., 2018; Van Deun et al., 2014). Accordingly, discriminating EV-specific structural and functional properties from background noise and confounding artifacts remains challenging. To tackle this issue, the EV community has been strongly encouraged to examine EV preparations in parallel with complementary control samples, selected according to the specific EV harvesting, preparation, and analytical workflows employed. These encompass: i) unprocessed input sample (i.e., original biofluid or cell conditioned media); ii) EV-depleted fraction of processed input sample, collected after EV harvesting [e.g., UC supernatant for UC-isolated EVs, TFF permeate for TFF-isolated EVs, and SEC-derived protein fraction for SEC-isolated EVs]; iii) negative or background control(s), equivalent to the non-conditioned medium processed identically to the conditioned medium; iv) procedural control(s), comprising all reagents and materials introduced during EV isolation, engineering, and analysis; v) formulation buffer used for EV resuspension and storage (Théry et al., 2018; Welsh et al., 2024). Nevertheless, the lack of consensus on methodological standardization continues to result in insufficient adherence to these guidelines or poor methodological reporting, thereby hindering progress toward an accurate and robust understanding of EV characteristics (Welsh et al., 2024).

1.6 Evaluating EV bioactivity beyond end-point measurements

1.6.1 Conventional approaches to EV bioactivity assessment

Any biological function of EVs is traditionally measured as a response of a certain biological system, either *in vitro* or *in vivo*, to EV preparations. In a clinically relevant setting, this may be linked to the concept of efficacy, defined as the maximum effect that is therapeutically beneficial at a specific dose, and potency, indicating the capacity to provide the intended effect, which is ascertained by appropriate laboratory assays (Nguyen et al., 2020). Conventional *in vitro* approaches to assess EV bioactivity rely on quantitative measurements of distinct aspects of cellular behaviour, using either cell-based or cell-free (e.g., enzymatic activity assays) analytical platforms (Adamo et al., 2025; Gimona et al., 2021; Nguyen et al., 2020; Théry et al., 2018). These can be broadly grouped as follows: i) cell fate regulation (e.g., proliferation assays, cytotoxicity assays, apoptosis assays, etc.); ii) cell metabolic and bioenergetic activity [e.g., MTT assay, MTS assay, cell counting kit-8 (CCK-8) assay, etc.]; iii) cell migration, invasion, and motility [e.g., scratch (wound healing) assay, Transwell® migration assay, Matrigel®-based assays, etc.]; iv) cell differentiation and phenotypic reprogramming [e.g., morphological differentiation assays, tube formation assays (angiogenesis), etc.]; v) cell immunomodulatory function (e.g., cytokine secretion by enzyme-linked immunosorbent assay (ELISA), analysis of activation markers by flow cytometry, macrophage polarization assays, T cell proliferation/suppression assays, etc.); vi) cell signalling pathway activation or mechanistic assays [e.g., reporter gene and transcriptional assays, (de)phosphorylation assays, etc.] (Nguyen et al., 2020; Pegtel et al., 2010; B. Zhang et al., 2015). Of these, the most commonly adopted methods are designed to provide functional readouts that are recorded at pre-defined, fixed time points. Despite being selected for either their technical simplicity, economical accessibility, higher throughput, or multiplexing capabilities, end-point measurements deliver only static snapshots of biological activity, thereby disregarding the dynamic, mechanistic nature of cellular processes, whose understanding is currently highly demanded in the EV field. If not complemented with orthogonal methods yielding kinetic insights into the assessed biological function, these approaches may result in functional interpretations that are oversimplified and misleading (Single et al., 2015; Tkach & Théry, 2016) (Figure 7).

1.6.2 Dynamic and kinetic evaluation of EV bioactivity

Cellular processes involve complex cascades of molecular events, triggered by individual or combined stimuli, that evolve in a spatiotemporal manner, rather than undergoing binary activation–deactivation transitions (Lehninger et al., 2013). This implies that signalling mediators, including EVs, may induce functionally equivalent outcomes, yet arising from mechanisms that are fundamentally different in terms of: i) onset time (*i.e.*, the time at which the response is induced by the stimulus); ii) rate of change (*i.e.*, the speed at which the response is activated or inactivated); iii) amplitude (*i.e.*, the magnitude or strength of the response, either in its transient state or steady state); iv) duration (*i.e.*, the length of the response); v) frequency (*i.e.*, the number of signal peaks per unit of time) (Behar & Hoffmann, 2010).

At present, deeper mechanistic insights into cellular functions can be obtained through dynamic functional assays evaluating their kinetics and temporal progression through continuous or longitudinal monitoring over time (Bonsignore et al., 2025). To date, a wide range of these methods have been applied to both mammalian and non-mammalian EVs

to primarily study the following processes: i) EV uptake mechanisms and intracellular trafficking [e.g., live-cell imaging, single-particle tracking of labelled EVs, continuous Förster resonance energy transfer (FRET) assays, imaging flow cytometry, etc.]; ii) reporter-based EV-mediated cargo delivery (e.g., Cre-loxP recombination-based, luciferase-based, organelle-targeted dual reporter-based, etc.); iii) EV-induced cellular response dynamics (e.g., live-cell imaging, in vivo live imaging, impedance-based real-time cell analysis and electric cell-substrate impedance sensing, computational/ systems-level continuous modelling, etc.); iv) EV-induced metabolic flux changes [e.g., real-time Seahorse extracellular flux (XF) analysis]; v) EV-induced signalling dynamics [e.g., live Ca^{2+} imaging, extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) pathway via phosphorylation time-course WB] (Barry et al., 2024; Bonsergent et al., 2021; Carter et al., 2022; Dave et al., 2023; Jurgielewicz et al., 2020; Liang et al., 2025; Music et al., 2026; Nguyen et al., 2020; O'Brien et al., 2022; O'Donoghue et al., 2017; Picciotto et al., 2022; Reclusa et al., 2020; Shedenkova et al., 2025; Somiya & Kuroda, 2021; Tian et al., 2010; Vanaja et al., 2016; Zhang et al., 2025; Zomer et al., 2016). The majority of these methods, which are typically pathway-specific, can suffer from sensitivity issues, require specific sample configurations or manipulation prior to assessment, and provide functional readouts over relatively short timescales. In this context, their integration with complementary approaches capable of overcoming these limitations, such as isothermal microcalorimetry and dynamic mass redistribution (DMR)/resonant waveguide biosensing, could provide an opportunity to uncover previously unexplored aspects of EV bioactivity (Fang et al., 2006; Wadsö, 2002) (Figure 7).

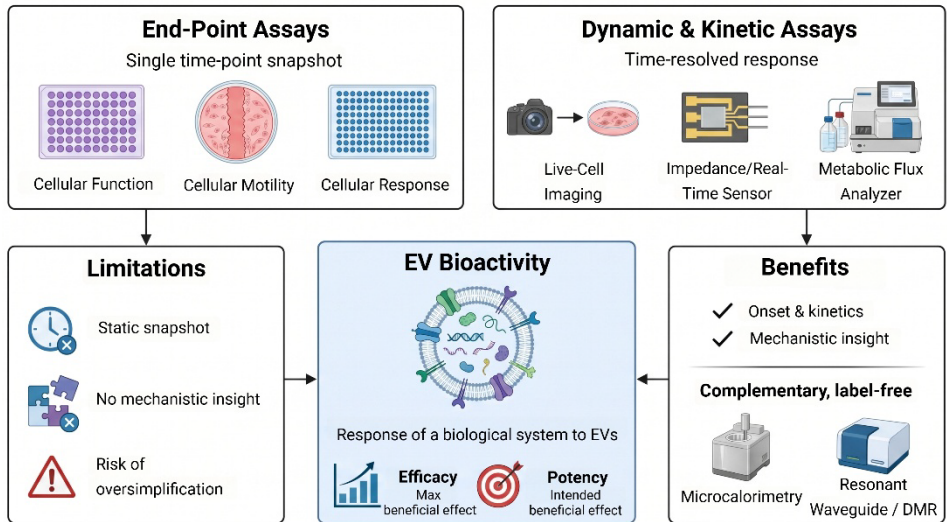


Figure 7. Overview of EV end-point versus dynamic bioactivity assays. Abbreviations: DMR = dynamic mass redistribution; EV = extracellular vesicle. The figure was created in BioRender. Loria, F. (2026) <https://BioRender.com/z85fj3y>.

1.7 The challenge of decision-making in EV research and product development

1.7.1 The standardization burden

While the EV field is exhibiting substantial potential and a constantly evolving range of applications, related biological and technical challenges continue to interfere with data reliability and reproducibility, thereby hindering proper translation of EV-based products into effective and marketable solutions (Loria et al., 2024; Zarovni et al., 2021). For more than a decade, in response to concerns about data and methodological harmonization, the EV community has turned increasing attention to experimental rigor and standardization efforts, encouraging researchers and developers to follow EV-specific guidelines (e.g., MISEV2018, MISEV2023) and adhere to various EV data reporting frameworks (e.g., EV-TRACK) (Couch et al., 2021; Nieuwland et al., 2020; Théry et al., 2018; Welsh et al., 2024; Zarovni et al., 2021). Despite these initiatives and the latest technological advancements in EV preparation and analytical approaches, the EV landscape continues to face either incomplete or biased understanding of EVs and their underlying properties, largely due to the persistent lack of universally accepted standards (Loria et al., 2024).

1.7.2 Structured decision-making

The EV landscape is a highly dynamic arena with a continuous interplay between rapid innovation and significant market risks, exposing researchers to both opportunities and uncertainties arising from lack of understanding, incomplete knowledge, and limited experimental control (Loria et al., 2024; Zarovni et al., 2021). In this challenging environment, the systematic integration of key responsible research and innovation (RR&I) principles into routine EV-related R&D practices may provide a structured approach for navigating the multidimensional decision-making challenges dominating the field, thereby fostering the translation of EV innovations into effective, sustainable, and commercially viable solutions (Loria et al., 2024).

RR&I is intended to reframe the Research and Innovation (R&I) process in response to challenging and unpredictable constraints, with a view to minimizing undesired impacts and reducing costs (Owen et al., 2013). To achieve this, it requires the harmonization of R&D operations with supporting activities related to quality, data, and risk management along the entire bioprocessing path (G. Liguori & Kisslinger, 2020; Owen et al., 2013). This functional convergence approach is particularly relevant in environments addressing complex and insufficiently characterized study objectives, such as EVs, which are often dominated by subjective and non-traceable decisions leading to inconsistent evaluations and suboptimal outcomes (Owen et al., 2013). In this regard, structured decision-making tools (DMTs) offer an opportunity to prioritize alternative options through systematic comparison of alternative solutions. Largely designed to be standardized, customizable, and cost-effective, these frameworks can enhance reproducibility, reliability, and traceability of decision-making processes, while also improving standardization and facilitating alignment with Good Research Practice (GRP) and Good Manufacturing Practice (GMP) standards (Loria et al., 2024).

The emergence of mathematical DMTs can be traced back to the 17th century, with the formulation of the probability theory, aiming at promoting fair compensations for uncompleted gambling games. Later, built upon this concept, the expected utility theory provided a mathematical framework accounting for individual preferences when

evaluating uncertain outcomes, which laid the foundation of modern quantitative decision-making approaches (Hacking, 1975). Since the beginning of the Industrial Revolution, under the influence of concurrent advances in mathematics, statistics, and computing, mathematical and computational DMTs have undergone a significant evolution, prompted by the greater complexity of industrial, military, and organizational operations. Across multiple industrial and societal sectors (*e.g.*, aerial and automotive vehicles, biotherapeutics, healthcare, digital and information systems), as well as diverse application domains (*e.g.*, industrial manufacturing and operations management, business management, policy-making and public administration, R&D), DMTs have been primarily employed to support strategic planning, while mitigating risks arising from technical, logistical, and economic constraints (Allen, 2009; Amrozi et al., 2020; Beniger, 1986; Chhatre et al., 2006; Rao, 2007; Rau, 2005; Rekhi et al., 2015; Shim et al., 2002; Sutton et al., 2020; T.-E. Tan et al., 2014; Tashkinov, 2025).

The first computational DMT dedicated to research-grade and clinical-grade EV product development was developed by Ng et al. in 2019. This system was specifically intended for assisting in the identification of best technological combinations for upstream (*i.e.*, human cell expansion) and downstream (*i.e.*, EV harvesting) EV manufacturing, thereby enabling upscaling and minimizing overall cost of goods (Ng et al., 2019). Furthermore, in 2021, Picciotto et al. pioneered the application of a mathematical DMT based on the weighted sum model (WSM), a widely used multi-criteria decision-making (MCDM) method, for selecting the microalgal sources best suited for microalgae-derived EV production (Picciotto et al., 2021).

1.7.3 Multi-criteria decision-making

MCDM frameworks enable objective and quantifiable rankings among complex sets of alternative options. By structuring the evaluation process around multiple, well-defined assessment criteria, and accounting for their relative importance (or importance weight) on the decision-making process, these strategies offer the opportunity to reduce the likelihood of either biased or erroneous decisions, while promoting cost and time savings. Therefore, they are instrumental in enhancing project performance, simplifying administrative processes, and helping prevent or resolve conflicts among stakeholders. Overall, by allowing decision-making activities to take precedence over decision-control activities, MCDM facilitates the effective realization of intended outcomes (Loria et al., 2024; Rao, 2007; Taherdoost & Madanchian, 2023; Zarghami & Szidarovszky, 2011).

MCDM platforms are numerous and share a common set of fundamental procedural steps, which are: i) definition of the specific alternatives to compare and criteria to follow for their evaluation; ii) determination of the numerical importance weight to assign to each assessment criterion; iii) data processing to grade alternatives. Despite the comparable working structure, they differ in their mathematical foundations, which can result in different rankings of the same alternative options. Their classification system is traditionally based on either i) the type of data they process or ii) the number of decision makers involved in the evaluation process. According to the first classification system, methods may be distinguished as deterministic, stochastic, or fuzzy. Deterministic methods [*e.g.*, WSM, weighted product model (WPM), analytic hierarchy process (AHP), elimination and choice expressing the reality (ELECTRE), technique for order of preference by similarity to ideal solution (TOPSIS), *etc.*] process input data that are assumed to be known with certainty and must be maintained fixed throughout the evaluation process. Conversely, stochastic methods [*e.g.*, stochastic multi-attribute

decision-making (MADM), sensitivity analysis-based approaches, stochastic dominance, Monte Carlo simulation-based MCDM] focus on guiding assessments in which values are uncertain, integrating their relative stochastic information into the evaluation process. On the other hand, fuzzy methods (e.g., Fuzzy MADM, Fuzzy AHP, Fuzzy ELECTRE, Fuzzy TOPSIS, etc.) are mostly designed for managing information that is inaccurate or misleading. The second classification system discriminates between single decision-making (SDM) approaches, in which a single evaluator runs the entire ranking process, and group decision-making (GDM) approaches, especially relevant in complex environments where consensus needs to be achieved, which incorporate the opinions and priorities of multiple individuals into a collective decision (Ceballos et al., 2017; Celik et al., 2019; Kahraman et al., 2015; Triantaphyllou et al., 1998).

1.7.3.1 Weighted Sum Model

When addressing single-dimensional problems, in which all decision criteria values are in the same units (e.g., dollars, feet, hours) or can be converted to a common scale, the WSM constitutes one of the oldest and most widely adopted MCDM methods due to its simplicity, ease of use, and effectiveness. The general mathematical formula that describes the construction of a WSM-based MCDM tool is the following:

$$A_{WSM}^* = \max_i \sum_{j=1}^N q_{ij}w_j \quad \text{for } i = 1, 2, 3, \dots, M$$

in which: A_{WSM}^* is the final WSM score of the (best) alternative; M is the number of alternatives to assess; N is the number of decision criteria; i is a single alternative; j is a single criterion; q_{ij} is the actual value corresponding to the i -th alternative in terms of the j -th criterion; W_j is the importance weight of the j -th criterion. This approach relies on the additive utility assumption, according to which the overall value of an alternative can be represented by a single numerical value calculated by adding weighted scores relative to all decision criteria. Specifically, for each alternative under evaluation, each decision criterion is given a performance score (i.e., q_{ij}), based on conformity to pre-defined acceptance criteria, which is multiplied by its corresponding importance weight (i.e., W_j), reflecting its influence on the decision-making process, to obtain a weighted performance score. The sum of the weighted performance scores across all decision criteria provides a final, cumulative score or grade (i.e., A_{WSM}^*) (Triantaphyllou et al., 1998).

2 Aim and objectives

The aim of the present thesis was to investigate how methodological choices shape EV data generation, comparison, interpretation, and translational use, while contributing to methodological advances that enhance the robustness of EV analysis, engineering, and applications, as well as foster responsible decision-making across EV research and translational settings. To achieve this aim, the thesis sought to examine innovative analytical, functional, and decision-support approaches addressing key methodological, biological, and translational challenges that currently limit the reliability, interpretability, and meaningful use of EVs. The specific objectives that were pursued are the following:

- examining how analytical resolution, sensitivity, and labelling strategies affect EV detection and subpopulation profiling (**Publication I**);
- exploring whether EV cargo loading can be enhanced without compromising EV structural integrity, cellular uptake, and biological activity (**Publication II**);
- evaluating how different normalization strategies influence the comparative assessment of EVs from distinct biological origins, while supporting the identification of more robust quality metrics for reliable EV comparison and meaningful application (**Publication III**);
- exploring dynamic approaches for EV functional assessment to enhance mechanistic interpretability of EV-mediated biological effects (**Publication IV**);
- assessing the applicability of a WSM-based MCDM tool for supporting responsible and sustainable decision-making in EV research and product development (**Publication V**).

3 Materials and Methods

This section outlines the methodologies adopted throughout this thesis, with detailed documentation provided in their respective appended publications. As Publication III is not included as an appendix to this thesis to protect the confidentiality of unpublished work, only the methods related to Publication III are described in this section.

3.1 Enrichment and separation of EVs and nanovesicles

3.1.1 Publications I, II, and IV

- EV enrichment from conditioned medium of:
 - the human colon cancer cell line HT29 (**Publication I**)
 - the human embryonic kidney 293 (HEK293) cell line (**Publications I and II**)
- Nanovesicle enrichment from ginger rhizome (**Publication IV**)

3.1.2 Publication III

- EV enrichment from conditioned media of human endometrial decidual tissue-derived mesenchymal stem/stromal cells (MSCs)

Human endometrial decidual tissue-derived MSCs were isolated from uterine decidual tissue and used as EV-producing cell line, as previously reported (Bortolotti et al., 2018; Rossignoli et al., 2013). Decidual endometrial blood was collected from healthy female volunteer donors during the first few days of the menstrual cycle. Written informed consent was obtained from each donor and material donation was approved for research purposes by the Local Ethics Committee (Emilia Nord Wide Area, Prot. AOU 0036565/20 issued on December 16, 2020). At passage 2 (P2) detachment, cells were characterized by fluorescence-activated cell sorting (FACS) for the expression of MSC identification markers (*i.e.*, CD90, CD105, CD73). Furthermore, the purity of the isolated and expanded population was assessed by evaluating negativity for human leukocyte antigen – DR (HLA-DR), CD45, and CD14 markers. Viability was also assessed at P2 using FACS and 7-amino-actinomycin D (7-AAD) staining. At each passage, cells were checked under the microscope for morphological evaluation. Furthermore, at each detachment, viability was assessed using Trypan blue staining and a Neubauer chamber. Cells, at three consecutive cell passages (P3-P5), were seeded in T75 flasks at 6,000-7,000 cells/cm² in MEM-Alpha medium (GibcoTM; #10712124) supplemented with 10% Exosome-depleted Fetal Bovine Serum (GibcoTM; #15434573), 1% L-glutamine (GibcoTM; #25030081), and 0.5% Ciprofloxacin (Fresenius Kabi). After 48-72 h of cell conditioning, cell conditioned media were harvested and stored at -80°C. Frozen media (1.5 L for each EV batch) were thawed at room temperature (RT), followed by centrifugation at 800 g for 30 min at 4°C to remove cell debris. The supernatant was transferred to a clean tube and further centrifuged at 10,000 g for 45 min at 4°C to pellet larger EVs. The newly generated supernatant was transferred to cold-sterilized (tubes: 10% H₂O₂ for 30 min; aluminum caps and O-rings: 70% ethanol for 30 min) UC 70 mL polycarbonate bottles (Beckman Coulter Inc.; #355655) and ultracentrifuged at 100,000 g for 4 h at 4°C, using an Optima XPN-80 floor-standing ultracentrifuge equipped with a Ty-45 rotor (Beckman Coulter Inc.). One mL of supernatant was collected and frozen at -80°C, before being freeze-dried overnight without excipients to be used as MSC-EV-

depleted control (MSC-CTL). The pellet was washed in 1 mL of sterile-filtered phosphate-buffered saline (PBS) (Corning; #21-040-CV), transferred to clean tubes (Beckman Coulter Inc., #355748), and again ultracentrifuged at 100,000 g for 90 min at 4°C, using an Optima MAX-XP benchtop ultracentrifuge equipped with a TLA-55 rotor. Lastly, the EV pellet was resuspended in sterile-filtered PBS supplemented with 5% (w/v) sucrose (Sigma-Aldrich; #S0389), frozen at -80°C, and freeze-dried overnight. All freeze-dried samples were sealed with Parafilm® and stored at 4°C in a sealed box filled with desiccant silica gel. Before analysis, MSC-EV samples were reconstituted with 300 µL of Milli-Q water, whereas the CTLs were reconstituted with 1 mL of Milli-Q water.

- EV enrichment from bovine milk

Bovine milk EVs (BM-EVs) were isolated as previously described (Ridolfi et al., 2020; Zonneveld et al., 2021). Briefly, raw BM (Tolakker dairy farm, Utrecht, The Netherlands) was centrifuged at 3,000 g for 10 min at RT, using a Beckman Coulter Allegra X-12R centrifuge (Beckman Coulter Inc.). After removal of the cream layer, the BM supernatant was harvested without disturbing the pellet and transferred to new tubes. After another spin at 3,000 g for 10 min at RT, the supernatant was stored at -80°C. Thawed BM supernatant was centrifuged at 5,000 g and 10,000 g for 30 min at 4°C in a Beckman Coulter Optima L-90K ultracentrifuge (Beckman Coulter Inc.) equipped with a SW40Ti rotor. Sodium citrate [20% (w/v) trisodium citrate dihydrate; Merck Millipore; #106448] was added to a final concentration of 2% to dissociate casein micelles. Next, BM supernatant was loaded on top of a 60% – 10% Optiprep gradient (Optiprep™; Progen Biotechnik GmbH; #1893) and ultracentrifuged at 197,000 g at 4°C, using the Beckman Coulter Optima L-90K ultracentrifuge (Beckman Coulter Inc.) with a SW40Ti rotor. To obtain the BM-CTL, the EV-depleted BM supernatant on top of the gradient was collected and loaded onto a new Optiprep gradient, following further UC and processing identically to BM-EVs. Optiprep gradient fractions with densities 1.06-1.19 g/mL were harvested, pooled, and applied to a Sephadex® G-100 (Sigma-Aldrich; #G100120) SEC columns to exchange Optiprep for PBS. One mL fractions were eluted and the EV-containing eluates 3 to 9 were pooled, followed by storage at -80°C until use.

- EV enrichment from the parasitic helminth *Ascaris suum*'s secretome

Adult *Ascaris suum* (AS) worms were acquired at a local abattoir from naturally infected pigs (DAT-Schaub A/S, Herning, Denmark). The worms were collected in 39°C saline [0.9% (w/w) sodium chloride (NaCl) (Sigma-Aldrich; #S8776)], which was exchanged during the harvesting process. Saline was further exchanged before transport to the laboratory in a Styrofoam box. Worms were distributed into T175 culture flasks in a ratio of three females and two males, and incubated for a total of 7 times over 4 h in an antibiotic-antimycotic mixture at 39°C. Worms were then incubated in 175 mL RPMI-1640 medium with the antibiotic-antimycotic mixture at 37°C and 5% CO₂ under sterile conditions, for a total of 3 days with media exchange every 24 h, as previously described (Borup et al., 2022). The collected condition medium is referred to as excretory/secretory product (ESP). To remove eggs, cellular debris, and potential aggregates, ESP was differentially centrifuged at 500 g for 5 min, 2,000 g for 15 min, and the cleared ESP was stored at -80°C for AS-EV enrichment until needed. Approximately 300 mL of cleared ESP, thawed overnight at 5°C, was concentrated to 500 µL, using protein concentrators with a molecular weight cut-off

(MWCO) value of 100,000 Daltons (Thermo Scientific; #88503). The concentrate was enriched for AS-EVs by SEC using qEVOriginal/70 nm columns (iZON Science Ltd), following the manufacturer's instructions. Fractions (0.5 mL each) 7-10 were pooled as the AS-EV enriched sample and fractions 16-24 were pooled to be used as AS-CTL. PBS (Biowest; L0616) from the batch used for SEC was used as a negative control. Samples were kept at -80°C until further analyses.

3.2 EV labelling

- Fluorescent membrane labelling (**Publications I, II, IV, and V**)
- Fluorescent cargo labelling (**Publication II**)

3.3 EV engineering

- EV loading with sodium carbonate treatment (**Publication II**)
- EV loading with electroporation (**Publication II**)

3.4 EV physicochemical characterization

3.4.1 Publications I, II, IV, and V

- Nanoparticle tracking analysis (**Publication I, II, IV, and V**)
- Fluorescence nanoparticle tracking analysis (**Publications I, IV, and V**)
- Nanoscale flow cytometry (**Publication I**)
- Zeta potential measurement (**Publication V**)
- Transmission electron microscopy (**Publications II and IV**)
- Atomic force microscopy (**Publications IV and V**)
- Single-particle interferometric reflectance imaging sensor analysis (**Publication II**)
- Acquisition, processing, and analysis of Raman spectra (**Publication II**)

3.4.2 Publication III

- Nanoparticle tracking analysis

Nanoparticle concentrations and size distributions were assessed NTA using Particle Metrix ZetaView® PMX-120 [software version 8.05.12 SP1]. For each sample, two to three measurements were conducted in scattering mode, as previously described (Fortunato et al., 2021). For each sample measurement, total particle surface area and volume per milliliter of sample were extrapolated from the raw data provided by the software of the instrument and calculated satisfying the following mathematical expressions. $A_{particles} = \sum_{i=1}^n C_i 4\pi r_i^2$ is the surface area of the total number of particles detected per milliliter of original sample (cm²/mL); i is a single particle with a specific diameter; n is the number of particles with a specific diameter; C is the original concentration of particles per milliliter of (undiluted) sample; r is the particle radius. $V_{particles} = \sum_{i=1}^n C_i \frac{4}{3}\pi r_i^3$ is the volume of the total number of particles detected per milliliter of original sample (cm³/mL); i is a single particle with a specific diameter; n is the number of particles with a specific diameter; C is the original concentration of particles per milliliter of (undiluted) sample; r is the particle radius.

3.5 EV biochemical characterization

3.5.1 Publications I, II, IV, and V

- Bicinchoninic acid assay (**Publications II, IV, and V**)
- Protein extraction, Western blot, and silver staining analysis (**Publication II**)
- Phospholipid assay (**Publication IV**)
- Cholesterol assay (**Publication II**)
- Total carbohydrate assay (**Publication IV**)
- Nucleic acid qualitative and semi-quantitative assessment (**Publication II**)
- Enzyme-linked immunosorbent assay (**Publications II and V**)
- Direct enzyme-linked immunosorbent assay-like lectin assay (**Publication II**)
- Esterase activity assay (**Publication V**)
- Colorimetric nanoplasmonic assay (**Publication V**)

3.5.2 Publication III

- Bicinchoninic acid assay

Protein concentrations were measured by BCA assay using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific; #23228), according to the manufacturer's instructions. Standards and samples were run in duplicate into transparent Nunc™ 96-Well polystyrene round bottom microwell plates (Thermo Scientific; #268152). Absorbance (A) was measured at 540 nm, using Tecan GENios Pro microplate reader.

- Phospholipid assay

Phospholipids were quantified using the fluorescent Phospholipid Quantification Assay Kit (HansaBioMed Life Sciences), according to the manufacturer's instructions. Standards, samples, blanks, and background controls were loaded into black polystyrene – no binding capacity – microplate wells (HansaBioMed Life Sciences) and assessed in duplicate. Fluorescence intensities (FIs) were read using Tecan GENios Pro microplate reader ($\lambda_{\text{ex}} = 540 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

- Cholesterol assay

Cholesterol quantification was performed using the Cholesterol Quantification Assay Kit (HansaBioMed Life Sciences), following the user's manual. Standards, samples, blanks, and background controls were loaded in duplicate into black polystyrene – no binding capacity – microplate wells (HansaBioMed Life Sciences). FIs were read using Tecan GENios Pro microplate reader ($\lambda_{\text{ex}} = 540 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

- Total carbohydrate assay

Total carbohydrate concentrations were measured by the Total Carbohydrate Colorimetric Assay Kit (Sigma-Aldrich; #MAK104-1KT), following the manufacturer's protocol. Standards, samples, and blanks were loaded into transparent polystyrene – no binding capacity – microplate wells (HansaBioMed Life Sciences) and assessed in duplicate. A was read at 492 nm, using Tecan GENios Pro microplate reader.

- Enolase activity assay

Enolase activity assessment was performed by the fluorometric EV-Enolase Kit (HansaBioMed Life Sciences; #HBM-K691-EN), following the manufacturer's

instructions. Standards, samples, blanks, and background controls were loaded in duplicate into black polystyrene – no binding capacity – microplates (HansaBioMed Life Sciences) and FIs were read in kinetic mode using Tecan GENios Pro microplate reader ($\lambda_{\text{ex}} = 540 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

- Peroxidase activity assay

Peroxidase activity was measured by the Peroxidase Assay Kit (Sigma-Aldrich; #MAK092), according to the manufacturer's guidelines. Standards, samples, and blanks were evaluated in duplicate in fluorescence mode, using black polystyrene – no binding capacity – microplates (HansaBioMed Life Sciences). FIs were measured using Tecan GENios Pro microplate reader ($\lambda_{\text{ex}} = 540 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

- Xanthine oxidase activity assay

Xanthine oxidase activity was assessed using the Xanthine Oxidase Activity Assay Kit (HansaBioMed Life Sciences), following the user's manual. Standards, samples, and blanks were evaluated in duplicate in fluorescence mode, using black polystyrene – no binding capacity – microplates (HansaBioMed Life Sciences). FIs were measured using Tecan GENios Pro microplate reader ($\lambda_{\text{ex}} = 540 \text{ nm}$; $\lambda_{\text{em}} = 590 \text{ nm}$).

- Leucine aminopeptidase activity assay

Leucine aminopeptidase activity was assessed by the Leucine Aminopeptidase Assay Kit (AssayGenie; #MAES0190), according to the manufacturer's instructions. Standards, samples, and blanks were run in duplicate and loaded into transparent polystyrene – no binding capacity – microplate wells (HansaBioMed Life Sciences). A was measured in kinetic mode at 405 nm, using Tecan GENios Pro microplate reader.

3.6 EV functional assessment

- Cell viability assay (**Publication II**)
- Flow cytometry-based uptake assay (**Publication II**)
- Confocal microscopy-based uptake assay (**Publication II**)
- Isothermal microcalorimetry (**Publication IV**)

3.7 Extracellular metabolite profiling

- High-performance liquid chromatography (**Publication IV**)
- Ultra-performance liquid chromatography (**Publication IV**)

3.8 Design and implementation of the EV decision-making grid (EV-DMG) (Publication V)

3.9 EV data analysis

3.9.1 Publications I, II, and IV

- Statistical analysis

3.9.2 Publication III

- Partial least squares discriminant analysis

Ordination was calculated and visualized in R 4.3.0 (The R Foundation for Statistical Computing, Vienna, Austria). PLS-DA was done using R package 'mixOmics' 6.22.0. The variables were centered and scaled.

- Statistical analysis

Statistical analysis was conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Depending on the evaluated dataset, statistical analysis was performed using either an ordinary unpaired t-test or ordinary one-way ANOVA with follow-up test for multiple comparisons, as described in each figure legend. Statistical significance was claimed when the p-values were < 0.05 [*], < 0.01 [**], < 0.001 [***], and < 0.0001 [****]. For each sample, data were expressed as mean $\pm$ standard error of the mean (SEM) from three independent biological batches.

4 Results and Discussion

Taking inspiration from an Aristotle's statement "the totality is not, as it were, a mere heap, but the whole is something besides the parts" (Martin, 2019; W D Ross, 1937), EVs may be portrayed as "known unknowns", constituting "parts" of specific sources carrying a "whole" that partially reflects the status of their cells of origin (Margolis & Sadovsky, 2019; Martin, 2019; W D Ross, 1937; Zarovni et al., 2021). Their multifaceted nature enables them to serve as multiparametric structures for exploring biological processes in a comprehensive manner, underpinning both their significant translational potential and the current Achilles' heel of the EV landscape: the lack of standardized methodologies capable of capturing EV complexity and heterogeneity, while improving the performance and reliability of EV study workflows (Zarovni et al., 2021). In this regard, working with EVs recalls the "blind men and the elephant" parable (DeCourcy Ireland, 1997), where single-domain or reductionistic study approaches enable the acquisition of only fragmented, biased aspects of highly complex entities, thereby increasing the demand for integrative, multi-dimensional frameworks to support research and decision-making. On this basis, to move forward, the EV field requires existing R&D approaches to be reassessed and refined (Loria et al., 2024).

The research presented in this thesis was conceptualized to gain a deeper understanding of EVs by looking at their heterogeneity as an "elephant" through the lens of "sighted" researchers, ultimately learning how to handle EV uncertainty in a responsible manner. In this work, a specific focus was placed on tackling key EV research gaps across three interconnected and interdependent domains: i) methodological (*i.e.*, how to generate reliable, reproducible, and comparable data); ii) biological (*i.e.*, how to interpret EV data); iii) translational (*i.e.*, how to support decision-making for effective and meaningful use). **Publications I** and **III** primarily contributed to addressing methodological and biological gaps, demonstrating that EV biological interpretation is affected by the analytical ability to resolve heterogeneity and the selection of normalization approaches to enable comparisons across studies and EV samples of different origin. **Publication II** provided an additional methodological contribution by presenting a novel EV exogenous engineering strategy balancing cargo loading efficiency with preservation of EV integrity and functionality. **Publication IV** further advanced the biological domain by showcasing isothermal microcalorimetry (IMC) as an innovative approach for conducting real-time, high-throughput mechanistic investigation of EVs *in vitro*. This study enabled the identification of new EV functional traits that were overlooked by conventional end-point bioactivity assays with limited temporal resolution, reinforcing the importance of extending functional interpretation using more dynamic mechanistic approaches. Building upon prior knowledge and the evidence generated in Publications I-IV, **Publication V** provided a valuable contribution to tackling translational gaps arising from fragmented, biased, and context-insensitive decision-making processes across research and translational settings. This was specifically achieved by introducing a systematic WSM-based MCDM framework that can promote responsible decisions regarding the management of EV sources and samples by supporting users in selecting the options under evaluation that best fit their specific purposes and context.

The following sections describe and discuss the most relevant findings of Publications I-V, which enabled the development of the narrative of the present thesis. Further details are provided in the appended papers.

4.1 EV quantification and identification are affected by the analytical context (Publication I)

Owing to their nanoscale dimensions, intrinsic heterogeneity, and physicochemical overlap with non-vesicular structures, true quantification and identification of EVs remain fundamental challenges in EV R&I, with direct implications for the downstream interpretation of EV functional outcomes and ultimate exploitation. To tackle this challenge, within the context of this thesis, we considered it essential to first explore how analytical choices shape EV detection and profiling. This was specifically addressed in **Publication I**, in which two well-established single-particle analytical technologies, NTA and nFCM, were systematically examined for their analytical capabilities, in combination with different EV fluorescent labelling approaches. Compared with other single-particle methodologies that are commercially available, NTA and nFCM offer greater flexibility and higher throughput, making them among the most widely adopted technologies for routine EV quantification, sizing, and phenotyping. Both platforms enable the detection and multiparametric analysis of individual particles in suspension, operating either in scatter mode or in fluorescence mode (Mladenović et al., 2025). Nevertheless, they are characterized by fundamental differences in instrumental configurations and detection principles, which makes them ideal analytical model systems for investigating how the analytical context shapes EV characterization outcomes (Fortunato et al., 2021; Mladenović et al., 2025). The analytical performance of NTA and nFCM was compared based on the following criteria: i) EV labelling efficiency and specificity; ii) resolution of EV subpopulations; iii) ability to assess how EV subpopulation profiling is affected by different excess-of-dye removal strategies [*i.e.*, SEC and UF]; iv) ability to detect EVs from non-purified cell conditioned media (CCM). To remain aligned with the scope of this thesis, the results related to EV labelling efficiency and resolution of EV subpopulations will be primarily described and discussed.

EV labelling efficiency was assessed on purified EVs derived from the human colon cancer cell line HT29 stained with the following dyes: i) CellMask™ Green (CMG) and CellMask™ Red (CMR) Plasma Membrane Stains, which are amphipathic molecules emitting, respectively, green and red fluorescence when inserted into the lipid membrane; ii) green CellTrace™ carboxyfluorescein succinimidyl ester (CFSE) and red CellTrace™ Red (CTR) proliferation dyes, which are non-fluorescent and membrane-permeable molecules preferentially binding to amino group-rich luminal molecules, such as proteins, after being converted into a fluorescent, amine-reactive form upon esterase cleavage of their two acetate groups. Owing to their longer excitation wavelengths, red fluorescent signals were not captured on our selected NTA instrument, as it was exclusively equipped with a single 488 nm laser, in contrast to the nFCM platform, which includes a compatible laser of 638 nm wavelength.

By nFCM, EVs stained with all dyes showed optimal labelling efficiency of approximately 90% (Figure 8A-B). A comparable EV staining performance, close to 88%, was also observed by (F-)NTA using CFSE, although 5-fold higher dye concentration was required for labelling, compared with that used for nFCM measurements (Figure 8A). An analytical discrepancy between the two platforms was highlighted upon (F-)NTA analysis of CMG-stained EVs, displaying a labelling efficiency around 32% (Figure 8A). This result was shown to depend on the nature of the CMG dye itself, as well as the fundamental differences characterizing the two analytical systems in terms of structural and operational settings. Unlike CFSE, whose binding directly correlates with the EV

intraluminal levels of esterase activity and amine groups, CMG, which is incorporated into EV membranes, stains EVs depending on their membrane surface area.

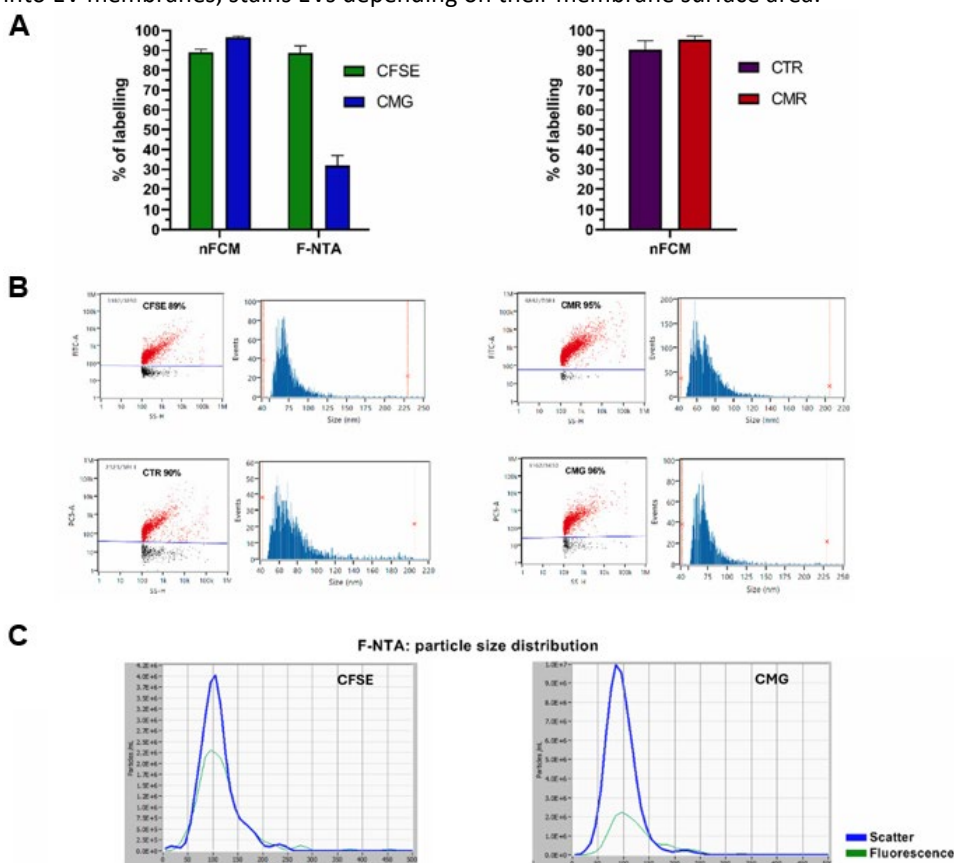


Figure 8. Scatter and fluorescence measurements of fluorescently labelled EVs by (F-)NTA and nFCM. The (A) bar graphs, (B) representative dot plots, and (B-C) representative size distribution histograms show (A-C) (F-)NTA and (B) nFCM-estimated HT29 EV labelling efficiency – expressed as ratio of fluorescent particle number to scattering particle number (%) – after staining with either CMG, CMR, CFSE, or CTR. In A, data are reported as mean $\pm$ SEM from three independent experiments, except for nFCM data of CMG- and CMR-labelled EVs, as well as (F-)NTA data of CFSE-labelled EVs, which were repeated twice. The figure was adapted from Figure 1 in **Publication I**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Modifications were made. Abbreviations: CFSE = CellTrace™ carboxyfluorescein succinimidyl ester; CMG = CellMask™ Green; CMR = CellMask™ Red; CTR = CellTrace™ Red; (F-)NTA = (fluorescence) nanoparticle tracking analysis; nFCM = nanoscale flow cytometry.

As a result, the smaller the EVs, the fewer CMG dye molecules are incorporated into their membranes, and the weaker the fluorescent signals emitted. This implies that, to be reliably detected, small CMG-labelled EVs would require a method equipped with a highly sensitive detection system, which can reduce false-negative events, while lowering background interference. For signal acquisition, the (F-)NTA system relies on a CMOS camera sensor, which is less sensitive than the APDs of the nFCM instrument, thereby limiting the detection of smaller particles. Consistent with this, CMG-stained particles detected by (F-)NTA showed size distributions shifted toward larger sizes (Figure

8C), confirming that only the largest and brightest particles could be captured. Furthermore, as the NTA instrument used in this study is equipped with a 488 nm laser that has a higher power than the corresponding laser included in the nFCM hardware (40 mW versus (vs.) 15 mW on nFCM), the resulting increase in generated background noise makes its distinction from weak fluorescent signals challenging. Conversely, nFCM is capable of lowering background noise and the consequent recording of false negatives by acquiring fluorescent signals only when the generating sources are confirmed to be particles by scatter.

EV subpopulation profiling was analyzed using two labelling approaches: i) antibody-based, using fluorescently labelled antibodies recognizing the tetraspanins CD9, CD63, and CD81, conventionally assessed as mammalian EV markers (Buzas, 2023); ii) RNA-specific, relying on the RNA-binding probes SYTO™ RNASelect™ (Syto) and Quant-iT™ RiboGreen™. As the latter provided poor analytical outputs in terms of sensitivity, especially on the (F-)NTA instrument, the results obtained using the first strategy were preferentially reported and discussed in this thesis. The study first aimed at selecting fluorophores showing the highest sensitivity for EV detection. To that end, we stained HT29 EVs with antibodies anti-CD9 conjugated to green AF488, red-orange phycoerythrin (PE), far-red AF647, and far-red allophycocyanin (APC). Because of the longer excitation wavelengths of the red fluorescent probes, their comparative assessment was conducted only by nFCM. AF488 and PE demonstrated the highest performance, leading to approximately 50% labelling efficiency, in contrast to AF647 and APC, which enabled the detection of up to 15% EVs (Figure 9A). The comparable performance of AF488 and PE was unexpected, as their theoretical brightness was supposed to be the lowest and the highest, respectively, among all evaluated fluorophores. Although brighter than AF488, APC and AF647 showed poor detection capability potentially due to self-quenching in the context of single-particle analysis. This suggests that, by accumulating in a localized surface area at a 1-10 nm distance from each other, fluorophores with longer emission wavelengths may be more prone to transfer energy to neighbouring molecules, instead of emitting photons. This process, known as FRET, may ultimately lead to dye self-quenching by reducing its quantum yield (i.e., the fraction of absorbed photons re-emitted as fluorescence) below its theoretical value. Based on these results, AF488 and PE were chosen as preferred fluorescent molecules to conduct subsequent antibody-based EV profiling. Because PE bleaches faster than AF488, only the latter was selected for (F-)NTA-based phenotyping due to its higher stability upon more powerful laser illumination and longer exposure/acquisition time.

On nFCM, purified EVs from the HT29 and HEK293 cell lines were used as model vesicular preparations and stained with antibodies anti-CD9, anti-CD63, and anti-CD81 conjugated to either AF488 or PE. Using antibodies anti-CD9 and anti-CD81, the labelling efficiency of both EV samples was comparable with both fluorophores. The sole discrepancy between AF488 and PE detection was observed in HT29 EVs stained with antibodies anti-CD63. This could be attributed to a methodological artifact, potentially associated with the use of two different fluorescently labelled antibody clones. It was also highlighted that the proportion of labelled EVs was different between EV sources (i.e., lower for HEK293 EVs, compared with HT29 EVs) and tested tetraspanins (i.e., CD81>CD9>CD63 for Batch A and CD9>CD81>CD63 for Batch B), confirming their biological heterogeneity (Figure 9B). On (F-)NTA, the same analysis was performed only on HT29 EVs. We highlighted lower detectability using all antibodies, consistently with the lower sensitivity of the CMOS camera sensor, compared with the APDs of the nFCM

system. Furthermore, unlike nFCM measurements, we observed a tetraspanin expression trend (i.e., CD9>CD81>CD63) that was maintained across multiple biological batches, confirming its capability to make reliable acquisitions of directional expression patterns under low sensitivity.

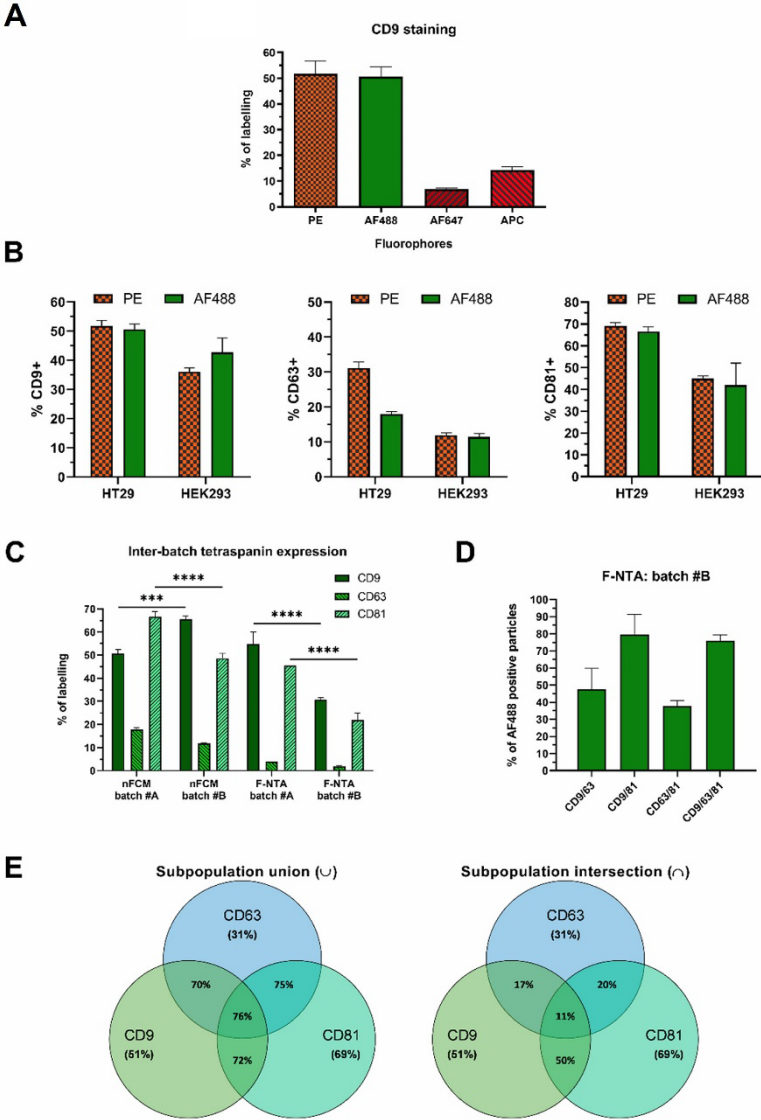


Figure 9. Subpopulation profiling of fluorescently labelled EVs by (F-)NTA and nFCM. The bar graphs show: (A) nFCM-estimated HT29 EV labelling efficiency (%) – expressed as ratio of fluorescent particle number to scattering particle number (%) – after staining with antibodies anti-CD9 conjugated to AF488, PE, AF647, and APC; (B) nFCM-estimated HT29 and HEK293 EV labelling efficiency (%) after staining with antibodies anti-CD9, anti-CD63, and anti-CD81 conjugated to AF488 and PE; (C) nFCM- and (F-)NTA-estimated inter-batch variability of HT29 EV labelling efficiency (%) after staining with AF488-conjugated antibodies anti-CD9, anti-CD63, and anti-CD81; (D) (F-)NTA-estimated HT29 EV labelling efficiency (%) after staining with double or triple mixtures of antibodies anti-CD9, anti-CD63, and anti-CD81 conjugated to AF488. (E) Venn diagrams

depicting HT29 EV subpopulations, either expressing 1, 2, or 3 tetraspanins (left) or co-expressing 2 or 3 tetraspanins (right), characterized by nFCM using individual or mixtures of PE-conjugated antibodies anti-CD9, anti-CD63, and anti-CD81. In A-E, data are reported as mean $\pm$ SEM from two to three independent experiments. In C, data statistical analysis was performed between (F-)NTA vs. nFCM and batch A vs. batch B using two-way ANOVA with Tukey's test for multiple comparisons (not reported p-value: not significant; <0.001 [***], <0.0001 [****]). The figure was adapted from Figure 2 in **Publication I**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: AF = Alexa Fluor; APC = allophycocyanin; (F-)NTA = (fluorescence) nanoparticle tracking analysis; HEK293 = human embryonic kidney 293; nFCM = nanoscale flow cytometry; PE = phycoerythrin.

However, batch-to-batch variability in terms of labelling efficiency was displayed using both platforms (Figure 9C). Staining of HT29 EVs was also evaluated using double (CD9+CD63; CD9+CD81; CD81+CD63) and triple (CD9+CD81+CD63) antibody combinations, which were PE- and AF488-conjugated for nFCM and (F-)NTA, respectively. By both nFCM and (F-)NTA, nearly 76% of all particles, stained with triple antibody mix, were found to express CD9, CD81, and CD63, either individually or in combination (Figure 9D-E). Venn diagram intersection analysis of nFCM data enabled us to quantify the subpopulations of HT29 EVs co-expressing different tetraspanins, which consisted of i) 17% co-expressing CD9 and CD63, ii) 50% co-expressing CD9 and CD81, iii) 20% co-expressing CD63 and CD81, and iv) 11% co-expressing all three tetraspanins. Importantly, nearly all CD9-positive particles, corresponding to 51% of all detected scattering particles, also expressed CD81, as 50% of the total number of scattering events were positive for both CD9 and CD81 (Figure 9E). This indicated that tetraspanin expression in EVs can occur individually or concomitantly, thereby resulting in potential overlapping phenotypes, which requires multiparametric analysis to be performed in order to appreciate the true complexity of their subpopulations. We also observed that the percentage of fluorescent particles stained with double antibody mixes CD9/CD63 and CD63/CD81 was higher upon detection with the (F-)NTA instrument, while individual or combined expression of CD9/CD81 was comparable between the two platforms (Figure 9D-E). These findings could derive from the limitation of the (F-)NTA's detection threshold, as well as the relative abundance of each tetraspanin in the evaluated EVs. Because of the low sensitivity of the CMOS sensor, (F-)NTA is able to detect only particles emitting a cumulative fluorescent signal that is detectable. In single labelling, CD63 demonstrated the lowest expression level among all tetraspanins, which implies that most CD63-positive particles, carrying a few epitopes per particle, potentially fall below the (F-)NTA detection threshold. In the case of CD63 co-expression with CD9 and/or CD81, the weak fluorescence emitted by its antibodies was enhanced by combination with the fluorescent signals emitted by the antibodies recognizing the other two tetraspanins, thus allowing the visualization of previously undetected particles. On the other hand, the percentage of particles stained with CD9/CD81 antibody mix was comparable between nFCM and (F-)NTA [i.e., 72% on nFCM vs. 79.4% on (F-)NTA] (Figure 9D-E). This indicated that all particles positive for CD9 and/or CD81 carried a sufficient number of epitopes per particle to generate fluorescent signals detectable by both analytical platforms, without falling below their respective detection thresholds.

Overall, these findings demonstrated that the biological interpretation of EV preparations strongly depends on the analytical ability to resolve their complexity and heterogeneity. Although numerous technologies have been thus far validated for EV quantification and phenotyping, to promote rigorous and reliable analyses, optimization

of analytical procedures should be carried out for each platform, controlling instrument settings and optimizing EV labelling strategies.

4.2 Alkaline treatment of EVs with sodium carbonate buffer enables efficient encapsulation of exogenous hydrophilic macromolecular proteins without compromising EV structural integrity, uptake, and cargo functional performance (Publication II)

Because of their natural cargo protection and delivery properties, low immunogenicity, efficient tissue penetration capability, and high amenability to manipulation, EVs have been extensively explored as attractive drug delivery systems for multiple healthcare applications (Sun et al., 2024). To enhance targeted EV functional outcomes, conventional exogenous EV engineering strategies have mainly focused on maximizing payload efficiency, despite causing substantial impairment of EV structural integrity, often leading to reduced potency (Somiya & Kuroda, 2021; Théry et al., 2018). These considerations laid the foundations for the conceptualization of our second methodological objective, aimed at determining how EVs can be engineered to make their biological activity truly meaningful in translational settings. A valuable contribution to addressing this challenge was provided by the case study described in **Publication II**, in which a novel procedure for exogenous drug loading of EVs was assessed for its impact on EV structural integrity and functional performance. This approach was compared with electroporation, selected as a gold standard method known to cause substantial structural damage to EVs with variable drug loading efficiency (Singh et al., 2025; Zuppone et al., 2024).

The strategy investigated in this study consisted of exposing EVs to temporary alkaline pH alteration, without affecting membrane structural integrity, to stimulate exogenous small molecule encapsulation by transient membrane perturbation. Its experimental design was based upon prior evidence demonstrating that, under basic pH conditions, biological membranes retain their inherent integrity, while experiencing a reduction of non-covalent protein–protein interactions, resulting in the detachment of weakly associated peripheral membrane proteins (Fujiki et al., 1982; Zuppone et al., 2024). As already employed in previous studies for effective isolation of intracellular/organellar membranes, 1 M sodium carbonate (Na_2CO_3) was selected as the preferred alkaline solution. Furthermore, purified HEK293 EVs were used as model vesicular preparations, given the common use of their parental cells for the development of either EV-based or non-EV-based biotherapeutics (Cully, 2021; E. Tan et al., 2021; Zuppone et al., 2024).

Alkaline treatment with Na_2CO_3 was initially tested for its effects on EV morphology and physicochemical properties. According to TEM and NTA analyses, untreated and Na_2CO_3 -treated EVs showed an overlapping, homogeneous size distribution, peaking at roughly 150 nm in diameter, which was associated with an expected yet minor particle loss (Figures 10A-B and 11A-G). WB analysis confirmed retention of typical EV markers (i.e., CD63, CD9, ALIX, and TSG101) after treatment (Figure 10C), although a slight reduction in total protein content was observed by both silver staining (Figure 10D) and BCA assay (Figure 11B-G). The latter finding was validated by Raman spectroscopy, which highlighted a modest decrease in the protein peak at 2930 cm^{-1} (relative to the protein - CH_2 groups), associated, however, with no shift in peak position, thereby indicating preservation of protein structures. In the same spectra, the lipid peak at 2930 cm^{-1} (relative to the lipid - CH_2 groups) showed no alteration upon treatment, demonstrating

the maintenance of membrane lipid integrity (Figure 10F). This was confirmed by quantification of the cholesterol content (Figure 11E-G), known to be enriched in mammalian EV membranes (Ghadami & Dellinger, 2023), which resulted in comparable levels between untreated and Na₂CO₃-treated EVs.

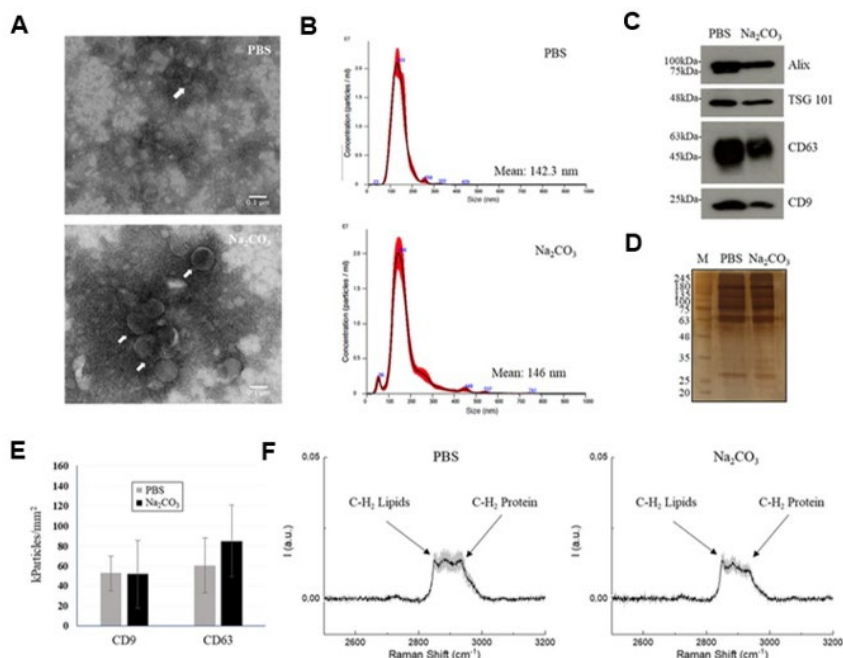


Figure 10. Assessment of Na₂CO₃-based alkaline treatment for its impact on EV morphology and membrane structure. The presented images and analytical outputs show comparative characterization of untreated and treated HEK293 EVs by (A) TEM, (B) NTA, (C) WB for EV markers (i.e., ALIX, TSG101, CD63, and CD9), (D) silver staining, (E) SP-IRIS analysis, and (F) Raman spectroscopy. The figure was adapted from Figure 1 in **Publication II**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: ALIX = ALG-2-interacting protein X; EV = extracellular vesicle; HEK293 = human embryonic kidney 293; M = marker; NTA = nanoparticle tracking analysis; PBS = phosphate-buffered saline; SP-IRIS = single-particle interferometric reflectance imaging sensor; TEM = transmission electron microscopy; TSG101 = tumor susceptibility gene 101; WB = Western blot.

In alignment with these observations, exposure to Na₂CO₃ did not affect EV surface immunoreactivity for tetraspanins CD63 and CD9 (Figures 10E and 11C-G), as well as EV surface glycosylation patterns (Figure 11D-G), while preserving luminal RNA content (Figure 11F-G). Overall, based on these data, it could be concluded that short-term exposure to a highly alkaline Na₂CO₃ solution could maintain EV integrity and potentially improve purification from protein contaminants, enabling EV membrane domains to be better exposed to the surrounding environment, potentially improving recognition and uptake by recipient cells.

After demonstrating its suitability for EV engineering without compromising EV structure, Na₂CO₃-based alkaline treatment was assessed against electroporation. To evaluate whether the Na₂CO₃-mediated drug loading efficiency can be influenced by the structural characteristics of the exogenous payload, two hydrophilic molecules with

distinct size and physicochemical properties were tested: i) fluorescein isothiocyanate (FITC)-labelled dextran, a complex and branched polysaccharide of 70 kDa size, widely employed as a benchmark macromolecular cargo in delivery studies (Li et al., 2015; Natarajan et al., 2017; Ramon et al., 2025; Zuppone et al., 2024); ii) saporin (SAP), a globular protein of 25 kDa size.

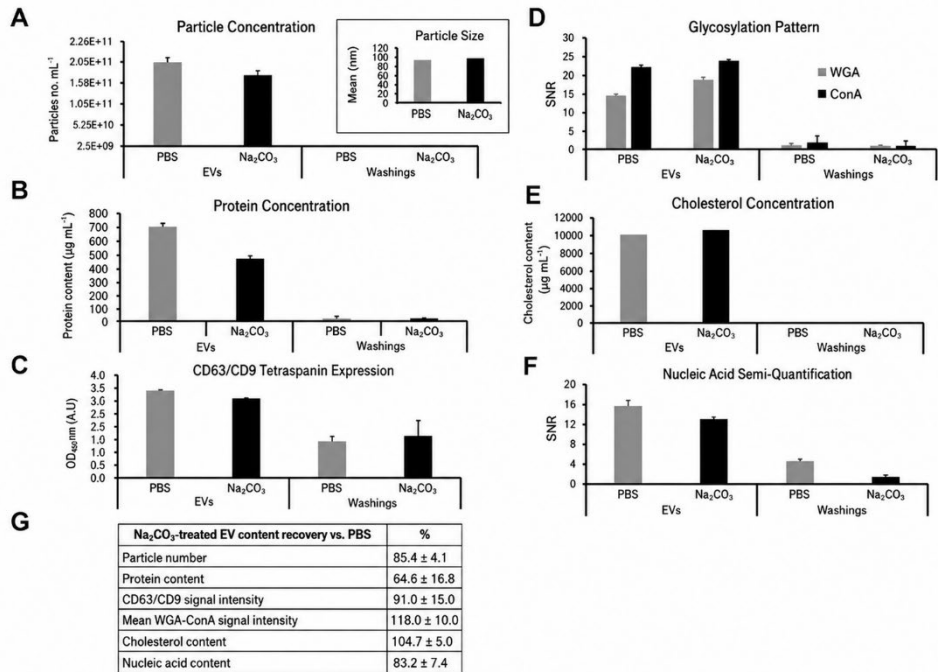


Figure 11. Assessment of Na₂CO₃-based alkaline treatment for its impact on EV biochemical and biophysical integrity. The bar graphs show comparative assessment of untreated and treated HEK293 EVs by (A) NTA, (B) BCA assay, (C) CD63/CD9 sandwich ELISA, (D) lectin assay, (E) cholesterol quantification assay, and (F) RiboGreen™-based nucleic acid semi-quantification assay. (G) Table summarizing EV maintenance (%) of assessed parameters after treatment relative to control (PBS). The figure was adapted from Figure 2 in **Publication II**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Modifications were made. Abbreviations: BCA = Bicinchoninic acid; ConA = concanavalin A; ELISA = enzyme-linked immunosorbent assay; EV = extracellular vesicle; HEK293 = human embryonic kidney 293; NTA = nanoparticle tracking analysis; PBS = phosphate-buffered saline; SNR = signal-to-noise ratio; WGA = wheat germ agglutinin.

The selection of the latter was also driven by its therapeutic potential, as it is a plant-derived ribosome-inactivating protein with important anticancer activity, as well as its limited cytotoxicity when used as a free molecule (Polito et al., 2013), making it an ideal molecule for verifying and validating the effectiveness of loading strategies on relevant functional models.

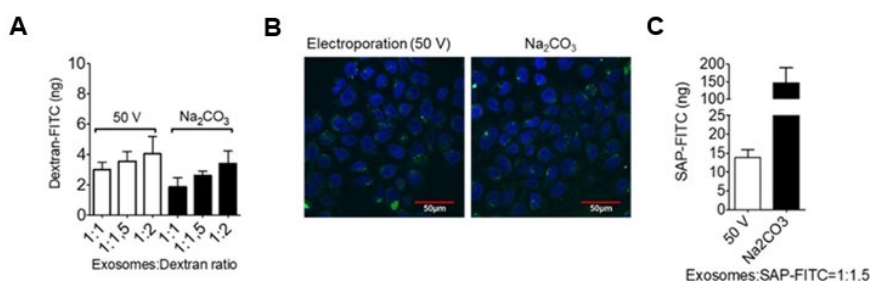


Figure 12. Comparative assessment of Na₂CO₃-based alkaline treatment and electroporation for cargo-dependent EV encapsulation efficiency and cellular uptake. (A) The bar graph shows Dextran-FITC content (ng) encapsulated in HEK293 EVs at various EV:Dextran-FITC ratios (1:1, 1:1.5, 1:2) following treatment with either Na₂CO₃-based alkaline treatment or electroporation (50 V). (B) The confocal microscopy images illustrate HeLa cells (blue) treated for 24 h with Dextran-FITC-encapsulating EVs (green) prepared by either Na₂CO₃-based alkaline treatment or electroporation (50 V) with EVs:Dextran-FITC ratio of 1:1.5. (C) The bar graph displays SAP-FITC content (ng) encapsulated in HEK293 EVs at EV:Dextran-FITC 1:1.5 ratio following treatment with either Na₂CO₃-based alkaline treatment or electroporation (50 V). The figure was adapted from Figure 3 in Publication II. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: EV = extracellular vesicle; FITC = fluorescein isothiocyanate; HEK293 = human embryonic kidney 293; SAP = saporin.

Assessment of different EV:FITC-dextran ratios (i.e., 1:1, 1:1.5, and 1:2) subjected to either Na₂CO₃-based alkaline treatment or electroporation at low voltage (i.e., 50 V) resulted in a comparable, low (i.e., 0.2-0.3%), and dose-dependent FITC-dextran encapsulation efficiency (Figure 12A). This finding was confirmed by confocal microscopy of HeLa cells, demonstrating equivalent intracellular dextran-FITC-associated signal after 24 h incubation with EVs loaded with both methods (Figure 12B). A striking performance difference in favour of the Na₂CO₃-based loading approach was observed when SAP was used as the targeted exogenous cargo. Using a fixed EV: FITC-SAP ratio of 1:1.5, Na₂CO₃-based alkaline treatment showed 10-fold higher SAP loading efficiency, compared with 50 V electroporation (i.e., 15% with Na₂CO₃ solution vs. 1.5% with electroporation). Furthermore, with both methods, drug encapsulation was more pronounced with SAP, compared with FITC-dextran (i.e., 50-fold higher with Na₂CO₃ solution vs. 4-5-fold higher with electroporation) (Figure 12C). This suggested that loading capacity depends on the steric hindrance and geometry of the exogenous molecule to be encapsulated, while being potentially maximized upon use of smaller molecules.

The Na₂CO₃-based loading approach with SAP was then assessed against EV passive incubation (PBS) and free SAP alone for its ability to promote effective SAP delivery and induce cytotoxicity in cancer cells, using the 5637 bladder cancer cell line as a cellular model. Confocal microscopy and flow cytometry of 5637 cells incubated for 24 h with two doses of fluorescently labelled EVs (i.e., 5 and 10 µg of EV proteins) loaded with either PBS or Na₂CO₃ revealed a comparable, EV dose-dependent uptake across conditions (Figure 13A-B).

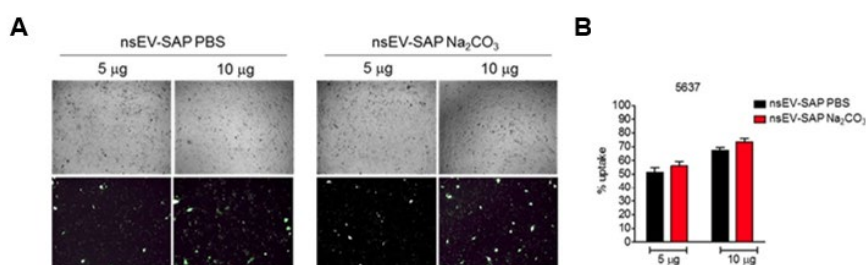


Figure 13. Assessment of SAP uptake in cancer cells mediated by EVs subjected to Na₂CO₃-based alkaline treatment. (A) The confocal microscopy images, acquired in bright-field (upper row) and fluorescence mode (lower row), illustrate 5637 cells treated for 24 h with 5 or 10 µg of fluorescently labelled HEK293 EVs prepared in the presence of SAP using either PBS (left) or Na₂CO₃ (right). (B) The bar graph shows cellular uptake – expressed as mean fluorescence (%) ± standard deviation (SD) (n = 3) – in 5637 cells treated for 24 h with 5 or 10 µg of fluorescently labelled HEK293 EVs prepared with PBS or Na₂CO₃ in the presence of SAP. The figure was adapted from Figure 4 in **Publication II**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: EV = extracellular vesicle; HEK293 = human embryonic kidney 293; nsEV = nano-sized extracellular vesicle; PBS = phosphate-buffered saline; SAP = saporin.

The same cell line was subsequently treated for 72 h with three doses (i.e., 5, 10, and 20 µg of EV proteins) of differently loaded SAP-bearing EVs to compare induced cytotoxicity effects. This assessment highlighted a significant, EV dose-dependent reduction in cell viability upon use of SAP-bearing EVs loaded with Na₂CO₃, indicating efficient SAP preservation and cellular internalization. Interestingly, a dramatic effect was induced already by the lowest EV dose (i.e., 1E+9 particles with SAP content estimated to be approximately 500–750 ng) exceeding the effective half maximal inhibitory concentration (IC₅₀). In contrast, no pronounced SAP-induced cytotoxicity was observed at any tested dose of passively loaded SAP-bearing EVs, confirming low SAP loading capacity (Figure 14A-B).

To estimate the amount of SAP encapsulated in EVs, differently loaded SAP-bearing EV samples and control samples (i.e., EVs alone and SAP alone) were subjected to NaCl washing steps, ultimately promoting the detachment of potential EV surface-bound SAP by disruption of weak electrostatic interactions.

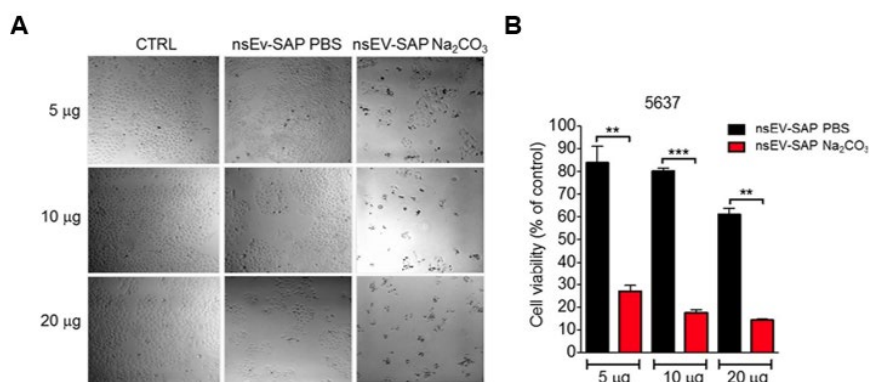


Figure 14. Assessment of SAP cytotoxicity in cancer cells mediated by EVs subjected to Na₂CO₃-based alkaline treatment. (A) The bright-field confocal microscopy images show 5637 cells treated for 72 h with 0, 5, 10, or 20 µg of HEK293 EVs prepared in the presence of SAP using either PBS (left)

or Na_2CO_3 (right). (B) The bar graph presents cell viability (%) of 5637 cells treated for 72 h with 5, 10, or 20 μg of SAP-bearing HEK293 EVs prepared with PBS or Na_2CO_3 . The figure was adapted from Figure 5 in **Publication II**. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: CTRL = control; EV = extracellular vesicle; HEK293 = human embryonic kidney 293; nsEV = nano-sized extracellular vesicle; PBS = phosphate-buffered saline; SAP = saporin.

By WB analysis, SAP expression was confirmed only in association with EVs loaded with Na_2CO_3 or in free form, yet at high doses (*i.e.*, 500 ng and 2 μg) (Figure 15A). WB band densitometric analysis enabled the estimation of 1 μg of SAP intravesicular content per 10 μg of EVs, consistent with approximately 15% loading efficiency measured with fluorescently labelled SAP. The cytotoxic effect of washed Na_2CO_3 -loaded SAP-bearing EVs showed to be lower (*i.e.*, IC_{50} at more than 10 μg of EVs), compared with unwashed samples (*i.e.*, IC_{50} at less than 5 μg of EVs), suggesting the cytotoxic effect to be partly induced by EV surface-bound SAP. Importantly, a comparable effect could be observed between roughly 8 μg of free SAP and 10 μg of Na_2CO_3 -loaded EVs containing approximately 8-fold less SAP (*i.e.*, $\sim 1 \mu\text{g}$), demonstrating the higher potency of SAP when delivered via Na_2CO_3 -treated EVs due to enhanced protection and cellular uptake (Figure 15B-D). Furthermore, the removal of residual free SAP or buffer components from Na_2CO_3 -treated SAP-bearing EVs by UF enabled to attribute the observed functional outcomes solely to the SAP encapsulated within EVs.

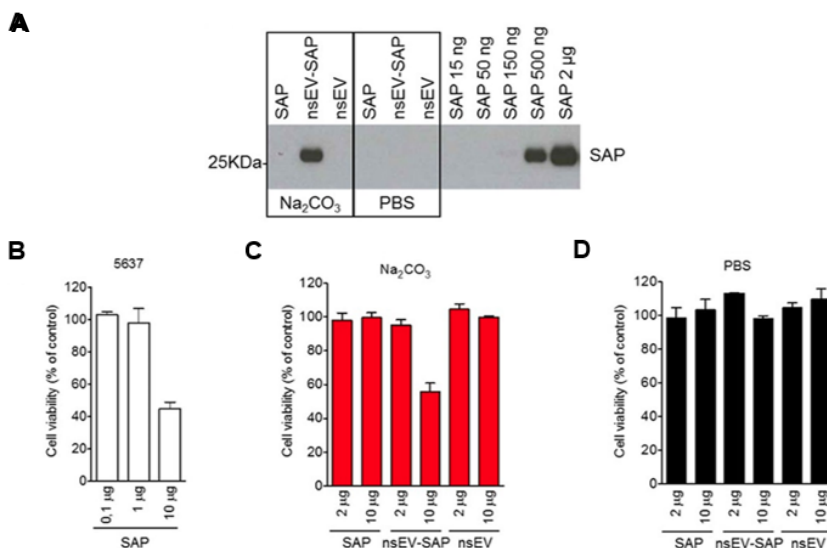


Figure 15. Assessment of intraluminal SAP encapsulation efficiency in Na_2CO_3 -treated EVs after removal of excess of EV membrane-associated SAP. (A) The image presents WB analysis of SAP in HEK293 EVs prepared using either PBS or Na_2CO_3 , followed by washing steps with NaCl. (B) The bar graph shows cell viability (%) of 5637 cells treated for 72 h with 0.1, 1, or 10 μg of SAP. The bar graphs depict cell viability (%) of 5637 cells treated for 72 h with 2 or 10 μg of SAP, SAP-bearing EVs, or EVs-only after treatment with either Na_2CO_3 (C) or PBS (D). Excess of unbound SAP was removed by UF, using 100 kDa MWCO filter units. The figure was adapted from Figure 6 in Publication II. The original figure is licensed under the Creative Commons Attribution 4.0 International License (CC BY 4.0) (<https://creativecommons.org/licenses/by/4.0/>). Abbreviations: EV = extracellular vesicle; HEK293 = human embryonic kidney 293; MWCO = molecular weight cut-off; nsEV = nano-sized extracellular vesicle; PBS = phosphate-buffered saline; SAP = saporin; UF = ultrafiltration; WB = Western blot.

Overall, this study showcased the temporary alkaline treatment of EVs with 1 M Na₂CO₃ as a novel and straightforward engineering method promoting efficient encapsulation of EVs with exogenous water-soluble macromolecular cargoes, namely polysaccharides and globular proteins, while preserving EV integrity, cellular uptake, and cargo functional activity. This suggests its potential applicability to a broader range of biomolecular cargoes, such as nucleic acids, peptides, antibodies, and other therapeutic molecules, although dedicated experimental evaluation and validation are still required.

4.3 Three-dimensional normalization strategies and multivariate analysis strengthen EV data comparability and interpretation (Publication III)

To promote rigorous EV research and exploitation, the EV community has increasingly emphasized the significance of applying Quality-by-Design (QbD) principles to EV processing practices from the earliest, explorative phases (Paolini et al., 2022). Within this framework, QbD aims at identifying and controlling measurable properties that define the target product, conventionally referred to as critical quality attributes (CQAs), ultimately ensuring desired product quality and performance [ICH Q8 (R2) Pharmaceutical Development - Scientific Guideline | European Medicines Agency (EMA), 2009; ICH Q11 Development and Manufacture of Drug Substances (Chemical Entities and Biotechnological/Biological Entities) - Scientific Guideline | European Medicines Agency (EMA), 2013]. In the EV field, the current lack of standardized normalization strategies underlies the use of inconsistently defined and insufficiently context-tailored quality metrics for comparing EVs across distinct sources and studies. This prevents the definition of robust EV CQAs, ultimately hampering the translation of EV R&I outputs into effective and marketable solutions (Li et al., 2026). Accounting for this, the work conducted within **Publication III** was focused on addressing the following objectives: i) to evaluate how different normalization strategies shape the comparative assessment of small EVs from different eukaryotic sources; ii) to identify core biochemical attributes that are either shared or source-specific across the examined EV samples, with a view to supporting the definition of EV quality metrics tailored to eukaryotic EV-based products and downstream uses. In this study, a specific focus was placed on EVs derived from human mesenchymal stem/stromal cells (MSC-EVs), bovine milk (BM-EVs), the helminth parasite *Ascaris suum* (AS-EVs), and ginger rhizome (G-NVs), which were assessed via surrogate properties for their quantity (*i.e.*, particle number), identity (*i.e.*, size distribution, protein content, phospholipid content, cholesterol content, carbohydrate content), and bioactivity (*i.e.*, enzymatic activities).

To mitigate inherent quantitative variability arising from the use of different protocols for the enrichment and purification of EVs of different origin, we paid particular attention to the identification and application of a suitable strategy for data normalization. Generated datasets were initially aligned based on two parameters that are ordinarily adopted by the EV community as indicators of EV abundance: particle number and protein content. The criterion for selecting the most appropriate normalization approach was based on identifying the parameter showing enrichment across all EV samples, while being absent or considerably reduced in the processed EV sources depleted of EVs and accumulating non-EV biocomponents, hereby defined as controls (CTLs). The comparative assessment of EVs against their relative CTLs confirmed particle enrichment in all EV preparations, while highlighting inconsistent protein levels with large trend

variations dependent on source-tailored purification used (Figure 16A-B). As a result, particle count was recognized as the quantitative parameter that best met our criteria for EV sample normalization.

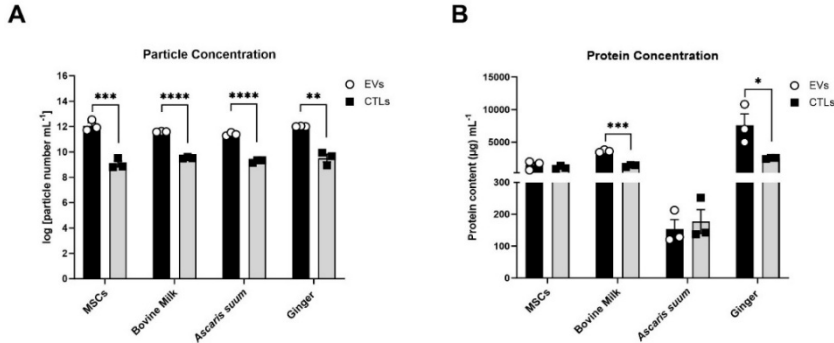


Figure 16. Setup of EV normalization strategy. The bar graphs highlight (A) particle concentrations – expressed as particle number per milliliter – and (B) protein concentrations – expressed as protein content (μg) per milliliter – of EVs and CTLs derived from MSCs, BM, AS, and G. The numerical values relative to particle concentrations are displayed in log scale. All data are reported as mean ± SEM from three biological batches corresponding to three independent sample preparations. Statistical analysis was performed between EVs and corresponding CTLs using ordinary unpaired t-test (not reported p-value: not significant; significant p-value: < 0.05 [*], <0.01 [**], <0.001 [***], <0.0001 [****]). Abbreviations: AS = *Ascaris suum*; BM = bovine milk; CTL = EV-depleted control; EV = extracellular vesicle; G = ginger; MSC = human mesenchymal stem/stromal cell.

Nevertheless, as our set of vesicular preparations showed overlapping but slightly divergent particle size distributions (Figure 17A-B), we decided to strengthen our normalization approach by integrating two additional factors, namely particle surface area and particle volume, to simultaneously account for differences in both particle count and size. This decision was driven by the awareness that, unlike particle size, particle surface area and particle volume scale in a non-linear manner [surface area with the square of the radius (r^2) and volume with the cube of the radius (r^3)], thereby implying that minor differences in particle size can translate into significant differences in particle surface area and volume.

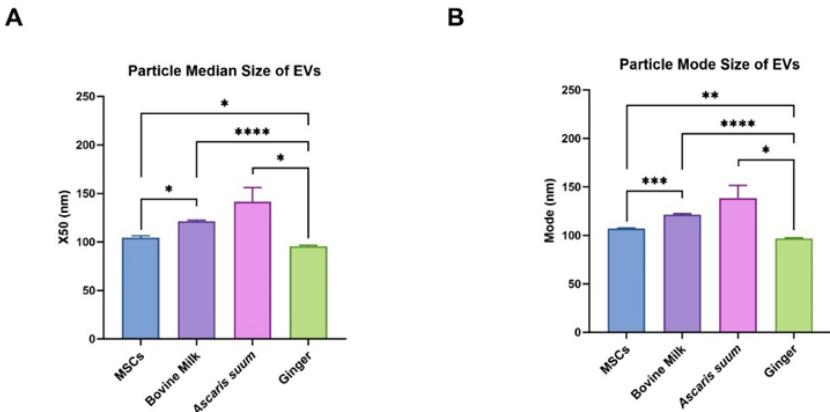


Figure 17. Particle size analysis of MSC-EVs, BM-EVs, AS-EVs, and G-NVs. Bar graphs showing EV particle sizes expressed as (A) particle median (X50; nm) and (B) particle mode (nm). Data are

reported as mean $\pm$ SEM from three biological batches correspondent to three independent sample preparations. Statistical analysis was performed between groups of two EV types using ordinary unpaired t-test (not reported p-value: not significant; p-value < 0.05 [], <0.01 [**], <0.001 [***], <0.0001 [****]). Abbreviations: AS = Ascaris suum; BM = bovine milk; EV = extracellular vesicle; G = ginger; MSC = human mesenchymal stem/stromal cell.*

AS-EVs displayed a protein content per particle that was consistent with prior evidence and estimations (Zarovni et al., 2025). In contrast, a higher payload per particle was revealed by the other EV preparations, either reflecting source-dependent heterogeneity or arising from technical limitations, such as the co-isolation of extravesicular proteins, as well as the NTA-based sub-detection of small particles, falling below the detection limit of the instrument (Chaudhuri, 2016; Théry et al., 2018) (Figure 18A). Importantly, sample normalization based on particle surface area and volume abolished the significant difference displayed by MSC-EVs upon comparison with BM-EVs and G-NVs, likely indicating a comparable protein density and protein surface density across these samples (Figure 18B-C).

Taxonomic divergence between EV samples was further underscored by lipid assessment, which was focused on choline-containing phospholipids (ChoPLs), highly abundant in EV membranes regardless of their origin, and cholesterol, an inherent constituent of animal EV membranes (Ghadami & Dellinger, 2023; Skotland et al., 2020). Both mammalian EVs showed different particle cholesterol levels, which were notably higher in MSC-EVs, yet a similar ChoPL amount per particle, which was the highest across EV samples (Figure 18A). The higher cholesterol content highlighted in our MSC-EV preparations could align with previous data demonstrating the active role exerted by cholesterol in promoting the functionality of bone marrow-derived MSCs (Sohn et al., 2018). The undetectable and reduced ChoPL content per particle observed in AS-EVs and G-NVs, respectively, (Figure 18A) may be potentially linked to different biological and technical factors: in AS-EVs, either the highly glycosylated, sterically hindered surface of helminth EVs, potentially interfering with the performance of the quantification assay used, or a particle concentration below the assay's sensitivity threshold; in G-NVs, the low abundance of ChoPLs in G-NVs, as previously proven in comparison with vesicles from other plant sources (Simbari et al., 2016; Teng et al., 2018; White et al., 2023). Importantly, relative lipid trends across EV samples were preserved upon normalization per particle surface area and volume, suggesting that the observed similarities and differences did not arise from normalization artifacts, but rather reflected actual biological divergence (Figure 18B-C).

With respect to carbohydrates, MSC-EVs exhibited the highest content across all EV samples, regardless of the normalization strategy (Figure 18A), which may reflect the important biological role exerted by their parental cells in promoting cellular processes that are highly supported by carbohydrates, such as tissue repair, regeneration, and immunomodulation (Du & Yarema, 2010; Margiana et al., 2022). Interestingly, normalization based on particle number and surface area highlighted a comparable carbohydrate content between BM-EVs and AS-EVs, which was the lowest across EV samples (Figure 18A-B). However, normalization per particle volume enabled to unmask the intrinsically lower carbohydrate density of AS-EVs (Figure 18C). This result may be biologically relevant, as potentially reflecting the metabolic flexibility of parasitic helminths, known to consume not only carbohydrates, but also other host-derived carbon sources (e.g., amino acids, fatty acids, and cholesterol) to meet their metabolic demands (Reed & Smith, 2024; van Die & Cummings, 2010).

As conventionally analyzed as proxies for EV bioactivity and integrity (Adamo et al., 2025; Gimona et al., 2021), multiple enzyme activities were assessed in this study to add a functional dimension to EV comparison. We decided to focus on enzymes involved in diverse cellular processes across eukaryotic organisms: enolase (ENO), peroxidase (POD), leucine aminopeptidase (LAP), and xanthine oxidase (XO). These were selected based on their potential to discriminate among the EV samples examined in this investigation, as supported by evidence from literature and database analyses, although not exclusively derived from EV-focused studies (Ancarola et al., 2023; Aoyagi et al., 1978; Bartling & Weiler, 1992; Chen et al., 2012; Davies et al., 2008; Eirin et al., 2018; Gadegaard et al., 2025; Nishino et al., 2008; Pandey et al., 2017; Qin et al., 2021; Rhoads & Fetterer, 1998; *UniProt*, 2025; Van der Straeten et al., 1991; Verma & Dutta, 1994; Xu et al., 2020). For all enzymatic activities tested, relative trends across EV samples were maintained regardless of the specific normalization strategy used (Figure 18A-C), suggesting that the observed discrepancies or similarities did not emerge as a consequence of normalization artifacts, but were likely due to EV source-dependent biological heterogeneity.

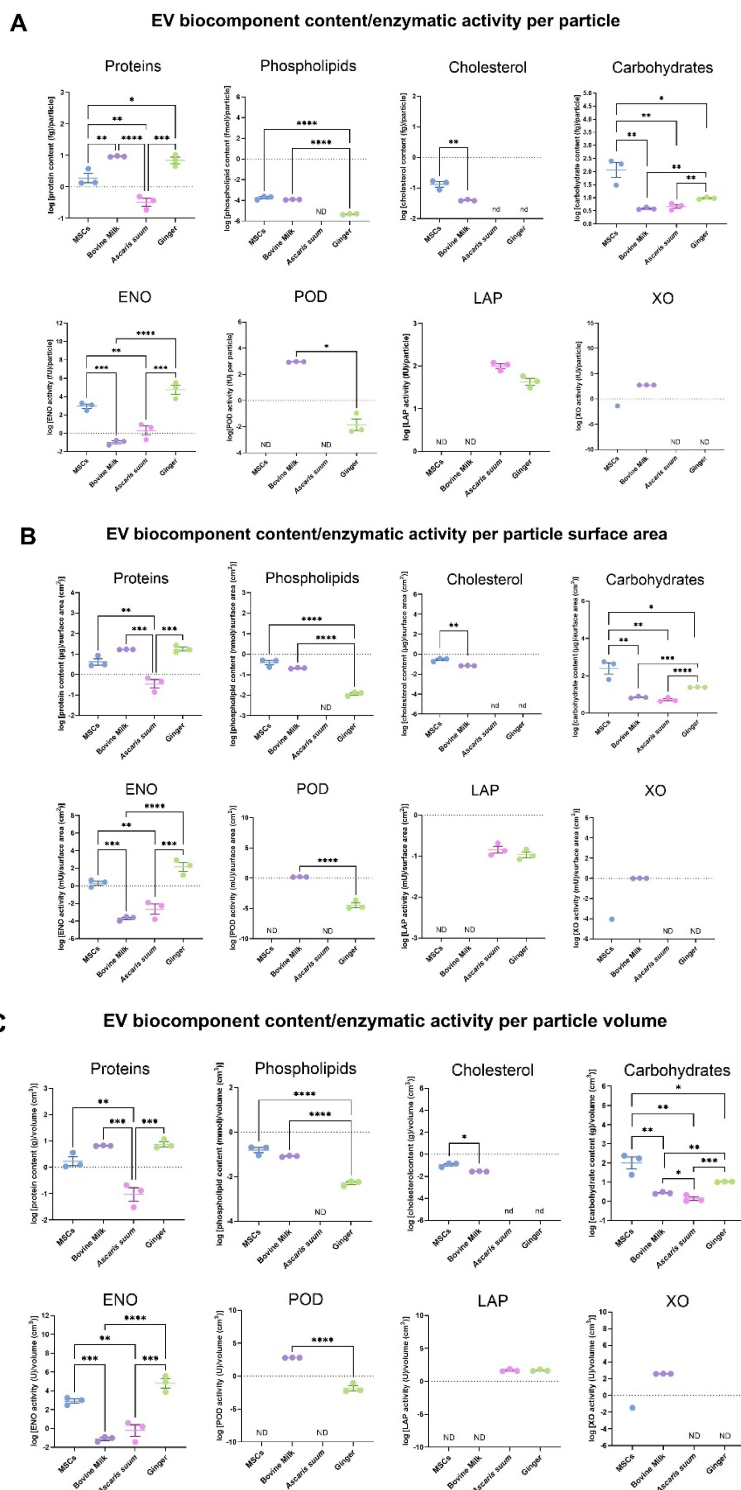


Figure 18. EV cargo analysis. The dot plots depict biocomponent contents and enzymatic activities of MSC-EVs, BM-EVs, AS-EVs, and G-NVs normalized per (A) particle, (B) particle surface area, and

(C) particle volume. Data are reported in log scale as mean $\pm$ SEM from three biological batches corresponding to three independent sample preparations. Statistical analysis of log-transformed carbohydrates data – comparing MSC-EVs with each of the other EVs – and log-transformed cholesterol data was carried out using ordinary unpaired t-test. Statistical analysis of all other data was performed using ordinary one-way ANOVA with follow-up test for multiple comparisons (not reported p-value: not significant; significant p-value < 0.05 [*], <0.01 [**], <0.001 [***], <0.0001 [****]). Abbreviations: AS = *Ascaris suum*; BM = bovine milk; ENO = enolase; EV = extracellular vesicle; G = ginger; LAP = leucine aminopeptidase; MSC = human mesenchymal stem/stromal cell; ND = not detected; nd = not determined; NV = nanovesicle; POD = peroxidase; XO = xanthine oxidase.

Specifically, ENO was the sole activity detected in all EV samples, reflecting its conserved role in metabolic processes (i.e., glycolysis and gluconeogenesis), as well as stress responses (Ji et al., 2016). Conversely, POD activity was highlighted only in BM-EVs and G-NVs, with the mammalian one showing a significantly higher level, potentially resulting from the combined activity of multiple types of PODs that have been so far reported in BM (Buys, 2011; Sharma et al., 2013). The detection of LAP activity was restricted to AS-EVs, correlating with its important role in helminth nutrient digestion and immune evasion. The unexpected detection of G-NVs for LAP activity, which should be absent in ginger, may originate from a functionally convergent enzyme that is structurally distinct from its mammalian and AS counterparts. As expected, XO activity was revealed by BM-EVs, rather than by AS-EVs and G-NVs, which was consistent with the known taxonomic distribution of this enzyme. Importantly, a minor activity was also detected in MSC-EVs, yet in a single batch, which was that exhibiting the highest particle concentration, thereby suggesting that its reliable detection was potentially limited by the sensitivity of the performed enzymatic activity assay.

To promote the visual sorting of EV samples into distinct clusters based on their composite biomolecular properties, our multivariate dataset was subjected to partial least-squares discriminant analysis (PLS-DA), a statistical method that enables dimensionality reduction and discriminant analysis of complex datasets to identify the primary variables contributing to the distinction of the sample groups under evaluation (Lee et al., 2018). Regardless of the normalization approach, this assessment highlighted protein and enzymatic activity levels as the primary source of variation between mammalian and non-mammalian EV preparations, as captured by dimension 1. Conversely, the differentiation of MSC-EVs from the other EV types, primarily driven by differences in their lipid and carbohydrate composition, was identified as the second largest source of variation, as captured by dimension 2. Furthermore, PLS-DA contributed to uncovering distinct associations between EV sample types and certain biochemical features analyzed in this study: BM-EVs with XO activity; AS-EVs with LAP activity; G-NVs with ENO activity; MSC-EVs with cholesterol and carbohydrate contents (Figure 19 A-C).

By evaluating each assessed biocomponent for its total content or total activity recovered in both EV samples and corresponding CTL preparations, the following emerged as the most robustly EV-confined features tested in this study: ChoPL, POD and XO activities in BM-EVs; cholesterol and ENO activity in MSC-EVs and BM-EVs; LAP activity in AS-EVs and G-NVs (Figure 20A-B).

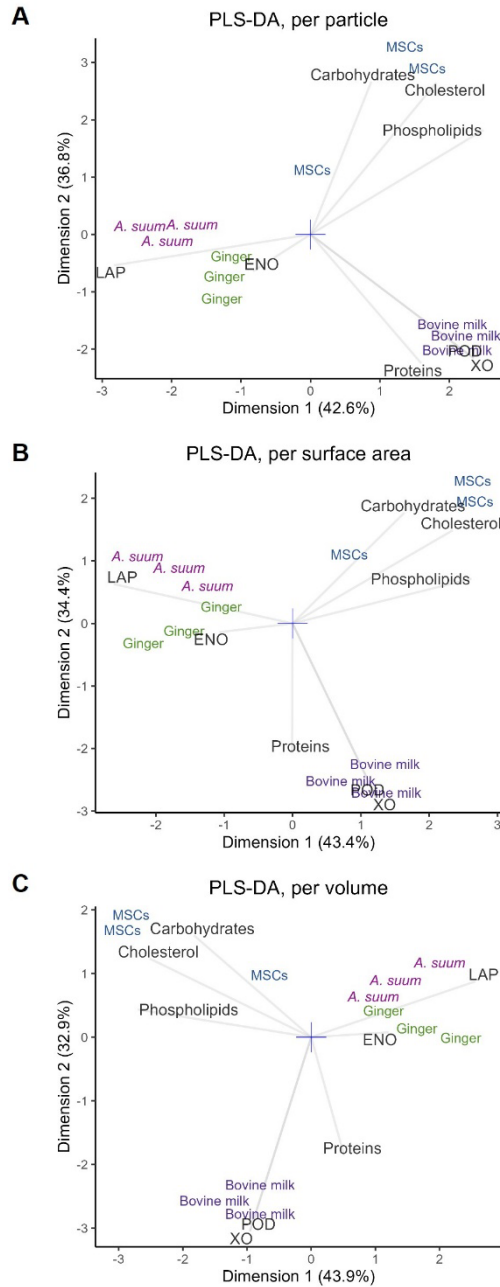
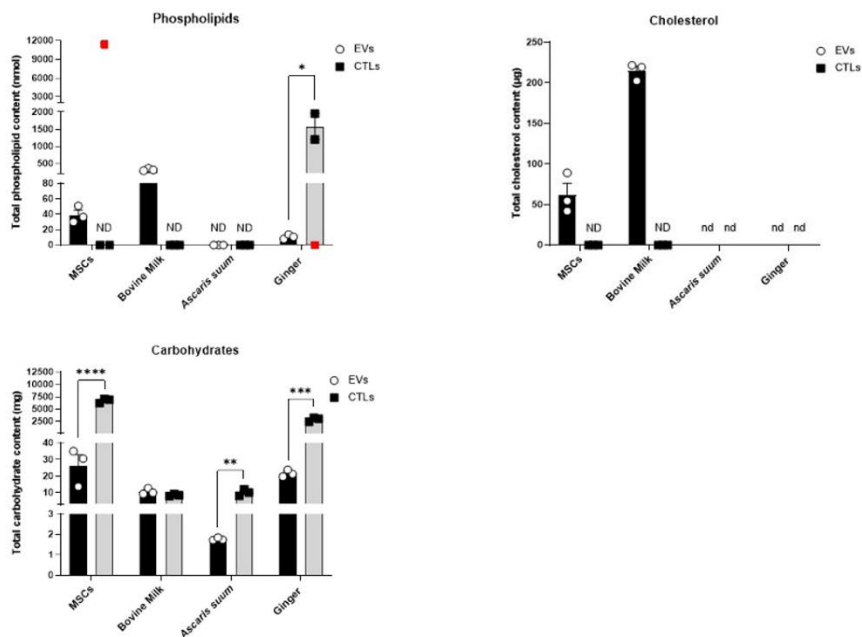


Figure 19. PLS-DA biplot of biochemical parameters identified in MSC-EVs, BM-EVs, AS-EVs, and G-NVs. The evaluated EV biochemical parameters were normalized per (A) particle, (B) particle surface area, and (C) particle volume. The colors corresponding to the different biological sources are the following: blue for MSCs; purple for BM; pink for AS; green for G. The ellipses indicate confidence region at 0.95 level. The number in parentheses shows variance captured by the component. Legend: AS = *Ascaris suum*; BM = bovine milk; ENO = enolase; EV = extracellular vesicle; G = ginger; LAP = leucine aminopeptidase; NV = nanovesicle; MSC = human mesenchymal stem/stromal cell; POD = peroxidase; XO = xanthine oxidase.

A

Total biocomponent content in EVs and CTLs



B

Total enzymatic activity in EVs and CTLs

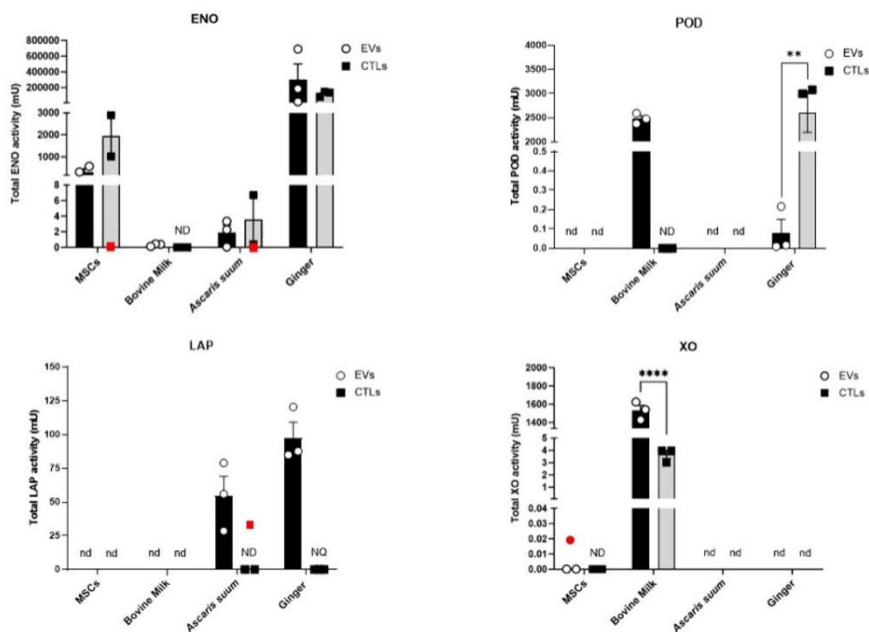


Figure 20. Determination of the association of choline-containing phospholipids, cholesterol, carbohydrates, and enzymatic activities with EVs. The bar graphs illustrate the biocomponent contents of EVs and CTLs derived from MSCs, BM, AS, and G, normalized per total volume. The total yield of phospholipids (nmol), cholesterol (μg), and carbohydrates (mg), and enzymatic activities (ENO, POD, LAP, and XO; mU) was calculated by multiplying their concentrations or activities by the total

recovery volume of the sample. All data are reported as mean $\pm$ SEM from three biological batches corresponding to three independent sample preparations. Statistical analysis was performed between EVs and their corresponding CTLs using ordinary unpaired t-test (not reported p-value: not significant; significant p-value < 0.05 [*], <0.01 [**], <0.001 [***], <0.0001 [****]). The red dot indicates an outlier, which was excluded from the statistical analysis. Abbreviations: AS = *Ascaris suum*; BM = bovine milk; CTL = EV-depleted control; ENO = enolase; EV = extracellular vesicle; G = ginger; LAP = leucine aminopeptidase; MSC = human mesenchymal stem/stromal cell; ND = not detected; nd = not determined; NQ = not quantified; POD = peroxidase; XO = xanthine oxidase.

Collectively, this study demonstrated that the adoption of normalization approaches accounting for the three-dimensional nature of EV particles is relevant for conducting robust comparisons across distinct EV samples and studies, especially in the presence of subtle differences in size distributions, which may introduce interpretive bias into the analysis. This work also identified biochemical parameters that are either common to the EVs investigated in this study or more specific to the selected EV sources. Comparing EVs with their respective CTLs enabled us to further classify them as i) EV-confined, ii) EV-associated but not dominant, and iii) non-EV-associated, underscoring the importance of CTL benchmarking in preventing co-isolated features from being strictly attributed to the EV cargo. Furthermore, our findings highlighted the potential of PLS-DA to delineate generic and source-specific EV fingerprints capable of supporting the definition of robust quality metrics tailored to EV-based products and intended uses, which is currently strongly demanded in the context of translational research and regulatory compliance.

4.4 Robust interpretation of EV functionality requires dynamic mechanistic approaches (Publication IV)

Biological entities with a multiscale nature — simultaneously operating at multiple, interconnected organizational levels of biological systems (*i.e.*, molecular, cellular, tissue, organ, systemic), each governed by its own spatiotemporal scale — and establishing non-linear interactions with the surrounding environment (*e.g.*, signalling cascade triggers, such as receptor-ligand binding or enzyme kinetics) require analytical approaches that can capture their functional effects in a dynamic manner (Banga & Villaverde, 2025). Although EVs fully fit this description, conventional approaches to EV bioactivity assessment have mostly relied on methods that provide readouts at pre-defined, fixed time points, thereby restricting functional interpretation to simplified, likely misleading measurements (Single et al., 2015; Tkach & Théry, 2016). This methodological limitation was demonstrated in **Publication IV**, where the adoption of a high-throughput technology providing real-time assessment of EV functionality *in vitro* enabled us to unveil EV functional traits that could not be captured by single end-point measurements. The specific case study described herein focused on implementing IMC to evaluate the biological response of the human gut bacterium *Bacteroides thetaiotaomicron* (*Bt*) following exposure to nanovesicles derived from ginger rhizome (G-NVs).

Any biological process results in either heat production or absorption (Becker & Kuznetsov, 2015; Wilkie, 1960). This fundamental principle constitutes the foundation of calorimetry, which quantifies the amount and rate of heat released by biological systems as a function of time and temperature, thereby providing an integrative readout of biological activity (Herwaarden, 1999). IMC has been historically implemented to measure and monitor real-time heat changes arising from complex biological processes in closed, small-scale systems maintained at a constant temperature (I. Wadsö, 2002). In

microbiological studies, it is well suited for exploring anaerobic bacterial activity under controlled conditions, converting the heat release associated with bacterial growth to biomass yield, amount of metabolized substrate, and efficiency of carbon source utilization (Braissant et al., 2010b; Kabanova et al., 2012, 2013; Nykyri et al., 2019; Stulova et al., 2015). In addition to enabling dynamic monitoring of microbial metabolic activity in a highly reproducible manner, IMC delivers many other benefits that distinguish it from traditional analytical approaches, including high sensitivity (i.e., LoD of $10E+4$ - $10E+5$ cells), high throughput (i.e., up to 48 parallel mini-calorimeters with 4 mL volume each), and independence from sample optical transparency or the need for specific sample preparation for proper assessment (Braissant et al., 2015; Mauerhofer et al., 2019; L. Wadsö, 2001). Our literature review revealed no studies employing IMC within the context of EV research prior to this investigation. This gap, together with the significant advantages of IMC, represented a primary driver in the selection of this method as an innovative functional approach to address our research objectives.

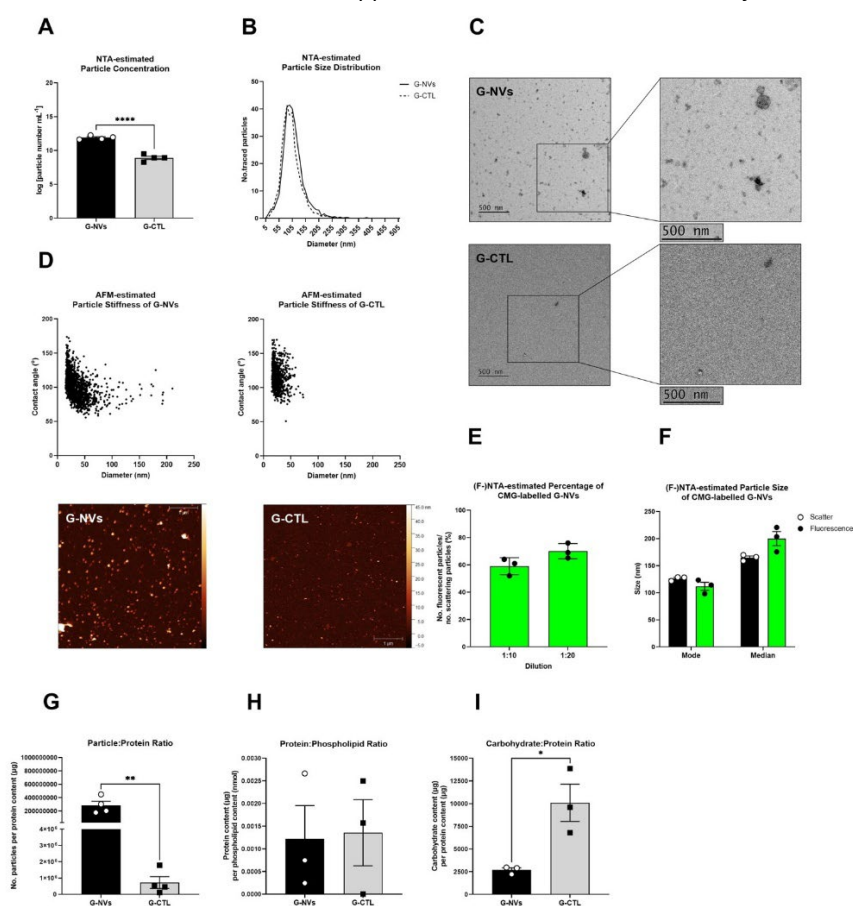


Figure 21. Physicochemical assessment of G-NVs and G-CTL. The bar graphs highlight: (A) NTA-estimated particle concentrations of four biological batches of G-NVs and G-CTL – expressed as particle number per milliliter in logarithmic scale; (E) (F-)NTA-estimated ratio of total number of fluorescent particles over total number of scattering particles detected in three biological batches of CMG-labelled G-NVs – expressed as percentage (%) and measured with two serial dilutions (1:10 and 1:20) in PBS; (F) (F-)NTA-estimated particle mode size (nm) and median size (nm) of three biological batches of CMG-labelled G-NVs measured in scattering and fluorescence mode with 1:10

dilution in PBS; (G) particle:protein ratio of four biological batches of G-NVs and G-CTL – expressed as particle number per protein content (μg); (H) protein:phospholipid ratio of three biological batches of G-NVs and G-CTL – expressed as protein content (μg) per phospholipid content (nmol); (I) carbohydrate:protein ratio of three biological batches of G-NVs and G-CTL – expressed as carbohydrate content (μg) per protein content (μg). The histograms in B depict the NTA-estimated particle size distributions of four biological batches of G-NVs and G-CTL – expressed as average of two to four technical measurements per sample. The (C) TEM and (D) liquid AFM images illustrate, respectively, the morphological and nanomechanical properties of one representative batch of G-NVs and G-CTL. The scatter plots in D show the contact angle ($^\circ$) and equivalent diameter (nm) of each particle detected and analyzed by liquid AFM. The bar graph data are reported as mean $\pm$ SEM. In A, E, G, H, and I, statistical analysis was performed using ordinary unpaired t-test (not reported p-value: not significant; significant p-value: < 0.05 [*], < 0.01 [**], < 0.0001 [****]). In F, statistical analysis was performed between matching scatter and fluorescence values using ordinary unpaired t-test (not reported p-value: not significant). The figure was adapted from Figure 2 in **Publication IV**. The original figure is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Abbreviations: AFM = atomic force microscopy; CMG = CellMask™ Green; G-CTL = G-NV-depleted control; G-NV = ginger-derived nanovesicle; (F-)NTA = (fluorescence) nanoparticle tracking analysis; TEM = transmission electron microscopy.

Our choice of G-NVs as a vesicular model for this investigation was motivated by their ability to modulate the composition of the human gut microbiota (HGM), as previously demonstrated in preclinical settings (Dad et al., 2021; Teng et al., 2018). Importantly, a study highlighted no significant effect of G-NVs on the growth of *Bacteroides fragilis*, used as a reference member of the Bacteroidaceae family. However, a significant stimulatory impact was observed *in vivo*, potentially as a result of positive feedback loops or interactions established within the gut microbial consortium (Dad et al., 2021; Fischbach & Sonnenburg, 2011). These findings provided a strong rationale for selecting *Bacteroides* as suitable microbial genus for our research, as it was assumed to be potentially influenced by G-NVs through mechanisms that might not be readily detectable through non-dynamic analytical approaches. The species *Bt*, an obligate, saccharolytic anaerobe with encouraging exploitable potential as next-generation probiotic (Chang et al., 2019; Jan et al., 2024), was preferentially included in our experimental setup because of its ease of cultivation under anaerobic conditions and its suitability for HGM-focused co-culture studies, as it exhibits predictable interactions with other commensal microbial species (Bacic & Smith, 2008; da Silva et al., 2024; Meslé et al., n.d.; Wexler & Goodman, 2017).

G-NVs were separated, enriched, and recovered from the original matrix as the retentate of a TFF system, characterized by hollow-fibre cartridges with a pore size of 50 nm. To comply with the MISEV guidelines (Théry et al., 2018; Welsh et al., 2024), we assessed a parallel control sample (G-CTL) obtained as the TFF permeate, corresponding to the ginger extract depleted of the G-NV sample. As both samples were sterilized with 0.22 μm filter units before storage and further use, we expected G-NV preparations to be enriched in particles with a size range of 50-200 nm and a typical vesicular structure, while the G-CTL was anticipated to be dominated by non-vesicular constituents smaller than 50 nm. This was confirmed by comprehensive physicochemical characterization, integrating single-particle and bulk analytical approaches. Specifically, according to NTA, G-NVs exhibited enrichment in particles within the expected size range, while the G-CTL revealed a substantially lower concentration of particles with a partially overlapping size

distribution, potentially attributable to hydrodynamic diameter overestimation (Figure 21A-B). (F-)NTA of G-NVs labelled with CMG confirmed the presence of lipid membranous particles (Figure 21E-F). TEM and AFM further revealed that G-NV particles displayed a typical vesicular morphology and membrane stiffness, coexisting with a minor subset of particles smaller than 50 nm, most of which likely corresponded to co-isolated contaminants (*e.g.*, protein aggregates and polysaccharide fragments), as featured by amorphous appearance and higher membrane stiffness. Conversely, the G-CTL displayed enrichment of amorphous and largely stiff particles smaller than 50 nm, indicating selective accumulation of non-vesicular impurities (Figure 21C-D). These observations were further supported by protein and phospholipid quantification, revealing enrichment of phospholipid-rich particles purified from soluble proteins in the G-NV sample, compared with the G-CTL (Figure 21H).

As recommended by the MISEV guidelines (Théry et al., 2018; Welsh et al., 2024), *Bt*'s exposure to G-NVs and G-CTL was conducted in a dose-response manner to determine whether the observed biological outcomes scaled linearly with G-NV sample quantity, thereby discriminating G-NV-mediated effects from those arising from non-vesicular confounding factors. The specific G-NV doses selected for this study were: 20,000 particles/cell (dose 1); 10,000 particles/cell (dose 2); 5,000 particles/cell (dose 3). For parallel G-CTL dosing, we corrected each condition to the equivalent protein content of the corresponding G-NV treatment. We excluded particle count as preferred normalization parameter due to the pronounced depletion and underestimation of particles in the G-CTL by NTA. Indeed, this strategy would have required impractically large G-CTL sample volumes to perform the experiments, while introducing substantial quantification bias, thereby making the experimental setup both unfeasible and unreliable. As proteins represent major constituents of G-NVs and can be reliably quantified also in the G-CTL, we considered protein normalization a practical proxy for total sample mass, as the direct measurement of the latter in G-NVs is technically challenging. This choice was also in line with the literature, where protein content is as widely adopted normalization factor in EV functional assessment studies, alongside particle number (Royo et al., 2020).

Consistently with previous findings showing no effect of G-NVs on the growth of *Bacteroides fragilis*, used as a representative member of the Bacteroidaceae family (Teng et al., 2018), our study demonstrated that our G-NV preparations, up to 20,000 particles per treated cell, did not exert any influence on *Bt*'s heat release either over the entire cultivation period (Q_{tot}) or during the exponential growth phase (Q_{exp}), indicating no change in total biomass production (Figures 22A-B and 23A-B). This observation was also confirmed by OD₆₀₀ measurements (Figure 23C), denoting no change in biomass production, and further enhanced by metabolite profiling, which enabled us to reveal no quantitative alterations in fermentation end-products, such as short-chain fatty acids (SCFAs), herein referred to as organic acids, and free amino acids (FAAs) (Figure 24B-C). In contrast, *Bt*'s treatment with the G-CTL led to a prominent stimulatory effect, as demonstrated by the resulting dose-dependent increase in heat-related readouts, both Q_{tot} and Q_{exp} , OD₆₀₀ measurements (Figures 22A-B and 23A-B), as well as secretion of acetic, succinic, and propionic acids (Figures 24B), overall indicating enhanced fermentation activity. Despite the evident differences between G-NVs and G-CTL in terms of total biomass and metabolite output, it was relevant to observe that both ginger preparations induced a pronounced increase in *Bt*'s specific growth rate (μ_{max}), with G-

NVs yielding a stronger effect, while enhancing maximum metabolic activity (P_{max}) and causing a greater anticipation of the latter (T_{Pmax}) (Figures 22A-B and 23D-F).

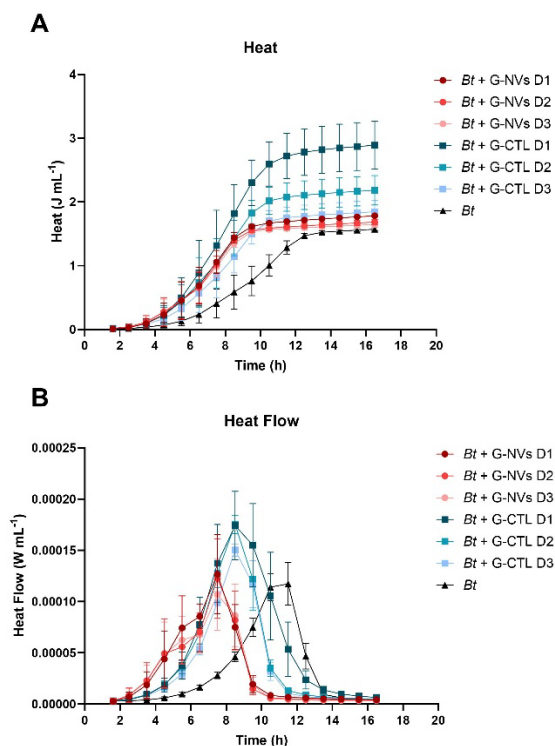


Figure 22. IMC-estimated thermograms of *Bt* grown with or without G-NVs and G-CTL. The line graphs show (A) heat and (B) heat flow curves of *Bt* grown at 37°C for 24 h in growth media containing or not containing either G-NVs or G-CTL at dose 1, dose 2 or dose 3. Data are reported as mean $\pm$ SEM from four biological batches. Abbreviations: *Bt* = *Bacteroides thetaiotaomicron*; D = dose; G-CTL = G-NV-depleted control; G-NV = ginger-derived nanovesicle; IMC = isothermal microcalorimetry. The figure was adapted from Figure 3 in **Publication IV**. The original figure is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) (<https://creativecommons.org/licenses/by-nc-nd/4.0/>).

From these observations, both G-NVs and G-CTL appeared responsible for triggering an earlier metabolic response in *Bt*, yet leading to distinct metabolic outcomes, which remained unaltered with G-NVs and increased with G-CTL. The functional discrepancy between the two samples may be attributable to the higher carbohydrate-to-protein ratio measured in the G-CTL (Figure 21I), compared with G-NVs, given that *Bt* primarily consumes indigestible, high-molecular weight carbohydrates (e.g., dietary fiber and host-derived glycans) (Rivera-Chávez & Bäumlér, 2015; Wexler & Goodman, 2017; Zocco et al., 2007). This interpretation was further reinforced by the absence of changes in FAA levels across all conditions, suggesting *Bt*'s preferential utilization of carbohydrates as its main carbon source (Figures 24A and 24C). On the other hand, we assumed that the higher specific growth rate promoted by G-NVs could be linked to multiple mechanisms that may lead to rapid and synergistic activation of metabolic pathways (Ginini et al., 2022; Y.-J. Liu & Wang, 2023): i) EV-mediated co-transport carbohydrates with other bioactive components (e.g., proteins, lipids, and nucleic acids); ii) potential activation of

signalling cascades by ligand–receptor interactions; iii) putative facilitation of cargo internalization bypassing the lag phase required by the starch utilization system. However, further investigations are warranted to confirm these hypotheses.

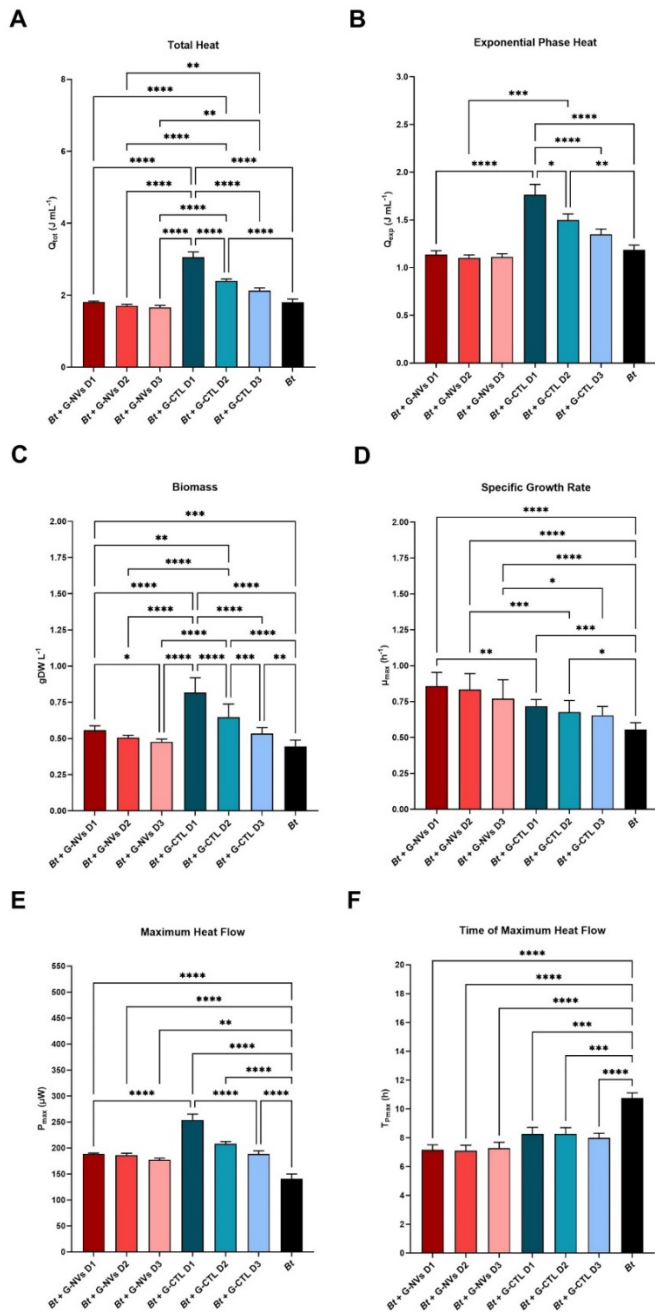


Figure 23. IMC-derived growth characteristics and OD_{600} of Bt grown with or without G-NVs and G-CTL. The bar graphs show (A) the cumulative heat produced during the whole cultivation period (Q_{tot}), expressed as Joule (J) per milliliter, (B) the cumulative heat produced during the exponential

phase (Q_{exp}), expressed as Joule (J) per milliliter, (C) biomass, expressed as grams of dry weight (gDW) per liter, (D) the maximum specific growth rate (μ_{max}), expressed per (h), (E) the maximum power output at the highest peak of the heat flow curve (P_{max}), expressed as microwatt (μW), and (F) the time at which the P_{max} occurs (T_{Pmax}), expressed as time (h), of Bt grown at 37°C for 24 h in growth media containing or not containing either G-NVs or G-CTL at dose 1, dose 2 or dose 3. Data are reported as mean $\pm$ SEM from four biological batches. Statistical analysis was performed using ordinary one-way ANOVA with follow-up test for multiple comparisons (not reported p-value: not significant; significant p-value: < 0.05 [*], < 0.01 [**], < 0.001 [***], < 0.0001 [****]). Comparisons between Bt + G-NVs doses and Bt + G-CTL doses were restricted to matching doses. The figure was adapted from Figure 4 in **Publication IV**. The original figure is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) (<https://creativecommons.org/licenses/by-nc-nd/4.0/>). Abbreviations: Bt = *Bacteroides thetaiotaomicron*; D = dose; G-CTL = G-NV-depleted control; gDW = grams dry weight; G-NV = ginger-derived nanovesicle; IMC = isothermal microcalorimetry.

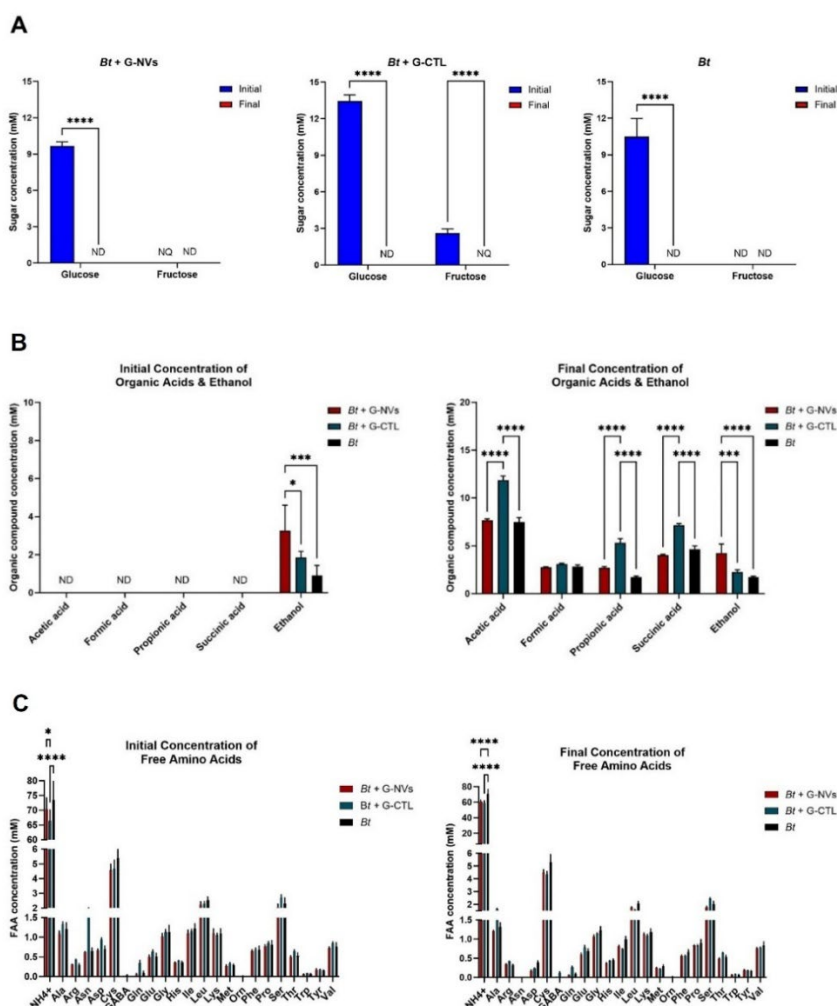


Figure 24. Metabolic profiling of Bt's growth media in the presence or absence of G-NVs and G-CTL. The bar graphs depict initial and final concentrations (mM) of (A) free sugars (glucose and fructose), (B) organic acids and ethanol, and (C) FAAs in non-inoculated and Bt-inoculated media

supplemented with either G-NVs (dose 1), G-CTL (dose 1), or PBS. Initial concentration data are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Final concentration data are reported as mean $\pm$ SEM from two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA followed by multiple-comparison testing (not reported p-value: not significant; significant p-value: < 0.05 [*], < 0.01 [**], < 0.001 [***], < 0.0001 [****]). The figure was adapted from Figures 5–7 in **Publication IV**, with permission from Wiley. Modifications were made. Abbreviations: Ala = alanine; Arg = arginine; Asn = asparagine; Asp = aspartic acid; Bt = *Bacteroides thetaiotaomicron*; Cys = cysteine; FAA = free amino acid; GABA = γ -aminobutyric acid; G-CTL = G-NV-depleted control; Gln = glutamine; Glu = glutamic acid; Gly = glycine; G-NV = ginger-derived nanovesicle; His = histidine; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; ND = not detected; NH_4^+ = ammonium; NQ = not quantified; Orn = ornithine; PBS = phosphate-buffered saline; Phe = phenylalanine; Pro = proline; Ser = serine; Thr = threonine; Trp = tryptophan; Tyr = tyrosine; Val = valine.

In summary, this work demonstrated that both G-NVs and G-CTL can modulate *Bt*'s metabolism, but through different mechanisms: G-NVs elicit a rapid, transient metabolic stimulation, without leading to an increase in total metabolic output, whereas the G-CTL induces a sustained, dose-dependent metabolic response, boosting overall metabolic output. By adopting IMC as a strategic analytical approach for the dynamic analysis of G-NV/G-CTL–*Bt* interactions, we were able to reveal functional traits attributable to G-NVs that could not be captured by end-point assessment of microbial growth at the end of the cultivation period, thereby underscoring the significance of implementing real-time, high-throughput mechanistic approaches to broaden the context-dependent interpretation of EV functionality.

4.5 Multi-criteria decision-making promotes informed, transparent, traceable, and responsible decisions in EV R&I (Publication V)

In a R&I landscape where uncertainties and risks prevail over opportunities, the EV field still lacks methodologies capable of supporting strategic planning and management across all stages of the EV research and product development pipeline. Due to this gap, decision-making is often biased and context-insensitive, ultimately limiting the translational potential of EVs and related processes. In this context, the implementation of MCDM frameworks may enable systematic decision-making for the selection of the most suitable EV sources, study methods, and potential applications, while accounting for multiple EV attributes and ultimately leading to optimization of resource use and performance (Loria et al., 2024). The potential of MCDM in assisting EV researchers in navigating these key decision domains was specifically investigated in **Publication V**, which aimed at exploring the use of the EV decision-making grid (EV-DMG), a WSM-based MCDM matrix, for promoting responsible decisions tailored to EV products and their intended uses. To provide a straightforward and accessible demonstration of the EV-DMG's applicability, we selected EV QC as a proof-of-concept application. This choice was further motivated by the growing attention of the EV community to improve quality management in EV R&D to address current rigor and standardization limitations (G. Liguori & Kisslinger, 2020; G. L. Liguori & Kisslinger, 2022; Paolini et al., 2022).

From our perspective, in EV R&D settings, especially in their early stages, QC activities should not serve solely to grade EV products for a single indication of use, but rather to allocate them into distinct use categories based on their quality grade. In this regard, the

context in which our work was developed constituted a strong pillar of our investigation. This was represented by a research-oriented context at a premature developmental phase, where different EV samples were explored for multiple uses, ranging from their application as substrates for engineering to their deployment as drug delivery systems or intrinsic effectors, either in vitro or in vivo, all supported by promising preliminary evidence (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021, 2022). Our case study was specifically focused on microalgae-derived EVs (i.e., nanoalgosomes), as they represented an EV type that, being newly emerging and relatively unexplored, was particularly vulnerable to limited standardization and high methodological uncertainty, thereby making them suitable for examining and verifying the effectiveness of our approach (Biogenic Organotropic Wetsuits | BOW | Project | Fact Sheet | H2020, n.d.; Loria et al., 2024).

According to recent considerations by the EV community, once gained a comprehensive understanding of the EV product to be formulated for specific applications, efforts should be directed toward scaling up EV QC across product development and manufacturing to maximize efficiency and cost-effectiveness. Prior to this study, nanoalgosomes were subjected to a comprehensive analytical assessment in several lines of research (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021, 2022). Therefore, to comply with these recommendations, the QC methods adopted in this study were restricted to a minimal but informative analytical package, providing robust and sensitive readouts of nanoalgosome quantity, identity, purity, and functionality — defining our QC assessment criteria — while requiring medium-to-low turnaround time and minimal sample consumption (Paolini et al., 2022).

Table 1. Setting up the EV-DMG metrics for nanoalgosome QC – alignment of assessment criteria, sub-criteria, importance weights, and analytical methods. The table was adapted from Figure 3 in Publication V. The original table is licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0) (<https://creativecommons.org/licenses/by-nc/4.0/>). Abbreviations: AFM = atomic force microscopy; BCA = bicinchoninic acid; CONAN = colorimetric nanoplasmonic; ELISA = enzyme-linked immunosorbent assay; (F-)NTA = (fluorescence) nanoparticle tracking analysis; ZP = zeta potential.

Assessment Criteria	Assessment Sub-criteria	Importance Weights	Analytical Methods
Quantity	Scattering particle number per volume unit	3	NTA
Identity	Scattering particle size distribution	2	NTA
	Morphology		AFM
	Membrane H ⁺ /ATPase expression		ELISA
	Surface charge		ZP measurement
Purity	Scattering particle number-to-protein content ratio	3	NTA/BCA
	Fluorescent particle number-to-scattering particle number ratio		(F-)NTA
	Aggregation of gold nanoparticles		CONAN assay
Functionality	Esterase activity	2	Esterase activity assay

Except for (F-)NTA and AFM, demanding the use of costly equipment that is complicated to move, the other analytical methods were selected as they were user-friendly, easily accessible, and affordable.

As envisaged in the construction of a WSM-MCDM matrix, we assigned an importance weight, ranging from 1 (i.e., low importance in the decision-making process) to 3 (i.e., high importance in the decision-making process), to each QC assessment criterion, according to the purposes of our R&D context: 3 out of 3 to quantity and purity, whereas 2 out of 3 to identity and functionality (Table 1).

Table 2. Setting up the EV-DMG metrics for nanoalgosome QC – alignment of assessment criteria, sub-criteria, and acceptance criteria with relative scoring. The table was adapted from Figure 4 in Publication V. The original table is licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0) (<https://creativecommons.org/licenses/by-nc/4.0/>). Abbreviations: x = the measure of the corresponding parameter; ZP = zeta potential.

Assessment Criteria	Assessment Sub-criteria	Acceptance Criteria			
		Score 0	Score 1	Score 2	Score 3
Quantity	Scattering particle number/mL	$x < 5E+10$	$5E+10 < x < 1E+11$	$1E+11 < x < 5E+11$	$x > 5E+11$
	Scattering particle size distribution (mode; nm)	$x < 60$ $x > 200$	$60 < x < 80$ $130 < x < 200$	$80 < x < 90$ $120 < x < 130$	$90 < x < 120$
Identity	Morphology (number of rounded object(s)/10 total objects)	0	1-2	3-5	> 6
	Membrane H ⁺ /ATPase expression (signal-to-noise ratio/1E+10 particles)	$x < 2$	$2 < x < 3$	$3 < x < 4$	$x \geq 5$
	Surface charge (ZP; mV)	$x < -45$	/	/	$-45 < x < -10$
		$x > -10$			
Purity	Scattering particle number-to-protein content (μ g) ratio	$x < 1E+8$ $x > 1E+11$	$x < 2E+8$ $x > 5E+10$	$x < 2E+9$ $x > 1E+10$	$2E+9 < x < 1E+10$
	Fluorescent particle number/ scattering particle number (%)	0	$0.5 < x < 3$	$3 < x < 10$	$x > 10$
	Aggregation of gold nanoparticles	/	NO	Proportional to sample dilution with aggregation index above the 20% threshold	Proportional to sample dilution with aggregation index below the 20% threshold
	Esterase activity (nmol/min/ 5E+10 particles)	$x < 0.5$	$0.5 < x < 1$	$1 < x < 2$	$x > 2$

To provide each nanoalgosome batch with a specific performance score — a parameter that, in our integrated EV-DMG, is essential to calculate the score to assign to each assessment sub-criterion — a relevant set of acceptance criteria and a corresponding scoring system were defined based on empirical data obtained from either high-quality or low-quality nanoalgosome preparations, which were previously

produced for preliminary characterization studies. Our scoring system featured the following evaluation scale: 0 = null; 1 = low; 2 = medium; 3 = high (Table 2).

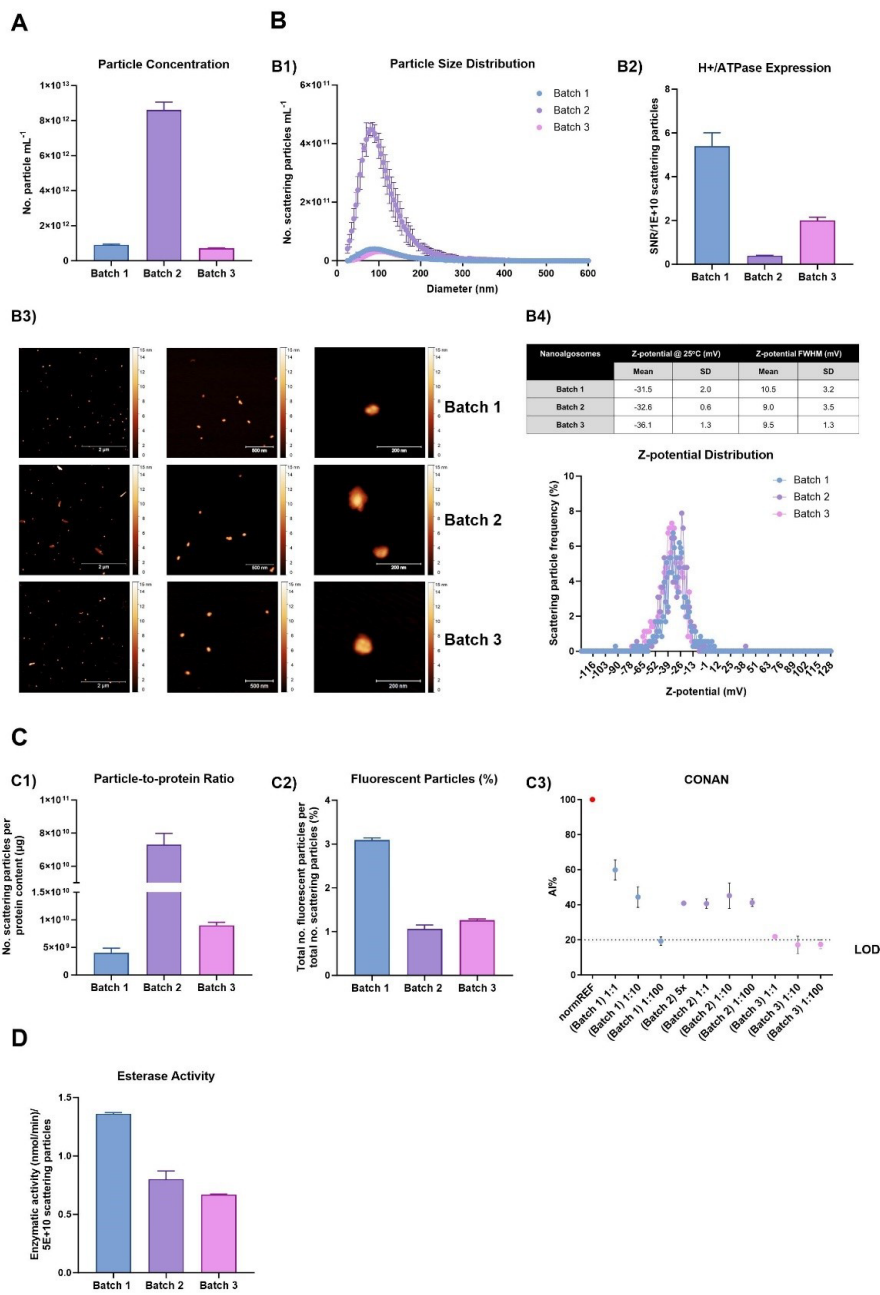


Figure 25. Nanoalgosome QC. (A) Quantity: particle concentration of nanoalgosome batches 1, 2, and 3 – expressed as particle number per milliliter – estimated by NTA in scattering mode. (B) Identity: B1) NTA-measured size distributions of scattering particles detected in nanoalgosome batches 1, 2, and 3; B2) H⁺/ATPase expression revealed by 1E+10 scattering particles in nanoalgosome batches 1, 2, and 3; data are expressed as average SNR ± SD of two technical replicates; B3) morphology analysis performed on nanoalgosome batches 1, 2, and 3 by in-air

tapping mode AFM; the colored scale on the right of each AFM image indicates the height of detected particles; B4) ZP of nanoalgosome batches 1, 2, and 3 – distribution profiles plotting ZP values (mV) against scattering particle frequency (%) and table displaying mean $\pm$ SD of ZP and FWHM values (mV). (C) Purity: C1) ratio of NTA-measured scattering particle number to protein content (μ g) of nanoalgosome batches 1, 2, and 3; C2) ratio of (F-)NTA-measured number Di-8-ANEPPS-labelled fluorescent particles to NTA-measured scattering particle number (%); C3) CONAN assay of nanoalgosome batches 1, 2, and 3 – data are expressed as mean $\pm$ SD of three technical replicates. (D) Functionality and membrane integrity: esterase activity of nanoalgosome batches 1, 2, and 3 – data are expressed as average amount of enzymatic reaction product (nmol) generated by $5E+10$ scattering nanoparticles per time unit (min) $\pm$ SD of two technical replicates. The figure has been adapted from Supplementary Figure 1 in **Publication V**. The original figure is licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0) (<https://creativecommons.org/licenses/by-nc/4.0/>). Abbreviations: AFM = atomic force microscopy; AI = aggregation index; CONAN = colorimetric nanoplasmonic; (F-)NTA = (fluorescence) nanoparticle tracking analysis; FWHM = full width at half maximum; LoD = limit of detection; normREF = pristine gold nanoparticles used for normalization; SNR = signal-to-noise ratio; ZP = zeta potential.

To test the validity of our EV-DMG, our analysis was conducted on three nanoalgosome batches, herein referred to as Batches 1, 2, and 3. QC revealed that they were characterized by comparable, NTA-derived particle concentrations, size distributions, and ZP (Figures 25A, B1, and B4), suggesting no differences in colloidal stability. Nonetheless, ELISA of plasma membrane-bound H⁺/ATPase provided evidence of a higher expression level in Batch 1 and a lower expression level in Batch 2 (Figure 25B2). This trend matched protein purity levels estimated by particle number:protein content ratios (Figure 25C1), providing Batch 1 and Batch 2, respectively, with the smallest and the largest deviation from the theoretical optimal ratio proposed by Sverdllov in 2012 (Sverdllov, 2012), which we adopted as reference value in our purity grading system. This was validated by colorimetric nanoplasmonic (CONAN) assay (Figure 25C3) and (F-)NTA of fluorescently labelled nanoalgsomes (Figure 25C2), which further revealed a lower concentration of non-vesicular particles in Batch 1 and a higher contamination by soluble proteins in Batch 2. AFM supported these observations (Figure 25B3), also enabling the visualization of elongated objects in Batch 2, which we attributed to the presence either of microalgal cell debris or artificial fibres detached from the TFF cartridges. Finally, by assessing EV esterase activity as an established proxy for EV functionality and membrane integrity, Batch 1 emerged as the EV preparation with the highest quality (Figure 25D). Furthermore, despite the substantial difference in particle-to-protein ratio between Batch 2 and Batch 3, their similar esterase activity per particle likely reflected the NTA-based overestimation of particle counts in Batch 2, potentially attributable to the detection of the artificial objects observed by AFM as false positives.

Based on the degree of conformity to our pre-defined acceptance criteria, the performance of each batch for each analytical assessment was assigned an analytical assessment score. Then, this was weighted according to the importance weight of its corresponding assessment criterion, ultimately generating an assessment criterion score. By summing up all assessment criterion scores, Batch 1, 2, and 3 were given, respectively, the following actual final grades: 59/66 (89.4% of the theoretical final grade), 36/66 (54.5% of the theoretical final grade), and 52/66 (78.8% of the theoretical final grade) (Table 3).

Table 3. EV-DMG for nanoalgosome QC. The table was adapted from Figure 5 in **Publication V**. The original table is licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0) (<https://creativecommons.org/licenses/by-nc/4.0/>). Abbreviations: AA = analytical assessment criterion score; W = importance weight.

Assessment Criteria	Assessment Sub-criteria	Importance Weights (W)	Nanoalgosome Batch 1		Nanoalgosome Batch 2		Nanoalgosome Batch 3	
			AA	AA x W	AA	AA x W	AA	AA x W
Quantity	Scattering particle number per volume unit	3	3	9	3	9	3	9
Identity	Scattering particle size distribution	2	3	6	3	6	3	6
	Morphology		3	6	2	4	3	6
	Membrane protein H ⁺ /ATPase expression		2	4	0	0	1	2
	Surface charge		3	6	3	6	3	6
Purity	Scattering Particle number-to-protein content ratio	3	3	9	1	3	3	9
	Fluorescent particle number/ scattering particle number (%)		2	6	1	3	1	3
	Aggregation of gold nanoparticles		3	9	1	3	3	9
Functionality	Esterase activity	2	2	4	1	2	1	2
Actual Final Grade				59/66		36/66		52/66
Actual Final Grade/ Theoretical Final Grade (%)				89.4		54.5		78.8

Based on these results, each batch could be systematically allocated into one of the following QC categories: category A, including batches with an actual final grade of 70–100% of the theoretical final grade, which could be considered high-quality and suitable for all applications, after being confirmed sterile; category B, comprising batches with an actual final grade $\geq 30\%$ up to $< 70\%$ of the theoretical final grade, which could be defined as intermediate-quality batches, thus compatible only for engineering purposes, but not for functional testing; category C, covering batches with an actual final grade lower than 30% of the theoretical final grade, thereby representing low-quality batches to be discarded or restricted to non-critical analyses (Table 4). Accordingly, Batch 1 and Batch 3 were ranked as suitable for all downstream applications, including in vitro and in vivo use, whereas Batch 2 was considered appropriate only for engineering applications. The reliability and practical utility of this structured QC ranking framework were further supported by feedback received from our project partners, who conducted independent assessments of the three batches, which were consistent with the performance predicted by the EV-DMG grading system.

Table 4. EV-DMG-based QC classification of nanoalgosomes. The table was adapted from Figure 6 in **Publication V**. The original table is licensed under the Creative Commons Attribution-NonCommercial 4.0 International License (CC BY-NC 4.0) (<https://creativecommons.org/licenses/by-nc/4.0/>). Abbreviations: x = the measure of the corresponding parameter.

Nanoalgosome QC Classification System						
Level	Description	Actual/Theoretical Grade (%)	Use(s)			
			No	Not Critical	Engineering	Functional Study
A	High Quality	$70 \leq x \leq 100$		x	x	x
B	Medium Quality	$30 < x < 70$		x	x	
C	Low Quality	< 30	x	x		

In summary, using QC as an illustrative application scenario, this work showcased the potential of our EV-DMG model in supporting informed, traceable, and responsible evaluations of EV-based products and processes. The strength of this framework lies in its capacity for customization, enabling the proactive adjustment of assessment criteria and relative weighting according to the specific study context, as well as in its ability to guide strategic, process-driven resource allocation, thereby supporting not only systematic, fit-for-purpose decision-making, but also performance and cost optimization. Furthermore, by providing documented and traceable decision records, the EV-DMG may serve as a valuable regulatory resource, which could be leveraged to enhance regulatory communication and lead to more efficient, faster-paced review and approval times by the national regulatory authorities. Collectively, the EV-DMG's capabilities underscore the importance and potential of fully embedding the core principles of RR&I into everyday EV R&I practices to promote the successful translation of EV innovations into marketable solutions that comply with regulatory requirements.

5 Conclusions

The present thesis contributed to expanding the current knowledge of EV heterogeneity and advancing the understanding of how methodological choices shape EV analytical assessment, comparison, functional interpretation, and translational implementation. Additionally, this work introduced novel methodological approaches that can strengthen EV assessment, engineering, and application, while supporting more informed, traceable, and responsible decision-making in EV research and translational settings. More specifically, the thesis addressed key EV research gaps along multiple interconnected and interdependent domains — methodological, biological, and translational — thereby demonstrating that meaningful progress in the EV field requires context-aware data interpretations and priority-driven decision-making.

At the methodological level, the thesis demonstrated that analytical, engineering, and normalization strategies fundamentally shape the reliability, reproducibility, and comparability of EV data, ultimately affecting EV biological interpretation. Specifically, it yielded the following findings.

1. Diverse analytical platforms and EV labelling approaches provide distinct outcomes in terms of EV quantification and subpopulation profiling. In particular, the comparison between NTA and nFCM proved that different analytical sensitivities and resolutions strongly influence the ability to detect and characterize EV subpopulations, resulting in platform-dependent representations of EV heterogeneity. These results highlight the need for rigorous, platform-specific, and fit-for-purpose optimization, implementation, and interpretation strategies when generating and comparing EV data (**Publication I**).
2. Upon EV engineering, we demonstrated that ensuring the biological activity of loaded cargo does not necessarily require compromising intrinsic EV integrity and functionality. This was exemplified by a novel sodium carbonate-based loading strategy. Compared with the commonly used electroporation, this approach achieved either comparable or even superior encapsulation efficiency of exogenous cargo, depending on the cargo type, while preserving EV physicochemical characteristics, cellular uptake, and cargo biological activity (**Publication II**).
3. We observed that, when comparing data from different EV samples, it is important to consider applying normalization strategies that account for the three-dimensional nature of EV particles, particularly when subtle differences in size distributions are apparent, as well as to rely on multivariate statistical approaches, such as PLS-DA, to better interpret the multidimensionality of EV datasets (**Publication III**).

At the biological level, the thesis highlighted the importance of adopting dynamic mechanistic approaches to obtain more comprehensive insight into EV-mediated processes, thereby strengthening translational decision-making. In the specific case study described herein, where G-NVs were explored for their effect on the growth and metabolic activity of the human gut microbe Bt, the implementation of IMC as a functional analytical tool enabled the identification of a broader spectrum of G-NV functional traits that would remain undetected using conventional end-point measurements performed at the end of the cultivation period, thereby opening new avenues for application (Publication IV).

At the translational level, the thesis contributed to highlighting a critical shift in the EV field, revealing that its primary bottleneck has progressively evolved from insufficient

methodological guidance to limited contextual interpretation and responsible decision-making. This work addressed such an emerging challenge by introducing a customizable, user-friendly, transferable, and regulatory-aligned MCDM framework, which was termed EV-DMG, enabling the systematic and informed evaluation of EV samples and related processes based on multiple weighted criteria, thereby supporting fit-for-purpose decision-making, while promoting performance optimization and cost efficiency (Publication V).

Collectively, these methodological observations and developments are expected to encourage EV researchers to reframe their approach to EV investigation and exploitation, fostering more rigorous scientific interpretation, greater comparability across studies, and more informed translational decision-making.

6 Ongoing Work and Future Perspectives

Ongoing experimental work aims at further expanding the EV normalization strategies adopted in **Publication III** by integrating fluorescence-based EV labelling approaches that promote selective staining of vesicular membranes, while reducing false-positive signals and nonspecific background derived from unbound dye. This activity is mainly intended to reinforce comparability of different EV samples across sources and study contexts, where bias may be introduced through the adoption of different EV enrichment procedures. Furthermore, the analytical and QC frameworks developed throughout this thesis are currently serving as the cornerstone for the management of a recently funded Applied Research and Experimental Development grant (“Rakendusuringu ja tootearenduse toetus”), awarded by Enterprise Estonia (Grant Agreement No. RES.5.04.25-0824, 2026-2028), which is entitled “Potency Assay and Novel Avenues for Clinical Evaluation of AngioARC or derived EV-based Therapeutics PANACEA/EV”. This project aims at advancing the clinical development of a patented autologous MSC therapy for Critical Limb Ischemia, named AngioARC, by setting up robust potency assays and exploring AngioARC-derived EVs as a stable, scalable, and off-the-shelf therapeutic alternative. In this setting, the methodologies described in **Publications I, III, and V** are being strategically applied to support responsible manufacturing, characterization, QC, and formulation development of AngioARC-derived EVs, in alignment with regulatory requirements.

The research of this thesis also lays the foundation for future R&D initiatives, all aimed at ensuring the long-term sustainability and translational relevance of the thesis’s outputs. These range from methodological developments to biological investigations and translational advancements, as follows.

- **Methodological perspectives** — Efforts will be focused on expanding the cross-taxa EV comparative analysis described in **Publication III** to EV sources that were not included in this study, such as bacteria and microalgae. The extended panel of EV sources will be also processed through an identical EV enrichment workflow, applying the same EV source pre-clearing step, when feasible, to enhance discrimination of EV-specific physicochemical traits from potential method-dependent artifacts (Poupardin et al., 2024). Moreover, the application of IMC as a strategic analytical tool for assessing cellular dynamic response to EV treatment, introduced in **Publication IV** using a microbial cell model, will be also explored in aerobic cell systems, such as mammalian cells, where it has been previously demonstrated to provide robust, real-time measurements of cell growth, metabolism, viability, pharmacological responses, and functional potency (Bokhari et al., 2021; Braissant et al., 2010a).
- **Biological perspectives** — The work conducted in **Publication IV** will be further expanded through supplementary analyses (*i.e.*, intermediate time-point IMC analysis, multi-omics characterization, carbohydrate-matched control experiments, bacterial EV profiling, uptake assays) and extended to additional microbial and plant-derived vesicle (PDV) model systems. This is expected to support mechanistic understanding of PDV–microbe interactions, enhance the commercial potential of PDVs as nutraceuticals and microbiome-targeted therapeutic solutions, while contributing to advancing current fermentation design and process optimization strategies (Di Renzo & Reale, 2025; Helal et al., 2019; Jardine & McDowell, 2023; Lemon et al., 2012; Loria et al., 2026).

- Translational perspectives — The EV-DMG showcased in **Publication V** is planned to be developed from its current static version into a computational framework, integrating advanced statistics and predictive modelling. This will ensure the enhancement of complex data interpretability, ultimately improving MCDM effectiveness and implementation readiness. Within the framework of a clinically oriented HORIZON-HLTH-2026-01-TOOL-05 grant application, this upgrade has been designed to support EV process optimization and the release of regulatory-compliant EV batch quality scores, predictive potency scores, patient stratification, and EV batch-to-patient matching.

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Abstract

Extracellular Vesicles: Novel Approaches for Characterization, Functional Assessment, and Translation

Over the past few decades, extracellular vesicles (EVs) have attracted growing scientific and commercial interest because of their potential to reshape multiple areas of nanoscience, providing competitive clinical and industrial solutions across diverse sectors. Their promise arises from their distinctive identity as naturally occurring biological particles, ranging from nanoscale to microscale dimensions, which are enclosed by a lipid bilayer and carry a variety of biomolecular cargo that can reflect the identity and status of their parental cells. Such properties confer upon EVs the fundamental ability to mediate cell-to-cell and organism-to-organism communication, thereby enabling a broad spectrum of applications, spanning their use as diagnostic tools and their adoption as delivery vectors with intrinsic or extrinsic effector molecules. However, regardless of their specific cell of origin, EVs are highly heterogeneous in their size, physicochemical, and functional attributes, which makes their study and exploitation particularly challenging to manage and standardize.

To unlock the full potential of EVs, recent debates within the EV community have largely revolved around implementing rigor and standardization approaches to address key constraints associated with EV manufacturing, analytical assessment, and quality management. Major methodological limitations comprise the lack of suitable reference materials, standardized protocols, and robust data normalization strategies, collectively contributing to method-dependent discrepancies in EV recovery and purity, insufficient resolution of EV subpopulations, significant variability in and poor comparability between datasets generated from different studies or samples. Another bottleneck arises from the common use of EV engineering approaches for loading exogenous molecules that focus on maximizing cargo encapsulation efficiency at the expense of EV structural integrity, thereby often resulting in impaired downstream functional performance. A biologically relevant constraint that is frequently overlooked is the widespread practice of evaluating EV bioactivity through assays that primarily generate end-point measurements, rather than reflecting the dynamic nature of EV-induced biological responses. As a result, this may oversimplify underlying EV mechanisms and potentially promote misleading biological conclusions. From a translational perspective, the EV field requires the adoption of methodological frameworks that help EV stakeholders navigate the multidimensionality of EV decision-making, accounting for the biases introduced by methodological choices and the study context. The current thesis aimed at exploring and tackling several of these constraints by developing and evaluating novel methodological approaches for reinforcing the robustness of EV analytical assessment, engineering, and translational use, as well as promoting more responsible and sustainable decision-making processes across both research and product development pipelines.

Using EVs from sources of distinct taxonomic classification (*i.e.*, human, bovine, parasitic helminth, or plant) and specific biological origin (*i.e.*, human cell conditioned media, bovine milk, parasitic helminth conditioned media, or ginger rhizome), this work implemented a comprehensive set of methodologies across diverse studies, each distinguished by specific objectives and experimental models. These encompassed: i) EV enrichment and separation tailored to both EV sources and study contexts; ii) EV labelling

with either fluorescent membrane or cytoplasmic dyes; iii) EV loading with either fluorescent or bioactive cargo by 1 M sodium carbonate treatment vs. electroporation; iv) physicochemical characterization, integrating imaging, single-particle, and bulk analyses; v) EV functional assessment, either end-point or real-time; vi) extracellular metabolite profiling; vii) EV data analysis, including partial least-squares discriminant analysis (PLS-DA).

At the methodological level, by comparing the performance of nanoparticle tracking analysis and nanoscale flow cytometry, two common methods used for EV particle concentration measurement and size distribution assessment, the thesis established that quantification and identification of heterogeneous EV subpopulations are fundamentally affected by the selected analytical and labelling approaches, thereby requiring rigorous, platform-specific, fit-for-purpose optimization strategies to ensure reliable measurements (**Publication I**). It was also demonstrated that, upon exogenous EV engineering by sodium carbonate-based alkaline treatment, efficient cargo loading, successful cargo delivery to recipient cells, and potent cargo biological activity can be achieved without disrupting EV physicochemical characteristics and inherent pleiotropic functional capabilities (**Publication II**). Furthermore, the comparative assessment of distinct EV samples was shown to be highly influenced by the dimensionality of the selected normalization strategy, showcasing metrics reflecting the three-dimensional nature of EV particles (*i.e.*, volume) as promising alternatives to traditional dimensionless (*e.g.*, particle number) and single-dimensional (*e.g.*, protein content) parameters. In this context, PLS-DA emerged as a powerful multivariate statistical tool for strengthening the interpretation of normalized EV datasets by reducing their multidimensionality (**Publication III**). At the biological level, the adoption of isothermal microcalorimetry, providing real-time functional readouts, to investigate the influence of ginger-derived nanovesicles (G-NVs) on the growth and metabolic activity of the human gut microbe *Bacteroides thetaiotaomicron* enabled to unveil G-NV functional traits that would remain undetected using conventional end-point measurements performed at the end of the cultivation period (**Publication IV**). Finally, at the translational level, the thesis introduced the EV decision-making grid (EV-DMG) as customizable, user-friendly, transferable, and regulatory-aligned multi-criteria decision-making (MCDM) framework designed to enable the systematic and informed evaluation of EV samples and related processes across multiple weighted criteria. In a specific case study focused on EV quality control, this tool demonstrated its ability to support fit-for-purpose decision-making, while fostering performance optimization and cost efficiency (**Publication V**).

In conclusion, this thesis addressed EV heterogeneity and related uncertainty as a multidimensional challenge, resulting in multiple methodological advances that strengthen the robustness and translational potential of EV data assessment, engineering, interpretation, and use. Overall, these contributions are anticipated to promote greater robustness of scientific practices, improve cross-study comparability, and facilitate more effective development and commercialization of EV-based products.

Lühikokkuvõte

Ekstratsellulaarsed vesiikulid: uudsed lähenemisviisid nende iseloomustamiseks, funktsionaalseks hindamiseks ja kliiniliseks rakendamiseks

Viimaste aastakümnete jooksul on ekstratsellulaarsed vesiikulid (EV-d) pälvinud üha suuremat teaduslikku ja kommertsiaalset huvi tänu nende potentsiaalile kujundada ümber mitmeid nanoteaduse valdkondi ning pakkuda konkurentsivõimelisi kliinilisi ja tööstuslikke lahendusi erinevates rakendusvaldkondades. Nende potentsiaal tuleneb nende ainulaadsest olemusest looduslikult esinevate bioloogiliste osakestena, mille mõõtmed ulatuvad nanoskaalast kuni mikroskaalani, nad on ümbritsetud lipiidse kaksikkihiga ning kannavad mitmekesist biomolekulaarset lasti, mis peegeldab ka nende lähterakkude identiteeti ja seisundit. Need omadused annavad EV-dele võime vahendada rakkudevahelist ja organismidevahelist kommunikatsiooni, võimaldades laia rakenduste spektrit, alates diagnostilistest rakendustest kuni kohtetoimetamisvektoriteni, mis kannavad kas päritolurakkude või eksogeenseid bioaktiivseid molekule. Sõltumata nende rakulisest päritolust on EV-d väga heterogeensed nii suuruse kui ka füüsikalise-keemiliste ja funktsionaalsete omaduste poolest, mis muudab nende uurimise, rakendamise ja standardiseerimise eriti keerukaks.

EV-de täieliku potentsiaali rakendamiseks on viimastel aastatel EV-de teaduskogukonnas keskendunud rangemate ja standardiseeritud lähenemisviiside väljatöötamisele, et lahendada peamised probleemid, mis on seotud EV-de tootmise, analüütilise hindamise ja kvaliteedijuhtimisega. Olulisemateks meetodilisteks kitsaskohtadeks on sobivate võrdlusmaterjalide, standardiseeritud protokollide ja usaldusväärsete andmete normaliseerimise strateegiate puudumine, mis kokkuvõttes põhjustavad meetodist sõltuvaid erinevusi EV-de saagistes ja puhtuses, EV alampopulatsioonide ebapiisavat eristamist ning märkimisväärset varieeruvust ja vähest võrreldavust erinevate uuringute või proovide vahel. Teiseks oluliseks piiranguks on EV-de eksogeensete molekulide laadimise meetodite puudulik arendus - nende eesmärk on sageli maksimeerida lasti kapseldamise efektiivsust EV-de struktuurse terviklikkuse arvelt, vähendades seeläbi nende järgnevat funktsionaalset võimekust. Sageli tähelepanuta jäetav bioloogiline piirang seisneb ka selles, et EV-de bioaktiivsust hinnatakse peamiselt lõpp-punkti mõõtmistel põhinevate analüüside abil, mis ei kajasta EV-de poolt esile kutsutud bioloogiliste protsesside dünaamilist olemust. Selle tulemusena võivad EV-de toimemehhanismid jääda liialt lihtsustatuks ning bioloogilised järeldused osutada eksitavateks. Translatsiooniliste rakenduste seisukohalt vajab EV-de valdkond meetodilisi arendusi, mis aitaksid sidusrühmadel teha teadlikumaid otsuseid, võttes arvesse nii meetodiliste valikute kui ka uuringukonteksti põhjustatud kallutatusi. Käesoleva doktoritöö eesmärk oli uurida ja käsitleda mitmeid neist piirangutest, töötada välja ja hinnata uusi meetodilisi lähenemisviise EV-de analüütilise hindamise, modifitseerimise ja translatsioonilise rakendamise usaldusväärsuse suurendamiseks ning süsteemsemate, vastutustundlikumate ja kestlikumate otsustusprotsesside edendamiseks nii teadusuuringutes kui ka tootearendustes.

Töös kasutati erineva taksonoomilise päritoluga (inimene, veis, parasiitne helmint, taim) erinevatest bioloogilistest allikatest (inimese rakukultuuri konditsioneeritud sööde, veisepiim, parasiitse helmindi konditsioneeritud sööde või ingveri risoom) eraldatud EV-sid, samuti erinevate uuringute meetodikate kogumit, mis hõlmas: i) EV-de rikastamist ja

eraldamist vastavalt nende päritolule ja uuringukontekstile; ii) EV-de märgistamist membraani- või tsütoplasmaatiliste fluorestseeruvate värvainetega; iii) EV-de laadimist fluorestseeruva või bioaktiivse lastiga, kasutades 1 M naatriumkarbonaadiga töötlemist või elektroporatsiooni; iv) füüsikalis-keemilist iseloomustamist, ühendades kuvamis-, üksikosakeste- ja koondanalüüse; v) EV-de funktsionaalset hindamist nii lõpp-punkti kui ka reaajas läbiviidud mõõtmiste abil; ning vi) rakuväliste metaboliitide profileerimist.

Metoodilisel tasandil näitas nanopartiklite jälgimisanalüüsi (NTA) ja nanoskaalalise voolutsütomeetria võrdlus, et EV-de osakeste kontsentratsiooni määramist ja suurusjaotuse hindamist mõjutavad oluliselt valitud analüütilised ja märgistamismeetodid. Seetõttu nõuab heterogeensete EV alampopulatsioonide usaldusväärne kvantifitseerimine ja tuvastamine rangelt optimeeritud, platvormispetsiifilisi ning konkreetseks eesmärgiks sobivaid analüüsistrateegiaid (**Publikatsioon I**). Samuti näidati, et EV-de eksogeensel modifitseerimisel naatriumkarbonaadil põhineva aluselise töötlemisega on võimalik saavutada tõhus lasti laadimine, efektiivne lasti kohaletoimetamine sihtrakkudesse ning tugev bioloogiline toime, kahjustamata seejuures EV-de füüsikalis-keemilisi omadusi ega nende loomupäraseid pleiotroopseid funktsioone (**Publikatsioon II**). Lisaks selgus, et erinevate EV-proovide võrdlev hindamine sõltub oluliselt kasutatud normaliseerimisstrateegia ruumigeomeetria mõõtmelisusest. Kolmemõõtmelise olemust kirjeldavad näitajad, näiteks EV-de ruumala, osutusid paljulubavateks alternatiivideks traditsioonilistele (nt osakeste arv) või ühemõõtmelistele (nt valgusisaldus) parameetritele. Selles kontekstis osutus osaliste vähimruutude diskriminantanalüüs (PLS-DA) tõhusaks mitmemõõtmeliseks statistiliseks meetodiks, mis võimaldab normaliseeritud EV-andmetike tõlgendamist ja kasutamist parandada nende mõõtmelisuse vähendamise kaudu (**Publikatsioon III**). Bioloogilisel tasandil võimaldas isotermilise mikrokaloremeetria kasutamine, mis annab reaajas funktsionaalseid mõõtmistulemusi, uurida ingverist pärinevate nanovesiikulite (G-NV-de) mõju inimese soolestiku mikroobi *Bacteroides thetaiotaomicron* (Bt) kasvule ja metaboolsele aktiivsusele ning paljastada G-NV-de funktsionaalseid omadusi, mis jäid tavapäraste lõpp-punkti bioaktiivsuse analüüside abil tuvastamata (**Publikatsioon IV**). Translatsioonilisel tasandil tutvustati doktoritöös EV otsustusmaatriksit (EV decision-making grid, EV-DMG) kui kohandatavat, kasutajasõbralikku, ülekantavat ja regulatiivsete nõuetega kooskõlas olevat mitmekriteeriumilise otsustamise (MCDM) metoodilist platvormi, mis võimaldab EV-proovide ja nendega seotud protsesside süstemaatilist ning teadlikku hindamist mitme kaalutud kriteeriumi alusel. EV-de kvaliteedikontrolli käsitlevas juhtumiuuringus näitas see tööriist oma sobivust eesmärgipärase otsustamise toetamisel ning protsesside tulemuslikkuse ja kulutõhususe parandamisel (**Publikatsioon V**).

Kokkuvõttes käsitles käesolev doktoritöö EV-de heterogeensust ja sellega seotud ebakindlust mitmemõõtmelise väljakutsena, mille tulemusena töötati välja mitmeid metoodilisi lahendusi EV-andmete hindamise, modifitseerimise, tõlgendamise ja rakendamise usaldusväärsuse ning translatsioonilise potentsiaali suurendamiseks. Lisaks demonstreeriti mitmekriteeriumilise otsustamise rakendamist EV-de valdkonnas uudse lähenemisviisina, mis soodustab süsteemsemat, teadlikumat, jälgitavamat, kontekstitundlikumat ja vastutustundlikumat otsustamist erinevates teadus- ja translatsioonilistes rakendustes. Kokkuvõttes eeldatakse, et käesoleva töö tulemused aitavad suurendada teaduspraktika usaldusväärsust, parandada eri uuringute tulemuste võrreldavust ning toetada EV-põhiste toodete tõhusamat arendamist ja kommertsialiseerimist.

Appendix 1

Publication I

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Article

Opportunities and Pitfalls of Fluorescent Labeling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms

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Abstract: The relevance of extracellular vesicles (EVs) has grown exponentially, together with innovative basic research branches that feed medical and bioengineering applications. Such attraction has been fostered by the biological roles of EVs, as they carry biomolecules from any cell type to trigger systemic paracrine signaling or to dispose metabolism products. To fulfill their roles, EVs are transported through circulating biofluids, which can be exploited for the administration of therapeutic nanostructures or collected to intercept relevant EV-contained biomarkers. Despite their potential, EVs are ubiquitous and considerably heterogeneous. Therefore, it is fundamental to profile and identify subpopulations of interest. In this study, we optimized EV-labeling protocols on two different high-resolution single-particle platforms, the NanoFCM NanoAnalyzer (nFCM) and Particle Metrix ZetaView Fluorescence Nanoparticle Tracking Analyzer (F-NTA). In addition to the information obtained by particles' scattered light, purified and non-purified EVs from different cell sources were fluorescently stained with combinations of specific dyes and antibodies to facilitate their identification and characterization. Despite the validity and compatibility of EV-labeling strategies, they should be optimized for each platform. Since EVs can be easily confounded with similar-sized nanoparticles, it is imperative to control instrument settings and the specificity of staining protocols in order to conduct a rigorous and informative analysis.

Keywords: extracellular vesicles; exosomes; flow cytometry; nanoparticle tracking analysis; fluorescent dyes; purification; isolation; subpopulations; tetraspanin; antibody

1. Introduction

For the past two decades, EV research has risen exponentially along with the outstanding discoveries that have revealed numerous biological functions mediated or directly executed by EVs. These membrane-enclosed nanoparticles virtually encapsulate any biomolecule type found in respective donor cells, namely: DNA, RNA, proteins, lipids, or metabolites [1–3]. Nearly all of the cells in the human body actively secrete EVs, which can circulate through all sorts of biological fluids such as plasma, urine, or saliva [1]. Recently, the scientific community realized that EVs were significantly promising non-invasive indicators of an individual's global health status. This sparked the race for the discovery of EV-specific biomarkers, with the goal of improving or enabling the detection of a number of diseases and translating novel non-invasive practices into routine clinical use. Since small EVs (sEVs), typically in a nanoscale range (30–200 nm), are highly heterogeneous and indistinguishable from other biological nanoparticles, the absolute need to

perform true single-vesicle discrimination, analysis, and characterization quickly emerged. For this purpose, high-resolution methodologies, such as nanoparticle tracking analysis (NTA) [4–8], tunable resistive pulse sensing (TRPS) [9,10], Raman spectroscopy [11–13], atomic force microscopy [14,15], super-resolution microscopy [16–19], or nanoflow cytometry [20–24], have been explored [25,26]. Techniques relying on label-free EV analysis can estimate particle size, concentration, and other physical parameters such as zeta potential. Methods focused exclusively on fluorescence measurements help to determine the nature or composition of labelled particles within the samples. Hence, it becomes clear that approaches incorporating both label-free and fluorescence measurements may provide the necessary robustness to discriminate true sEVs in complex samples and to quantitatively characterize relevant subpopulations, often present in extremely low abundance.

In conventional flow cytometers, light scatter measurements alone enable a high-throughput multiparametric analysis of microscopic particles, which can be combined with fluorescent labeling to pinpoint and characterize specific components or biological processes. However, due to the physical properties of particles and light, conventional flow cytometers fail to detect events under 200 nm [27]. Summarily, the intensity of scattered light decreases by orders of magnitude (sixth power), for particles with size smaller than the wavelength of the incident light [28,29]. In order to accurately detect sEVs, dedicated state-of-the-art systems have been developed to increase the sensitivity of nanoparticle profiling in sheathed flow.

NTA has been extensively used for counting and estimating the size of particles based on their Brownian motion in suspension. This platform has a high-resolution capability, detecting biological particles as small as 30 nm; however, measurements of larger particles (>1 μ m) tend to be less accurate due to their slower movement [25,30]. Aside from size and concentration, multiple additional parameters can be analyzed, such as zeta potential, volume, surface area, light intensity, and aspect ratio of particles, providing a multifaceted biophysical assessment of polydisperse samples. More recently, NTA platforms developed optimized fluorescence modes (F-NTA), which permit a phenotypic characterization of analyzed particles within a sample [4,31,32]. Fluorophore stability and intensity pose as crucial factors for precise measurement of smaller particles, while the sensitivity required for the reliable capture of such signals renders instruments more susceptible to background noise and contaminants.

All sEVs are structurally similar. They are enclosed by a lipidic membrane, within a well-defined size range and carry different types of biomolecules found in respective donor cells. Cargo loading and release, though not fully elucidated yet, are actively regulated mechanisms that form the unique identity of each sEV and are thus responsible for the wide heterogeneity between vesicle subpopulations [33–37]. Identification and characterization of true sEVs requires a biochemical analysis of their content, often relying on the presence or absence of surface proteins on the lipidic membrane (i.e., classical tetraspanins CD9, CD63, and CD81). Subpopulations containing internal markers of interest can also be identified with specific membrane-permeable dyes.

Recently, several strategies for EV labeling have been proposed. These often consist of adapting staining protocols of fluorescent dyes intended to be applied in cells [20]. Dyes used for EV labeling were selected based on their specificity for different EV components such as proteins, lipids, or nucleic acids. Moreover, we applied fluorescently labeled antibodies to probe for specific outer membrane molecules. sEVs were characterized on nFCM, a dedicated high-resolution nanoflow cytometry platform that combines single-particle fluorescence detection with respective scattered light, suitable for biological particles down to 40 nm. To understand whether optimized staining protocols for purified EVs or cell-conditioned media (CCM) could be transversally applied between different platforms, we further tested them in F-NTA. Finally, we underline some of the main hurdles to single sEV discrimination, which are often related to potential co-isolated contaminants, such as large protein complexes, soluble proteins, or cell culture media components.

2. Results

2.1. EV Labeling with Membrane and Cytoplasmic Dyes

Different staining approaches were evaluated in this study. One consisted of staining the lipidic membrane constituents of EVs using amphipathic molecules, such as the CellMask™ (CM) Plasma Membrane Stains, CMG and CMR, which emit green and red fluorescence, respectively. Secondly, we exploited the properties of CellTrace™ cell proliferation dyes, in this case, carboxyfluorescein succinimidyl ester (CFSE) and CellTrace™ Red (CTR). These dyes differ only in the wavelength of emitted fluorescence (CFSE: green; CTR: Red), but their mechanism of action is identical. Briefly, dye molecules easily penetrate the lipidic bilayer of EVs, becoming activated by an enzymatic cleavage and covalently bound to proteins present in the EV lumen. This process effectively traps dye molecules inside every single EV, generating a stable fluorescent signal [38,39]. Over 90% of the particles in the HT29 sEV samples were stained by CMG, CMR, CFSE and CTR and detected on nFCM (Figure 1A). Larger sEVs displayed notably higher fluorescence intensities on dot plots, which was likely elicited by the incorporation of more dye molecules (Figure 1B). Staining protocols were also tested on F-NTA, with the scope of validating them on a state-of-the-art orthogonal platform. Staining performance with CFSE was ~88% on F-NTA, comparable to the one detected on nFCM (Figure 1A), although a higher concentration of dye was required to reach a staining plateau (50 µM in F-NTA, with respect to 10 µM used in nFCM measurements). On the other hand, CMG displayed a consistently lower staining efficiency, with a maximum of ~32% obtained at the concentration of 20× CMG (Figure 1A). Particle size distribution (PSD) histograms demonstrated a prevalent detection of larger particles on F-NTA, as they harbored more dye (Figure 1C—CMG). Bulk fluorescence signal-to-background ratios measured on a microplate reader varied linearly with the number of stained particles for all dyes, although the highest sensitivity was obtained with both green dyes, CFSE and CMG (Figure 1D).

To control dye specificity, besides the compulsory use of blanks and unstained controls (Supplementary Figure S1), a protein called thyroglobulin was included in this study. Due to its large size, single events could be picked up in the side scatter channel. Hardly any thyroglobulin particles were stained by CMG, however, after CFSE staining the number of detected fluorescent events in F-NTA surpassed the scattered ones by orders of magnitude, even after 300 kDa ultrafiltration (UF) washing (Supplementary Figure S5). In nFCM, CFSE-labelled thyroglobulin particles were also detected (data not shown). A comparable degree of dye activation was observed in F-NTA with CFSE-stained BSA controls, while UF successfully removed all fluorescent events (Supplementary Figure S5).

2.2. Characterization of sEV Subpopulations Using Single Fluorophore Tetraspanin Labeling Strategies

To identify the fluorophores conferring maximum sensitivity for sEV detection on nFCM, we compared the staining of HT29-derived sEVs obtained with anti-CD9 primary antibodies conjugated to PE, AF488, AF647, and APC. Maximal CD9 staining was achieved with PE and AF488, as both allowed detection of similar percentages of the CD9 subpopulation (close to 50%). The red fluorophores, AF647 and APC, did not stain CD9+ sEVs to a comparable degree (Figure 2A). Henceforth, in nFCM experiments we opted for PE and AF488-conjugated antibodies, having PE as reference fluorophore due to its higher brightness. Despite its extreme brightness, the high photobleaching rate of PE renders it unsuitable for the F-NTA platform, where precise measurements rely on signal acquisition for longer time periods. Therefore, AF488 was chosen for sEV phenotyping on F-NTA, since it is a more stable fluorophore.

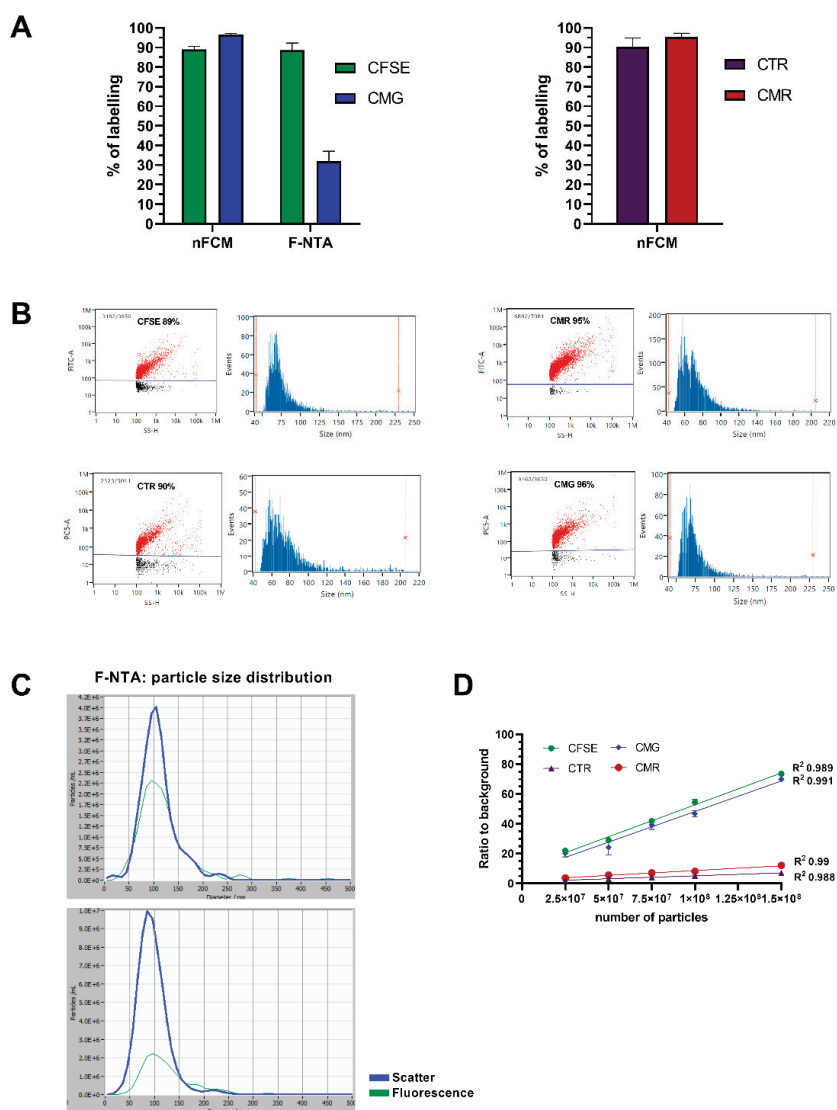


Figure 1. Fluorescence measurements on nFCM, F-NTA and plate reader after sEV staining with membrane and cytoplasmic dyes. **(A)** HT29 sEVs were stained with membrane-specific (CMG or CMR) or cytoplasmic dyes (CFSE or CTR) and analyzed on nFCM and F-NTA to evaluate labeling %. Data is presented as mean $\pm$ SEM of at least three independent experiments (except for nFCM-CMG and CMR, and F-NTA-CFSE, which were repeated twice). **(B)** Representative nFCM dot-plots and PSD histograms, and **(C)** histograms obtained after analyzing EVs by F-NTA in scatter and fluorescence mode. **(D)** Bulk fluorescence intensity of a dilution series of stained HT29 EVs was measured by plate reader. Signal over background (PBS) ratios are represented and trendlines drawn for the assessment of correlations. Additional data from procedural controls is provided in Supplementary Figure S1.

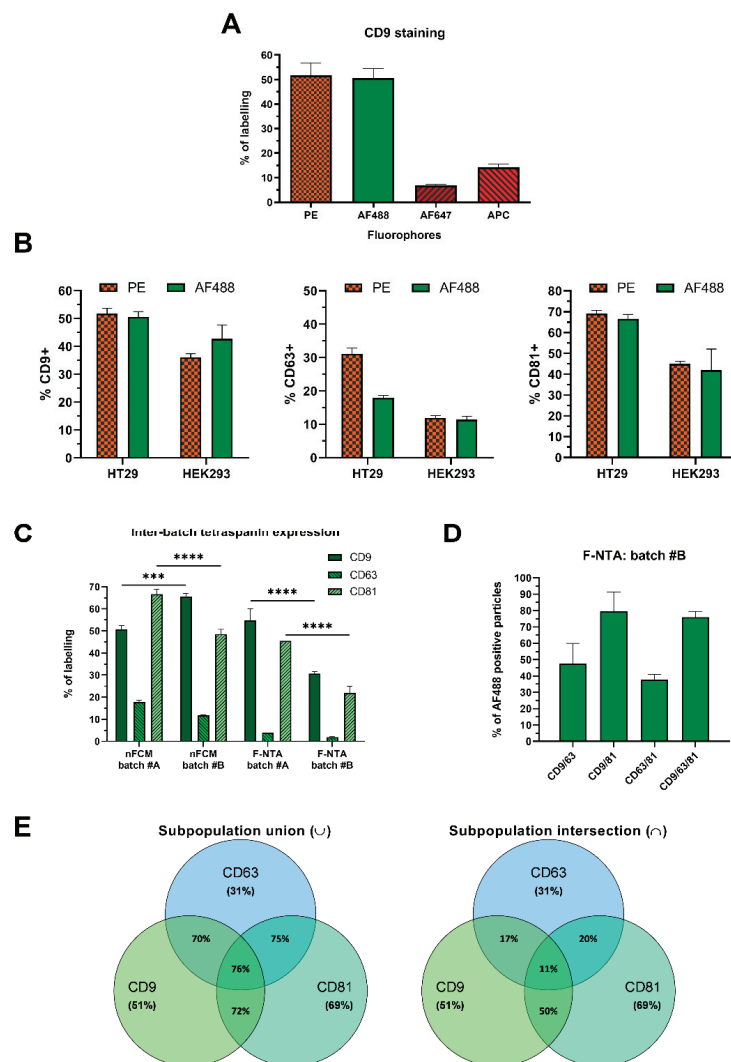


Figure 2. Characterization of purified sEV subpopulations based on their surface markers. (A) Antibody staining efficiency was evaluated by nFCM using anti-CD9 antibodies conjugated with different fluorophores. (B) HT29 and HEK293 sEVs were stained with anti-tetraspanin (CD9, CD63, and CD81) antibodies, conjugated with either PE or AF488, and analyzed on nFCM ($n \geq 3$; mean $\pm$ SEM). (C) Cross-platform and inter-batch variability was assessed by single-staining HT29 sEV (batches #A and #B) with anti-tetraspanin AF488 antibodies ($n = 3$; mean $\pm$ SEM). Differences in tetraspanin expression between nFCM and F-NTA, and batches #A and #B, were assessed using two-way ANOVA with Tukey's test for multiple comparisons ($\alpha = 0.05$, $p = 0.1234$ (ns), 0.0002 (**), <0.0001 (****)). Detailed results of the statistical analysis are provided as supplementary material. (D) To evaluate single, as well as co-expressing, events, HT29 sEVs were stained with a mix of 2 and 3 different anti-tetraspanin AF488 antibodies and analyzed on F-NTA ($n = 3$; mean $\pm$ SEM), or on (E) nFCM using PE-conjugated antibodies ($n = 3$; mean $\pm$ SEM). HT29 sEV subpopulations expressing either 1, 2, or 3 markers are represented in the Venn diagram on the left. The Venn diagram on the right refers to the same HT29 sEVs, however it depicts subpopulations co-expressing both 2 or 3 markers simultaneously. Additional data from procedural controls, as well as the F-NTA PSD histograms, are provided in Supplementary Figure S1.

To evaluate whether the chosen fluorophores indeed performed equally well, HT29 and HEK293 sEVs were stained with PE or AF488-conjugated anti-CD9, -CD81 and -CD63 primary antibodies, followed by nFCM analysis (Figure 2B). In HT29 sEVs, CD9 was detected at 51% (PE)/50% (AF488), CD63 at 31% (PE)/18% (AF488) and CD81 at 69% (PE)/66% (AF488). For HEK293 sEVs, detected CD9 events accounted for 36% (PE)/42% (AF488), CD63 for 12% (PE)/11% (AF488) and CD81 for 45% (PE)/42% (AF488) (Figure 2B). Both fluorophores generally allowed for the detection of similar proportions of EV subpopulations, with a major discrepancy observed only for CD63 detection in HT29 sEVs. The latter may be due to the fact that different anti-CD63 antibody clones labeled with two fluorophores were available and used in this study. To assess antibody specificity, in addition to the blank reactions, where sEVs were absent (Supplementary Figure S1), thyroglobulin was also stained as a negative control, since it is a protein particle and does not carry tetraspanin epitopes. All anti-tetraspanin antibodies caused negligible labeling, especially PE-tagged ones. CD63-AF488 revealed the highest degree of unspecific staining (Supplementary Figure S5).

Tetraspanin expression in EVs is known to vary across cell types, as well as across EV batches. When two independently harvested and purified batches of HT29 sEVs (batch #A and batch #B) were compared on both nFCM and F-NTA, we identified variations in tetraspanin expression (Figure 2C). Purified sEVs, from two different batches of HT29 CCM were stained with AF488-labelled antibodies and showed a significantly different expression level of CD9 (50.6% vs. 65.7%) and CD81 (66.6% vs. 48.7%) in nFCM, while for CD63 (17.9% vs. 11.9%) difference was not statistically significant (two-way ANOVA, Tukey's multiple comparisons test, $\alpha = 0.05$). Staining and analysis of batch #B sEVs in F-NTA resulted in 30.7%, 22% and 1.9% for CD9, CD81 and CD63, respectively, substantially lower percentages compared to batch #A (CD9 = 54.9%, CD81 = 45.5%; CD63 = 3.9%). Although the expression of each single tetraspanin significantly differed between batches (particularly CD9 and CD81), in F-NTA their relative trend of expression was maintained: CD9 >> CD81 >> CD63 (F-NTA batch #A and batch #B—Figure 2C). Instead, in nFCM the relative expression of tetraspanins slightly differed between the batches (batch #A CD81 >> CD9; batch #B CD9 >> CD81). From the perspective of inter-platform comparison, tetraspanin expression was significantly different across the two platforms, except for CD9 in batch #A (two-way ANOVA, Tukey's multiple comparisons test, $\alpha = 0.05$; detailed statistical analysis in supplementary material). A consistent trend of relative expression between platforms was obtained only in batch #B (CD9 >> CD81 >> CD63).

To analyze the single and concomitant expression of these tetraspanins, HT29 sEVs were stained with double (CD9 + CD63; CD9 + CD81; CD81 + CD63) and triple (CD9 + CD81 + CD63) antibody combinations, conjugated with PE for nFCM and AF488 for F-NTA. Nearly 76% of all particles observed on nFCM contained either CD9, CD81, CD63, or a combination of each, thereof (Figure 2E, left). The percentage of events detected upon double or triple antibody staining were not purely cumulative, which is consistent with the expectation that each sEV may express one, two, or even three tetraspanins, concomitantly. The overall portion of sEVs positive for all tetraspanins could be reproduced on F-NTA, with 76% of the particles stained by a triple antibody mix (Figure 2D). The proportion of double-positive sEVs detected on F-NTA, showed a more prominent increase when compared to previously measured single staining events, with 47.6% of particles stained with a mix of anti-CD9/anti-CD63, 79.4% with anti-CD9/anti-CD81, and 37.8% with anti-CD63/anti-CD81. Even though the signal was much higher than the sum of individually stained reactions (e.g., CD9/63 > CD9 + CD63), the patterns of expression maintained the trend expected from individual tetraspanin expression—CD9/81 >> CD9/63 >> CD63/81.

After double and triple antibody staining and understanding the expression of each single tetraspanin, sEV subpopulations co-expressing these markers could be calculated through a Venn diagram intersection analysis. Since on nFCM 70% of events co-stained with anti-CD9/anti-CD63, 72% with anti-CD9/anti-CD81 and 75% with anti-CD63/anti-CD81, we could estimate that 17% of all HT29 sEVs co-expressed CD9 and CD63, 50%

co-expressed CD9 and CD81, around 20% co-expressed CD63 and CD81, and only 11% expressed all three tetraspanins simultaneously (Figure 2E). Interestingly, we noticed that CD9+ accounted for 51% and that CD9 + CD81+ for 50% of total events, which meant that all CD9+ events expressed also CD81, in our HT29 sEV samples (Figure 2E, right).

2.3. SEC Purification and Ultrafiltration Do Not Change the Overall Composition of sEV Subpopulations

Optimized sEV staining protocols included a filtering step for buffer exchange, which was able to retain sEVs and to remove dyes and antibodies in excess. After testing several filtration devices, Nanosep 300 kDa ultra-filters and SEC were chosen for this purpose. One major concern was that background fluorescence could mask the mild fluorescent signal derived from fluorescently labeled nanoparticles and impede the acquisition of true positive events, resulting in skewed measurements. Indeed, free dye and antibodies impinged on sEV analysis, as clearly demonstrated on F-NTA where the fluorescence background led to overestimations of labeling efficiency (Figure 3A). PSDs of fluorescently labeled sEVs were also affected, as the size distributions shifted to the left, revealing a peak below 50 nm (Supplementary Figure S2). Similarly, on nFCM the excess of free fluorophores in solution led to increased thresholds in fluorescence channels. Therefore, filtering proved to be a critical step for the reliable detection of labeled vesicles, as even traces of free dyes and/or antibodies generated artifacts and overwhelming fluorescence noise on both instruments.

Nonetheless, we questioned whether these buffer exchange methods could inadvertently select specific sEV subpopulations and thus result in biased analysis. To this end, HEK293 sEVs (10^8 particles/ μ L) were stained with PE-conjugated anti-CD9, -CD63, or -CD81 and detected subpopulations compared after sample over-dilution, SEC and UF at nFCM. Sample over-dilution consisted of diluting a complete staining reaction in PBS until the concentration of the unbound fluorescent antibody was low enough to avoid background fluorescence, while sEV concentration was maintained at the optimal range for measurement. Since the over-dilution approach is inherently unbiased, it functioned as reference staining. Regardless of the method applied, we observed no significant difference between tetraspanin expression levels (Figure 3B), suggesting that neither SEC nor UF alters the composition of sEV subpopulations. This was also supported by the mean and median values of sample PSDs (Figure 3C,D).

2.4. Identification and Quantification of sEV Populations upon RNA Staining

Aiming to further characterize HT29 sEV subpopulations, we evaluated their nucleic acid content with membrane-permeable dyes, specific for RNA-SYTOTM RNASelectTM and Quant-iTTM RiboGreenTM. Since both dyes only exhibit their full brightness upon binding to RNA, the background signal generated by unbound dye molecules should be drastically reduced compared to PE or AF488. Such reasoning prompted us to explore the suitability of the sample over-dilution approach in this context. After staining reaction over-dilution, fluorescent subpopulations detected by nFCM averaged around 10.2% and 10.6% for Syto and RiboGreen, respectively (Figure 4A). However, UF washing caused a consistent drop in fluorescent events for both dyes, with respect to the sample over-dilution reference. Loss of fluorescent events after UF also reflected on PSDs, as a noticeable reduction in particles with smaller diameters was observed (Figure 4B).

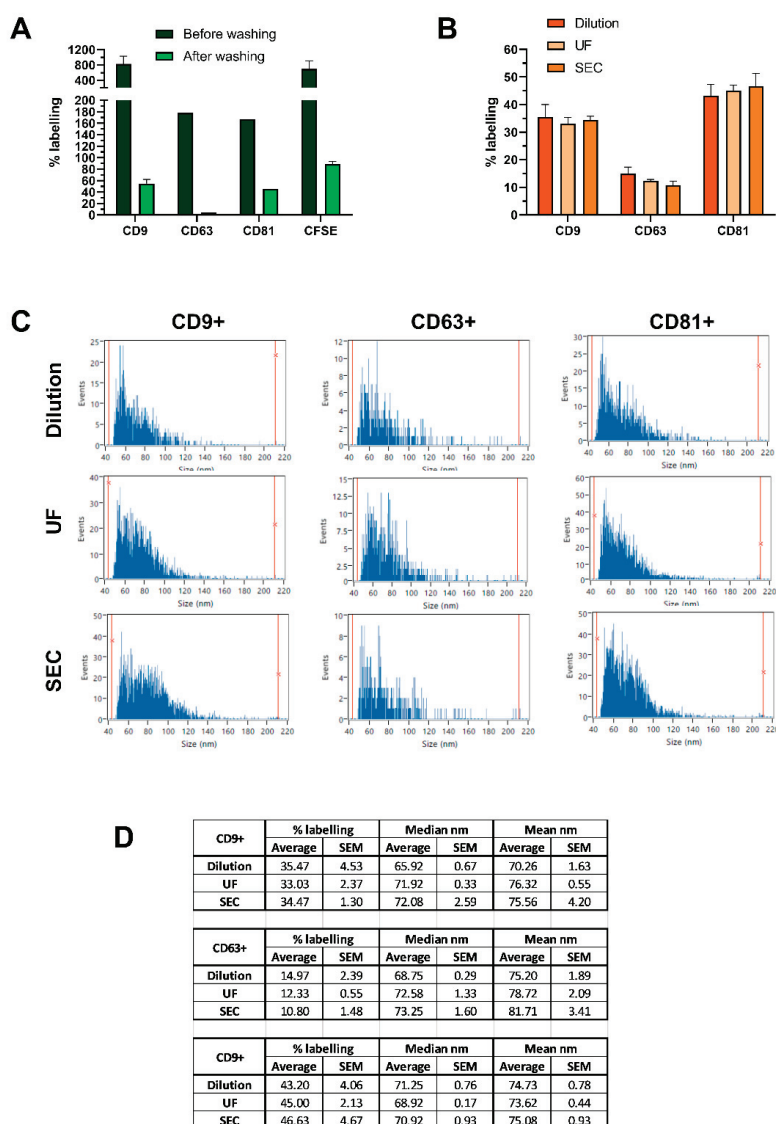


Figure 3. Suitability of SEC and UF as methods for clearing dyes in excess after sEV fluorescent labeling and their effect on subpopulation ratios. **(A)** Comparison of labeling % for stained HT29 sEVs before and after the removal of excess dye. 1×10^9 or 5.5×10^9 sEVs were incubated with antibodies (aCD9 1:12.5; aCD63 1:12.5; aCD81 1:25) or CFSE (50 μ M), respectively, and measured by F-NTA. To remove the excess dye, UF washing strategy was applied, followed by F-NTA detection. **(B)** To compare different strategies for the removal of fluorescent antibodies in excess, labeling %, **(C)** PSD histograms, and **(D)** median and mean particle diameter were assessed for CD9+, CD63+ and CD81+ HEK293 sEVs, on nFCM. 2×10^9 sEVs, at a concentration of 10^8 particles/ μ L, were incubated with PE-labelled antibodies (aCD9 1:500; aCD63 1:25; aCD81 1:500) and unbound antibodies were removed by SEC or UF. Sample dilution (500–1000-fold) served as staining reference. Data is presented as mean $\pm$ SEM of at least three independent experiments. F-NTA PSD histograms of samples analyzed before and after washing are provided in Supplementary Figure S2.

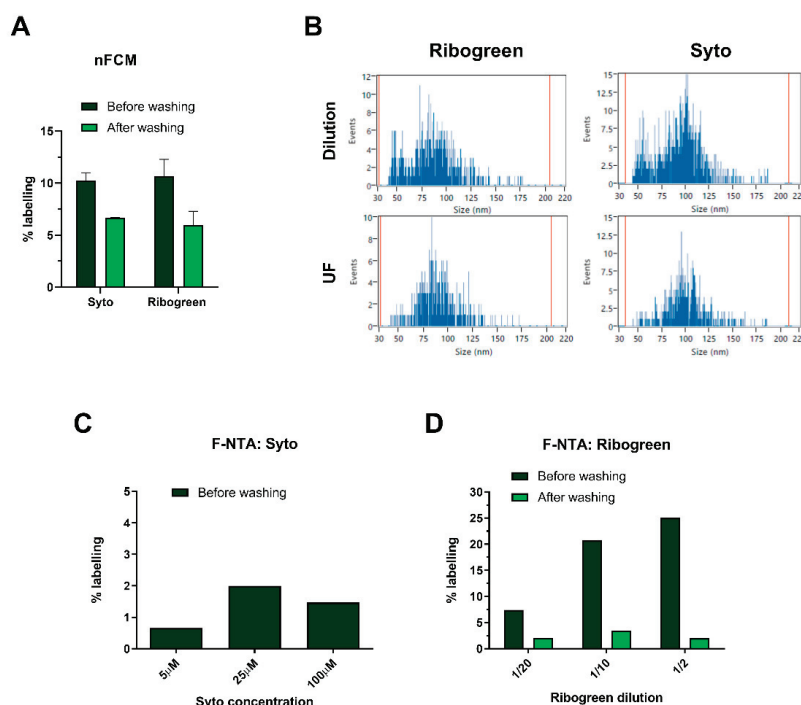


Figure 4. Identification and quantification of sEV populations based on their RNA content. (A) HT29 sEVs were stained with the RNA dyes Syto (25 μ M) and RiboGreen (1:50). After incubation, staining reactions were washed by UF and fluorescent particles subsequently measured, with respective (B) PSDs analyzed on nFCM. (C) HT29 sEVs were incubated with Syto at increasing concentrations and the % of positive events was read on F-NTA, without applying further washing steps. (D) The impact of UF washing was evaluated on labeled HT29 sEVs, at increasing concentrations of RiboGreen. Fluorescent subpopulations were detected using F-NTA. Staining for nFCM was performed in three independent experiments ($n = 3$; mean $\pm$ SEM), while for F-NTA, representative results of titration experiments are shown for both dyes. Additional data from procedural controls, as well as the F-NTA PSD histograms of analyzed samples, are provided in Supplementary Figure S3.

Similar observations were made after analyzing Syto and RiboGreen-stained HT29 sEVs on F-NTA. No significant fluorescence signal was detected with Syto (<2%), even at the highest concentration of dye used (100 μ M) (Figure 4C). Conversely, sEVs stained with different concentrations of RiboGreen reached up to 25% of labeling efficiency (Figure 4D). Notably, detected fluorescent particles showed a tendency towards larger PSDs (Supplementary Figure S3). Nevertheless, after washing the samples with UF, the fluorescent signal was completely lost (Figure 4D). Blank controls (without sEVs) showed a negligible number of fluorescent events on both instruments, indicating that free dye alone did not generate false-positive counts, either before or after washing (Supplementary Figure S3).

2.5. Identification and Quantification of sEVs Directly in Cell-Conditioned Media

Purified EVs are ideal for a single-particle characterization. However, purification can be lengthy, labor-intensive, and biased if the process enriches certain EV subpopulations. To evaluate the possibility of avoiding sEV purification from CCM, while still accurately detecting sEV subpopulations, previously optimized staining protocols were directly applied in HT29 CCM and particles were measured by nFCM and F-NTA. As shown in Figure 5A, CFSE and CMG labeled ~90% of events when analyzed on nFCM, well recapitulating the results obtained with purified HT29 sEVs (Figure 1A). On the contrary,

CFSE and CMG staining on HT29 CCM resulted in only 33% and 27% of labeling on F-NTA, respectively (Figure 5A). Peak and overall PSD of fluorescent subpopulations leaned towards higher values, as opposed to total particles measured in a scatter mode (Supplementary Figure S4).

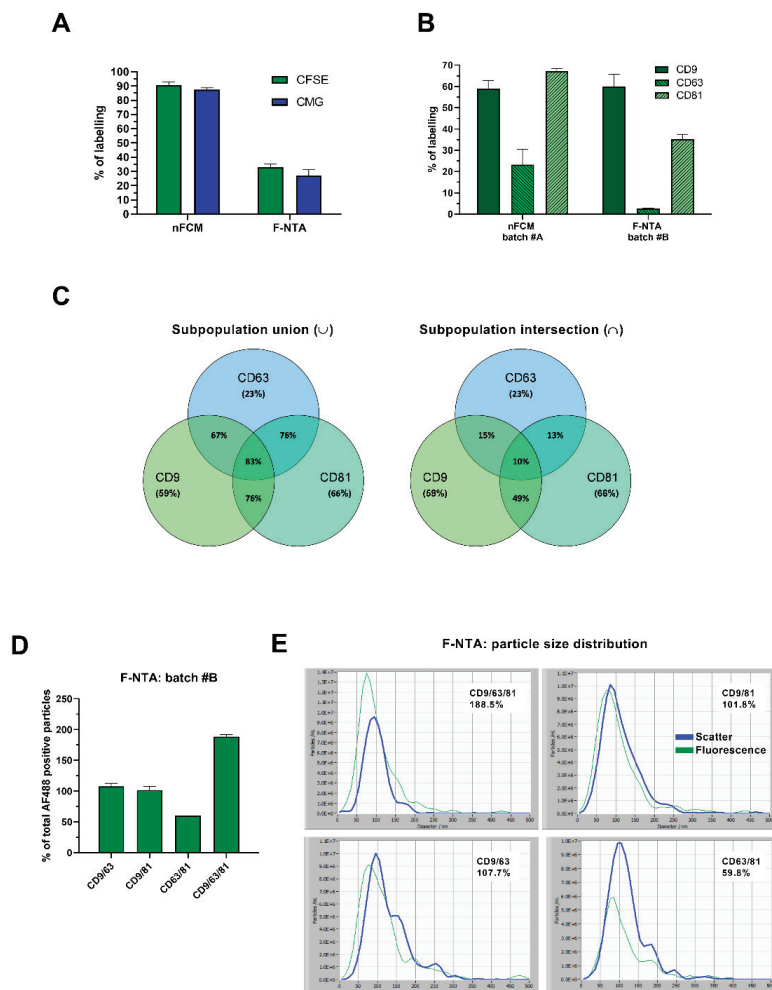


Figure 5. Characterization and quantification of sEVs directly from non-purified cell-conditioned media. HT29 CCM particles were stained and analyzed following the optimized protocols for sEV labeling with CMG, CFSE or fluorescently labeled anti-tetraspanin antibodies. (A) Staining efficiencies of CMG-, CFSE-, and (B) single antibody-labeling were assessed on both platforms. (C) Co-expressing markers were also evaluated on HT29 CCM by applying a mix of 2 and 3 anti-tetraspanin, PE-conjugated antibodies for nFCM measurements. The Venn diagram on the left depicts subpopulations that carried 1, 2, or 3 tetraspanins, while the one on the right represents sEV subpopulations which expressed 2 or 3 markers simultaneously. (D) HT29 CCM staining employing mixes of 2 and 3 AF488-labelled anti-tetraspanin antibodies was carried out for F-NTA, and staining efficiencies, as well as the (E) PSDs in scatter and fluorescence modes were assessed. Data represents triplicate experimental points ($n = 3$; mean $\pm$ SEM), except for (C) which was performed in two independent experiments ($n = 2$; mean $\pm$ SEM). F-NTA PSD histograms of CCM samples after CFSE-, CMG- and single antibody-labeling, are provided in Supplementary Figure S4.

Direct incubation of anti-tetraspanin antibodies in HT29 CCM and subsequent nFCM analysis revealed slight variations in the tetraspanin expression profiles, with respect to those reported for purified HT29 sEVs. Percentages of positive events were 59% for CD9, 67% for CD81 and 23% for CD63 (Figure 5B). On F-NTA, percentages of staining with AF488 antibodies were 59.8% for CD9, 35.3% for CD81, and 2.6% for CD63 (Figure 5B), higher than in purified HT29 sEVs from the corresponding batch (Figure 2C, F-NTA). Nevertheless, the same trend of expression was maintained between HT29 CCM and HT29 purified sEVs (CD9 >> CD81 >> CD63). Detailed statistical analysis by two-way ANOVA, comparing variability in tetraspanin expression levels between purified (sEVs) and non-purified (CCM) samples, is provided in the supplementary material.

In line with the purified sEV experiments reported above, we evaluated the concomitant expression of the three tetraspanins in HT29 CCM, applying combinations of CD9, CD81, and CD63 antibodies, PE-conjugated for nFCM and AF488-conjugated for F-NTA. Nearly 83% of the detected particles on nFCM displayed either CD9, CD81, or CD63, slightly more than the 76% previously determined for purified sEVs from the same CCM batch (Figure 5C). Regarding tetraspanin co-expression on nFCM, 15% of events carried both CD9 and CD63, almost 50% displayed both CD9 and CD81 and 13% were positive for both CD63 and CD81, while 10% resulted positive for all the three markers. The profile of tetraspanin expression was identical to the one obtained in purified sEVs, evidencing a maximum fluctuation of only 7% (Figures 2E and 5C).

AF488-conjugated antibody combinations did not reproduce the same values on CCM, as observed for purified EVs on F-NTA. Double and triple staining reactions evidenced unrealistic fluorescent event numbers, yielding over 100% of events detected in scatter mode. The combination of multiple antibodies in a complex, non-purified biofluid likely resulted in poor removal of unbound antibodies in excess (Figure 5D). Background fluorescence hampered correct analysis, as shown by the shift in PSD histograms between scatter and fluorescence mode (Figure 5E).

2.6. Characterization of sEV Subpopulations Using Multicolor Fluorescence Labeling Strategies

Depending on the complexity of biological samples, a wide range of contaminants can co-purify with sEVs, adding up to the already high heterogeneity of subpopulations. Consequently, it is crucial to pinpoint and discriminate true sEVs from confounding particles, whilst extracting additional information about their nature and/or contents. This prompted us to attempt multiple labeling strategies, combining different dyes and antibodies. Firstly, purified HT29 sEVs were stained with CD9-AF488 and CD81-PE alone, to individually determine the expression of each marker. Then, both antibodies were combined, and the staining efficiency was compared. CD9-AF488 alone stained 50% of particles and in combination with CD81-PE, this number slightly increased to 55%. The labeling efficiency obtained with CD81-PE was 70% in single staining and 68% after incubation together with CD9-AF499 (Figure 6A). As for the double-positive CD9+/CD81+ subpopulation, 48% of the particles displayed a double fluorescent signal, which perfectly matched the co-expression level of CD9 and CD81, previously identified in HT29 sEVs using a combination of strictly PE antibodies (Figures 2E and 6A). In line with the goal of further distinguish the nature of antibody-labeled particles, we attempted to optimize a double staining protocol applying CTR and CD81-AF488. The labeling obtained for each individual dye matched well with single stain controls. It was observed that the entire CD81+ subpopulation could be simultaneously stained with CTR, thereby supporting the presence of true sEVs (Figure 6B). Surprisingly, HT29 sEVs expressed nearly 40% of CD81 in this experiment, whereas in previous ones it was detected at 65–70% (Figure 2B).

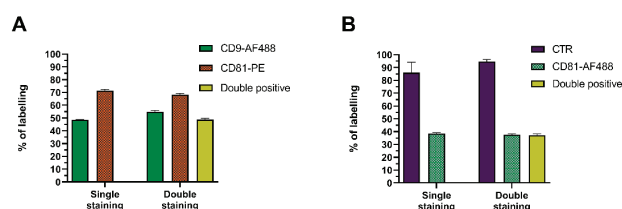


Figure 6. Multiplex fluorescence analysis for enhanced sEV identification and characterization. (A) Purified HT29 sEVs were labeled with anti-CD9-AF488 and anti-CD81-PE either individually (single staining) or in combination (double staining). (B) HT29 sEVs were stained with CTR and anti-CD81-AF488 either individually or in combination. Results are shown as % of labeled particles detected on nFCM, in two fluorescence channels ($n = 3$; mean $\pm$ SEM).

3. Discussion

In the present study, we compared single nanovesicle profiling platforms, aided by fluorescent labeling with different dyes and antibodies. To this end, we evaluated the capabilities of two platforms, the recent nFCM and the more established NTA, which has been recently upgraded for compatibility with fluorescence measurements—F-NTA. Although both instruments perform single-particle analysis, there are crucial differences in their hardware components and mode of operation. nFCM gathers scattered light to estimate particle size, while F-NTA calculates their hydrodynamic diameter by tracking particle diffusion motion.

The accuracy of nFCM particle size estimations relies on calibrating the instrument using silica beads with refractive properties similar to those of sEVs. The resulting calibration curves are in accordance with the Rayleigh scattering theory as they fit the expected model for the light scattering of particles smaller than the wavelength of incident light [28]. However, the refractive index (RI) of silica beads (1.46) does not exactly match the RI of EVs (1.36–1.4), and given the heterogeneity of EV sizes and biomolecular scaffolding, even greater differences in refractive properties between subpopulations of particles could arise [40,41]. To account for these limitations, size estimation in nFCM has implemented Mie scattering theory calculations, which adjust calibration curves to minimize any potential errors stemming from differences between size standards and EVs [42–44].

On the other hand, NTA requires a longer acquisition time window to determine particle size and the analysis of polydisperse samples imposes protocol readjustments to encompass a wider range of sizes [30]. Additionally, accurate size estimation based on Brownian motion becomes challenging with larger particles due to their slower diffusion, which could be affected by the EV surface composition, medium viscosity, and temperature [5,45–47]. The strength of NTA lies in the fact that it is a well-established method and does not rely on RI, which provides great flexibility for measuring nanoparticles of different compositions without the need for reference material in each analysis. However, when it comes to fluorescent labeling and detection, NTA poses certain limitations (bright and stable fluorophores; longer signal acquisition time) and requires further development and optimization. Furthermore, avalanche photodiodes (APD) in nFCM might allow for higher resolving power and better signal detection, especially in fluorescence mode, when compared to CMOS camera sensors [48].

In this study, F-NTA measurements required more washing cycles to completely eliminate background fluorescence, than nFCM. Stronger laser power (15 mW in nFCM vs. 40 mW in F-NTA), the fact that F-NTA acquires fluorescent signal from a stationary liquid in a cell for a longer fraction of time, compared to a fast detection in continuous flow on nFCM, and that nFCM applies SSC-triggered measurements of fluorescence, might be some of the reasons for a higher susceptibility to background noise on F-NTA. This was especially the case when analyzing more complex biofluids, such as CCM, drawing attention towards limited EV analysis in non-purified matrices.

Despite the need for more washing cycles, the analysis of purified sEVs could be carried out on both instruments. Cytoplasmic dyes, particularly CFSE, performed comparably well on both instruments, representing an optimal balance between fluorescence intensity and photostability. CFSE and cell-trace dyes theoretically require enzymatic cleavage by esterases to covalently bind to intraluminal proteins and become fluorescent. Several studies employing various nanoparticle profiling platforms have applied CFSE as a way to selectively stain EVs [20–24,49,50]. Nonetheless, CFSE and cell-trace dyes should be used with caution, since in our hands they became activated independently of intraluminal esterases. The presence of non-vesicular proteins and potential contamination with soluble esterases may cause CFSE activation—as evidenced by the staining of thyroglobulin particles and by the significant number of fluorescent artifacts in BSA controls reported in this work. Cell membrane dyes (CMG, CMR) did not exert the same extent of background fluorescence; however, their staining efficiency was sub-optimally detected in F-NTA experiments.

For ideal single nanoparticle profiling, it was important to choose widely available, photostable, and high brightness fluorophores. On nFCM, AF488 and PE performed equally well and surprisingly, allowed for increased sEV staining efficiencies over the red fluorophores AF647 and APC, even though AF488 theoretically should have the lowest brightness (extinction coefficient $\times$ quantum yield) out of them all (see the Supplementary Table S1). Generally, red fluorophores may also be more prone to self-quenching, consequently diminishing their quantum yield [51]; therefore, it would be relevant to address the properties of such dyes within the scope of single nanoparticle analysis. For F-NTA, AF488 provided the optimal balance between stability and brightness. Despite being one of the brightest commercially available dyes, PE was omitted from F-NTA measurements due to its fast bleaching, which could result in the underestimation of truly stained particles.

The fact that total particle counts (scatter mode) on F-NTA were nearly 4–5 times higher than they were on nFCM might be explained by differences in laser power, as the strength of incident light sources and the composition of illuminated particles directly correlate with the intensity of scattered light and ultimately, with the number of detectable nanoparticles. On the other hand, CMOS sensors might not be as sensitive as APDs, which could lead to poorer detection of faintly expressed epitopes on the surface of sEVs, limiting the number of fluorophores associated per fluorescent event. This could help to explain the significantly lower labeling percentages detected with F-NTA during antibody staining experiments. Such reasoning is further corroborated by double and triple antibody staining experiments, where the number of fluorescent events was higher than the sum obtained after each single staining (Figure 2C,D). EVs displaying few CD9, CD63 or CD81 epitopes on their surface would remain undetectable on F-NTA until multiple tetraspanins are labeled (Supplementary Figure S6). Nevertheless, F-NTA still provided consistent results between batches, revealing a trend in tetraspanin expression that was comparable in the case of single (CD9 >> CD81 >> CD63) as well as multiple antibody reactions (CD9/CD81 >> CD9/CD63 >> CD63/CD81).

These limitations did not seem to occur in nFCM, however, CD63-PE and CD63-AF488 resulted in 31% and 18% of staining on HT29 sEVs, respectively (Figure 2B). The fact that suppliers and clones were different between PE and AF488 antibodies could explain this discrepancy, though it was noticed only for CD63 and on HT29 samples; CD63 staining efficiencies using PE or AF488-conjugated antibodies were equal on HEK293. Notably, CD63 protein is reported to have different isoforms deriving from different splicing variants or post-translational modifications that may have functional or morphological implications and affect their partnering with other membrane molecules [52]. Therefore, the potential specificity of certain Ab clones for cell types or conditions must be better understood. Another possibility aligns with the phenomenon described just above, since CD63 was the least abundant tetraspanin in this study, AF488 staining may miss events carrying very few epitopes. Generally, nFCM was able to better discern fluorescently labeled EVs and also enabled the characterization of multiple surface markers through a single-color fluorescent

analysis, which is extremely valuable, especially in dedicated high-resolution platforms that are limited to a few channels for fluorescence detection. Therefore, we can argue that nFCM is more sensitive and consistent for fluorescent measurements.

For applications where sEV purification is not feasible, or minimal sample processing is a concern, we questioned whether our protocols for sEV fluorescent labeling could be applied directly in more complex biological samples such as CCM. Results between purified sEVs and CCM sEVs were surprisingly similar on nFCM. On the other hand, F-NTA was more promiscuous—lower fluorescent signal with CFSE in CCM could mean the presence of many non-EV particles, however, antibody staining gained percentages that were significantly higher than those obtained with purified sEVs. Behind this contradiction may be a reduced antibody washing efficiency, attributed to the richness of CCM. Double and triple antibody staining experiments further supported this hypothesis, where fluorescence background was even higher, leading to the conclusion that for background-free F-NTA measurements, purified material is preferred, or alternative washing procedures should be used instead of UF. It should be noted that the efficiency of UF washing was reduced when presented with CCM samples and multiple-antibody staining reactions.

We also assessed the feasibility of a sample over-dilution approach as an alternative staining protocol that avoids further processing for dye removal. Staining reactions had been previously optimized, maintaining a fixed range of sEVs (10^8 – 10^9), while titrating both dyes and antibodies to determine their optimal concentrations (at which a staining plateau was reached). For dyes, the dilution approach was not feasible for either of the instruments, as at the optimal dilution for the sample measurement, background fluorescence levels were still massive. On F-NTA, this problem also persisted when the over-dilution was applied to anti-tetraspanin antibody staining, confirming the need for a washing step after optimized staining reactions. Conversely, this protocol could be applied in nFCM, though it required an elevated concentration of purified sEV input (up to 10^8 per microliter). This allowed for staining reactions in lower volumes, minimizing the amount of antibody while maintaining optimized concentrations. Samples could be directly analyzed after a 500–1000-fold dilution, without any loss in sEV staining efficiency, with respect to UF or SEC.

The sample dilution-based protocol also featured as a reference method to evaluate possible biases introduced by UF or SEC, employed in this study for sEV buffer exchange and as a benchmark separation method for EV isolation. We confirmed that regardless of the dye removal method, there were no significant differences between CD9, CD81, or CD63 subpopulations. In this way, we demonstrated that neither SEC nor UF alters the composition of sEV subpopulations (Figure 3B). Similar mean and median size values also supported this claim (Figure 3C,D). Throughout the study, we favored the use of 300 kDa UF devices, as it is a more practical method than SEC and also confers higher sample processing throughput.

Overall comparison of washed and unwashed sEV samples proved to be useful in the early assessment of the efficacy and specificity of sEV staining protocols, including nucleic acid-specific dyes. The use of RNA-specific dyes for EV characterization has been reported in earlier works [53–55]. In this study, both SYTOTM RNASelectTM and Quant-iTTM RiboGreenTM revealed a low abundance of RNA-containing HT29 sEVs. As the activation of these dyes is dependent on nucleic acid binding, interference caused by background fluorescence signal seemed unlikely, hence the sample over-dilution approach was successfully applied. The low percentage of stained events questions not only the overall amount and accessibility of EV RNA but also the brightness of hereby employed dyes.

Following the MIFlowCyt-EV guidelines [56], procedural and assay controls, as well as washing steps, were included in all the experiments. Similarly, staining reactions with RNA dyes were also subjected to UF. This reduced the percentage of fluorescent events, leading to the conclusion that RNA staining may not be as specific or stable as with other dyes and antibodies. Such a decrease in fluorescent events after UF washing raises concerns

on the possible artifacts generated by dyes, as well as on the nature and strength of nucleic acid association to EVs. Given that RNA dyes can minimally bind to DNA molecules, the overall weak fluorescent signal could be justified by a low affinity binding to extravesicular DNA, which would then be lost upon UF washing. The presence and topology of nucleic acids on EVs have been the topic of discussion in previous publications [35,36,57–59], which might pose more critical approaches to assess the true EV nucleic acid content, location, and their usefulness as biomarkers. It is also possible that fluorescent events are not sEVs, but rather large individual ribonucleoprotein complexes [35,54,60,61]. This could be addressed through co-staining experiments with RNA-specific dyes and anti-tetraspanin antibodies. Although their blank controls show a low presence of fluorescent events, we cannot exclude the possibility that SYTO and RiboGreen may nonspecifically adsorb to EVs.

Since nFCM is equipped with two fluorescence channels, it allows for colocalization analysis. Dual fluorophore labeling proved to be a viable strategy for sEV subpopulation assessment, using either a combination of two antibodies or one antibody with another dye, as long as their emission spectra are sufficiently far apart to avoid fluorescence bleed-through between channels. The downside of this approach lies in its prolonged incubation time, which may have resulted in lower staining efficiency—HT29 sEVs expressed nearly 40% of CD81 after double-staining, whereas after single-staining CD81 was detected at 65–70% (Figures 2B and 6B). In conclusion, after careful optimization of staining protocols, it is possible to combine multiple fluorophores efficiently, to enable multiparametric sEV characterization.

4. Materials and Methods

4.1. Cell Culture and sEV Isolation

Cell lines HT29 (ATCC[®] HTB-38[™], Manassas, VA, USA) and HEK293 (ATCC) were expanded in McCoy's and DMEM growth media (Euroclone, Pero, Italy), respectively, supplemented with 10% FBS (Euroclone) and 1% pen/strep (Sigma, St. Louis, MO, USA). Cells were grown in T75 or T150 Flasks and maintained in a humid atmosphere of 5% CO₂ and at 37 °C. Once expanded to the desired confluence (80%), cells were washed 2 times with 1× PBS and conditioned in a serum-free medium (to avoid serum-derived confounding particles) for 48–72 h. CCM was harvested and clarified by differential centrifugation at (1) 300× *g* for 10 min, (2) 1200× *g* for 20 min, and (3) 10,000× *g* for 30 min at 4 °C. Pre-cleared CCM was directly used for experiments, further processed for EV purification and isolation, or stored at −80 °C.

For sEV isolation, CCM was concentrated using an Amicon Stirred Cell ultrafiltration unit (Ultracel 100 kDa Ultrafiltration Discs, Merck Millipore, Burlington, MA, USA). A maximum volume of 500 mL of CCM was concentrated down to 10 mL for each isolation. Concentrated CCM was fractionated in size-exclusion chromatography (SEC) columns (Sephacrose CL-4B bed volume 70 mL, GE Healthcare, Chicago, IL, USA), pre-equilibrated with 1 × 0.22 µm filtered PBS. Fractions of 1 mL were collected and EV-containing fractions 16 to 40 were pooled (total volume ≈ 25 mL). SEC-purified sEVs were concentrated down to 0.5–1 mL by 100 kDa ultrafiltration (Amicon[®] Ultra-15 Centrifugal Filter Unit, Merck Millipore).

4.2. Staining Protocols

4.2.1. Staining with Cytoplasmic, Membrane, or RNA-Specific Dyes

To ensure the optimal working concentrations of dye for our platforms, incremental concentrations were tested on a fixed number of particles (2×10^9). Dye concentrations at which the percentage of stained events reached a plateau were henceforth applied. After determining particle concentration (10^7 to 10^8 particles/µL), between 5×10^8 to 2×10^9 particles (purified sEVs or CCM) were loaded in each staining reaction with CellTrace[™] CFSE (CFSE; 10 µM; Thermo Fisher Scientific, Waltham, MA, USA), CellTrace[™] Far Red (CTR; 15 µM; Thermo Fisher Scientific), CellMask Green (CMG 20×; Thermo

Fisher Scientific) and CellMask Red (CMR 20 $\times$; Thermo Fisher Scientific), in filtered PBS. Reactions were incubated for 1.5 h at 37 °C under shaking, protected from light. After incubation, reaction volumes were brought up to 500 μ L with filtered PBS and loaded into SEC columns (Sephacrose CL-4B bed volume 10 mL, GE Healthcare) for the removal of unbound dye in excess. Fractions of 500 μ L were collected and the EV-containing fractions (7, 8 and 9) were pooled, resulting in a total of 1.5 mL of stained sEV samples, which were immediately analyzed. SYTOTM RNaselectTM (Syto; Thermo Fisher Scientific) and Quant-iTTM RiboGreen[®] (RiboGreen; Thermo Fisher Scientific) were added at 25 μ M and diluted 1:50, respectively. For Syto and RiboGreen, samples were incubated for 1.5 h and 30 min, respectively, both at 37 °C under shaking, protected from light. Afterward, they were washed 3–4 times with PBS using ultrafiltration centrifugal devices (Nanosep[®] 300 K, Pall Corporation, Port Washington, NY, USA; hereinafter referred to as UF) and analyzed.

As the ZetaView PMX-120 NTA instrument (Particle Metrix, Inning am Ammersee, Germany; in this text referred to as F-NTA) has only one laser (488 nm), red dyes were not used on this platform due to their longer excitation wavelengths. Furthermore, F-NTA detected 4–5.5 times more total particles than the Flow NanoAnalyzer (model U30, nanoFCM Inc., Xiamen, China; in this text referred to as nFCM), hence the particle amount and dye concentration for staining reactions were additionally optimized on this platform. For F-NTA analysis, 1×10^9 – 5.5×10^9 of particles (purified sEVs or CCM) were incubated with CFSE (50 μ M) and CMG (20 $\times$ concentrated) for 1.5 h at 37 °C. Afterward, the excess dye was removed by repeated washing (6–8 times) with 500 μ L PBS and UF. Washed samples were measured in both scatter and fluorescence mode. Titration was also conducted for Syto (5, 25, and 100 μ M per reaction) and RiboGreen (1/20, 1/10, and 1/2 dilution per reaction), samples were incubated for 1.5 h and 30 min at 37 °C, respectively, after which only RiboGreen stained samples were washed 8 times with PBS using UF. Samples were then analyzed in both scatter and fluorescence mode.

In both platforms, unstained samples and blanks (PBS and dye) were used as procedural and assay controls. The number of thyroglobulin (Merck/Sigma-Aldrich) particles was equivalent to the number of sEVs per staining reaction and the same protocols applied. Additionally, 20 μ g of purified bovine serum albumin (BSA) standards (Thermo Fisher Scientific) were used in a control reaction of CFSE specificity.

4.2.2. Single Fluorophore Antibody Staining

Optimal working dilutions for antibodies were determined following the same approach described above. For each staining reaction in nFCM, 2×10^8 – 2×10^9 particles (purified sEVs or CCM) were incubated with fluorescent primary antibodies for 1 h at 37 °C under shaking, protected from light. Next, PBS was added up to 500 μ L, unbound antibodies were removed by 3 to 4 rounds of UF or SEC and stained samples were characterized. The following fluorescently-labeled primary antibodies were used: Phycoerythrin (PE)-conjugated mouse anti-human CD9, CD63 and CD81 (dilution 1:10 for all; Exbio, Vestec, Czech Republic), Alexa Fluor[®] 488 (AF488)-conjugated mouse anti-human CD9, CD63 and CD81 (1:500, 1:25 and 1:500, respectively; R&D Systems, Minneapolis, MN, USA), allophycocyanin (APC)-conjugated mouse anti-human CD9 (1:10; Exbio), Alexa Fluor[®] 647 (AF647)-conjugated mouse anti-human CD9 (1:10; Exbio). Since PE is quite susceptible to photobleaching and APC and AF647 require a 600–640 nm excitation wavelength, only AF488-conjugated anti-human CD9, CD63 and CD81 were used in F-NTA, diluted 1:12.5, 1:12.5, and 1:25, respectively. 1×10^9 particles (purified sEVs or CCM) were used for the staining reaction at 37 °C, for 1.5 h. Unbound antibodies were removed by repeated washing (6–8 times) with 500 μ L PBS and UF, prior to analysis.

4.2.3. Multicolor Fluorescence sEV Staining

It is important to avoid the overlap between the emission spectra of dyes to be used in combination, which results in the detection of false-positive events. With that in mind, AF488 and PE were combined for dual-color fluorescent labeling experiments

with antibodies. Between 2×10^8 – 2×10^9 particles were incubated with CD9-AF488 and CD81-PE at optimal working dilutions indicated in the section above, for 1 h at 37 °C under shaking, covered from light. Antibody in excess was eliminated by 3 to 4 rounds of UF or SEC and labeled sEVs directly analyzed on nFCM. To avoid interference of dyes in the specific binding of antibodies when combining both for sEV double staining, 5×10^8 to 2×10^9 particles were firstly labeled with CD9-AF488 for 1 h at 37 °C under shaking, followed by incubation with CTR (15 µM) for 1 h 30 min at 37 °C under shaking. The volume of double staining reactions was brought to 500 µL with filtered PBS, unbound antibodies and dyes were eliminated by SEC, and samples were examined right after. Since our F-NTA instrument is equipped with only one laser, dual-color labeling was omitted on this platform.

4.3. nFCM: Instrument Setup and EV Analysis

Conventional flow cytometers are not designed to characterize small nanoparticles such as sEVs and thus may provide dubious information. For this reason, nFCM, a dedicated nanoflow cytometry platform, was employed in this study. Our instrument is equipped with two lasers (488 and 638 nm), three single-photon counting modules (SPCM) and enables simultaneous detection in three independent channels. Light is first detected in the SSC channel (bandpass filter: 488/10) and then directed by two dichroic beam splitters (DicF495; DicF555) towards the green channel (bandpass filter: 525/40) and finally to the orange/red channel (bandpass filter: 580/40 or 670/30). Before each experiment, the NanoAnalyzer was aligned using polystyrene QC beads (nanoFCM Inc.). Size and concentration standard nanospheres (nanoFCM Inc.) were read directly after in order to calibrate the instrument for sEV analysis. Once nFCM was aligned and calibrated, sEV samples were diluted in filtered PBS (blank) to the optimal range for measurement (10^8 particles/mL). Samples and blanks (200–800 events) were measured for 1 min, applying a laser power of 15 mW as excitation source, constant pressure of 1 kPa, and at an event rate between 2500 to 12,000 events/min (as recommended by manufacturers), to avoid particle swarm detection^{14,15}. Since SSC was set as the trigger channel, each particle that generated a signal above the SSC threshold was acquired as an event. For each event that also generated a signal above thresholds set in the fluorescent channels, the fluorescence intensity was registered. Thresholds were automatically set for each sample, accounting for background signal throughout the run (thresholds = average background measurements + $2 \times$ their standard deviation). Any sample with an SSC threshold 10% higher than the reference (size standard nanospheres) was excluded from the study. Similarly, strict criteria were set for the acceptance of fluorescently labeled samples, relying on unstained sEV controls as a reference for background fluorescence. Empty staining reactions (without sEVs) were performed as a control for all fluorescent reagents and measured under the same conditions as complete reactions (with sEVs).

4.4. F-NTA: Instrument Setup and EV Analysis

Particle Metrix ZetaView PMX-120 (software version 8.05.12 SP1) is a nanoparticle tracking analysis instrument equipped with a 488 nm laser (40 mW of power) and CMOS camera sensor which enables enumeration, physical characterization, and fluorescence measurement of particles in suspension. Measurements can be performed in liquid samples with a minimal volume of 500 µL, containing particles as low as 10^6 . PMX-120 was set up according to the manufacturer's instructions. Cell check was performed after each start-up, followed by camera and laser alignment using 100 nm polystyrene size standard beads and optimizing profile auto-symmetry. Upon instrument setup, daily performance was conducted with the same beads in order to assess the accuracy and precision. Biological samples were then diluted in PBS to reach the optimal particle count per frame in scatter mode (50–200 particles) and analyzed throughout 11 positions of the cell, with camera sensitivity 85, shutter speed 100, high video quality (capturing 60 frames) at 30 frames/s (1 cycle), minimal area 10, maximal area 1000 and brightness 25.

For fluorescence measurements, a 500 nm cut-on long-pass filter was used with camera sensitivity adjusted to 95 and video quality to low (capturing 15 frames). “Low Bleach” technology was enabled during acquisition in order to reduce the laser exposure time for the fluorophore and capture fluorescence signal at its maximum intensity. 11-position tables and histograms were used in the data analysis.

4.5. Bulk Fluorescence Measurements

After labeling, 200 µL of fluorescent sEV samples were loaded in black 96-well plates (PerkinElmer, Waltham, MA, USA). Plates were inserted in a fluorometer plate reader (CLARIOstar Plus; BMG Labtech, Ortenberg, Germany) and for each fluorescence measurement, optimal gain and focal height were adjusted to the brightest well. Raw fluorescent signals of samples were normalized to a blank (PBS) and data was presented as signal-to-noise.

4.6. Statistical Analysis

Experiments were performed in triplicate unless stated otherwise. All results are presented as average with SEM. GraphPad Prism 9 (San Diego, CA, USA) and Two-way ANOVA with Tukey test for multiple comparisons were used to examine inter-batch (batch #A and batch #B) and cross-platform (nFCM and F-NTA) variability in tetraspanin expression, as well as the correlation between purified and non-purified samples.

5. Conclusions

This work addresses some of the main benefits and limitations of working with two different single-nanoparticle profiling platforms. We propose protocols for optimal sEV characterization on both instruments, but also raise concerns regarding the value of certain dyes, affinity reagents, or methodologies commonly employed. Additionally, composite sEVs characterization addressed in our study represents an important information feed for downstream applications, such as uptake studies, in which the importance of parameters such as size, aggregation status, maintained integrity of sEVs, as well as surface display of molecules that actively mediate the cell uptake (e.g., tetraspanins), has been proven fundamental for correct experimental design (i.e., EV dosage) and results elaboration. Hereby used analytical protocols consume a very small sample fraction, thus leaving a majority of well characterized sEV isolates intact for further testing and use. Throughout this report, we aimed to highlight the aspects and considerations that are often understated but highly important, where a balance between obtaining trustworthy data and pushing instruments to operate at the edges of current technological limits must exist. It is fundamental to underline that a critical attitude must drive experimental works and reports aimed at accurately dissecting the EV field, where irrefutable evidence over major topics is still lacking.

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nFCM controls

Blank reaction	Average conc. Fluo (p/uL)	SEM
CFSE	3.69E+03	1.05E+03
CMG	4.39E+04	7.35E+03
CTR	3.69E+02	5.22E+02
CMR	3.69E+02	5.22E+02

Blank reaction	Average conc. Fluo (p/uL)	SEM
CD81-PE	4.03E+02	7.45E+01
CD9-PE	6.62E+02	1.39E+02
CD63-PE	7.43E+02	3.20E+02

Blank reaction	Average conc. Fluo (p/uL)	SEM
CD81-488	9.20E+02	1.02E+02
CD9-488	2.34E+03	5.33E+02
CD63-488	8.18E+03	3.86E+03

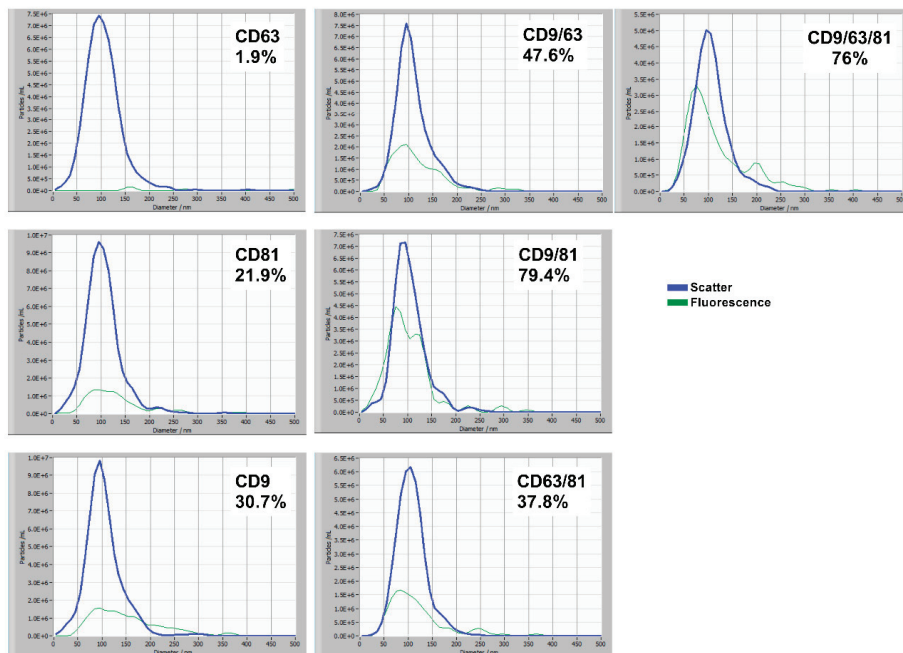
F-NTA controls

Blank reaction	Average number of fluorescent particles per position	SEM
CFSE	1.75	0.55
CMG	1.77	0.83
Unstained	2.58	0.57

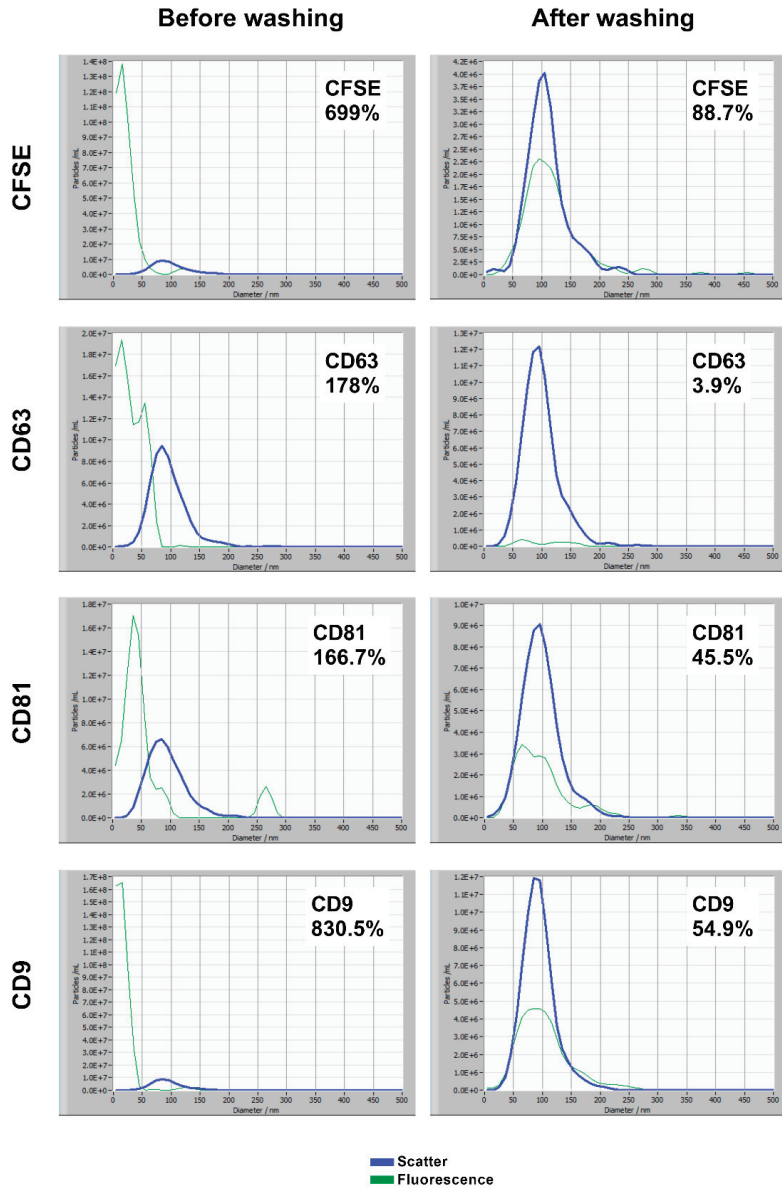
Blank reaction	Average number of fluorescent particles per position	SEM
CD81-AF488	0.95	0.05
CD9-AF488	1.50	0.10
CD63-AF488	0.85	0.15
Unstained	0.60	0.20

Blank reaction	Average number of fluorescent particles per position	SEM
CD9/CD81	1.40	-
CD9/CD63	2.10	-
CD81/CD63	0.65	0.05
CD9/CD81/CD63	2.00	0.80

F-NTA: Antibody staining PSD



Supplementary Figure S1. Procedural controls and PSD histograms of antibody-stained sEVs. Blank (dye + buffer) and unstained reactions (sEVs + buffer) were used as controls in experiments. None of the membrane (CMG, CMR), cytoplasmic (CFSE, CTR) nor antibody labels (anti-CD9, anti-CD63 and anti-CD81; single, double and triple) showed fluorescent artefacts on nFCM and F-NTA, proving efficient dye removal prior to measurements. PSD histograms represent concentration and size of particles measured in scatter and fluorescence mode on F-NTA after single, double and triple staining with different anti-tetraspanin antibodies conjugated to AF488. Such results showed lower staining percentage with single antibodies, while PSD of fluorescent particles leaned more toward higher values. Double and triple staining reactions labelled more sEVs, with apparently smaller diameter particles being more visible in fluorescence mode.



Supplementary Figure S2. Effect of excess dye on fluorescence measurements. Stained sEVs analyzed before UF washing showed very high fluorescent background, which interfered with accurate analysis of true positive events. UF efficiently removed all of the unbound dye, enabling proper fluorescence measurement, while maintaining total sEV subpopulation composition (scatter PSD before and after washing remains unchanged).

nFCM controls

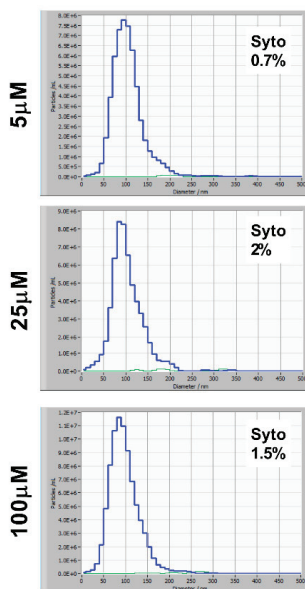
Blank reaction	Average conc. Fluo (p/uL)	SEM
Ribogreen	1.87E+05	9.27E+04
Syto	4.71E+05	9.20E+04

F-NTA controls

Blank reaction	Average number of fluorescent particles per position	SEM
Ribogreen [1:2]	0.30	-
Syto [100μM]	0.90	-
Unstained	0.50	-

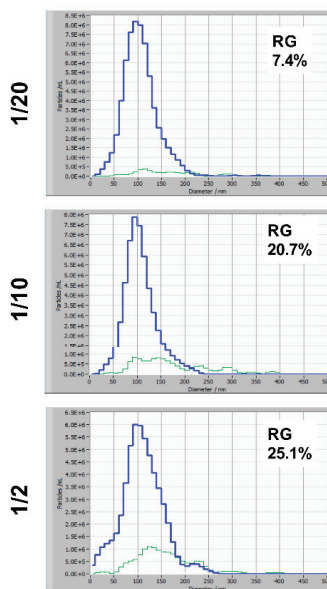
F-NTA: Syto PSD

Before washing

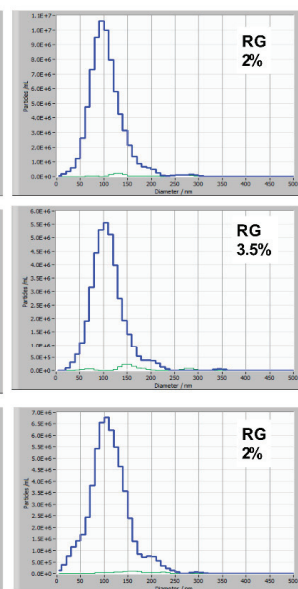


F-NTA: Ribogreen PSD

Before washing



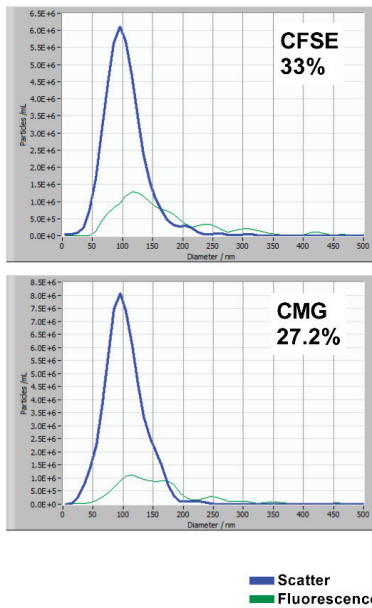
After washing



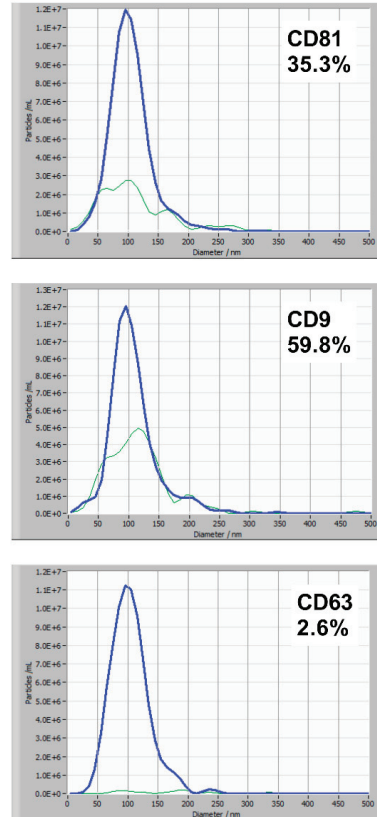
■ Scatter
■ Fluorescence

Supplementary Figure S3. Procedural controls and PSD histograms of sEVs stained with RNA dyes. Blank controls for both Syto and Ribogreen expressed negligible number of fluorescent events even without UF washing, showing that the dye on its own did not create artefacts. Reactions with Syto resulted in very low staining efficiency even with the highest concentration of 100μM. On the other hand, Ribogreen had a dose dependent increase in signal, with PSD showing preferential dye loading into larger particles. Nevertheless, signal was completely removed with UF washing.

F-NTA: CFSE and CMG staining PSD



F-NTA: Single antibody staining PSD



Supplementary Figure S4. F-NTA PSDs of sEVs stained directly in cell conditioned media. Staining of non-purified sEVs gave lower percentage of labelling for CFSE and CMG, with fluorescent PSD shifting to the right. However, single-antibody labelling had higher number of fluorescent events, compared to analogous reactions with purified sEVs.

nFCM: thyroglobulin control

Thyroglobulin staining				
	Staining reaction	Average conc. Fluo (p/μL)	% staining	% SEM
PE	CD9	1.16E+03	0.55	0.05
	CD63	1.05E+03	0.35	0.05
	CD81	2.25E+03	0.65	0.15
AF488	CD9	4.21E+03	2.10	0.00
	CD63	4.58E+04	12.05	3.85
	CD81	2.44E+04	4.25	1.65

F-NTA: thyroglobulin control

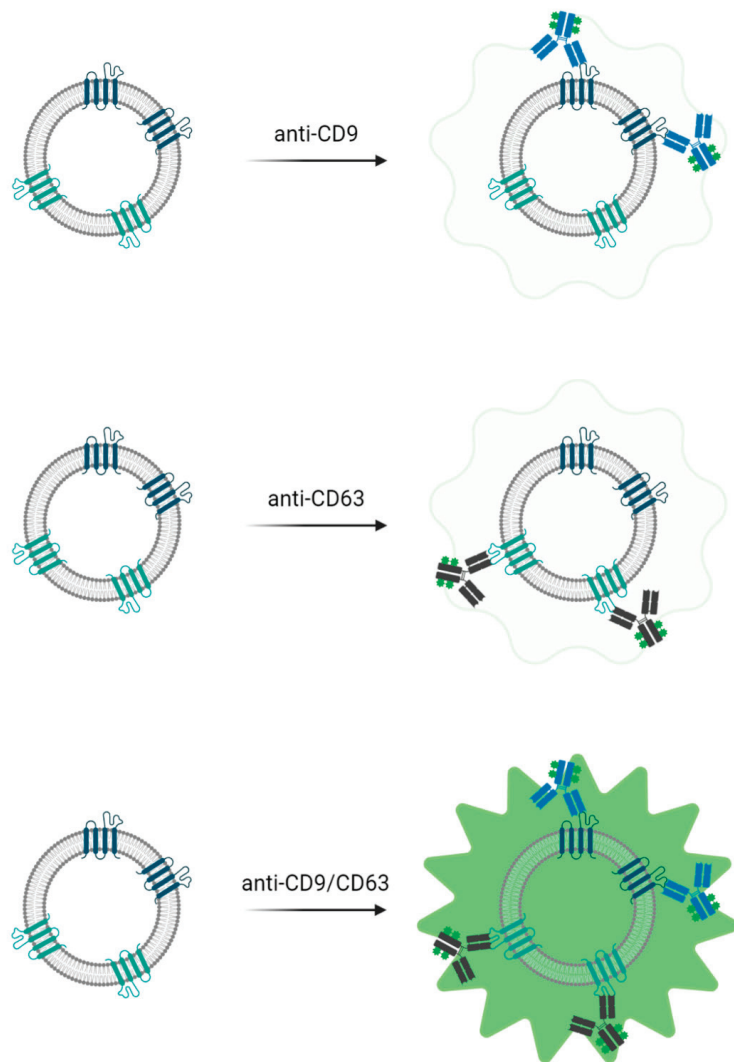
Thyroglobulin staining			
Staining reaction	Average number of fluorescent particles per position	% staining	% SEM
CFSE	1449.40	1265.75	2.15
CMG	15.20	13.13	0.63

F-NTA: BSA control

BSA staining		
Staining reaction	Average number of fluorescent particles per position	SEM
Before washing	1404.7	155.0
After washing	1.1	0.2

Blank reaction	Average number of fluorescent particles per position	SEM
Before washing	0.6	0.1
After washing	0.4	0.0

Supplementary Figure S5. Procedural controls of dye specificity. Membrane and cytoplasmic dyes, as well as antibodies were tested for their specificity using large globular protein, thyroglobulin. While majority of antibodies (except for anti-CD63 conjugated to AF488) showed very high specificity, CFSE exerted strong non-specific activation in presence of other proteins. In case of thyroglobulin, activated CFSE was detectable even after UF washing, as the protein was large enough to remain in the UF retentate. This was not the case with BSA, where all of the activated CFSE was successfully washed out. Spontaneous activation of CFSE in buffer only (blank reactions) was not observed.



Supplementary Figure S6. Schematic representation (created on BioRender.com) of sEV staining by fluorescently labeled primary antibodies, proposing that epitope scarcity on the surface of sEVs may limit the complete detection of certain subpopulations upon single staining, due to a lack of sensitivity of the platform used. If the labeling of multiple tetraspanins with the same dye renders visible previously undetected events after single staining, a correct co-expression analysis is not possible due to an underestimation of single marker expression.

Table S1. Fluorescence properties of different fluorophores.

Fluorophore	Extinction coefficient (ϵ) [$\text{M}^{-1}\text{cm}^{-1}$]	Quantum yield (Φ)	Brightness ($\epsilon \times \Phi$)
AF488	73,000	0.92	67,160
AF647	270,000	0.33	89,100
APC	700,000	0.68	476,000
PE	1,960,000	0.84	1,646,400

2 way Anova, Tukey's multiple comparisons test, alpha 0.05								
Within each column, compare rows (simple effects within columns)								
Number of families	3							
Number of comparisons per family	6							
Alpha	0,05							
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value			
CD9								
NanoFCM batch #A vs. F-NTA batch #A	-4,285	-12.94 to 4.373	No	ns	0,5253			
NanoFCM batch #A vs. NanoFCM batch #B	-15,08	-23.73 to -6.417	Yes	***	0,0005			
NanoFCM batch #A vs. F-NTA batch #B	19,9	12.27 to 27.54	Yes	****	< 0.0001			
F-NTA batch #A vs. NanoFCM batch #B	-10,79	-20.79 to -0.7930	Yes	*	0,0314			
F-NTA batch #A vs. F-NTA batch #B	24,19	15.06 to 33.31	Yes	****	< 0.0001			
NanoFCM batch #B vs. F-NTA batch #B	34,98	25.85 to 44.10	Yes	****	< 0.0001			
CD63								
NanoFCM batch #A vs. F-NTA batch #A	13,96	3.009 to 24.91	Yes	**	0,0094			
NanoFCM batch #A vs. NanoFCM batch #B	6	-2.364 to 14.36	No	ns	0,2199			
NanoFCM batch #A vs. F-NTA batch #B	16	8.703 to 23.30	Yes	****	< 0.0001			
F-NTA batch #A vs. NanoFCM batch #B	-7,96	-20.20 to 4.284	No	ns	0,2957			
F-NTA batch #A vs. F-NTA batch #B	2,043	-9.500 to 13.59	No	ns	0,9597			
NanoFCM batch #B vs. F-NTA batch #B	10	0.8773 to 19.13	Yes	*	0,0284			
CD81								
NanoFCM batch #A vs. F-NTA batch #A	21,19	10.24 to 32.14	Yes	***	0,0001			
NanoFCM batch #A vs. NanoFCM batch #B	17,99	9.626 to 26.35	Yes	****	< 0.0001			
NanoFCM batch #A vs. F-NTA batch #B	44,68	37.38 to 51.98	Yes	****	< 0.0001			
F-NTA batch #A vs. NanoFCM batch #B	-3,2	-15.44 to 9.044	No	ns	0,8846			
F-NTA batch #A vs. F-NTA batch #B	23,49	11.95 to 35.04	Yes	****	< 0.0001			
NanoFCM batch #B vs. F-NTA batch #B	26,69	17.57 to 35.82	Yes	****	< 0.0001			
Test details								
	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF
CD9								
NanoFCM batch #A vs. F-NTA batch #A	50,58	54,86	-4,285	3,106	4	2	1,951	21
NanoFCM batch #A vs. NanoFCM batch #B	50,58	65,65	-15,08	3,106	4	2	6,864	21
NanoFCM batch #A vs. F-NTA batch #B	50,58	30,67	19,9	2,739	4	3	10,27	21
F-NTA batch #A vs. NanoFCM batch #B	54,86	65,65	-10,79	3,587	2	2	4,255	21
F-NTA batch #A vs. F-NTA batch #B	54,86	30,67	24,19	3,274	2	3	10,45	21
NanoFCM batch #B vs. F-NTA batch #B	65,65	30,67	34,98	3,274	2	3	15,11	21
CD63								
NanoFCM batch #A vs. F-NTA batch #A	17,9	3,94	13,96	3,929	5	1	5,025	21
NanoFCM batch #A vs. NanoFCM batch #B	17,9	11,9	6	3,001	5	2	2,828	21
NanoFCM batch #A vs. F-NTA batch #B	17,9	1,897	16	2,619	5	3	8,641	21
F-NTA batch #A vs. NanoFCM batch #B	3,94	11,9	-7,96	4,393	1	2	2,563	21
F-NTA batch #A vs. F-NTA batch #B	3,94	1,897	2,043	4,141	1	3	0,6978	21
NanoFCM batch #B vs. F-NTA batch #B	11,9	1,897	10	3,274	2	3	4,321	21
CD81								
NanoFCM batch #A vs. F-NTA batch #A	66,64	45,45	21,19	3,929	5	1	7,627	21
NanoFCM batch #A vs. NanoFCM batch #B	66,64	48,65	17,99	3,001	5	2	8,478	21
NanoFCM batch #A vs. F-NTA batch #B	66,64	21,96	44,68	2,619	5	3	24,13	21
F-NTA batch #A vs. NanoFCM batch #B	45,45	48,65	-3,2	4,393	1	2	1,03	21
F-NTA batch #A vs. F-NTA batch #B	45,45	21,96	23,49	4,141	1	3	8,022	21
NanoFCM batch #B vs. F-NTA batch #B	48,65	21,96	26,69	3,274	2	3	11,53	21

2 way Anova, Tukey's multiple comparisons test, alpha 0.05									
Within each column, compare rows (simple effects within columns)									
Number of families	3								
Number of comparisons per family	6								
Alpha	0,05								
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value				
CD9									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	-8,425	-19.63 to 2.783	No	ns	0,1921				
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	19,9	10.02 to 29.79	Yes	****	< 0.0001				
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	-9,255	-19.14 to 0.6296	No	ns	0,0725				
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	28,33	16.51 to 40.14	Yes	****	< 0.0001				
NanoFCM batch #A CM vs. F-NTA batch #B CM	-0,83	-12.64 to 10.98	No	ns	0,9974				
F-NTA batch #B sEVs vs. F-NTA batch #B CM	-29,16	-39.72 to -18.59	Yes	****	< 0.0001				
CD63									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	-5,35	-16.18 to 5.478	No	ns	0,5374				
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	16	6.552 to 25.45	Yes	***	0,0005				
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	15,32	5.865 to 24.77	Yes	***	0,0008				
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	21,35	9.539 to 33.17	Yes	***	0,0002				
NanoFCM batch #A CM vs. F-NTA batch #B CM	20,67	8.852 to 32.48	Yes	***	0,0003				
F-NTA batch #B sEVs vs. F-NTA batch #B CM	-0,6867	-11.25 to 9.880	No	ns	0,9979				
CD81									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	-0,51	-11.34 to 10.32	No	ns	0,9992				
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	44,68	35.23 to 54.13	Yes	****	< 0.0001				
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	31,37	21.92 to 40.82	Yes	****	< 0.0001				
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	45,19	33.38 to 57.01	Yes	****	< 0.0001				
NanoFCM batch #A CM vs. F-NTA batch #B CM	31,88	20.06 to 43.69	Yes	****	< 0.0001				
F-NTA batch #B sEVs vs. F-NTA batch #B CM	-13,32	-23.88 to -2.750	Yes	**	0,0096				
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	N1	N2	q	DF	
CD9									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	50,58	59	-8,425	4,086	4	2	2,916	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	50,58	30,67	19,9	3,603	4	3	7,811	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	50,58	59,83	-9,255	3,603	4	3	3,633	26	
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	59	30,67	28,33	4,307	2	3	9,302	26	
NanoFCM batch #A CM vs. F-NTA batch #B CM	59	59,83	-0,83	4,307	2	3	0,2726	26	
F-NTA batch #B sEVs vs. F-NTA batch #B CM	30,67	59,83	-29,16	3,852	3	3	10,7	26	
CD63									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	17,9	23,25	-5,35	3,947	5	2	1,917	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	17,9	1,897	16	3,445	5	3	6,569	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	17,9	2,583	15,32	3,445	5	3	6,287	26	
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	23,25	1,897	21,35	4,307	2	3	7,012	26	
NanoFCM batch #A CM vs. F-NTA batch #B CM	23,25	2,583	20,67	4,307	2	3	6,787	26	
F-NTA batch #B sEVs vs. F-NTA batch #B CM	1,897	2,583	-0,6867	3,852	3	3	0,2521	26	
CD81									
NanoFCM batch #A sEVs vs. NanoFCM batch #A CM	66,64	67,15	-0,51	3,947	5	2	0,1827	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B sEVs	66,64	21,96	44,68	3,445	5	3	18,34	26	
NanoFCM batch #A sEVs vs. F-NTA batch #B CM	66,64	35,27	31,37	3,445	5	3	12,88	26	
NanoFCM batch #A CM vs. F-NTA batch #B sEVs	67,15	21,96	45,19	4,307	2	3	14,84	26	
NanoFCM batch #A CM vs. F-NTA batch #B CM	67,15	35,27	31,88	4,307	2	3	10,47	26	
F-NTA batch #B sEVs vs. F-NTA batch #B CM	21,96	35,27	-13,32	3,852	3	3	4,889	26	



Correction

Correction: Fortunato et al. Opportunities and Pitfalls of Fluorescent Labeling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms. *Int. J. Mol. Sci.* 2021, 22, 10510

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In the original publication [1], there was an error regarding the affiliation for Francesca Loria. In addition to affiliation 2, the updated affiliation should include the following: Department of Chemistry and Biotechnology, Tallinn University of Technology, 12618 Tallinn, Estonia. The authors state that the scientific conclusions are unaffected. This correction was approved by the Academic Editor. The original publication has also been updated.

Reference

1. Fortunato, D.; Mladenović, D.; Criscuoli, M.; Loria, F.; Veiman, K.-L.; Zocco, D.; Koort, K.; Zarovni, N. Opportunities and Pitfalls of Fluorescent Labeling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms. *Int. J. Mol. Sci.* **2021**, *22*, 10510. [[CrossRef](#)] [[PubMed](#)]

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Appendix 2

Publication II

Zuppone, S., Zarovni, N., Noguchi, K., **Loria, F.**, Morasso, C., Löhmus, A., Nakase, I., & Vago, R. (2024). Novel loading protocol combines highly efficient encapsulation of exogenous therapeutic toxin with preservation of extracellular vesicles properties, uptake and cargo activity. *Discover Nano*, 19(1). <https://doi.org/10.1186/s11671-024-04022-8>.

Research

Novel loading protocol combines highly efficient encapsulation of exogenous therapeutic toxin with preservation of extracellular vesicles properties, uptake and cargo activity

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Abstract

Extracellular vesicles (EVs) have mostly been investigated as carriers of biological therapeutics such as proteins and RNA. Nevertheless, small-molecule drugs of natural or synthetic origin have also been loaded into EVs, resulting in an improvement of their therapeutic properties. A few methods have been employed for EV cargo loading, but poor yield and drastic modifications of vesicles remain unsolved challenges. We tested a different strategy based on temporary pH alteration through incubation of EVs with alkaline sodium carbonate, which resulted in conspicuous exogenous molecule incorporation. In-depth characterization showed that vesicle size, morphology, composition, and uptake were not affected. Our method was more efficient than gold-standard electroporation, particularly for a potential therapeutic toxin: the plant Ribosome Inactivating Protein saporin. The encapsulated saporin resulted protected from degradation, and was efficiently conveyed to receiving cancer cells and triggered cell death. EV-delivered saporin was more cytotoxic compared to the free toxin. This approach allows both the structural preservation of vesicle properties and the transfer of protected cargo in the context of drug delivery.

Keywords Extracellular vesicles · Cargo loading · Drug delivery · Saporin · Therapeutic molecules · Nanovectors

1 Introduction

Recently, increasing efforts have been made to exploit nanovectors for drug delivery. They represent a privileged system that offers several advantages over the use of drugs as such. In fact, as the drug is “protected” inside the carrier, it is not degraded by serum components; therefore, encapsulation often enhances its solubility and enables higher accumulation in target cells. In this context, naturally secreted extracellular vesicles (EVs), which have the intrinsic ability to carry and deliver diverse bioactive cargoes, appear to be a preferable choice compared to synthetic particles, mostly due to the lack of immunogenicity and innate stability in biological fluids. In particular, exosomes represent a class of EVs that

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1186/s11671-024-04022-8>.

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have attracted a special attention because of their nanoscale size (30–150 nm), which allows them to easily penetrate almost all body areas and cross biological barriers, as well as their cargo protection and customizable surface, making them ideal vectors for targeted drug delivery in medical applications [1, 2]. They originate from inward budding of the late endosome membrane, resulting in the formation of small vesicles inside the lumen. The neo-formed structures, called multivesicular bodies, can either fuse with lysosomes for degradation or with the cell membrane, releasing exosomes in the external environment. The molecular composition of thus formed vesicles strongly depends on the cell of origin, containing different biomolecules, such as proteins, carbohydrates, nucleic acids, and lipids, which are finely sorted and transferred to the receiving cells to mediate intercellular communication and regulate many biological processes [2]. Although we base our attachment to the term “exosome” to more than just a vesicle size (for instance we consider the presence of typical ESCRT proteins from the exosome biogenesis pathway), in congruence with the latest suggestions on EV nomenclature [3] we refer to exosomes as nano-sized EVs (nsEVs) throughout this article.

To date, multiple research groups have been developing an efficient approach to incorporate bioactive molecules into nsEVs. The use of active loading methods that exploit mechanical (extrusion), chemical (detergent permeabilization), or physical stimuli (sonication, electroporation, freeze and thaw cycles), for instance, has yielded positive results in terms of increased encapsulation efficiency. However, the major drawback of these techniques is that the structural architecture of vesicles is critically affected, often triggering vesicle aggregation and alteration in size [4–11]. Electroporation, in particular, has been widely used to incorporate a broad spectrum of druggable molecules into EVs, including nucleic acids such as DNA [12], siRNA [13], and miRNA [14], proteins [6, 8, 15], and small molecules [16, 17], claiming to have achieved increased loading efficiency compared to other methods. Noteworthy, its loading efficiency varied consistently in different studies [18], fundamentally depending on type of exogenous cargo; it was typically reported high for small RNAs and drugs (0.5–60%) and very low for proteins (< 0.6%). Consequently, fine optimization of the electroporation protocol is required taking into consideration the properties of the drug used, in order to allow efficient particle engineering, whereas the efficacy of the resulting complexes is significantly hampered by molecular aggregation and structural damage of the vesicles [19]. The passive loading strategies, modeled on the incubation of EVs/cells with free drugs, are simple and do not compromise the vesicles membrane integrity but, on the other hand, often lead to a very low cargo-incorporation [9, 11, 18], with active loading methods being generally considered superior.

To overcome these challenges, in the present study, we tested the efficiency of a new strategy based on the use of a highly concentrated alkaline buffer to induce conspicuous incorporation of exogenous molecules into nsEVs with a higher loading efficiency compared to electroporation, while preserving the structural and functional features of the vesicles. In this study, we applied our protocol to hydrophilic molecules, with the ultimate goal of loading into EVs a small protein proposed as an anticancer agent. Encapsulation was shown to protect the cargo from enzymatic degradation and to favor the efficient delivery of a toxic payload, which promotes the death of recipient cancer cells. Altogether, we designed a simple protocol to efficiently load nsEVs with functional exogenous therapeutic molecules and preserve their integrity and uptake.

2 Results

2.1 A pH alteration via sodium carbonate-based exposition enhances exogenous molecules incorporation and preserves nano-sized EVs structure and uptake properties

Since extracellular vesicles reflect the cell-of-origin molecular composition, it is very important to select the proper cell line to produce safe carriers for therapeutic applications. In this study, we opted for commercially available HEK293 cells as vesicle donors because they have been reported to be one of the most frequently used systems for this purpose, including EV-based therapeutics that are currently in clinical trials [20]. Besides, HEK293 cells and their subtypes are widely used in the production of biotherapeutics such as recombinant proteins and viruses [21].

We set up and optimized the protocol for the isolation and purification of EVs from HEK293 culture medium, consisting of sequential centrifugation and ultracentrifugation steps, and a 0.22 µm filtration to enrich for nsEVs [22]. We then evaluated the impact of incubation in a carbonate buffer on the physiological properties of the collected vesicles. We performed accurate molecular and structural characterization of EVs, as much as possible in accordance to the updated guidelines of the International Society for Extracellular Vesicles (MISEV2018 and 2023) [23, 24]. After recovery, nsEVs appeared to be perfectly preserved in their morphological and biophysical properties, as well as in their overall number,

as shown by TEM and nanoparticle tracking analysis (Fig. 1A and B). Independent of the incubation of isolated EVs in PBS or carbonate buffer, TEM analysis revealed vesicles to be homogeneous in shape, with a size distribution picking at 150 nm in diameter, as determined also by NTA. Typical EV markers (CD63 and CD9) and exosome markers (Alix and TSG101) were still present in nsEVs after treatment (Fig. 1C). Notably, the treatment did not alter the reactivity of single vesicles towards anti-CD9 or -CD63 antibodies, which were still able to capture them on the chip (Fig. 1E) in a comparable manner with respect to a control sample. The slight reduction in these proteins observed in WB was in line with silver staining, which showed a general but modest decrease in total proteins (Fig. 1D). Accordingly, in the Raman spectra, the peak at 2930 cm^{-1} , relative to CH_2 in proteins, appeared to be slightly reduced [25], confirming that treatment with sodium carbonate affects the protein content of recovered nsEVs, even if not significantly (Fig. 1E). At the same time, no shift in peak position was observed that would indicate a change in the protein structure. Notably, we did not observe any variation in the intensity of the peak at 2851 cm^{-1} which corresponds to methyl groups in lipids (Fig. 1F).

These initial results were double-checked and confirmed by additional analytical package assessing, in addition to particle numbers and overall protein content (Fig. 2A and B), also the content of cholesterol content, which is known to be enriched in EV membranes from mammalian cells (Fig. 2E). We also assessed the preserved immunoreactivity of surface-displayed proteins (CD63 and CD9 in ELISA) (Fig. 2C) and surface glycans (by assessing lectin binding) (Fig. 2D). Finally, the overall nucleic acid content of the recovered EVs was assessed using the RiboGreen assay (Fig. 2F). Overall

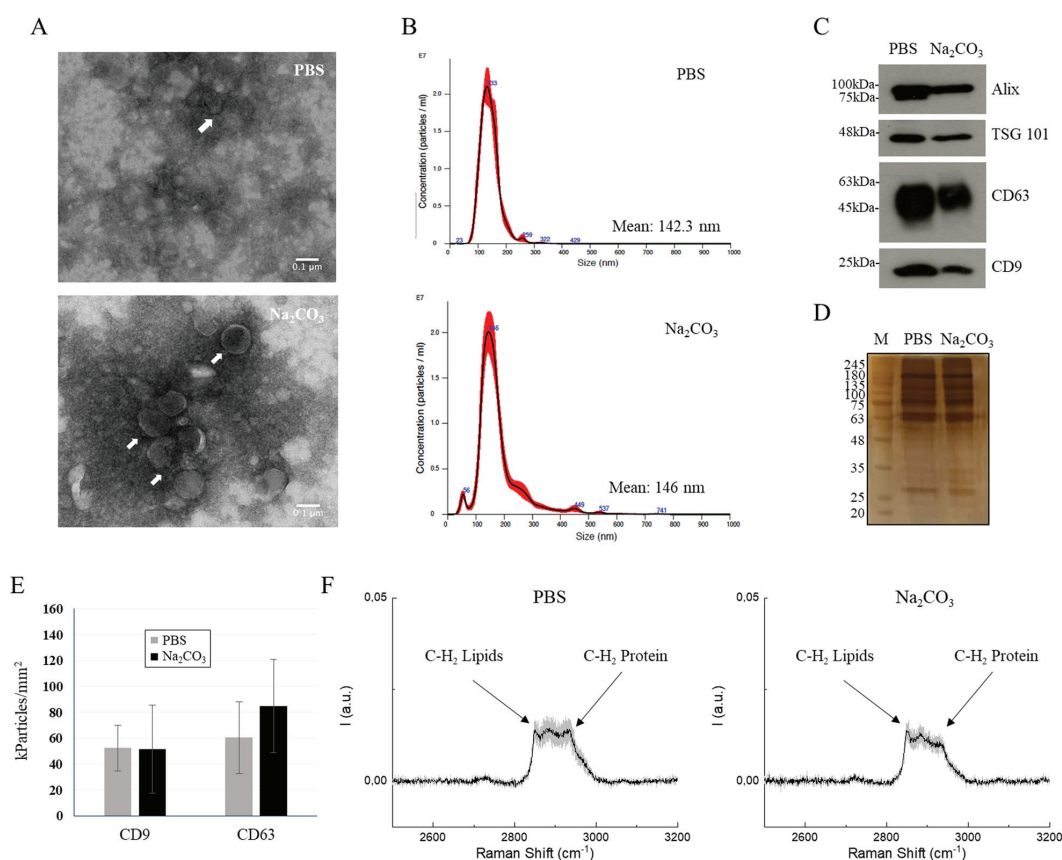


Fig. 1 Evaluation of structural modifications of nsEVs after sodium carbonate treatment. Transmission electron microscopy (TEM) image (**A**) and nanoparticle tracking analysis (**B**) of HEK293-derived nsEVs after sodium carbonate treatment. Western blot analysis (**C**) of Alix, TSG101, CD63 and CD9 before (pre) and after (post) sodium carbonate treatment. Comparison of nsEVs treated with phosphate buffer saline (PBS) or sodium carbonate (Na_2CO_3) through silver staining (**D**) single-particle interferometric reflectance imaging sensor (SP-IRIS) analysis (**E**) and Raman spectroscopy (**F**). M: molecular weight marker

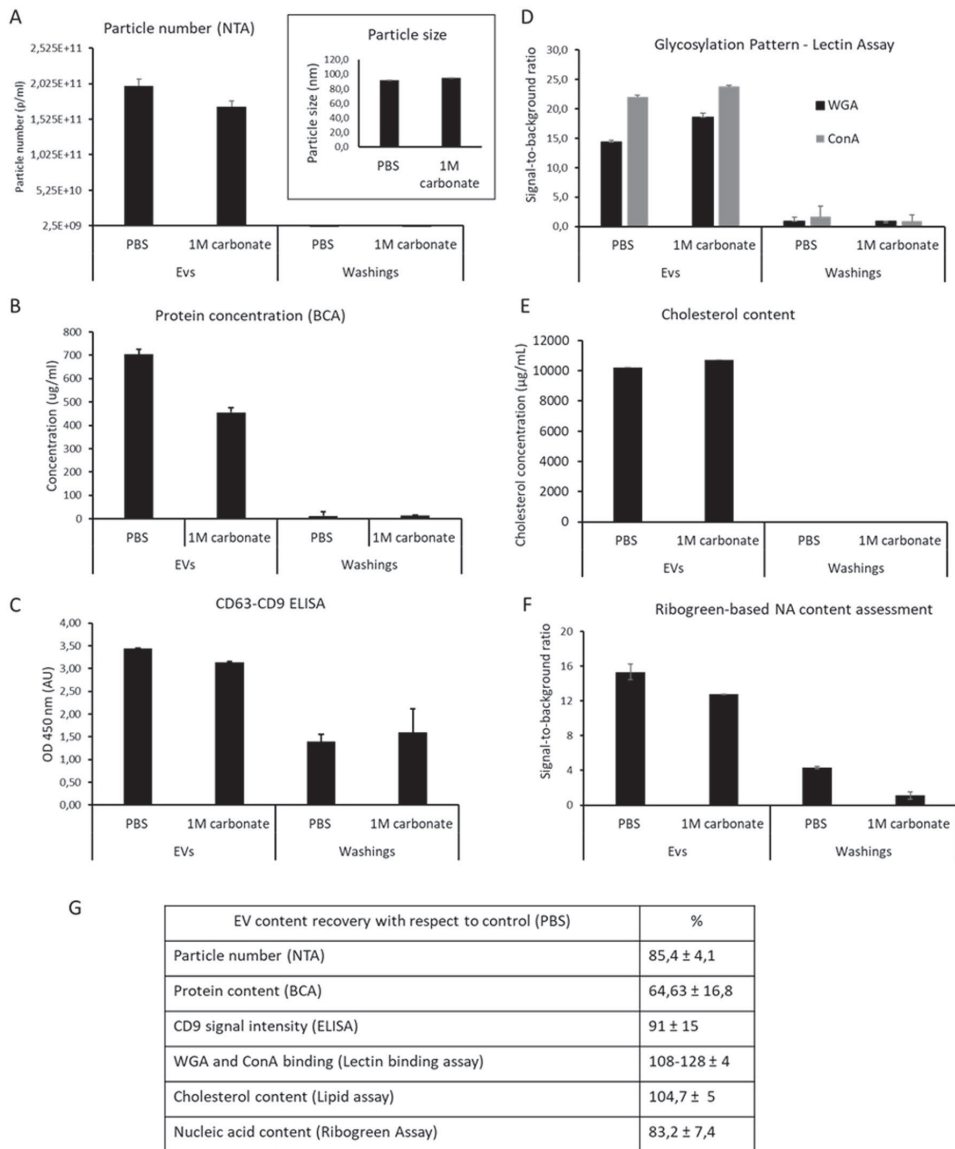


Fig. 2 Evaluation of nsEVs content and integrity after sodium carbonate treatment. The number and size of particles recovered upon nsEV incubation in either PBS or in carbonate buffer was assessed by NTA (**A**). The overall protein concentration was measured by Bicinchoninic Acid Assay (BCA) (**B**). 10^9 of particles were loaded per well of 96-well microplate and following assays are performed for the measurement of cholesterol content (**E**), assessment of surface glycans by lectin (ConA and WGA) binding (**D**), and quantification of total nucleic acids content via Ribogreen Assay (**F**). All assays are performed according to manufacturer's indications for commercial kits or otherwise detailed in Material and Methods. The fluorescent signal measured at the plate reader fluorimeter and signal expressed either as a ratio over assay background control (PBS) or as analyte concentration using kit provided standard calibration curve (for cholesterol). Surface tetraspanin expression was assessed by sandwich immunoassay (ELISA) featuring precoated aCD63 microplate and aCD9 detection antibody according to a protocol provided within the kit (see Material and Methods) (**C**). The absorbance (OD 450 nm) is measured on a bench top microplate reader

Table 1 Assessment of EV content recovery after sodium carbonate treatment expressed as percentage	EV content recovery with respect to control (PBS)	%
	Particle number (NTA)	85.4 ± 4.1
	Protein content (BCA)	64.63 ± 16.8
	CD9 signal intensity (ELISA)	91 ± 15
	WGA and ConA binding (lectin binding assay)	108–128 ± 4
	Cholesterol content (lipid assay)	104.7 ± 5
	Nucleic acid content (ribogreen Assay)	83.2 ± 7.4

results showed a high integrity of vesicular membranes and content, with a decrease in particle number being less pronounced than that of overall proteins (recovery of 85% and 65%, respectively), and in line with maintained nucleic acid content (83%) (Table 1).

The drop-in particle number was not associated with a change in particle size; therefore, aggregation was unlikely to occur. Moreover, alkaline buffers are known to decrease the rate of aggregation by removing impurities and are therefore used for this purpose in various purification methods. Given the highly preserved cholesterol content, it is possible that the measurement of particle number and protein content overestimates the loss of nsEVs and instead indicates the loss of non-EV contaminants, possibly protein aggregates that can give a signal in NTA, and/or material loosely associated with the EV membrane. Indeed, it is known that the use of a carbonate buffer disrupts the association of peripherally associated proteins or soluble proteins without disrupting the membrane [26]. It is therefore possible that higher lectin binding (> 100% with respect to PBS treated EVs) is due to a cleaner preparation and better exposure of surface glycans.

Finally, to illustrate the preservation of both intrinsic nsEV structure and, possibly, the integrity of engineered membrane proteins upon carbonate buffer exposure, we took advantage of fluorescent vesicles produced in-house from a recombinant HEK293 model expressing the CD9-RFP fusion protein [22]. As shown in Supplementary: Fig. 1, western blot analysis demonstrated that incubation in neither PBS nor sodium carbonate altered the CD9-RFP protein structure, as only CD9 associated RFP and no free fluorescent proteins was detectable. Concordantly, recombinant vesicle fluorescence was well appreciated in a fluorimeter (not shown). Therefore, both intrinsic and engineered EV membrane molecules are well-preserved and functional.

2.2 Comparative assessment of nsEV loading efficiency upon alkaline buffer treatment or electroporation

The effect of sodium carbonate treatment on vesicle loading efficiency was compared to that of electroporation as a benchmark. To this end, we initially used FITC-labelled dextran with a molecular size of 70 kDa as a trackable fluorescent

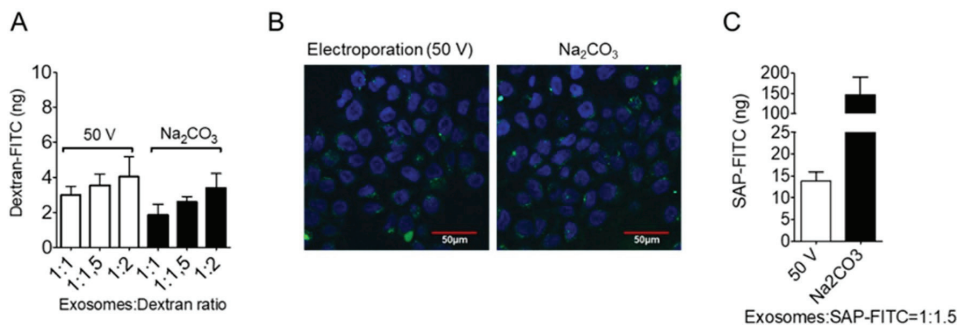


Fig. 3 Encapsulation efficiency evaluation and comparison between electroporation and sodium carbonate treatment. **A** Spectrofluorometer analysis for quantification of Dextran-FITC incorporation after 50 V electroporation (see material and methods) or sodium carbonate treatment of HEK293-derived nsEVs (25 µg/ml) in various nsEVs:Dextran-FITC ratios (1:1, 1:1.5 and 1:2). **B** Confocal microscopy images of HeLa cells treated for 24 h with Dextran-FITC bearing nsEVs (25 µg/ml) after electroporation (50 V) or sodium carbonate incubation in nsEVs:Dextran-FITC = 1:1.5 molecular ratio (blue = DAPI; green = Dextran-FITC bearing nsEVs). **C** Spectrofluorometer analysis for quantification of SAP-FITC incorporation after 50 V electroporation (see material and methods) or sodium carbonate treatment of HEK293-derived nsEVs (25 µg/ml) in nsEVs:SAP-FITC = 1:1.5 ratio

payload. This small sugar molecule is commonly used to study the in-cell transport, cell permeation, and *encapsulation efficiency*. No significant differences in loading capacity in terms of total cargo encapsulation could be observed between the two treatments; notably, the cargo loading efficiency remained low, around 0.2–0.3%. In both cases, the dextran-FITC incorporation capacity was shown to be directly dependent on the amount of payload used when different nsEVs:dextran ratios were used (Fig. 3A). The similar performance of the two treatments was further confirmed by the uptake assay performed on HeLa cells, in which a comparable green punctuation was detectable after 24 h on confocal microscopy (Fig. 3B).

Next, we investigated whether sodium carbonate encapsulation efficiency was dependent on the structural properties of the payload. To achieve this goal, we used a 25 kDa plant derived globular protein, saporin (SAP), whose enzymatic activity is directed at inhibiting protein synthesis [27–30]. First, we used a fluorescently labelled protein, SAP-FITC, and an nsEV-to-cargo ratio of 1:1.5 (w:w). Strikingly, while in the case of electroporation, SAP-FITC incorporation was 4–5 times improved compared to that of dextran, reaching a loading efficiency of 1.5%, sodium carbonate treatment reached a 50 folds higher loading capacity of SAP-FITC with respect to dextran, significantly overperforming the electroporation (Fig. 3C) and reaching a loading efficiency of 15%. This demonstrates the relationship between encapsulation efficiency and steric hindrance of the molecule used. The use of higher electroporation voltages (100 V and 150 V) together with higher nsEV:SAP ratios (1:2) seemed to poorly improve SAP-FITC encapsulation, but instead caused vesicle damage, as shown by reduced total protein recovery after treatment (Supplementary Fig.2). Overall, in our settings, the carbonate buffer-based strategy resulted in a tenfold more efficient loading of a 25 kDa protein into nsEVs with respect to electroporation.

2.3 Sodium carbonate treated-SAP-encapsulating nsEVs exert a high cytotoxic activity on cancer cells

The ultimate way for assessing the success of a loading strategy involves a specific in vitro or in vivo biological outcome. Hence, we investigated whether SAP loaded nsEVs could deliver and efficiently release their cargo in vitro to recipient cells. To answer this question, we first assessed whether sodium carbonate treatment affects cellular uptake. Upon exposure to PBS or carbonate buffer, vesicles were labelled with a lipophilic fluorescent dye and co-incubated with 5637 bladder cancer cells for 24 h. As shown by microscopy and flow cytometry analysis, no significant differences were observed between the fluorescence signals observed in cells receiving PBS-treated or sodium carbonate-treated nsEVs, further indicating that short-term pH modification did not alter the capacity of the nanovesicles to be captured by cells (Fig. 4). As expected, the fluorescence signal depended on the dose of nsEVs used.

5637 bladder cancer cells were then treated for 72 h with increasing doses of SAP-bearing HEK nsEVs (corresponding to 5, 10, and 20 μ g of protein) obtained by incubation with the toxin after prior incubation with sodium carbonate or PBS, as previously described. Sodium carbonate-treated SAP-bearing nsEVs (termed nsEV-SAP Na_2CO_3) strikingly affected cell viability in a dose-dependent manner and to a significantly improved extent compared to the nsEVs recovered after

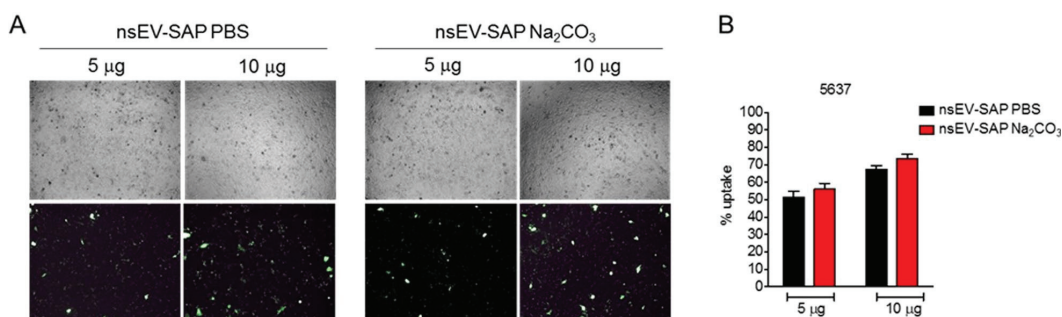


Fig. 4 Evaluation of sodium carbonate-treated SAP-bearing nsEVs uptake. **A** Bright field (upper row) and fluorescent (lower row) microscopy images of 5637 cells 24 h after incubation with HEK293-derived nsEVs treated with phosphate buffer saline (PBS, left) or sodium carbonate (Na_2CO_3 , right) in the presence of SAP and stained with Vybrant Dio lipophilic dye. Different concentrations of nsEVs (5 and 10 μ g) were used for comparison. Representative images of three independent experiments. **B** Flow cytometry analysis of 5637 cells 24 h after treatment with 5 or 10 μ g of PBS or Na_2CO_3 -treated SAP-bearing nsEVs. Results are shown as mean fluorescence percentage + SD from 3 independent experiments

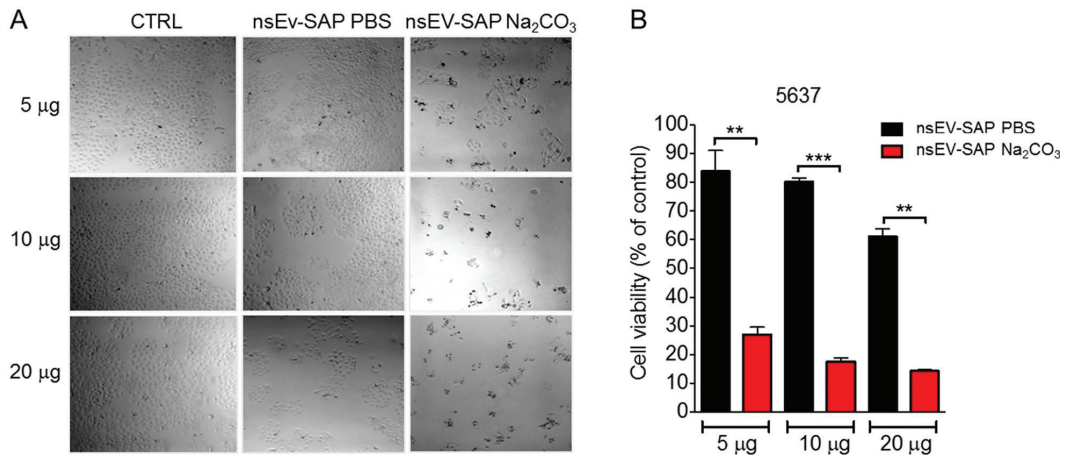


Fig. 5 SAP-loaded nsEVs toxic activity evaluation on 5637 cancer cells. **A** Bright field microscopy images of 5637 cells 72 h after treatment with 5, 10 or 20 µg of SAP-bearing nsEVs (nsEVs-SAP) following sodium carbonate or PBS incubations. **B** MTT assay on 5637 cells after 72 h incubation with 5, 10 or 20 µg of SAP-bearing nsEVs treated with sodium carbonate (red) or PBS (black). Results are shown as mean percentage residual viability to control + SD from three independent experiments. Test t $^{**}p < 0.01$, $^{***}p < 0.001$

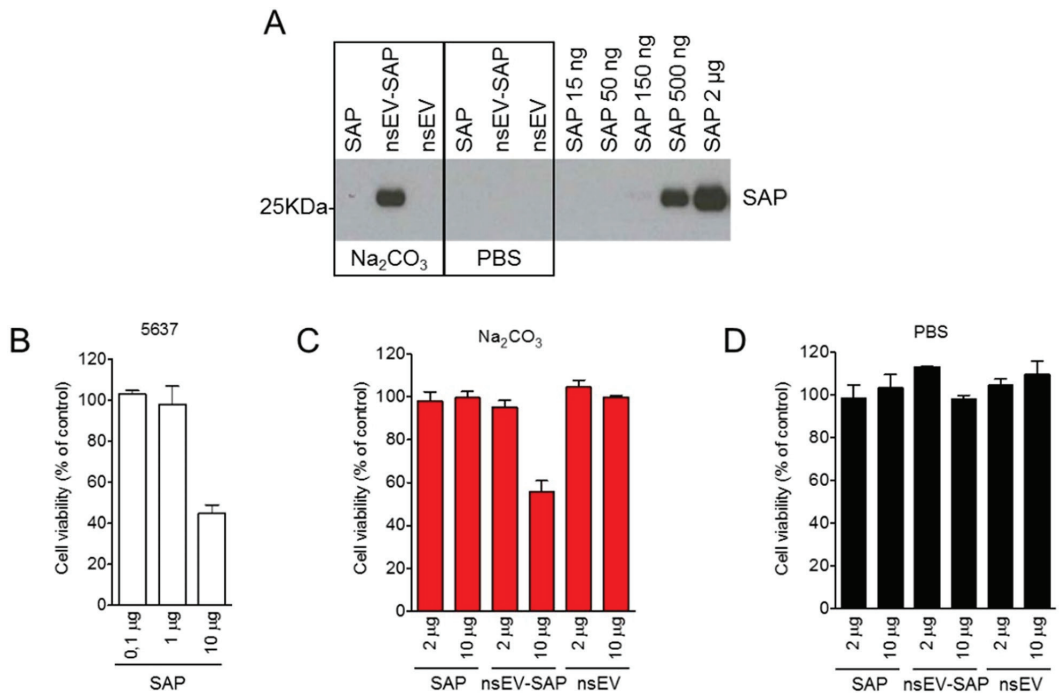


Fig. 6 Elimination of SAP excess from nsEVs surface and evaluation of sodium carbonate encapsulation efficiency. **A** Western blot analysis of SAP from sodium carbonate (Na₂CO₃) or phosphate buffer saline (PBS)-treated nsEVs. Excess of SAP was removed by washing steps with sodium chloride. 5 µg of nsEV proteins are loaded per lane. Non-loaded nsEVs and increasing concentrations of SAP alone were used as control. **B** MTT assay on 5637 bladder cancer cells after 72 h incubation with scalar concentrations of SAP. MTT assay on 5637 bladder cancer cells after 72 h incubation with 2 and 10 µg of SAP, SAP-bearing nsEVs (nsEV-SAP) or nsEVs alone after Na₂CO₃ (**C**) or PBS (**D**) treatments. Samples were filtered with 100 kDa MWKO centrifugal devices to remove any unincorporated SAP. Results are shown as mean percentage residual viability to control + SD from three independent experiments

PBS treatment (nsEV-SAP PBS). Indeed, it can be appreciated that already the lowest dose of nsEV-SAP Na_2CO_3 used (corresponding to 10^9 particles, with an estimated SAP content of 500–750 ng, based on observed loading capacity, Fig. 3B and C) is higher than the effective IC_{50} dose (a dose that kills over 50% of recipient cells) (Fig. 5). This observation is consistent with successful intravesicular protection and intracellular delivery of SAP.

We could also observe, although much smaller, a drop of cell viability upon treatment with a highest dose of nsEV-SAP PBS and hypothesized that this could be due to the saporin sticking to the nsEV surface. To test this hypothesis and reduce this surface association, accurately estimate the amount of encapsulated drug, and to guarantee limited potential by-stander toxic effects of the unincorporated fraction, we added sodium chloride washing steps following SAP encapsulation. As evidenced by western blot analysis (Fig. 6A) of the processed nsEV samples, the SAP band was well detected in the carbonate-treated sample, whereas no SAP signal was retained in the PBS-treated control. By comparing the intensity of the nsEV-SAP band to that of serial dilutions of free protein, we estimated that 10 μg of loaded nsEVs contained approximately 1 μg SAP, which is in line with the loading efficiency estimated using fluorescently labelled protein in a prior experiment.

At the same time, our hypothesis that some saporins bind to the nsEV surface was confirmed by the fact that the addition of sodium chloride washing resulted in lower cytotoxicity of nsEV-SAP Na_2CO_3 on tumor cells (Fig. 5B) with an effective toxic dose, measured as IC_{50} , corresponding to more than 10 μg of nsEVs bearing SAP for washed samples, with respect to less than 5 μg of unwashed nsEVs used in a prior experiment (Fig. 5A).

Notably, we demonstrated that a conspicuously higher concentration of SAP toxin alone (approximately 8 μg of free toxin with respect to 1 μg of nsEV-encapsulated toxin) was needed to reach the IC_{50} under the same conditions (Fig. 6B and C). This is likely due to the easier internalization of nsEV-delivered SAP than that of toxin alone. Furthermore, the toxic activity on cancer cells could not be influenced by the presence of the buffer, nor by the presence of the excess of non-encapsulated SAP, as both of them are completely eliminated during the washing procedure with centrifugal filter columns (Fig. 6C and D).

3 Discussion

In the last decades, the use of EVs as drug nanocarriers has revealed as a challenge for many research groups. Owing to their natural origin and morphological characteristics, the biomedical applications of these vesicles can overcome many of the drawbacks faced by artificial nanoparticles. Many strategies have been explored to either actively or passively load exogenous therapeutic cargoes into EVs, including genetic engineering of parental cells or direct application of mechano-physical stress to purified vesicles to perturb membrane integrity [6]. One of the most effective and commonly employed approaches is electroporation, widely used by many research groups to incorporate a variety of molecules, including siRNAs, small molecules, and enzymes [4, 6, 7, 31]. However, when used to load exogenous molecules into EV lumen, it results in a very low loading efficiency (0.2% in the case of SAP), accompanied by a severe structural alteration of the EV morphology [6, 15]. Overall, the exposure of vesicles to an electric field strongly affects their properties, causing aggregation and damage with a significant loss of the starting material.

In the present study, we took advantage of a highly concentrated alkaline buffer, sodium carbonate, to establish a method that, throughout pH alteration, can reversibly perturb nsEV membranes and favor the encapsulation of exogenous molecules. Sodium carbonate was first used in 1982 by Fujiki et al. to isolate intracellular/organellar membranes [32]. They demonstrated that incubation of purified fractions of rat liver endoplasmic reticulum, peroxisomes, or mitochondria in sodium carbonate induced the conversion of membranes into membrane sheets, which retained only integral membrane proteins, but not lipid-anchored ones. In fact, alkaline pH decreases noncovalent protein–protein interactions, releasing weakly attached peripheral membrane proteins without affecting membrane integrity. Here, we first proved that the use of a high concentration of sodium carbonate (1 M) can preserve the morphological and biochemical properties of vesicles. For this purpose, we combined several assays to assess the independent vesicle parameters that are indicative of their amount and integrity. The decrease in particle count and protein content to 85–65% respectively, with respect to PBS treated control indicated a moderate loss of nsEVs due to the loading protocol. However, it is possible that this loss is overestimated, as alkaline washing is likely to clear up the EV sample of associated proteins and decrease the amount of protein aggregates that may appear as particles. If we use the lipid content as an indicator of the amount of EVs in our preparation, the loss of nsEVs is negligible. Therefore, it is likely that this alkaline wash adds value to the purity of EV preparations. Moreover, the preserved integrity of nsEVs recovered after carbonate treatment is supported not only by the well-maintained protein or lipid content per particle but also by the preserved recognition of surface proteins

and glycans in ELISA and lectin binding assays. Therefore, rather than interfering with the vesicle corona, this treatment ensured the fine display and functionality of the EV membrane domains, potentially mediating recognition and uptake. Finally, the fully preserved functionality of the recombinant protein (CD9-RFP fusion) in engineered HEK nsEVs upon carbonate buffer incubation is a fine proof that this loading strategy might be applied to EVs with modified membrane domains, and thus, is compliant and complementary with other strategies for tailored nsEV fabrication.

We envisaged that carbonate treatment would prompt exogenous molecule encapsulation by co-incubation depending on the type and size of the molecule used. This hypothesis was confirmed by the observation that molecules with different structural properties (dextran and SAP) are incorporated in a dose-dependent manner. However, the distinctive steric hindrance of the two molecules was reflected in the encapsulation efficiency, which was higher when globular enzyme SAP was used. Nonetheless, we showed that the sodium carbonate-induced loading capacity appears to be significantly improved compared to electroporation under our experimental conditions. Importantly, we excluded SAP aggregation on the surface by introduction of washing with saline solution. The retention of the SAP signal in carbonate buffer-treated nsEVs, but not in PBS-incubated nsEVs, clearly indicated that a significant fraction of endogenous protein was indeed encapsulated and protected by the vesicular membrane. The difference between sodium carbonate-treated SAP-bearing nsEVs and PBS control treatment was unambiguously evident in the *in vitro* cytotoxic assay, where the former was able to strongly affect cell viability at a very low dose. Conversely, PBS-treated SAP-bearing nsEVs did not produce any marked toxic effects even at the highest concentration used. It is worth mentioning that in our study, an effective *in vitro* dose of SAP-loaded nsEVs corresponded to 10^9 – 10^{10} vesicles, being thus in a reported dose range for EV based functional assays [33] and bearing an effective concentration of SAP to cause striking, tumor cell-killing, activity. Importantly, the effective SAP IC_{50} was in the nanomolar range, a dose significantly lower than the necessary IC_{50} of a free toxin and comparable to the efficacy of targeted SAP variants reported in the literature [29, 30]. Type I RIPs like SAP are single chain toxins that lack specific targeting moieties. Their internalization capacity and resulting toxicity are thousand folds lower compared to type II RIPs, like ricin, double-chain toxins equipped with binding domains. SAP cytotoxicity varies dramatically between different mammalian cell lines, likely due to different expression of surface proteins involved in the toxin internalization [28]. SAP encapsulated into EVs is subjected to cell uptake through different mechanisms, which are driven by the carrier: EVs are known to be internalized by clathrin-dependent or caveolin-mediated endocytosis, macropinocytosis, phagocytosis, lipid raft-mediated uptake and direct fusion [34]. EV-carried SAP resulted effective in reducing bladder cancer cell viability, while SAP alone did not exert any cytotoxicity, possibly due to easier toxin internalization and efficient release into the cytoplasm, where its inhibitory activity on translation is exerted. This method greatly broadens the potential targets of SAP and its use as anti-cancer therapeutic.

Finally, we demonstrated that the use of a physiological concentration of sodium chloride in the washing step allows the surface-attached portion of SAP to be released, lowering the weak protein interactions with the vesicle membrane. Thus, the biological effect is confined to the encapsulated toxin, rendering its delivery to recipient cells safer and more controlled. We could observe that, while for 75 kDa dextran the efficiency of carbonate buffer treatment resembles that of electroporation (around 0.2–0.3%), its efficiency in incorporation of 25 kDa globular protein reaches 10–15%, which is about 10 times higher than that of the commonly used electroporation protocol (the efficiency of loading by electroporation in our hands was 1–1.5%, higher than that typically reported in other studies) [15]. Our results also showed that pre-treatment of EVs with carbonate buffer increased the loading efficiency of the protein payload compared to that reported for passive incubation in other studies. For instance, the efficiency of loading of the antioxidant enzyme catalase into macrophage EVs via passive incubation was reported to be < 5% [6]. The addition of membrane permeabilization agents, such as detergents (i.e., saponin), has been proposed to increase the efficacy of passive incubation loading strategies, but they pose additional downstream steps and problems [35]. They can hamper the downstream analytics or reactivity of EV surface proteins and necessitate additional time-consuming steps that augment material loss and decrease reproducibility. Our method is fully reversible, does not leave any residual additives or reactives, and fully preserves EV structure and function.

4 Conclusions

We demonstrated that pH alteration induced by high concentrations of sodium carbonate is a safe and effective method for arranging nsEVs as drug nanocarriers, preserving their size and structure, allowing conspicuous loading of a therapeutic protein, and mediating the desired biological outcome in recipient cells. Further investigations will

confirm the transferability of our method to a broad spectrum of EV sources. Nevertheless, to achieve the effective intracellular delivery of EV contents, future experiments will be performed in order to assess the compatibility of this loading protocol with preserved functionality of different types of exogeneous cargo of interest and, on the other side, to stably customize EV membrane with chemically conjugated targeting moieties, which must enhance a selective and preferential cellular uptake by target cells exploiting a receptor-mediated endocytosis uptake rather than micropinocytosis.

5 Methods

5.1 Cell culture

HEK293 (human embryonic kidney-derived) cells were purchased from ATCC. HeLa (human cervical cancer-derived) cells were purchased from the Riken BRC Cell Bank (Ibaraki, Japan). 5637 human bladder cancer cell line was purchased from Cell Lines Service. Cells were cultured in DMEM (Gibco, Life Technologies Corporation, Grand Island, NY, USA) (HeLa and HEK) or RPMI (5637) + Glutamax supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin-sulphate). Cells were grown in incubation at 37 °C in 5% CO₂.

5.2 NsEV isolation

Human embryonic kidney cell line HEK293T (5×10^6) was seeded in 150 mm dishes in DMEM medium supplemented with EV-depleted 10% FCS, 2 mM L-Glutamine, and antibiotics. After 72 h of incubation, the cell culture medium was collected and centrifuged at 300 g for 25 min to remove cell debris. For nsEV isolation, the obtained supernatant was filtered through a 0.22 µm filter before ultracentrifugation at 150,000g for 2 h at 4 °C (Beckman Coulter). The nsEV-containing pellet was resuspended in phosphate buffer saline (PBS) [22].

5.3 Nanoparticle tracking analysis (NTA)

Nanoparticle tracking analysis (NTA) was performed on the isolated vesicles using a NanoSight LM10-HS microscope (NanoSight Ltd., Amesbury, UK) as previously described. The NanoSight system was calibrated by using polystyrene latex microbeads. The videos were analyzed using NTA software version 2.3 to determine the concentration and size of the measured particles with the corresponding standard error (SEM) [36].

5.4 Transmission electron microscopy (TEM) analysis

Freshly purified nsEVs from culture media of CD9/CD63-RFP stably expressing HEK293 were absorbed on glow discharged carbon coated formvar copper grids, washed with water, contrasted with 2% uranyl acetate, and air-dried. The grids were observed under a Zeiss LEO 512 transmission electron microscope (Zeiss). Images were acquired using a 2 k × 2 k bottom-mounted slow-scan Proscan camera, controlled by EvisiionPro 3.2 software [30].

5.5 Total EV protein quantification

The amount of proteins was quantified for each sample and time point using Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Fisher Scientific). Samples were loaded at 10 µL per well in duplicate, and the protein content was determined by alignment with the standard calibration curve provided by the kit.

5.6 Lectin binding assay

Ten microliters of HEK nsEVs from PBS and carbonate buffer treatments, corresponding to 10^9 particles, were loaded into flat-bottom black plates with a high binding capacity (Biomat, Cat. No.: MGB01F2-HB8) and diluted with PBS to a final volume of 50 µL. After overnight incubation at room temperature, the plates were washed three times with 300 µL of PBS + 0.05% Tween20 washing buffer, and 50 µL (20 µg/mL) of ConA-FITC and WGA-FITC (Vector Laboratories) were loaded

into each well. The 30 min incubation with fluorescently-labelled lectins was carried out at room temperature, after which the washing step with PBS + 0.05% Tween20 was repeated, and 200 μ L of PBS was added to each well for measurement of the fluorescence intensity (excitation 485 nm / emission 535 nm) on microplate reader GENios Pro (Tecan).

5.7 Enzyme linked immunoassay (ELISA) for EV tetraspanins quantification

The surface marker expression of common EV tetraspanins (CD63 and CD9) was assessed using Exo-TEST, an in-house sandwich enzyme linked immunoassay (ELISA) kit (HansaBioMed Life Sciences), following the manufacturer's protocol provided with the kit. After incubation with PBS or carbonate buffer, 10^9 HEK nsEVs were loaded onto anti-CD63 coated plate and incubated overnight at 37 °C. Subsequently, biotinylated anti-CD9 antibody (diluted 1/500) included in the kit was used for the detection of bound EVs in combination with streptavidin-HRP (diluted 1/5000). The absorbance at 450 nm was measured on microplate by a GENios Pro microplate reader (Tecan).

5.8 Cholesterol assay

The lipid content of HEK nsEVs after incubation with PBS or carbonate buffer was quantified using a Cholesterol assay (Hansabiomed Life Sciences, Estonia). For this purpose, 10^9 particles were loaded onto black flat-bottom plates with no binding capacity (Biomat, Cat. No.: MGB03F2-NB) and diluted with PBS to a final volume of 20 μ L. The reaction mix preparation, incubation, and fluorescence intensity measurements (excitation 540 nm/emission 590 nm) were performed according to the manufacturer's guidelines.

5.9 Nucleic acid qualitative and semi-quantitative assessment

Qualitative and semi-quantitative assessment of nucleic acid content was performed using a fluorescence microplate assay, a black 96-well microplate with no binding capacity (HansaBioMed Life Sciences) and Quant-iT™ RiboGreen™ RNA reagent (Invitrogen; Cat. R11491; 1/1000 working dilution). Briefly, samples, 10X diluted in DPBS (Sigma-Aldrich; Cat.: D8537-500ML) to a final volume of 50 μ L per well, were loaded onto microplate wells, followed by incubation with 50 μ L per well of dye stock solution (1/500 dilution in DPBS) for 30 min at RT. Fluorescence intensity (λ_{ex} = 485 nm, λ_{em} = 535 nm, gain:50) was measured using a Tecan GENios Pro microplate reader. The results were reported as signal-to-background ratios.

5.10 Single-particle interferometric reflectance imaging sensor (SP-IRIS) analysis

Interferometric analyses of EVs were performed exploiting the SP-IRIS technique as described [37]. Briefly, anti-CD9 and -CD63 antibody-DNA conjugates were spotted on SP-IRIS chips. The chips were incubated with a 1.25×10^9 particles/mL solution of isolated EVs treated with sodium carbonate or PBS as control, for 1.5 h at RT. Chips were then washed 5 min in PBS, rinsed with 2 mM MgCl_2 and dried. Finally, they were analyzed using ExoView R100 reader for image acquisition and nanoViewer 2.6.0 software for analysis (NanoView Biosciences Inc., MA). ExoView R100 measures the number of single particles ranging from 50 to 200 nm in diameter captured on the chip surface.

5.11 Fluorescent labelling of nsEVs and in vitro uptake tracing

NsEVs isolated from the cell culture media were resuspended in PBS and combined with a PBS solution containing Vybrant™ DiO (1 μ L for 100 μ g nsEVs, Molecular Probes, Eugene, OR, USA). The samples were incubated at 37 °C for 30 min (protected from light) under stirring and then passed through Exosome spin columns (MW 3000, Invitrogen) to remove any incorporated dye. PBS containing the dye alone was used as a negative control. The samples were then ultracentrifuged (150,000 $\times g$, 2 h at 4 °C) and the pellet was resuspended in PBS. For the in vitro tracing assay, 5637 were seeded (up to 50–60% confluence) in a 24 multiwell plate and incubated at 37 °C with 5% CO_2 . 24 h later, labelled nsEVs (5 μ g or 10 μ g/well) were added to recipient cells. The uptake of the NsEVs was evaluated after 24 h. Fluorescent images were captured using Axio Vision Imaging Software (Axiovision Rel 4.8®) on an Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany) [30].

5.12 Sodium carbonate-based molecule encapsulation into isolated nsEVs

HEK293 derived-nsEVs were resuspended with dextran-FITC (Sigma-Aldrich) or seed saporin (SAP) (Sigma-Aldrich) in 1 M Na₂CO₃ (pH 11.5) or PBS and incubated for 30 min at 37 °C. The amount of nsEVs used was calculated as protein concentration. A ratio of 1 µg nsEVs to 1/1.5/2 µg dextran/SAP was used to define the optimal yield of exogenous protein encapsulation. The pH was neutralized by dropwise addition of HCl, and the sample was filtered by centrifugation through Exosome spin columns 100 kDa MWKO (Merck Millipore) to remove any incorporated proteins and washed with PBS or NaCl 150 mM to reduce protein aggregation. Dextran, SAP or nsEVs alone were used as negative controls.

5.13 Encapsulation of fluorescently-labelled dextran and SAP into nsEVs by electroporation

To load fluorescently-labelled dextran or SAP into nsEVs, nsEVs (25 µg) were mixed with FITC-labelled dextran (70 kDa) or SAP (25 kDa) in PBS (100 µl) at a total protein ratio (w/w) of 1:1, 1:1.5, or 1:2. After electroporation (poring pulse: two pulses (5 ms), transfer pulse: five pulses (50 ms)) in a 1 cm electroporation cuvette at room temperature using a super-electroporator NEPA21 Type II (NEPA genes, Tokyo, Japan), the unencapsulated FITC-dextran or FITC-SAP was removed by washing and filtration using Amicon Ultra centrifugal filters (100 K device, Merck Millipore). The incorporation of FITC-dextran and FITC-SAP into the nsEVs was confirmed by spectrofluometer analysis (FP-6200, JASCO, Tokyo, Japan). The incorporation of FITC-dextran and FITC-SAP into vesicles was quantified by interpolation based on a calibration curve generated using scalar dilutions of FITC dextran/SAP. The electroporation method was optimized for poring pulses (50, 100, and 150 V), resulting in the encapsulation of FITC-dextran (15–20 ng/ml in 50 µg/ml nsEVs), respectively. Therefore, we used an experimental poring pulse of 50 V for electroporation [8].

5.14 Confocal microscopy

HeLa cells (2 × 10⁵ cells, 2 ml) were plated on a 35 mm glass dish (Iwaki, Tokyo, Japan) and incubated in the cell culture medium. After complete adhesion, cells were washed with culture medium and treated for 24 h with nsEV samples (25 µg/ml), either treated with electroporation or sodium carbonate buffer protocol to load dextran-FITC. Cells were then washed with fresh cell culture medium and analyzed using an FV1200 confocal laser scanning microscope (Olympus, Tokyo, Japan) equipped with a 40 × magnification objective [8].

5.15 Flow cytometry analysis

5637 cells were cultured in 24-well microplates until 60% confluence. 24 h later, the cells were treated with labelled nsEVs (15 µg/well) and incubated at 37 °C, 5% CO₂ for 48 h. The cells were detached by incubation with 0.01% trypsin for 5 min at 37 °C, collected, washed with 1% FBS (in PBS), and subjected to flow cytometric analysis using Accuri™ flow cytometer. Analysis was performed for 20,000 gated events per sample [22].

5.16 Protein extraction, western blot and silver staining analysis

Cells were washed twice with cold PBS, collected by scraping, centrifuged 5 min at 1200 rpm, and lysed for 30 min in ice-cold buffer (150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1 mM PMSF, 75 mU/ml aprotinin-Sigma, 50 mM Tris-HCl, pH 7.5) containing 1% TX-100, and a cocktail of protease inhibitors (Sigma-Aldrich). Cell lysates were centrifuged at 10,000 × g at 4 °C for 10 min. Proteins contained in the supernatant were quantified by BCA assay, resuspended in sample buffer (62.5 mM Tris-HCl (pH = 6.8), 2% SDS, 10% glycerol, 0.002% bromophenol blue), and boiled at 95 °C for 5 min. The same amount of total protein was loaded unless otherwise indicated. Proteins were then separated via SDS-PAGE under non-reducing conditions for the detection of the CD9, CD63, and CD81 or under reducing conditions (with 5% 2-mercaptoethanol) for the detection of the other proteins. Proteins were silver stained or transferred onto a nitrocellulose membrane for western blot analysis incubated with 5% non-fat powdered milk in TBS-T (0.5% Tween-20) for 1 h and then with the following antibodies: saporin (1:5000), flotillin (1:5000, Sigma), Alix (1:800, Santa Cruz Biotechnology), anti-CD9 (1:10,000; BD Pharmingen), anti-CD63 (1:20,000, BD Pharmingen), anti-CD81 (1:5000, BD Pharmingen), anti-TSG101 (1:1000; Novus Bio). Secondary horseradish peroxidase-conjugated antibodies (anti-mouse/

rabbit IgG HRP-linked whole antibody donkey; GE Healthcare) were used, and the immunoreactive bands were visualized using Enhanced Chemiluminescence (Merck Millipore) [38].

5.17 Acquisition of Raman spectra, data processing and analysis

Raman spectra were acquired using an InVia Reflex confocal Raman microscope (Renishaw plc, Wotton-under-Edge, UK) equipped with a solid-state laser light source, operating at 785 nm. We visualized lipids and proteins in the sample by imaging the Raman peaks at 2935 and 2853 cm^{-1} originating from methyl groups in proteins and methylene groups in fatty acids. The intensity of the peak indicates the overall protein and lipid content, whereas a shift in the peak would indicate conformational alterations. The Raman spectrometer was calibrated daily using 520.7 cm^{-1} of a silicon wafer. Typically, a 3 μL drop of sample was dropped onto the surface of Raman-compatible CaF_2 discs (Crystran, UK) and dried for 20 min at room temperature. The Raman study was performed using a 785 nm excitation laser with 100% power (approximately 42 mW at source), a 1200 l/mm grating and a 100 $\times$ objective. Spectra were acquired as the sum of nine 10 s acquisitions. The spectral resolution was $\sim$ about 1 cm^{-1} . For each sample, four different spectra were collected at different positions of the drop. The software package WIRE 5 (Renishaw, UK) was used for spectral acquisition and to remove the cosmic rays. Background fluorescence was removed using an asymmetric least-squares smoothing method. [39] The spectra acquired for each sample were vector-normalized and averaged. Spectrum normalization and data analysis were performed using OriginPro, Version 2019 (OriginLab Corporation, Northampton, MA, USA) [40].

5.18 Cell viability assay

5637 cells (5×10^3 cells/well) were seeded in 96 wells plates and incubated for 72 h with 2, 10, or 20 μg SAP-bearing nsEVs or 0.1, 1, or 10 μg SAP alone (seed SAP). After 72 h of incubation at 37 $^\circ\text{C}$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) was added (0.5 mg/ml working concentration). After 1-h incubation at 37 $^\circ\text{C}$, the supernatants were removed, and 100 μL /well of dimethyl sulfoxide was added to dissolve the formazan crystals. Cell viability was assessed by measuring the absorbance at 570 nm and expressed as a percentage of the untreated control. SAP toxicity was evaluated as IC_{50} , shown as the mean $\pm$ SEM from three independent experiments [41].

5.19 Statistical analysis

Three biological replicates were performed for all in vitro experiments. When appropriate, statistical significance was determined using 2-tailed Student's *t* tests. Test symbols mean: ***p* < 0.01; ****p* < 0.001.

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Author contributions SZ, KN, FL, CM and AL performed the experiments and acquired data; SZ, NZ, IN and RV analyzed and discussed the results; SZ, NZ and RV drafted the manuscript; RV supervised and coordinated the project; All authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Competing interests The authors declare no competing interests.

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Appendix 3

Publication III

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All relevant methods and results related to Publication III are derived from an original research manuscript that is currently in preparation and has not yet been published. To protect the confidentiality of unpublished work, the manuscript is not included as an appendix to this thesis. Nevertheless, to comply with the regulations of Tallinn University of Technology for doctoral studies and provide the basis for a thorough assessment of the thesis, a copy of this manuscript is made available to the preliminary reviewers, the opponents, as well as the appointed members of the Defense Committee

Appendix 4

Publication IV

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RESEARCH ARTICLE OPEN ACCESS

Investigating the Effect of Ginger-Derived Nanovesicles on the Growth and Metabolic Activity of *Bacteroides thetaiotaomicron*: An Isothermal Microcalorimetric Study

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ABSTRACT

Plant-derived extracellular vesicles (EVs) have shown numerous health benefits, including modulation of the human gut microbiota. Herein, we employed isothermal microcalorimetry (IMC) to explore the effects of ginger-derived nanovesicles (G-NVs) on the growth and metabolic activity of *Bacteroides thetaiotaomicron* (*Bt*), a dominant saccharolytic commensal with promising next-generation probiotic potential. *Bt* was exposed to either G-NVs or the ginger extract separated from G-NVs (G-CTL) in defined media under anaerobic conditions. Both ginger samples enhanced bacterial specific growth rate and maximum metabolic activity, inducing the latter earlier. However, higher biomass production and greater secretion of acetic, succinic and propionic acids occurred only in response to the G-CTL. Complete sugar depletion and unchanged free amino acid levels indicated preferential carbohydrate utilisation by *Bt*. Overall, these findings revealed that *Bt*'s metabolic state is shaped by both G-NVs and G-CTL, yet through distinct mechanisms, with G-NVs inducing rapid stimulation without increasing total metabolic output and G-CTL providing a sustained, dose-dependent effect. To our knowledge, this is the first study applying IMC to monitor in real-time the impact of EVs on microbial growth and metabolism, underscoring IMC's utility for mechanistic studies of EV-microbe interactions. Furthermore, this research sets the ground for innovative strategies in nutraceutical and microbiome-targeted therapy development.

1 | Introduction

The human gastrointestinal tract hosts a diverse collection of coexisting microorganisms, including bacteria, archaea and viruses, whose composition and functionality critically regulate human health (Hillman et al. 2017). The microbial communities

residing in the intestine, collectively termed human gut microbiota (HGM), are considered key regulators of host homeostasis due to their high microbial load, metabolic activity, immune functions and systemic impact (Fan and Pedersen 2021; Ruan et al. 2020). Indeed, in addition to supporting nutrient digestion, metabolism and gut barrier integrity, the HGM has shown to

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play an important role in promoting direct regulation of the host immune cells and fostering protection against the action of pathogens and toxins (Bemark et al. 2024; Mörbé et al. 2021; Ubeda et al. 2017; Yoo et al. 2020). Recent studies have also provided evidence of its prominent action in modulating the development and proper functioning of the nervous system via the gut–brain axis. The HGM is mainly composed of the following phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia (Yang et al. 2009). In adult individuals, the Gram-negative Bacteroidetes and the Gram-positive Firmicutes constitute more than 80% of the entire microbial community (Rios-Covian et al. 2017). Within the Bacteroidetes phylum, the genus *Bacteroides* has been extensively studied due to its dominance over other genera and its long-term adaptability to the changing host's gut environment across human populations (Wexler and Goodman 2017).

Bacteroides thetaiotaomicron (*Bt*) is a non-motile, obligate anaerobe primarily found in the large intestine. By virtue of its prevalence, functional relevance, metabolic flexibility and genetic tractability, *Bt* is considered an ideal model organism for studying the HGM. Its biological significance mainly relies on its direct regulation of the host gene expression pertaining to metabolism, immunomodulation and maintenance of the gut barrier integrity (Zocco et al. 2007). *Bt* shares versatile metabolic capabilities characteristic of all *Bacteroides* spp. Upon carbohydrate fermentation, *Bt* secretes several organic and inorganic products that support its primary biological functions, including short-chain fatty acids (SCFAs) (e.g., acetate, propionate and formate), succinate, ethanol, lactic acid, CO₂ and H₂. As *Bt* is capable of fermenting proteins and amino acids under specific conditions, it has also been demonstrated that *Bt* releases SCFAs, along with branched-chain fatty acids, phenolic/indolic compounds, amines and ammonia (Rivera-Chávez and Bäumlér 2015). Technically, *Bt* is easy to cultivate under anaerobic conditions and serves as an excellent candidate for HGM-focused co-cultured studies, as it interacts with other commensal species in a predictable manner (Bacic and Smith 2008; da Silva et al. 2024; Meslé et al. 2023; Kattel et al. 2024). With its whole genome sequenced, *Bt* can also be genetically manipulated, thus contributing to facilitate fast-paced advancements in the synthetic biology and microbiome (engineering) fields (Xu et al. 2003). Because of these important attributes, *Bt* shows significant potential as a next-generation probiotic (NGP) (Chang et al. 2019; Jan et al. 2024). Probiotics are viable and beneficial microorganisms exerting health-enhancing and health-protecting properties when administered in sufficient quantities. Compared with traditional probiotics, including *Lactobacillus* and *Bifidobacterium* spp., NGPs demonstrate superior metabolic benefits and offer the competitive advantage of targeting specific disease conditions, rather than managing overall health (Helal et al. 2019).

Extracellular vesicles (EVs) have recently been documented in plants as bioactive components exhibiting health-protecting and enhancing properties (e.g., anti-cancer, anti-inflammatory, antioxidant and prebiotic) (Munir et al. 2020; Record 2013; Lian et al. 2022). Here, plant-derived EVs and nanovesicles [i.e., these last indicating co-isolates of both natural EVs and artificial particles resulting from mechanical and destructive pre-processing of the plant source (Pinedo et al. 2021)] are collectively referred to as plant-derived vesicles (PDVs). PDVs are lipid-

bilayer membranous particles with nanometre and micrometre size: ~50–150 nm, defined as 'small EVs'; ~200–800 nm, defined as 'medium EVs' and ≥ 1000 nm, defined as 'large EVs' (Lian et al. 2022; Pinedo et al. 2021; Buzas 2023). By carrying diverse bioactive components (e.g., proteins, lipids, nucleic acids and metabolites), PDVs have been shown to mediate intercellular communication within and across organisms. Additionally, they have been demonstrated to be involved in plant cell proliferation and differentiation, cell wall remodelling and defence mechanism against abiotic and biotic stresses. Due to their inner composition and functional properties, PDVs are rapidly gathering momentum as competitive clinical-grade and commercial-grade tools that can be deployed between several industrial markets (e.g., healthcare, personal care and agrifood) (Zarovni et al. 2021). Among the PDVs that have thus far promoted important health benefits in preclinical settings, small ginger rhizome (*Zingiber officinale* Roscoe)-derived nanovesicles (G-NVs) have exhibited significant potential in regulating and fostering a healthy HGM (Teng et al. 2018; Bahri et al. 2024).

Isothermal microcalorimetry (IMC) enables real-time monitoring of heat changes associated with complex biological processes in closed systems (e.g., ampoules) of small calibre at a constant temperature (Bahri et al. 2024; Wadsö 2001, 2002). For decades, IMC has been widely applied to study bacterial growth and metabolism, providing quantitative and thermodynamic insights into growth dynamics, kinetics and metabolic activity. This technique measures the heat generated by microbial metabolic activity and quantitatively relates it to biomass formation, substrate consumption and carbon source utilisation efficiency (Braissant et al. 2010; Nykyri et al. 2019; Kabanova et al. 2009; Kabanova et al. 2012; Kabanova et al. 2013; Stulova et al. 2015). Compared with traditional methods, IMC offers high robustness, throughput, sensitivity (i.e., a limit of detection ranging from 10⁴ to 10⁵ cells), and reproducibility. Furthermore, it does not require sample optical transparency, specific physical forms or specialised preparation before assessment (Mauerhofer et al. 2019; Wadsö 2001, 2002; Braissant et al. 2015). Despite these advantages, IMC has not yet been reported as a method for investigating the influence of EVs on microbial growth.

The primary aim of this study was to apply IMC to explore the effect of G-NVs on the growth and metabolic activity of the human gut bacterium *Bt*. This was analysed by measuring the dose-dependent functional activity of G-NVs, enriched in the sterile retentate of a tangential flow filtration (TFF) system with 50 nm pores. The ginger extract depleted of the G-NV sample, equivalent to the TFF permeate enriched in constituents smaller than 50 nm and mostly non-vesicular, was assessed in parallel as a procedural control sample (G-CTL). The metabolic response of *Bt* to the treatment with G-NVs and G-CTL was further evaluated by high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) with ultraviolet (UV)/visible or refractive index (RI) detection analysis of non-vesicular extracellular metabolites [i.e., sugars, organic acids, ethanol and free amino acids (FAAs)] secreted throughout microbial growth (Figure 1). Our data provided evidence of the great potential of IMC to unravel the dynamic metabolic regulation of *Bt* upon exposure to ginger sample fractions, specifically showing that both G-NVs and G-CTL affected *Bt*'s growth and metabolism, yet through different mechanisms and kinetics.

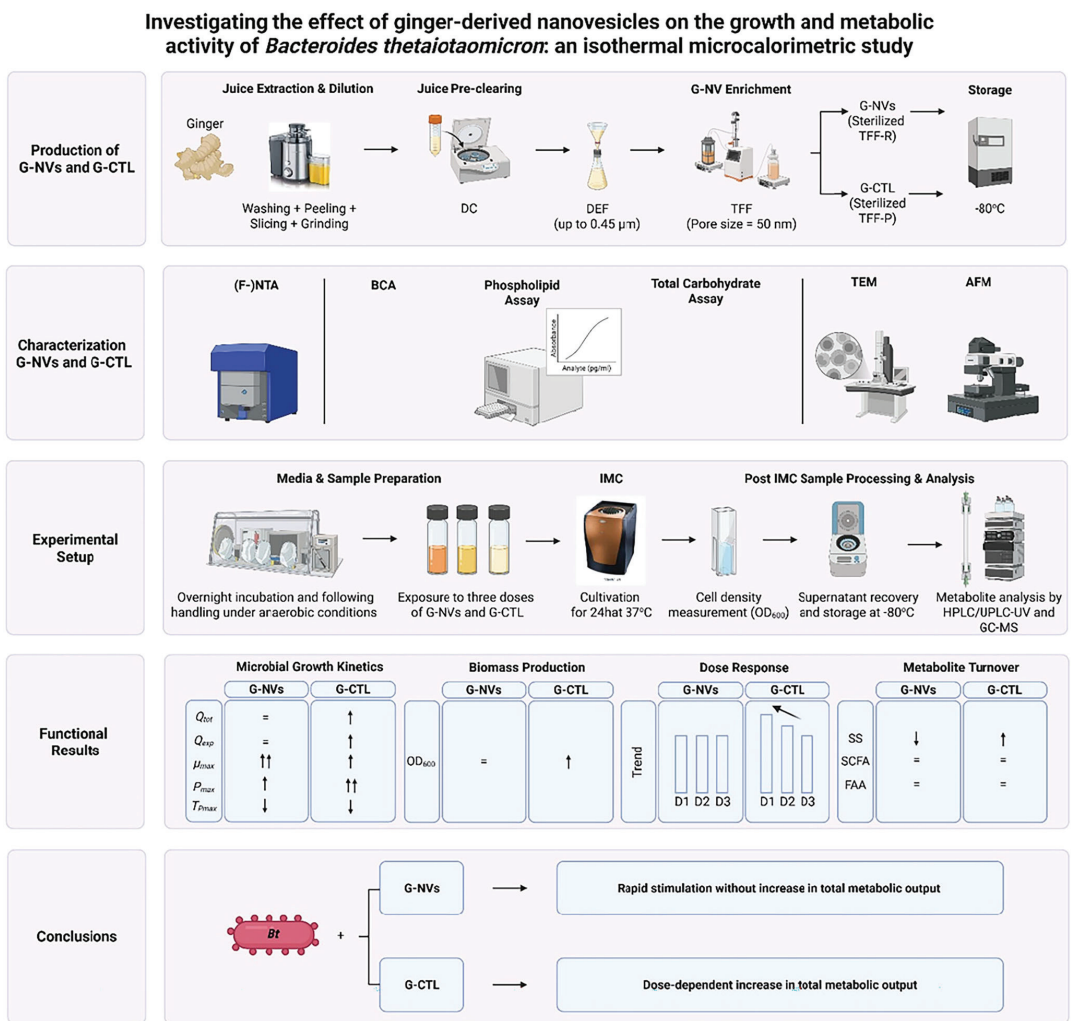


FIGURE 1 | Graphical abstract of the study. Abbreviations: AFM, atomic force microscopy; BCA, bichinchonic acid assay; D, dose; FAA, free amino acids; (F-)NTA, (fluorescence) nanoparticle tracking analysis; GC-MS, gas chromatography-mass spectrometry; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; HPLC, high-performance liquid chromatography; IMC, isothermal microcalorimetry; μ_{max}, maximum specific growth rate; OD₆₀₀, optical density at a wavelength of 600 nm; P_{max}, maximum power output or metabolic activity level; Q_{exp}, cumulative heat produced during the exponential phase; Q_{tot}, cumulative heat produced during the whole cultivation period; SCFA, short-chain fatty acids; SS, simple sugars; TEM, transmission electron microscopy; T_{Pmax}, time at which the P_{max} occurs; UPLC-UV, ultra-performance liquid chromatography with ultraviolet detection system. This figure was created in BioRender. Loria (2026) <https://BioRender.com/m55s48z>.

2 | Materials and Methods

2.1 | Production of G-NVs and G-CTL

Ginger rhizome (*Zingiber officinale Roscoe*) of Chinese origin was obtained from a local Estonian market. Ginger rhizome was washed with Milli-Q water, followed by peeling, slicing and grinding with a centrifugal juicer (Severin; #ES3566) to extract juice and dry residues. For each batch, 30 g of dry residues

were resuspended in 60 mL of pre-extracted juice supplemented with protease inhibitor cocktail (SigmaFast™ Protease Inhibitor Tablets; Sigma-Aldrich; #S8820; 50X working dilution) and diluted with phosphate buffered saline (PBS) (Corning; #21-031-CV) up to 300 mL. After 30 min of gentle shaking at room temperature (RT), residues were discarded using a funnel and a drainer. Then, samples were centrifuged twice at RT, using a Ohaus FC5718R centrifuge with fixed rotor: (i) at 300 r.c.f. for 20 min and (ii) at 3000 r.c.f. for 20 min. The resulting

supernatant was subsequently subjected to vacuum-based glass filtration with a glass fibre prefilter (Merck; #APFD09050). After dead-end filtration through a 0.45 µm pore size filter and a further centrifugation step at 10,000 r.c.f. for 30 min at RT, samples were processed by TFF, using TFF-EVs-Small device (HansaBioMed Life Sciences; #HBM-TFF-EVs-S). Recovered TFF retentate and permeate were finally sterilised by dead-end filtration using 0.22 µm pore size filter units (Sartorius; #16532). Finally, the filtered TFF retentate and permeate were stored at −80°C to be subsequently analysed as small ginger-derived nanovesicles (G-NVs) and G-NVs procedural control (G-CTL), respectively.

2.2 | Characterisation of G-NVs and G-CTL

2.2.1 | Nanoparticle Tracking Analysis (NTA)

Nanoparticle concentrations and size distributions were analysed by NTA, using Particle Metrix ZetaView PMX-120 (software version 8.05.12 SP1), as previously described (Fortunato et al. 2021).

2.2.2 | Fluorescence Labelling

Fluorescence labelling was optimised and conducted using CellMask™ Green (CMG) Plasma Membrane Stain (Thermo Fisher Scientific; #C37608) [peak excitation wavelength (λ_{ex}) = 522 nm; peak emission wavelength (λ_{em}) = 535 nm], as shown prior (Fortunato et al. 2021). Briefly, for each G-NV biological batch, 5E+9 particles—quantified by NTA in scatter mode—were incubated with CMG (1:50 dilution or 20X working concentration), in a final reaction volume of 25 µL, for 90 min at 37°C in the dark. The G-CTL was stained at the same dilution as its corresponding G-NV sample. After staining, the excess of unbound dye was removed by size-exclusion chromatography (SEC) using miniPURE-EVs SEC columns (HansaBioMed Life Sciences; #HBM-mPEV-##), according to the manufacturer's datasheet. The eluate corresponding to the EV fraction (500 µL) was then assessed by fluorescence NTA (F-NTA). Three replicates of a fluorescence labelling control sample, equivalent to the buffer-only (PBS) supplemented with 20X CMG, were analogously processed and analysed in parallel to assess the effectiveness of SEC in removing the excess of unbound dye.

2.2.3 | Fluorescence Nanoparticle Tracking Analysis (F-NTA)

F-NTA was conducted using Particle Metrix ZetaView® Evolution (software version 1.0) equipped with a CMOS-camera sensor and lasers of 405 nm (violet), 488 nm (blue), 520 nm (yellow/orange) and 640 nm (red) wavelengths. Instrument setup was conducted according to the manufacturer's instructions. Briefly, alignment of the optical system (camera and lasers), focus optimisation and temperature equilibration (22–25°C) were performed using 100 nm polystyrene beads (Applied Microspheres; #75009-03) and fluorescent PS OR-520 beads for 520 nm laser (Particle Metrix; #700093). Before measurement, CMG-labelled samples and procedural controls were diluted 1:10 and 1:20 in PBS to achieve an optimal scattering particle count of 50–200 particles

per cell position throughout a total of 11 cell positions. Scatter measurements were performed in the scatter channel illuminated with a 488 nm (blue) laser (SC488), selecting a camera gain of 40 and a shutter speed of 200. These operational settings were selected to align readouts with those previously obtained using Particle Metrix ZetaView PMX-120. Fluorescence measurements were conducted in the fluorescence channel illuminated with a 520 nm (yellow/green) laser (FL520) and coupled with a 550 nm cut-on long-pass fluorescence filter, adjusting the camera gain to 80 and maintaining the shutter speed 200. Each size and concentration measurement, both in scatter and fluorescence mode, was performed in triplicate. Data were presented as mean ± standard error of the mean (SEM) from the three technical measurements.

2.2.4 | Bicinchoninic Acid (BCA) Assay

Proteins were quantified by BCA assay, using the Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific; #23228), according to the manufacturer's instructions. Standards and samples were loaded in duplicate into transparent Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates (Thermo Scientific; #268152). Optical densities (ODs) were measured at 540 nm using Tecan GENios Pro microplate reader.

2.2.5 | Phospholipid Assay

Phospholipid concentrations were measured using fluorescent Phospholipid Quantification Assay Kit (HansaBioMed Life Sciences), following the manufacturer's protocol. Standards, samples, blanks and background controls were assessed in duplicate into black polystyrene—no binding capacity—microplate wells (HansaBioMed Life Sciences). Fluorescence intensities (FIs) were read using Tecan GENios Pro microplate reader (λ_{ex} = 540 nm; λ_{em} = 590 nm).

2.2.6 | Total Carbohydrate Assay

Total carbohydrate quantification was performed by Total Carbohydrate Colorimetric Assay Kit (Sigma-Aldrich; #MAK104-1KT), according to the manufacturer's datasheet. Standards, samples and blanks were analysed in duplicate, using transparent polystyrene—no binding capacity—microplate wells (HansaBioMed Life Sciences). ODs were read at 492 nm, using Tecan GENios Pro microplate reader.

2.2.7 | Transmission Electron Microscopy (TEM)

For morphological assessment, TEM was performed as done prior (Puhka et al. 2017). Briefly, samples were prepared by loading to carbon coated and glow discharged 200 mesh copper grids with pioloform support membrane. Then, samples were fixed with 2.0% PFA in NaPO₄ buffer, stained with 2% neutral uranyl acetate, further stained and embedded in uranyl acetate and methyl cellulose mixture (1.8%/0.4%). Finally, samples were visualised using TEM Hitachi HT7800 operating at 100 kV. Images were

taken with Gatan Rio9 bottom mounted CMOS-camera, model 1809 (Gatan Inc., USA) with 3072×3072 pixels image size and no binning.

2.2.8 | Atomic Force Microscopy (AFM)

AFM and subsequent quantitative morphometry were performed on vesicular samples as previously described (Ridolfi et al. 2020; Ridolfi et al. 2023). Briefly, images were taken in PeakForce mode on a Multimode 8 microscope (Bruker, USA) equipped with Scanasyt Fluid+ probes (Bruker), a Nanoscope V controller, a JV-type piezoelectric scanner and a sealed fluid cell. For each sample, 5 µL aliquots were loaded on poly-L-lysine-coated glass coverslips for 30 min at 4°C to promote adsorption, followed by direct introduction into the fluid cell without prior drying. To maximise the number of isolated particles, thus facilitating automatic image analysis, sample concentration was adjusted through successive depositions. For proper comparative assessment, G-NV and G-CTL samples, at the same dilution, were deposited, scanned and analysed following an identical pipeline. Quantitative morphometric analysis was performed using Gwyddion 2.61 (Nečas and Klapetek 2012) and custom Python scripts. Each particle on the surface was assessed for its spherical diameter in solution (D), as well as for its surface contact angle (CA), which has previously demonstrated to directly correlate with the mechanical stiffness of intact EVs. For each sample, all measured particles were plotted on CA versus D scatterplots. Intact vesicles and non-vesicular particles were previously shown to cluster into characteristic zones of these plots (Ridolfi et al. 2020; Ridolfi et al. 2023).

2.3 | Assessment of Bacterial Growth and Metabolic Activity

2.3.1 | Media Preparation

The experiment was conducted using a pre-defined mineral medium, whose composition consisted of: D-(+)-glucose monohydrate (2.2 g/L), L-amino acids [(g/L) Ala 0.11, Arg 0.0575, Asn 0.095, Asp 0.095, Glu 0.09, Gln 0.045, Gly 0.08, His 0.0675, Ile 0.15, Leu 0.3, Lys-HCl 0.2, Met 0.0575, Phe 0.125, Pro 0.1025, Ser 0.2375, Thr 0.1025, Trp 0.0225, Val 0.15, Tyr 0.0375], mineral salts [(mg/L) MgSO₄ × 7H₂O 36, MgCl₂ 2, FeSO₄ × 7H₂O 0.1, CaCl₂ 9, MnSO₄ × H₂O 3, ZnSO₄ × 7H₂O 1, CoSO₄ × 7H₂O 1, CuSO₄ × 5H₂O 1, (NH₄)₆Mo₇O₂₄ × 4H₂O 1, NaCl 527], (NH₄)Cl 3.5; NaHCO₃ 1], vitamins [(mg/L) biotin 6.02, riboflavin 0.9, pyridoxamine-2HCl 5, niacin 0.9, thiamine-HCl 0.56, pyridoxine-HCl 4.8, Ca-pantothenate 1.2, folic acid 0.56, p-aminobenzoic acid 0.05, lipoic acid 1.05, ascorbic acid 500, vitamin B12 0.001, vitamin K (menadione) 1], hemin (5 mg/L). Media were prepared using 1 M dipotassium hydrogen phosphate buffer (16.8 mL/L) and 1 M potassium dihydrogen phosphate buffer (34.2 mL/L), and supplemented with L-Cysteine hydrochloride (1 g/L) as a sulphur source and reducing agent. Media were filtered with filter units having 0.22 µm pore size, followed by overnight incubation in an anaerobic chamber (COY box, Coy Laboratory Products Inc.) with an atmosphere of 2.5% ± 0.5% H₂, 10% CO₂ and balanced N₂. If needed, the pH of the media was adjusted to 7.0 ± 0.1 before performing the experiment.

2.3.2 | Assessment of Bacterial Growth Activity by Isothermal Microcalorimetry (IMC) and Optical Density Measurement

Bt stock cultures (strain DSM 2079) were prepared from cells harvested during the exponential phase and stored at −80°C, as previously described (Kattel et al. 2023). For the IMC experiments conducted in this study, inoculation was initiated from a frozen stock culture, which was thawed on ice and centrifuged at 19,000 r.c.f. for 3 min at RT. The pellet was resuspended in PBS supplemented with 1% L-cysteine hydrochloride, and cell concentration was adjusted to 1–1.2E+9 colony-forming units (CFU)/mL. For each sample supplemented with or without G-NVs and G-CTL, 10 or 15 µL of inoculum [1% (v/v)]; final cell concentration of 1–1.2E+7 CFU/mL was added to 990 or 1485 µL of medium, respectively. Treated bacteria were exposed to three distinct doses of G-NVs (i.e., 2E+11, 1E+11 and 5E+10 scattering particles) and G-CTL, which was equivalent to the corresponding G-NV dose based on protein content. To exclude microbial contamination, equal doses of non-inoculated G-NVs and G-CTL were tested in parallel. These contained 990 or 1485 µL of medium with either G-NVs or G-CTL, which was supplemented with 10 or 15 µL of PBS [1% (v/v)], respectively. All samples were run in triplicate in four independent experiments. Each experiment assessed a distinct biological batch of both G-NVs and G-CTL. After being hermetically sealed, the vials were placed into a TAM IV-48 (48-channels, TA Instruments) heat conduction multi-channel microcalorimeter to assess bacterial growth and growth kinetics. The experiment was run at 37°C up to 24 h. Subsequently, the samples were transferred into new Eppendorf tubes, and the cell density was measured as OD at a wavelength 600 nm (OD₆₀₀) using an Ultrospec 10 Cell Density Meter (Biochrom). As described elsewhere (Kattel et al. 2023), the biomass concentration (gDW/L) was estimated using OD₆₀₀, according to the following formula:

$$X = OD_{600} \cdot 0.3$$

Finally, after centrifugation of the samples at 19,000 r.c.f. for 5 min at 4°C to separate the cells from conditioned media, the resulting supernatants were transferred to new Eppendorf tubes. Both cell pellets and supernatants were stored at −80°C for subsequent analyses.

2.3.2.1 | Analysis of IMC Power-Time Curves and Calculation of Growth Characteristics. A typical power-time (or heat flow) curve relative to the growth of individual microbial species, such as *Bt*, describes the evolution of heat released by bacteria throughout the entire growth process. Specifically, it illustrates a thermogram plotting the rate of heat against the unit of growth time (W). As in any microbial growth curve, which plots the biomass concentration against the unit of growth time, primary, consecutive growth phases can be discriminated: (i) lag phase (i.e., no rise in number of living bacterial cells due to initial adjustment to the new environment); (ii) exponential phase (i.e., exponential rise in number of living bacterial cells); (iii) deceleration phase (i.e., plateau in number of living bacterial cells due to equal rate of bacterial cell division and death, followed by metabolic slowdown).

The bacterial metabolic evolution displayed by the power-time curve may be described through thermodynamic and kinetic parameters that can be analytically determined through mathematical analysis of the curve: (i) the maximum specific growth rate ($\mu_{\max}$), which is the rate at which a microbial population increases in size per unit of time; (ii) the maximum power output or metabolic activity level ($P_{\max}$) at the highest peak of the calorimetric curve; (iii) the time at which the $P_{\max}$ occurs ($T_{P_{\max}}$); (iv) the cumulative heat produced during the exponential phase ($Q_{\exp}$); (v) the cumulative heat produced during the whole cultivation period (Q_{tot}).

The $\mu_{\max}$ is determined based on the direct relationship between the biomass concentration (X), the rate of biomass concentration ($\frac{dX}{dt}$) and the specific growth rate (μ), satisfying the following mathematical equation (Maskow and Babel 2003):

$$\frac{dX}{dt} = \mu X$$

During the exponential growth phase, the biomass increases exponentially with time, as described below:

$$X(t) = X_0 e^{\mu t}$$

in which $X(t)$ is the biomass at time point t of the exponential growth phase and X_0 is the biomass at time point 0 of the exponential growth phases.

Based on the assumption that the rate of biomass production is directly proportional to the rate of heat production ($\frac{dQ}{dt}$), the $\mu_{\max}$ can be derived from the slope of the exponential phase of the relative power-point curve, as follows:

$$\ln \frac{dQ}{dt} = \frac{dQ}{dt_0} + \mu_{\max} t$$

in which $\frac{dQ}{dt}$ is the rate of heat production at the time point t of the exponential growth phase and $\frac{dQ}{dt_0}$ is the rate of heat production at a time point 0 of the exponential growth phases.

The $Q_{\exp}$ and Q_{tot} are mathematically expressed as the integrals of the power-time curve over specific growth intervals: (i) between the initial time point and the final time point of the exponential growth phase ($Q_{\exp}$); (ii) between the initial time point and the final time point of the entire course of the microbial growth (Q_{tot}). $Q_{\exp}$ is depicted by the area under the power-time curve comprised between the baseline and an imaginary vertical line passing through the peak of the power-time curve ($P_{\max}$) and intersecting the x-axis at time point $T_{P_{\max}}$. In contrast, Q_{tot} corresponds to the area comprised between the power-time curve and the baseline over the total duration of the microbial cultivation (Kabanova et al. 2009; Kabanova et al. 2012; Kabanova et al. 2013; Stulova et al. 2015).

Raw data were collected from the TAM Assistant program (version 0.9.1012.40, SciTech Software AB, Thermometric AB, Järfälla, Sweden). Heat evolution was recorded at a frequency of 1 s^{-1} and then resampled at 1-h intervals. All parameters were estimated through Microsoft Excel (Kabanova et al. 2009;

Kabanova et al. 2012; Kabanova et al. 2013; Stulova et al. 2015). Baseline-corrected integrals of the power-time curve, which were automatically provided by the software, were used to calculate $Q_{\exp}$ and Q_{tot} .

2.3.3 | Analysis of Bacterial Extracellular Metabolites

2.3.3.1 | Liquid Chromatography (LC). Fermentation supernatants were centrifuged at 21,100 r.c.f. for 6 min at 4°C . The supernatant was transferred into the prerinsed with Milli-Q Amicon Ultra 0.5 mL 3 kDa Molecular Weight Cut-off spin filters (Merck Millipore Ltd; #UFC5003) and centrifuged at 6150 r.c.f. for 30 min at RT. Filtered samples were 10 times diluted with Milli-Q water and assessed by HPLC to determine the content of sugars [i.e., glucose (Glc), fructose (Fru), mannose (Man), *N*-acetyl-D-glucosamine (GlcNAc) and rhamnose (Rha)], organic acids [i.e., acetate, butyrate, propionate, malate, formate, fumarate, glucuronic acid, isobutyrate, isovalerate, lactate and succinate] and ethanol, using Aminex HPX-87H columns (Bio-Rad Laboratories; #1250140). UPLC-UV was adopted to examine and quantify FAAs [i.e., alanine (Ala), arginine, (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val)]. Ammonium ion (NH_4^+) levels were also analysed in parallel, as being a key indicator of amino acid metabolism. Both HPLC and UPLC-UV analyses were conducted as previously described (Kattel et al. 2023).

2.4 | Statistical Analyses

Statistical analysis was conducted using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Data groups were compared by either ordinary unpaired T test, one-way ANOVA or two-way ANOVA with follow-up test for multiple comparisons, as outlined in the descriptive captions of each figure. Statistical significance was claimed when the p values were < 0.05 [*], < 0.01 [**], < 0.001 [***] and < 0.0001 [****]. For each sample, data were presented as mean $\pm$ SEM from three technical replicates, calculated across three to four independent biological batches, as described in the descriptive captions of each figure.

3 | Results

3.1 | Physicochemical Assessment of G-NVs and G-CTL

Before the IMC study, we assessed G-NVs and G-CTLs for their physicochemical properties. NTA revealed that the G-NV preparation was enriched in particles with median and peak sizes of 97.5 ± 4.4 and 98.9 ± 6.0 nm, respectively. Particles have also been detected in the G-CTL, yet at significantly lower concentration and revealing a size distribution shifting towards smaller sizes (Figure 2A, B and Figure S1A, B).

TEM of G-NVs showed that most of the imaged structures had morphology and size typically attributable to small vesicles,

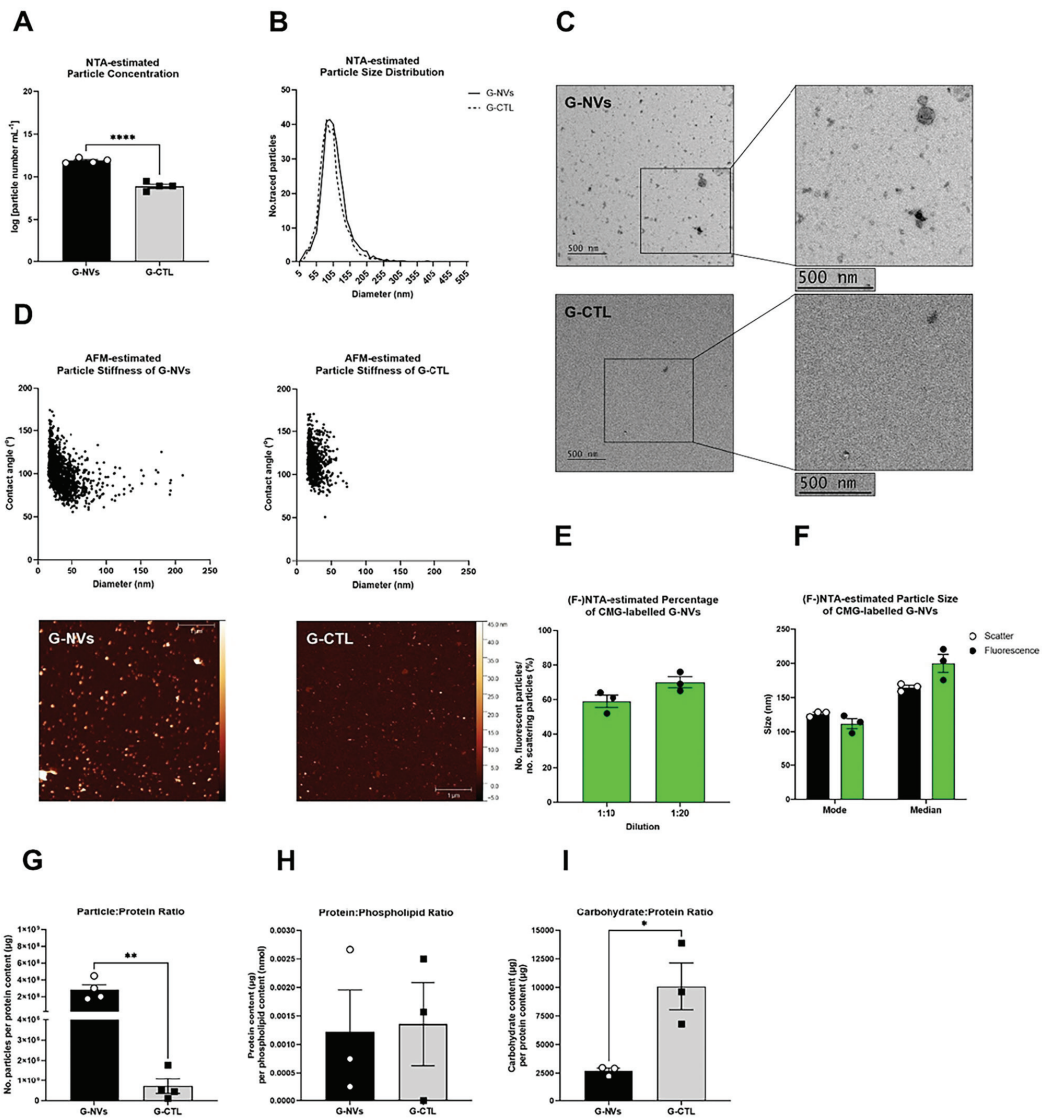


FIGURE 2 | Physicochemical assessment of G-NVs and G-CTL. The bar graphs show: (A) NTA-estimated particle concentrations of four biological batches of G-NVs and G-CTL—expressed as particle number per millilitre in logarithmic scale; (E) (F-)NTA-estimated ratio of total number of fluorescent particles over total number of scattering particles detected in three biological batches of CMG-labelled G-NVs—expressed as percentage (%) and measured with two serial dilutions (1:10 and 1:20) in PBS; (F) (F-)NTA-estimated particle mode size (nm) and median size (nm) of three biological batches of CMG-labelled G-NVs measured in scattering and fluorescence mode with 1:10 dilution in PBS; (G) particle:protein ratio of four biological batches of G-NVs and G-CTL—expressed as particle number per protein content (μg); (H) protein:phospholipid ratio of three biological batches of G-NVs and G-CTL—expressed as protein content (μg) per phospholipid content (nmol); (I) carbohydrate:protein ratio of three biological batches of G-NVs and G-CTL—expressed as carbohydrate content (μg) per protein content (μg). The histograms in B depict the NTA-estimated particle size distributions of four biological batches of G-NVs and G-CTL—expressed as average of two to four technical measurements per sample. The (C) TEM and (D) liquid AFM images illustrate, respectively, the morphological and nanomechanical properties of one representative batch of G-NVs and G-CTL. The scatterplots in D show the contact angle (°) and equivalent diameter (nm) of each particle detected and analysed by liquid AFM. Bargraph data are reported as mean ± SEM. In (A, E, G, H and I), statistical analysis was performed using ordinary unpaired *T* test (not reported *p* value: not significant; significant *p* value: < 0.05 [*], < 0.0001 [****]). In (F), statistical analysis was performed between matching scatter and fluorescence values using ordinary unpaired *T* test (not reported *p* value: not significant). Abbreviations: AFM, atomic force microscopy; CMG, CellMask™ Green; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; (F-)NTA, (fluorescence) nanoparticle tracking analysis; TEM, transmission electron microscopy.

well distinguished from the appearance of the G-CTL, which displayed only a handful of amorphous entities, aggregates, along with a few G-NV-like bodies (Figure 2C). AFM quantitative morphometry (Welsh et al. 2024) also supported these findings (Figure 2D). Although both samples showed abundant globular particles in the AFM images, the vast majority of those found in the G-CTL sample displayed morpho-mechanical characteristics incompatible with intact EVs (Ridolfi et al. 2020; Ridolfi et al. 2023). Conversely, most of the particles contained in G-NVs clustered around a plot region described as characteristic for EVs (Ridolfi et al. 2023). In particular, in the G-NV sample, particles above 50 nm diameter (i.e., the size that corresponds to the cutoff of the TFF device used for G-NV enrichment) showed an average CA of $88.0^\circ \pm 14.3^\circ$, falling within the expected range for EVs (i.e., average CAs of 80° – 120°). In the same sample, we also observed a cluster of smaller objects showing a much higher dispersion of CAs and extending to higher mechanical stiffnesses (i.e., $CA > 130^\circ$), which were not previously associated with EVs (Ridolfi et al. 2023), thus suggesting the residual presence of non-vesicular co-isolates. As expected, the G-CTL sample only exhibited the latter type of particles, confirming the selective enrichment of particles smaller than 50 nm and incompatible with the nanomechanical characteristics of intact EVs. AFM morphometry was also employed to assess the relative contribution of different particle diameter ranges to the total particle volume, further emphasising the difference between G-NV and G-CTL. This analysis clearly showed the presence of a substantial fraction of particles larger than 100 nm in the G-NV sample, accounting for more than half of the total particle volume. In contrast, the G-CTL contained only the particles smaller than 100 nm and at much lower prevalence (Figure S1C).

Data collected from the G-NV and G-CTL samples were also validated by fluorescence NTA (F-NTA), which was performed to verify the presence of lipid membranous nanoparticles in our G-NV sample. G-NV particles were labelled with CMG Plasma Membrane Stain, a lipophilic dye that emits bright and uniform green fluorescence upon insertion into the outer leaflet of lipid membranes. An optimised staining protocol was followed as previously described (Fortunato et al. 2021) and SEC was used to purify stained vesicles from the excess of unbound dye to avoid signals due to dye molecules self-aggregation (Welsh et al. 2024; Théry et al. 2018). The sensitivity of the F-NTA instrument required for reliable detection and quantification of fluorescent nanoparticle signals makes the analysis more susceptible to background noise derived from impurities, especially unbound dye (Fortunato et al. 2021). As a control, a procedural blank sample, corresponding to the buffer (PBS) supplemented only with CMG, was processed analogously and assessed in parallel. Averaging the data obtained from the F-NTA analysis of two serial sample dilutions in PBS (1:10 and 1:20), approximately $65\% \pm 8\%$ of scattering particles was fluorescent, suggesting that a modest fraction of the total G-NV particle population detected by NTA is non-vesicular. This result is consistent with AFM analysis, which showed that 72% of the detected G-NV particles had a nanomechanical behaviour typical for intact vesicles. Using both sample dilutions, the proportion of fluorescent particles relative to total scattering particles did not significantly differ and showed consistent size distribution (Figure 2E). This confirmed that fluorescent labels were stably associated with intact vesicles. Scattering and fluorescently labelled G-NV particles showed

overlapping size distributions, indicating stable incorporation of the dye into G-NV vesicle membranes without altering particle size or aggregation state (Figure 2F). Analysis of the blank showed negligible particle count in scatter mode, while revealing a detectable particle population in fluorescence mode, exhibiting a large hydrodynamic diameter (Figure S1D). On one hand, this proved that SEC was effective in removing most of the unbound CMG. On the other hand, it also highlighted that a fraction of the free dye potentially formed self-aggregates or micelle-like structures, which was expected in a neutral and hydrophilic environment as PBS. The wider size distribution of the detected fluorescent particles (Figure S1E) can be potentially attributed to stronger brightness resulting from the clustering of multiple dye molecules, which may lead to size overestimation. F-NTA of the G-CTL yielded particle counts that were comparable to those observed in the PBS (Figure S1D). Nonetheless, size estimation did not follow the same trend, showing no significant differences between scattering and fluorescent particles (Figure S1F). This may confirm the presence of a small subset of residual lipid particles that may sequester CMG, thereby limiting its self-aggregation or the formation of large micelle-like structures. In contrast to scatter detection, the sensitivity of which depends on particle size and RI, fluorescence detection mainly relies on the brightness, stability and intensity of the fluorophore in use. Therefore, particles that are too small or weak in terms of RI to be revealed in scatter mode may be detected in fluorescence mode if conjugated to a proper fluorophore (Braissant et al. 2015). This explains a higher number of fluorescent particles relative to scattering particles in CMG-labelled G-CTL sample, which may indicate the detection of lipid particles emitting scattering signals falling below the resolution of the instrument that become visible only upon staining.

In alignment with these observations, the G-NV sample exhibited a higher particle number-to-protein ratio, compared with the G-CTL, which is indicative of greater sample purity from protein contaminants (Figure 2G) (Théry et al. 2018). As EVs are fundamentally characterised by the presence of a phospholipid bilayer, the protein-to-lipid ratio has been introduced in the EV field as an additional metric that could be used to evaluate the degree of contamination of EV samples from soluble proteins (Théry et al. 2018; Osteikoetxea et al. 2015). We specifically focused on analysing the ratio between protein content and phospholipid content, which was observed to be comparable between G-NV and G-CTL preparations, confirming the presence of small phospholipid particles also in the G-CTL, but primarily without typical EV connotation (Figure 2C, D). Furthermore, as *Bt* preferentially utilises sugars as a primary carbon source, we were also interested in quantifying total carbohydrate content in both samples, which highlighted a higher carbohydrate enrichment per protein content in the vesicular preparation (Figure 2I). Overall, these data proved that our manufacturing protocol was efficient in enriching our G-NV sample with particles having typical nanovesicular features and a suitable degree of purity.

3.2 | IMC-Based Assessment of *Bt*'s Growth Under Treatment With G-NVs and G-CTL

To evaluate the effect of G-NVs on *Bt*'s growth, untreated bacteria were analysed in parallel with cultures treated with three doses

TABLE 1 | *Bt* treatment conditions with three doses of G-NVs and G-CTL quantified by NTA.

Sample name	Dose number	Particle concentration	Particle mode	Particle median	Inoculum	Particle number/ treated cell
G-NVs	1	2.0E+ 11/mL	98.9 ± 7.6 (nm)	97.5 ± 6.0 (nm)	1E+ 7 CFU/mL	20,000
	2	1.0E+ 11/mL				10,000
	3	5.0E+ 10/mL				5,000
G-CTL	1	6.0E+ 08/mL	91.0 ± 7.6 (nm)	90.1 ± 6.4 (nm)		60
	2	3.0E+ 08/mL				30
	3	1.5E+ 08/mL				15

Note: The summary table shows the treatment conditions of *Bt* 1E+7 CFU/mL with three doses of G-NVs and G-CTL in terms of NTA-estimated particle concentration—expressed as number of particles per millilitre of treatment reaction, particle size—expressed as mode (nm) and median (nm), and number of particles per treated *Bt* cell. Data are presented as the average of four biological batches of G-NVs and G-CTL.
Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles.

of G-NVs: 20,000 particles/cell (Dose 1); 10,000 particles/cell (Dose 2); 5,000 particles/cell (Dose 3) (Table 1). To distinguish the effects specifically attributable to the G-NV preparation from those caused by the ginger matrix separated from it, bacteria were also challenged with the G-CTL at doses normalised to equivalent protein amount of the respective G-NV sample dose. As proteins constitute predominant component of both vesicles and their respective procedural controls, total protein content was selected as normalisation factor between samples, while particles were observed to be highly enriched in the G-NV preparation. Indeed, the NTA-estimated number of detected particles in G-CTL Dose 1, Dose 2 and Dose 3 corresponded to 60, 30 and 15 particles per cell, respectively. Bacterial growth was monitored via IMC for up to 24 h (Table 1).

Figure 3A, B illustrate the total heat and heat flow curves of untreated *Bt* and was used as a reference. A parallel analysis of non-inoculated ginger samples confirmed the absence of microbial contamination (Figure S2A, B). The total heat produced by *Bt* ($Q_{\text{tot}} = 1.81 \pm 0.09$ J/mL) was not affected by G-NV supplementation. In contrast, addition of the G-CTL induced a dose-dependent increase in Q_{tot} (i.e., Dose 1: +69%; Dose 2: +33%; Dose 3: +18%), reaching statistical significance at the two highest doses (Figure 4A). A similar trend was observed for the heat released during the exponential phase (Q_{exp}), although no statistical difference was detected between bacteria treated with G-NVs Dose 3 and its corresponding G-CTL (Figure 4B). IMC data were complemented by biomass measurements obtained via OD_{600} (Figure 4C). The specific growth rate of *Bt* ($\mu_{\text{max}} = 0.56 \pm 0.01$ h⁻¹) increased significantly in cultures supplemented with either G-NVs or G-CTL, except for the G-CTL Dose 3. The increase of μ_{max} was more pronounced for cultures exposed to G-NVs (i.e., Dose 1: +54%; Dose 2: +50%; Dose 3: +39%), whereas G-CTL treatments exhibited a clearer dose-dependent pattern (i.e., Dose 1: +29%; Dose 2: +22%) (Figure 4D). A higher maximum metabolic activity level (P_{max}) was exhibited by all treated cultures (i.e., G-NVs Dose 1: +34%; G-NVs Dose 2: +32%; G-NVs Dose 3: +26%; G-CTL Dose 1: +80%; G-CTL Dose 2: +47%; CTL Dose 3: +34%), compared with control bacteria ($P_{\text{max}} = 141 \pm 8.91$ μ W/mL). A statistically significant difference between G-NV samples and their respective G-CTL was observed only at the highest dose, with a stronger response induced by the G-

CTL (i.e., +34% with G-NVs Dose 1 vs. +80% with G-CTL Dose 1) (Figure 4E). The time at which P_{max} occurred ($T_{P_{\text{max}}} = 10.8 \pm 0.4$ h in control bacteria) was significantly shorter with all doses of both G-NVs and G-CTL (i.e., G-NVs Dose 1: 7.2 ± 1.2 h; G-NVs Dose 2: 7.1 ± 1.3 h; G-NVs Dose 3: 7.3 ± 1.4 h; G-CTL Dose 1: 8.3 ± 1.6 h; G-CTL Dose 2: 8.3 ± 1.6 h; CTL Dose 3: 8.0 ± 1.1 h), indicating accelerated growth. This was in line with the thermogram profiles shown in Figure 3. Although no significant difference was found between G-NV- and G-CTL-treated cultures, G-NVs tended to promote slightly faster bacterial growth (Figure 4F).

3.3 | Assessment of *Bt*'s Extracellular Metabolites Released in Response to Treatment With G-NVs and G-CTL

To evaluate the potential metabolic alterations induced in *Bt* by treatment with either G-NVs or G-CTL, extracellular fermentation substrates and products were quantified in both unconditioned and conditioned growth media by HPLC or UPLC-UV. A specific focus was given to sugars [i.e., Glc, Fru, Man, GlcNAc and Rha], ethanol and organic acids (i.e., acetate, butyrate, propionate, malate, formate, fumarate, glucuronic acid, isobutyrate, isovalerate, lactate and succinate), as well as FAAs (i.e., Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val), the classes of metabolites that serve as key indicators of microbial metabolism. To facilitate data acquisition and interpretation, the assessment of the samples containing G-NVs and G-CTL was restricted to the highest doses, as these induced the most pronounced and measurable biological effects.

3.3.1 | Sugars

Among tested sugars, only Glc and Fru could be reliably quantified. Other saccharides were excluded due to insufficient chromatographic resolution on the analytical column used; specifically, disaccharides co-eluted as a single peak, while others showed excessive peak broadening. Furthermore, dual-detector analysis (RI and UV) revealed that peaks appearing at the retention times of certain sugars, such as Rha, contained interfering

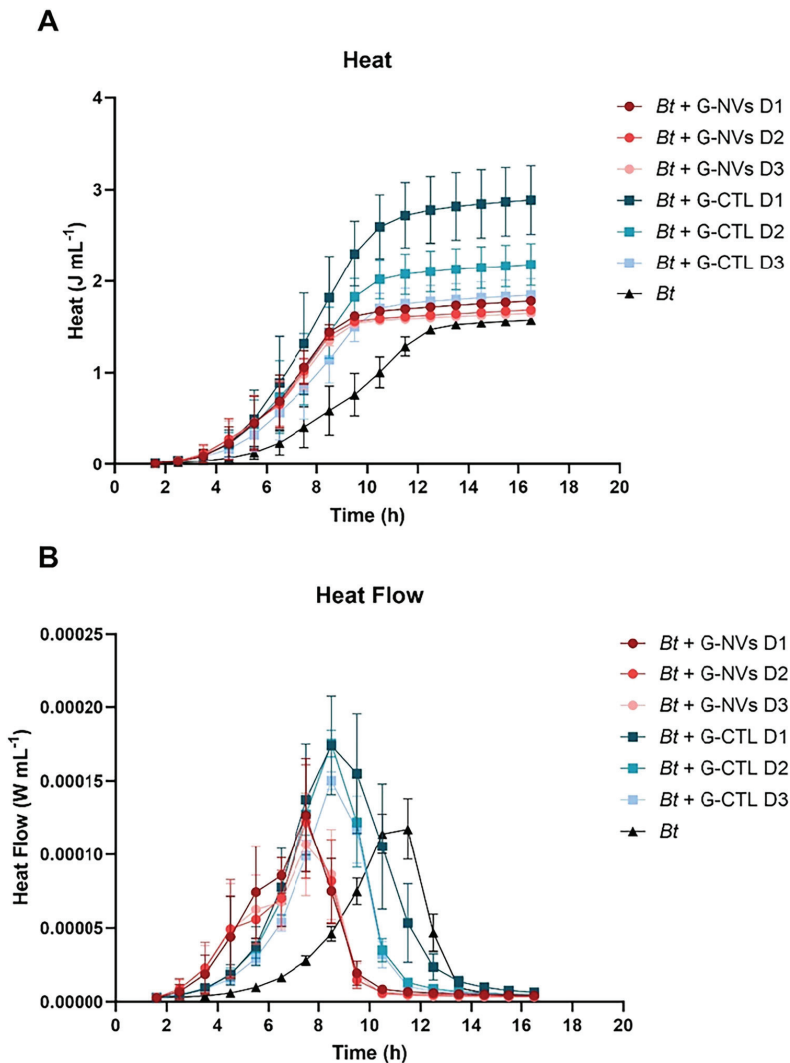


FIGURE 3 | IMC-estimated thermograms of *Bt* grown with or without G-NVs and G-CTL. The line graphs show (A) heat and (B) heat flow curves of *Bt* grown at 37°C for 24 h in growth media containing or not containing either G-NVs or G-CTL at Dose 1, Dose 2 or Dose 3. Data are reported as mean $\pm$ SEM of four biological batches. Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; D, dose; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; IMC, isothermal microcalorimetry.

components. Since the Rha standard is UV-inactive, the presence of a UV signal in the sample at that same retention time indicated a lack of peak purity, preventing accurate quantification (Figure S3).

During preparation, growth media were supplemented with Glc to provide a primary carbon source for promoting *Bt*'s growth and metabolism (Section 2.3.1). Therefore, before bacterial inoculation and IMC-based cultivation, all media, either containing or not containing G-NVs and G-CTL, showed detectable levels of Glc. While the control media and the media including G-

NVs had comparable Glc concentrations consistent with the originally added Glc, the media formulated with the G-CTL displayed significantly higher Glc amount (Figure 5 and Figure S4), indicating the contribution to Glc supplementation by the G-CTL content. Similarly, although Fru was not supplemented to the original media, it could be measured in the media containing the G-CTL (Figure 5B and Figure S4B). At the end of the IMC experiment, complete sugar consumption upon fermentation was confirmed in all inoculated samples, while the sugar level in non-inoculated samples remained unaltered with respect to the original media composition (Figure 5 and Figure S4).

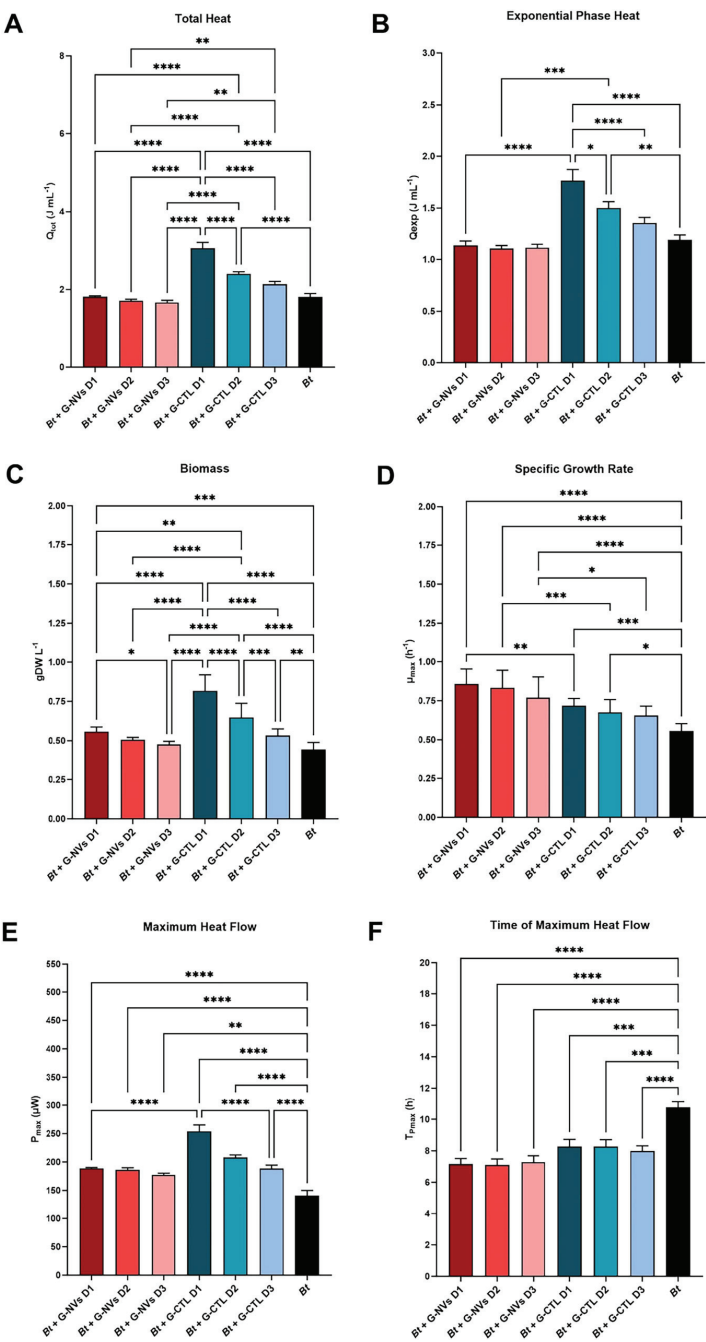


FIGURE 4 | Legend on next page.

3.3.2 | Organic Acids and Ethanol

Organic acids and ethanol represent primary bacterial fermentation products. Therefore, they were expected to be measured only in the conditioned media collected at the end of bacterial growth. This was confirmed for all tested organic compounds, except for ethanol, which was surprisingly detected in the starting media supplemented with both G-NVs and G-CTL before inoculation with bacteria (Figure 6A, B and Figure S5). To exclude the association of this readout to any artefact, a confirmative GC-MS analysis was performed on replicates of two samples collected at the end of the IMC-based cultivation period, validating our observations by HPLC: (i) blank media (not inoculated) enriched with G-NVs; (ii) conditioned media, depleted of grown bacteria, that were originally supplemented with the G-CTL (Figure S6). Fermentation of *Bt* induced measurable secretion of ethanol, acetic, formic, propionic and succinic acids. Among them, acetic, succinic and formic acids revealed the highest levels. A significantly greater production and release of acetic, succinic and propionic acids was observed in samples supplemented only with G-CTL, while enrichment of growth media with G-NVs did not provide any added value in boosting bacterial metabolic activity (Figure 6B and Figure S5). The end concentrations of ethanol, normalised with initial concentrations and formic acid did not reveal any significant differences between control and treated bacteria (Figure S7).

3.3.3 | FAAs

Initial concentrations of all tested FAAs were shown to be comparable across all growth media before inoculation (Figure 7A and Figure S8). Interestingly, a significantly lower level of NH_4^+ was observed in the media containing G-CTL, compared with the control media and the media supplemented with G-NVs (Figure 7A). Although *Bt* is known to be both a saccharolytic and a proteolytic microbial species (Rivera-Chávez and Bäumlér 2015), no changes in FAA concentrations were observed in any sample at the end of the cultivation period (Figure 7B and Figure S8). Unexpectedly, a significant reduction in NH_4^+ was also highlighted in the non-inoculated medium containing G-NVs post IMC experiment (Figure S8D).

4 | Discussion

In the present study, we explored the growth and metabolic response of *Bt*, an obligate anaerobe of the HGM with encouraging exploitable potential as NGP (Chang et al. 2019; Jan et al. 2024), to the exposure with small nanovesicles derived from ginger rhizome (*Zingiber officinale Roscoe*), herein indicated as G-NVs. In preclinical models, G-NVs have been shown to modulate the microbial composition of the HGM, actively inducing anti-inflammatory and hepatoprotective effects (Dad et al. 2021; Teng

FIGURE 4 | IMC-derived growth characteristics and OD_{600} of *Bt* grown with or without G-NVs and G-CTL. The bar graphs show (A) the cumulative heat produced during the whole cultivation period (Q_{tot}), expressed as Joule (J) per millilitre, (B) the cumulative heat produced during the exponential phase (Q_{exp}), expressed as Joule (J) per millilitre, (C) biomass, expressed as grams of dry weight (gDW) per litre, (D) the maximum specific growth rate (μ_{max}), expressed per (h), (E) the maximum power output at the highest peak of the heat flow curve (P_{max}), expressed as microwatt (μW) and (F) the time at which the P_{max} occurs ($T_{P_{\text{max}}}$), expressed as time (h), of *Bt* grown at 37°C for 24 h in growth media containing or not containing either G-NVs or G-CTL at Dose 1, Dose 2 or Dose 3. Data are reported as mean $\pm$ SEM of four biological batches. Statistical analysis was performed using ordinary one-way ANOVA with follow-up test for multiple comparisons (not reported p value: not significant; significant p value: < 0.05 [*], < 0.01 [**], < 0.001 [***], < 0.0001 [****]). Comparisons between *Bt* + G-NVs doses and *Bt* + G-CTL doses were restricted to matching doses. Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; D, dose; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; IMC, isothermal microcalorimetry; OD_{600} , optical density at 600 nm wavelength.

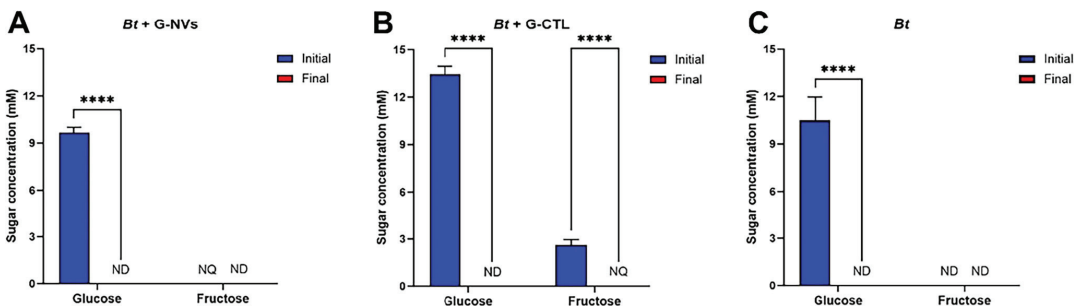


FIGURE 5 | Initial and final free sugar concentrations in *Bt*'s growth media with or without G-NVs and G-CTL. The bar graphs show initial and final sugar concentrations (mM) in *Bt*'s growth media supplemented with (A) G-NVs (Dose 1), (B) G-CTL (Dose 1) and (C) PBS. Initial concentration data are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Final concentration data are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow-up test for multiple comparisons (not reported p value: not significant; significant p value: < 0.0001 [****]). Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; ND, not detected; NQ, not quantified.

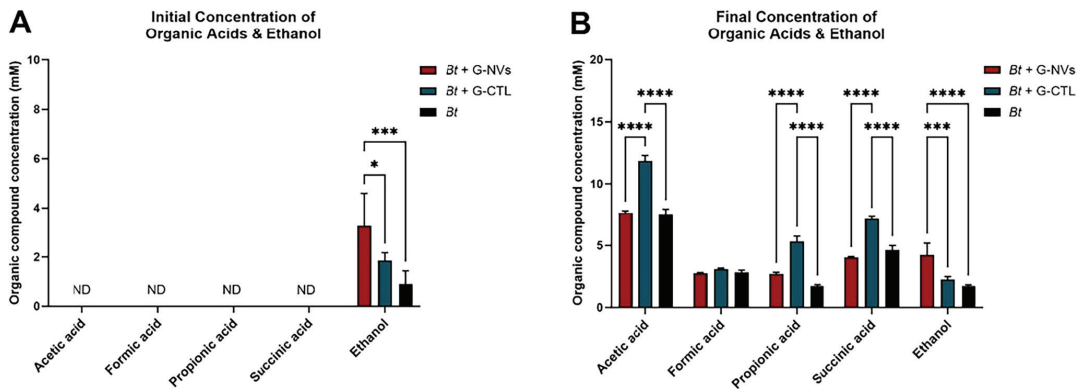


FIGURE 6 | Initial and final concentrations of organic acids and ethanol in *Bt*'s growth media with or without G-NVs and G-CTL. The bar graphs show (A) initial and (B) final concentrations (mM) of organic acids and ethanol in (A) non-inoculated media and (B) inoculated media. Data in (A) are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Data in (B) are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow-up test for multiple comparisons (not reported *p* value: not significant; significant *p* value: < 0.05 [*], < 0.001 [***], < 0.0001 [****]). Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles; ND, not detected.

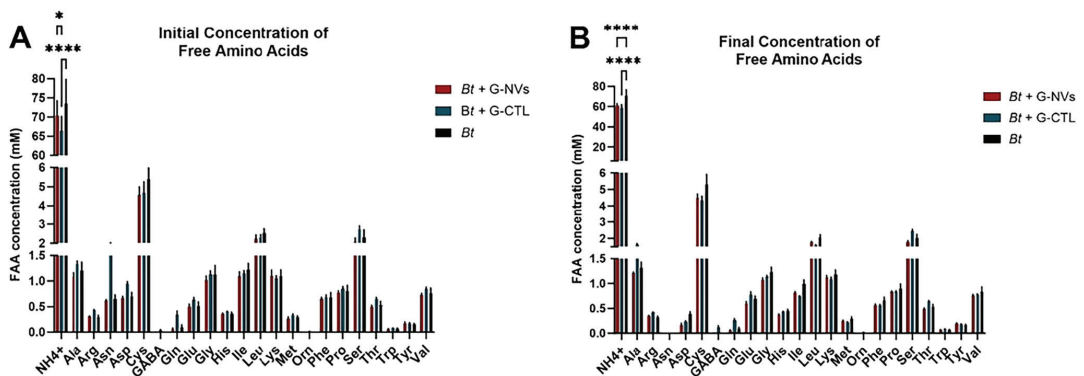


FIGURE 7 | Initial and final FAA concentrations in *Bt*'s growth media with or without G-NVs and G-CTL. The bar graphs show (A) initial and (B) final concentrations (mM) of FAAs in (A) non-inoculated media and (B) inoculated media. Data in (A) are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Data in (B) are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow-up test for multiple comparisons (not reported *p* value: not significant; significant *p* value: < 0.05 [*], < 0.0001 [****]). Abbreviations: *Bt*, *Bacteroides thetaiotaomicron*; G-CTL, G-NVs procedural control; G-NVs, ginger-derived nanovesicles.

et al. 2018). However, to our knowledge, this work showcases the first application of IMC to probe the impact of G-NVs, and EVs in general, on bacterial growth dynamics, enabling kinetic measurement of biomass production and real-time monitoring of metabolic activity. Such methodology may provide an appealing tool for a currently unmet need in studying EV roles and applications in microbiology. As recommended by the 'Minimal Information for Studies of EVs' (MISEV) 2018 and 2023 guidelines (Welsh et al. 2024; Théry et al. 2018), we have assessed G-NVs against a procedural control sample, which we named G-CTL, to distinguish the biological activity attributable to the 50–

200 nm sized particles enriched in our G-NV preparation from the residual functionality of the ginger extract depleted of the G-NV and containing constituents smaller than 50 nm.

Ginger samples were prepared using a combination of state-of-the-art methodologies for EV production, including differential centrifugation, dead-end filtration and TFF. In the final processing step, both G-NVs and G-CTL were recovered as the retentate and permeate, respectively, from a TFF device with a 50 nm pore-size membrane, followed by subsequent sterilisation by passage through a 0.22 μ m pore-sized dead-end filter unit.

For their physicochemical characterisation, we adopted complementary biophysical and biochemical approaches, integrating single-particle, imaging and bulk biochemical analyses. NTA, selected as a widely used and well-established high-throughput method for single-particle analysis and quantification (Welsh et al. 2024), showed that the G-NV sample had a substantial concentration of particles within the expected size range for nanovesicles. NTA is measuring the hydrodynamic diameter of particles in solution based on their Brownian motion, thus potentially leading to particle size overestimation (Welsh et al. 2024). This may account for the apparent, though significantly reduced particle concentration (nearly 800-fold lower), detected in the G-CTL preparation, exhibiting a size range partially overlapping with the particle size distribution estimated in the G-NV sample. To validate either the presence or the absence of lipid membranous particles in our G-NV and G-CTL preparations, respectively, both were stained with CMG, a lipophilic dye emitting green fluorescence upon insertion into lipid membranes, and analysed by F-NTA. Additionally, low-throughput imaging technologies, specifically TEM and AFM, were adopted to complement F-NTA analysis by enabling the detection of particles falling below the detection limit of the NTA instrument and providing insights into the morpho-nanomechanical properties of particles. With respect to the G-NV sample, these methods confirmed the enrichment of the G-NV sample in particles displaying characteristic vesicular morphology and membrane stiffness within the anticipated size distribution of 50–200 nm. It also revealed the presence of a residual subset of particles, either vesicular or showing amorphous appearance and increased stiffness, likely corresponding to co-isolated contaminants, such as protein aggregates and polysaccharide fragments. Likewise, these analyses corroborated the NTA measurements of the G-CTL, highlighting the selective accumulation of small particles, especially those falling below the 50 nm diameter threshold, most of which exhibited higher stiffness than vesicles, as well as amorphous shape. Protein and phospholipid quantification were in line with the observed enrichment of nanovesicles in the G-NV sample, which exhibited higher concentrations of phospholipidic particles purified from soluble proteins, compared with the G-CTL. We observed minimal retention of particles smaller than the ultrafilter cut-off, as well as passage of particles bigger than the filter cutoff into the permeate, a phenomenon largely documented in ultrafiltration processes, likely due to diverse physicochemical and operational factors. The correlation between the nominal cut-off attributed to ultrafiltration systems and their actual retention efficiency is indeed governed not only by physical size exclusion but also by the hydrodynamic behaviour of particles and the hydrodynamic drag force, which regulate particle-particle and particle-surface interactions, ultimately affecting flow dynamics, filtering membrane fouling and particle separation efficiency (Lee et al. 2020; Busatto et al. 2018; Wolf et al. 2022). Furthermore, it is known that particles characterised by softer membranes and greater flexibility, such as EVs, may deform and squeeze through smaller pores into the permeate (Ridolfi et al. 2023; Chernyshev et al. 2022). Overall, our isolation protocol yielded G-NV samples with a significant enrichment in EV-like nanoparticles with a suitable degree of purity and exhibiting distinct physicochemical features, compared with the G-CTL preparation.

Our experimental design consisted in evaluating the dose-dependent response of *Bt* to ginger samples by treating bacterial

cells with G-NV and G-CTL preparations at corresponding doses normalised to equivalent total protein amount. The deselection of particle number as preferred normalisation factor between the two samples was driven by the pronounced depletion of NTA-estimated particles in the G-CTL, which would have required an extensively large sample volume to run the experiment, making the setting unfeasible. Furthermore, as shown by AFM and TEM, the G-CTL contained a few particles that were undercounted by NTA, as falling below the limit of detection of the instrument. Therefore, normalisation of G-NVs and G-CTL based on particle count would have introduced quantification bias into our experiment. On the contrary, total protein content can be reliably quantified in both samples, reflecting the molecular sample makeup, as proteins, along with lipids, are the predominant components of EVs, forming their structure and carrying out functions (Royo et al. 2020; Yáñez-Mó et al. 2015). Moreover, along with EV particle number, this parameter is among the most common normalisation factors used for EV functional assessment *in vitro* and *in vivo* (Skotland et al. 2020).

Bt's treatment with G-NVs, at concentrations of up to 20,000 particles per cell, did not affect the heat released during either the whole cultivation period (Q_{tot}) or solely the exponential growth phase (Q_{exp}), suggesting no alteration of the total biomass output. This was validated by traditional OD_{600} measurement and further supported by metabolite profiling, which showed no changes in fermentation end-products, such as SCFAs and FAAs. These findings concur with evidence from a recent study, reporting no impact of G-NVs on the individual growth of *Bacteroides fragilis*, used as a reference microbial strain of the Bacteroidaceae family (Teng et al. 2018). The G-CTL was analysed in parallel to assess *Bt*'s response to ginger non-vesicular components. G-CTL elicited a specific boost in fermentation yield, as reflected by the induced dose-dependent increase in heat outputs, both Q_{tot} and Q_{exp} and OD_{600} readings. The greater and dose-dependent secretion of acetic, succinic and propionic acids promoted by the G-CTL confirmed its stimulatory effect. Although microbial fermentation under G-NV conditioning resulted in unaltered cellular expansion and release of extracellular metabolites, we observed that both G-NVs and G-CTL enhanced *Bt*'s specific growth rate (μ_{max}), with G-NVs exerting a more substantial effect, raising the maximum metabolic activity output (P_{max}) and inducing earlier occurrence of the P_{max} (T_{Pmax}). This indicates that both ginger samples triggered an early metabolic response, but resulting in different metabolic output, which was steady with G-NVs while increased by the G-CTL. As *Bt* is preferentially involved in the decomposition of indigestible, high molecular weight (HMW) carbohydrates (e.g., dietary fibres and host mucus-derived glycans) (Wexler and Goodman 2017; Zocco et al. 2007; Rivera-Chávez and Bäuml 2015), the greater carbohydrate-to-protein ratio quantified in the G-CTL, with respect to G-NVs, likely contributed to the observed functional discrepancy between the two samples. In support of this, no consumption of FAAs was observed under any tested condition, confirming *Bt*'s prioritisation of carbohydrates as a primary carbon source for meeting metabolic demand.

The higher specific growth rate promoted by G-NVs, in comparison with the G-CTL, is potentially attributable to differences in multiple mechanisms, including cargo delivery and activation of cell signalling pathways. The G-NV-mediated co-transport of

carbohydrates with other active biomolecules, such as proteins, lipids and nucleic acids, as well as the potential induction of signalling cascades by ligand-receptor interactions on bacterial cell surface without internalisation, may exert rapid and synergistic effects, leading to the simultaneous activation of complementary metabolic and regulatory pathways, thus accelerating overall bacterial cellular response (Ginini et al. 2022; Liu and Wang 2023). In *Bacteroides* spp., the internalisation of soluble polysaccharides (PSs) is primarily mediated by the starch utilisation system (Sus), which is localised in the bacterial outer membrane. This apparatus functions in coordination with cell surface and periplasmic glycosidases, which digest HMW-PS, and the inner membrane transport system, which promotes the final entry of oligosaccharides and monosaccharides into the cytoplasm (Porter et al. 2018; Rogers et al. 2013). Due to the complex and diversified nature of this mechanism, the rate of carbohydrate processing and uptake mediated by the Sus may be highly affected by various factors. These include the PS MW, structure, local concentration and cellular energetic state, as transport of carbohydrates against their electrochemical gradient is energy intensive. The process may also be influenced by prior exposure history, as cells previously exposed to carbohydrates maintain the Sus ready for new exposure, thus shortening its induction latency (Nishiyama et al. 2021).

EVs are reported to induce rapid functional and metabolic changes through mechanisms that do not require full cargo internalisation by the recipient or interacting cell, such as binding to surface receptors or cargo delivery that directly modulates the cell surface or the surrounding extracellular environment. However, EV-mediated metabolic changes also and frequently rely on vesicle uptake via membrane fusion or endocytosis, processes that enable the internalising cell to access the EV's internal cargo (Liu and Wang 2023; Polónia et al. 2023). While EV uptake can occur rapidly (within minutes), the rate can also vary substantially depending on the interacting EV types and recipient cells. The rapid and transient 'kiss-and-run' contacts between EVs and target cell membranes have been described in human cell systems. These events can mediate either cell signalling or fusion accompanied by cargo release, both potentially influencing downstream cellular responses (Hermann et al. 2024). In gram-negative bacteria, EV uptake has been shown to be relatively slow, occurring after 2 h of treatment. This rate is slower than the bacteria division rate and is influenced by factors such as vesicle composition, size, bacterial growth phase and the specific mechanisms used for uptake, which, in the case of PDVs, remain uncertain (Bose et al. 2020; La Rosa et al. 2022). Although both membrane fusion and endocytosis are energy-dependent processes, EVs, including PDVs, may increase the rate of cargo internalisation and utilisation bypassing the lag phase required by the Sus to sense and pre-digest the substrates (La Rosa et al. 2022; Mulcahy et al. 2014). Nevertheless, this hypothesis is currently not supported by robust evidence and warrants further investigation.

Beyond glycosylated biomolecules, vesicles may be already loaded with soluble carbohydrates or contain active glycosidases that catalyse localised substrate hydrolysis in specific microenvironments—either in proximity to cells, within vesicles or at contact zones between vesicles and substrate (Woith

et al. 2019; Chou 2007; Williams et al. 2018). Furthermore, by enabling the direct delivery of enclosed and concentrated cargo by fusogenic or endocytotic pathways, they can ensure controlled intracellular release and prevent random diffusion to the surrounding environment (Nemati et al. 2022; Sanderson et al. 2019; Du et al. 2023). It was interesting to observe that the addition of either G-NVs or G-CTL to the non-inoculated incubation media induced a significant and comparable reduction of NH_4^+ , which was faster with the G-CTL. This suggests that our ginger samples are inherently capable of influencing nitrogen source availability, potentially attributable to, for instance, the presence of active metabolic enzymes incorporating NH_4^+ into organic compounds (e.g., glutamine synthetase), or, alternatively, the ability of sequestering NH_4^+ by dynamic electrostatic interactions with macromolecules, such as nucleic acids, making it undetectable in free form (Dang et al. 2020; Yu et al. 2020; Nelson et al. 2012). Although direct evidence supporting these hypotheses or describing alternative explanatory mechanisms is still lacking, these data highlight the intrinsic bioactivity of our ginger preparations as a putative confounding factor in data interpretation relative to the nitrogen metabolic responses mediated by *Bt*, thereby calling for more cautious analytical approaches to draw reliable conclusions in this regard.

The unexpected detection of ethanol in the same set of samples, not linked to microbial contamination, as demonstrated by both IMC and OD_{600} measurements, could be the result of partial contamination by microbial biomolecules, such as active enzymes, and substrates, involved in microbial fermentation. Because of the known symbiotic relationship of ginger with other microorganisms (Valderrama-Martin et al. 2023), these could have been co-isolated with ginger components upon sample preparation, although the contamination with viable bacteria was excluded. Alternatively, ethanol production could have been directly promoted by ginger in response to the activation of an endogenous plant ethanolic fermentation pathway, which typically involves two key enzyme, pyruvate decarboxylase and alcohol dehydrogenase, that may have been collected in our ginger samples in association with vesicles or in soluble form (Zhang et al. 2016). Overall, these assumptions warrant further investigation for confirmation.

5 | Conclusions

IMC demonstrated that both G-NVs and G-CTL affect *Bt*'s growth and metabolic response, although to varying extents and mediating different cargo delivery mechanisms. G-NV-enriched preparations showed to provide faster and more efficient metabolic stimulation, without increasing total metabolic output. Conversely, the soluble biocomponents enriched in G-CTL caused a sustained, dose-dependent metabolic effect, due to the higher cargo availability and potentially promoting cargo uptake through alternative pathways. The obtained data also highlighted the competitive advantage of IMC over conventional methods for assessing changes in bacterial growth dynamics and kinetics following incubation with EVs, including PDVs, thereby offering a novel and strategic approach to advance research and development focusing on (plant)EV/NV-microbe interactions.

The use of IMC in this study revealed a critical gap in the current EV research landscape, requiring elucidation of the mechanisms of action of PDVs with cells and, in general, EVs with bacteria. The analysis of the dynamic metabolic effects induced by EV-microbe interactions is often overlooked. From the parallel physico-chemical and functional characterisation of G-NVs and G-CTL within the selected microbiological model for bioactivity testing, we identified novel insights into the structural and functional differentiation between vesicular and non-vesicular components derived from ginger. The observed increase in *Bt*'s specific growth rate in response to treatment with both G-NVs and G-CTL, in association with a greater fermentation yield induced by the latter, advances our understanding of how ginger extracts exert different metabolic effects, depending on the relative contribution of bioactive constituents, as well as whether they are delivered in soluble form or within vesicles. Our approach and findings can also integrate present methods for fine-tuning *Bt* and overall gut microbial activity, thereby advancing current fermentation design and process optimisation strategies, while also fostering the development of nutraceutical products—not only NGPs, but also prebiotics (i.e., substances providing health benefits after utilisation by microorganisms) and synbiotics (i.e., combined probiotics and prebiotics)—and microbiome-targeted therapies (Helal et al. 2019; Jayan et al. 2022; Jardine and McDowell 2023; Di Renzo and Reale 2025; Lemon et al. 2012). For instance, our ginger preparations, or other samples with similar properties, may be exploited to prime and accelerate bacterial metabolism. Moreover, PDVs sharing characteristics with G-NVs may serve as efficient delivery systems of active nutrients or for therapeutics targeting of the HGM. On the other hand, systematic profiling of substrates promoting greater microbial metabolic output, such as our G-CTL, may support prebiotic design.

To support mechanistic understanding, our future studies will be dedicated to extending biomass and metabolic assessment to IMC intermediate time-points, while also conducting high-throughput multi-omics profiling of tested ginger samples and *Bt*'s secretome. This is intended to correlate the observed biological effects to specific molecular constituents, as well as assess the extent of contribution of G-NV co-isolates. A control experiment matching G-NVs and G-CTL based on total carbohydrate content will be performed to validate our hypothesis that the higher carbohydrate content contained in the G-CTL is a major driver of *Bt*'s enhanced metabolic response, compared with G-NVs. Furthermore, various targeted and non-targeted enzyme inactivation strategies will be investigated to determine whether the observed metabolic response of *Bt* to G-NVs are driven by active enzymes inherently expressed in G-NVs or co-isolated with G-NVs. To deepen *Bt*'s metabolic response efficiency and dynamics, we will also explore how the secretion and composition of *Bt*'s own EVs, conventionally known as Outer Membrane Vesicles (Kulp and Kuehn 2010), are modulated by G-NVs and G-CTL, along with elucidating triggered uptake mechanisms. Finally, we plan to expand IMC research to additional PDVs and NGP models tested either in monocultures or within synthetic, multi-strain consortia (Kattel et al. 2024; Bryant et al. 2017), thereby enhancing our understanding not only of PDV-mediated plant-microbe interactions but also of microbial cross-feeding mechanisms and community stability.

Author Contributions

Francesca Loria: conceptualization, methodology, investigation, validation, formal analysis, data curation, visualization, writing – original draft. **Anna Kattel:** methodology, investigation, validation, visualization, writing – review and editing. **Marina Junusova:** investigation, validation, data curation. **Marco Brucale:** resources, investigation, writing – review and editing. **Francesco Valle:** resources, investigation. **Paolo Bergese:** writing – review and editing. **Eeva-Gerda Kobrin:** resources, investigation, validation, data curation, writing – review and editing. **Antonio Chiesi:** funding acquisition, resources. **Paolo Guazzi:** writing – review and editing. **Sirin Korulu:** writing –review and editing, supervision. **Irina Stulova:** resources, formal analysis, data curation, writing –review and editing. **Nataša Zarovni:** conceptualization, methodology, resources, writing – review and editing, supervision. **Raivo Vilu:** funding acquisition, conceptualization, methodology, resources, writing – review and editing, supervision.

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Conflicts of Interest

The company HansaBioMed Life Sciences Ltd, current employer of Francesca Loria, Sirin Korulu, Paolo Guazzi and Antonio Chiesi is the manufacturer of some of the products used in this study: TFF-EVs-Small device, Phospholipid Quantification Assay Kit, black and transparent PS microplate wells with no binding capacity. The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information: jex270163-sup-0001-SuppMat.docx

1. Supplementary Information

1.1. Supplementary Figures

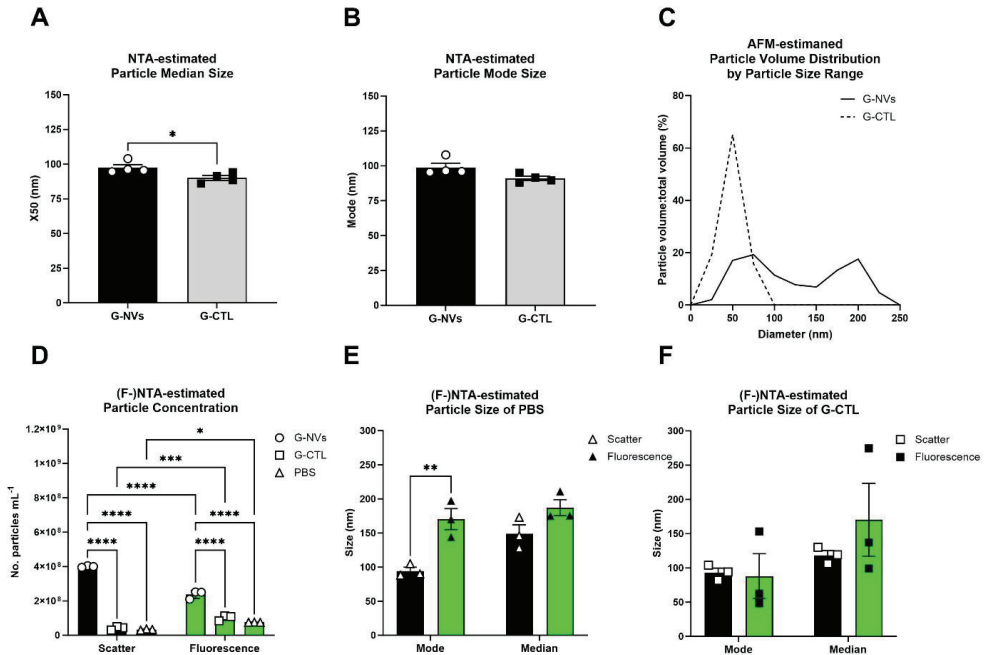
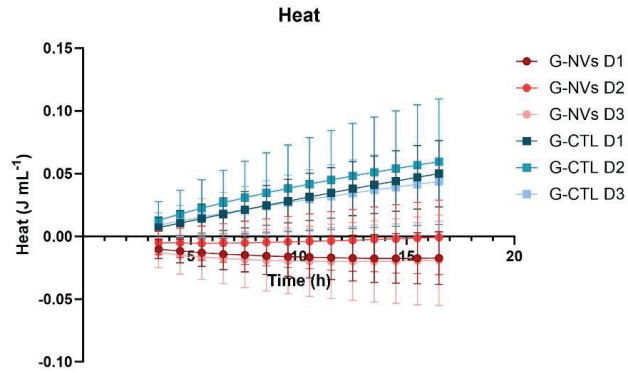


Figure S1: Physicochemical assessment of G-NVs, G-CTL, and procedural controls. The bar graphs show: A) NTA-estimated particle median size (nm) of four biological batches of G-NVs and G-CTL; B) mode size (nm) of four biological batches of G-NVs and G-CTL; D) (F-)NTA-estimated particle concentrations of three batches of CMG-labelled G-NVs, G-CTL, and PBS – expressed as particle number per milliliter – measured with 1:10 dilution in PBS; E) (F-)NTA-estimated particle mode size (nm) and median size (nm) of three biological batches of CMG-labelled G-CTL measured in scattering and fluorescence mode with 1:10 dilution in PBS; F) (F-)NTA-estimated particle mode size (nm) and median size (nm) of three replicates of CMG-labelled PBS measured in scattering and fluorescence mode with 1:10 dilution in PBS. The histograms in C depict the percentage fractions of total particle volume divided by particle diameter range of one representative batch of G-NVs and G-CTL, estimated by liquid AFM. Data in A and B are reported as mean $\pm$ SEM of four biological batches of G-NVs and G-CTL. In A and B, statistical analysis was performed using ordinary unpaired T test (not reported p-value: not significant; significant p-value: < 0.05 [*], < 0.0001 [****]). In D, statistical analysis was performed between matching scatter and fluorescence values, as well as between different samples within either scatter or fluorescence measurements, using ordinary two-way ANOVA with follow up test for multiple comparisons (significant p-value: < 0.01 [**], < 0.001 [***], < 0.0001 [****]). In E and F, statistical analysis was performed between matching scatter and fluorescence values using ordinary two-way ANOVA with follow up test for multiple comparisons (significant p-value: < 0.01 [**], < 0.001 [***]). Legend: AFM = atomic force microscopy; CMG = CellMask™ Green; G-CTL = G-NVs procedural control; G-NVs = ginger-derived nanovesicles; (F-)NTA = (fluorescence) nanoparticle tracking analysis.

A



B

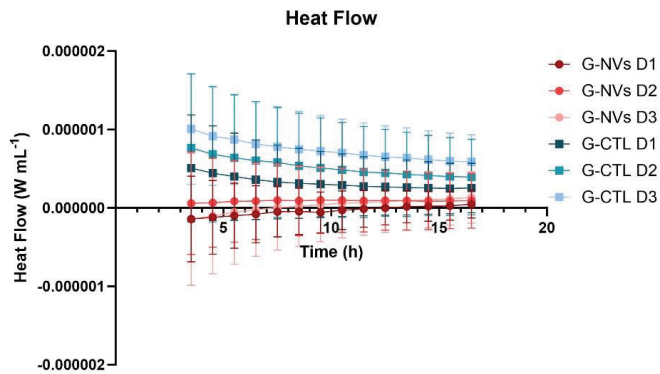


Figure S2: IMC-estimated thermograms of non-inoculated *Bt*'s growth media with G-NVs or G-CTL. The line graphs show (A) heat and (B) heat flow curves of non-inoculated *Bt*'s growth media supplemented with G-NVs or G-CTL, incubated at 37°C for 24h. Data are reported as mean $\pm$ SEM of four biological batches. Legend: D = dose; G-CTL = G-NVs procedural control; G-NVs = ginger-derived nanovesicles; IMC = isothermal microcalorimetry.

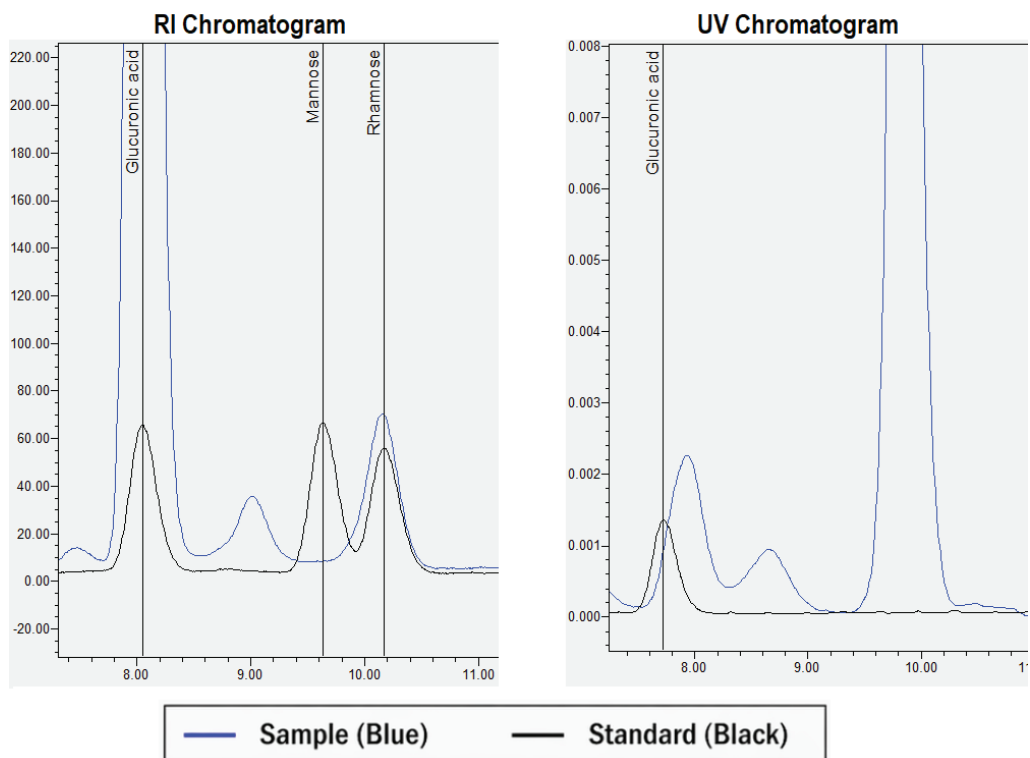


Figure S3: Dual-detector (RI and UV) HPLC analysis of sugar standard mixture vs. *Bt* + G-NVs D1. HPLC chromatograms relative to the lowest sugar concentrations of sugar standard mixture (black) and the supernatant of a *Bt* + G-NVs D1 technical replicate (blue) revealed by (A) RI and (B) UV detector systems. In the UV chromatogram of the standard mixture, only a single peak corresponding to glucuronic acid is detected, whereas peaks attributable to mannose and rhamnose are not observed. If present, these compounds would be expected to exhibit a retention time (RT) shift between the RI and UV detectors comparable to that observed for glucuronic acid (approximately 8.0 min in RI versus ~7.8 min in UV). In the sample chromatogram, a prominent RI peak elutes at a RT of 10.2 min, which is consistent with the RT of the rhamnose standard detected by RI. The same component (or a co-eluting mixture of components) is also detected in the UV chromatogram, showing an analogous RT shift relative to the RI signal, with an elution time of approximately 10.0 min in the UV detector. Legend: *Bt* = *Bacteroides thetaiotaomicron*; D1 = dose 1; G-NVs = ginger-derived nanovesicles; HPLC = high performance liquid chromatography; RI = refractive index; RT = retention time; UV = ultraviolet.

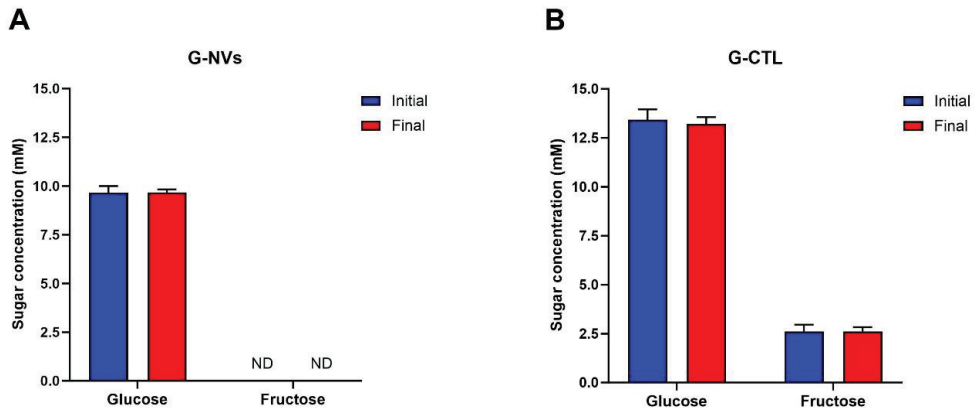


Figure S4: Initial and final free sugar concentrations in non-inoculated *Bt*'s growth media with G-NVs or G-CTL. The bar graphs show initial and final sugar concentrations (mM) in non-inoculated *Bt*'s growth media supplemented with (A) G-NVs (dose 1) or (B) G-CTL (dose 1). Initial concentration data are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Final concentration data are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow up test for multiple comparisons (not reported p-value: not significant). Legend: G-NVs = ginger-derived nanovesicles; G-CTL = G-NVs procedural control; ND = not detected.

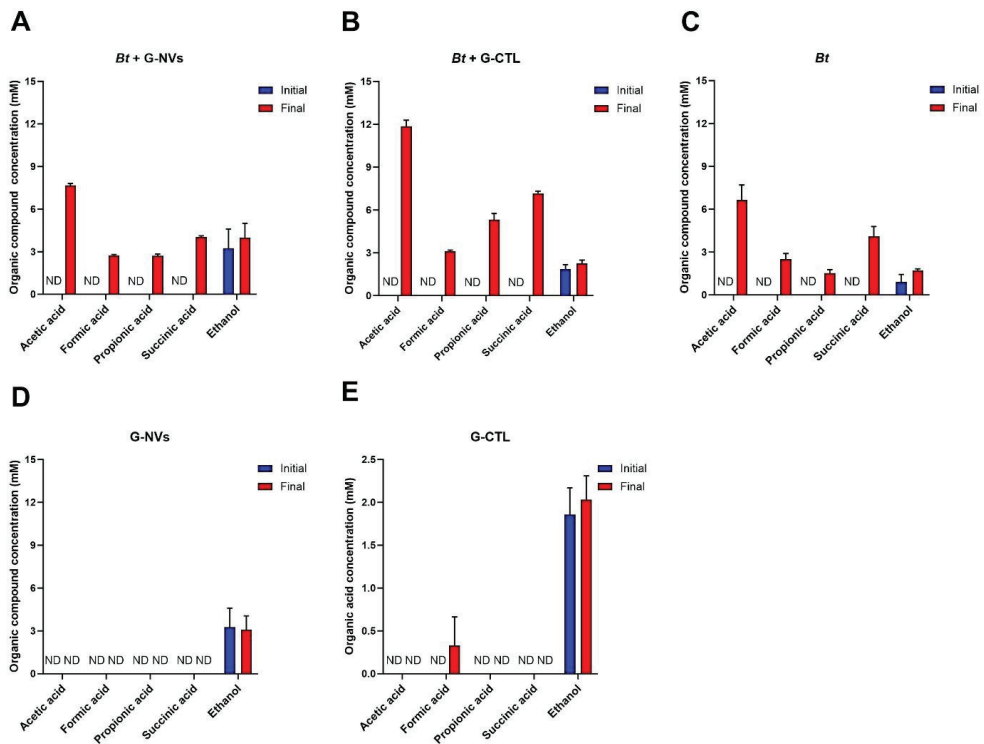


Figure S5: Initial and final concentrations of organic acids and ethanol in inoculated and non-inoculated *Bt*'s growth media with or without G-NVs and G-CTL. The bar graphs show initial and final concentrations (mM) of organic acids and ethanol in inoculated (A-C) and non-inoculated (D-E) *Bt*'s growth media supplemented with G-NVs (dose 1) or (B) G-CTL (dose 1). Initial concentration data are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Final concentration data are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow up test for multiple comparisons (not reported p-value: not significant). Legend: *Bt* = *Bacteroides thetaiotaomicron*; G-NVs = ginger-derived nanovesicles; G-CTL = G-NVs procedural control; ND = not detected.

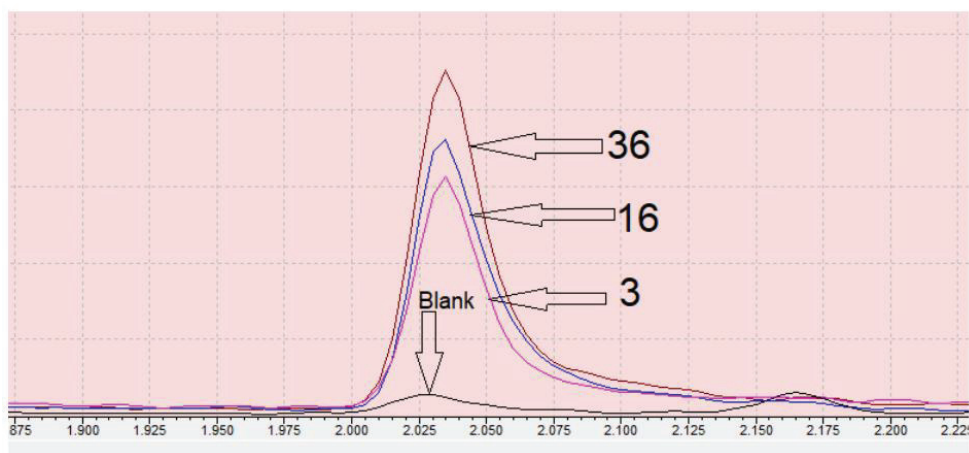


Figure S6. Control GC-MS analysis. The GC-MS chromatograms show detectable levels of ethanol in non-inoculated *Bt*'s growth media with G-NVs (dose 1) (sample no. 36) and in fermented *Bt*'s growth media with G-CTL (dose 1) (sample no. 3 and 16). Ethanol was identified with NIST17 library, which gave hit of 98% accuracy. Legend: D = dose; GC-MS = gas chromatography-mass spectrometry; G-CTL = G-NVs procedural control; G-NVs = ginger-derived nanovesicles; IMC = isothermal microcalorimetry.

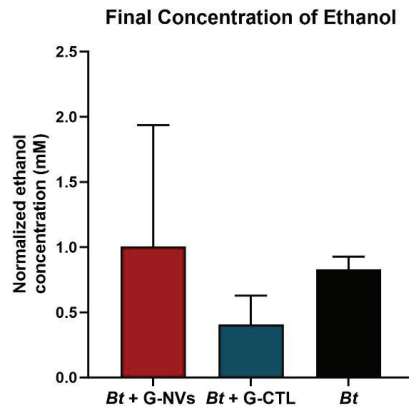


Figure S7: Normalized ethanol concentrations in fermented *Bt*'s growth media with or without G-NVs and G-CTL. The bar graph depicts final ethanol concentrations (mM) in *Bt*'s growth media with or without G-NVs (dose 1) and G-CTL (dose 1) after subtraction of initial concentrations. Data are reported as mean $\pm$ SEM of two technical replicates relative to four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary one-way ANOVA with follow up test for multiple comparisons (not reported p-value: not significant). Legend: *Bt* = *Bacteroides thetaiotaomicron*; G-CTL = G-NVs procedural control; G-NVs = ginger-derived nanovesicles.

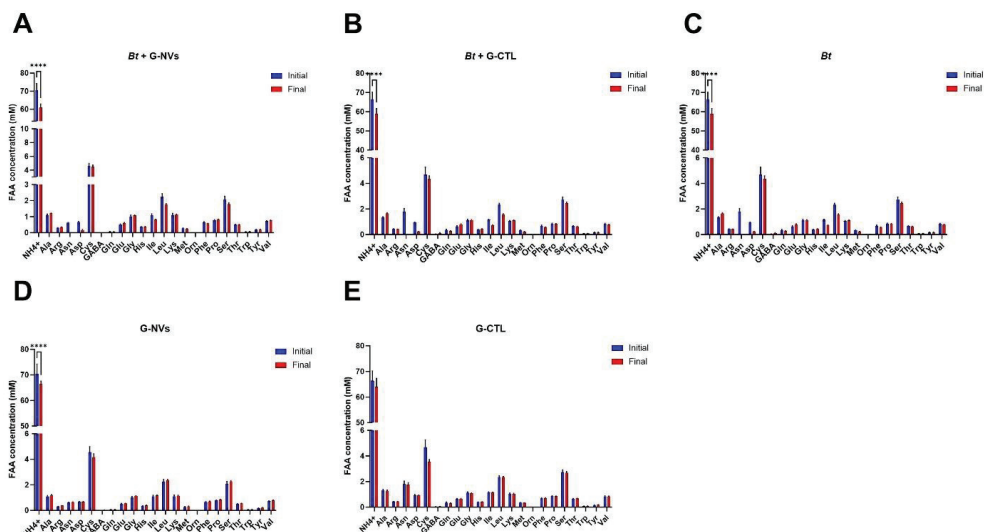


Figure S8: Initial and final FAA concentrations in *Bt*'s growth media with or without G-NVs and G-CTL. The bar graphs show initial and final concentrations (mM) of FAAs in (A-C) inoculated and (D-E) non-inoculated *Bt*'s growth media supplemented with G-NVs (dose 1), (B) G-CTL (dose 1), or PBS. Initial concentration data are reported as mean $\pm$ SEM of one technical replicate across four independent experiments, each performed using a distinct biological batch of ginger sample. Final concentration data are reported as mean $\pm$ SEM of two technical replicates across four independent experiments, each performed using a distinct biological batch of ginger sample. Statistical analysis was performed using ordinary two-way ANOVA with follow up test for multiple comparisons (not reported p-value: not significant; significant p-value: <0.0001 [****]). Legend: *Bt* = *Bacteroides thetaiotaomicron*; G-CTL = G-NVs procedural control; G-NVs = ginger-derived nanovesicles.

1.2. Supplementary Materials and Methods

1.2.1. Gas chromatography–mass spectrometry (GC–MS)

Volatile compounds were extracted using solid-phase microextraction (SPME). A 100 µL aliquot of the sample was transferred into a 10 mL glass vial. A 1 cm SPME fiber coated with 30/50 µm DVB/Car/PDMS (Stableflex) was exposed to the headspace for 40 minutes at 40°C to allow adsorption of volatiles. The collected compounds were then thermally desorbed in the injection port of a gas chromatograph for 5 minutes. Ethanol identification was performed using a Shimadzu GC-2030 system coupled with a 8050NX Triple Quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Separation was achieved on a ZB5-MS column (30 m × 0.25 mm i.d., 1.0 µm film thickness; Phenomenex, Torrance, CA, USA) with helium as the carrier gas at a linear velocity of 35 cm/s. The oven temperature program started at 40°C, increased at 5°C/min to 190°C, then ramped at 25°C/min to 280°C, with a final hold of 4 minutes, resulting in a total run time of 36 minutes. Mass spectra were acquired using electron ionization at 70 eV, scanning a mass-to-charge (m/z) range of 35–250. Each sample was analyzed in duplicate. Ethanol was identified in a non-targeted manner using GC-MS solution software (Shimadzu) and retention indices (RI), which were calculated based on the retention times of adjacent n-alkanes. Compound identification was confirmed by comparing experimental RI values and spectra with those in the NIST17 and FFNSC spectral libraries.

Appendix 5

Publication V

Loria, F., Picciotto, S., Adamo, G., Zandrini, A., Raccosta, S., Manno, M., Bergese, P., Liguori, G. L., Bongiovanni, A., & Zarovni, N. (2024). A decision-making tool for navigating extracellular vesicle research and product development. *Journal of Extracellular Vesicles*, 13(12). <https://doi.org/10.1002/jev2.70021>.

TECHNICAL NOTE

A decision-making tool for navigating extracellular vesicle research and product development

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Abstract

Due to their intercellular communication properties and involvement in a wide range of biological processes, extracellular vesicles (EVs) are increasingly being studied and exploited for different applications. Nevertheless, their complex nature and heterogeneity, as well as the challenges related to their purification and characterization procedures, require a cautious assessment of the qualitative and quantitative parameters that need to be monitored. This translates into a multitude of choices and putative solutions that any EV researcher must confront in both research and translational environments. In this respect, decision-making tools may help assess various options, weigh pros and cons, and ultimately arrive at a thought-out decision that considers both the best fit-to-source and fit-to-scope EV application(s). Here, we present a multi-criteria EV decision-making grid (EV-DMG) as a novel, efficient, customizable, and easy-to-use tool to support EV research and innovation. By identifying and weighing key assessment criteria for comparing distinct EV-based preparations and related processes, our EV-DMG may assist any EV community member in making informed, traceable, and reproducible decisions regarding the management of EV sources or samples. Ultimately, this EV-DMG may guide the adoption of the most suitable EV production and analytical pipelines for targeting a defined aim or application.

KEYWORDS

decision-making tools, extracellular vesicles, multi-criteria decision-making, product development, quality control, research and development, responsible research and innovation

1 | INTRODUCTION

Extracellular vesicles (EVs) are nanosized (i.e., ~50–150 nm, defined as “small EVs”; 200–800 nm, defined as “medium EVs”) and micro-sized (i.e., ≥ 1000 nm, defined as “large EVs”) membranous particles secreted and taken up by either eukaryotic or prokaryotic cells. By intrinsically conveying a multitude of biologically active components, including proteins, lipids, nucleic acids, and metabolites, EVs have been shown to mediate cell-to-cell and organism-to-organism communication, thus governing the functionality of organisms and their interplay with the surrounding environment (Buzas, 2023; Raposo & Stoorvogel, 2013; Yáñez-Mó et al., 2015).

The complex and heterogeneous nature of EVs underlies both their enormous potential and the significant challenges related to their exploitation. The spotlight on EVs has raised high expectations, mostly relying upon the promise of EV-based technologies to revolutionize nanoscience, as well as offer competitive clinical and commercial solutions within broad industrial landscapes

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(e.g., healthcare, personal care, and agrifood) (Zarovni et al., 2021). In line with this, substantial public funding, alongside corporate and venture investments, has supported the prominent academic and industrial engagement in Research and Development (R&D), paving the way to the first Product Development (PD) attempts. However, the shortage of reference materials, quality standards, and standardized methodologies for EV R&D and PD still challenges the ability to unravel the full spectrum of EV constituents and mechanisms of action. Consequently, this has posed a burden not only on comprehending the content and physio-pathological role of EVs, but also on the ability to manage and control their manufacturing reliably and sustainably.

Currently, EV bioprocessing in research and industrial settings is mostly dominated by non-standardized manufacturing systems. This enables researchers to keep up with the rapid innovations in the field. Nevertheless, it may also lead to the inevitable introduction of variability into EV-based products, resulting in inconsistencies in their structural identity (e.g., EV subset collection), quality, and safety, and accounting for reported discrepancies among different studies. By potentially and severely affecting data reliability and reproducibility, this overall lack of standardization and robustness has had a strong detrimental impact on the EV landscape. It also poses challenges to the rationale by which scientists select the most appropriate and tailored applications for any given EV source, as well as to the way they formulate and dose EVs. On top of that, the predominant EV bioprocessing procedures are still mostly research-compliant, characterized by considerable cost intensiveness and low-throughput performance (Agrahari et al., 2019; Clayton et al., 2019; Lener et al., 2015; Liguori & Kisslinger, 2021; Liguori & Kisslinger, 2022; Lötvall et al., 2014; Nieuwland et al., 2020; Paolini et al., 2022; Théry et al., 2018). Furthermore, so far, they have been able to address only small-scale preclinical development and/or integration into clinical trials featuring small patient cohorts (e.g., 10–500 enrollments), even at advanced study phases (i.e., phases 3 and 4) (clinicaltrials.gov, n.d.). Proper support for EV manufacturing and analytical assessment at an industrial scale, which requires extremely controlled standards of precision and has limited space for errors, remains an open issue.

In this challenging scenario, to prompt the translation of EV innovations into effective and marketable solutions, we likely need to make our efforts more streamlined and responsible. The delivery of Responsible Research and Innovation (RR&I) within the EV landscape should involve an increasing interconnection of R&D operations with supporting activities related to quality, data, and risk management, both beforehand and along the bioprocessing path (Liguori & Kisslinger, 2021; Owen et al., 2013). In this context, the strategic adoption of standardized and customizable working methodologies since early developmental phases could be crucial for ensuring reproducibility and reliability in compliance with Good Research Practice (GRP) and Good Manufacturing Practice (GMP) regulations (Hollmann et al., 2022).

Hereby, we describe the application of a multi-criteria decision-making (MCDM) matrix that can serve as an efficient, customizable, user-friendly, and transferable decision-making tool (DMT) model, specifically dedicated to EV features and related bioprocesses. In this manuscript, we showcase the adoption of our EV decision-making grid (EV-DMG) to streamline and improve the quality control (QC) of EVs derived from microalgae (i.e., nanoalgaosomes). We demonstrate its effectiveness in promoting application-tailored EV classification, leading to the minimization of erroneous and cost-intensive misuses. Furthermore, we describe the great potential of implementing our EV-DMG to address the following: (i) maximization of EV bioprocess cost and time efficiency, targeting the achievement of desired quality and quantity performances; (ii) provision of traceable records of the whole EV PD life cycle, which can be later used for the compilation of the regulatory dossier relative to the final EV-based product. Overall, we underline the validity of the EV-DMG as a tool for steering complex decision-making throughout the entire EV value chain, from research to PD, with the ultimate goal of delivering responsible and sustainable EV R&I.

2 | METHODOLOGY

The workflow for the design of the EV-DMG is displayed in Figure 1. Overall, the process comprises four main steps: (i) identification of “alternatives,” which are the EV-based preparations or processes to compare for evaluation and decision-making; (ii) definition of EV-related alternative assessment criteria and relative importance weights; (iii) determination of EV-related acceptance criteria and relative scoring system; iv) implementation of the MCDM method to integrate EV-related alternative assessment into a weighted sum model (WSM) matrix to rank the alternatives. The elements and concepts at the basis of our MCDM approach are elucidated in the following sections.

2.1 | Step 1–Identification of EV-related alternatives

An MCDM approach aims at generating preferences from different options or conditions under comparison (i.e., “alternatives”) by considering and integrating more than one assessment criterion in the selection process (Triantaphyllou et al., 1998). Therefore, the first step defines the decision to be made and requires the listing of the alternatives to choose from. In both EV R&D and PD settings, there are some cautious decisions to make that are of core importance for the conduction of any upstream and downstream EV bioprocess. These can be allocated into the following categories: (i) EV sources; (ii) EV production, handling, characterization, and QC methods; (iii) EV applications. To maximize the performance of any EV-based product and process, optimal decision-making within each of these categories needs to be driven by specific assessment criteria set in advance.

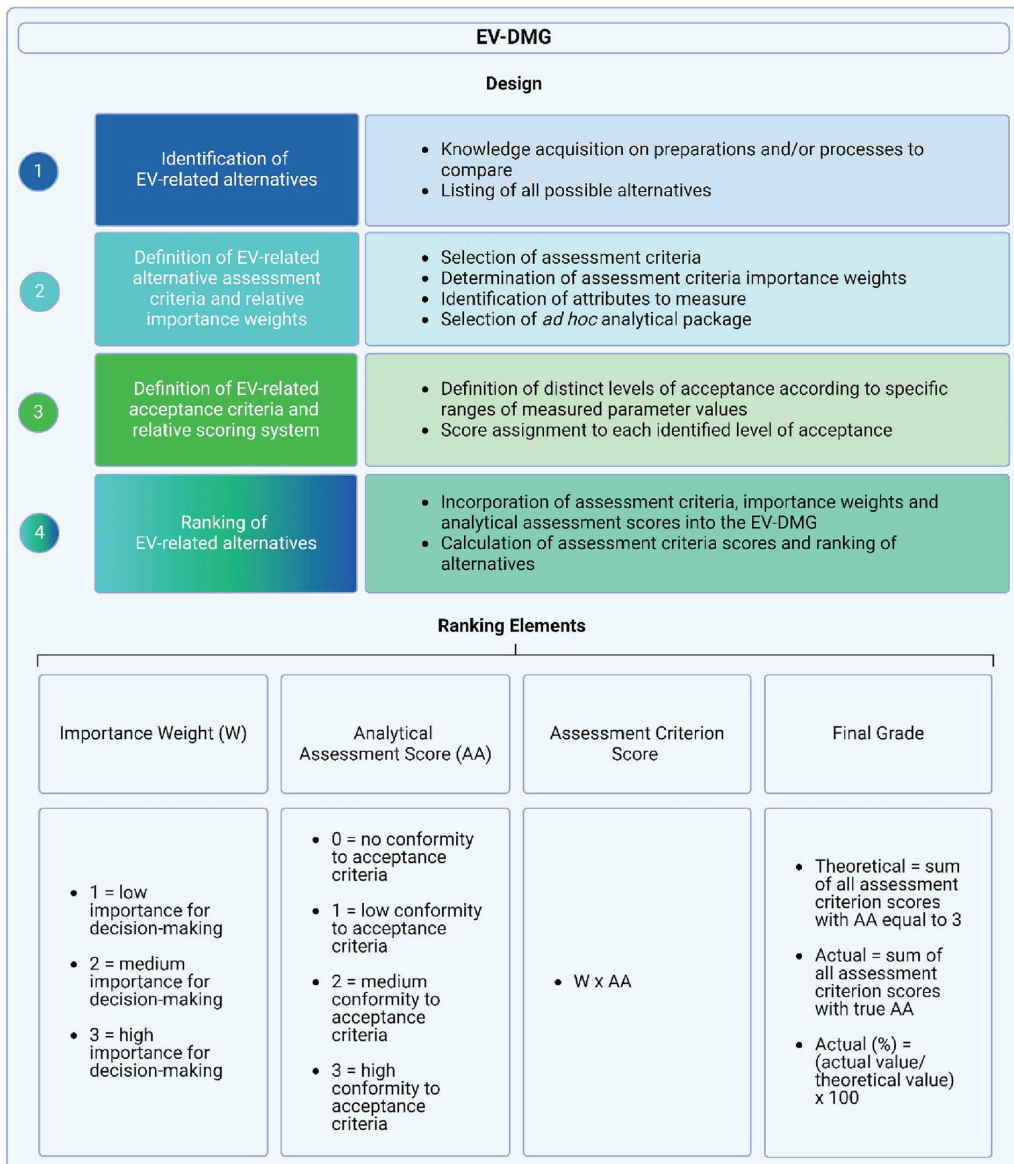


FIGURE 1 Proposed workflow for EV-DMG design and implementation. This figure was created with BioRender.com. EV-DMG, extracellular vesicle decision-making grid.

2.2 | Step 2—Definition of EV-related alternative assessment criteria and relative importance weights

To drive the selection of the best “alternative(s)” under evaluation, a set of qualitative and quantitative EV-related assessment criteria needs to be formulated. Each assessment criterion addresses a specific question and ultimately yields clear and measurable assessment results. Figure 2 outlines six major assessment criteria considered in R&D contexts to qualify EV-based products and processes, most of them adapted from the “checklist” reported in the “Minimal Information for Studies of EVs” (MISEV) 2018 guidelines (Théry et al., 2018). Newly released MISEV2023 guidelines do not re-propose an analogue of this checklist, but

Assessment Criteria	Assessment Sub-criteria	Importance Weights	Analytical Methods
Quantity	Particle number [per volume unit or original (tissue) mass/number of cells]	+++	(F-)NTA, (high resolution) FC, TRPS, EM, AFM, etc.
	Total protein content [per volume unit or original (tissue) mass/ number of cells]	+++	BCA, Bradford assay, SDS-PAGE, etc.
	(Total) lipid amount [per volume unit or original (tissue) mass/number of cells]	++	Sulfophosphovanillin assay, FTIR, TLC, phospholipid assay, cholesterol assay (depending on the source), etc.
	Ratios of two quantification indicators (e.g., total particle number/total protein amount)	+++	/
Identity	Particle size distribution	+++	(F-)NTA, (high resolution) FC, DLS, EM, AFM, SRM, FCS, AF4-MALS, TRPS, etc.
	Structure/morphology	+++	(F-)NTA, (high resolution) FC, DLS, EM, AFM, SRM, FCS, AF4-MALS, TRPS, SAXS, etc.
	EV plasma membrane-derived or endosomal transmembrane or GPI anchored protein	+++	WB, ELISA, FC, etc.
	EV cytosolic protein with membrane binding or association capacity	+++	WB, ELISA, FC, PCR, etc.
	Presence/absence of expected contaminants	+++	WB, ELISA, FC, PCR, etc.
	Topology of the relevant functional components	+	Mild digestions, detergent permeabilization, antibody studies, etc.
	Surface charge	/	ZP measurement, etc.
Purity/Enrichment	Ratios of two quantification indicators (e.g., total particle number/total protein amount)	+++	Assays for determining quantity and identity
	Presence/absence of expected contaminants	+++	
Potency/Functionality	Dose-response assessment	+++	<i>In vitro</i> [cell-based, cell-free (enzymatic assays)] assays, <i>in vivo</i> assays
	Negative control assessment (conditioned medium, biofluid/ tissue from control donors, as applicable)	+++	
	Quantitative comparison of functional activity of total input material vs EV-depleted control vs EVs	+++	
	Quantitative comparison of functional activity of EV subtypes	+	
Integrity/Stability	Testing changes in quantity or quality attributes (identity and potency/functionality)	+++	Assays for determining quantity, identity and potency/functionality
Sustainability	Environmental footprint	+++	Life cycle assessment study
			Market analysis
	Costs of goods		Evaluation of economic feasibility
			Regulatory assessment and guidelines

FIGURE 2 Setting the EV-DMG metrics for EV R&D—alignment of assessment criteria, sub-criteria, importance weights, and analytical methods inspired by and adapted from MISEV2018. AF4-MALS, asymmetric flow field-flow fractionation-multiangle light scattering; AFM, atomic force microscopy; BCA, bicinchoninic acid assay; DLS, dynamic light scattering; EM, electron microscopy; ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle; EV-DMG, extracellular vesicle decision-making grid; FC, flow cytometry; FCS, fluorescence correlation spectroscopy; (F-)NTA, (fluorescence) nanoparticle tracking analysis; FTIR, Fourier transform infrared spectroscopy; PCR, polymerase chain reaction; SRM, selected reaction monitoring; SAXS, small-angle x-ray scattering; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TRPS, tunable resistive pulse sensing; WB, western blot; ZP, Z-potential.

maintain the continuity with recommendations on the key qualitative and quantitative criteria to consider for upgrading EV study design and execution (Welsh et al., 2024). These criteria include quantity, identity, purity/enrichment, potency/functionality, stability/integrity, and sustainability. Each of these may be further subdivided into various sub-criteria addressed by different analytical methods and can be given a specific importance weight depending on its impact on the decision-making process. MISEV2018 proposed the following system for criteria scoring: +++ for mandatory criteria; ++ for mandatory criteria, if applicable to follow; + for encouraged criteria (Figure 2). We assumed that this weighing system would be equivalent to numerically assigning an importance weight ranging from 3 to 1, as later applied in our showcase and reported in Figure 3. It is important to specify that the list of assessment sub-criteria presented in Figure 2 is not exhaustive; rather, it includes some of the assessment sub-criteria that we deemed most relevant and commonly considered by the EV community.

2.3 | Step 3—Definition of EV-related acceptance criteria and relative scoring system

Formatting an EV-DMG based on MCDM principles necessarily requires the establishment of appropriate acceptance criteria and correspondent scoring. Acceptance criteria represent “numerical limits, ranges, or other suitable measures” (Elder, 2017), such as those based on non-numerical, qualitative assessments (e.g., visual analysis), that are relative to the qualitative and quantitative features of the alternative options under assessment. These criteria define the requirements that the products or processes under evaluation must meet to satisfy stakeholders’ expectations and needs for an intended use. In an EV R&D environment, for each EV-related assessment (sub-)criterion, the acceptance value range needs to be empirically determined through specific, pre-validated analytical procedures. The performance of the samples or processes under assessment can be then ranked through a systematic scoring system, reflecting the different degrees to which the performance conforms to the pre-established requirements. The description of the acceptance criteria and respective scoring system selected for showcasing the application of our EV-DMG are reported in Section 3.3.

2.4 | Step 4—Ranking of EV-related alternatives

In our study, the relevant criteria selected for assessing alternatives had been systematically incorporated into a WSM matrix (Triantaphyllou et al., 1998). The general construction of a WSM matrix satisfies the following mathematical equation:

$$A_{WSM}^* = \max_i \sum_{j=1}^N q_{ij} w_j \text{ for } i = 1, 2, 3, \dots, M \quad (1)$$

in which: A_{WSM}^* is the WSM of the final score of the (best) alternative; M is the number of the alternatives to assess; N is the number of decision criteria; i is a single alternative; j is a single criterion; q_{ij} is the actual value relative to the i th alternative in terms of the j th criterion; w_j is the importance weight of the j th criterion (Triantaphyllou et al., 1998). Upon generation of the WSM matrix, for all alternatives under evaluation, an analytical assessment score (corresponding to q_{ij}) is given to each assessment criterion, based on its conformity to acceptance criteria. This is subsequently multiplied by its relative importance weight (w_j) to obtain an overall assessment criterion score. The sum of all assessment criterion scores provides a final, cumulative grade or score (A_{WSM}^*) relative to each alternative evaluated, which leads to the selection of the best alternative for a specific downstream application or to discarding. In the following subsections, we present an illustrative example demonstrating the generation and utilization of the WSM matrix, showcasing the EV-DMG.

3 | ILLUSTRATIVE EXAMPLE OF EV-DMG DESIGN AND APPLICATION FOR EV R&D

In this manuscript, we exemplify the setting, adoption, and utility of our EV-DMG to streamline the QC assessment and grading of microalgae-derived EVs (viz., nanoalgosomes) (Adamo et al., 2021), chosen as model EVs. Its result addresses the consequent destination of our EV preparations to different uses within an exploratory scenario.

3.1 | Identification of nanoalgosome-related alternatives

Nanoalgosomes were extensively characterized and evaluated in several lines of research, exploring their potential as intrinsic effectors or drug delivery vehicles (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021; Picciotto et al., 2022). Our EV-DMG is suitable for a QC evaluation that allows EV batches of different quality to be shunted towards the most appropriate application, or to be discarded if failing to reach the desired standards. In this work, we showcase the application of the EV-

Assessment Criteria	Assessment Sub-criteria	Importance Weights	Analytical Methods
Quantity	Scattering particle number per volume unit	3	NTA
Identity	Scattering particle size distribution	2	NTA
	Morphology		AFM
	Membrane H+/ATPase expression		ELISA
	Surface charge		ZP measurement
Purity	Scattering particle number-to-protein content ratio	3	NTA/BCA
	Fluorescent particle number-to-scattering particle number ratio		(F-)NTA
	Aggregation of gold nanoparticles		CONAN assay
Functionality	Esterase activity	2	Esterase activity assay

FIGURE 3 Setting the EV-DMG metrics for nanoalgosome QC—alignment of assessment criteria, sub-criteria, importance weights, and analytical methods. AFM, atomic force microscopy; BCA, bichinchoninic acid assay; CONAN, colorimetric nanoplasmonic assay; ELISA, enzyme-linked immunosorbent assay; EV-DMG, extracellular vesicle decision-making grid; (F-)NTA, (fluorescence) nanoparticle tracking analysis; QC, quality control; ZP, Z-potential.

DMG for the comparative analysis and classification of three distinct nanoalgosome batches. Our case study was conducted within the frame of the Biogenic Organotropic Wetsuits (BOW) project [funded within the Horizon 2020 Future and Emerging Technologies (FET) Proactive Program in 2021], which aims at exploiting nanoalgosomes as scaffolds for the production of engineered EV membrane-coated magnetic bead devices (CORDIS—EU research results, n.d.). In this context, we have chosen (i) engineering and (ii) *in vivo* functional testing as primary nanoalgosome applications, tailored to meet the immediate needs of the BOW project Partners/users.

3.2 | Definition of nanoalgosome assessment criteria and relative importance weights

Our QC system was based upon a minimal but informative analytical package, comprising a feasible number of EV assessment methods, all having the characteristic of being user-friendly, time-effective, mostly transferable, and requiring low sample consumption. The following EV properties were assessed: (i) EV quantity [determined and expressed in terms of particle concentration by nanoparticle tracking analysis (NTA)]; (ii) identity [assessing scattering particle size distribution by NTA, morphology by atomic force microscopy (AFM), expression of nanoalgosome biomarkers (i.e., the plasma membrane protein H+/ATPase) by enzyme-linked immunosorbent assay (ELISA), and surface charge by particle Z-potential measurement]; (iii) purity [labelling samples with an EV-specific lipidic dye (i.e., di-8-ANEPPS) and measuring the percentage of fluorescent particles over the total number of scattering particles by Fluorescence NTA (F-NTA); determining the ratio between total particle number and total protein content (Adamo et al., 2021; Paterna et al., 2022; Picciotto et al., 2021; Picciotto et al., 2022; Sverdllov, 2012); estimating purity from soluble co-isolated proteins by Colorimetric NANoplasmonic (CONAN) assay (Maiolo et al., 2015; Zendriani et al., 2020)]; (iv) functionality [determining EV esterase activity as a proxy of EV functionality and membrane integrity by a proprietary EV functional enzymatic activity assay (Adamo et al., 2023)]. Considering the importance of the weighing system described in Section 2.2, we provided an importance weight equivalent of 3 out of 3 to EV quantity and purity, whereas an importance weight of 2 out of 3 was assigned to EV identity and functionality (Figure 3).

3.3 | Definition of nanoalgosome acceptance criteria and determination of relative scoring

To assign each nanoalgosome batch a specific quality score, a set of acceptance criteria was defined based on the published MISEV-inspired checklist of minimal information for studies of nanoalgosomes, which identified characteristics of

Assessment Criteria	Assessment Sub-criteria	Acceptance Criteria			
		Score 0	Score 1	Score 2	Score 3
Quantity	Scattering particle number/mL	$x < 5E+10$	$5E+10 < x < 1E+11$	$1E+11 < x < 5E+11$	$x > 5E+11$
	Scattering particle size distribution (mode; nm)	$x < 60$	$60 < x < 80$	$80 < x < 90$	$90 < x < 120$
Identity	Morphology (number of rounded object(s)/10 total objects)	0	1-2	3-5	> 6
	Membrane H ⁺ /ATPase expression (signal-to-noise ratio/1E+10 particles)	$x < 2$	$2 < x < 3$	$3 < x < 4$	$x \geq 5$
	Surface charge (ZP; mV)	$x < -45$	/	/	$-45 < x < -10$
		$x > -10$			
	Scattering particle number-to-protein content (μ g) ratio	$x < 1E+8$	$x < 2E+8$	$x < 2E+9$	$2E+9 < x < 1E+10$
Purity	Fluorescent particle number/scattering particle number (%)	0	$0.5 < x < 3$	$3 < x < 10$	$x > 10$
	Aggregation of gold nanoparticles	/	NO	Proportional to sample dilution with aggregation index above the 20% threshold	Proportional to sample dilution with aggregation index below the 20% threshold
	Esterase activity (nmol/min/5E+10 particles)	$x < 0.5$	$0.5 < x < 1$	$1 < x < 2$	$x > 2$

FIGURE 4 Setting the EV-DMG metrics for nanoalgosome QC—alignment of assessment criteria, sub-criteria, and acceptance criteria with relative scoring. EV-DMG, extracellular vesicle decision-making grid; QC, quality control; x, the measure of the corresponding parameter; ZP, Z-potential.

nanoalgosome preparations (Adamo et al., 2021). Next, we empirically classified nanoalgosome preparations as either high-quality (positive) or low-quality (negative), according to their performance and suitability for our desired applications. The set of acceptance criteria relative to each nanoalgosome assessment sub-criterion was complemented by a scoring system. This system assigned a specific analytical assessment score to the obtained measurement results, describing to what extent the sample met the defined requirement for a specific assessment criterion and sub-criterion. Specifically for the work presented here, we defined a scoring system featuring an evaluation scale as follows: 0 = null; 1 = low; 2 = medium; 3 = high (Figure 4).

Assessment Criteria	Assessment Sub-criteria	Importance Weights (W)	Nanoalgososome Batch 1		Nanoalgososome Batch 2		Nanoalgososome Batch 3	
			AA	AA x W	AA	AA x W	AA	AA x W
Quantity	Scattering particle number per volume unit	3	3	9	3	9	3	9
Identity	Scattering particle size distribution	2	3	6	3	6	3	6
	Morphology		3	6	2	4	3	6
	Membrane protein H ⁺ /ATPase expression		2	4	0	0	1	2
	Surface charge		3	6	3	6	3	6
Purity	Scattering Particle number-to-protein content ratio	3	3	9	1	3	3	9
	Fluorescent particle number/scattering particle number (%)		2	6	1	3	1	3
	Aggregation of gold nanoparticles		3	9	1	3	3	9
Functionality	Esterase activity	2	2	4	1	2	1	2
Actual Final Grade				59/66		36/66		52/66
Actual Final Grade/Theoretical Final Grade (%)				89.4		54.5		78.8

FIGURE 5 EV-DMG for nanoalgososome QC. EV-DMG, extracellular vesicle decision-making grid; QC, quality control.

3.4 | Ranking of nanoalgososome-related alternatives

Figure 5 represents the EV-DMG compiled for the three analyzed nanoalgososome batches. The results obtained from the NTA analysis on nanoalgososome Batch 1, Batch 2, and Batch 3 showed differences in terms of particle concentration (Batch 1 = $9.1\text{E}+11 \pm 4.1\text{E}+10$ particles/mL; Batch 2 = $8.6\text{E}+12 \pm 4.6\text{E}+11$ particles/mL; Batch 3 = $7.2\text{E}+11 \pm 2.2\text{E}+10$ particles/mL; Figure S1-A), but a quite overlapping size distribution, peaking at approximately 100 nm (Figure S1-B1). No significant differences between the three batches were revealed by Z-potential measurements, indicating an analogous particle membrane surface charge (Figure S1-B4). However, the expression of nanoalgososome plasma membrane protein H⁺/ATPase, measured by ELISA, showed a greater signal-to-noise ratio (SNR) per particle in Batch 1 and Batch 3, with Batch 1 displaying the highest readout (Figure S1-B2). Batch 1 and Batch 2 exhibited a particle-to-protein ratio (Figure S1-C1) that deviated the least and the most, respectively, from the theoretical optimal ratio suggested by Sverdllov (2012) [Batch 1 = $\sim 3.9\text{E}+9$ particles/protein content (μg); Batch 2 = $\sim 7.3\text{E}+10$ particles/protein content (μg)]. This ratio states that 1 μg of EV preparation proteins corresponds to $2\text{E}+9$ EVs and served as the basis for our nanoalgososome purity grading system. In line with these observations, F-NTA of fluorescently labelled samples demonstrated the presence of an inferior content of non-EV particles in Batch 1, compared with the other batches. This was highlighted by the different percent ratio of fluorescent particles over total particles detected in scattering mode, which corresponded to 3.3% for Batch 1, 1% for Batch 2, and 1.3% for Batch 3 (Figure S1-C2). This was further validated by the CONAN assay (Figure S1-C3), showing that Batch 1 and Batch 3 could be considered pure from protein co-isolates within the limit of detection (LOD) of the assay (20–50 ng/ μL). Indeed, the Aggregation Index (AI) % of Batch 1, which is initially

Nanoalgosome QC Classification System						
Level	Description	Actual/Theoretical Grade (%)	Use(s)			
			No	Not Critical	Engineering	Functional Study
A	High Quality	$70 \leq x \leq 100$		x	x	x
B	Medium Quality	$30 < x < 70$		x	x	
C	Low Quality	< 30	x	x		

FIGURE 6 EV-DMG-based QC classification of nanoalgosomes. EV-DMG, extracellular vesicle decision-making grid; QC, quality control; x, the relationship indicator of two corresponding parameters.

above the threshold, decreased below assay LOD upon dilution. This behaviour is typical in pure samples rich in EVs (dilution is needed to match the assay working range). In Batch 3, the EV concentration was already in the CONAN assay optimal range, with the AI% stabilizing below the LOD even without diluting the sample. On the contrary, Batch 2 displayed a constant AI%, regardless of the dilution tested. Therefore, such samples either (i) did not contain enough EVs to trigger complete pristine gold nanoparticle aggregation or (ii) carried a significant amount of protein co-isolates. To verify option (i), increased amounts of sample volume (up to 5X the amount normally used to run the assay) were tested, leading to the same result. Therefore, Batch 2 could be considered contaminated by co-isolated proteins. In support of these results, AFM highlighted elongated objects in Batch 2, potentially originating from microalgal cells or the detachment of tangential flow filtration (TFF) cartridge fibres (Figure S1-B3). Consistently with these highlights, Batch 1 showed the highest esterase activity per particle (1.36 ± 0.01 nmol/min/5E+10 particles), indicating its superior functionality and membrane integrity, compared with the other two batches. Nevertheless, the particle enzymatic activity assessed in Batch 2 and Batch 3 revealed a comparable readout, with a slightly higher value detected in Batch 2 (Batch 2 = 0.80 ± 0.07 nmol/min/5E+10 particles; Batch 3 = 0.67 ± 0.00 nmol/min/5E+10 particles; Figure S1-D).

As shown in Figure S2, the maximum theoretical final grade, corresponding to 66, was calculated by summing up all assessment criterion scores obtained by multiplying the importance weight of each assessment criterion by the maximum assignable analytical assessment score (i.e., 3). The observed experimental discrepancies between the three tested batches translated into different analytical scores on our EV-DMG (Figure 5). The sum of all assessment criterion scores resulted in an actual final grade equivalent to 59/66 for Batch 1 (89.4% of the theoretical final grade), 36/66 for Batch 2 (54.5% of the theoretical final grade), and 52/66 for Batch 3 (78.8% of the theoretical final grade). As illustrated in Figure 6, depending on the final grade obtained, each batch could be classified into one of three QC classification levels: level A, for batches of good quality, appropriate for all uses, once confirmed to be sterile (actual final grade equal to 70%–100% of the theoretical final grade); level B, for batches of medium quality, for engineering and other uses, excluding functional testing (actual final grade equivalent to a value $\geq 30\%$ up to $< 70\%$ of the theoretical final grade); level C, for batches of poor quality, to be discarded or used only for non-critical analyses (actual final grade inferior to 30% of the theoretical final grade). Accordingly, the use of our EV-DMG to streamline our nanoalgosome QC assessment highlighted, straightforwardly and objectively, the suitability of Batch 1 and Batch 3 to be exploited for any downstream uses, including *in vivo* applications, and the potential for Batch 2 to be used only for engineering and not functional analyses. The accuracy and actionability of this ranking system have been proven through feedback from the BOW project Partners/users, who had an opportunity and a mandate to test the three batches, and report the results that were in line with the EV-DMG-predicted nanoalgosome performance.

4 | DISCUSSION

Decision-makers have historically adopted computational or mathematical DMTs to manage and mitigate the technical, logistic, and economic risks associated with the complex set of factors affecting the dynamics of systems in numerous industrial manufacturing contexts (i.e., from aerial and automotive vehicles to biotherapeutics) (Chhatre et al., 2007; Rao, 2007; Rekhi et al., 2015; Sutton et al., 2020; Tan et al., 2014). DMTs have also been applied in public research environments to help identify the most promising project aims, directions, and activities (Bongiovanni et al., 2015; Digilio et al., 2016). In early 2019, the EV landscape first embraced the notion of implementing DMTs for assisting research- and clinical-grade EV-based PD. Specifically, Ng and co-authors developed a novel computational DMT to identify combinations of technologies for upstream cell expansion and downstream EV harvesting to promote upscaling, while addressing the minimization of the cost of goods (COGs) (Ng et al., 2019). More recently, Picciotto et al. (2021) have adopted a WSM-based DMT for addressing the selection of the microalgal sources best suited to produce microalgae-derived EVs (*viz.*, nanoalgosomes) (Picciotto et al., 2021). Nevertheless, a comprehensive and straightforward decision-support framework to drive and streamline complex decision-making at each stage of EV R&D and PD has not been proposed yet.

Assessment Criteria	Assessment Sub-criteria	Degree of Recommendation		
		R&D (MISEV2018)	PD of MSC EV-based medicinal products (Witwer et al., 2019)	PD of EV-based medicinal products (Silva et al., 2021)
Quantity	Particle number [per volume unit or original (tissue) mass/number of cells]	+++	+++	+++
	Total protein content [per volume unit or original (tissue) mass/number of cells]	+++	+++	+++
	(Total) lipid amount [per volume unit or original (tissue) mass/number of cells]	+	+	+
	Ratios of two quantification indicators (e.g., total particle number/total protein amount)	+++	+++	+++
Identity	Particle size distribution	+++	+++	+++
	Structure/morphology	+++	+++	+++
	Plasma membrane-derived or endosomal transmembrane or GPI anchored protein	+++	+++	+++
	Cytosolic protein with membrane binding or association capacity	+++	/	/
	Presence/absence of expected contaminants	+++	+++	+++
	Topology of the relevant functional components	+	/	+++
Purity/Enrichment	Surface charge	/	/	+ / +++
	Ratios of two quantification indicators (e.g., total particle number/total protein amount)	+++	+++	+++
Potency/Functionality	Presence/absence of expected contaminants	+++	+++	+++
	Biological activity testing	+ / +++ / +++	+++	+++
Integrity/Stability	Testing changes in quantity or quality attributes (identity and potency/functionality)	+++	+++	+++
	Biophysical destabilization tests	/	/	++
Sterility	Microbiological security check and clearance	/	+++	+++
Sustainability	Environmental footprint	/	+++	+++
	Costs of goods			

FIGURE 7 Setting the EV-DMG metrics for EV PD—alignment of assessment criteria, sub-criteria, and importance weights deduced from the MISEV2018 guidelines (Théry et al., 2018), Silva et al. (2021), and Witwer et al. (2019). EV-DMG, extracellular vesicle decision-making grid; PD, product development.

To address this need, in the current work, we targeted the development and implementation of a DMT suitable for managing the multidimensionality of the decision-making issues challenging EV R&D and PD. Specifically, we focused our attention on a DMT based on the MCDM approach, which is known to provide a valid, reliable, and straightforward RR&I-tailored approach to make unbiased selections and classifications within complex sets of alternative options. This strategy is also noted for creating opportunities for cost and time savings, besides contributing to increased data reliability and reproducibility. Overall, it allows decision-making to take the lead over decision-control activities, enhancing project performance and simplifying project administrative work (Rao, 2007; Silva et al., 2021; Stilgoe et al., 2020; Taherdoost & Madanchian, 2023; Zarghami et al., 2011).

The EV-DMG outlined in this work was conceived to offer a user-friendly and transferable MCDM matrix model for addressing any uncertainty related to EV bioprocessing decisions. The design and working principles of the EV-DMG (described in Section 2) are applicable to any EV type, use, and bioprocessing context. However, the selection of the specific assessment criteria and importance weights to base the construction of the EV-DMG upon must be adjusted to the specific context of use, as

well as be built on empirical observations related to the key features of the products or processes that are targeted for evaluation (showcased in Section 3).

To demonstrate the implementation of the EV-DMG in a practical working scenario, we decided to present a case study illustrating its use as a straightforward and effective QC-support tool. This work was conducted within the frame of a multicentric and multidisciplinary research project at an early developmental phase, in which we were projected to tackle several potential usage venues for the EV types that were under investigation, ranging from their employment as substrates for engineering to their application as nano-vehicles or intrinsic effectors. Among the EV types studied in this early-stage research context, we selected nanoalgosomes as a less explored model EVs, suitable to validate our approach. Our findings showed that the adoption of the EV-DMG system to streamline nanoalgosome QC allowed us to go beyond the simple nanoalgosome grading for a single intended use. Rather, this method demonstrated the potential to maximize the full potential of the QC process by sorting the tested nanoalgosome preparations towards different potential applications that best suited their quality and quantity. Furthermore, this study exhibits the potential of the EV-DMG-based approach to tailor EV production design, scale, and performance to evolving goals and priorities concerning specific products and/or uses to target.

The design of the EV-DMG exemplified in this study was originally conceived taking into consideration the MISEV2018 guidelines. In a summary “checklist”, reported in the final section of the document, MISEV2018 piloted the first attempt to assign specific importance weights to a comprehensive set of methodological criteria addressing the key EV features to study for conducting good EV science (Hendrix et al., 2023; Théry et al., 2018; Van Deun et al., 2017). However, it did not diversify the importance of each methodological criterion according to chosen study scopes, adopted EV sources, and envisaged EV-based application(s). Rather, it proposed a “one-scoring-fits-all-or-most” model, where the importance weights associated with the proposed methodological framework were averaged based on prevailing opinions within the EV community (Théry et al., 2018). Thus far, these metrics have served as a cornerstone for improving the quality of EV R&D results and provided an excellent benchmark to early industrial EV innovators. An updated version of the MISEV guidelines has been recently published, mostly focusing on strengthening recommendations for upgrading EV study design and execution (Welsh et al., 2024). MISEV2023 did not offer a checklist as the one reported by MISEV2018. Nevertheless, its basic principles appear in agreement with the fundamental notions contemplated in MISEV2018. Despite being inspired by the MISEV guidelines, we realized that the methodological criteria and weighing systems are likely to be determined in a case-by-case manner. Certainly, it would be ideal and wise to include as many EV assessment criteria as possible in a research-grade EV-DMG, especially at an early developmental stage (Silva et al., 2021). However, it is intuitive that their selection, weighing, and technical feasibility would still depend on the specific research objectives, study context, and logistics. For instance, it is more likely that preserving EV integrity and potency/functionality is more critical for researchers intending to use their model EVs as intrinsic effectors, than for those who are going to engineer them with heterologous moieties or cargos. Conversely, quantity is likely to be critical for dosing EVs in general and might be universally weighted as a highly impactful parameter. Furthermore, high purity and the absence of any other non-vesicular contaminants may have a very different degree of importance in distinct applications. Finally, the selection of technologies is often guided by pragmatic criteria of accessibility, cost, or time restraints, which can considerably vary across the research community. This supports the conduction of exploratory studies within multidisciplinary consortia with pooled expertise and infrastructure.

The integration of the EV-DMG into routine EV bioprocessing practices may arise as a strategic move to dynamically frame PD and manufacturing, with early reference to the later submission of a product Common Technical Document (CTD) or dossier. The EV-DMG could be aligned with and merged into the Target Product Profile (TPP) of the prospective product to be developed. The TPP is an evolving document, the formulation of which is recommended from the product design phase to guide benchmarking, provide a comprehensive recapitulation of the targeted product's attributes, and improve regulatory dialogue for more efficient regulatory review and approval times (Breder et al., 2017). National Regulatory Authorities (NRAs), including the European Medicines Agency (EMA) and the United States Food and Drug Administration (US FDA), have been increasingly exploiting Multi-Criteria Decision Analysis (MCDA) to regulate approval decisions to optimize trade-offs between risks and benefits (Chisholm et al., 2022). This suggests that the adoption of MCDM-based DMTs to target clinical validation and acceptance by the NRAs could improve communication and understanding between innovators and regulatory bodies about product value propositions. This would ultimately enhance the likelihood of shortening the PD life cycle and speeding up market approval. Moreover, the EV-DMG template that we propose may be easily handled by common software, and its associated numerical values could be turned into algorithms, providing an interface for unbiased and clear gatekeeping decisions.

5 | LIMITATIONS OF THE STUDY

In this study, we propose the EV-DMG as a flexible and evolving tool, which not only can, but must be adjusted and empirically validated in the context of the specific application scope that is envisaged. Therefore, we prompt its integration into additional case studies, with a view to illustrating its applicability to other, possibly any EV type, use, and bioprocessing setting, both in R&D and PD environments. Verifying reproducibility was not included as part of the current work, and translating the EV-DMG into a PD context will likely require the collection of empirical data from a larger number of replicates from independent production cycles. The selection of our analytical package for QC testing was minimal and specifically tailored to nanoalgosome

Considerations for setting up the EV-DMG metrics for EV product development

MISEV2018 principles have been a cornerstone for early EV-DMG design. However, recent position papers focusing on the development of next-generation EV-based therapeutics have identified a need to comply with regulatory and economic requirements to be met in clinical and industrial settings (Silva et al., 2021; Witwer et al., 2019). These prompt new considerations regarding the blueprint for a clinical- or industrial-grade EV-DMG (Figure 7), many of which have been included in our nanoalgosome specifications for increasing the maturity of nanoalgosome-based PD.

Purity

The requirement of high purity at the expense of potency should be revisited when targeting EV-based therapeutics, as some “co-isolates”, possibly associated with the EV “protein corona”, are known to potentially correlate with desired sample biological activity. Furthermore, it is widely recognized that a completely pure EV preparation is unlikely to be produced (Palviainen et al., 2020; Tóth et al., 2021). Therefore, in the construction of an EV-DMG, this may translate into the reduction of the importance weight relative to purity (e.g., importance weight of 2/3 instead of 3/3) or into the delineation of alternative acceptance criteria and relative scoring systems.

Functionality

If the evaluation of EV functionality is not imperative in a research-grade scenario, depending on the investigational purposes, fulfilling specific potency requirements represents a very critical aspect when developing products for in-human use (Silva et al., 2021; Witwer et al., 2019). This could require the adjustment of the importance weight attributed to the assessment of EV functionality in research settings, raising it to 3/3 in an EV-DMG for use in clinical environments (e.g., therapeutics, vaccines, and drug delivery).

Sterility and sustainability

The EV community is currently encouraging to encounter sterility and economic sustainability from the premature research phases to minimize safety concerns and avoid developmental setbacks at more advanced therapeutic development and manufacturing stages (Committee for Medicinal Products for Human Use, 2012; European Medicines Agency, n.d.; Guideline IH, 2011). In this scope, early adding microbiological security, cost, and time management to the panel EV assessment criteria configuring the EV-DMG (potentially with a high importance weight score) could help allocate resources into fair and best-suited categories of use, enabling us to make financial trade-offs and prevent cost-intensive misuses.

assessment, as well as to the needs and requirements of our project Partners. Although in basic and discovery research it is useful to include diverse alternative and orthogonal methodologies—and the design of the EV-DMG lends itself to the addition of any new emerging ones—the adoption of a comprehensive set of analytical tools to perform nanoalgosome QC did not fall within the scope of this study. To select our panel of EV assessment methodologies, we aimed at balancing the requirement for proper evaluation of nanoalgosome characteristics with method accessibility and cost-effectiveness. Specifically, we opted for a concise but informative analytical panel that meets the following criteria: (i) robustness; (ii) sensitivity; (iii) affordable sample consumption, in order not to allocate more than 30% of the total produced EV batch to QC; (iv) ease of use; (v) medium-to-low turnaround time; (vi) cross-site transferability. This rationale was in line with recommendations diffused and accepted across the EV community, suggesting to upscale the analytics along with EV manufacturing development (Paolini et al., 2022).

6 | CONCLUSIONS

The EV-DMG proposed in this work offers a user-friendly MCDM matrix model for addressing a wide range of uncertainties inherent in EV bioprocessing decisions. These include, but are not limited to, selecting the best “fit-to-the-purpose” EV sources and starting materials, choosing the most suitable methods for optimal EV sample extraction, purification, handling, and QC, as well as determining the best-fitting EV applications based on sample properties. Our approach emphasizes the significance of integrating RR&I principles into routine EV R&I practices. Specifically, we propose early conception, development, and adoption of multi-dimensional methodological strategies, such as our EV-DMG, for responsible EV R&I decision-making. This is intended to promote robust, sustainable, ethically acceptable, and socially desirable outcomes of EV R&I. On the one hand, the EV-DMG is presented as a tool that facilitates accountable and impartial qualification of EV-based products and bioprocessing decisions. On the other hand, we highlight its potential for tailoring resources and products towards *ad hoc* applications based on specific requirements, enabling early adjustments in designs and scales to maximize performance and cost-effectiveness. This is especially relevant for enhancing the prospects of successfully translating R&D efforts into regulatory-compliant marketable

solutions. Indeed, by providing traceable records throughout the EV PD life cycle, the EV-DMG will support the compilation of comprehensive regulatory dossiers for EV-based products.

AUTHOR CONTRIBUTIONS

Francesca Loria: Conceptualization (lead); data curation (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing—original draft (lead). **Sabrina Picciotto:** Conceptualization (supporting); data curation (equal); investigation (equal); methodology (equal); validation (equal); visualization (equal); writing—original draft (supporting); writing—review and editing (supporting). **Giorgia Adamo:** Conceptualization (supporting); data curation (equal); investigation (equal); methodology (equal); validation (equal); writing—original draft (supporting); writing—review and editing (supporting). **Andrea Zendrini:** Investigation (equal); validation (equal); writing—review and editing (supporting). **Samuele Raccosta:** Investigation (equal); validation (equal). **Mauro Manno:** Investigation (equal); resources (equal). **Paolo Bergese:** Funding acquisition (lead); resources (equal); writing—review and editing (supporting). **Giovanna L. Liguori:** Conceptualization (supporting); writing—review and editing (supporting). **Antonella Bongiovanni:** Conceptualization (lead); methodology (lead); resources (equal); supervision (equal); writing—review and editing (lead). **Nataša Zarovni:** Conceptualization (lead); methodology (lead); resources (equal); supervision (equal); writing - original draft (supporting); writing—review & editing (lead). Nataša Zarovni and Antonella Bongiovanni equally contributed to this work.

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CONFLICT OF INTEREST STATEMENT

The authors declare the following financial competing interests: Antonella Bongiovanni and Mauro Manno have filed a patent (PCT/EP2020/086622) related to microalgae-derived extracellular vesicles. Antonella Bongiovanni, Sabrina Picciotto, and Giorgia Adamo have filed an Italian patent (Patent: No. 102023000004503) related to the Esterase Activity Assay. Antonella Bongiovanni and Mauro Manno are co-founders, and Antonella Bongiovanni is CEO, of EVEBiofactory s.r.l. Nataša Zarovni is an independent researcher that currently acts as CTO of EVEBiofactory s.r.l. The remaining authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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1 1. Supplementary Information

2 1.1. Supplementary Figures

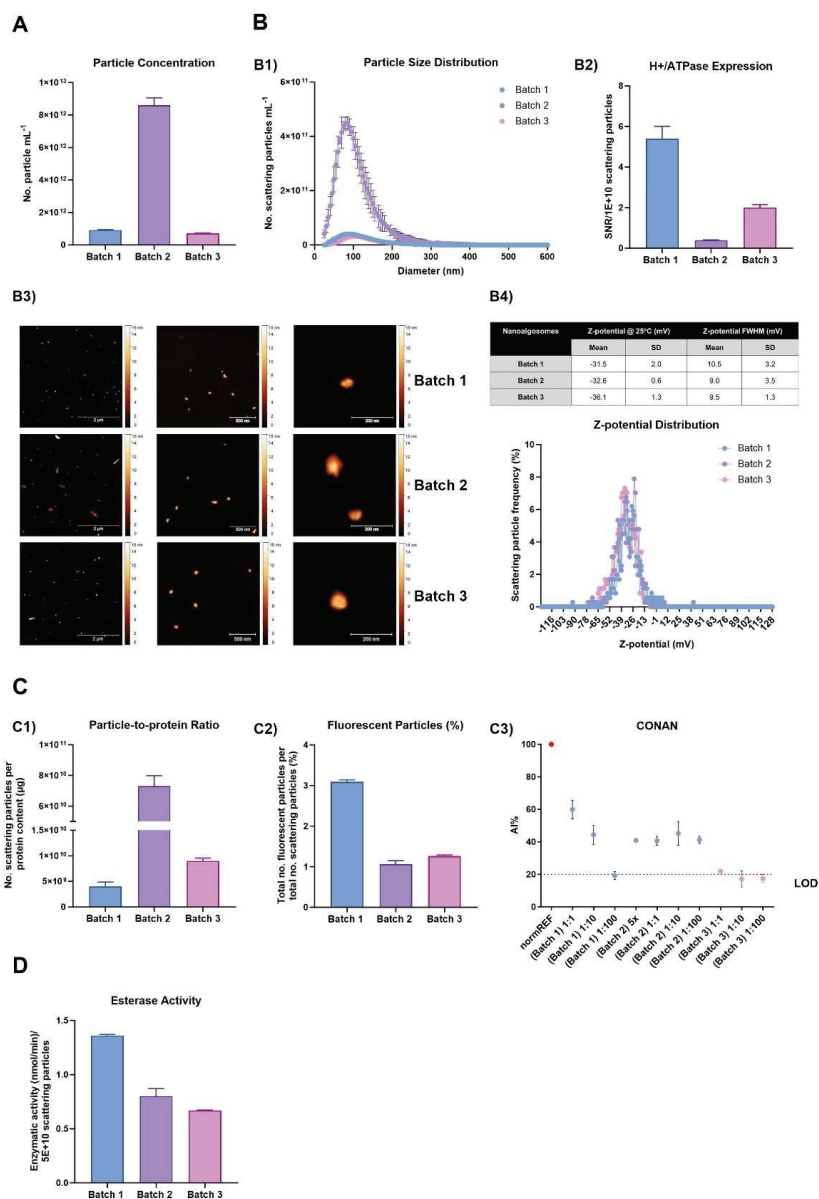


Figure S1. Nanoalgosome QC. (A) Quantity: particle concentration of nanoalgosome batches 1, 2, and 3 (particle number per mL) estimated by Nanoparticle Tracking Analysis (NTA) in scattering mode. (B) Identity: B1) NTA-measured size distributions of scattering particles detected in nanoalgosome batches 1, 2, and 3; B2) H⁺/ATPase expression revealed by 1E+10 scattering particles in nanoalgosome batches 1, 2, and 3; data are expressed as average signal-to-noise ratio (SNR) ± standard deviation of two technical replicates; B3) morphology analysis performed on nanoalgosome batches 1, 2, and 3 by in-air tapping mode Atomic Force Microscopy (AFM); the colored scale on the right of each AFM image indicates the height of detected particles; B4) Z-potential of nanoalgosome batches 1, 2, and 3 – distribution profiles plotting Z-potential values (mV) against scattering particle frequency (%) and table displaying mean ± standard deviation of Z-potential and Full Width at Half Maximum (FWHM) values (mV). (C) Purity: C1) ratio of NTA-measured scattering particle number to protein content (µg) of nanoalgosome batches 1, 2, and 3; C2) ratio of total Fluorescent NTA (F-NTA)-measured number Di-8-ANEPPS-labelled fluorescent particles to total NTA-measured scattering particle number (%); C3) Colorimetric Nanoplasmonic (CONAN) assay of nanoalgosome batches 1, 2, and 3 – data are expressed as mean ± standard deviation of three technical replicates. (D) Functionality and membrane integrity: esterase activity of nanoalgosome batches 1, 2, and 3 – data are expressed as average amount of enzymatic reaction product (nmol) generated by 5E+10 scattering nanoparticles per time unit (min) ± standard deviation of two technical replicates. Legend: normREF = pristine gold nanoparticles used for normalization; LOD = limit of detection; AI = Aggregation Index.

Assessment Criteria	Assessment Sub-criteria	Importance Weights (W)	Alternative X (Nanoalgosome Batch X)	
			Analytical Assessment Score (AA)	Assessment Criterion Score (AA x W)
Quantity	Scattering particle number per volume unit	3	3	9
Identity	Scattering particle size distribution	2	3	6
	Morphology		3	6
	Membrane protein H ⁺ /ATPase expression		3	6
	Surface charge		3	6
Purity	Scattering particle number-to-protein content ratio	3	3	9
	Fluorescent particle number/scattering particle number (%)		3	9
	Aggregation of gold nanoparticles		3	9
Functionality	Esterase activity	2	3	6
Actual Final Grade				66
Actual Final Grade/ Theoretical Final Grade (%)				100

Figure S2. Calculation of EV-DMG's theoretical final grade. Legend: X = unknown nanoalgosome batch.

1.2. Supplementary Materials and Methods

1.2.1. (Fluorescent) Nanoparticle Tracking Analysis

To determine particle concentrations and size distributions, scattering and Di-8-ANEPPS (Sigma)-labelled nanoalgosomes were analyzed by Nanoparticle Tracking Analysis (NTA) and Fluorescence NTA (F-NTA), as previously described [1], using a NanoSight NS300 (Malvern Panalytical, UK). In terms of particle size diameter, the limit of detection of the instrument relatively to nanoalgosome preparations ranges between 20 and 1000 nm.

1.2.2. Analysis of H⁺/ATPase expression by Enzyme-Linked Immunosorbent Assay (ELISA)

H⁺/ATPase expression was assessed in nanoalgosome batches 1, 2, and 3 by direct Enzyme-Linked Immunosorbent Assay (ELISA). Briefly, 1E+10 scattering particles of each sample was loaded in duplicate onto an uncoated microplate with high-binding capacity (HansaBioMed Life Sciences) for overnight nanoalgosome capture at 37°C. After three washing steps with 300 µL/well of washing buffer (HansaBioMed Life Sciences), microplate wells were blocked with 300 µL/well of sample buffer (HansaBioMed Life Sciences) for 2 hours at room temperature (RT). Following a second washing step performed as described above, microplate wells were incubated with primary rabbit antibodies anti-plasma membrane H⁺/ATPase (Agrisera; #AS07 260; 1/1000 dilution in sample buffer) for 2 hours at 37°C. A subsequent third washing step, performed as above, was followed by a microplate well incubation with Horse Radish Peroxidase (HRP)-conjugate secondary antibodies (HansaBioMed Life Sciences; 1/5000 dilution in sample buffer) for 1 hour at 37°C. The final procedural steps were performed according to the ExoTEST™'s protocol provided by the manufacturer (HansaBioMed Life Sciences). Optical Densities were measured at 450 nm, using Tecan GENios Pro microplate reader. Data are expressed as average signal-to-noise ratio (SNR) ± standard deviation of two technical replicates.

1.2.3. Atomic Force Microscopy (AFM)

Nanoalgosome morphology analysis was performed by in-air tapping mode Atomic Force Microscopy (AFM), as previously described [1].

1.2.4. Z-potential measurement

Nanoalgosome Z-potential measurement was assessed using the ZetaView PMX-120 instrument by Particle Metrix (Germany), which is equipped with a 488 nm laser (40 mW power) and a CMOS camera sensor. Instrument initialization and camera alignment were executed according to the

manufacturer's guidelines, utilizing 100 nm polystyrene size standard beads. Measurements were performed in pulsed mode, diluting samples with 0.1X concentrated PBS solution in order to have having a conductivity equal to or below 2000 $\mu\text{S}/\text{cm}$. Three technical measurements were performed for each sample. Z-potential measurements were verified by measuring a suitable Z-potential reference suspension of $-50 \text{ mV} \pm 5 \text{ mV}$ (Applied Microspheres; #70325C; 1/1000 dilution in NaOH solution pH 10), following the manufacturer's instructions.

1.2.5. Bicinchoninic Acid (BCA) assay

Nanoalgosome protein concentrations were measured by Bicinchoninic Acid (BCA) assay, as previously described [1].

1.2.6. Colorimetric NANoplasmonic (CONAN) assay

Colorimetric NANoplasmonic (CONAN) assay was performed to evaluate nanoalgosome purity from protein contaminants, as previously described [2].

1.2.7. Esterase activity assay

Nanoalgosome esterase activity relative to $5\text{E}+10$ scattering particles was assessed by a recently patented Esterase Activity Assay, as prior reported [3].

1.3. References

1. Adamo G, Fierli D, Romancino DP, Picciotto S, Barone ME, Aranyos A, Božič D, Morsbach S, Raccosta S, Stanly C, Paganini C. Nanoalgosomes: Introducing extracellular vesicles produced by microalgae. *Journal of extracellular vesicles*. 2021 Apr;10(6):e12081.
2. Zandrini A, Paolini L, Busatto S, Radeghier A, Romano M, Wauben MH, Van Herwijnen MJ, Nejsun P, Borup A, Ridolfi A, Montis C. Augmented COlorimetric NANoplasmonic (CONAN) method for grading purity and determine concentration of EV microliter volume solutions. *Frontiers in bioengineering and biotechnology*. 2020 Feb 12;7:452.
3. Adamo G, Picciotto S, Gargano P, Paterna A, Rao E, Raccosta S, Salamone M, Romancino DP, Manno M, Bongiovanni A. Functional enzymatic assays to predict the potency of extracellular vesicles. *bioRxiv*. 2023 Oct 25:2023-10.

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2018	Early-Stage Researcher - PhD Candidate in the Marie Skłodowska-Curie Innovative Training Network (ITN) "Training in Extracellular Vesicles: for benefit in Health and Disease" (TRAIN-EV) under the European Union's Framework Programme for Research and Innovation Horizon 2020 (Grant Agreement ID: 722148)

Publications

Luddi, A., Zarovni, N., Maltinti, E., Governini, L., Leo, V. D., Cappelli, V., Quintero, L., Paccagnini, E., **Loria, F.**, & Piomboni, P. (2019). Clues to Non-Invasive Implantation Window Monitoring: Isolation and Characterisation of Endometrial Exosomes. *Cells*, 8(8), 811. <https://doi.org/10.3390/cells8080811>.

Ridolfi, A., Brucale, M., Montis, C., Caselli, L., Paolini, L., Borup, A., Boysen, A. T., **Loria, F.**, van Herwijnen, M. J. C., Kleinjan, M., Nejsun, P., Zarovni, N., Wauben, M. H. M., Berti, D., Bergese, P., & Valle, F. (2020). AFM-Based High-Throughput Nanomechanical

Screening of Single Extracellular Vesicles. *Analytical Chemistry*, 92(15), 10274–10282. <https://doi.org/10.1021/acs.analchem.9b05716>.

Fortunato, D., Mladenović, D., Criscuoli, M., **Loria, F.**, Veiman, K.-L., Zocco, D., Koort, K., & Zarovni, N. (2026). Correction: Fortunato et al. Opportunities and Pitfalls of Fluorescent Labelling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms. *Int. J. Mol. Sci.* 2021, 22, 10510. *International Journal of Molecular Sciences*, 27(7), 3199. <https://doi.org/10.3390/ijms27073199>.

Zarovni, N., **Loria, F.**, Zenatelli, R., Mladenović, D., Paolini, L., Adamo, G., Redeghier, A., Bongiovanni, A., & Bergese, P. (2021). Standardization and commercialization of extracellular vesicles. In *Extracellular Vesicles. Applications to Regenerative Medicine, Therapeutics and Diagnostics*. Royal Society of Chemistry publishing. <https://doi.org/10.1039/9781839164552-00303>.

Zuppone, S., Zarovni, N., Noguchi, K., **Loria, F.**, Morasso, C., Löhmus, A., Nakase, I., & Vago, R. (2024). Novel loading protocol combines highly efficient encapsulation of exogenous therapeutic toxin with preservation of extracellular vesicles properties, uptake and cargo activity. *Discover Nano*, 19(1). <https://doi.org/10.1186/s11671-024-04022-8>.

Loria, F., Picciotto, S., Adamo, G., Zandrini, A., Raccosta, S., Manno, M., Bergese, P., Liguori, G. L., Bongiovanni, A., & Zarovni, N. (2024). A decision-making tool for navigating extracellular vesicle research and product development. *Journal of Extracellular Vesicles*, 13(12). <https://doi.org/10.1002/jev2.70021>.

Governini, L., Haxhiu, A., Shaba, E., Vantaggiato, L., Mori, A., Bruttini, M., **Loria, F.**, Zarovni, N., Piomboni, P., Landi, C., & Luddi, A. (2025). Unraveling the Multi-Omic Landscape of Extracellular Vesicles in Human Seminal Plasma. *Biomolecules*, 15(6), 836. <https://doi.org/10.3390/biom15060836>.

Loria, F., Kattel, A., Junusova, M., Brucale, M., Valle, F., Bergese, P., Kobrin, E.-G., Chiesi, A., Guazzi, P., Korulu, S., Stulova, I., Zarovni, N., & Vilu, R. (2026). Investigating the Effect of Ginger-Derived Nanovesicles on the Growth and Metabolic Activity of *Bacteroides thetaiotaomicron*: An Isothermal Microcalorimetric Study. *Journal of Extracellular Biology*, 5(7), e70163. <https://doi.org/10.1002/jex2.70163>.

Conference Presentations

- | | |
|------|---|
| 2021 | Oral presentation, Annual Meeting 2021 organized by the International Society for Extracellular Vesicles (ISEV2021), Remote |
| 2022 | Oral presentation, International Symposium organized by the Consortium of the European Commission-funded FET Proactive project MARVEL (Grant Agreement ID: 951768), Lugano, Switzerland |
| 2022 | Poster presentation, Annual Meeting 2022 organized by the International Society for Extracellular Vesicles (ISEV2022), Lyon, France |
| 2023 | Poster presentation, XVII School of Pure and Applied Biophysics on “Extracellular Vesicles: From Biophysical to Translational Challenges” organized by the European |

2024	Commission-funded FET-Proactive project BOW (Grant Agreement ID: 952183), Venice, Italy Poster presentation, Annual Meeting 2024 organized by the International Society for Extracellular Vesicles (ISEV2024), Melbourne, Australia
Teaching & Supervision	
2023-2024	Kaisa Rõngas, MSc student at Tallinn University, Tallinn, Estonia. Laboratory practice Report “Optimizing the Isolation and Purification of Extracellular Vesicle-like Nanovesicles from Ginger Rhizome (<i>Zingiber officinale</i>)”
Certifications & Professional Training	
2014	International English Language Testing System (IELTS), Cambridge English Language Assessment, the British Council, IDP Education, Palermo, Italy
2019	FET2RIN workshop, Brussels, Belgium
2020	Innovation Training Workshop, European Innovation Council (EIC), Remote
2021	Pathfinder Bootcamp, European Innovation Council (EIC), Remote
2021	Summer School “Valorization of Food Industry Wastes and By-product”, ERA Chair in European Commission-funded Valortech (Grant Agreement ID: 810630), Remote
2022	“Process Validation for Biotechnological Products” MasterClass, GLC Europe, Remote
Extra-curricular activities	
2018–2018	Marie Skłodowska-Curie Actions (MCSA) Fellow Volunteer Exhibitor, School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin, Ireland

Elulookirjeldus

Isikuandmed

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Sünniaeg: 13.08.1990
Sünnikoht: Palermo, Itaalia
Kodakondsus: Itaalia

Kontaktandmed

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Hariduskäik

2021–2026	Tallinna Tehnikaülikool, keemia ja biotehnoloogia doktoriõpe (PhD), Tallinn, Eesti
2018–2018	Trinity College Dublin, farmaatsia ja farmaatsiateaduste doktoriõpe (PhD), Dublin, Iirimaa
2014–2016	Groningeni Ülikool, biomeditsiiniteaduste magistrikraad (MSc), Groningen, Madalmaad
2009–2013	Palermo Ülikool, biotehnoloogia bakalaureusekraad (BSc), Palermo, Itaalia
2004–2009	Gümnaasium “G. Meli”, keskharidus, Palermo, Itaalia

Keelteoskus

Inglise keel	Kõrgtase
Itaalia keel	Emakeel

Teenistuskäik

2024–...	Rakendusteadlane (Applied Scientist), HansaBioMed Life Sciences Ltd, Tallinn, Eesti
2019–2024	Teadur (Research Scientist), HansaBioMed Life Sciences Ltd, Tallinn, Eesti
2018–2018	Laboritehnik, Exosomics Siena SpA, Siena, Itaalia

Praktilised kursused

2017–2017	Ettevõtte praktikant, Exosomics Siena SpA, Siena, Itaalia
2015–2016	Teaduspraktikant, Meditsiinilise onkoloogia osakond, University Medical Center Groningen, Groningen, Madalmaad
2014–2015	Teaduspraktikant, Cardiovascular Regenerative Medicine (CAVAREM), Groningeni Ülikooli Meditsiinikeskus, Groningen, Madalmaad
2013–2013	Teaduspraktikant, Biopatoloogia ja meditsiinilise ning kohtubioloogia osakonna bioloogia ja geneetika üksus, Palermo Ülikool, Palermo, Itaalia

Teadusgrandid ja rahastused

2026-2028	PANACEA/EV – „Potency Assay and Novel Avenues for Clinical Evaluation of AngioARC or Derived EV-Based “Therapeutics”, rakendusuuringu ja tootearenduse toetus (Grant Agreement No. RES.5.04.25-0824), EIS. Juhtis granditaotluse koostamist, mille tulemusena projekt sai
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rahastuse, ning määrati HansaBioMed Life Sciences Ltd juhtivteaduriks (Principal Investigator)

Teadusprojektid

2018-2022	Merck KGaA, Grant for Fertility Innovation (GFI) 2016/17 projekt "Exosomal Profile of the Receptive Endometrium: A Source of Non-Invasive Biomarkers for Guiding a Successful Embryo Implantation". Projekti koordinaator: prof Paola Piomboni, Siena Ülikool, Siena, Itaalia
2018-2022	Euroopa Komisjon, H2020-FETOPEN-2016-2017 projekt "The Extracellular Vesicle Foundry" (evFOUNDRY) (Grant Agreement No. 801367). Projekti koordinaator: prof Paolo Bergese, Brescia Ülikool, Brescia, Itaalia
2020-2024	Euroopa Komisjon, H2020-FETPROACT-2019-2020 projekt "Biogenic Organotropic Wetsuits" (BOW) (Grant Agreement No. 952183). Projekti koordinaator: prof Paolo Bergese, Brescia Ülikool, Brescia, Itaalia
2025-2028	Euroopa Komisjon, HORIZON-HLTH-2024-TOOL-05-two-stage projekt "Non-Animal Platform for Nanoparticle-Based Delivery across the Blood-Brain Barrier Interface with Vehicle Evolution" (NAP4DIVE) (Grant Agreement No. 101155875). Projekti koordinaator: prof Cecilia Sahlgren, Åbo Akademi, Turu, Soome
2026-2028	Ettevõtluse ja innovatsiooni sihtasutus (EIS), rakendusüritingu ja tootearenduse toetus projektile "Potency Assay and Novel Avenues for Clinical Evaluation of AngioARC or Derived EV-Based Therapeutics" (PANACEA/EV) (Grant Agreement No. RES.5.04.25-0824). Projekti koordinaator: Cellin Technologies OÜ, Tallinn, Eesti

Autasud, tunnustused ja stipendiumid

2013	Palermo Ülikool, biotehnoloogia bakalaureusekraad, Palermo, Itaalia. Lõpetas kiitusega (110/110 cum laude) ning pälvis tunnustuse Academic Career Distinction
2018	Marie Skłodowska-Curie uuendusliku koolitusvõrgustiku (Innovative Training Network, ITN) "Training in Extracellular Vesicles: for Benefit in Health and Disease" (TRAIN-EV) varajase etapi teadlane (Early-Stage Researcher) ja doktorant Euroopa Liidu teadus- ja innovatsiooniprogrammi Horizon 2020 raames (Grant Agreement No. 722148)

Publikatsioonid

Luddi, A., Zarovni, N., Maltinti, E., Governini, L., Leo, V. D., Cappelli, V., Quintero, L., Paccagnini, E., **Loria, F.**, & Piomboni, P. (2019). Clues to Non-Invasive Implantation Window Monitoring: Isolation and Characterisation of Endometrial Exosomes. *Cells*, 8(8), 811. <https://doi.org/10.3390/cells8080811>.

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Screening of Single Extracellular Vesicles. *Analytical Chemistry*, 92(15), 10274–10282. <https://doi.org/10.1021/acs.analchem.9b05716>.

Fortunato, D., Mladenović, D., Criscuoli, M., **Loria, F.**, Veiman, K.-L., Zocco, D., Koort, K., & Zarovni, N. (2026). Correction: Fortunato et al. Opportunities and Pitfalls of Fluorescent Labelling Methodologies for Extracellular Vesicle Profiling on High-Resolution Single-Particle Platforms. *Int. J. Mol. Sci.* 2021, 22, 10510. *International Journal of Molecular Sciences*, 27(7), 3199. <https://doi.org/10.3390/ijms27073199>.

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Loria, F., Picciotto, S., Adamo, G., Zandrini, A., Raccosta, S., Manno, M., Bergese, P., Liguori, G. L., Bongiovanni, A., & Zarovni, N. (2024). A decision-making tool for navigating extracellular vesicle research and product development. *Journal of Extracellular Vesicles*, 13(12). <https://doi.org/10.1002/jev2.70021>.

Governini, L., Haxhiu, A., Shaba, E., Vantaggiato, L., Mori, A., Bruttini, M., **Loria, F.**, Zarovni, N., Piomboni, P., Landi, C., & Luddi, A. (2025). Unraveling the Multi-Omic Landscape of Extracellular Vesicles in Human Seminal Plasma. *Biomolecules*, 15(6), 836. <https://doi.org/10.3390/biom15060836>.

Loria, F., Kattel, A., Junusova, M., Brucale, M., Valle, F., Bergese, P., Kobrin, E.-G., Chiesi, A., Guazzi, P., Korulu, S., Stulova, I., Zarovni, N., & Vilu, R. (2026). Investigating the Effect of Ginger-Derived Nanovesicles on the Growth and Metabolic Activity of *Bacteroides thetaiotaomicron*: An Isothermal Microcalorimetric Study. *Journal of Extracellular Biology*, 5(7), e70163. <https://doi.org/10.1002/jex2.70163>.

Konverentsi ettekanded

2021	Suuline ettekanne, International Society for Extracellular Vesicles (ISEV2021) aastakoosolek, veebikonverents
2022	Suuline ettekanne, Euroopa Komisjoni rahastatud FET Proactive projekti MARVEL (Grant Agreement No. 951768) rahvusvaheline sümpoosion, Lugano, Šveits
2022	Posterettekanne, International Society for Extracellular Vesicles (ISEV2022) aastakoosolek, Lyon, Prantsusmaa
2023	Posterettekanne, XVII School of Pure and Applied Biophysics "Extracellular Vesicles: From Biophysical to Translational Challenges", Euroopa Komisjoni rahastatud FET-Proactive projekti BOW (Grant Agreement No. 952183) raames, Veneetsia, Itaalia
2024	Posterettekanne, International Society for Extracellular Vesicles (ISEV2024) aastakoosolek, Melbourne, Austraalia

Õppetöö ja juhendamine

2023-2024

Tallinna Ülikooli magistrandi Kaisa Rõngase laboripraktika juhendamine. Praktikaaruanne "Optimizing the Isolation and Purification of Extracellular Vesicle-like Nanovesicles from Ginger Rhizome (*Zingiber officinale*)"

Täienduskoolitus ja kutsealane enesetäiendamine

2014

International English Language Testing System (IELTS), Cambridge English Language Assessment, British Council ja IDP Education, Palermo, Itaalia

2019

FET2RIN töötuba, Brüssel, Belgia

2020

Innovation Training Workshop, European Innovation Council (EIC), veebikonverents

2021

Pathfinder Bootcamp, European Innovation Council (EIC), veebikonverents

2021

Suvekool „Valorization of Food Industry Wastes and By-product“, Euroopa Komisjoni rahastatud ERA Chair VALORTECH projekti (Grant Agreement No. 810630) raames, veebikonverents

2022

"Process Validation for Biotechnological Products" MasterClass, GLC Europe, veebikonverents

Ühiskondlik tegevus

2018–2018

Marie Skłodowska-Curie Actions (MSCA) vabatahtlik eksponent Euroopa Teadlaste Ööl, Trinity College Dublini farmaatsia- ja farmaatsiateaduste kool, Dublin, Iirimaa

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