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Department of Materials and Environmental Technology

Development of a synthetic receptor-based chemosensor for label-free detection of erythromycin in aqueous media

MASTER THESIS

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AUTHOR'S DECLARATION

I declare that this thesis is based on my own work and has not been submitted for any degree of examination in other universities. All ideas, major views and results of researches by other authors are used only with a reference to the source.

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(signature and date)

Master Thesis meets the established requirements.

Supervisor: *Dr. Vitali Sõritski*
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Board of Examiners
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Department of Materials and Environmental Technology

THESIS TASKS

Student: Ciocan Valeriu 177215KAYM
Study programme: KAYM09/09 - Materials and Processes for Sustainable Energetics
Supervisor(s): Senior Research Scientist, Dr. Vitali Sõritski

Researcher, Dr. George Ayankojo

Thesis topic:
(in English) Development of a synthetic receptor-based chemosensor for label-free detection of erythromycin in aqueous media

(in Estonian) Sünteetilise retseptoriga modifitseeritud keemiline sensor erütromütsiini märgisevabaks määramiseks vesikeskkondades

. **Thesis main objectives**:

1. Selection of a suitable electropolymerizable functional monomer;
2. Determination and optimization of parameters for the synthesis of a polymer from the selected functional monomer in the presence of the template, Erythromycin (Ery) on the working electrode of a screen printed electrode (SPE);
3. Removal of Ery from the polymer in an appropriate washing out solvent to generate an Erythromycin-molecularly imprinted polymer (MIP) on SPE (Ery-MIP/SPE)
4. Characterization of Ery-MIP/SPE in terms of selectivity and limit of detection toward Ery;

Thesis tasks and time schedule:

Nr	Task description	Deadline
1.	Selection of the best suited electopolymerizable functional monomer	December, 2018
2.	Determination of optimal parameters for the polymer film synthesis from the selected functional monomers in the presence of the template on the working electrode of a screen printed electrode	January, 2019
3.	Determination and optimization of the washing out step parameters to generate an Ery-MIP on SPE	January, 2019
4.	Characterization of Ery-MIP/SPE in terms of selectivity and limit of detection toward Ery	February, 2019

Language: English **Deadline for submission of thesis:** "29" May 2019

Student: Ciocan Valeriu……...…...15" December2018

/signature/

Supervisor: Dr. Vitali Sõritski……..…..…"15" December 2018

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Table of Contents

Preface

This work was conducted at Tallinn University of Technology, Department of Materials and Environmental Technology, laboratory of Biofunctional Materials.

I am grateful to my supervisor of this master thesis Dr. Vitali Sõritski whose leadership, guidance and patience have created an excellent environment for a successful completion of the thesis objectives. He provided invaluable help and support in the thesis write-up and continuous constructive criticism in academic life and laboratory work.

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I would like to thank my colleagues Dr. Roman Borozнjak and Anna Kidakova for the good advice and permanent readiness to help.

Introduction

The discovery and use of antibiotics in the treatment of bacterial infections are considered as the single most important event in the history of medicine, improving the human condition and pushing development to our current levels[1]. While deaths from infectious diseases diminish every year, the use of antibiotic drugs is consistently rising. As a result of this wide usage, these drugs find their way into the environment where they contribute to the development of antibiotic resistance in bacteria that contacts sublethal doses of the drug[1,2]. This poses a great challenge to medical practices for the benefit of human development as antibiotic resistance increasingly makes it difficult to treat patients with infectious diseases resistant to some previously effective drugs[3,4,5]. In an effort to combat the development of antibiotic resistance, otherwise a naturally occurring process[4], great efforts are made to optimize antibiotic use in medicine and agriculture with a special accent on detecting and eliminating antibiotics from the environmental sources - being considered as pollutants[6]. To eliminate antibiotics from natural waters, the scientific community is exploring methods for better detection of these compounds[7,8,9]. Today, sample analysis is done in laboratory conditions using high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), mass spectrometry, gas chromatography, and their pairing variations. These methods are known for their high precision measurements, however their use requires special conditions, analytical grade solvents for analysis and maintenance, an experienced operator for handling the device and analysis of the results, and thus, great efforts are put into developing sensors with similar or higher detection precision, ease of use and improved portability. An answer may be in sensors modified with synthetic receptors as the recognition element as they have good physical and chemical stability, maintain high sensitivity, and selectivity for the molecule they were designed.

Synthetic receptors, generated through “molecular imprinting technology (MIT)”, are known as Molecularly Imprinted Polymers (MIPs)[9]. The technology of molecularly imprinting is very promising. The technology of molecularly imprinting is very promising, MIP based sensors have been showed to recognize investigated molecules at rates close to biological receptors. This is due to their high sensitivity, and excellent stability, all while being more sturdy than biological receptors. MIT relies on the formation of mixtures of the analyzed compound, the template, and specially selected monomers which will surround the template due to an alignment of functional moieties of the compounds in the prepolymerization mixture, assuring the formation of specific

binding sites after the removal of the molecule from the hardened polymer. The binding sites formed can recognize the analyzed compound, with excellent affinity, and discriminate between molecules with a similar structure. Sensors modified with synthetic receptors, MIPs, as the recognition element can be investigated by electrochemical methods, showing a fast response time and excellent sensitivity[10,11,12]. This is a label free method, it does not require the utilization of special pigments for labeling of the target, which may influence MIPs performance. Therefore employment of a label-free sensor platform integrated with MIP may allow more rigorous monitoring of rebinding events in the polymer matrix, making possible the detection of very small molecules with very high accuracy.

Employing MIP modified transducers for small analyte detection does not require the usage of special solvents, they are stable at moderate temperature variations and resistant to considerable changes in acidity[9]. However, the preparation of analyte-MIPs modified sensors requires substantial consideration in developing the synthesis protocol and its consequent optimization.

In the last decade, a number of attempts have been made to imprint erythromycin (Ery) for subsequent quantification of the antibiotic in complex aqueous matrices[10,13,14,15], however, most fall short of achieving a limit of detection (LoD) equal or lower than levels detected in environmental samples[16].

This thesis describes the formation of an Ery-MIP thin film on a gold working electrode (WE) through electropolymerization. A conductive layer of Poly m-Phenylenediamine (PmPD) was formed and deposited from a monomer solution of m-Phenylenediamine (mPD) onto the conductive electrode. Electropolymerization seems a proper method for the synthesis of a thin MIP film on a WE, providing satisfactory control over the film's thickness and ensuring the integration of the film with the electrode surface. Electropolymerization can be carried out at room temperature and does not suffer from local overheating, it doesn't require initiators, catalysts, or other additives.

This work investigates the synthesis method of developing a thin polymer film with imprinted cavities capable of selective rebinding-recognition of Ery in aqueous media. The described method may be compatible with a range of label-free detection methods ensuring a sturdy integration of the MIP with the WE. The screen printed electrode (SPE) was selected as the transduction device as it may be the most advantageous electrochemical sensor for in-situ investigations for real-time monitoring of rebinding events taking place in the Ery-MIP matrix, with simultaneous quantification of MIPs selectivity and the LoDfor the target analyte. While considered a novelty, MIP/SPEs may

be the gateway to simple electrochemical analysis, it has low power requirements, fast response and may represent an alternative for electrochemical analysis with bulky laboratory equipment.

1. Literature Review

1.1 Antibiotics as environmental pollutants

Antibiotics are compounds with antimicrobial activity, considered a very successful class of pharmaceuticals employed by humans in medicine[17,18] celebrated for saving countless lives; today it is used in animal feed, livestock production and in food preservation[3]. The first antibiotic to be revealed was penicillin, and it is regarded as the single most important advance in the history of medicine, making previously incurable diseases treatable. Ever since its discovery, countless lives have been saved, and economic prosperity is ensured for a society no longer afraid of regular flu outbursts[3]. Both human and livestock antibiotics can be found in the environmental samples, they promote the development of antibiotic resistance, and this threatens human survivability, by making existing antibiotics ineffective[5,19]. Due to large use and insufficient levels of control, antibiotics can be detected in most environmental samples being actively released into the environment and resulting in serious adverse effects on the ecosystem and on people[20]. Thus, eliminating antibiotics from wastewater is increasingly gathering the attention of the international community, regarding them as environmental pollutants of significant interest[6,21].

Antibiotics have an inhibitory effect on the biodegradation of organic compounds in wastewater, hence limiting the capabilities of water treatment plants[22,23]. These pollutants are usually found in very low concentrations (ng/L) and are referred to as contaminants of emerging concern (CEC), and traditional methods of wastewater treatment are insufficient and ineffective in the removal of most CECs[24]. While science and technology have considerably advanced in the last decades, the consequence of these contaminants, once introduced, remains relatively unresolved and it was established that wastewater treatment plants represent hotspots for horizontal antibiotic-resistance genes transfer[25]. World Health organizations (WHOs) Global Action Plan on Antimicrobial Resistance (AMR) recommends that all countries implement a system of surveillance to combat AMR[23]. The beginning of the 21st century was characterized with an increased concern related to bacterial resistance as a consequence of the occurrence of various antibiotic groups such as macrolides (ML), fluoroquinolones (FQ), Sulfonamides (SA), etc.; in major water bodies across the world, except Antarctica[26,27]. This discovery plays a key role, considering the development of drug resistance as a natural evolutionary function of most microbes that is aided by exposure to sublethal drug concentrations[4,28].

Increased development of antibiotic resistance evidence the need to better understand the key features of antibiotic action and use[4]. In this direction, great efforts are being put towards understanding AMR and preventing its spreading. Antibiotic resistance genes are complex and usually not necessarily developed due to mutations in bacteria. They are mostly carried by the existing genes of some bacteria in the environment and later acquired by other bacteria[4]. Antibiotic resistance genes are regularly identified in municipal wastewater[25], bacteria resistant to antibiotics heighten the costs of healthcare, which leads to lower productivity and slower economic growth of a nation.

Loss of potency of modern antibiotics is a great concern for the international community[3,5] and understanding how to use antibiotics more effectively and preserve their efficacy for future generations is the path the international community has taken very seriously[4]. In the last 4 decades the US Food and Drug Administration is consistently approving less antibiotic drugs each year, 16 new compounds in 1983-1987, 7 in 1998-2002 and 2 in 2008-2012, illustrating the need to preserve current antibiotic solutions as efficient and lucrative as possible[43], simultaneously the amount of research done is constantly increasing[29]. New drug discoveries become more infrequent, drug usability in healthcare is increasingly difficult and seems to become more challenging - modelings propose that between 2010 and 2030 the global antibiotics consumption will increase by 67% in agriculture alone, from 63000 tons to 105000 tons per year indicating a possible increase in antibiotic resistance[4]. There already exist fungi with+ developed resistance to all known antifungal therapies[30].

Today the emerging contaminants in wastewater systems are studied through analysis of liquid samples, this ignores the complex interaction of the pollutants in the environmental matrix - bioactive compounds are adsorbed on microparticles and have a potentially greater risk to aquatic organisms and AMR development than previously thought[22], this is particularly important considering that most antibiotic drugs have a low solubility in aqueous environments and antibiotic families such as macrolides have a potentially higher proclivity to adsorb to hydrophobic surfaces. This makes very clear the urgency for continuous monitoring of municipal wastewaters and timely detection of the CEC much before they have a chance of entering the environment.

1.2 Macrolides

Since their discovery in 1950, macrolides have become a valuable family of first choice antibiotics with increased utility in therapy, notably in pediatrics[9]. They are characterized by the presence of a macrocyclic lactone ring with one or more deoxy-sugar

or amino sugar residues attached. They represent a large family of protein synthesis inhibitors binding to bacterial ribosomal subunit and hindering their protein synthesis[31]. These antibiotics are considered the highest priority on the WHO critically important antimicrobials list (CIA list) and carry a high risk of selection of bacterial resistance. Resistance to macrolides has become a major issue for most of the species originally described as susceptible[4], the first and most notable member of this family is Erythromycin.

1.2.1 Erythromycin

Erythromycin is the first representative of the class of macrolide antibiotics introduced for clinical use[4]. It has a molecular weight of 733.9 g/mol and a structural formula shown in Fig. 1. Erythromycin and macrolides, in general, are characterized by a moderately broad spectrum of activity, which includes most Gram-positive and a few selected Gram-negative organisms, as well as several bacteria.

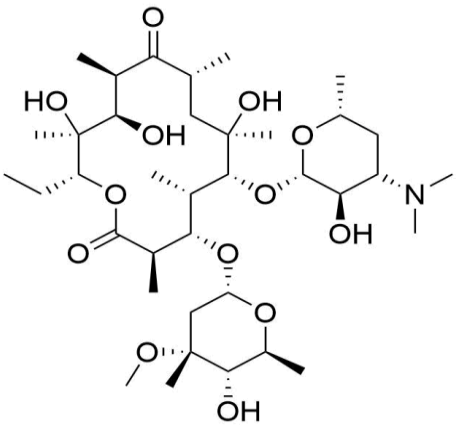

Figure 1. Molecular structure of Erythromycin.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ery for 90% of organisms are between 0.13 and 2.0 mg/L, respectively, much higher than in environmental samples[4]. We can assume from these findings alone that environmental concentrations of Ery are sub-lethal for microorganisms, hence represent a great risk for AMR. Most hospital-acquired Methicillin-resistant Staphylococcus aureus (MRSA) strains have now acquired resistance to macrolides[4,32].

It was reported that Ery is found predominantly dissolved in the aquatic environment rather than bound to particulates; this indicates consistent results for Ery analysis in all water bodies[2]. Although Ery is very effective, the availability of derived

compounds with increased potency and bioavailability like clarithromycin, roxithromycin, and azithromycin has substantially reduced its use over the last decade.

1.2.2 Azithromycin

Azithromycin (AZM) differs from Ery in possession of a methylated nitrogen atom at the ninth position of the macrolide lactone ring (Fig. 2). This improves its activity against gram-negative bacteria[33]. It is frequently used as a single dose medication for sexually transmitted diseases (STD) therapy and short duration therapy of soft tissue and respiratory tract infections as a result of the concentration of the drug remaining high in these tissues for lengthy periods of time[34]. Azithromycin has only 37% oral bioavailability and is excreted unchanged[34], thus finding its way to wastewater treatment facilities and is further discarded into the environment. Given its remarkably long half-life, azithromycin carries a higher risk for antibiotic resistance among the macrolides[29].

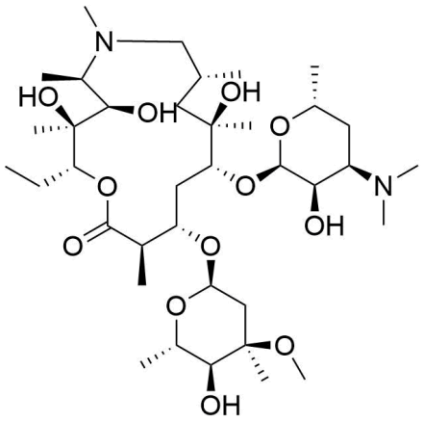

Figure 2. Molecular structure of Azithromycin.

1.2.3 Clarithromycin

It is a semi-synthetic compound with improved stability in an acidic environment and oral bioavailability, as well as markedly improved pharmacologic profiles[4]. It possesses a substituted methoxy group at the sixth position of the macrolide ring (Fig. 3). Clarithromycin is listed as a first-choice option for Helicobacter pylori, community-acquired severe pneumonia, and as a second-choice option for pharyngitis[29]. Derived from erythromycin with an increased bioavailability (50%) it has the highest absorption among all macrolides and is routinely used to treat respiratory tract infections[34].

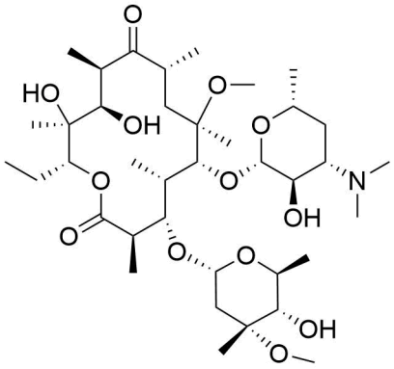

Figure 3. Molecular structure of Clarithromycin.

1.3. Quinolones

These antibiotics are considered highest priority critically important antimicrobials on the WHO CIA list and carry a high risk of selection of bacterial resistance (in particular MRSA, ESBL, and resistance to fluoroquinolones)[29]. Discovered in the early 1960s, quinolones have grasped the attention of clinicians for having many aspects of a "perfect antibiotic" with great potency, good bioavailability, and broad spectrum. It exists in both oral and intravenous formulations and potentially possesses a low incidence of side-effects. In this group of compounds over 10,000 molecules have been derived and patented, and are mainly used to treat urinary tract infections. One of the most well-known fluoroquinolones with better systemic activities is Ciprofloxacin[35].

1.3.1 Ciprofloxacin

Ciprofloxacins structural formula shown in Fig. 4. It is used in the treatment of gram-negative bacterial infections and does so by inhibiting the reproduction and reconstruction of their DNA. It has a bioavailability of 70% and a half-life of 3.5 hours[34].

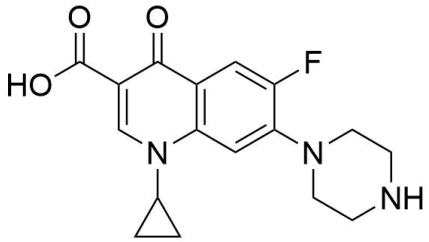

Figure 4. Molecular structure of Ciprofloxacin.

Ciprofloxacin is listed on the WHOs Essential Medicines List (EML) as the first-choice option for acute invasive bacterial diarrhea/dysentery, low-risk febrile neutropenia,

pyelonephritis or prostatitis (mild to moderate), and as a second-choice option for cholera and complicated intra-abdominal infections (mild to moderate)[29].

1.4 Molecularly imprinted polymers (MIP)

MIPs are gaining attention from researchers developing biomimetic solutions, taking advantage of their low preparation cost and ease of use[36]. Referred to as synthetic receptors due to their capacity to recognize and interact with analytes. In the last decade, MIPs greatly advanced from plain molecular extraction to efficient molecule recognition[37,38].

Molecular imprinting consists of the design and synthesis of the biomimetic receptors, capable of binding the target compound with great capacity and selectivity, previously thought possible only to their biological counterparts. A target molecule is used to guide the formation a complex with the highest stability[39], by the alignment of monomers and templates functional groups in a complex[40] with the lowest entropy. Following the polymerization step, the hardened mold is then washed out, to remove the

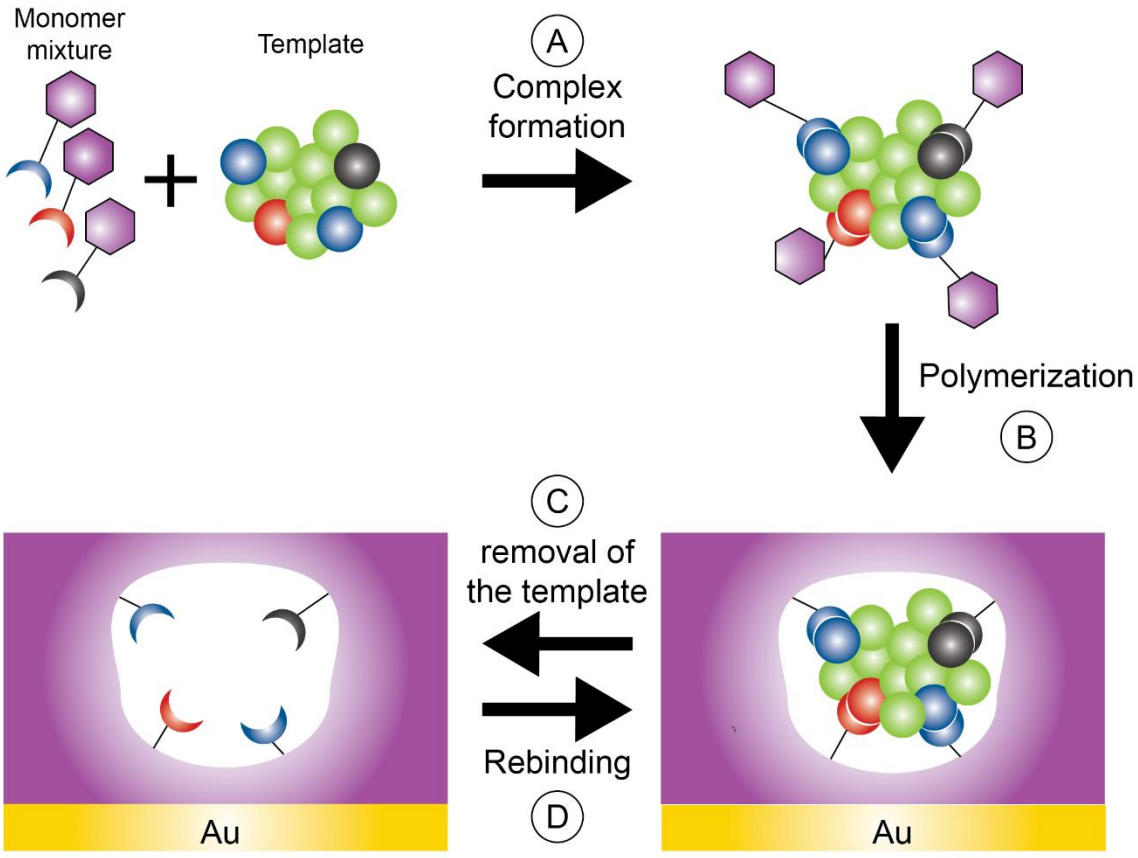

Figure 5. Principle of molecular imprinting (a)complex formation (b) polymerization, (c)template removal, (d) template recognition-rebinding.

template and obtain cavities molded for the sole purpose of rebinding the template molecule.

MIPs show a greater physical and chemical stability, longer shelf life, more robust format[8,41], ease of synthesis, simplicity of use compared to their biological counterpart[38]. Noteworthy is the possibility of tailoring MIPs characteristics to best suit a particular task and environment. They are very attractive candidates for taking over biochemical analysis[37].

A fascinating aspect of MIP employment is the potential use in automated monitoring using electrochemical methods of analysis[42,40], measuring currents with high accuracy even in turbid samples[12]. Combined with the miniaturization of the transducer and the relative simplicity using electronic hardware for measuring the detection output[39].

Designing a MIP with good template recognition and distinguished stability, we must take in to account many factors, markedly the polymer format.

1.4.1 MIP formats

MIP format is selected with consideration of the intended application[36,43]. Some applications require specific MIP architecture and properties are interconnected most notable: nanoparticles have a high surface area to weight ratio, a monolith is relatively simple to synthesize, membranes and thin films can be formed directly on a transducer. The following is a concise description of each format.

Nanoparticles

Nanoparticles are commonly known as beads with less than 100 nanometers in diameter and polymers are exemplary materials used for the fabrication of nanoparticles[44]. The particle size can be controlled via polymerization chemistry, the most common preparation methods for MIP nanoparticles include precipitation polymerization, core-shell emulsion polymerization and living radical polymerization such as transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer polymerization (RAFT)[44]. MIP nanoparticles have been applied in drug delivery systems, capillary electrophoresis, and chromatography columns among others[45,46]. Molecularly imprinted nanoparticles are of great interest for their potential in vitro applications: diagnostics, therapeutics, and separation[46,47]. Figure 6a shows a principle representation of the process of template rebinding for MIP nanoparticles.

Monolith

Also referred to as bulk polymerization or mass polymerization it is a technique of creating a large volume polymer block with template molecules entrapped in the polymer mass. This method sacrifices particle size precision for its simplicity of preparation; however, it is time-consuming, labor-intensive, and wasteful since only about 30-40% of the polymer is recovered as usable material[47]. Bulk polymerization generates a monolith polymer which must be ground down and passed through a sieve to separate particles in the 25-50µm size range[48]. The obtained particles are then washed to remove the template and can be used for rebinding experiments.

Bulk polymerization is a very attractive method due to its relative simplicity, yet its industrial scaling is difficult in part due to uneven heat distribution during polymer curing and obtainment of heterogeneous particles[47], and a more judicious MIP design is required to meet industrial scale.

Membrane

Molecularly imprinted membrane (MIM) shown in Fig. 6c is another MIP format investigated by research groups for analyte detection[41,49]. A membrane is an interphase between two bordering phases, regulating the transport of compounds between two chambers, acting as a selective barrier[50]. Passive transport through the MIM results following the effect of a driving force: i.e., a gradient across the membrane (e.g., concentration, pressure), a difference in chemical potential, or by an electric field[50]. Researchers are increasingly working on the improvement of the separation mechanisms, notably affinity interactions and electrochemical potentials[41,50]. Figure 6c shows a principle representation of the process of template rebinding for MIP membranes.

Polymer membranes have been used for pharmaceutical enrichment, food processing, water purification, among others[51]. Combined with an electrochemical transducer, MIP shows great promise for future point of care solutions. Today these membrane-modified electrodes present good results and a consistent gain in the detection limit[41].

Thin Films

Thin films are a very promising configuration for the development of biomimetic sensors. Thin films can be employed on the surface of diverse transducers for the monitoring of binding interactions close to the film surface, potentially increasing the sensors specific rebinding. Thin films demonstrate a shorter diffusion path for the

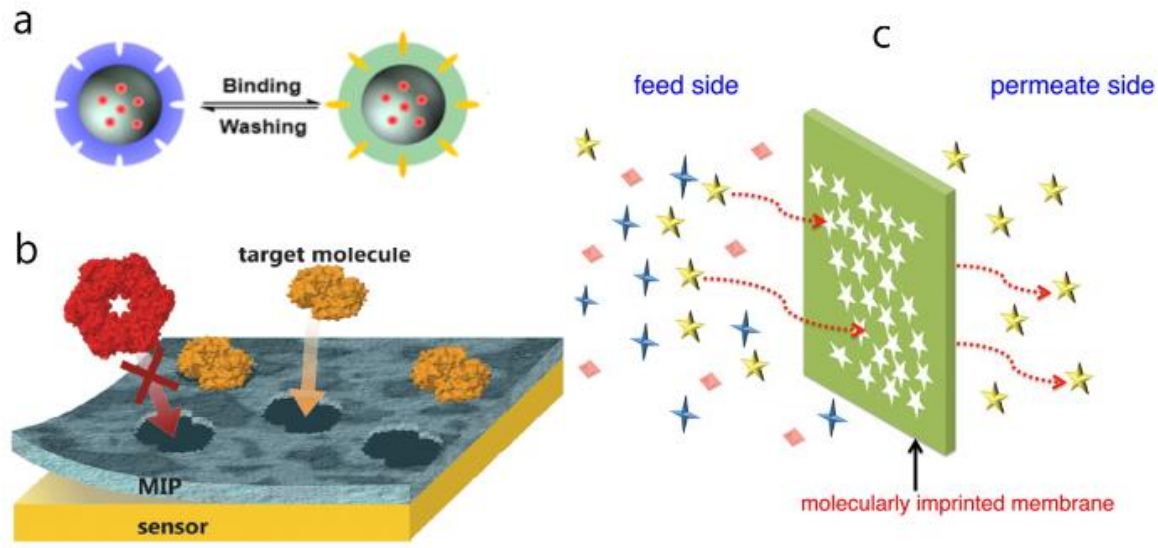

Figure 6. Principle representation of the process of template rebinding for different MIP formats: membrane(c)[86], thin film (b)[87], nano-particle (a)[88].

template to travel from the analyte solution towards the rebinding cavity; thus, thin films permit a faster detection method for small molecules. This format shows great promise for environmental monitoring and small molecule detection[52]. Figure 6b shows a principle representation of the process of template rebinding for MIP thin films.

1.5 MIP sensor for antibiotics detection

Microbial resistance to antibiotics represents a growing concern for the WHO, promising to be a great challenge for human civilization by reducing the effectiveness of some pharmaceuticals and making entire families of antibiotics - fully inefficient against bacterial strains combating which they were once the first line of drugs. To better combat antibiotic resistance, the pharmaceuticals presence in the environment must be actively monitored[53]. While essential, environmental monitoring has significant costs like sampling, sample transportation to a specialized laboratory, and costly analysis. Most antibiotic molecules are small, increasing the difficulty of their recognition and quantification. Molecularly imprinting technology is laying the path to overcoming these shortcomings, already today, MIPs can be employed for antibiotics detection with very high accuracy.

While there are many approaches to MIP design and synthesis, the most widely employed and simplest one is the bulk polymerization where the Template-MIP is formed as a monolith and is later grounded down to achieve a smaller particle size. This method allows the formation of irregularly shaped particles, and most of the material is lost in the

grinding process[13]. The particles can later be packed in an HPLC column and used for analytical purposes. Worth noting is the more novel approach of MIP preparation, such as surface imprinting with more control over imprinted cavities distribution on the surface[8], precipitation polymerization grants the ability of forming well defined particles with a more controlled polymerization, and thin film synthesis on a variety of support materials[13], directly on some transducers, most notably quartz crystal microbalance and Surface Acoustic Wave transducers (SAW). showing great synergic compatibility for MIP technology[54]. All mentioned methods are considered of great importance to the field of bio-sensorics. However, they all require specialized equipment for the measurements performed through them, analytical grade solvents, and a skilled operator to interpret the data. A promising approach is the employment of screen-printed electrodes (SPEs) combined with a "pocket" potentiostat, representing a fast and easy method of remote monitoring and analysis.

1.5.1 Erythromycin as an antibiotic target molecule

Antibiotics are used in a variety of applications saving countless lives each day, increasing life expectancy, and reducing mortality from infectious disseases. Antibiotics have considerably increased the efficiency of agriculture[14], together compounding for greater development of society. With their wider use, they can be found in higher concentrations in natural waters[55], more and more pose a danger for all human civilization due to the development of antibiotic resistance as a direct consequence of their presence in the environment[6].

Guided by the Water Framework Directive (WFT), the Surface Water Watch List (SWWL) is a list of likely water pollutants that require monitoring by EU member states to determine the risk they pose to the environment and to the general community[53]. On the SWWL Erythromycin has its place as a member of the larger, macrolide group. Different methods of monitoring this antibiotic are employed, most popular is the High-Performance Liquid Chromatography (HPLC) and Solid Phase Extraction (SPE) being very well known, delivering high analysis precision while being costly and resource intensive requiring a specialized laboratory with ultrapure solvents to be used by an experienced operator[14]. A number of research groups work on finding a better solution with the possibility of deployment of the newly developed technology for fast, reliable, and cheap detection of Ery, especially in remote areas[56]. Attempting to achieve all these, great work is done by research groups, employing a range of methods for detection of Ery in aqueous solutions from mini-emulsion polymerization[57] and synthesis of nanoparticles,

radiation-induced reversible addition−fragmentation chain transfer (RAFT)-mediated grafting on polymer tubes[13], magnetic imprinted sorbents prepared by picking emulsion polymerization[58], many research groups seek to enhance the existing methods performance by adding special materials and forming complex matrices[14], enhancing surface plasmon response of gold electrodes by adding gold nanoparticles[57], and nanoparticle decorated microcantilever sensor[55] for the fast detection and precise quantification of Ery.

Despite having congruent objectives, most currently developed MIP sensors suited for Ery detection are complex, costly, laborious, and rarely support online monitoring, consequently not addressing the need for a highly sensitive and selective Ery detection method with real-time monitoring support and low-cost[56]. With this objective in mind, we have prepared a reliable MIP sensor for the selective detection of Erythromycin with excellent recognition of the template and selectivity.

1.6 Experimental Methods

Electrochemical methods of analysis are a more promising method of analyte detection using molecular imprinting technology, where the current flow through the polymer is measured before and after the analyte rebinding, and the difference in current can be quantified as the amount of analyte rebinded[12]. Most notably cyclic voltammetry, electrochemical impedance spectroscopy, and differential pulse voltammetry.

1.6.1 Cyclic Voltammetry

Cyclic Voltammetry (CV) is considered one of the most adaptable electroanalytical techniques for the study of electroactive species[59]. Its versatility and simplicity of measurement have resulted in the extensive use of CV as the first experiment performed in an electrochemical study of an electrode surface or a compound.

In CV to obtain information about the redox potential and electrochemical reaction rates the voltage is swept between two values at a fixed rate (Fig. 7 A-D) i.e. once the higher voltage limit (D) is achieved the scan is reversed, and the voltage is swept back to (Fig. 7 D-A) to its lower limit[60].

The equilibrium established between the oxidized (Ox) and reduced (Red) species is described by the Nernst equation (Eq. 1). The potential of an electrochemical cell (E) to the standard potential of a species (E^0) and the relative activities of the oxidized (Ox) and reduced (Red) analyte in the system at equilibrium[61]. In Equation 1. F is Faraday's

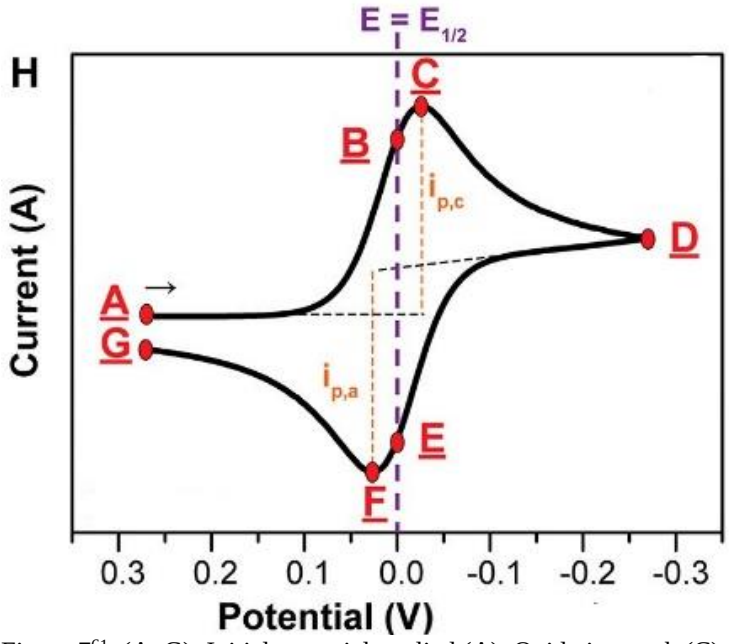

Figure 7[61]. (A–G): Initial potential applied (A), Oxidation peak (C), Higher potential limit (D), Reduction peak (F).

constant, R is the universal gas constant, n is the number of electrons, and T is the temperature. The resulting voltammogram is comparable to a traditional spectrum that represents information as a function of an energy scan[59].

$$\mathrm{E} = E^{o} + \frac{\mathrm{RT}}{\mathrm{nF}} \ln \frac{(\mathrm{Ox})}{(\mathrm{Red})} = E^{o} + 2.3026 \frac{\mathrm{RT}}{\mathrm{nF}} \log \frac{(\mathrm{Ox})}{(\mathrm{Red})} \qquad (1)$$

1.6.2 Electrochemical Impedance Spectroscopy

Electrochemical Impedance Spectroscopy (EIS) is considered a strong tool for probing the features of surface modified electrodes[62,63], a non-invasive technique that does not require bulky or complex instrumentation, is easy to operate, known for high precision and sensitivity[21,64]. This technique has been successfully employed in designing MIP-sensors as an analytical tool for measuring the electric properties of the integrated recognition element with the transducer. EIS involves the analysis of materials with dominant ionic conduction, where conduction can involve motion of ion vacancies[60,63], it is used to investigate the performance of batteries, fuel cells, semiconductors, monitoring corrosion, etc.

EIS measures the impedance of a system (Z, the ratio of the applied voltage to measured current Z=E/I, measured in Ω) by applying an alternating signal of small amplitude over a wide frequency range to the electrode. The initial perturbation applied is then compared to the response obtained from the electrode, by measuring the current phase change (φ) and voltage components and by the measurement of their amplitudes.

Impedance is a complex resistance that materializes through a circuit made of a combination of resistors, inductors, and capacitors. In the complex equation, the real part is attributable to resistors (in phase with applied voltage), and the imaginary part is attributable to the input of capacitors and/or inductors (out of phase with applied voltage by +π/2, -π/2 respectively).

$$Z = Z' + jZ'' = R - jX;\ X = \frac{1}{\omega C};\ j = \sqrt{-1}; \qquad (2)$$

In Eq. (2) R is the resistance (measured in Ω), C the capacitance (measured in Farads, F), X the reactance, and ω is the applied angular frequency (measured in rad/s, $\omega = 2\pi f$ where f is the frequency, Hz).

Experimental Impedance data can be fitted to the impedance of an equivalent circuit, comprised mainly of capacitors and resistors. In the equivalent circuit - resistance generally describes the conductive path while the capacitance describes space-charge-polarisation and modification of an electrode surface due to adsorption, within the system.

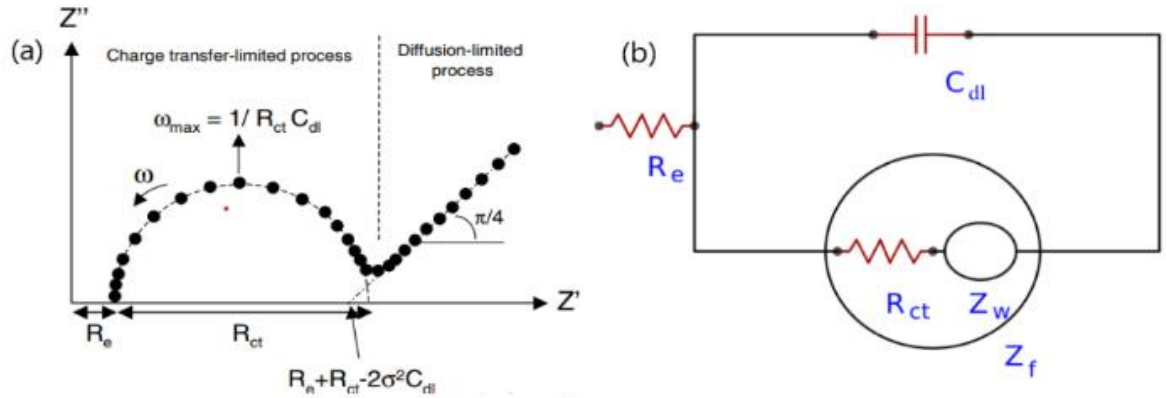

Figure 8. (a) Nyquist Plot[65], each dot of the Nyquist Plot represents the impedance at a given frequency. (b) Randles equivalent circuit.

There is no universal circuit to suit all EIS data, while the Randles circuit (Fig. 8b) is the simplest one, describes a cell where a single-step Faradaic process may take place, where R_e is the electrolyte resistance between working and reference electrodes, C_{dl} is the double-layer capacitance, Z_f is Faradaic impedance due to the charge-transfer process at the working at the electrode-electrolyte interface. Zf is subdivided into charge-transfer resistance (R_{ct}) and Warburg Impedance (Z_w). Warburg Impedance exhibits the influence of mass transport of electroactive species on the total impedance of the electrochemical cell and may become dominant for diffusion-limited processes.

A prevalent method of representing the obtained data is the Nyquist Plot (Fig 8a) where the real components (Z') are plotted versus the imaginary (Z''). In this plot, two distinct processes differentiated: a semicircle relating to a charge-transfer-controlled process, the intercept of the semicircle with the X-axis gives R_e and R_{ct} values, and a line

with a slope of 1 for the reason that Z_w extrapolated to the X-axis allows the calculation of the Warburg coefficient (σ), which permits the estimation of the electroactive species diffusion coefficients to be estimated[65].

EIS is extensively used as a standard characterization technique for many material systems and applications such as corrosion, film porosity, coatings, sensors, and others[66].

1.6.3 Differential Pulse Voltammetry

Differential pulse voltammetry (DPV) is a prominent pulse technique due to its high sensitivity, great resolution of the peak-shaped response, and minimization of background effects[67]. Pulses of fixed amplitude superimposed on a slope of increasing potential are applied to the WE[68]. The current is measured before the application of the pulse and after the second pulse. By sampling the current at the end of the successive potential pulses and plotting the current difference versus the potential, we obtain a peak-shaped response - a Gaussian graph, area of this peak is directly proportional to the concentration of analyte[67,68]. DPV technique is extensively used in electroanalysis for the determination of electrode processes.

1.7 Screen-printed Electrode

Screen-printed Electrode (SPE) represent a planar electrochemical substrate with all electrodes "printed" on its surface. In screen printing, the conductive ink is pushed through a mesh stencil on to a substrate, plastic, ceramic, polymer, other. After thermal curing and forming the specific pattern on the substrate surface, another layer of isolating material is applied forming the end product. SPE consists of a working electrode (WE), a reference electrode (RE) (Ag+/Ag) and a Counter (CE) electrode all positioned on a miniature polymer support (Fig 9). SPE substitutes the traditional electrochemical cell for a new, less cumbersome format, "disposable" printed electrodes and gives many advantages being a fast, small, portable and easy to use disposable electrode system[69,70,71]. Screen-printed electrochemical platforms form the basis of converting laboratory-based studies to "in-the-field" experiments. These platforms are fabricated on a large scale ensuring in low cost, reproducible sensors for a single use or can be modified, this being a great advantage, including the adaptability of the design and the possibility of using different inks that can include organic compounds, nanomaterials for the easy modification of their surface[72]. SPE provides a gateway into simple electrochemical analysis with low cost of materials, possibility of on-site testing[73,74,75]. for example for

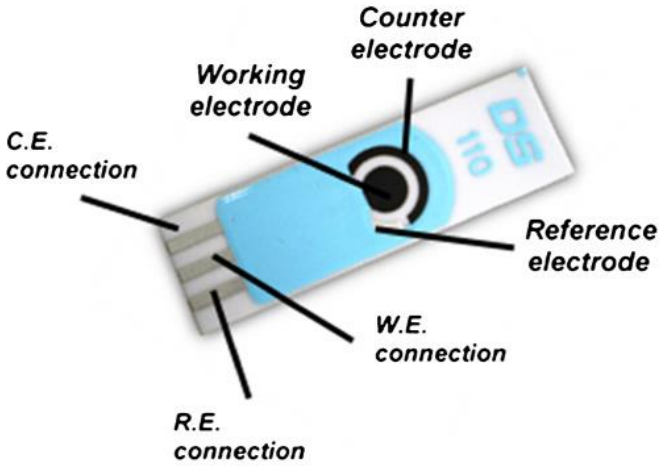

Figure 9. Screen-printed electrode main elements[72].

the detection of herbicides[70], DNA methylation[76], antibiotics[77] and other small molecules[77,78]. Relationship between electrode surface morphology and electrochemical activity of the electrode was established and the choice of the WE relies on the technique used and the electroanalytical application[75], and there are a whole host of commercially available working electrodes with a large variety of materials as the working electrode, notable: carbon, platinum, and gold.

1.8 Summary of the literature review and objectives of the study

Antibiotics discovery may be the single greatest contribution to human civilization, paving the way to greater productivity and quality of life. Antibiotics are increasingly becoming redundant due to the development of antibiotic-resistant bacteria as a result of their wide use. The pharmaceuticals, after being excreted with human waste, find their way into the environment - where they contribute to the development of antibacterial resistance in microbes, contacting sublethal doses of the drugs. Bacterial resistance to antibiotics makes it increasingly difficult to treat patients with infectious diseases.

Antibiotic resistance development must be interrupted, and different research groups are exploring methods for simple, fast, and reliable detection of antibacterials in environmental samples. Methods used today are insufficient, very costly, hard to operate, and requiring the use of analytical grade solvents. Chemists and Engineers alike are investigating new methods for a cheaper, faster, and more reliable method of antibiotic detection in environmental samples.

Synthetic receptors may be the solution for a remote, cheap, and reliable instrument for antibiotic detection in the environment having previously shown good detection for investigated compounds in environmental samples. Molecular imprinting technology (MIT) is widely perceived as a strong technique for the preparation of custom-made biomimetic materials, notably synthetic receptors. MIT has not reached its maturity, yet its potential can be judged by the increasing number of publications each year. A great number of research groups are working on the development of MIPs for solid phase extraction, remote pollutant detection, and fast analyte recognition, among others. A considerable limitation in the development of a robust and reusable biosensor is derived from the poor chemical and physical stability of traditionally used biological receptors (e.g., enzymes). A promising way of overcoming this limitation is the employment of molecularly imprinted polymers (MIP) with similar target recognition capacities while considerably more sturdy, stable, and resistant to different environmental changes. The MIP can be synthesized directly on the working electrode as a thin film via electrochemical polymerization to obtain a label-free biosensor for the fast analyte recognition and further quantification of the compound in a mixture. This method of detection does not require the usage of special solvents; it is stable at temperature variations and is resistant to great changes in acidity. Employing this technology on a simple and reusable transducer, such as screen-printed electrodes, we obtain a synergistic effect securing a simple, cheap, and reliable sensor for remote analyte detection with good reproducibility.

The aim of this thesis is to prepare erythromycin imprinted polymer film (Ery-MIP) on the working electrode of SPE for the electrochemical detection of Ery and to analyze the performance of the resulting Ery-MIP/SPE in terms of sensitivity to and selectivity for the target. To achieve this aim, the following objectives were formulated:

• Selection of a suitable electropolymerizable functional monomer;

• Determination and optimization of parameters for the synthesis of a polymer from the selected functional monomer in the presence of the template (Ery) on the working electrode of SPE;

• Removal of Ery from the polymer in an appropriate washing out solvent to generate Ery-MIP on SPE (Ery-MIP/SPE);

• Characterization of Ery-MIP/SPE in terms of selectivity and limit of detection toward Ery;

2. Experimental

2.1 Chemicals and Materials

All chemicals were obtained from Sigma-Aldrich, except acetic acid, which was provided by Lachner. The chemicals were used as received without further purification. Phosphate buffered saline (PBS) solution (0.01 M, pH 7.4) was used to prepare the synthesis and analyte solutions. Au-SPEs were purchased from BVT technologies, a.s, Czech Republic with a circular gold working electrode of 1 mm in diameter, a silver covered by AgCl as a reference and a gold counter electrode.

2.2 Functional Monomer Selection

Functional monomer for preparing Ery-MIP film with optimal performance was selected based on the prediction of binding energy potential existing between the monomer and Ery using theoretical, computational modeling. Several electropolymerizable monomers containing complementary functional groups were randomly selected. These include Dopamine (DOPA), Pyrrole (Py), 2-Aminopyridine (2-AP), 1H-pyrazolo[3,4-b]pyridine-3-carboxylic acid (Pyrazo), 1,8-Diaminonaphthalene (1,8-DAN), m-Phenylenediamine (mPD) and 2-mercaptonicotinic acid (MNA). To calculate binding energies for a monomer-antibiotic pair, structures of their pre-polymerization complexes were prepared and geometrically optimized by GaussView 5.0.9 software and semiempirical PM3 method respectively. Binding energies were then calculated by density functional theory (DFT) method using Gaussian'09 software.

2.3 Protocol for Ery-MIP film preparation

The preparation of erythromycin MIP on the gold surface of screen-printed electrode involves different stages (Fig. 10) including electrodeposition of polymer film in the presence of the template molecules (A) and subsequent removal (washing out) of the template molecules from the polymer matrix to reveal binding sites (B) for subsequent rebinding (C).

2.3.1 Electrodeposition of Ery-PmPD film on SPE

Electropolymerization of mPD and simultaneous deposition of PmPD on the gold working electrode of SPE was achieved by applying a constant potential (0.63 V vs

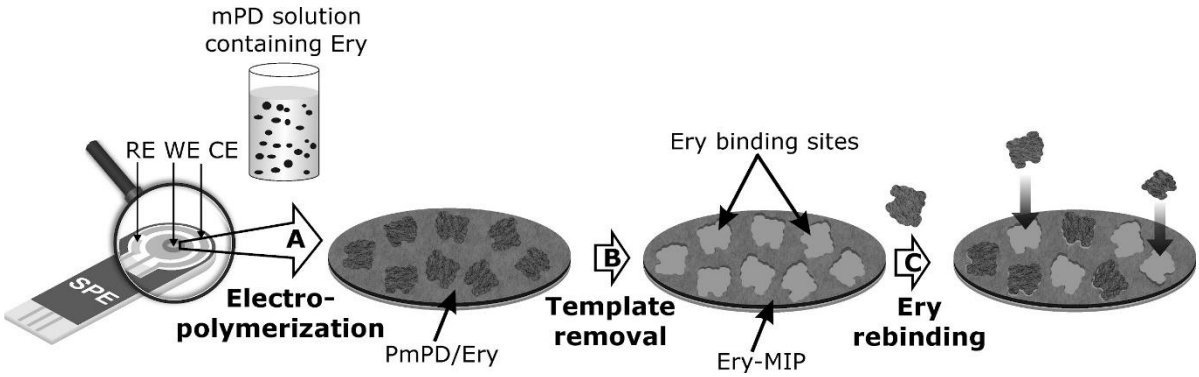

Figure 10. Schematics of the protocols for Ery-MIP film formation on the Au working electrode of SPE.

Ag/AgCl of SPE) to the working electrode of the SPE dipped in an aqueous solution of the monomer (5 mM). PBS (pH 7.4) solution was used as both the aqueous buffer and the electrolyte. Prior to film electrodeposition, activation of the working electrode of the SPE was achieved by electrochemical cleaning in 0.1 M sulfuric acid solution, cycling the potential between 0.1 and 1.15 V at a scan rate of 100 mV/s for 15 cycles. The electrodeposition of PmPD containing Ery film (PmPD-Ery) was achieved by dissolving Ery molecules in mPD solution prior to electropolymerization. Ery concentration used in the PmPD-Ery film formation is based on its solubility in the buffer solution; hence, concentrations ranging from 1 to 5 mM were used. PmPD and PmPD-Ery thicknesses were controlled by the amount of the electrical charge passed through the WE.

2.3.2 Template removal

Template molecules entrapped within the polymer were removed through a washing out process. This involves exposing the SPE coated film to appropriate washing out solvent or solvent mixture. Different solvents and mixtures were examined for removing Ery from PmPD-Ery film. These include acetic acid-methanol (1:9), dimethyl sulfoxide (DMSO), acetone, and ethanol.

2.4 Characterization and Monitoring of MIP preparation stages

Electrochemical techniques, including cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS), were employed in monitoring the different stages of the MIP preparation. All electrochemical measurements were made using the screen-printed electrode provided by BVT technology (a.s, Czech Republic) which was connected to an electrochemical workstation (Reference 600, Gamry

Instruments, USA) through a connector purchased from Palmsens BV (PS-CONN, the Netherlands). All potentials quoted in the text are against the Ag/AgCl reference of the SPE.

2.4.1 Electrochemical Impedance Spectroscopy (EIS)

EIS measurements were performed in a 1 M KCl solution containing 4 mM redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. An alternating potential with amplitude 10 mV and frequency range between 0.1 to 100 kHz were applied. The experiments were repeated for at least three times. The impedance spectra were fitted to an equivalent electrical circuit by using the equivalent circuit program provided by Gamry Echem Analyst software.

2.4.2 Cyclic Voltammetry (CV)

Similar to the EIS measurements, CV measurements were performed in a 1 M KCl solution containing 4mM redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$. The potentials were cycled between 0 and 0.5 V at a scan rate of 50 mV/s. At least 3 repeated scanning cycles were applied on each electrode.

2.5 Rebinding Study

The recognition properties of prepared Ery-MIP towards Ery were studied by differential pulse voltammetry electrochemical technique in 1 M KCl solution containing 4 mM of a redox probe ($K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$). Prior to rebinding measurement, the Ery-MIP SPE sensor was incubated in PBS buffer (pH 7.4) for 30 min followed by stabilization by DPV in probe solution to establish a stable baseline for the following analyte recognition measurements. Different analyte solutions were prepared in PBS buffer solution (pH 7.4) containing different concentrations of Ery: 12.8 nM, 64 nM, 320 nM, 1.6 μM, 8 μM and 40 μM. After baseline stability, subsequent incubation of the sensor in analyte solution and DPV measurements were carried out starting with the lowest analyte concentration. Analyte incubation was performed for 30 min followed by a further incubation in PBS for 5 min to remove loosely bound target before DPV measurements.

DPV measurements were recorded in the potential window between 0 V and 0.4 V with a pulse amplitude of 0.025 V, a pulse width of 0.1 s, a step potential of 0.005 V and a sample period 0.5s. DPV current peaks recorded were normalized according to Eq. 3, to

obtain response signals (I_n) of the Ery-MIP SPE sensor. Where (I_0) is the initial current response and (I) is the current response after analyte incubation:

$$I_n = (I_0\text{-}I)/I_0 \quad (3)$$

2.6 Selectivity Study

The selective recognition of Ery-MIP for Ery in the presence of other antibiotics were examined. This was achieved through a comparison of Ery-MIP responses towards Ery and other interfering antibiotics at the same concentration of 40 μM. Antibiotics used in the selectivity experiments include azithromycin, clarithromycin, ciprofloxacin, sulfamethizole, and amoxicillin (Fig. 11). Selection is based on close similarities with the target[79] e.g. azithromycin, clarithromycin; and the possibility of being located in the same environment together with Ery.

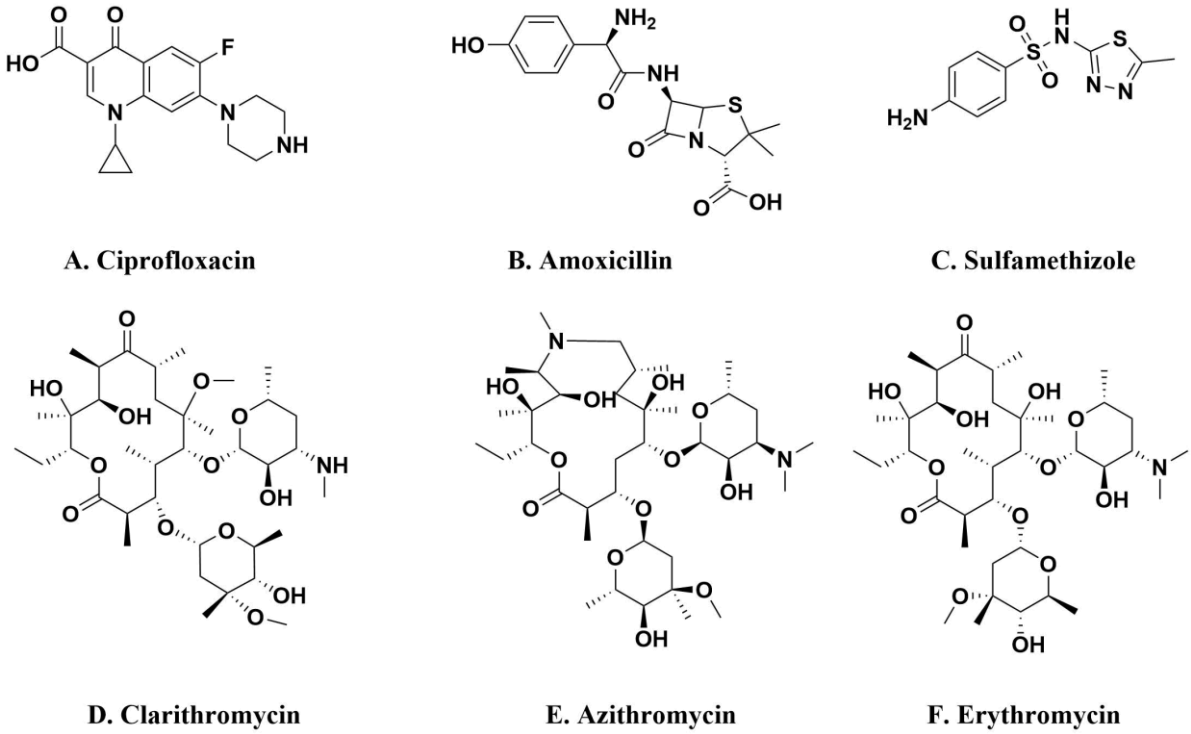

Figure 11. Molecular structures of antibiotic pollutants, ciprofloxacin (A), amoxicillin (B), sulfamethizole(C), clarithromycin (D), azithromycin (E), and Erythromycin (F).

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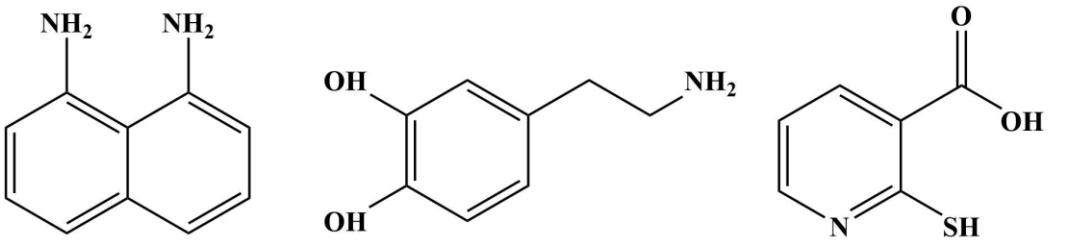
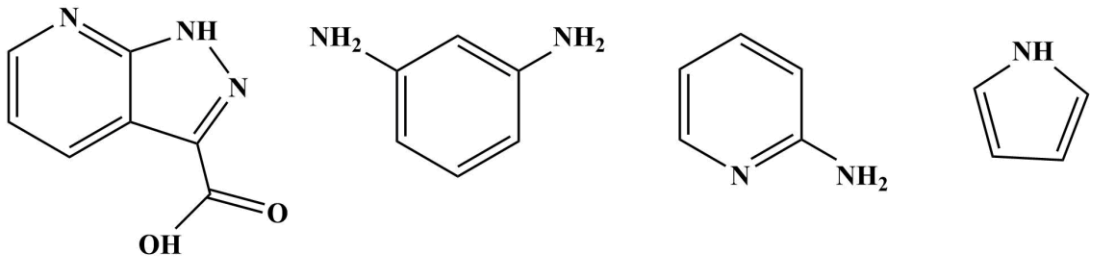
3. Results and Discussion

3.1 Functional Monomer Selection

A major factor that contributes to the optimal performance of an imprinted polymer is the selection of functional monomer. This is important since MIP performance is based on the strength of the interaction between the template molecule and a functional monomer that helps to ascribe molecular memory into the imprinted matrix by a combination of both shape and arrangement of functional groups. In this work, a suitable functional monomer would possess functional groups complementary to the functional moiety of Ery, good solubility in the porogenic solvent, and the possibility to synthesize stable homogeneous film in the presence of the template. Thus, to ensure an adequate selection of functional monomer that will produce an Ery-MIP with improved functionality, theoretical modeling was adopted. Theoretical, computational modeling has demonstrated great importance and application in MIP research with continuously increasing scientific interest.



1,8-Diaminonaphthalene (1,8-DAN) **Dopamine (DOPA)** **2-Mercaptonicotinic acid (MNA)**



***Pyrazo** **m-Phenylenediamine (mPD)** **2-Aminopyridine (2-AP)** **Pyrrole (Py)**

Figure 12. Randomly selected electropolymerizable monomers (*Pyrazo - 1H-pyrazolo[3,4-b]pyridine-3-carboxylic acid).

Several electropolymerizable monomers consisting of complementary functional groups to that of Erythromycin were examined as potential monomers for Ery-MIP

synthesis. Since Ery (Fig.1) is rich in hydroxyl groups that can participate in a hydrogen bond, monomers possessing functional groups that can interact with Ery through hydrogen bond formation were selected. Included in this list are Dopamine (DOPA), Pyrrole (Py), 2-Aminopyridine (2-AP), 1H-pyrazolo[3,4-b]pyridine-3-carboxylic acid (Pyrazo), 1,8-Diaminonaphthalene (1,8-DAN), m-Phenylenediamine (mPD) and 2-mercaptonicotinic acid (MNA). Their structural details are shown in Figure 12.

Table 1. Summary of QCC calculation.

Antibiotics	Antibiotic-Monomers H-bond energy (mH)						
	mPD	DOPA	PY	Pyrazo	MNA	2-AP	1,8-DAN
Erythromycin	**650**	323	96	208	190	**`663**	632

Table 1 shows a summary of the binding energy derived from theoretical calculations. As observed, 2-AP, mPD, and 1,8-DAN show very high binding energy towards Ery, indicating their capacity to form strong non-covalent interaction with Ery. This observation could be due to the improved activity of arylamines as proton donors coupled with the presence of non-sterically hindered primary amino groups in these monomers that could freely participate in hydrogen bond formation with the hydroxyl groups of Ery. Thus, according to the computational calculations, the two monomers with the highest binding energies, 2-AP and mPD were potentially suitable functional monomers for Ery-MIP synthesis. However, experimental results indicated that electrodeposited poly(2-AP) films on gold electrode demonstrate high instability on exposure to any organic solvents as depletion of the layer is easily observed while PmPD films are very homogeneous and stable. Consequently, further experimental work was carried out using mPD as an electropolymerizable functional monomer.

3.2 Ery-MIP film preparation

3.2.1 Electropolymerization and PmPD-Ery deposition

To prepare the PmPD-Ery film on the gold working electrode of SPE, electrosynthesis was carried out at a constant potential of 0.63 V. To preserve the structure of the template molecule, it is essential to ensure that the template does not oxidize at the electrodeposition potential of the chosen monomer. Thus, a prior CV scan was conducted to determine the oxidation potential of the template. As see in Fig. 13a, Ery exhibit electrochemical oxidation at approximately 0.72 V vs Ag/AgCl of SPE, thereby indicating its stability during PmPD-Ery deposition.

.Figure 13b indicates the time dependence of the applied charge density on the electrodeposition of both PmPD and PmPD-Ery films. As seen, the electrodeposition in the presence of the template takes longer time to achieve the applied charge density as compared to that of PmPD without template inclusion indicating that the presence of Ery in the synthesis solution affects the polymerization rate of mPD.

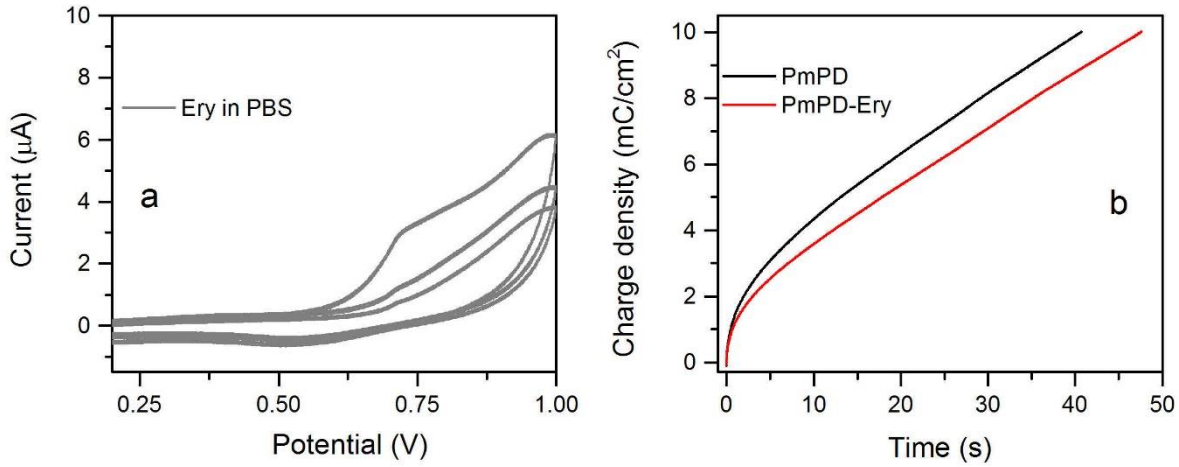


Figure 13. (a) CV scan in 5 mM Erythromycin in PBS pH 7.4 solution showing one irreversible current peak at ca. 0.72 V vs Ag/AgCl of SPE. Scan rate of 100 mV/s. (b). Charge-time dependence of PmPD and PmPD-Ery synthesized on the Au electrode of SPE at a constant.

3.2.2 Template removal and Ery-MIP formation

To ensure rebinding of the target, the imprinted template molecules must be removed from the polymer film. Solvent extraction is the common method used in achieving this aim in a process referred to as the washing out. This comprises exposing the SPE coated film to an appropriate washing out solution. For this purpose, consideration is given to different solvents or mixtures that allow solubility of Ery hence, faster elimination of entrapped molecules without affecting the polymer film. Thus, for removing Ery from PmPD-Ery films, we incubated the SPE modified films in acetic acid-methanol (1:9), dimethyl sulfoxide (DMSO), acetone and ethanol and the changes in charge transfer resistances (R_p) were monitored by EIS. Using this approach, it was assumed that the removal of template molecules should reveal cavities that enable improved diffusion of probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ $[Fe(CN)6]^{3-/4-}$ ions leading to a corresponding increase in charge transfer (i.e a reduction in charge transfer resistance) between the underlying gold substrate and the ionic species of redox probe solution[80].

Although acetone was reported to offer the best solubility for Erythromycin[81], it shows a very strong negative effect on the stability of PmPD polymer film dissolving the film in less than 30 min. Thus, acetone was excluded from the washing out solvent list.

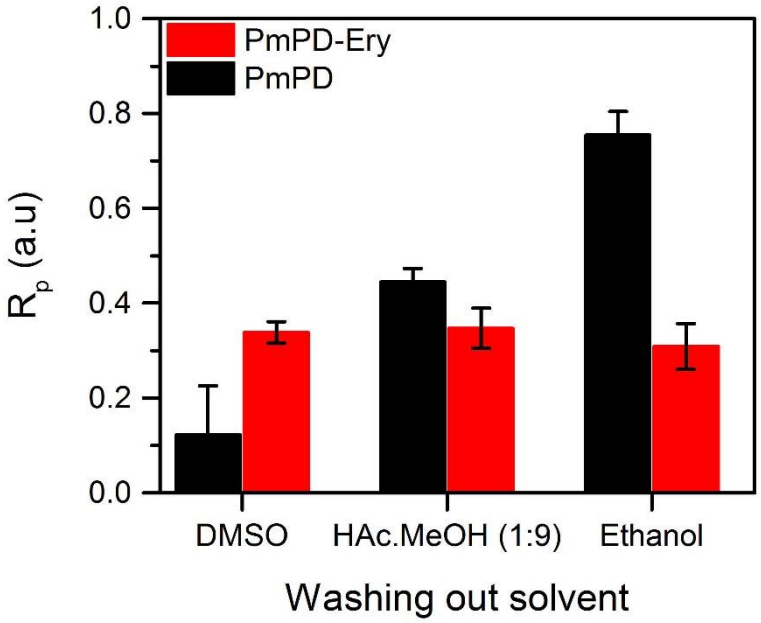


Figure 14. Polarization resistance (R_p), rescaled to 1, of the as-prepared PmPD and PmPD-Ery films after washing out in different solvents, DMSO, HAc.MeOH (1:9) and Ethanol.

Figure 14 shows the EIS R_p responses (rescaled to 1) after each solvent treatment. As observed, although DMSO shows a decrease in charge transfer resistance after MIP formation from PmPD-Ery, a look at the corresponding NIP signal however indicates that it has a very significant damaging effect on the polymer matrix as it also shows much lower resistance. Nonetheless, a comparison of acetic acid methanol mixture and ethanol reveals that ethanol is a better washing out solvent for Ery-MIP formation from PmPD-Ery. This conclusion stems from two obvious observations after ethanol treatment relative to that of acetic acid methanol: the lower charge transfer resistance observed on the MIP, and the less damaging effect (less significant change in resistance) observed on the NIP. Therefore, ethanol was more suitable in removing more template molecules from PmPD film and was selected as the appropriate solvent for Ery-MIP formation.

3.2.3 Characterization of electrode modification

To monitor each step of the Ery-MIP preparation, EIS and CV measurements were carried out (Fig. 15). As shown, after PmPD-Ery film formation, the diameter of the semicircle greatly increased, indicating an increasing charge transfer resistance. However, Ery-MIP formation, a quite significant decrease in the charge transfer is

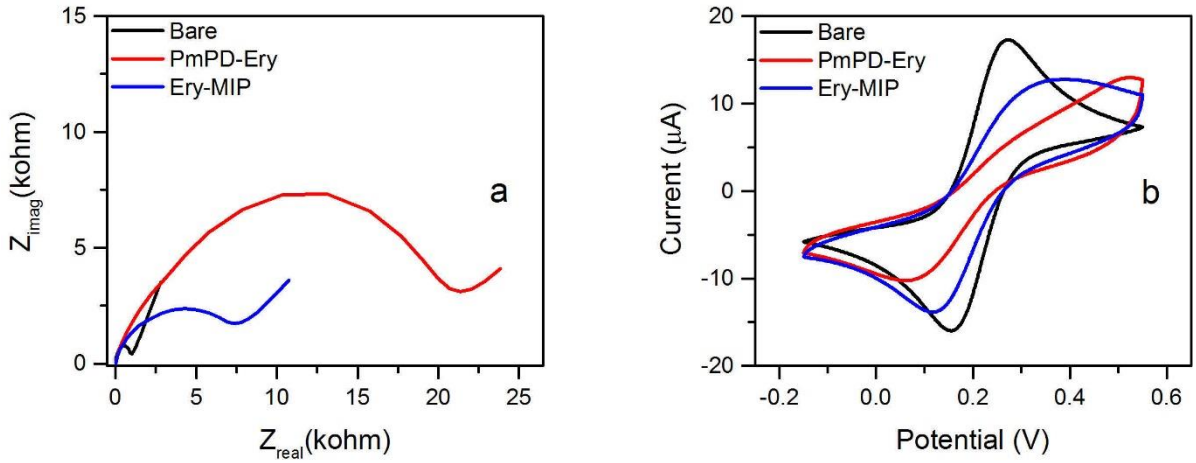


Figure 15. EIS (a) and CV (b) characterization of MIP preparation on WE of SPE in 1 M KCl solution containing 4 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ redox probe solution.

observed. A similar result is observed by CV where the redox peaks of the bare Au electrode was greatly decreased after PmPD-Ery formation but was improved after washing out procedure.

3.3 Optimization of MIP performance

3.3.1 Washing out time

Following the selection of the washing out solvent, the required incubation time for efficient template removal was examined. This was achieved by following the increase in charge transfer ($-R_p$) after each successive treatment in ethanol for 30 min and overnight (16 hrs). As shown in Fig. 16, charge transfer increases drastically up to 1.0 hr and steadily

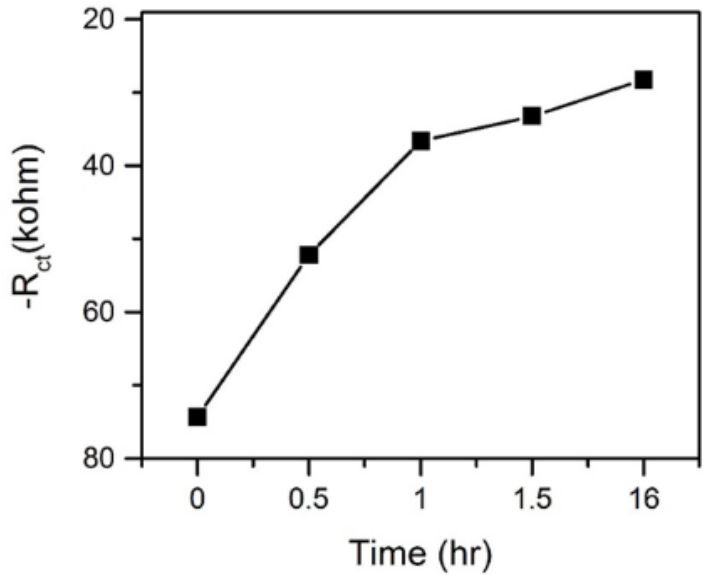


Figure 16. Time-dependent increase in charge transfer ($-Rp_{ct}$) due to the effect of washing out PmPD-Ery film in ethanol for 30 min time interval and overnight (16 hrs).

increased up to 1.5 hrs; however, overnight treatment further indicates an increased charge transfer resulting from more template removal.

For a more efficient washing out and optimal MIP performance, overnight treatment for 16 hrs was selected as an appropriate template removal time and was subsequently used in this work.

3.3.2 Template-Monomer concentration ratio

One of the factors determining the success of molecular imprinting is the stoichiometric ratio of template to monomer. This is of high importance in non-covalent molecular imprinting in which the arrangement of the pre-polymerization complex formed between the monomer and template molecules dictates the MIP performance. Thus, to ensure optimal performance, we examined different combinations of monomers-template concentrations ratio by fixing mPD concentration at 5 mM and varying Ery concentration from 1 to 5 mM.

As shown in Fig. 17, signals corresponding to the rebinding of Ery on Ery-MIP decreases with increasing template/monomer ratio from 0.2 to 1. This result is in agreement with previously reported works on non-covalent imprinting[82] in which the optimum MIP performance was said to be obtained at a template-monomer ratio less than 1, as a further increase in template concentration fails to optimize the MIP performance. In this work, although, 1:5 template-monomer ratio gives the highest signal, however, using a large number of monomers may also result in higher non-specific sites thus

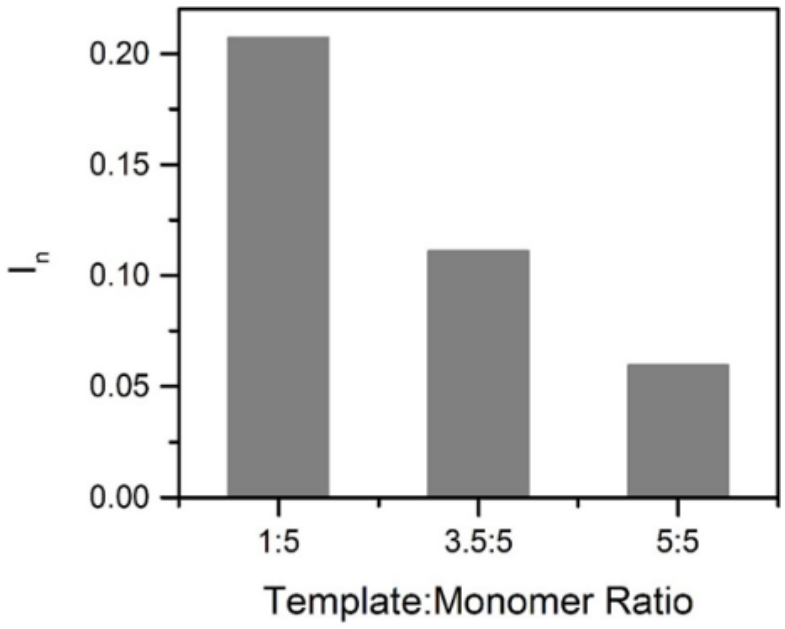


Figure 17. Normalized DPV signals, resulting from rebinding of 40 µM Ery on Ery-MIP prepared using different ratios of the template:monomer (1:5, 3.5:5 and 5:5).

leading to an increase in non-specific interactions during rebinding. Consequently, we have selected 3.5:5 as the safe template-monomer ratio for Ery-MIP formation.

3.3.3 Thickness Optimization

To ensure the synthesis of film layers with reproducible thicknesses, the forming PmPD and PmPD-Ery films were electrodeposited on the WE of SPE by controlling the amount of electrical charge passing through WE.

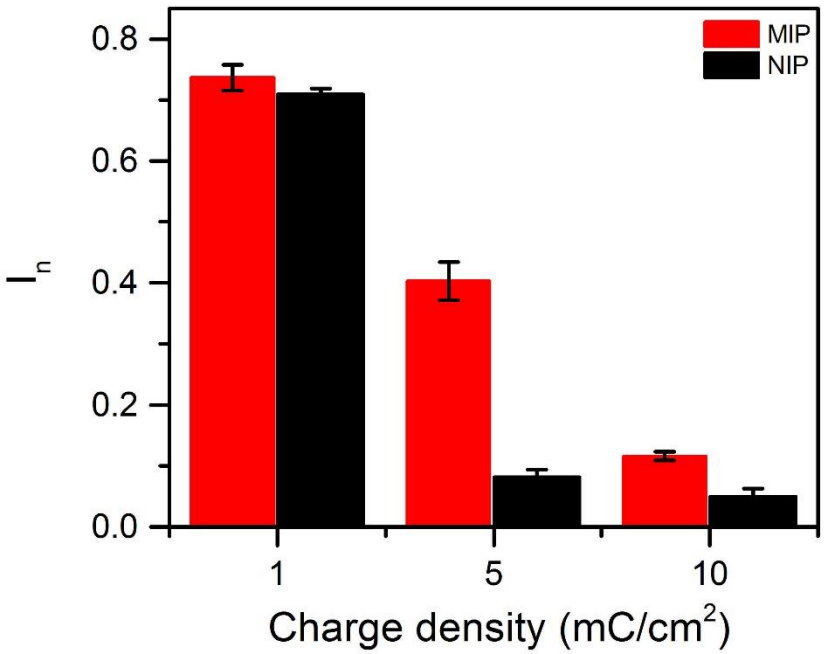


Figure 18. Normalized DPV current peaks, resulting from rebinding of 40 µM Ery on Ery-MIP prepared using different charge density through the WE of SPE (1, 5 and 10 mC/cm^2).

To determine the thickness that gives the optimal performance, films having different thicknesses were electrodeposited by applying different charge densities (1, 5 or10 mC/cm^2) to WE. As observed in Fig. 18, although the thinnest Ery-MIP film prepared by 1 mC/cm^2 gives the highest signal, the corresponding NIP signal is also very high indicating a great contribution from non-specific binding that compromises the performance of this MIP. Vice-versa Ery-MIP prepared by 10 mC/cm^2 shows a relative lower contribution from non-specific interaction, but the lower response is obtained. Notwithstanding, much higher response and comparatively lower contribution from non-specific interaction are achieved from film prepared by 5 mC/cm^2, thereby indicating its better suitability. Thus, it can be concluded that although little is known about the imprinting mechanism, the effect of thickness on imprinting efficiency should not be underestimated when building a PmPD-based MIP sensor for the detection of Ery.

3.4 Rebinding Study

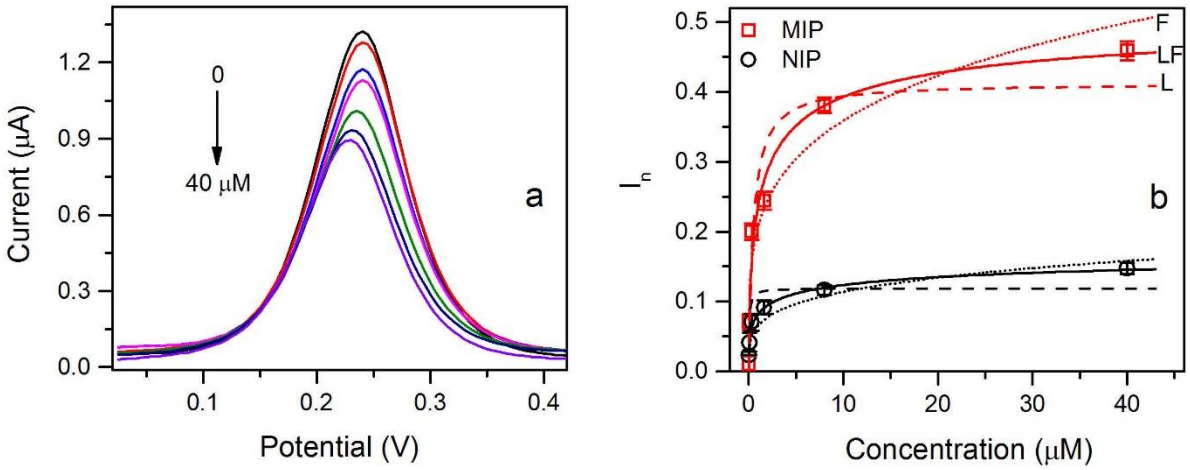


Figure 19. (a) DPV curves recorded in 1 M KCl solution containing 4 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ on the Ery-MIP modified SPE after incubating in Ery solutions of increasing concentration (12.8 nM, 64 nM, 320 nM, 1.6 μM, 8 μM and 40 μM); (b). Binding isotherms of Ery.

The recognition capacities of Ery-MIP towards Ery was determined through a rebinding study achieved by DPV in the redox probe solution after incubation in increasing analyte concentration. To properly evaluate the recognition properties, the responses to the increasing analyte concentration were plotted as an adsorption isotherm and fitted to an appropriate mathematical model of adsorption. For this purpose, common models were tested, including Langmuir (Eq. 4), Freundlich (Eq. 5) and Langmuir-Freundlich (Eq. 6):

$$I_n = I_{max} * C/(K_D + C) \quad (4)$$

$$I_n = I_{max} * C^m \quad (5)$$

$$I_n = I_{max} * C^m/(K_D + C^m) \quad (6)$$

Where I_n and I_{max} are the sensor responses (Eq. 3) upon Ery rebinding and saturation, respectively, m is the heterogeneity, and C is Ery concentration in solution. R^2 is the coefficient of determination and provides a measure of how well the observed outcomes are replicated by the model ranging from 0 to 1, the higher the value, the better the fit to the analyzed model. K_D is the equilibrium dissociation constant. The parameters derived from the fitting are shown in Table 2.

Table 2. Parameters derived from fitting the adsorption isotherms to different models.

	Langmuir		Freundlich		Langmuir -Freundlich	
	MIP	NIP	MIP	NIP	MIP	NIP
I_{max}	0.40±0.03	0.11±0.02	0.20±0.02	0.07±0.01	0.5±0.1	0.2±0.1
K_D	0.5±0.2	0.05±0.01	-	-	1.2±0.4	1.4±0.2
m	-	-	0.20±0.03	0.20±0.02	0.6±0.1	0.4±0.1
R^2	0.961		0.963		0.987	

Figure 19b and R^2 values in Table 2 indicate that the LF model gives a more accurate fit to the experimental data measuring the binding interactions between Ery and Ery-MIP surface. Thus, from the point view of the LF model, Ery-MIP shows a much higher binding capacity than the corresponding NIP (0.52 vs 0.19) revealing probably increased porosity in Ery-MIP vs NIP due to the formed binding sites as a result of the imprinting protocol.

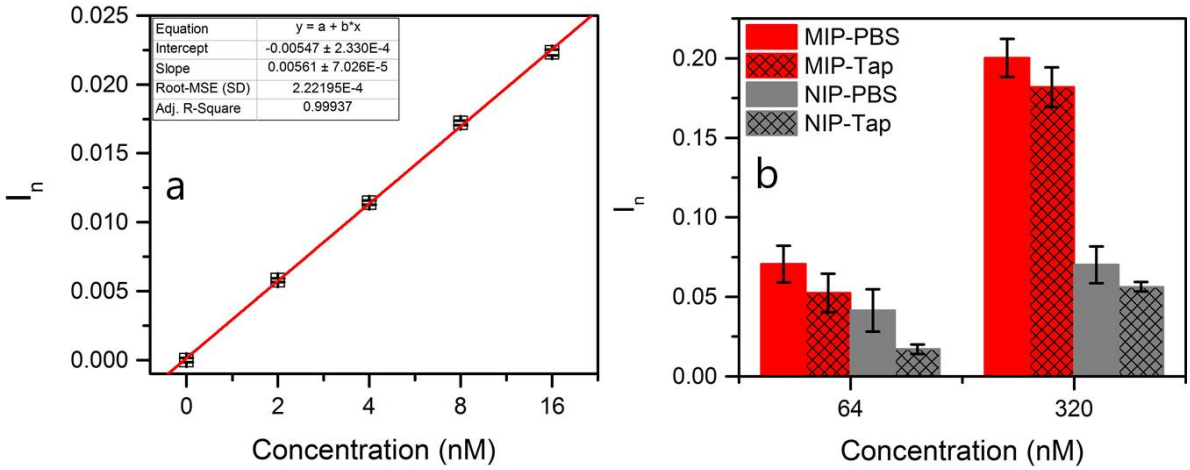


Figure 20. (a) Linear rgression plot of Ery-induced response on Ery-MIP after low analyte concentration injection (2-16 nM), (a) normalized DPV current peaks, obtained from incubation of Ery-MIP and NIP sensors in different Ery concentrations in tap water.

When incubated in low concentration template solution, Ery-MIP exhibits an LoD of, 0.12 nM determined from Fig. 20a with Eq. (7)[83], wheres $S_{x/y}$ and b are the estimated standard deviation and the slope of the analytical calibration function:

$$\mathrm{LoD} = 3 * S_{x/y}/\mathrm{b} \qquad (7)$$

Furthermore, to determine the behavior of the sensor in environmental water, similar rebinding experiments were repeated in tap water samples. For this purpose, two analyte concentrations (64 nM and 320 nM) were prepared in tap water, and the responses were monitored by DPV in the probe solution following Ery-MIP and NIP sensors incubation as previously described. As observed in Fig. 20b, rather similar responses are

obtained in PBS and tap water. Although Ery-MIP responses on the tap water prepared samples are relatively lower than those on prepared from PBS, they are still within the range of error. It is assumed that this lower response observed for both Ery-MIP and NIP could be related to the less conductivity of tap water when compared to the buffered PBS solution. Thus, the sensor has displayed potential suitability for its use in environmental media such as tap water.

3.5 Selectivity of Ery-MIP

The environment represents a blend of different molecules and compounds. Thus, an environmental sample for antibiotic detection may contain other antibiotic molecules different from the target. These interfering molecules can affect the performance of a MIP sensor through cross-reactivity with the MIP binding sites. Thus, one of the factors

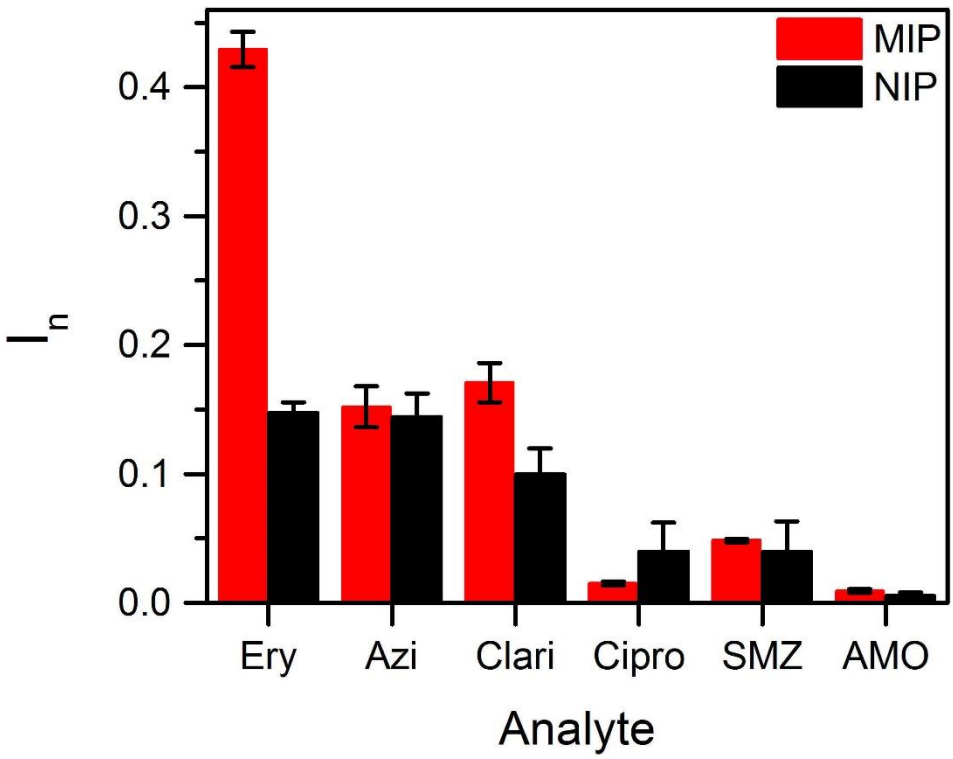


Figure 21. Normalized DPV current peaks, obtained from incubation of Ery-MIP and NIP sensors in similar concentrations (40 µM) of different antibiotics (Azithromycin, Azi; Clarithromycin, Clari; Ciprofloxacin, Cipro; sulfamethizole, SMZ; and amoxicillin, AMO).

determining the quality of a MIP sensor is its ability to show preferential binding towards the target in the presence of other similar or interfering molecules. This property is called selectivity[84]. The selectivity of Ery-MIP was tested in samples containing an antibiotic compound with and without close structural similarities to Ery. These include azithromycin, clarithromycin, ciprofloxacin, sulfamethizole, and amoxicillin (Fig. 11). The identical concentration of each compound (40 µM) were prepared in PBS, and

following incubation, the resulting DPV signals were compared to the signal resulting from the target at the same concentration. To appreciate how selective the biosensor I, the selectivity factor (α) was calculated according to Eq. (8).

$$\alpha = I_{n\,Target}/I_{n\,interferent} \quad (8)$$

Figure 21 shows the result of the selectivity study. As seen, Ery-MIP shows the highest response towards Ery as compared to other analytes. Although Clari and Azi have very close structural similarities to Ery (Fig. 11), Ery-MIP demonstrated about three times preferential recognition towards Ery than these analogs.

Table 3. Selectivity factor values of different interfering antibiotics used in the selectivity experiment

Analyte	I_n	α
Ery	0.43 ± 0.01	-
Azi	0.15 ± 0.01	2.9
Clari	0.17 ± 0.01	2.5
Cipro	0.015 ± 0.001	28.7
SMZ	0.050 ± 0.001	8.6
AMO	0.010 ± 0.001	43

Furthermore, Ery-MIP displayed more recognition of structurally related molecules. Thus, the result indicates a good capacity of Ery-MIP in discriminating between Ery among other antibiotics.

Summary

Antibiotics represent a major contributor to our modern way of living, being used in both medicine and agriculture, considered an essential part of modern life. However, since their discovery, they are being overused and misused. In recent decades, they started being considered as environmental pollutants and became a subject of a more thorough investigation into their presence in environmental waters as pollutants. In the antibiotic-contaminated environment, bacteria encounter sublethal doses of the antibiotics, causing the development of antimicrobial resistance (AMR), i.e., the ability of the bacteria to withstand the antibiotics working against it. Obviously, AMR is a source of concern for the medical community, making it increasingly difficult to treat patients with infectious diseases. Thus, environmental monitoring for the presence of antibiotics must become a regular activity in order to prevent the formation and spreading of AMR. Currently used monitoring methods like chromatography, mass spectrometry, solid phase extraction, and ELISA show great precision and reproducibility. However, they require resources and conditions often unattainable in remote areas and therefore, can not be employed in routine monitoring of the environment. A promising method of monitoring the presence of pollutants in the environment is the employment of sensors modified with synthetic receptors as recognition elements.

Synthetic receptors, generated through "molecular imprinting", so-called Molecularly Imprinted Polymers (MIPs), are known for their excellent recognition of the target molecules while being cheap and reproducible in manufacturing, demonstrate excellent physical and chemical stability. MIPs are generated through the polymerization of a mixture of functional monomers in the presence of an analyte, that acts as a template. In the process of the polymerization, the analyte molecules are fixed in the growing polymeric matrix and after their removal binding sites capable of selectively recognizing the analyte-similar molecules are formed. MIPs have shown great stability and operability for environmental monitoring and recognition of small molecule pollutants, like antibiotics.

The purpose of this thesis was to develop a robust and highly selective MIP film interfaced with a screen-printed electrode (SPE) for electrochemical detection of erythromycin in a label-free manner. Erythromycin (Ery) was selected as the target analyte due to its consistent presence in the EU's Surface Water Watch List (SWWL) as part of the macrolide class of antibiotics. SPE was selected as a cost-effective platform for real-time monitoring of molecular binding events happening in the MIP. The thesis

describes in detail the preparation of the Ery-MIP/SPE and its characterization in terms of selective detection of Ery. The prepared Ery-MIP/SPE showed encouraging data in terms of recognition of Ery demonstrating nanomolar LoD and considerable selectivity versus similarly structured antibiotic molecules. The described method for the preparation Ery-MIP shows great promise for the future development of a portable device for real-time monitoring of antibiotic pollutants in aqueous environments.

Conclusion

The thesis has demonstrated a possibility to use a robust and highly selective Ery-MIP film interfaced with a SPE (Ery-MIP/SPE) for electrochemical detection of Ery in a label-free manner. Erythromycin (Ery) was selected as the target analyte due to its consistent presence in the EU's Surface Water Watch List (SWWL) as part of the macrolide class of antibiotics. SPE was selected as a cost-effective platform for real-time monitoring of molecular binding events happening in the MIP. The following conclusions can be drawn from the study:

• The computational studies supported the selection of 2-Aminopyridine (2-AP) and meta-phenylenediamine (mPD) as suitable functional monomers for the electropolymerization of thin Ery-containing polymer films on the gold surface of a SPE. However, the following experimental studies revealed that a film prepared from 2-AP monomer could not withstand the washing out step. Therefore, mPD was used to prepare Ery-MIPs;

• The capacity of Eri-MIPs to recognize Ery was found to have substantial dependence on the ratio of Ery to the functional monomer in the prepolymerization mixture. The optimal ratio was found to be 1:0.7;

• Binding of Ery to Ery-MIP was best described by the Langmuir-Freundlich adsorption model suggesting a heterogeneous nature of the binding process

• Ery-MIP/SPE showed significant selectivity towards Ery as compared to compounds with related structures Cipro, Amo, SMZ, Clari, and Azi, in both PBS and tap water. In particular, in PBS, selectivity was determined to be 2.5, 2.9, 8.6, 28.7 and 43 for Clari, Azi, SMZ, Cipro, and Amo respectively;

• Ery-MIP/SPE showed noteworthy LoD of 0.12 nM, for Ery;

The integration of Ery-MIP with SPE showed great promise for the future development of a portable device for real-time monitoring of antibiotic pollutants in aqueous environments.

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Figure 9[72],[85]; Figure 6 (c)[86], (b)[87], (a)[88]