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Review

Artificial Biosensors: How Can Molecular Imprinting Mimic Biorecognition?

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Receptors generated by natural evolution in living organisms show an astonishing capacity for specifically recognizing target molecules. If applied as recognition units of biosensors, these receptors provide very high selectivity. However, they suffer from instability under measurement conditions, and low durability. Devising alternative robust artificial receptors circumvents these deficiencies. For instance, an antibody can be successfully replaced by a corresponding molecularly imprinted polymer (MIP), sometimes called a 'plastic antibody'. Therefore, MIPs used as recognition units in chemical sensors are gaining increasing interest. In this review, we survey selected examples of MIPs used for determining target bioanalytes by mimicking natural recognition. For scientists working with biosensors, MIPs might be considered as alternatives to natural receptors, such as antibodies, enzymes, or histones.

Molecularly Imprinted Polymers in Chemosensors

MIPs (see Glossary) have received significant attention for nearly half a century (Box 1). Molecular imprinting results in synthetic intelligent materials that mimic biological recognition. Designing such biomimetic materials is one example of 'learning from nature'. Most applications of MIPs are limited to preparing column-packing materials for flow analytical techniques, such as solid-phase extraction (SPE) and liquid chromatography (LC). These applications have been reviewed extensively elsewhere [1–6]. Herein, we survey recent research publications involving the application of MIPs as recognition units in chemosensors selective for chosen analytes of biological importance.

To avoid confusion, different definitions of chemosensors and biosensors have been recommended by IUPAC. Specifically, a chemosensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal; this information may originate from a chemical reaction of the analyte or from a physical property of the system investigated [7]. By contrast, a biosensor is an integrated receptor-transducer device, which provides selective quantitative or semiquantitative analytical information using a biological recognition element [8]. Artificial recognition units are almost as selective as natural ones, such as antibodies, enzymes, and histones. However, artificial recognition units outperform natural ones, with significant advantages including low cost, long-term stability, and resistance to harsh environmental conditions. Therefore, biological recognition units are being increasingly replaced with artificial recognition units (Figure 1, Key Figure).

Most previous reviews on MIPs have surveyed the general literature referring to MIP fabrication. Here, we provide a new perspective on this topic. We discuss examples of MIP-based chemosensors that are the most similar to biosensors to highlight the former to scientists working with biosensors and to encourage them to consider MIPs as an alternative to the antibodies or

Trends

MIPs can recognize target analytes not only by their shape and size, because introducing a dedicated set of recognizing sites into the imprinted cavity increases both the affinity of the cavity for the analyte and its selectivity with respect to interferences.

Different functional monomers have been introduced to provide selective chemical recognition that involves the formation of covalent bonds, hydrogen bonds, and coulombic and supramolecular interactions, as well as metal chelation and π-π stacking.

MIPs are prepared as bulk polymers, which can then be ground, or, alternatively, can be prepared as beads with the desired shape and size. However, for chemosensor fabrication, these MIPs should be deposited as thin films. Electropolymerization or electrochemically inducted polymerization is best suited for depositing both conducting and nonconducting MIP films.

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Box 1. Historical Background of Molecular Imprinting

Artificial recognition materials based on the molecular imprinting concept date back to Dickey's pioneering work in 1955 [78]. He modified selective silica adsorbents with analytes of organic dyes, such as methyl orange and its homologs, by acidifying silica solutions of these dyes. However, low repeatability in the preparation of these adsorbents and their moderate selectivity with respect to the dye target analytes resulted in little interest from the analytical chemistry community. During the early 1970s, Wulff *et al.* [79] and Klotz *et al.* [80] introduced the concept of molecular imprinting. Illustrated by imprinting templates in organic polymers, this concept opened new horizons for designing molecular cavities in a precise manner. A real breakthrough came in 1993, when Mosbach *et al.* [81] introduced a radiolabeled ligand-binding assay proving that MIPs can replace antibodies in biosensors.

enzymes that they currently use in their research. We also provide the first published summary of strategies of molecular recognition. Therefore, this review not only gives a brief overview of this field for nonspecialists, but constitutes a convenient summary for a researcher working in the MIP field. We also emphasize currently underappreciated molecular interactions, such as supramolecular interactions or π-π stacking, which should be taken into account when selecting appropriate functional monomers to achieve selective recognition.

How Do MIPs Mimic Biological Systems?

The main sensing feature of MIPs comprises selective recognition of the target analyte because of the dedicated architecture of cavities embedded in the polymer matrix. Formation of these cavities involves three steps (Figure 1): (i) prearrangement of functional monomer molecules around the template molecule in solution; (ii) polymerization of the resulting complex in the presence of cross-linking monomers and porogens (e.g. suitable solvents or ionic liquids); and finally (iii) template removal from the synthesized MIP. The first step is crucial for generating highly selective molecular cavities. This prearrangement can be accomplished either by forming covalent bonds or by self-assembling with noncovalent bonds (Box 2).

Artificial Immunosensors: Macromolecule Chemosensors based on MIPs

MIPs can recognize analytes of the molecules that fit the shape and size of the imprinted molecular cavities (Figure I in Box 2). Therefore, this recognition can be applied only for macromolecular compounds that differ from each other much more than low-molecular-weight compounds do, e.g., native and denatured proteins. There are several ways of preparing MIPs that discriminate analytes by size and shape. These polymers comprise monomers capable of only weak and rather nonspecific interactions with target analyte molecules, (i.e., hydrophobic and van der Waals forces, or weak hydrogen bonds). For instance, three different procedures were proposed and compared for preparing **acrylamide** (AA) polymers with generated cavities recognizing trypsin protein by shape [9]. The proposed procedures were: (i) polymerization from a solution of the protein template and monomers; (ii) pressing the protein crystal stamps against a thin layer of the prepolymerization dense solution; and (iii) polymerization with a layer of the lyophilized protein on a transducer surface. Each procedure allowed for **Au-quartz crystal resonators** (Au-QCRs) coating with trypsin-templated MIPs. These MIP film-coated Au-QCRs were then used for piezoelectric microgravimetry (PM) determination of trypsin with a quartz crystal microbalance (QCM). The first procedure was determined to be superior to the other two.

Moreover, films of shape-recognizing MIP materials can be deposited by electropolymerization of the phenol or aniline monomers in the presence of the target proteins. The deposited films are only dozens of nanometers thick because of the low conductivity of the resulting polyphenol or polyaniline films. This control of film thickness yields MIP films that are thinner than the diameter of the target macromolecule. Therefore, all of the imprinted cavities are located directly on the transducer surface and are all open, thus facilitating the diffusion of the analyte from the solution. For example, IgG was covalently immobilized on the Au-QCR surface with a linker containing the cleavable disulfide bond [10]. Then, a

Glossary

2,4-Dichlorophenol (DCP): a photodegradation product of triclosan.
Acrylamide (AA): a functional monomer.
ATP: a coenzyme transporting chemical energy within cells.
Bovine serum albumin (BSA): protein often used as a concentration standard.
Capacitive impedimetry (CI): an electrochemical technique allowing for determination of capacity of a solution–electrode interface.
Differential pulse voltammetry (DPV): an electroanalytical technique used for faradaic current measurement.
Electrochemical impedance spectroscopy (EIS): an alternating current electrochemical technique used for investigation of electrochemical processes.
Enzyme-linked immunosorbent assay (ELISA): a test using antibodies and an enzymatic reaction to detect a biological substance.
Ethylene glycol dimethacrylate (EGDMA): a cross-linking monomer.
Flow-injection analysis (FIA): a method of flow analysis involving sample solution injection to a flowing carrier solution.
Gold film-coated quartz crystal resonator (Au-QCR): a transduction element of a quartz crystal microbalance (QCM) used in piezoelectric microgravimetry (PM). Any change in its mass, viscosity, or density, caused by analyte adsorption on its surface, changes its resonant frequency.
Hemoglobin (Hb): protein responsible for oxygen and carbon dioxide transport in blood.
Horse apoferritin (ahsFtn): horse ferritin (hsFtn) not combined with the iron cation. Generally, this protein is responsible for iron storage in liver and for controlled iron release.
Horseradish peroxidase (HRP): a redox enzyme.
Human serum albumin (HSA): constitutes nearly half the protein fraction in blood serum; mainly responsible for transport of hormones, fatty acids, and xenobiotics.
Imprinting factor (IF): enables one to distinguish the binding of the analyte inside molecular cavities from nonspecific adsorption; defined as the ratio of the calibration plot

polydopamine film was deposited by electropolymerization over the immobilized template protein molecules. Given that this electropolymerization allowed precise control of the amount of the deposited polymer, several films of different thicknesses were tested and the 17.7 nm-thick film was determined to be optimal (IgG molecules range in size from 8.5 to 18 nm). Moreover, the thickness of the deposited MIP is compatible with electropolymerization on nanoelectrodes. For the purpose of electropolymerization, tips of arrayed carbon nanotubes [11], an array of gold nanoelectrode ensembles (GNEEs) [12], or a graphene/(carbon nanotube)-modified carbon electrode [13], were coated with polyphenol or polyaniline MIP films imprinted with human ferritin, human papillomavirus E7 protein (type-16), the Ca^{2+}-calmodulin complex [11], CA 125 protein [12], or **bovine serum albumin** (BSA) [13]. The current detection signal increased significantly at these arrays because of semispherical analyte diffusion to these nanoelectrodes. Therefore, it was possible to reach a **limit of detection** (LOD) as low as 10 ag/ml of human papillomavirus E7 protein.

MIPs can recognize the target analyte not only by its shape and size. Introducing a dedicated set of recognizing sites into the imprinted cavity increases both the affinity of the cavity to the target analyte and its selectivity with respect to interferences. These sites can interact with the template or analyte molecules in a noncovalent way, such as via coulombic interactions, hydrogen bonding, ion coordinating, π-π stacking, hydrophobic and van der Waals forces, or by reversible formation of covalent bonds (Table 1).

For affording coulombic interactions, functional monomers containing acidic or basic groups capable of dissociation are often used. These monomers are usually capable of hydrogen bonding and, most likely, both coulombic interactions and hydrogen bonds are responsible for analyte binding inside the imprinted cavity. This noncovalent imprinting is straightforward and flexible. **Methacrylic acid** (MAA) **1** and acrylamide **2** are the most popular functional monomers of this type (Table 1). Several review articles provide selected examples of their application (e.g., [3–6]). These monomers are usually copolymerized with cross-linking monomers, such as ***N,N′*-methylene bis-acrylamide** (MBAA) and **ethylene glycol dimethacrylate** (EGDMA). Typically, the resulting MIPs are prepared as bulk polymers, which can then be ground, or, alternatively, they can be prepared as beads of the desired shape and size [5,6]. For chemosensor fabrication, these MIPs should preferably be deposited as thin films. Electropolymerization appears to be the technique best suited for depositing these films. Therefore, there is increasing interest in engaging electropolymerization in the preparation of MIP films, both conducting and nonconducting [14,15]. For this purpose, MIP films are deposited from solutions containing functional monomers (e.g., monomers **3**–**11** with embedded electropolymerizable moieties and functional carboxy, amine, or hydroxy groups), template molecules, and suitable cross-linking monomers [16–27].

Noncovalent imprinting is efficient for low-molecular-weight compounds. However, for macromolecular compounds (e.g., proteins), it is difficult to indicate which and how many charged binding sites on the template molecule are accessible for interactions with the recognizing sites of the imprinted cavity. Therefore, time- and labor-consuming optimization of the molar ratio of the functional monomers to a peptide in the prepolymerization solution is crucial. Recently, this hurdle was circumvented [16]. That is, semicovalent imprinting allowed the deposition of a film of conducting MIP that was selective to **human serum albumin** (HSA). This selectivity was achieved by molecular cavities containing recognizing groups of both quaternized amine **8** and carboxylate **3**. A high **imprinting factor** (IF) of this MIP (≥20) proved that semicovalent imprinting resulted in a large number of well-defined molecular cavities with high affinity to HSA molecules.

Moreover, recognizing sites of imprinted cavities can capture analyte molecules by supramolecular interactions. In one example [28], a functional monomer **12** containing the crown ether-recognizing moiety was designed that enabled imprinting of melamine, which contains three

constructed for an MIP and that for an NIP; considered to be high if >10.
Indium-tin oxide (ITO): an optically transparent conductive (glass) material.
Limit of detection (LOD): concentration of an analyte for the signal-to-noise ratio usually assumed to be not less than 3.
Magnetic nanoparticle (MNP): usually comprises a magnetic core coated with a chemically resistant shell.
Methacrylic acid (MAA): a functional monomer.
Molecularly imprinted polymer (MIP): a polymer containing molecularly imprinted cavities capable of selective recognition of compounds whose molecules fit these cavities with respect to shape, size as well as position and orientation of recognition sites.
***N,N′*-methylene bis-acrylamide (MBAA):** a cross-linking monomer.
Nanoparticle (NP): a particle of a nanometer size.
Non-imprinted polymer (NIP): a control polymer similar to an MIP but not imprinted.
Quantum dot (QD): a fluorescent semiconductive NP.

Key Figure

Molecularly Imprinted Polymers (MIPs) Can Mimic Natural Recognition Units.

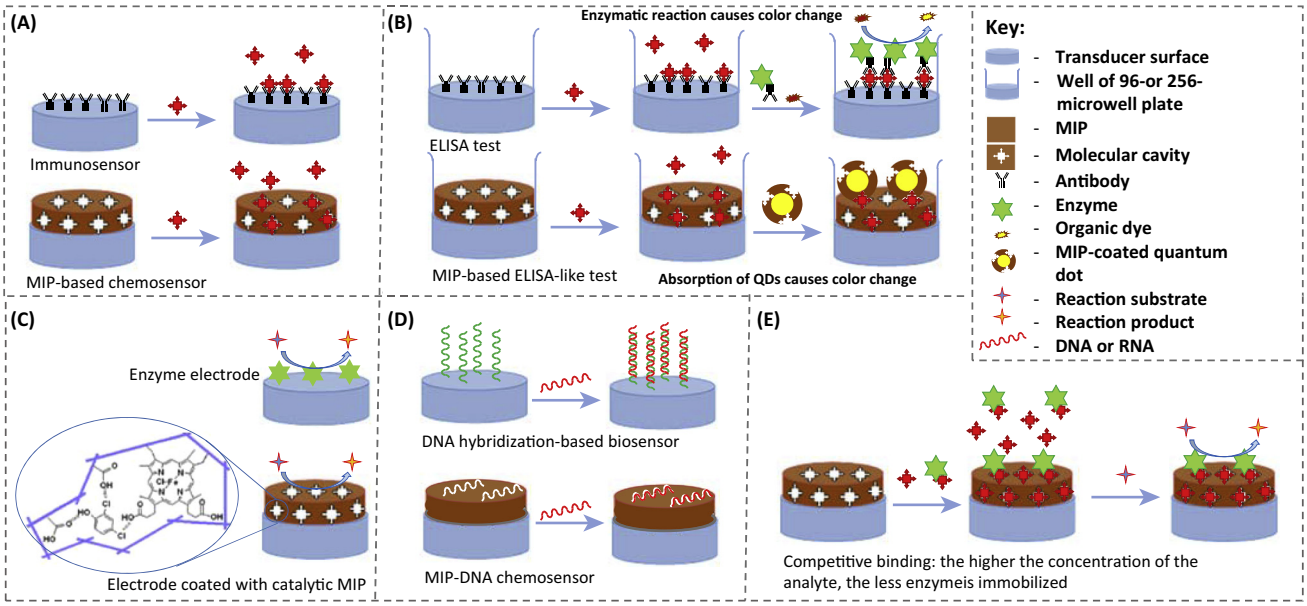


Figure 1. Some applications of MIPs include: (A) immunosensors; (B) enzyme-linked immunosorbent assay (ELISA) tests; (C) enzyme electrodes; and (D) DNA chips. Selective analyte binding by bio- or chemosensors (A,D) can be determined by the change in mass deposited on the gold film-coated quartz crystal resonator (Au-QCR) (piezometric microgravimetry; PM), electrochemical properties of the electrode surface [differential pulse voltammetry (DPV) or electrochemical impedance spectroscopy (EIS)] or optical properties [surface plasmon resonance (SPR) spectroscopy, fluorescence quenching]. For (C), the reaction rate and, thus, analyte concentration, of enzyme electrodes and catalytic MIP-coated electrodes may be estimated by determining the electroactive substrate/product consumption/production during the catalytic reaction, or electron transfer from the electrode surface to the active canter of the enzyme/MIP. Moreover, MIP films may serve as selective platforms for enzyme immobilization, and can be used for (E) competitive binding of the analyte and the analyte labeled enzyme for example. Adapted from [66] (C).

primary amine groups in its structure. The complex of all-amine-groups quarternized melamine with the functional monomer **12** with a 1:3 stoichiometry was electropolymerized in the presence of a suitable cross-linking monomer, resulting in an MIP film deposited on the Au-QCR surface. This film served as the recognition unit of a PM chemosensor in the determination of melamine under **flow-injection analysis** (FIA) conditions. Another example of supramolecular interactions adopted for imprinting involved the formation of inclusion complexes of phenyl substituent guest moieties with monomers containing calixa[6]rene [29] or cyclodextrin macrocycle host moieties [30,31]. Moreover, functional monomer **8** was used to selectively recognize the explosive 1,3,5-trinitrotoluene compound via π-π stacking interactions between the electron-deficient aromatic ring of the target analyte and the electron-rich aromatic ring of monomer **8** [32].

Coordination of metal ions is another way of selectively recognizing target analytes. In one example, engineered *N*-(4-vinylbenzyl)iminodiacetic acid **14** was used as the functional monomer [33]. This monomer was capable of ligating Cu(II) ions. The resulting complex bound the protein (RNase A) template via Cu(II) coordination. Polymerization of this complex led to deposition of an MIP film on silica beads, which were surface modified with vinyl groups. Then, these beads were used as a column-packing material for RNase A separation by HPLC. Moreover, the [*N*-(4-vinylbenzyl)diethylenetriamine]Cu(II) diformate **15** functional monomer was used for imprinting several mono- and disaccharides [34,35].

Box 2. How to Prepare a Polymer with Molecularly Imprinted Cavities

The formation of a molecular cavity, and then selective recognition of the target analyte can be accomplished via two different chemical routes (Figure I). One involves covalent imprinting, as introduced by Wulff [79]. In covalent imprinting, functional monomers are bound to template molecules with covalent bonds. After polymerization, the latter are removed from the resulting polymer by cleaving these bonds. The analyte is recognized via the formation of similar covalent bonds. The main advantage of this procedure is the well-defined structure of these cavities. However, MIP preparation is complicated, and time and labor intensive; moreover, drastic conditions are required for template removal from this MIP. In addition, the functional monomers applied must readily react with the template. Therefore, the selection of these monomers is mostly limited to boronic acid derivatives.

Another strategy involves noncovalent imprinting, as proposed by Mosbach and Sellergren in 1988 [82]. In this strategy, template molecules and functional monomers preorganize themselves in solution solely by noncovalent interactions, including chelation, hydrogen bonding, and van der Waals or hydrophobic interactions. After subsequent MIP preparation, analytes are recognized by the same noncovalent interactions. These MIPs are easily prepared and templates are readily removed under mild conditions. Reversibility of prepolymerization complex formation is the major disadvantage of noncovalent imprinting. In effect, the imprinted molecular cavities may not be homogenous and part of the functional monomers may not be located inside the cavities but randomly distributed in the polymer matrix, thus lowering the imprinting factor and, hence, the MIP selectivity with respect to the analyte.

To circumvent the above deficiencies, in 1990 Sellergren introduced a semicovalent strategy for imprinting [83]. This strategy involves covalent binding a template with functional monomers. After subsequent polymerization, the template is removed by cleaving the formed covalent bonds. Then, the analyte attaches with its binding sites to recognition sites of the imprinted cavity only in a noncovalent fashion. The semicovalent imprinting strategy combines advantages of covalent imprinting with the flexibility of noncovalent imprinting.

Introduced by Whitcombe *et al.* in 1995, a strategy using a 'sacrificial spacer' is a useful modification of semicovalent imprinting [84]. In this strategy, a functional monomer and a template are joined together with a short linker, such as a carbonate, and then polymerized. Next, two bonds are cleaved, thus releasing both the linker and the template. Thus, the distance between the functional monomer molecule and the template molecule is precisely controlled.

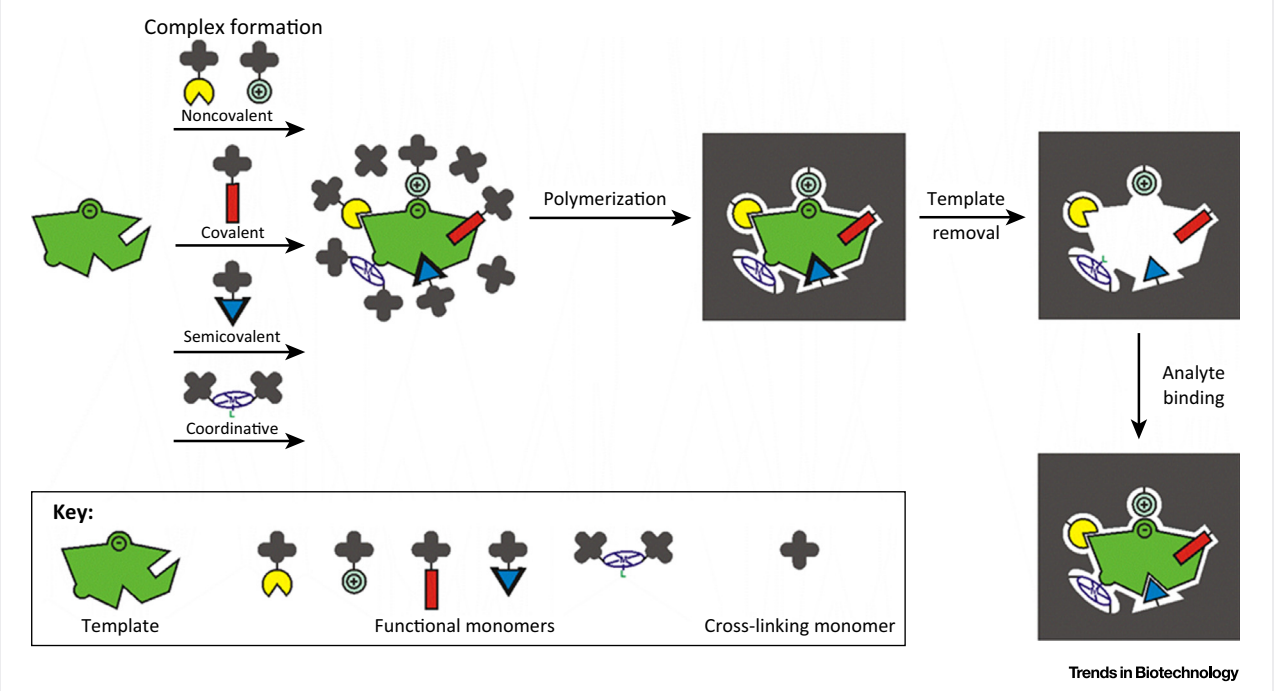


Figure I. Different Routes of Molecular Cavity Formation in a Molecularly Imprinted Polymer. Adapted from [85].

The strategy of recognition by reversible formation of covalent bonds enables the generation of molecular cavities with well-defined structures in an MIP. Therefore, it results in MIPs with high affinity for the molecule of a target analyte and high selectivity with respect to interfering compounds. However, designing and preparing such an MIP is challenging because the covalent bonds should stay intact during monomer polymerization, but this bond should also be easily cleaved during template extraction. Therefore, covalent imprinting is so far limited to MIPs prepared by using boronic acid derivatives, such as 3-aminophenyl boronic

Table 1. Selected Examples of Functional Monomers Providing Defined Recognizing Sites in Imprinted Molecular Cavities

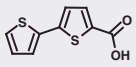
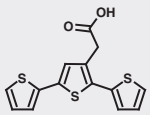
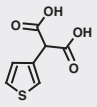
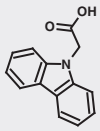
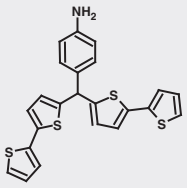
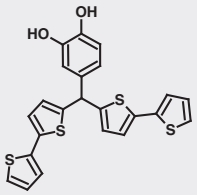
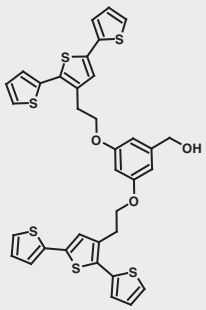
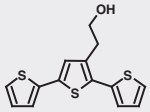
No.	Chemical Name	Structural Formula	Possible Interactions with Target Molecule	Comment	Refs
1	Methacrylic acid		Hydrogen bond donor or acceptor, or electrostatic interactions in a dissociated form	Deposition of MIP films by induced free-radical polymerization	Reviewed in [3–6]
2	Acrylamide		Hydrogen bond donor or acceptor		
3	2,2′-Bithiophene-5-carboxylic acid		Hydrogen bond donor or acceptor, or electrostatic interactions in a dissociated form	Deposition of MIP films by oxidative electropolymerization	[16]
4	3-Thiophene acetic acid				[17–19]
5	2-[2,5-Di(thiophen-2-yl) thiophen-3-yl] acetic acid				[20–23]
6	3-Thiophene malonic acid				[24]
7	2-(9H-Carbazol-9-yl)acetic acid				[25]
8	*p*-Bis(2,2′-bithien-5-yl) methylalanine		Hydrogen bond donor or acceptor, or electrostatic interactions in a protonated form		[16,32]
9	*p*-Bis(2,2′-bithien-5-yl)methyl-*o*-catechol		Hydrogen bond donor		[26]
10	3,5-Bis{[2-(2,5-dithiophen-2-ylthiophen-3-yl)ethoxy] phenyl}methanol				[27]
11	2-[2,5-Di(thiophen-2-yl)thiophen-3-yl]ethanol				[25]

Table 1. (continued)

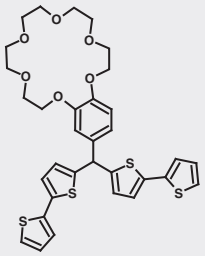
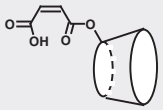
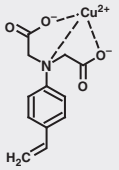
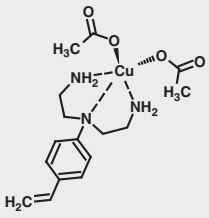
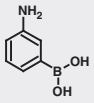
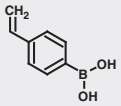
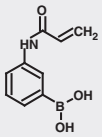
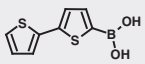
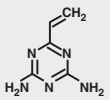
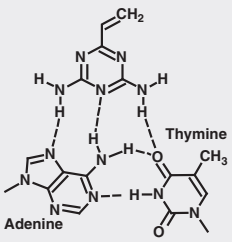
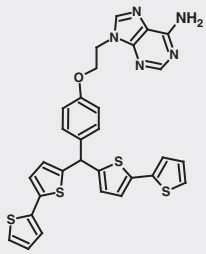
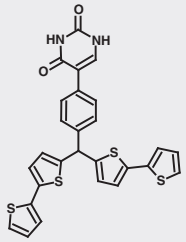
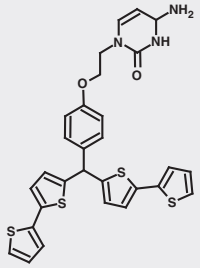
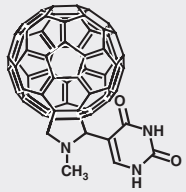
No.	Chemical Name	Structural Formula	Possible Interactions with Target Molecule	Comment	Refs
12	Bis(2,2′-bithien-5-yl)-methylbenzo-18-crown-6		Coordination of protonated primary amines		[28]
13	*β*-Cyclodextrin-6-monomaleinate		Formation of inclusion complexes with hydrophobic moieties		[30]
14	*N*-(4-Vinylbenzyl) iminodiacetic acid		Coordination of diols via the copper ion		[33]
15	[*N*-(4-Vinylbenzyl) diethylenetriamine] Cu(II) diformate				[34,35]
16	3-Aminophenyl boronic acid		Reversible formation of esters with vicinal diols	Formation of boronic acid esters can be reversed by controlling pH of the solution	[36–39]
17	4-Vinylphenyl boronic acid				[40–45]
18	3-Acrylamido phenylboronic acid				[46]
19	2,2′-Bithiophene-5-boronic acid				[47,48]
20	2-Vinyl-4,6-diamino-1,3,5-triazine				[53]

Table 1. (continued)

No.	Chemical Name	Structural Formula	Possible Interactions with Target Molecule	Comment	Refs
21	*p*-Bis(2,2′-bithien-5-yl)methyl phenol 2-(adeni-9-yl)ethyl ether		Formation of Watson-Crick pairs with complementary nucleobases	Deposition of MIP films by oxidative electropolymerization	[54]
22	*p*-Bis(2,2′-bithien-5-yl)methyl phenyluracil				[55]
23	2-(Cytosin-1-yl)ethyl *p*-bis(2,2′-bithien-5-yl) methylbenzolate				[48,56]
24	5-(1-Methyl fulleropyrrolidin-2-yl)-uracil			Deposition of MIP films by reductive electropolymerization	[57]

acid **16** [36–39], 4-vinylphenylboronic acid **17** [40–45], 3-acrylamidophenylboronic acid **18** [46], and 2,2′-bithiophene-5-boronic acid **19** [47,48], as functional monomers. The boronic acid group reversibly forms esters with two adjacent hydroxyl groups of vicinal diols and catechols. This strategy should be well suited for imprinting carbohydrates and polysaccharides in particular. Despite many examples of successful monosaccharide [36,37,44–47] and glycoprotein [38–43] covalent imprinting, oligo- and polysaccharides appear to be more demanding and currently there are no published examples of the successful covalent imprinting of these saccharides and only a few examples of noncovalently imprinted oligo- and polysaccharides [49,50].

MIPs for Aptamer–DNA Recognition

Aptamers are short oligonucleotides or peptides complementary to target compounds of the same type. Typically, aptamer oligonucleotides are immobilized on a solid substrate to fabricate biosensors for the determination of the DNA or RNA of a complementary nucleotide sequence [51].

There have been several attempts to use molecular imprinting for oligonucleotide detection (Figure 1D). Recently, a highly selective MIP-based amperometric chemosensor for short (17-mer), single-stranded DNA (ssDNA), associated with the point mutation of the human prostate cancer susceptibility gene *p53*, was devised [52]. The *o*-phenylenediamine functional monomer was electropolymerized on an **indium-tin oxide** (ITO) electrode in the presence of this oligonucleotide. Not only was the resulting poly(*o*-phenylenediamine) matrix wrapped around the oligonucleotide, but its recognizing amine groups also provided a set of complementary hydrogen bonds and electrostatic interactions matching the corresponding binding sites of the imprinted oligonucleotide template. Advantageously, the sensitivity of this amperometric chemosensor was 20 times lower to the oligonucleotide with only one mismatched nucleotide than to the oligonucleotide analyte.

Furthermore, highly selective oligonucleotide recognition was accomplished by designing monomers capable of specific nucleobase recognition. In one example, the 2-vinyl-4,6-diamino-1,3,5-triazine (VDAT) **20** monomer was specifically designed for binding the adenine–thymine pair in double-stranded DNA (dsDNA) [53]. The imprinted polymer was prepared by chemical polymerization in a solution of VDAT, acrylamide, a cross-linking monomer, and the verotoxin gene dsDNA template. Then, the resulting polymer was deposited on a silanized glass surface. This polymer was subsequently used for the determination of a fluorescently labeled dsDNA. Unfortunately, the selectivity of the resulting biosensor with respect to interference [the oligo(dG)–oligo(dC) nucleotide of the same length] was unsatisfactory. However, when a similar MIP film was deposited on the surface of a surface plasmon resonance (SPR) chip, the selectivity of the resulting chemosensor was higher. In the latter case, changes in the SPR signal in the presence of the oligo(dG)–oligo(dC) nucleotide interference were negligible. Moreover, the dissociation constant, K_D, of the MIP-verotoxin gene dsDNA complex was determined with SPR. The determined value was high, equaling 1.4×10^{-9}. This value was similar to that ($K_D = 10^{-10} - 10^{-11}$) for Cro regulatory protein, which binds λ-DNA.

In addition, bis(2,2′-bithiophen-5-yl)methane derivative functional monomers based on the nucleobases adenine **21** [54], uracil **22** [55], and cytosine **23** [48,56] were synthetized. They were successfully used for imprinting low-molecular-weight compounds, including neopterin [48], 5-fluorouracil [54], ATP [55], and 6-thioguanine [56]. Here, thin MIP films were deposited by electropolymerization of bis(2,2′-bithiophen-5-yl)methane derivative functional monomers with appropriate cross-linking monomers in the presence of their respective templates. These films served as recognition units in PM, **differential pulse voltammetry** (DPV), and **capacitive impedimetry** (CI) determinations. In another study, the C_{60} functional monomer bearing a uracil addend **24** was introduced for ATP imprinting [57]. It enabled the preparation of the MIP-ATP film by electropolymerization and deposition in the negative potential range. Films of this MIP-ATP served as recognition units in the PM and CI determination of ATP. However, oligonucleotides appeared to be more demanding targets for imprinting. The susceptibility of DNA and RNA to environmental factors, such as pH, temperature, and particularly the presence of organic solvents, made applying the above monomers to oligonucleotide imprinting impossible.

What if the Template Is Too Fragile?

The imprinting technique is now so well developed that devising selective MIPs is relatively easy for a large number of templates featuring binding sites. However, there are classes of template that still resist imprinting. These mainly include macromolecular compounds, such as oligonucleotides or oligo- and polysaccharides. Their sensitivity to harsh environmental conditions, including unfavorable pH, temperature, and particularly the presence of aggressive organic solvents, is why they have not yet been successfully imprinted. A solution may involve the use of a 'dummy template,' or imprinting a surrogate template of a similar shape and location of binding sites but of different chemical structure than that of the target analyte. This imprinting strategy has been successfully used in the case of low-molecular-weight liable compounds. For instance,

trinitrophenol was used instead of highly explosive trinitrotoluene to deposit an MIP film on the surface of **quantum dots** (QDs) [58]. The fluorescence of those QDs was quenched in the presence of trinitrotoluene.

Recently, a TATAAA (T, thymine; A, adenine) hexamer of peptide nucleic acid (PNA) was used as a 'dummy template' for DNA imprinting [59]. The high chemical stability of the resulting duplex allowed for deposition by electropolymerization of an MIP film from an acetonitrile solution using A- and T-substituted bis(2,2′-bithien-5-yl)methanes as functional monomers. Although the imprinted nucleobase sequence was short, this new procedure opens routes to imprinting longer oligonucleotides.

Catalytic MIPs as Artificial Enzymes

A large group of biosensors involves enzymatic electrodes. These biosensors combine electrochemical transduction with recognition reactions catalyzed by immobilized enzymes [60,61]. Numerous MIPs mimic enzyme active centers, exhibiting selective catalytic properties [62,63]. These MIPs, particularly those catalyzing redox processes, can readily be applied for electrochemical determinations.

For instance, electrodes coated by tyrosinase-mimicking MIP films were devised [64,65] that contained two Cu(II) cationic centers ligated by imidazole monomers. These centers were capable of catalyzing the oxidation of catechols. The catechol concentration was determined by voltammetry or using oxygen electrodes.

Another example involved an electrode coated with the film of a polymer imprinted with **2,4-dichlorophenol** (DCP). This MIP mimicked the dehalogenative activity of the chloroperoxidase enzyme [66]. Chlorochemin, used to prepare this MIP, had a dual role. Together with methacrylic acid, it both served as the functional monomer responsible for selective DCP binding and operated as the oxidation catalyst (Figure 1C). A microsuspension of the resulting MIP microgel was drop-cast on the surface of the chitosan/Nafion film-modified glassy carbon electrode (GCE). For determination, the DCP analyte was first catalytically oxidized to chloroquinone by the MIP in the presence of hydrogen peroxide, and then this chloroquinone was determined by DPV.

Moreover, hemin, the 4-vinylpyridine functional monomer, and the ethylene glycol dimethacrylate cross-linking monomer were deposited in the presence of 5-hydroxyindole-3-acetic acid on magnetic particles [67]. After polymerization, the resulting core/shell magnetic/MIP particles were capable of determining 5-hydroxyindole-3-acetic acid as follows: (i) the devised artificial MIP enzyme was attached to the surface of a carbon screen-printed electrode by an external magnetic field; (ii) the target analyte, 5-hydroxyindole-3-acetic acid, was bound in MIP molecular cavities; and (iii) these molecular cavities operated as the active centers of peroxidase. Therefore, 5-hydroxyindole-3-acetic acid was oxidized in the presence of oxygen peroxide to oligomeric products. Finally, (iv) these oligomeric products were released from the MIP and electrochemically reduced on the electrode surface.

Combination of Molecular Imprinting and Biosensing

The imprinting strategy can be combined with the **enzyme-linked immunosorbent assay** (ELISA) protocol [68] because MIPs can mimic biorecognition units in biosensors. That is, antibodies used in a typical ELISA may be replaced by MIPs. MIPs can have a dual role in these protocols. That is, they can be deposited on a solid substrate to provide selective accumulation of the target analyte on the one hand and MIP-coated **nanoparticles** (NPs) can be used for selective labeling the absorbed analyte on the other. In one study, NPs of an acrylamide polymer imprinted with histone or fibrinogen were prepared that contained monomers derivatized with the biotin moiety [69]. These moieties enabled these MIP NPs to be labeled with biotinylated

horseradish peroxidase (HRP) using avidin-biotin interactions. The target proteins were physically adsorbed on walls of the Nunc-Immuno^{TM} MicroWell^{TM} 96-well microplate. Then, they were contacted with a suspension of MIP-HRP NPs. Moreover, a solution of the commercial *o*-phenylenediamine substrate for HRP determination was added to the microwells to determine the HRP activity with a dedicated microplate spectrophotometer.

In the most extreme example, MIPs replaced both antibodies in the ELISA setup. In this example, a thin film of poly(ethylene-co-vinyl alcohol) (pEVAL) imprinted with the protein template was deposited on both walls of the 96-well microplate and on the surface of QDs [70]. After binding target proteins in microplate wells, a suspension of QD@MIP was added. Next, all of the unbound molecules and QDs were washed out, and the fluorescence intensity in all of the wells was then measured using a hybrid microplate reader at the excitation wavelength of 430 nm. This procedure was adopted for the determination of proteins such as amylase and lipase (both from hog pancreas), and lysozyme.

Another interesting example of indirect detection of protein binding involved fabricating NPs of $Cu(OH)_2$ wrapped with a lysozyme-imprinted TiO_2 shell [71]. These NPs had a dual role: they functioned both as supports for immobilizing the imprinted TiO_2 and as the catalyst for *o*-phenylenediamine oxidation. As more molecules of the protein target compounds were bound to the NPs of $Cu(OH)_2$@TiO_2, the surface of the NPs became more hydrophobic, and more of these NPs were physically adsorbed on the surface of the polystyrene 96-well strongly binding ELISA plate. Finally, after washing, and then treating with a solution of *o*-phenylenediamine, which was subsequently oxidized, the solution turned intensively yellow and fluorescence appeared with an intensity maximum at 568 nm.

In another example, **magnetic NPs** (MNPs), each coated with a film of an acrylic polymer imprinted with **hemoglobin** (Hb), were combined with antibodies labeled with Ru@SiO_2@Au [72]. These MNPs, Hb, and labeled antibodies formed a 'sandwich' complex. After the sandwich was formed, an external constant magnetic field was applied to pull the MNPs to the electrochemiluminescence detector surface, thus enhancing the detection signal.

Moreover, molecular imprinting can be combined with enzymatic electrode sensing. For instance, electrode surfaces were coated with films of MIPs imprinted with antibiotic templates, such as streptomycin [73] or oxytetracycline [74,75] (Figure 1E). In both examples, the analytical protocol involved competition of the antibiotic analyte with glucose oxidase labeled with the respective streptomycin or oxytetracycline template. The analyte concentration was determined amperometrically by monitoring the level of hydrogen peroxide originating from glucose oxidation. This oxidation was catalyzed by the glucose oxidase absorbed on the surface of the MIP film-coated electrodes. The more molecular cavities occupied by the analyte, the fewer glucose oxidase molecules were absorbed and, hence, the lower the amount of hydrogen peroxide produced in the electrode vicinity.

Miscellaneous Applications

Molecularly imprinted polymer NPs have recently attracted much interest [76]. This form of MIPs is most promising for both fundamental research and practical applications. For example, NPs of MIP imprinted with melittin (a component of bee venom) injected into mouse protected the animal from the bee venom [77]. Moreover, NPs of SiO_2 coated with a sialic acid (SA)-imprinted MIP film bearing nitrobenzoxadiazole (an organic dye) moieties in the polymer matrix were prepared [45]. These NPs were used for fluorescent labeling cells with SA-terminated glycan motifs on their surface. The presence of SA-terminated glycans correlated with diseases such as cardiovascular and neurological diseases and cancer. Therefore, absorption of fluorescent core/shell NPs enabled cancer cell detection and visualization using fluorescence microscopy.

Table 2. Analytical Performance of MIP Chemosensors Prepared with Selected Functional Monomers

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
1	Aspartame	MIP chemosensor based on colloidal sphere-patterned polyterthiophene thin film with **5** as the functional monomer	QCM piezoelectric microgravimetry (PM)	12.5–200 μM	31 μM	High selectivity with respect to similarly structured analog molecules, such as Ala–Gln, Gly–Gly, Ala–Phe and Arg–Gly–Asp	[23]
2		Bienzymatic biosensor comprising alcohol oxidase, carboxyl esterase, and BSA immobilized with glutaraldehyde onto screen-printed electrodes modified with cobalt phthalocyanine	Amperometry	5–600 μM	0.2 μM	(i) Applied in flow-injection analysis (FIA); (ii) high cost of fabrication and difficult to maintain; (iii) no selectivity with respect to methanol	[86]
3	HSA	MIP-HSA chemosensor based on semi-covalent poly-bis(2,2′-bithien-5-yl)methane film prepared using functional monomers **3** and **8**	**Electrochemical impedance spectroscopy** (**EIS**)	4–80 μg/ml	0.8 μg/ml	(i) Straightforward fabrication; (ii) high stability; its sensitivity remained unchanged for 3–4 weeks of everyday use (at least 40–50 measurements); (iii) applied for determination of HSA in several spiked artificial human serum samples	[16]
			Differential pulse voltammetry (DPV)	0.8–20 μg/ml	16.6 ng/ml		
4		Si-p/SiO_2/Si_3N_4 film-based immunosensor	EIS	10^{-13}–10^{-7} M (6.6 pg/ml to 6.6 μg/ml)	10^{-14} M (0.66 pg/ml)	(i) Laborious and expensive fabrication; (ii) disposable immunosensor; its sensitivity dropped by as much as 40% after the first use	[87]

Table 2. (continued)

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
5a	Human ferritin	MIP chemosensor comprising imprinted polyphenol, deposited on arrayed carbon nanotubes	EIS	1 fg/ml to 100 pg/ml	10 fg/ml	Highly selectively insensitive to BSA, **horse apoferritin** (**ahsFtn**), and horse ferritin (hsFtn)	[11]
5b	Human papillomavirus E7 protein (type 16)			10 ag/ml–100 ng/ml	10 ag/ml	Highly selective; very similar human papillomavirus E6 protein (type 16) was not detected	
6	Ferritin	Field-effect transistor immunosensor based on horn-like polycrystalline-silicon nanowire with ferritin antibodies immobilized in a modified 3-aminopropyl triethoxysilane deposited on a SiO_2/Si_3N_4/SiO_2 stacked surface	Field-effect transistor	50 pg/ml to 500 ng/ml	–	(i) Complicated fabrication procedure; (ii) selectivity was not determined	[88]
7	CA 125	MIP chemosensor comprising an array of gold nanoelectrode ensembles coated with imprinted polyphenol film	DPV	0.5–400 U/ml	0.5 U/ml	(i) Used for determination of CA 125 in several spiked human serum samples and in two real samples (i.e., one originating from a healthy individual and one from a sick patient); (ii) sensitivity, reproducibility, and accuracy were slightly higher than that of a commercial ELISA CA 125 analysis kit (BioCheck, Inc. CA)	[12]

Table 2. (continued)

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
8	BSA	MIP chemosensor of carbon nanotubes/ graphene composite-modified carbon electrode coated with BSA-imprinted polyaniline film	DPV	100 pg/ml to 100 μg/ml	62 pg/ml	Reusable MIP chemosensor; average recovery after six measurements was 99.5%	[13]
9	L-Phenyl alanine	Chemosensor with an MIP film deposited in empty spaces of the colloidal polystyrene photonic crystal; acrylic acid and **13** served as functional monomers	Swelling of MIP caused shift of diffraction wavelength	10 nM to 10 mM	–	Highly selective to D-phenylalanine, L-tyrosine, and L-tryptophan	[30]
10	Horseradish peroxidase (HRP)	MIP film coated CdTe quantum dots (QDs)	Fluorescence quenching	1–10 μM	–	Used for determination of HRP in several spiked human urine samples	[40]
11a		Chemosensor with an MIP film deposited on a glass slide was used together with boronic acid-decorated AgNPs	Surface-enhanced Raman spectroscopy (SERS)	1 ng/ml to 10 μg/ml	–	Highly selective to glucose, RNase B, transferritin, and BSA	[42]
11b	∝-Fetoprotein (cancer biomarker)			1 ng/ml to 10 μg/ml	–	Used for determination of ∝-fetoprotein in several spiked human serum samples	
12	Sialic acid (SA)	NPs of SiO_2 coated with an MIP film bearing nitrobenzoxadiazole (an organic dye) moiety; **17** was used as the functional monomer	Fluorescence microscopy	–	–	(i) NPs displayed strong affinity for SA in methanol/water mixtures varying from $K = 6.6 \times 10^5\ M^{-1}$ to $5.9 \times 10^3\ M^{-1}$ depending on the water:methanol ratio; (ii)	[45]

Table 2. (continued)

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
						applied for fluorescent labeling of cells with SA-terminated glycan motifs on their surface; (iii) an expression of glycans terminated with SA on the cell surface correlated with cardiovascular and neurological diseases as well as with cancer	
13	Parathion (an organophosphate herbicide)	An MIP film-coated electrode; the functional monomer contained a *p-tert*-butylcalix[6]-1,4-crown-4 moiety	DPV	5 nM to 100 μM	1 nM	Used for determination of parathion in rice samples; results obtained were similar to those of HPLC	[29]
14	Heparin	Chemosensor with an ITO electrode grafted with an MIP film	Cyclic voltammetry (CV)	50–500 ng/ml	–	Used for determination of heparin in 50-μl whole-blood samples diluted 100 times with a redox probe solution; a commercial heparin-monitoring device (Hepcon® Medtronic Co. Ltd. Minneapolis, MN) using an activated clotting time test is not used widely because of its high cost and complicated operation. Moreover, this test requires collecting very large samples (~1.5 ml) that cannot be diluted	[50]
15	*p53* Gene single-stranded oligodeoxyribonucleotide	Chemosensor with an ITO electrode coated with a poly(*o*-	Amperometry	10 aM to 300 fM	10 aM	Reusable MIP chemosensor; no significant signal change after 10 uses	[52]

Table 2. (continued)

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
		phenylenediamine) MIP film					
16	2,4-Dichlorophenol	Chemosensor with a catalytic film of an MIP of chloroperoxidase-like activity deposited on the electrode surface	Amperometric determination of hydrogen peroxide consumption in MIP-catalyzed reaction	5–100 μM	1.6 μM	Used for determination of 2,4-dichlorophenol in spiked samples of tap, river, and drinking water	[66]
17	5-Hydroxyindole-3-acetic acid	Catalytic MIP chemosensor of peroxidase-like activity deposited on magnetic particles immobilized on a carbon screen printed electrode by an external magnetic field	Amperometric determination of 5-hydroxyindole-3-acetic acid oligomers resulting from the MIP-catalyzed reaction	1–50 μM	1.4 μM	Used for determination of 5-hydroxyindole-3-acetic acid in spiked urine samples of healthy individuals; high recovery and reproducibility for normal and elevated concentrations	[67]
18	Amylase	96-Well microplates coated with an MIP film were used along with MIP film-coated QDs	High-throughput fluorescent determination of amylase; fluorescence originated from MIP film-coated QDs absorbed on walls of the wells	100 pg/ml to 10 ng/ml	1 pg/ml	Used for amylase determination in saliva samples with results similar (20–25% accuracy) to those obtained with the automated ARCHITECT ci 8200 system	[70]
19	Lysozyme	MIP chemosensor featuring a TiO_2 sol–gel film deposited on Cu $(OH)_2$ NPs	High-throughput fluorescent determination of lysozyme in ELISA plates; fluorescence changes originated from *o*-phenylenediamine, oxidation [$Cu(OH)_2$ catalyzed] product	1–100 μg/ml	1 μg/ml	Selectivity with respect to trypsin, ovalbumin, BHb, cytochrome *c*, and BSA was 11.53, 2.96, 2.52, 9.00, and 2.06, respectively	[71]
20			High-throughput spectrophotometric	3–800 ng/ml	2.7 ng/ml		[89]

Table 2. (continued)

No.	Target Analyte	Biosensor/MIP Chemosensor	Transduction Method	Linear Dynamic Concentration Range	LOD	Comment	Refs
		Enzyme-linked immunosorbent assay (ELISA).	determination of lysozyme; change of solution color originated from 3,3′,5,5′-tetramethyl benzidine dihydrochloride hydrate oxidation (catalyzed by peroxidase-labeled antibody) products (Figure 1B, main text)				
21	Streptomycin	MIP chemosensor based on competitive binding of streptomycin and streptomycin-labeled glucose oxidase on the MIP-streptomycin film-coated electrode	Amperometric determination of hydrogen peroxide, a product of glucose reduction by streptomycin-labeled glucose oxidase	10 pg/ml to 10 ng/ml	7 pg/ml	Possible application for testing real food samples, as illustrated by determination of streptomycin in ten spiked honey and milk samples	[73]

Concluding Remarks

Given the high selectivity of molecularly imprinted polymers, they are sometimes called 'plastic antibodies'. These 'smart materials' can recognize target molecules in a similar manner to natural receptors; that is, physically by size and shape, and chemically with a set of recognition sites, located in molecularly imprinted cavities, complementary to binding sites of the analyte molecules. This selective chemical recognition involves the formation of covalent bonds, hydrogen bonds, coulombic interactions, coordination by metal ions, π-π stacking, and supramolecular interactions. This binding is then directly monitored by PM or SPR, or indirectly manifested by changes in the electrochemical or optical properties of the MIP film. Additionally, MIPs can be applied in more sophisticated analytical systems; for example, they can replace antibodies in ELISA tests or enzymes in enzymatic determinations involving electrodes. These advantages encouraged the development of different selective MIP chemosensors. Increasing research interest in this field leads us to hope that selective MIP chemosensors will be found on the market as commercial products in the near future (see Outstanding Questions).

The potential practical use of MIP chemosensors is emphasized by the numerous examples of their use as determinants of biological compounds, such as antibiotics, neurotransmitters, and disease biomarkers in spiked or real body fluid samples (Table 2). Moreover, molecular imprinting readily complies with device miniaturization and nanotechnology. Therefore, MIP NPs exemplify a form of nanostructuring that has recently attracted great interest [76]. This form of MIPs is most promising for both fundamental research and practical applications.

In this review, we briefly summarized imprinting strategies of artificial molecular recognition, highlighting underappreciated interactions, such as supramolecular interactions or π-π stacking, which should be taken into account when selecting the most appropriate functional monomers. In most published research papers, derivatives of acrylamide and methacrylic acid are exclusively used for that purpose. However, an increase in the diversity of functional monomers used would result in the improved performance of the MIP-based chemosensors, thus increasing the scope of target analytes for imprinting and offering attractive replacements for natural receptors in biosensors.

Outstanding Questions

Is it feasible to design MIPs with selectivity and affinity to an analyte as high as that of natural receptors, including enzymes, antibodies, and histones?

Can the analytical performance of MIP chemosensors be improved by combining molecularly imprinted polymers with nanotechnology, such as by using nanostructured MIP films, entrapping NPs in MIP polymer matrices, or surface developments, to match that of biosensors?

How can molecularly imprinted polymers be prepared for fragile templates such as nucleic acids, oligo- and polycarbohydrates, and proteins?

Can combining molecular imprinting with electrocatalysis lead to signal amplification and higher selectivity of chemosensors with molecularly imprinted polymer recognition units?

When will inexpensive, user-friendly, sensitive, and selective (with respect to chosen analytes) devices, capable of routine use in clinical analysis, such as for early disease diagnosis, be produced and appear on the market?

Acknowledgment

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