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Approaches to the Rational Design of Molecularly Imprinted Polymers Developed for the Selective Extraction or Detection of Antibiotics in Environmental and Food Samples

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The World Health Organisation (WHO) reported antimicrobial resistance (AMR) as a global threat comparable to terrorism and climate change. The use of antibiotics in veterinary or clinical practice exerts a selective pressure, which accelerates the emergence of antimicrobial resistance. Therefore, there is a clear need to detect antibiotic residues in complex matrices, such as water, food, and environmental samples, in a fast, selective, cost-effective, and quantitative manner. Once problematic areas are identified, can extraction of the antibiotics then be carried out to reduce AMR development. Molecularly imprinted polymer (MIPs) are synthetic recognition elements produced through the biomarker of interest being used as a template in order to manufacture tailor-made ligand selective polymeric recognition sites. They are emerging steadily as a viable alternative to antibiotics, especially given their low-cost, superior thermal and chemical stability that facilitates on-site detection, simplified manufacturing process, and avoiding the use of animals in the production process. In this paper, the authors critically review literature from primarily 2010–2020 on rational design approaches used to develop MIPs for sensing and extraction of antibiotics, providing an outlook on crucial issues that need to be tackled to bring MIPs for antibiotic sensing to the market.

1. The Need for Detection of Antibiotic Residues

1.1. Antimicrobial Resistance (AMR) and Its Acceleration through Water Contamination

The World Health Organization (WHO) predicts ten million annual deaths, globally, due to antibiotic-resistant infections, by 2050.[1] The rise in antimicrobial resistance (AMR) bacteria is therefore considered a global threat. Cassini et al.[2] studied deaths linked to drug-resistant infections in Europe and concluded that ≈700 000 infections in 2015 were related to resistant bacteria, of which 5% were fatal. Similarly, a study by Ventola[3] demonstrated that two million patients in the USA developed hospital-acquired infections (HAIs), of which 99 000 cases proved fatal, a significant proportion of which were also caused by resistant bacteria. In addition to their impact on mortality, these resistant bacteria also pose a serious financial burden. Misurski et al.[4] demonstrated that incorrect antibiotic prescription was estimated to be a $211 million burden,

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per year, in the USA alone. These costs are ascribed to prolonged hospitalization, increased morbidity, greater requirement for critical care support, delayed return to the workforce, and consequent economic impact associated with reduced productivity.

Moreover, recent events have led to concerns about a new surge in AMR due to the use of antibiotics in patients with COVID-19.[5] While antibiotics are ineffective against viruses, they can be used in patients with confirmed COVID-19 to prevent or treat secondary bacterial infections: an early study from China showed that secondary infections and HAIs were present in half of all deceased COVID-19 patients.[6] Surprisingly, no current inclusions of antimicrobial stewardship programs (ASPs) in disaster planning or emergency response preparedness efforts have been legislated, but there is a strong drive from the medical and scientific community to integrate ASPs into disaster planning and appoint stewards in a more formal role after the COVID-19 outbreak.[7]

Another concerning issue is the possibility of antibiotics leaching into water systems, via the effluents of the pharmaceutical industry, agriculture, and hospitals (**Figure 1**). However, these routes are all critical to the continuation of modern-day living so a solution is needed that does not impact them. Behera et al.[8] studied the introduction of pharmaceuticals, including antibiotics, into the environment in an industrial city in South Korea. Among the antibiotics screened, standard water treatments achieved antibiotic removal between only 10 and 70%. Sulfamethazine was found to have a removal efficiency below 30%, suggesting that the majority still entered further waterways. Conversely, caffeine, a common contaminant and a standard indicator molecule, showed a 99% removal efficiency under the same conditions. This exemplifies how using caffeine as an anthropogenic marker[9] may therefore yield false confidence in current purification processes.

Effluents from modern-day infrastructure are not the only route of antibiotics into the water systems. Alternative entry routes include livestock feed and excrement. Watanabe et al.[10] led a study into the waste outputs of two dairy farms in the USA. It was found that monensin, a polyether antibiotic commonly found in animal feeds, leached into ground water assumedly due to relatively high monensin concentrations found in manure. Tasho et al.[11] highlighted veterinary antibiotic use in livestock and the resulting antibiotic residue in livestock manure, which can be as high as 216 mg L^{-1}. Given that 0.9% of farming worldwide is conducted organically,[12] which equates to a significant amount of natural fertilizer use, the critical role of agricultural practices in the leaching of antibiotics into the environment is highlighted.

Watkinson et al.[13] traced the journey of drinking water all the way back to hospital effluent, evaluating the concentration of 28 antibiotics at different stages in Southeast Queensland, Australia. Some antibiotics were detected at concentrations of up to 64.0 μg L^{-1} in Waste Water Treatment Plant (WWTP) influent, compared with the maximum concentration in the effluent being 3.4 μg L^{-1}. It was shown that rivers having no effluents from WWTPs in their watershed have a significantly lower residual antibiotic concentration, demonstrating the broader impact of hospital pharmaceutical waste. A study based in Ter River, Spain, by Rodriguez-Mozaz et al.,[14] incorporated a wider range of antibiotics, showing that fluoroquinolones had the highest residual concentration in hospital effluents up to 14.4 mg L^{-1}, which is most likely due to their enhanced stability compared with other common antibiotics. Both studies show significant presence of antibiotics in water systems from developed counties, portraying that even countries with modern wastewater treatment infrastructure are being impacted by antibiotic presence in water systems.

The European Water Framework Directive is a legislative department set up in 2000, to ensure water safety. Carsten von der Ohe et al.[15] aimed to improve the existing European Water Framework Directive by classifying chemicals of critical importance in terms of their potential to cause harm. With more in-depth understanding of the harm AMR is causing, improvements to the legislation can lead to more appropriate restrictions on antibiotic presence in the environment. Despite this, Carvalho et al.[16] subsequently noted the lack of relevant regulations

Figure 1. Routes of entry of antibiotics into the environment. Green arrows displaying antibiotic removal from the environment, red arrows showing the entry routes of antibiotics, and orange arrows explaining that extraction methods are not fully efficient at antibiotic removal.

regarding the same issue in the European environmental water quality standards. It is therefore of critical concern that carriers of antibiotics, such as meat or milk, and common water micropollutants are regulated; however, there remains limited scrutiny of antibiotics in water systems; therefore, a system to accurately measure and follow antibiotics from a variety of media, specifically and critically including aqueous media, must be agreed upon.

The current lack of antibiotic regulation and therefore a need for detection within Europe's waterways could have dire consequences and certainly contribute to exacerbating AMR in the future. Hence, there is a need to identify and more closely monitor waste streams that may contain antibiotics and ultimately enforce new regulations for their processing and removal. However, these testing and analyses methods must be cost effective and financially viable if they are to be broadly implemented.

1.2. Limitations of Current Antibiotic Detection Platforms in Different Media

Extensive efforts are required for the research and development of novel detection systems for different families of antibiotics and to be able to do so in different media. In a study, Khaskheli et al.[17] displayed a procedure for the screening of β-lactam antibiotics in milk using a qualitative field disc assay. Despite encouraging results, the biggest drawbacks of the systems were 1) the 24 h turnaround time and 2) the extensive sample preparation required. The first issue was originally addressed by Knecht et al.,[18] with the use of an automated microarray for simultaneous detection of ten antibiotics in milk; however, this system needed the use of costly infrastructure and apparatus. Wang et al.[19] expanded the scope to meat and aquatic products; after these were minced and extracted, ultraperformance liquid chromatography was used to study residual levels in the range of 0.05 ng g^{-1} in meat and 0.2–5.0 ng mL^{-1} in milk. A more detailed review of antibiotics detection was released by Pikkemaat.[20] From this work, and the studies provided beforehand, it emerges that the most common analysis technique in the first decade of the 2000s involved microbial screening assays: these are cost effective, despite being time-consuming, and do not offer quantitative results.

Baquero et al.[21] gave an oversight as to the main ways in which antibiotics are detected in water samples. Depending on the analyte of interest, common detection systems include electrophoretic and chromatographic techniques as well as voltammetry and amperometry detection systems. This study shows that several antibiotics can be accurately monitored in a wide range of media and by different detection methods. However, all these techniques required 1) a lab environment, 2) time-consuming procedures, and 3) skilled personnel. Smith et al.[22] used a commercially available test kit that was modified to enable detection of antibiotics in water systems, trying to optimize the detection. The study provided a qualitative test for antibiotics but lacked the critical quantification. While these in-field testing kits are of promise due to the rapid and on-site detection of antibiotics that will yield more accurate information and simplicity of operation even for nontrained users, further development is still required to enable crucial quantification.

Colorimetric bacterial inhibition approaches and lateral flow immunoassays are common for on-site detection of antibiotics. However, colorimetric bacterial inhibition requires large sample volumes and is limited by poor sensitivity and complex user protocols, whereas lateral flow immunoassays require user intervention for quantifying results.[23] All these limitations can be worked around with the use of molecularly imprinted polymers (MIPs).

1.3. Potential of Using MIPs for Antibiotic Detection and Extraction

MIPs are custom-built, synthetic recognition sites, designed for a specific target molecule.[24] These synthetic receptors can be used in place of antibody–antigen, enzyme–substrate, or ligand–receptor interactions and indeed can be fabricated to have similar, if not better, affinity and selectivity than their naturally occurring counterparts[25] and crucially when no naturally occurring antibodies exist. The synthesis of MIPs can be accomplished in nonspecialist laboratories, with nondedicated equipment, and is often seen as a relatively simple process, with only little formal training in polymer chemistry required. However, its simplicity might be misleading and can mask the fact that it involves multiple and often interdependent variables. The ways in which these affect and change the properties of the resulting polymers are, in fact, quite complex and require a good understanding of molecular recognition theory, thermodynamics, and polymer chemistry.[26]

The concept of MIPs and their potential usefulness in multiple fields of scientific research have been gaining significant interest since the early 1990s.[27–29] Despite their obvious benefits over naturally occurring recognition biomolecules, particularly their increased stability and specificity, as well as their low cost, ease of production, and ability to target molecules for which natural receptors do not exist,[30,31] they are yet to garner widespread and commercial success.

MIPs were found to be highly suitable first for application in chromatography,[32–34] especially liquid chromatography.[35,36] Curti et al.[37] developed the first truly functional silica-derived-imprinted polymer systems, which subsequently became a well-established technique in the chromatography field. Significant advances in imprinting techniques and new synthetic methodologies, along with their excellent recognition specificity and structural predictability, make them a valuable alternative in the recognition systems landscape. This in turn has opened their potential use to a wide variety of applications.[38] After the development of a noncovalent fabrication method by Mosbach and coworkers[39], and modifications thereof, the use of these synthetic ligands has continued to grow. This is evident by the exponential increase in research papers over the past 20 years.

To date the use of MIPs has mostly been limited to academic research. Although MIPs have found their way into several commercial markets (**Table 1**), with further exploration, they could be implemented even further. Whitcombe and coworkers[40] stated that MIPs could seize 1–3% of the separation techniques market, worth $1.19 billion, based predominantly in the chromatography column sector alone.

Table 1. Summary of commercial MIPs that have been developed.

MIP	Company	Description
MIP cartridges for extraction[224]	Acros (SupelMIP)	SPE of 14 aminoglycosides (environmental contaminants) in foodstuffs, e.g., meat, milk, and fish
Epitope-imprinted MIPs[227]	Aspira Biosystems	Specific and selective uptake of micro-organisms
MIPs as model drug targets[228]	Semorex Inc.	Incorporation into drug discovery, being used to test drug leads by acting as synthetic drug targets
Biotage AFFINILUTE MIP columns	Biotage	Incorporation of MIPs into Biotage columns to afford significant sample clean-up
High-affinity nanoparticles	MIP diagnostics	High-affinity nanoparticles (nanoMIPs) are produced for extraction and sensing

MIPs can be easily synthesized by bulk polymerization, ground, mechanically sieved, and packed in a column.[41] This method, though crude, is simplistic and versatile. More specifically, monolithic MIP columns have been later prepared directly inside stainless steel columns or capillary columns to solve the problems of nonhomogeneousness of the binding sites and particle size.[42,43] High performance liquid chromatography (HPLC) has often seen the use of MIPs as a stationary phase in the racemic resolutions of several species[44] including amino acid derivatives[45,46] and drugs,[47,48] though often excessive tailing and peak broadening are the limiting factors for their use and commercialization for this purpose.[49] Another important area of analytical chemistry, where imprinted polymers have established themselves, is solid-phase extraction (SPE).[49–54] Sigma-Aldrich and Biotage sell MIPs for the highly selective extraction of trace analytes from complex matrices.[55–58]

Use in commercialization of MIPs has been limited as their integration into the sensor platforms is not straightforward. In earlier development stages, MIP microparticles prepared by free-radical polymerization were lacking in affinity and the grafting-on and in situ synthesis techniques were not as refined as they are now.[59–62]

1.3.1. Synthesis of MIPs

A typical MIP synthesis protocol contains a template, one or more functional monomers, a crosslinker monomer, a polymerization initiator, and a solvent.[63] **Figure 2** shows a simplistic schematic of the molecular imprinting principle.

However, the challenge of designing and synthesizing an MIP involves the selection of each of these variables: 1) monomer(s), 2) crosslinker(s), 3) solvent(s), and 4) initiator and the selection of initiation method along with the duration of the polymerization. Determination of the appropriate monomer(s) and the optimal stoichiometric ratios of each of the components often requires extensive empirical studies and testing to maximize target recognition.[64] The most common method of fabrication is known as self-assembly or noncovalent imprinting, often chosen for its ease and flexibility. It requires only a small number of synthesis steps, is compatible with a vast majority of target molecules, and template removal is facile postfabrication. The template and the monomers interact through noncovalent interactions and polymerization takes place with the system (interaction between functional monomer and template) at an equilibrium, although it depends on the choice of reagents and the conditions (temperature, solvent) applied. Interactions at interplay involve hydrogen bonding, ionic interactions, Van der Waals forces, and π–π interactions. MIPs are often synthesized (and optimized) in organic solvents, whereas it has been established that binding characteristics are highly dependent on the solvent. In particular, binding in water is complicated as target molecules can bind in a specific manner to the MIP matrix due to hydrophobic effects.[65] Another drawback is that generally an excess of functional monomers has to be added to increase the chance of binding, which leads to various configurations and heterogeneous binding site distributions.[66] The nonhomogeneity of the binding sites in MIPs resulting from noncovalent imprinting is comparable with that of polyclonal antibodies.[67] This might still be useful when a family of related compounds needs to be analyzed, especially if it is a class of antibiotics. If the MIP would be able to rebind to them, especially when a screening is sufficient and a very precise measure is not required, it would have a sizeable advantage over a biosensor that would have to include different antibodies for each analyte. However, though these types of MIPs can work well within a laboratory research setting, their sensitivity, accuracy, and limits of detection are not suitable for commercial devices.

A solution to the issue earlier is represented by the covalent method: the functional monomers are chosen following the criteria that they are able to form a reversible covalent bond with the template, which will be cleaved after the polymerization process, allowing for the template to be recovered. Covalent fabrication methods yield a homogeneous population of binding sites and minimize nonspecific sites, with selectivity comparable with

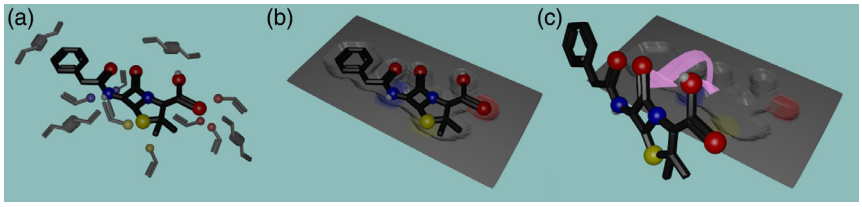

Figure 2. The several steps of molecular imprinting with a) precomplex with the example template of penicillin G surrounded by crosslinker and generic acrylate functional monomers, b) polymerized MIP, and c) amoxicillin molecule extracted, leaving an empty cavity to be used for rebinding.

monoclonal antibodies.[25,68,69] Often, the aforementioned reversible bonding uses boronate esters, ketals/acetals, and Schiff bases. Readily reversible condensation reactions are typically chosen to cleave the covalent bond responsible for the interaction, for its extraction to be successful. However, this type of polymerization introduces an additional step in the fabrication process. Moreover, the covalent strategy poses a major challenge as the covalent bond leads to slow dissociation,[40] which limits its practical application, particularly in the area of sensing. Dummy templates, which are very similar to the target molecule, in size and shape but, importantly, not present in the system, are a useful workaround for this issue.

Certain hybrid approaches have emerged, one of which is known as semicovalent imprinting.[70] Semicovalent imprinting exploits covalent interactions to form the prepolymerization complex between functional monomers and template; however, rebinding to the MIP produced will be solely due to noncovalent interactions that, by nature, will occur with faster kinetics. Another similar imprinting technique is hierarchical imprinting, which uses scaffold molecules, which eventually are eliminated, to produce pores that act as microreactors. This approach is hence termed "sacrificial spacer" method and was first introduced by Klein et al.,[31] but it has since been used in multiple areas of research. This work demonstrates the preparation of MIP shells, an example of the sacrificial support approach, with fast absorption kinetics (≈10 min) for detection of the antibiotic enrofloxacin (ENR), as shown in **Figure 3**, in fish samples, with limits of detection well below the legal maximum residual level.

The $K_2Ti_4O_9$ matrix was chosen because of its nontoxicity, low cost, and easy removal. Silica is indeed another popular choice as sacrificial support as the polymers can be embedded into the pores of the particles. The group of Moreno-Bondi developed MIPs that were able to recognize six cephalosporins below the maximum residual levels set for these antibiotics in raw milk.[71] Silica etching is a more time-consuming process and also involves harsh chemicals, as the particles were treated with an ammonium hydrogen difluoride mixture for 24 h. A similar approach, where silica beads were functionalized with MIPs according to the procedure by Yilmaz et al.,[72] was used to synthesize selective recognition elements for structurally related penicillins. This antibiotic family base structure, 6-aminopenicillanic acid, acts as a dummy template enabled to detect the entire class of target analytes from milk with high recovery rates.

Another dominant restriction to MIP synthesis is that only a limited number of functional monomers are suited for this task. Methacrylic acid (MAA) was already used by Mosbach et al.[73] and remains to this date the most common one, given its ability to serve as H-bond donor and acceptor. Lately, several approaches have been attempted where combinations of functional monomers have been used at the same time to enhance binding affinity.[63,74–78] Although a wide range of amino acids can be used, several restrictions reduce their availability. While this is certainly interesting, it exposes the fact that only a limited number of functional monomers is still available to this day.

The approach of using a "dummy" template when discussed earlier is advantageous as it would not involve working with target molecules that are expensive, dangerous, unstable, or toxic,[79,80] while also allowing to detect classes of compounds which can be of particular interest in the case of antibiotics. Many successful cases of epitope imprinting have already been reported,[81,82] as well as computational simulations that use molecular modeling[83,84] to select the epitope that yields the highest specificity.[85] The recent COVID-19 outbreak has triggered the interest in the epitope approach, considering one could imprint with antigens of the virus or particles with similar size and shape as the virus.

The known difficulties in extracting the template led to a problem known as template leaching, which might interfere with the analysis of a given target. While the extraction of residual template molecule is more exhaustive after every use, it might be possible to avoid it with the use of a dummy template.[86] However, this leads to less-selective binding cavities as a major drawback.

A common drawback of MIPs is the difference in sensing performance between lab testing, when samples are spiked with the target analyte, and field testing, when a complex matrix is used. Often, even when state–of-the-art biosensors are utilized, the sample is pretreated and concentrated to avoid the interference of several other components. Interestingly, often the separation and removal of unwanted, interfering analytes from a solution is achieved via the use of MIPs in chromatographic methods, as mentioned previously. Therefore, a more complex sensor, first

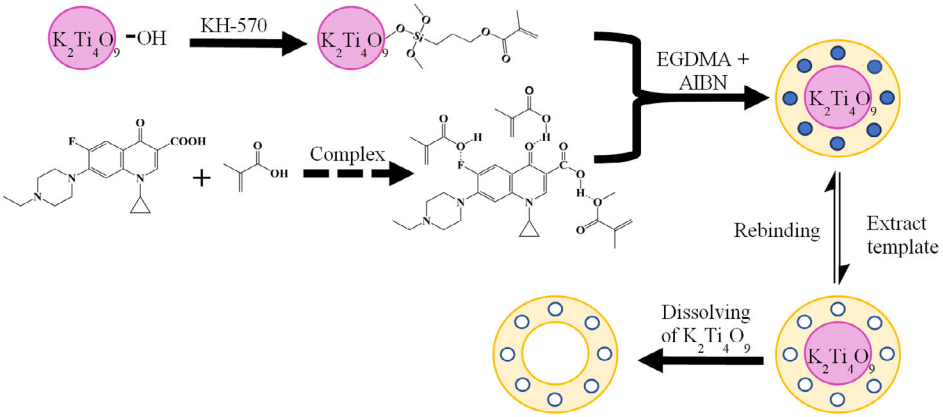

Figure 3. Preparation of hollow MIPs using covalent and noncovalent imprinting for an amino acid sequence, demonstrating the "sacrificial spacer" method using $K_2Ti_4O_9$ as a spacer molecule. Adapted with permission.[31] Copyright 1999, Wiley-VCH.

using a variety of MIPs in a separation procedure, prior to MIPs sensing of the analyte, may resolve the aforementioned problem.

Even with the drawbacks mentioned earlier, noncovalent imprinting, given the reduced number of steps and the ease of template extraction, is often the most widely used method of MIPs fabrication during the development of new sensors. Although sometimes less specific than other MIP methods, specificity of the binding sites can be increased via a low-temperature, light-induced polymerization process. The synthetic methodology, in contrast, can be chosen depending on the destination of use of the polymer; whereas monolith synthesis and consecutive grinding offers a very simplistic approach, and its use is destined to be abandoned in favor of those able to yield more homogeneous binding sites and a better yield. In the future, methods that will also ease scalability such as MIP beads, membranes, in situ-prepared monoliths, surface imprinting, and molecularly imprinted monolayers will likely be preferred, as they ease the rebinding kinetics and offer further improvement in the homogeneity of the binding sites.

2. Rational Design of MIPs for Antibiotics

2.1. Computational Modeling

As mentioned previously, determining the correct functional monomer, crosslinker, and solvent for the chosen template is one of the most important considerations when approaching MIP design and often the most time- and resource-consuming task, which is not eased by the availability of a considerable amount of these components. The experimental optimizations in both type and ratio may require the time-consuming fabrication of many imprinted polymers, whose difference in composition only slightly varies from each other, to obtain the most specific cavities. While this might be a big hindrance in terms of experimental work, such a task lends itself to the use of rational design through computational modeling, offering substantial advantages in both time and cost to the experimental counterpart.[87] The majority of computational modeling in relation to MIP design is centered around the prepolymerization complex. The nature of these interactions is a key step in obtaining high-affinity binding sites. With the use of varying computational techniques, these interactions can be investigated and optimized.[88]

While the adoption of simple computational methods toward MIP design was first seen toward the end of the 1990s,[89] their application toward direct rational design was not fully acknowledged until work by Piletsky et al.[90] This work utilized monomer screening, similar to experimental combinatorial screening, to predict the correct choice of monomer. This demonstrated the potential power of computational software for monomer selection. This work, besides many others, considers thermodynamic interactions between template and functional monomer. When the computational program shows increased stability of these interactions, the quality of the template-specific cavity being produced experimentally is usually improved.[91] These energetics-based template-monomer studies are characterized by the determination of binding scores (Equation (1)), comparisons of which identify stronger interactions.

Equation 1: The binding energy/score (ΔH_B) between a template and functional monomer based on heat of formation (ΔH_f).

$$\Delta H_B = \Delta H_f \text{ Final complex} - (\Delta H_f \text{ Monomer} + \Delta H_f \text{ Template}) \quad (1)$$

The energy difference between an independent template and a monomer compared with the final complex, template, and monomer bound by a new bond indicates the strength of this newly formed bond. There has been a considerable decrease in cost and increase in computing power available over the past 20 years. Not only has this increased the number of papers including rational MIP design, but it has also led to the development of more advanced techniques. A search of the available literature (Web of Science) shows a sharp increase in the number of papers since 2000 (**Figure 4**).

Quantum mechanical (QM) techniques, namely, semiempirical methods such as density functional theory (DFT), have become more widespread. QM-based methods offer a more advanced approach to predicting molecular energies. Such energies are calculated through the use of electronic structure-based techniques, allowing for determinations of interaction energies as well as structural predictions. For MIP design, QM methods are mainly used in monomer screening approaches and offer a more accurate method for determining energies for use in Equation (1).

While many studies consider only template−monomer interactions, many others have investigated this further with more in-depth structural analysis, considering not only changes in ratios but also interactions of both crosslinker monomers and solvent. Again, QM methods have been used in this area, considering crosslinker interactions to the template; however such techniques are still computationally expensive and limited to only a few molecules. Molecular mechanics (MM) and molecular dynamics (MD) are commonly implemented when a more dynamic system is wanting to be defined, with both solvent

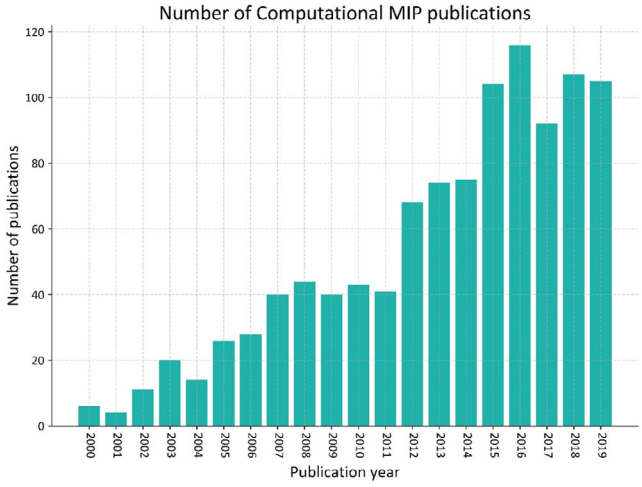

Figure 4. Representation of the increased research in the field via the number of publications including computational methods in MIP design between 2000 and 2019. The data obtained through Web of Science using the keywords "Molecularly Imprinted Polymer," "MIP," and "Computational."

and larger systems able to be represented. MD techniques allow these interactions to be investigated over time, analyzing the motion of individual molecules. MM focuses on the observed properties of these molecules, with bond lengths, angles, and dihedrals along with nonbonding interactions considered. Most commonly, these two methods are used in combination.

Specifically, in relation to computational rational design for antibiotics, there have been several papers offering many different techniques outlined previously. A relatively recent paper by Kong et al.[92] investigated norfloxacin-imprinted polymers using an MD--based approach. They considered changes in the ratio of functional monomer (MAA) and crosslinker (EGDM) while keeping the solvent (acetonitrile) constant. Computational analysis was completed using radial distribution functions (RDF), which allow for two specific atom pairs to be analyzed as a system interacts throughout a simulation. A ratio of 1:8:40 (template: monomer: crosslinker) produced the best pre-polymerization interactions, namely, the carbonyl to alcohol of norfloxacin to MAA. The experimental rebinding studies also supported this particular polymer composition ratio, with the computationally predicted one offering greater rebinding and specificity than the other MIPs analyzed.

The majority of papers on the subject, however, use the quantum mechanics-based approach, specifically for monomer selection. This is mainly due to the accuracy of the energies predicted, but also the relative speed and ease when determining template-functional monomer bonds in vacuum. Again, they use semiempirical methods, mostly DFT, to determine the energies required for Equation (1). Such work has been completed on the antibiotics norfloxacin,[93] ciprofloxacin,[93,94] ENR,[95] and tetracycline.[96] The studies found that the energies derived computationally were consistent with the experimental data produced and that the binding scores generated correlated with the experimental rebinding. The nature of antibiotics, with their relatively small size and presence of multiple, accessible functional groups, makes them a good candidate for computational modeling, as a wide range of techniques can be applied to them, depending on the requirements. Previous work in the area has shown good support for this, with complimentary computational and experimental data.

The rational design of MIPs through computational methods is still a growing area of research, with varying approaches and more advanced techniques being developed since its establishment in the early 2000s. Such methods were applied depending on the research, whether that were simple functional monomer predictions using QM or more in-depth structural analysis utilizing MD. Already, with many papers showing the ability for strong predictions, the application of such aided design looks set to become commonplace in future MIP production.

2.2. NMR Techniques Aiding MIP Production

NMR is an essential technique for structure determination, for a wide range of molecules. The plethora of techniques associated with proton and carbon NMR, which allows investigating spatial and electronic interactions between different nuclei, can offer very detailed structural information even in the case of complex molecules. MIPs are composed of crosslinked and macroscopic chains, which complicate the characterization due to their intractable and insoluble nature. A pioneering NMR study on MIPs was reported by Sellergren, Lepisto, and Mosbach.[97] Following a ^{1}H-NMR study, combined with a chromatographic technique, involving titration of the print molecule (phenylalanine anilide) with carboxylic acid, it was found that the results were consistent with the existence of multimolecular complexes by means of electrostatic and hydrogen-bonding interactions.[97]

NMR has been mostly used to determine the extent of the template to functional monomer association equilibrium in the pre-polymerization stage; the shift of the relative signals, compared with those of the template and monomer alone, can determine the extent of the interaction. Wang et al. used NMR to identify the best template-to-functional monomer ratio,[98] a procedure that is often very time-consuming, for the preparation of MIPs for the extraction of a valuable compound. The signals of the protons involved in hydrogen bonding and that of the adjacent carbon were used to gauge the strength.

A study by Mattos dos Santos et al. reported on the synthesis of MIPs targeting tegafur (an anticancer 5-fluorouracil prodrug) and used ^{1}H-NMR titration to study solution association between tegafur and 2,6-bis(acrylamido)pyridine (BAAPy).[99] This confirmed the formation of a 1:1 complex of template and functional monomer, in MIPs being prepared using stoichiometric imprinting. Interestingly, an affinity constant of $574 \pm 15\ \text{M}^{-1}$ in $CDCl_3$ was calculated using a previous work by Fielding, who reviewed the topic with a section dedicated to diffusion experiments.[100] Hydrophobic effects and their contribution to the selectivity of the resulting MIP were investigated with NMR spectroscopy by O'Mahony et al. to identify the interactions occurring in the prepolymerization mixture.[101]

Sánchez-González et al. used ^{1}H-NMR and, importantly as it represents a novelty, nuclear overhauser effect (NOE) to study the prepolymerization interaction between the cocaine (COCH) template and the functional monomers MAA and ethylene dimethacrylate; in particular, 1D selective NOE experiments were conducted to assess MAA-COCH and EDMA-COCH hydrogen-bonding interactions, which were contextually confirmed by in silico studies.[102]

Commonly, the other time-consuming step is the quest to identify the most appropriate functional monomer. Konishi and coworkers addressed this using ^{1}H-NMR to evaluate the influence of several monomers on the potentiometric performance of histamine-imprinted polymer-modified sensors.[103] Not only was ^{1}H-NMR able to assess the interaction between histidine and acrylamide (AA) and atropic acid (AT) and MAA, but it was even possible to see the influence of its imidazole ring on the pyridine ring of 4-vinylpyridine (4-VP).

The nature of MIPs makes their characterization usually harder, but solid-state NMR has been shown to evaluate the degree of the binding of the template when the interactions are strong. Less recent works already demonstrated that this technique is useful to provide insights. Andersson et al. used this technique to optimize the template-functional monomer proportion.[104]

Simple ^{13}C-NMR alone had not been used for the evaluation of the template-functional monomer interaction, until Zhang et al.[105] reported a study where it was utilized to evaluate the interactions between antibiotic erythromycin (ERY) and a set

of functional monomers, with the choice of MAA as the optimal one. The rational binding sites were predicted based on chemical shifts changes in ERY structure. DFT theoretical calculations of Lewis basicity of the O/N atoms located at the sites proposed by a sequence regarding their interaction force confirmed its reliability. Solid-state NMR was used by Annamma et al. to design a 2,4-dichlorophenoxyacetic (2,4-D) acid-imprinted polymer with 4-vinylpyridine (4-VP) as the functional monomer,[106] intriguingly showing the effect of increasing concentrations of 4-VP on the equilibrium; see **Figure 5**.

2.3. Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a microcalorimetry technique that measures the heat released or absorbed during a chemical reaction, generally used to determine binding affinity, enthalpy changes, and stoichiometry of interactions between molecules in solutions.[107] The advantage of this technique is that no immobilization or modification of the starting reagents is required. In addition, ITC has the ability to detect changes in the low mK range that corresponded to noncovalent interactions such as hydrogen bonds.[108] However, ITC is not widely available in laboratories and requires expensive instrumentation, which limits the application in the field of MIPs[109]. To the best of our knowledge, there have been no reports about the use of ITC for the characterization or rational design for MIPs produced for antibiotics. There are few reports in literature about using ITC to determine binding affinity of MIPs for small molecules[110,111] and predicting the optimum ratio of target-to-functional monomer.[112,113] Due to ITC providing thermodynamic data, it gives fundamental insight into the binding mechanism of MIPs that has been proven strongly dependent on pH,[114,115] showing that pH can be a considerable variable in achieving optimum MIP function. Considering ITC is routinely used in biomedical research including enzyme kinetics, it might play a crucial role in optimizing MIPs for antibiotic detection and understanding the influence of external parameters (T, pH, etc.) on binding to polymers.

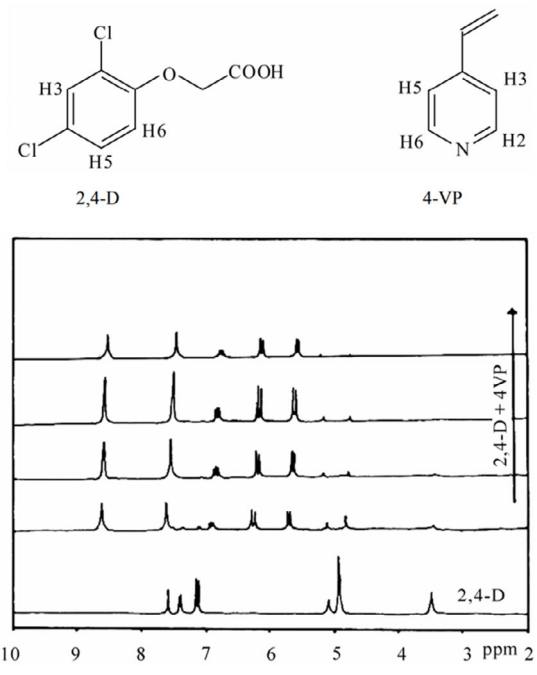

Figure 5. H NMR spectra of 2,4-D with increasing concentration of 4-VP showing changes in the chemical shifts used to rationalize a low cross-linked system that allows for higher levels of specificity and selectivity. Reproduced with permission.[106] Copyright 2011, Springer.

2.4. Infrared Spectroscopy

Infrared (IR) spectroscopy is ubiquitously used in laboratories as it is versatile, offers fast and straightforward analysis, and is inexpensive. The main disadvantage of IR is that it is difficult to analyze complex mixtures or aqueous solutions, as the corresponding spectra provides only limited information about individual peaks of chemicals of interest. Therefore, IR is not routinely used to study interactions in the prepolymerization complex prior to MIP formation as the stretching frequency of hydrogen bond donors or acceptors is generally in the same range as that of the solvent peaks.[116] However, IR measurements can also be collected from solid particles and this method of IR can provide useful information about the resulting solid MIP on the expected performance of the material as the peak intensity of the carbonyl group provides quantitative information about the amount of polymer present on the surface.[116] Thin films of poly(*N*-isopropylacrylamide) revealed systematic changes in IR peaks associated with the amide bond, that could serve to bind molecules via hydrogen bonding. In addition, the study also demonstrated that the molecular architecture can significantly vary depending on what solvent was used to cast the film.[117] IR measurements are fast and straightforward and therefore have scope for this technique to be used for high-throughput screening of performance of solid polymeric materials.

MIP materials are often heavily crosslinked and have limited solubility, which can complicate characterization. IR is often used on solid materials to establish the presence of the polymer on surfaces and determine whether the template has been fully removed from the MIP cavities. Chen et al., reported on the separation of tetracycline antibiotics from egg and tissue samples using magnetic MIPs as the solid phase.[116] Peaks from the carbonyl and hydroxyl group of the polymer were present in the recorded IR spectra, which confirmed presence of the polymer on the magnetic particles. Wei et al. developed dual-imprinted MIPs for the rapid determination of amphenicol antibiotics in water, serum, and food samples. The presence of the polymer was verified with IR measurements. Furthermore, IR spectra of the reference non-molecularly imprinted polymer (NIP) and its corresponding MIP were compared and significant frequency shifts in the peak corresponding to methacrylic acid were observed.[118] It was hypothesized that the observed shift in this peak was due to hydrogen bond interactions between functional monomer and target. A similar approach was followed by the group from Mizaikoff and coworkers[119] that developed gravimetric sensors for detection of the antimalarial drug artemether. Upon inspecting the IR spectra of the polymers, a spectral shift of the peaks corresponding to the carboxyl groups of methacrylic

acid to the shorter wavelength was reported. Besides these peaks, there was also a distinct intensity change at a wavelength of 3000 cm^{-1} that was attributed to additional interactions of functional monomers with the template.

Over recent years, there has been a move toward the use of the aforementioned "dummy" templates in Section 1.3, which avoid the use of high-cost or toxic target molecules. Zhang et al., used a simple sugar, raffinose, as a dummy template to develop MIPs for antiglycoside antibiotic detection.[120] This molecule resembles the size and shape of the antibiotic of interest and is able to selectively extract six antibiotics from the family of antiglycoside antibiotics from environmental samples. In research by Liu et al.[121] roxithromycin was used an example of a macrolide antibiotic to develop MIPs for the extraction of this entire family of macrolides. The interesting aspect of this work was that a simple wooden tip was used, that makes it extremely suitable for work in developing countries. IR was used to monitor extraction of the template from the MIP cavities.

IR measurements are fast and straightforward and, as shown via the earlier example, there is certainly scope for this technique to be used for high-throughput screening for the performance of MIPs.

3. MIP Morphologies

In the early years of imprinting, the most common approach involved free radical polymerization, often achieved using azobisisobutyronitrile (AIBN). The reason this method is often used is due to its simplicity as it can either be initiated with UV light or increasing the temperature; unfortunately, given the fast chain propagation and the fact that the associated termination reactions are irreversible, it yields inhomogeneous cavities and particle size.[122] This results in MIPs with heterogeneous binding sites, with cavities directly on the surface and others that are partly inaccessible, which hinder the mass transfer and limit selectivity. This problem has been addressed with the use of controlled polymerization techniques including reversible addition fragmentation chain transfer (RAFT) polymerization,[123,124] ring-opening metathesis polymerization,[125] and atom transfer radical polymerization (ATRP),[126] which lead to particles uniform in size with homogeneous binding sites. In this section, we will review a couple of methodologies which improve upon sensor specificity.

3.1. Use of Copolymers

One way to improve upon the specificity and selectivity of MIPs is the inclusion of more than one functional monomer, resulting in what is referred to as a copolymer.[127,128] Chullasat et. al.[129] demonstrated an amoxicillin detection system using a copolymer along with quantum dots (QDs). The resulting system proved capable of detecting amoxicillin in complex media such as milk and honey with a limit of detection (LoD) of 0.14 μg L^{-1}, outcompeting the HPLC standards it was tested against. It was also shown that the use of copolymer MIPs can increase the accuracy of results against modern, industrial detection systems but also decrease the time taken and reduce the need for expensive infrastructure. Tunc et al.[130] demonstrated optimizing copolymer MIPs through synthesis and comparative testing. This provided insights into optimum monomer selection for the theophylline-imprinted monomers; however, planning monomer selection via rational design, e.g., computational modeling, would lend aid as to which monomers to test. Valtchev et al.[131] tested a vast range of MIPs, including six cofunctional monomer polymers. The study resulted in the synthesis of many significantly optimized MIPs for the detection of the antibiotic sulfamethoxazole in wastewater. Although this type of "trial by error" study is not time efficient, it does give certain, quantified values to the efficiencies of all the MIPs tested and shows the positive impact that using copolymers can have. Wang et al.[132] developed inorganic−organic cofunctional monomer-imprinted polymers for fluoroquinolones in milk and observed an LoD in the ng/mL range, which exceeds the standards required by the EU. As sample matrices as complex as milk have benefited from the use of copolymers, the eventual use of copolymers for detection in other sample matrices, especially simple matrices such as water, would have presumptive benefits.

3.2. Integration of Fluorescent Moiety

Fluorescence detection of antibiotics has been a crucial analytical tool for many years because of its versatility, simplicity, and accuracy.[133–135] It can be used across a range of different recognition elements, including MIPs. However, it often requires the introduction of a secondary molecule to either bind to the target[136] or serve as a competitor[137] for a measurable fluorescent response to be obtained. Recent studies, therefore, have looked to develop MIPs, which are inherently fluorescent by introducing certain elements into the polymer itself.[138,139] This reduces the number of preparation steps needed to analyze samples and allows for the polymer to be adapted to a variety of targets. There have been two primary focuses to achieve this functionality: QDs embedded within or surrounded by the polymer matrix or a fluorescent moiety copolymerized into the backbone.[140,141] These integrated QDs and fluorophores typically rely on energy or electron transfer from the target molecule to achieve their fluorescence change (**Figure 6**). This transfer is strongest when the target rebinds into the imprinted sites as this is in closest proximity to the fluorescence element that can be achieved.

Fluorescence quenching is most commonly seen in both cases, although enhancement can be facilitated by tailoring the molecular system to achieve specific interactions upon rebinding.[60] Shi et al. utilized CdSe QDs to introduce fluorescence into a bulk MIP for kanamycin.[142] An increase in fluorescence was seen for both NIP and MIP upon addition of kanamycin, with a greater increase seen for the latter. The fluorescence response was greatly influenced by composition, pH, temperature, and required optimization. The system was able to detect kanamycin with a detection limit of 0.013 μg mL^{-1} and a linear range of 0.05–10 μg mL^{-1} in PBS. Furthermore, it was tested with real samples (including lake water and urine) and showed good recoverability of spiked concentrations of the drug. This methodology also utilized aptamers to increase binding affinity to the polymer layer and click chemistry to provide a more simplified chemical route for polymer attachment to the QDs compared with conventional methods. Zhang et al. integrated ZnS QDs into a mesoporous silica network containing

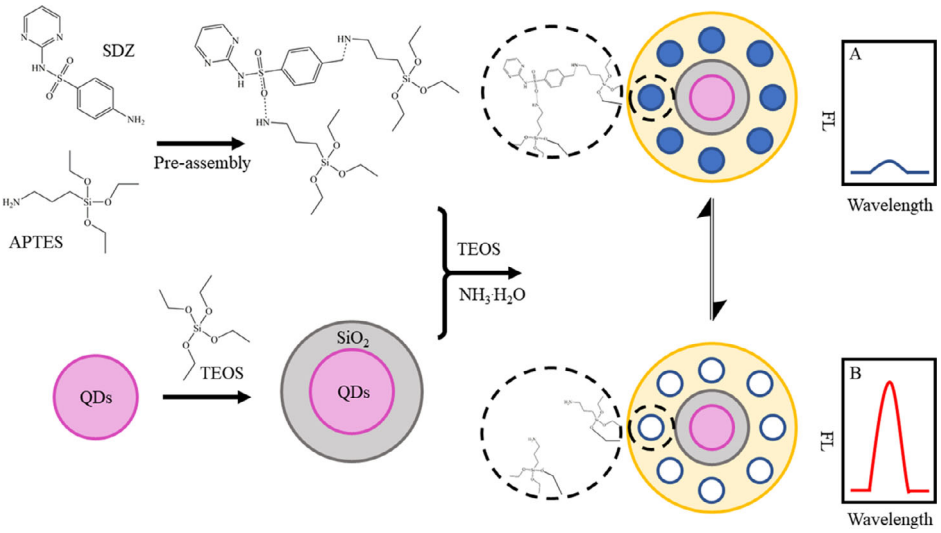

Figure 6. Formation of an MIP layer on the surface of QDs for an antibiotic, sulfadiazine (SDZ). The polymer layer can then have the target extracted, breaking the electron transfer between SDZ and the QDs, which results in a decrease in the fluorescence signal. Reproduced with permission.[141] Copyright 2018, Elsevier.

an imprinted polymer for tetracycline.[143] Quenching of the fluorescence was seen upon addition of tetracycline to the MIPs only, which they propose is due to an electron transfer between the target and the QDs. The particles showed high selectivity for tetracycline compared with similarly structured compounds, and an LOD of 15.0 ng mL^{-1} was obtained. Similar quenching probes for antibiotics have been produced using graphene,[144] ZnS,[145] and CdTe QDs,[142] yielding comparable LODs and selectivity.

Integrated fluorophores offer a unique alternative to QDs, where the interactions between the fluorescent moiety and the target can be more specifically tailored.[146,147] In addition, the use of heavy metal atoms can be avoided and integration into the polymer backbone significantly reduces leaching. However, these molecules often require a multistep synthesis and extensive optimization of the polymer composition as quantifying the fluorescence change requires one-to-one interactions. Niu et al. introduced an anthracene-based monomer into MIP nanoparticles for the detection of tetracycline.[148] The fluorescence of the RAFT-polymerized nanoparticles would be quenched in the presence of the target, with the imprinted particles exhibiting a stronger change than the nonimprinted ones. The polymers were able to detect tetracycline at an LOD of 0.26 μm and were able to perform in a more complex medium (bovine serum). UV−vis analysis demonstrated that the underlying mechanism for quenching was based on electron transfer, rather than energy transfer. Sunayama et al. used attached fluorophores to explore the binding activities of a cephalexin-imprinted polymer.[136] The template was functionalized and crosslinked into the polymer network and then removed using two different chemical reactions. This method is unconventional compared with common imprinting techniques but allows for direct functionalization of the imprinting sites; in this case, two fluorophores were introduced via Schiff base and disulfide reactions. Ampicillin was used to monitor rebinding due to solvent constraints, and an increase in fluorescence intensity was observed with corresponding concentrations. An LOD of 5.0 μM was observed and the fluorescence change was highly specific due to the nature of the interaction. Ashley et al. were able to produce fluorescent doxycycline-imprinted microparticles using an acrylated fluorescein derivative.[149] The moiety was introduced into a thin polymer layer surrounding FeO_x nanoparticles and would exhibit quenching upon rebinding of the target. Although the interaction between fluorophore and target appears less specific, the polymer demonstrated strong selectivity toward doxycycline compared with similarly structured antibiotics based on fluorescence readings. Recently, our group has explored the use of a fluorescein-based MIP as an optical detection platform for beta lactam antibiotics.

3.3. Using Redox Probes to Monitor MIP/Target Binding Phenomena

If the target analyte is not electrochemically active, a redox-active probe can suffice for the detection. A redox reaction involves the transfer of electrons, which is facilitated through the working of an auxiliary (counter) electrode, whereby if an oxidation process occurs at the working electrode, the corresponding reduction process will take place at the auxiliary electrode. In electrochemistry, redox probes can be used to follow interfacial changes in a system, such as adsorption, electrode modification, and binding phenomena. There is a vast array of redox probes and care must be taken when choosing one for use in characterization of a system or for use as an indicator for sensing applications. All of these systems are classified under two main categories, outer-sphere and inner-sphere redox probes, summarized by McCreery et al. (see **Figure 7**).[150]

Outer-sphere probes come close to the electrode surface, but do not directly contact it, to allow the electrons to tunnel/hop across the solvent monolayer, and as such are only influenced

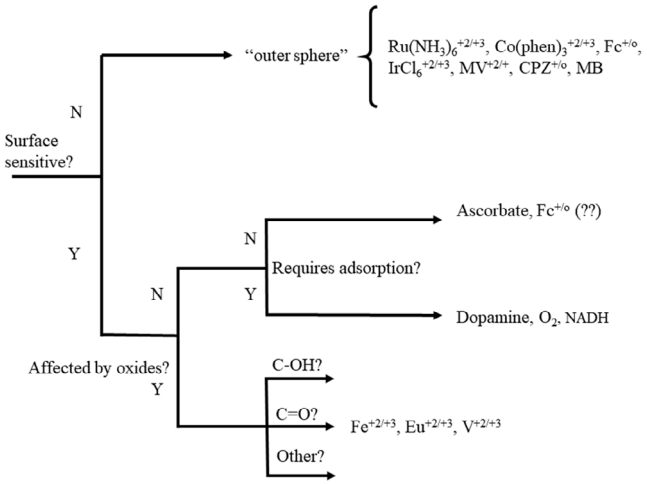

Figure 7. Flow diagram showing classification of outer- and inner-sphere probes. Reproduced with permission.[150] Copyright 2008, American Chemical Society.

by the electronic structure of the electrode surface. Ruthenium hexamine (RuHex) is the best example of a near-ideal outer-sphere redox probe; it does not exhibit any variation in electron transfer rate for any changes other than the electronic structure of the electrode (density of states and the Fermi level). Alternatively, inner-sphere redox probes require contact with the electrode surface to facilitate the electron transfer; as such, they are affected by both the electronic structure of the electrode surface and the surface chemistry at play (i.e., surface functional groups and adsorption sites).[151] When using MIPs as recognition elements, the desire is typically to record and track changes in binding phenomena between the imprinted polymers (typically on the surface of an electrode) and a target analyte (in solution that diffuses to the electrode surface and binds to the MIP). Most papers in literature combine MIPs and redox probes, where the use of an inner-sphere redox probe in solution is as an indirect detection method, where the binding of the target to the polymer blocks the access of the redox probe to the electrode surface, producing a reduction in measured response. As such, the most common redox probe in this area of research, as an indirect detection method, is potassium ferri/ferrocyanide ($K_3/K_4[Fe(CN)_6]^{3-/4-}$). This can be done utilizing various electro(analytical) techniques such as cyclic voltammetry (CV),[152] electrochemical impedance spectroscopy (EIS),[153] and differential pulse voltammetry (DPV).[154–156] CV typically will not reach the same levels of detection as other electroanalytical techniques; however, the analytical response can be amplified by adding extra components to the system. Lian et al.[157] accomplished this for the detection of the antibiotic kanamycin through the addition of horseradish peroxidase and H_2O_2, where potassium ferricyanide acts as a mediator for the reaction. This reaction mechanism gives a large amplification in the measured current; therefore, when the electrode surface is blocked through the binding between kanamycin and MIP, there is large reduction in current as the reaction can no longer be mediated. These methodologies typically require two-step analysis, consisting of an incubation step in the analysis solution and a subsequent measurement step in the probe solution, which is not ideal for production, reproducibility, and analysis time.

Instead of using a free in-solution redox probe, an alternative strategy is to incorporate the redox probe directly into the polymer matrix. In this process, known as redox tagging, a redox probe such as ferrocene is attached to a moiety capable of taking part in the polymerization process. Mazzotta et al.[158] reported this for the detection of the antibiotic vancomycin through using two ferrocene-derived monomers, vinylferrocene and ferrocenylmethyl methacrylate, in conjunction with the solid-phase synthesis approach,[159] for the development of nanoMIPs that produce homogeneous binding sites and pseudomonoclonal binding properties. The direct anchoring of the redox probe into the polymer allows for a reduction in analysis time and a more direct interaction between the probe and target, which does not rely on diffusion of the probe in solution. The main drawback of using ferrocene-derived redox probes covalently attached to the system is a natural reduction in the current signal when cycling. As seen from the cyclic voltammograms in the manuscript from Mazzotta, over the course of 250 scans, there is a significant reduction in the measured current; this needs to be taken into account when developing a sensor platform using this methodology and may require significant calibration steps to produce a reliable system.

4. Different MIP Production Methods

There are many different production methods for MIPs, depending on the desired function of the polymer. MIPs intended for use in chromatography are usually prepared by free-radical polymerization, which results in the production of heterogeneous microparticles. Research into other MIPs has elevated their use, such as, their synthesis via solid-phase imprinting to generate homogeneous high-affinity nanoparticles, which have the added benefit of biocompatibility. The evolution has led them to compete with their biological counterparts—antibodies. In fact, they can also offer benefits over antibodies because they are far more stable. MIPs can be stored in dry conditions and ambient temperatures for many years, without loss of recognition capability, whereas antibodies denature rapidly unless they are kept frozen. This provides advantages for manufactured MIPs sensing devices as they can have appropriate shelf life for use outside of lab settings. In addition, MIPs are able to withstand many adverse conditions (heat, cold, changes in pH, etc.), which allow them to be used for a variety of in-field, real-time sensing applications.[156] For our interest, MIPs can easily be implemented into sensors for food and water analysis for antibiotics.

One of the biggest stumbling blocks, however, for broad-scale commercial use of MIPs as sensors has been reproducible mass manufacture. The advent and recent development of nanoparticles, and especially core–shell and gel nanoparticles, whose solubility can finely be tuned, is promising for the next generation of sensors.[160] Recently, photolithography has been used to pattern MIPs for the wafer-scale production of biochips. This technique allows for the control of shape and size (1.5 μm) of the patterns and the deposition of different MIPs on the same

chip.[161] Some of the advancements to mass manufacture will be discussed later.

4.1. Screen-Printing Techniques

Screen printing is a well-established methodology for the mass production of biosensors.[162] The most common commercial example is the glucose sensor, used in the treatment of diabetes, that utilizes a screen-printed electrode (SPE) and is responsible for a billion-dollar market annually.[163] Screen printing offers the ability to mass produce disposable platforms that offer high reproducibility, flexibility, versatility, and high sensitivity at a low production cost. This technique involves the spreading of a thixotropic fluid, containing a mixture of predominantly graphite, solvents, and binder, through a predesigned mesh that will produce a printed pattern of a defined shape and size.[164] As such, as vast array of electrodes has been designed utilizing this methodology to produce different shapes such as microbands,[165] shallow recessed arrays, and[166] back-to-back sensors[167–169] and produce electrodes containing a wide range of constituents for various applications in biosensors.[170] MIPs can be incorporated into and used in conjunction with screen-printed platforms in a variety of ways, such as the direct formation of MIPs on the surface through processes like electropolymerization,[171] incorporation of MIPs into the screen-printing ink,[172–174] or depositing onto nanomaterials decorated on the surface.[175]

Direct formation of MIPs onto the surface of an unmodified SPE was reported by Ayankojo et al.,[156] who used computational modeling to choose *m*-phenylenediamine as their chosen monomer for the detection of erythromycin. In strategies such as this, where direct electrochemical detection is used, the thickness of the MIP layer becomes a priority. They explain that with the thinnest MIP layer deposited, a better detection signal is acquired; however, there is lower specificity due to nonspecific interactions, such as that directly with the surface. Conversely, when the layer was too thick, there was a significant decrease in the electroanalytical signal for both the MIP and NIP regimes. Therefore, when using MIP layers on SPEs, each system must be optimized for electrochemical detection.

Electropolymerization (discussed later) is the most obvious route for MIP formation on SPE surfaces; however, this methodology can struggle from a limited selection of suitable monomers and poor scalability. Within our group, Jamieson et al.[176] reported a sensor for amoxicillin through the direct formation of MIPs onto the SPE surface through UV polymerization. This offers better prospects in terms of mass production; however, more work has to be done to increase the synergy between the transducer, MIP, and detection methodology.

The enhancement of electroanalytical output for SPE platforms is typically done through the incorporation of nanomaterials. These materials are either incorporated in the ink, on the surface of the electrode, or on top of the MIP and help to overcome problems with poor conductivity and electroanalytical response. This can be seen in the work by Devkota et al.,[177] who reported a sensor platform for the detection of tetracycline, in which the formation of the MIP was achieved using the conductive polymer pyrrole. Following MIP formation, the polypyrrole layer was overoxidized, a process that while stabilizing also removes the conductivity associated with the polymer layer. Therefore, to increase the efficacy of the sensor, gold nanoparticles (AuNPs) were deposited on the surface of the MIPs. Although this helps to counteract the loss of conductivity, the multiple electrochemical modification steps do not lend the system ability for simple mass production, which is a great advantage when using SPEs.

This can be further seen through the work of Moghadam et al.[178] using a combination of gold nanourchins and graphene oxide for the detection of oxacillin. In this case, the nanomaterials were deposited onto the surface of the electrode prior to the MIP. Although the use of drop casting to modify electrodes is standard practice and scalable, using multiple drying conditions followed by electropolymerization and template removal provides difficult productions steps. Instead of multiple electrochemical functionalization steps, Dechrirat et al.[155] utilized inkjet printing of a nanocomposite layer, containing AuNPs and poly(3,4-ethylenedioxythiophene)/poly(styrene sulfonate) (PEDOT:PSS), onto the surface of the SPE. This synergetic approach to the incorporation of nanomaterials onto the electrode allowed for the detection of nitrofurantoin at two orders of magnitude lower than the bare sensor platform. This sensor exhibited the advantages of using screen printing and MIPs in combination, producing a sensor platform that is both highly reproducible and stable over a long lifetime.

4.2. Electrodeposition of MIP Layers on Transducer Surfaces

The electrodeposition of MIPs is concerned with the immobilization or formation of MIPs directly onto an electrode surface using electrochemical methodology.[179] The vast majority of MIPs that fall into this category is electrosynthesized MIPs (eMIPS). A more detailed overview of this subsection of MIPs, including their formation and application to the detection of biologically important molecules, including antibiotics, up to 2019, can be found in a recent past review.[171] Briefly, the formation of eMIPs takes place through electropolymerization, where a polymer layer is formed upon an electrode/substrate in the presence of the desired template. This can be achieved through a variety of electrochemical techniques, such as voltammetry,[180] potentiometry,[181] and galvanostatic techniques.[182] It is vital when using this methodology to define the working material, counter and reference electrodes, monomer composition, solvent, supporting electrolyte, electropolymerization methodology, and time as these variables will greatly affect the binding affinities, layer sizes, conductivities, and surface morphologies of the polymeric films.[183]

Good recent examples, presenting the optimization required for the production of a sensor array for the detection of β-lactam antibiotics, were reported by Moro et al.[184] and Bottari et al.[185] Both explain the rationale behind the design of their eMIP-based sensor platform. Moro et al. discussed the importance of utilizing a conductive polymer (4-aminobenzoic acid, 4-ABA) to synergize with their chosen squarewave voltammetric (SWV) detection method and the use of modifiers, in this example multiwalled carbon nanotubes (MWCNTs), to enhance the electroanalytical properties of the device.

The overwhelming majority of sensor platforms utilizing electrodeposition also use electrochemistry as their chosen detection method. This can be achieved either through indirect detection, such as the redox probes described earlier, or by direct detection of the binding between the target and recognition element. One such way to monitor the interfacial changes at the MIP/electrode surface is through EIS, where a change in the measured charge transfer resistance (R_{CT}) can signify the binding of a target molecule. Roushani et al.[186] demonstrate this using a design that utilizes a combination of electrosynthesized poly(resorcinol) MIP and a silver nanoparticle (AgNP)/reduced graphene oxide (RGO)/aptamer system. Although this methodology uses a large amount of preparation steps, it was able to detect chloramphenicol (CAP) in milk samples. Another effective choice of monomer for antibiotic detection through eMIP layers, due to its ease of polymerization and favorable structure, is represented by resorcinol. This presents an improved chance of advantageous hydrogen bonding occurring between the hydroxyl groups present and the functional groups on the antibiotic molecules. Although electropolymerization techniques lend themselves toward fast single-sensor production, it does not scale well for mass production due to the often multistep production schemes and varying conditions.[187]

Electropolymerization offers a promising method of fast and varied single-sensor production; however, in its current form, the scalability ready for mass production of sensor platforms is not readily available. In particular, this production methodology does not lend itself to array sensor development for multiple analytes. The use and development of more conductive polymers with an array of functional groups will allow for great improvement on the sensor efficacy of eMIP-based sensor platforms for the detection of antibiotics, allowing for improvements in the sensitivity and selectivity.

4.3. Grafting from/to Core–Shell Nanoparticles

There has been considerable increase in the use of MIPs based on core–shell nanoparticles that can overcome the drawbacks generally associated with monoliths. The formation of thin imprinted polymer layers on a solid support enhances binding kinetics, mass transfer, and facilitates easy template removal. Among the materials used for MIP functionalization, Fe_3O_4 magnetic nanoparticles (MNPs) are most often used due to their paramagnetic properties. A comprehensive review on magnetic particles for MIPs in analytical chemistry, including for the extraction of antibiotics from environmental and food samples, is provided by Chen et al.[188] The use of controlled polymerization techniques allows to devise the molecular architecture of interest. Atom transfer radical emulsion polymerization (ATREP) was used to functionalize a molecularly imprinted layer for tetracycline onto magnetic particles,[189] leading to a material that can extract the antibiotic tetracycline with very high specificity from a food sample.

AuNPs have the advantages of excellent optical, electronic, and catalytic properties[190] and are therefore often used for sensor applications. Gold structures combined with silica nanoparticles were used for the specific detection of ENR. The presence of gold core branches[191] acted as intrinsic hot spots that strongly enhance the electric magnetic field, thereby significantly augmenting the Raman scattering and thus leading to a higher specificity. It was shown that the combination of silica and AuNPs increased the signal by a factor of 2 compared with the use of AuNPs on its own.

It is expected that different cores, such as polystyrene microspheres[192] and chitosan microspheres, which possess excellent biocompatibility, will be explored in the future. However, it has to be noted that the use of a thin MIP layer can limit the number of recognition sites, which will hamper the sensor sensitivity. Therefore, currently, the preferred method of choice is suspension or emulsion polymerization, which has proven to yield MIPs with high adsorption capacity while maintaining excellent binding kinetics. The key issues with the current methodology include precise controlling of the thickness, as buried templates can limit the extraction process, and a multistep process that is often not scalable, even though examples of one-pot synthesis of MIP particles are present. The group of Niu developed a range of MIP particles bearing hydrophilic polymer brushes via controlled polymerization[148] techniques. A one-pot synthesis method, based on hydrophilic macromolecular chain-transfer agent (macro-CTA)-mediated reversible addition–fragmentation chain transfer precipitation polymerization, was used to prepare fluorescent MIP nanoparticles for tetracycline. The use of hydrophilic polymers ensured that measurements are compatible with biological samples, which enabled direct quantification of tetracycline in complex biological samples.[148]

Instead of a solid support, it is possible to use as a sacrificial support matrix. The approach chosen by Tang et al.,[193] shown in Figure 3, demonstrates the preparation of hollow MIPs with fast absorption kinetics (≈10 min) for ENR. This method was used to determine the levels of this antibiotic in fish samples, with LoDs well below the legally maximum residual level.

The sacrificed support matrix, $K_2Ti_4O_9$ was chosen because of its nontoxicity, low cost, and easy removal process. Silica is one of the popular choices as sacrificial support as the polymers can be embedded into the pores of the particles. The group of Moreno-Bondi[71] developed MIPs that were able to recognize six cephalosporin below the maximum residual levels set for these antibiotics in raw milk. Silica etching, in contrast, is a more time-consuming process and involves harsh chemicals, as the particles are treated with an ammonium hydrogen difluoride mixture for 24 h. A similar approach, where silica beads were functionalized with MIPs according to the procedure by Yilmaz et al.,[194] was used to synthesize selective recognition elements for structurally related penicillins. The base structure of this family of antibiotics, 6-aminopenicillanic acid, as a dummy template enabled to detect the entire class of target analytes from milk with high recovery rates.[195]

4.4. Solid-Phase Imprinting: Immobilization of the Template on a Solid Support

NanoMIPs have the potential to become cost-efficient and robust alternatives to natural antibodies in diagnostics. However, intrinsic problems associated with the imprinting technique have limited their adoption at an industry level. In particular, the most evident drawbacks are 1) the presence of residual template in

the MIP; 2) high binding site heterogeneity; and 3) lengthy or labor-intensive methodologies required for MIP production.

The solid-phase imprinting approach allows to overcome these drawbacks. In this method, the template is covalently immobilized on the surface of a suitable solid support (such as solid glass beads, magnetic particles, or similar). This support bearing the immobilized template is then placed in contact with the monomer mixture, and polymerization is initiated under conditions that promote the formation of polymeric nanoparticles. After the polymerization, the solid support acts as an affinity medium: by means of a temperature-based affinity step, unreacted monomers, oligomers, and low-affinity particles are eluted. Then, the temperature of the system is increased and this leads to the disruption of the stronger interactions between high-affinity particles and template, allowing to selectively collect high-affinity nanoMIPs only (**Figure 8**).

As previously demonstrated,[196] this process can be easily automated and executed in a matter of a few hours. Because of the affinity purification step, nanoMIPs possess high affinity and specificity toward their targets, exhibit a homogeneous distribution of binding site affinities, and do not contain any residual template (as it was covalently attached to the solid-support). However, template leaching may occur if the bond used to immobilize the template onto the solid phase gets cleaved (usually by hydrolysis) during the elution process.

It should be noted that high-affinity nanoMIPs can also potentially be collected by means of changes in pH and/or solvent. Haupt and coworkers[197] use the transition temperature of NIPAm to collect the high-affinity nanoMIPs, conducting imprinting at 37 °C and the elution at lower temperature (i.e., at 25 °C—which is below the lower critical solution temperature of the MIP polymer). This allows the nanoMIPs to swell, detach from the immobilized template, and be eluted. The proposed approach is generic in nature as virtually any molecule can be imprinted, and it can be conducted both in water and in organic solvents (via a UV-triggered process). However, the template is required to bear at least one functional group for immobilization on the solid phase, and this may be problematic especially for small molecules (<500 Da). Polymerization in the organic solvent has been successfully used to imprint small molecules,[196,198,199] whereas aqueous polymerization has proven effective for imprinting of peptides and proteins.[200–203]

Several works have demonstrated that nanoMIPs produced via solid-phase imprinting can be used as a direct replacement for natural antibodies in diagnostic or bioanalytical assays.[199,204–206]

In one such example, the solid-phase approach was used for the manufacture of these imprinted polymers against vancomycin.[207] They were then successfully immobilized on a novel sensor type capable of measuring tiny variations in temperature upon target binding. Thanks to the high affinity, the LoD of the sensor was lowered by three orders of magnitude compared with MIP microparticles developed without solid-phase extraction. Such improvement can also be attributed to the enhanced conductivity and increased surface area. The developed thermal sensors were capable of measuring samples with a turnaround time of a few minutes (< 5), which potentially enable real-time detection of biomolecules.

In a similar example, antivancomycin nanoMIPs produced by solid-phase synthesis were doped with ferrocene derivatives to make them electroactive.[158] This allowed the indirect electrochemical detection of vancomycin due to the change of redox properties of the ferrocene label upon binding. The authors claimed that the observed behavior is likely due to hindering of the electron transfer process of ferrocene in the nanoMIPs by their interaction with vancomycin.

As mentioned earlier, nanoMIPs have also replaced antibodies in assays. In one such example, antigentamicin-imprinted

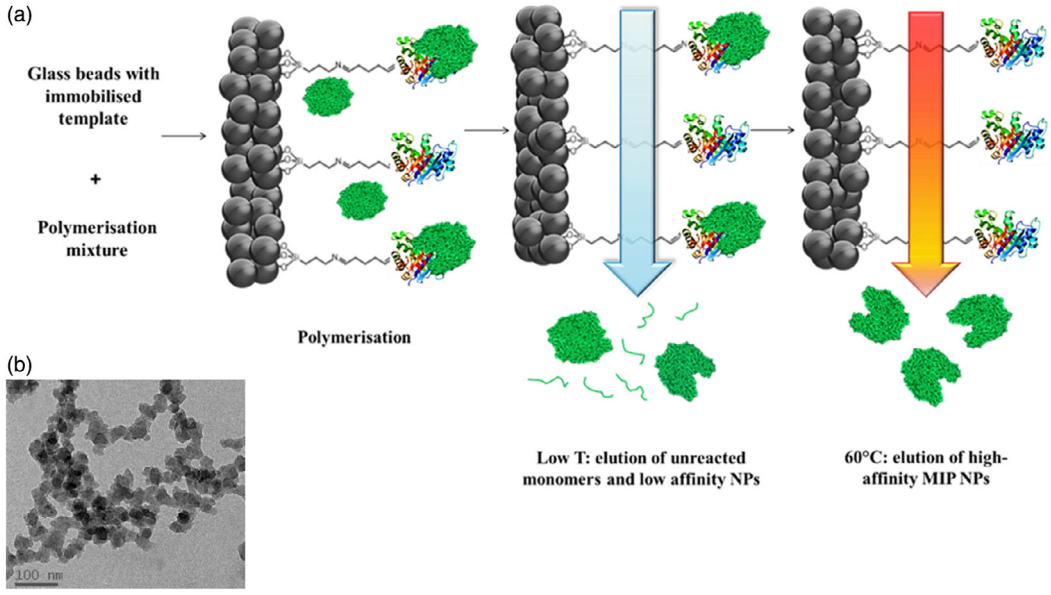

Figure 8. a) Solid-phase synthesis of nanoMIPs. In this example, a protein is shown as the template molecule. b) Representative TEM of nanoMIPs. Reproduced with permission.[207] Copyright 2018, Royal Society of Chemistry.

nanopolymers were used in a pseudo-enzyme-linked immunosorbent assay assay in spiked milk,[208] as a synthetic capture antibody, whereas target detection was achieved in competitive binding tests with a horseradish peroxidase−gentamicin conjugate. The developed polymers showed superior selectivity over other antibiotics (streptomycin and ampicillin) and were capable of detecting gentamicin in milk at clinically relevant concentrations.

5. Outlook on the Future of Commercial MIP Sensors for Antibiotics

The straightforward approach, ease of synthesis, and, more importantly, great chemical stability means that MIP integration within sensors is an appealing prospect as they can function in a variety of different environments. In contrast, a natural receptor that might have to be used instead often suffers from poor stability and lower specificity to the target compounds. In our research scenario, where the target/analyte is an antibiotic, an antibody or receptor might not be available,[209] and in cases such as this, MIPs have a clear advantage over naturally occurring ligands. Due to their relative ease of fabrication, researchers have utilized imprinted polymers for separation purposes, in the development of complex matrix pretreatment strategies,[210,211] and as artificial antibodies.[196,212–214]

While the imprinting techniques have improved greatly over the course of the years, it is easy to see how their use in combination with novel smart and nanomaterials greatly benefits the selectivity and the specificity of the sensors as the interest in exploring the integration of MIPs into chemo-[215–218] and biosensors[219–222] keeps rising. As a multidisciplinary technology, the use of MIPs will greatly benefit from the developments in polymer and material sciences, drug, and environmental research: in contrast, they can be tailored to get maximum advantage from the existing techniques e.g., microfluidic, nanotechnology, biotechnology, and stimuli−responsive technology among others.[223]

In recent years, the threat of AMR has been gathering much interest as the adverse consequences of such resistance are better understood. The manufacture of cheap, reliable sensors able to monitor antibiotic presence in a variety of environments and conditions will provide an avenue of determining the level of human exposure to antibiotics in our everyday life and what implications and impacts this may present for current and future increasing antibiotic resistance. The intrinsic stability of MIPs would allow for a long shelf life and robustness regardless of the conditions they might be used in. The adaptability of MIPs toward a target of interest and their accessibility make them great candidates for this very delicate and important task.

Despite their unequivocal usefulness and practicability, a few factors limit the commercial use of MIPs, including 1) limited sensitivity of microparticles, which makes them unable to compete with commercial antibodies; 2) integration of MIPs into electrodes that is not an evident task; and 3) mass production of these sensors, which is complicated with standard methods of producing nanoMIPs including electropolymerization or lithographic techniques, even though we have shown several examples where these problems are partially overcome. Currently, only a few companies are present in the market and the commercialization of MIPs has mostly been confined to laboratory research, mostly focused on SPE of several environmental contaminants.[224]

MIPs are usually prepared in organic solvents (water affects mostly H-bond-driven template-functional monomer equilibrium) but it has been shown that rebinding is usually more efficient when synthesis takes place in the same solvent used for synthesis.[225] Water and food analysis might thus be a challenge for the obvious presence of water; however, if practices such as taking hydrophilicity of monomers into consideration during monomer selection and conducting controlled radical polymerization to control surface modification are practiced, then, compatibility issues can be reduced. Another issue is the number of functional monomers available that are appropriate for their synthesis, which is a hurdle to overcome. However, this limitation has pushed several researchers to explore alternatives, including the combination of different monomers and the exploitation of amino acids. The issue can also be tackled by the design of new monomers with multiple and varied functional groups, as they can facilitate the ability to offer several interactions with the target, a highly desirable development at the current stage.[63]

The exceptional circumstances in which the COVID-19 pandemic has significantly accelerated the recent growing interest in AMR have drawn the attention to the mass production of in-field sensors, whose main features include long shelf life, stability, and ease of use. Despite the increase in academic output recorded in the last 20 years, industrial application has not followed a similar trend mostly due to the limitations mentioned earlier. Nonetheless, the recent breakthroughs in the synthesis are likely to ignite interest in novel MIPs. Mass production of polymers imprinted with antibiotics, especially if produced using the noncovalent method and mild temperatures, can benefit the great advantage of reusing the template and make sustainability a great selling point. The EU has banned animal-derived antibodies,[226] which boosts the appeal of MIP usage as their design and production does not require animal exploitation, constituting a significant ethical advantage. Moreover, their low cost and robustness make them a safe bet for the construction of field-deployable and in vivo sensors.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

antimicrobial resistance, environmental monitoring, food quality, molecularly imprinted polymers, solid-phase extractions

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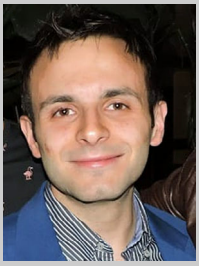

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