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REVIEW

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Molecular imprinting: a dynamic technique for diverse applications in analytical chemistry

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Abstract Continuous advances in analyzing complex matrices, improving reliability and simplicity, and performing multiple simultaneous assays with extreme sensitivity are increasing. Several techniques have been developed for the quantitative assays of analytes at low concentrations (e.g., high-pressure liquid chromatography, gas chromatography, immunoassay and the polymerase chain reaction technique). To achieve highly specific and sensitive analysis, high affinity, stable, and specific recognition agents are needed. Although biological recognition agents are very specific and sensitive they are labile and/or have a low density of binding sites. During the past decade molecular imprinting has emerged as an attractive and highly accepted tool for the development of artificial recognition agents. Molecular imprinting is achieved by the interaction, either noncovalent or covalent, between complementary groups in a template molecule and functional monomer units through polymerization or polycondensation. These molecularly imprinted polymers have been widely employed for diverse applications (e.g., in chromatographic separation, drug screening, chemosensors, catalysis, immunoassays etc.) owing to their specificity towards the target molecules and high stability against physicochemical perturbations. In this review the advantages, applications, and recent developments in molecular imprinting technology are highlighted.

Keywords Molecular imprinting · Analytical chemistry · Catalysis · Solid-phase extraction · Sensors

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Introduction

In view of the challenges in environmental, medicine, food process industries, molecular biology, security, and defense areas there is a great need for analytical methods with high sensitivity and good veracity. Biological recognition agents such as antibodies, enzymes, and other receptor molecules have been widely employed in analytical and diagnostic practices [1, 2]. Although these agents are highly specific and sensitive, they are labile, expensive, and have a low density of binding sites. Hence there is a significant demand for robust and stable receptor molecules that can mimic biorecognition elements such as antibodies and enzymes. The developing technique of molecular imprinting provides a promising and advantageous alternative to overcome the problems associated with biomolecules. Molecular imprinting is a dynamic methodology used to design new polymeric receptors against to wide range of target molecules. It was first reported in 1949 by adsorbing different dyes in silica [3, 4]. Originally it was introduced as a means to create binding sites in synthetic polymers. The technique has now matured and has become established in several disciplines owing to its ability to form stable, robust materials with molecular selectivity for a wide variety of compounds. It has also become an attractive method for the preparation of sensor components and catalysts [5–7]. Generally molecularly imprinted polymers (MIPs) are synthesized from functionalized monomers through processes such as radical polymerization or polycondensation. Compared to biomolecules, molecular imprints (MIs) are highly stable and convenient for the development of new analytical methods, which can even perform under relatively harsh conditions. Since the 1990s, great progress in analytical methods, sensors, and catalytic and membrane materials has been made, in which MIPs provide opportunities for advancements. MIs have been successfully developed for the binding of drugs [8], herbicides [9, 10], amino acids and their derivatives [11], peptides [12], proteins [13–19], nucleo-

tides [20], and nucleotide bases [21]. MIPs have been used as tailor-made separation materials, antibody and receptor mimics in assay systems, biomimetic recognition elements in biological sensors, and artificial enzyme systems for catalytic applications. In recent years several reviews on MIPs have appeared with specific applied areas (e.g., catalysis, chromatography, and biomimetic sensors) [19, 22–38]. Here we present an overview of molecular imprinting. It is extremely difficult to include all aspects of molecular imprinting and cover every report; however, we have tried to incorporate all important aspects. This article highlights the advantages, applications, and recent developments in MIP technology.

Advantages of molecular imprints over biomolecules

Biological recognition agents such as antibodies [39–43], enzymes [44–46], plant or animal tissues [47], and cells [48, 49] have been widely used as the affinity sensing agents in analytical biochemistry, biosensors, diagnostic kits, and other applications. These molecules are highly specific to the target molecules, but are highly fragile and unstable. The production of homogeneous biomolecules is difficult, and sometimes requires animals (i.e., in antibody production). Maintenance of animals and production of antibodies are laborious and complicated, and technical skills are necessary [50]. Purification of antibodies and enzymes is tedious and expensive, and they often require special handling methods. Regeneration of biomolecules is also difficult and limited. Sometimes they lose their activity within few reuse cycles, which leads to inconsistency of accuracy and increases cost per analysis. A wider acceptance of biosensor technology is particularly impaired by the limited stability of the biological component immobilized onto a transducer surface. MIs are synthetic receptor sites and have high physical and chemical resistance towards various external degrading factors. Hence MIs are highly appealing alternatives. The functional groups of MIs can easily be modified (present inside the cavity) to increase the affinity or catalytic activity of receptor sites. They can be easily regenerated without loss of affinity, and hence are highly useful for continuous use and flow injection analysis. MIs are stable towards a wide range of solvents, metal ions, and acid treatments [51, 52]. MIs with homogeneous quality and affinity can be synthesized in short span of time without any immunization or involvement of animals. MIPs are highly thermostable, can be used over a range of temperatures [53, 54], and are stored at ambient temperature and in dry state without loss of performance, whereas biomolecules are usually used under controlled conditions and their storage is also difficult. Owing to their advantageous properties over natural biological recognition agents, MIPs therefore offer great potential for various applications in diagnostics, separation, purification, and quantification processes etc.

However, MIPs possess many disadvantages: for example, it is hard to completely remove the template from MIPs; the imprinted polymer is insoluble; and the polymer contains many imprinted cavities of which only some are really good and match the template molecule [55]. Although many developed polymers perform effectively in separation and sensing, there has been no real rationality in the design and synthesis of MIPs. There is no ideal or effective procedure for the design of MIPs against microbial cells and macromolecules (biopolymers) [26]. To overcome these limitations new functional monomers and polymerization methods are being investigated. These improvements would be highly beneficial for nearly all potential applications. In this direction, new MIP formats such as hierarchical imprinting and grafting techniques have been developed [56]. More recently, combinatorial and computational approaches have also appeared for the development of highly specific MIPs. Some of the developments in molecular imprinting techniques are discussed in detail in the section.

Imprinting

In the past few years, molecular imprinting technology has developed into a viable approach for mimicking natural recognition entities, such as antibodies [57] and biological receptors such as enzymes [58, 59]. MIPs have been utilized as a molecular recognition membrane or layer on chemical-sensing systems in combination with transducers such as quartz crystal microbalances (QCMs) [60], surface plasmon resonance devices [61], field-effect devices [62], conductometry [63], or impedometric determination [64]. Imprinting of molecules occurs by the polymerization of functional and cross-linking monomers in the presence of a template/target ligand. In this process, the template is initially allowed to establish bond formation with polymerizable functionality, and the resulting complexes are subsequently copolymerized with cross-linkers into a rigid polymer (Fig. 1). Finally the polymer is ground into micrometer-sized particles, and the template molecules are removed from the imprint by using selective solvent systems. The resultant imprints possess a steric (size and shape) and chemical (spatial arrangements or complementary functionality) memory for the template. These enable MIPs to rebind the same target molecule from a mixture [14] provided that the binding sites of the molecular receptors and the guest molecules complement each other in size, shape, and chemical functionality. Delaney et al. [64] reported a MIP for desmetryn and characterized this MIP by impedance spectroscopy. The electrodes coated with the MIP displayed fairly specific binding of desmetryn, which was detected by the decrease in the electrode capacitance. Only small capacitative effects were observed on addition of terbumeton or atrazine. The same kind of specific recognition abilities were also observed in the case of parathion and

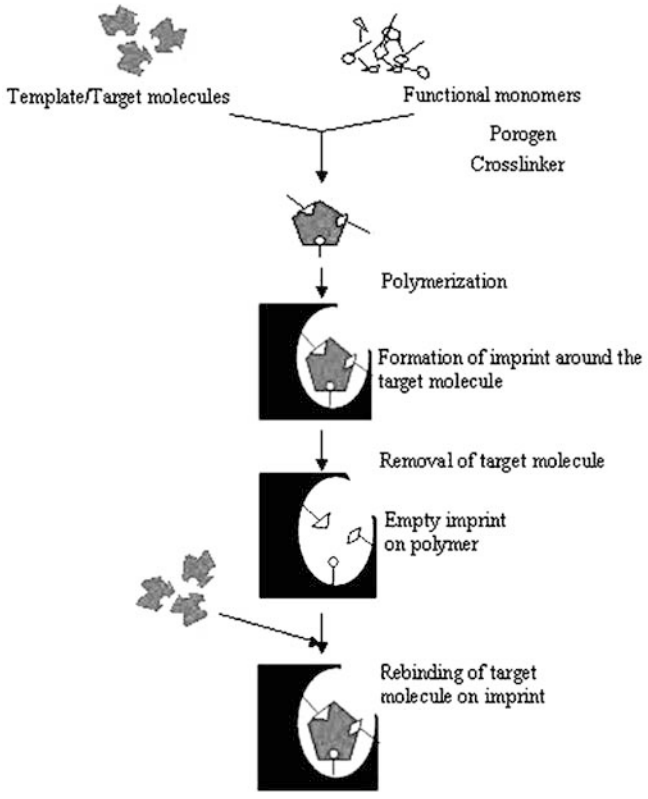

Fig. 1 Schematic representation of the preparation of MIs

penicillin MIPs [64–68]. Imprinting can be achieved by either noncovalent or covalent interactions. The most widely used strategy, pioneered by Mosbach [69, 70], is based on noncovalent interactions between specific functional groups on the polymerizable monomers and the template in order to position the monomers in a specific spatial orientation prior to polymerization. After polymerization and removal of the template, the functional groups of the polymeric matrix can then bind the target through the same noncovalent interactions. High-affinity binding sites can be generated using the noncovalent imprinting strategy; however, the limitation is that the template and target must form a sufficient number of noncovalent intermolecular interactions. Wulff [71, 72] introduced a covalent molecular imprinting method, which utilized reversible covalent bonding between a polymerizable monomer and a template molecule. After polymerization, these bonds were cleaved to liberate the template and subsequently reformed in order to selectively bond the target. Covalent imprinting strategy is very stable and selective. However, the number of functional groups to react with template in the imprint is limited. At high concentrations very rigid imprint formation occurs. For practical repetitive use the cleavage and rebinding may be limited and problematic due to the limited interactions.

To overcome problems associated with covalent and noncovalent approaches, Whitcombe et al. [73] reported an alternative approach, which had advantageous characteristics of both covalent and noncovalent imprinting strategies. In this approach, they employed (4-vinyl)phenylcarbonate ester as a functional monomer, which functioned as the covalently bond template monomer but was easily and efficiently cleaved hydrolytically with the loss of CO_2. Different functional monomers, such as methacrylic acid (MAA) [74, 75] and Cu[1–4-vinylbenzyl]-1,4,7-triazacyclononane]SO_4 (STACNCu) [76], have been employed in order to get highly specific affinity and stable MIPs. A combination of allylamine and tris(4-vinylphenyl)boroxine (TVPhB) was used in the development of a biomimetic receptor system for the determination of sialic acid based on molecular imprinting. Allylamine can interact with sialic acid through an ionic or hydrogen bond and the formation of a fluorescent compound [77]. Piletsky et al. [36] used MAA and diethyl aminoethylmethacrylate (DEAEM) as a functional monomers for the design of noncovalently interacted MIPs against atrazine. The DEAEM MIP showed good conductometric measurements and low detection limits [78]. Parathion molecularly imprinted sol–gel thin films were developed using aminopropyltriethoxysilane (APTES) and phenyltrimethoxysilane (PTMOS) [79]. Some of the monomers were specifically designed to allow fluorescence self-signaling in the presence of analytes, for example, a mixture of *trans*-4-[*p*-(*N*,*N*-dimethylamino)styryl]-*N*-vinylbenzylpyridinium chloride (DMASVBP, a signaling monomer) and 2-hydroxyethyl methacrylate (HEMA) [80]. Acryloyl–cyclodextrins were synthesized as functional vinyl monomers, and various antibiotics and oligopeptides were molecularly imprinted in water [81].

Although several monomers have been used, the most widely used functional monomer is MAA, because it can form hydrogen bonds with a number of chemical structures, and the binding and removal of guest molecules can be performed under mild conditions. The functional monomer usually includes two functional groups. At one end, it interacts with the template through noncovalent interactions (e.g., hydrogen bonding, van der Waals forces, or hydrophobic interactions) or reversible covalent interactions. At the other end of the monomer (i.e., the end that is not interacting with the template) there is a group that is able to bind covalently with the cross-linker. The cross-linker polymerizes the monomers around the template with covalent binding and holds them in place after the template is removed. If the entire binding sites are covered with polymer, then it may be difficult or impossible to remove the template from the imprint. This problem could be minimized by selecting the appropriate cross-linker and concentration [82, 83]. An excess of cross-linking agent is generally used to increase the rigidity of the imprinted matrix, but this sometimes has an adverse effect on the interaction between the template and the matrix, resulting in low recognition capacity [84]. The widely employed cross-linkers are ethyleneglycol dimethacrylate (EDMA) and trimethylolpropane trimethacrylate.

In addition to high-affinity binding sites, the number of binding sites is also very important to achieve the best

MIs. The successful in vitro preparation of molecularly imprinted monoliths is primarily dependent upon the choice and molar ratios of monomer, cross-linker, porogen, and template molecule, and the polymerization temperature. The optimum levels of these ingredients are very important for obtaining finely cross-linked, high affinity, and stable MIPs. Moreover, the selectivity of the imprints depends both on the orientation of the functional groups inside the cavities and the shape of the cavities [85–87]. The dominant factor, however, is the former [86]. The relationships between the imprints and template molecules have been reported by studying 43 compounds in their respective MIs [88]. The good MIPs exhibit high levels of binding affinity and selectivity that are typical of antibodies. Katz and Davis [89] reported organic-functionalized, amorphous, microporous silicas for molecular imprinting. In their study, the triethoxysilane was incorporated into the silica framework during sol–gel synthesis, and subsequent removal of the aromatic core created a cavity with spatially organized aminopropyl groups covalently anchored to the pore walls. Zimmerman et al. [90] reported a molecular imprinting inside dendrimers, which are macromolecules of highly regular structure consisting of a polyfunctional central core covalently linked to layers of repeating units. The template molecules and core used were tetrakis-*meso*(3,5-dihydroxyphenyl)-porphyrin in which dendrons were covalently attached through ester links. The outer shell of the dendrimer was polymerized intramolecularly, and the template was then removed. The binding site contained eight precisely positioned carboxyl groups. This imprinted dendrimer showed selectivity to rebind structural isomers of the template such as tetrakis-*meso*(2,6-dihydroxyphenyl)-porphyrin. The main advantage with dendrimer imprints is that the template can be easily removed completely, which is not always possible with traditional, highly cross-linked MIPs.

To obtain the optimized MIPs for a given target analyte, combinatorial approaches to MIP synthesis have been developed [25, 91, 92], in which the ingredients of the imprinting recipe, in particular the kind and molar ratio of the functional monomers, are varied using automated procedures. Cederfur et al. [92] developed a MI library against the β-lactam antibiotic penicillin G. This library was constructed by combining various functionalized monomers and cross-linkers and varying the stoichiometry and the concentration of the components in the prepolymerization mixtures. Among these polymers, one synthesized from MAA and trimethylolpropane trimethacrylate as the functionalized monomer and cross-linker, respectively, showed the highest selectivity for penicillin G. The best MIP against penicillin G was screened through radioligand competitive binding assay. The cross-reactivities of penicillin G MIP with other β-lactam antibiotics were also studied: cross-reactivity was shown to be 15, 16, and 19% with penicillin-V, ampicillin, and amoxicillin, respectively. Nafcillin, cephapirin, chloramphenicol, tetracycline, dapsone, and erythromycin showed much lower cross-reactivity (0.01–0.1%). The selection of MIPs from the MI library is highly convenient and effective for the isolation of high-affinity MIPs against target molecules. Recently Subrahmanyam et al. [93] developed a method that included computational screening (Bite-and-Switch) of a virtual library of functional monomers against a target molecule followed by selection of those able to form the strongest complex with the template. The computationally designed polymer showed superior selectivity in comparison to the polymer prepared by using a traditional approach. These polymers exhibited an affinity comparable with antibodies. This approach can be used for the design of assays and sensors exhibiting greater selectively for amino-containing substances.

Advances in molecular imprinting technology

There are only a few examples of non-cross-linked MIPs in the literature. Recently, Kobayashi et al. [94, 95] reported a phase-inversion molecular imprinting method, in which linear polymer was used as a matrix, and the template molecule information could be encoded by the phase-inversion process. Phase-inversion molecular imprinting of theophylline was performed by using acrylonitrile (AN) copolymers with acrylic acid (AA) (P(AN-*co*-AA)) and MAA (P(AN-*co*-MAA). These copolymers were synthesized in the presence of different concentrations of the theophylline template. The polymer solutions were cast and phase-inversed in water to prepare theophylline-imprinted copolymers. To confirm the polymer specificity towards the template, different structurally related compounds such as 2-hydroxyethyl-theophylline, theobromine, lumazine, purine, and uracil were checked. MAA imprinted polymer showed high selectivity for the theophylline. Recently Lim et al. [96] reported another non-cross-linked MIP for estrone. In this study, an aromatic polyimide was used as the imprinted matrix. Polyimide imprint exhibited good thermal stability and mechanical strength. The urethane bond formed between the template and polyimide matrix was highly stable at room temperatures and could be cleaved at elevated temperatures. This type of thermally reversible interaction between template and MIs is highly useful for the development of highly sensitive sensors and effective separation processes.

Shi et al. [97] reported a novel imprinting method for protein molecules. This method employed radio-frequency glow-discharge plasma deposition to form polymeric thin films around proteins coated with disaccharide molecules. The disaccharides were covalently attached to the polymer film, creating polysaccharide-like cavities that exhibited highly selective recognition for a variety of template proteins, including albumin, immunoglobulins G, lysozyme, ribonuclease, and streptavidin (Fig. 2). In this process, initially desired protein molecules were adsorbed on a mica surface and a sugar layer of 10–50 Å was spin-casted over the

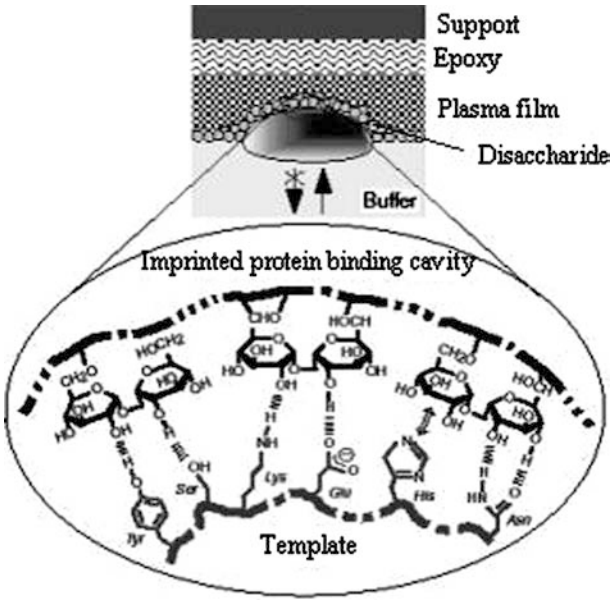

Fig. 2 Mechanism for the design of MIs for proteins (adapted from ref. [97])

adsorbed protein molecules. Finally, plasma deposition of C_3F_6 was used to form a 10- to 30-nm-thick fluoropolymer thin film. The resulting plasma was fixed to a glass cover slip with epoxy resin and oven-cured. The mica was pealed off and the obtained sample was soaked in NaOH/NaClO solution to remove the template molecules (protein). The amount of protein adsorbed was higher on the protein imprints than on a mica-imprinted control surface (without template protein). Through competitive adsorption, AFM, and time-of-flight secondary ion mass spectrometry, the binding efficiency and selectivity were confirmed. By using this method it could be possible for the development of highly selective MIPs for the proteins and high molecular weight compounds [97].

In general molecular imprinting methods, the template removal results in MIs containing nanometer-sized binding sites in addition to larger sized pores. In order to access the host-binding site the guest molecules must penetrate pores whose sizes are difficult to control independently from the generation of the imprinted site. Another possible limitation is aggregation of templates in the pre-polymerization mixtures [98, 99]. To overcome these limitations, an advantageous alternative is the immobilization of template on a surface of porous disposable solid material that acts as a mold to create a desired and homogeneous porosity. In addition to this, an oriented immobilization of the imprinted molecule also results in a better orientation homogeneity of the binding sites. In this field, Yilmaz et al. [100] developed the first MIP against theophylline. 8-Carboxypropyltheophylline was immobilized on a silica surface functionalized with aminopropyl groups through solid-phase peptide synthesis. Acetic anhydride was added at the end of coupling reaction to block remaining free aminopropyl groups. Imprints were designed by using immobilized theophylline with trifluoromethylacrylic acid as a negatively charged monomer, divinylbenzene as a nonpolar cross-linker, and 2,2′-azobis(2,4-dimetylvaleronitirile) as an initiator (Fig. 3). Finally silica was completely removed by treatment with aqueous hydrofluoric acid. The porosity of the polymer was determined by nitrogen adsorption and desorption analysis. A narrow pore size distribution was obtained with a mean pore diameter of 254 and 257 Å and pore surface area of 35 and 31 $m^2\ g^{-1}$ for the imprinted and nonimprinted polymers, respectively. No larger pores or mesopores were detected. This theophylline-imprinted polymer showed high selectivity over the related compounds such as theobromine and caffeine. The Sellergren group [101, 102] reported hierarchically imprinted polymers against nucleotides and peptides. In recent years, hierarchically imprinted polymers have gained considerable importance, as they allow the preparation of uniformly shaped and sized MIs with narrow pore size distribution and surface-confined binding sites. Template precursors such as 9-(2-bromoethyl) adenine and 6-chloro 2,4-diaminopyrididine were immobilized by reaction with amino groups of porous silica particles (11.5-nm average pore diameter). The resulting nucleotide immobilized pore silica particles were completely filled with MAA, EDMA, and initiator by repeated vacuum–nitrogen purge cycles. The polymerization was then carried out by heating the particles at 60°C (for 20 h), and the silica template was subsequently dissolved by treatment of the composite particles in $(NH_4)HF_2$ solution for 96 h. The resulting polymer imprinted particles were similar to the mirror image of the original silica template (Fig. 4). This imprinted polymer showed mesoporosity, narrow pore size distribution, and low bulk swelling for the template

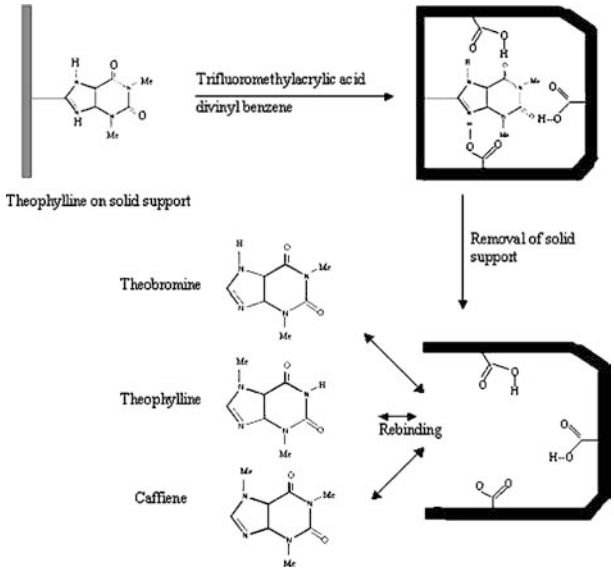

Fig. 3 Schematic representation of the molecular imprinting approach employing immobilized templates and a sacrificial solid support (adapted from ref. [100])

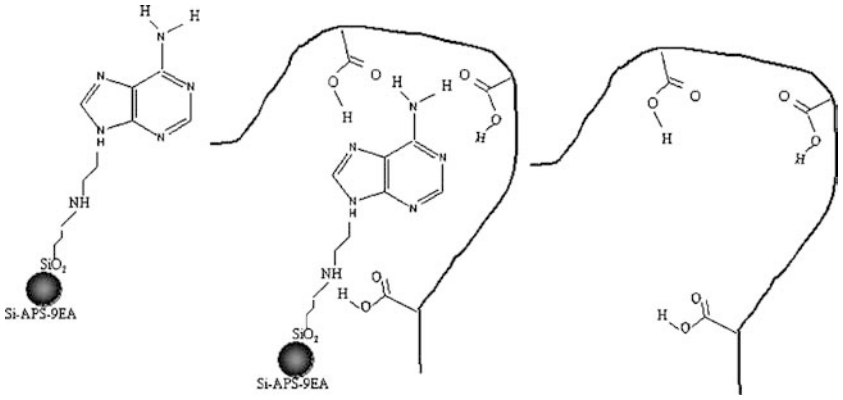

Fig. 4 Schematic representation of hierarchically imprinted polymer against adenine (adapted from ref. [101])

and analogues in acetonitrile/acetic acid (99:1 v/v) mobile phase [101]. Most of the molecular imprinting reports are confined to the small molecules such as drugs, amino acids, and nucleotides. Recently Titirici and Sellergren [102] reported hierarchically imprinted polymers for the peptide recognition. In this approach the peptide epitopes were synthesized by standard solid-phase synthesis on the surface of porous silica support. These immobilized peptides were used as templates for the generation of hierarchically imprinted polymer matrices. Dai et al. [103] developed a hierarchically imprinted sorbent for the separation of metal ions.

Applications of molecular imprinting technology

Purification and separation

In the pharmaceutical industry there is a great deal of interest in and necessity for the separation of enantiomerically pure compounds and other molecules. Most of the work on MIPs has focused on the separation of compounds [104], particularly from mixtures of racemates [105], using these polymers as stationary phases in HPLC and in capillary electrochromatography [106] owing to their inherent stability, low cost, and ease of preparation [107]. In addition, MIPs have demonstrated showed high stereoselectivity and enantioselectivity for a number of compounds such as amino acid derivatives [108], peptides [12, 109], amino alcohol [110], and anti-inflammatory agents [111].

The extraction and concentration of specific residues from complex mixtures like milk, serum, and urine are very tedious and laborious processes, and accuracy is also limited. For the isolation of residues from biological and food samples, specific and stable artificial receptor molecules are very convenient and advantageous. Muldoon and Stanker [112] developed an atrazine MIP for the extraction of atrazine from beef liver extracts. The column had a retention binding capacity of 19 μM of atrazine per gram of MIP from chloroform. For comparative analysis, purified and unpurified beef liver extracts were analyzed by both reversed-phase HPLC and ELISA. The use of molecular imprinted solid-phase extraction (MISPE) improved the accuracy and precision of the HPLC method and lowered the limit of detection (5 ng mL^{-1}). The average atrazine recovery was determined by HPLC in beef liver homogenates spiked to levels of 5–500 ng mL^{-1} and found to be 88.7% following MISPE and 60.9% for the unpurified extracts, whereas the recovery determined by ELISA was 92.8% following MISPE and 79.6% for the unpurified extracts. Unpurified sample extracts interfered with both the HPLC and ELISA methods [112].

Solid-phase extraction (SPE) is quite convenient compared with liquid/liquid extraction (LLE) owing to low solvent consumption and the possibility of automation. Sellergren [113] reported a pentamidine (PAM)-selective imprinted dispersion polymer (MAA and EDMA) for selective enrichment and analysis of PAM present in real samples at low concentrations. Sample sorption, determination, and desorption studies were carried out using HPLC, in which the column was filled with PAM-imprinted polymers. At the physiological concentration of 30 nM, an enrichment factor of 54 was obtained by using a PAM-selective polymer, whereas the enrichment factor on a benzamidine-imprinted reference polymer was only 14. MIP-based SPE appears to be an attractive alternative to the LLE techniques presently employed, and the separation of closely structurally related enantiomers with great efficiency could also be possible.

Dela et al. [114] developed two MIs for 2,5-dimethylpyrazine (DMP) and 2,3,5-trimethylpyrazine (3MP), which are major flavor compounds associated with various seed and grain food-processing operations, including roasting and drying. These imprints are useful for the selective isolation and enrichment of flavor compounds. SPE of flavonoids was reported by Molinelli et al. [115]. Flavonoids and related compounds, present in fruits or finished products such as wine, are responsible for the color, fragrance, and, to some extent, the taste and quality of the wine. In this study, MIPs selective for quercetin were prepared and applied as the material for SPE in offline separations. The MIPs showed excellent selectivity toward quercetin and a binding capacity of 0.4 mg quercetin per gram polymer with a recovery of 98.2%. The produced MIPs were cheap and thermally and mechanically stable.

Ochratoxin is a well-known natural contaminant on cereals, rice, peanuts, coffee beans, cottonseed, and decaying vegetation, which eventually enters the food chain. As it is a toxic compound even at very low concentrations, there are great need to separate and quantify it from food materials. Baggiani et al. [116] reported a molecular imprinted polymer that recognized mycotoxins such as ochratoxin-A. MIPs were prepared using the mimic *N*-(4-chloro-1-hydroxy-2-naphthoylamido)-L-phenylalanine as a template. The binding properties of the template towards the ochratoxin-A and several related molecules were measured by eluting with acetonitrile and chloroform in an HPLC column packed with the imprinted polymer. Molecular imprint polymer bound both the template and ochratoxin-A through hydrogen bond interactions and a steric fit effect in chloroform and acetonitrile. This molecular imprint polymer may be used for the solid-phase extraction of ochratoxin-A from real samples. In another study [117], ochratoxin-A MIP particles were synthesized from *N*-phenylacrylamide and slurry-packed into a microcolumn for selective SPE of ochratoxin-A from wheat extracts. Pulsed elution (PE) using methanol/triethylamine (99:1 v/v) allowed good quantitative desorption of ochratoxin-A. The MISPE–PE method, with fluorescence detection at $\lambda_{ex} = 385$ and $\lambda_{em} = 445$ nm, afforded a detection limit of 5.0 ng mL^{-1}. The recovery of ochratoxin-A from wheat extracts was $103 \pm 3\%$. Each molecularly imprinted SPE–PE analysis took less than 5 min to complete.

Molecularly imprinted polymers have been used for the SPE and quantification of environmental pollutants such as pesticides, herbicides, and phenolic compounds. Sulfonylureas are a class of herbicides which are highly toxic compounds for mammals. Recently, Zhu et al. [118] reported an MIP for the solid-phase extraction of sulfonyl ureas. MIP cartridges were developed by using metsulfuron-methyl (MSM; as template), 2-(trifluoromethyl)-acrylic acid (TFMAA; as monomer), DVB, and 2,2′-azobisisobutyronitrile (AIBN). About 96% recoveries were reported from river water and rainwater spiked with sulfonlyureas at 50 ng L^{-1}. The MIPs exhibited good stability and selectivity even after 200 enrichment and desorption studies. In another study, 4-nonylphenols (4-NP) were extracted from environmental water samples by using noncovalently imprinted MIP as a selective SPE sorbent, coupled online to a liquid chromatographic system. 4-NP is highly toxic, even at low concentrations, especially to aquatic organisms. In this study 4-NP was selectively extracted from river water samples, spiked with the 11 environmental protection agency phenolic compounds at microgram per liter levels [119].

Membrane-based chemical separations of biomedical and pharmaceutical constituents are an emerging research area and industrial technology. By using ultrathin films of MIPs, the target compounds can be separated specifically and quickly. Hong et al. [120] designed an ultrathin film composite across the microporous support for the separation of the bronchodilator theophylline. This thin film exhibited good selectivity and separation for the print molecule (theophylline) relative to a chemically very similar competing molecule (caffeine). MIs have also been used for the removal of secondary metabolites or by-products by specific adsorption from fermented broths. The pigments, *N*-glutamyl-rubropuctamine and *N*-glutamyl-monascorubramine, were successfully separated from a fermented broth of *Monascus sp*. by extracting into ethyl acetate [121]. The imprints showed very fast binding and desorption capacity and can be reused. Succinyl L-tyrosine is a by-product from the fermentation-based synthesis of clavulanic acid, a β-lactamase inhibitor [122]. A noncovalent MIP against succinyl L-tyrosine was designed and used successfully to separate succinyl L-tyrosine from the fermented broth.

Naproxen [(+)-2-(6-methoxynaphthyl)propanoic acid] is a nonsteroidal anti-inflammatory drug. Since this propanoic acid contains one chiral center, it has two enantiomers which must be considered separately in terms of pharmacokinetics and toxicology. Kempe and Mosbach [123] separated racemic naproxen by using a molecular imprinting strategy in which 4-vinylpyridine was used as a functional monomer. Lei and Tan [124] reported another chiral stationary phase for the enantioselective separation of naproxen using molecularly imprinted columns in which acrylamide and EDMA were co-polymerized in the presence of (*S*)-naproxen. Racemic naproxen was efficiently resolved on the MIP with affinity chromatography equilibrium constants of 34.45 and 17.69 in the mobile phase of THF/heptane and acetonitrile, respectively (for (*S*)-naproxen). Successful separation of nicotine from tobacco smoke has been reported [125], in which the MI was prepared using MAA and EDMA and chloroform as a porogen. This imprinted polymer showed good binding capacity of 90 mg g^{-1} for nicotine and is much more capable of removing nicotine from tobacco smoke than commercial filter tips.

Schweitz et al. [126] reported the separation of the (*S*)-ropivacaine enantiomers, bupivacaine and mepivacaine, using MIP CEC columns. The influences of several parameters on the ability of the resultant imprinted capillary column to resolve *rac*-ropivacaine were investigated. Highly selective MIP was obtained using MAA and EDMA as monomer and cross-linker respectively. The parameters affecting the characteristics of the MIP, including the type and amount of functional and cross-linking monomers, the molar ratio of the imprint molecule to the monomers, and the type of porogen, were thoroughly studied. Optimization of the volume ratio of toluene to isooctane in the porogen was reported to be important for obtaining columns with optimal flow-through properties. In the absence of isooctane the polymer became very dense, and hydrodynamic pumping was not possible. Higher amounts of isooctane led to the polymer having a soft gel-like appearance rather than being a stable monolith [126].

Cheong et al. [127] developed a noncovalent polymer imprint for the separation of testosterone. In this study, the influences of various polymerization conditions on the binding strength and selectivity of the template were examined. The efficacy of covalent and noncovalent imprinting methods was also compared. After preparation of methacrylate imprints, particles were wet-sieved in EtOH and the 25- to 45-μm fraction was packed into 100×4.6-mm (i.d.) HPLC column, and analyses was performed with a UV-Vis detector (set at 225 nm for these compounds) with MeCN as eluent. This MI showed the highest selectivity towards the testosterone among closely related molecules like β-estrodiol.

Silica particles have been widely used as a stationary phase in thin-layer chromatography (TLC). Krilz et al. [128] reported a MIP-based TLC method for the separation of D and L-phenylalanine anilide as model compounds. MIPs were prepared in acetonitrile by photo-initiation at 4°C using a system of EDMA as a cross-linker and MAA as a functional monomer. Nonimprinted polymers were prepared in the absence of target molecules. The polymers were ground for 20 min in a mechanical mortar, and the particles were slurred in acetonitrile and then left to allow sedimentation. The particles were again washed with 20% acetic acid in methanol and finally in acetone. After drying, fine white polymer particles were used as a stationary phase in TLC. Polymer particles and plaster of Paris were slurred in a solvent containing distilled water and ethanol. After sonication, the slurry was poured on a carborundum-sanded microslide glass (76×26 mm^2) and allowed to dry at room temperature overnight. The TLC plates coated with imprinted particles showed clear separation of D- and L-phenylalanine anilide [128]. No separation occurred TLC plates coated with nonimprinted particles. This study clearly indicated that MIP could be effectively used for the separation and manufacture of optically pure drugs.

Haginaka and Kagawa [129] prepared uniformly sized MIPs for *tert*-butoxycarbonyl-L-Trp (Boc-L-Trp) using EDMA as the cross-linker, and MAA and/or 4-vinylpyridine (4-VPY) as the functional monomers or without use of a functional monomer. The Boc-L-Trp-imprinted EDMA polymers were able to recognize Boc-L-Trp by virtue of its molecular shape and separate Boc-Trp enantiomers in the hydro-organic mobile phase. Besides the molecular shape recognition, the hydrogen-bonding interaction of Boc-Trp with a functional group in the polymers and hydrophobic interactions with the polymer backbones were also involved in the recognition of Boc-Trp enantiomers on the imprinted MAA-*co*-EDMA and 4-VPY-*co*-EDMA polymers when a mixture of phosphate buffer and acetonitrile was used as the mobile phase. The hydrogen-bonding interaction was dominant when acetonitrile only was used as the mobile phase. The Boc-L-Trp-imprinted 4-VPY-*co*-EDMA polymers showed the highest retention and enantioseparation factors for Boc-Trp under optimized HPLC conditions. The baseline separation of Boc-Trp enantiomers was obtained within 10 min on the imprinted 4-VPY-*co*-EDMA polymers. Rachkov et al. [130] prepared a MIP (MAA, EGDMA, 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV)) by an epitope approach for peptide separation in aqueous-rich mobile phases. Equimolar mixtures of the template Tyr-Pro-Leu-Gly amide (YPLG) and Gly-Leu-Tyr (GLY) were efficiently resolved in peptide concentrations in the range of up to 75 μM. It is noteworthy that oxytocin (a peptide bearing the same three-amino-acid C-terminus PLG sequence as the template) can be also recognized by the MIP and efficiently separated from its mixture with tocinoic acid, which has a structure containing the same cyclic part as that of oxytocin, but without the key C-terminus moiety.

Yilmaz et al. [131] reported a chromatographic enantioseparation of racemic isoproterenol, a β-adrenergic agonist, and then compared the results to those obtained with conventional irregularly shaped MIP particles. Both materials (i.e., silica-MIP composite and spherical MIP) showed lower back-pressures, higher mass transfers, and higher plate numbers than the traditional particles. However, a significant peak band broadening and tailing was still observed. Sellergren and Shea have studied how various factors affecting the stability of the monomer template assembly (e.g., choice of solvent, polymerization temperature, and type of cross-linking) influence the morphology and separation performance of the polymers imprinted with L-phenylalanine anilide. These authors also studied the equilibrium isotherms of the two enantiomers of phenylalanine anilide (PA) by conventional frontal analysis at three different pHs on a thermally treated imprinted stationary phase selective for the L enantiomer [132]. The saturation capacity is smaller for D-PA than for L-PA, the template. The analytical separation of D-PA rather than for L-PA is best at pH 3.0 (separation factor 2.82) with a short retention time. A good compromise between the resolution and the saturation capacity is obtained at pH 5.8. Poor analytical and preparative results are obtained at pH 7.0 (separation factor 1.32). At this pH, the isotherm remains nearly linear in the whole concentration range accessible to measurement. The authors found that the number of nonselective sites increased with increasing mobile phase pH slightly faster than the number of selective sites as a result of the different pK ranges for the two types of sites. Moreover, the binding energy and the homogeneity of the selective sites decreased with increasing pH. The same group [133] developed a method for online cleanup and high-throughput analysis of triazines in real samples. The MIP column selectively retains the triazine analytes and the residual matrix is separated completely. The cleaned and enriched extract is subsequently eluted to an HPLC column and analyzed by LC-MS. The setup includes a complete online analysis cycle including multidimensional SPE and separation. The detection time is less than 15 min. Terbuthylazine, atrazine, propazine, simazine, ametryn, prometryn, irgarol, and also the

metabolites deethylatrazine and deisopropylatrazine have been determined without any matrix interferences. Nonspecific interactions with the polymer are also very much less ($<1\%$). More than 300 enrichment and desorption cycles MIP showed good performance. Sellergren et al. developed MIPs for the selective solid-phase extraction and separation of nicotine [134], chlorotriazine [135], terbutylazine [136], and phenytoin [137] from real samples.

Most of the reports on MIPs are confined to those involving preparation of cross-linked macroporous monolith which are then ground and sieved to obtain small particles (μm). This preparation is time-consuming and yields of only moderate amounts of useful imprinted particles. One of the disadvantages with these particles is their irregular shape, which is inconvenient for efficient packing in the chromatographic separation (e.g., HPLC and LC) columns. Sometimes the cavities may be ruptured in the grinding process. A good alternative to this approach is the preparation of MIP microspheres. Ye et al. [138] presented MIP microspheres with good selectivity and affinity against theophylline, caffeine, and 17-estradiol. The microspheres were prepared using these three molecules as templates with dilute solutions of MAA and trimethylolpropane trimethacrylate. Their selectivity and binding specificity were confirmed using radioligand binding analysis. Very high binding specificity was retained when the molar ratio of functional monomer cross-linker was controlled between 1:1 and 2.3:1. Molecularly imprinted microspheres are highly desirable and could be employed in many applications such as competitive ligand binding assays, solid-phase microextraction (SPME), sensor development using deposited microspheres, and capillary electrochromatography. The ion-imprinting method is a useful technique for the preparation of adsorbents for the separation of metal ions. Recently Say et al. reported preconcentration of copper (II) ions from aqueous solutions using ion-selective imprinted polymer microbeads [139]. Microbeads were prepared using poly(ethylene glycol dimethacrylate–methacryloylamidohistidine/Cu(II)) and metal complexing though a dispersion polymerization technique. A maximum of 48 mg g^{-1} Cu(II) was adsorbed onto the imprinted beads. The pH significantly affected the adsorption capacity of the imprinted microbeads. Under acidic conditions, the beads showed very low affinity, whereas the maximum affinity was reported at pH 7.0. The imprinted microbeads showed excellent selectivity for the target ion, Cu(II), even in the presence of Zn^{2+}, Ni^{2+}, and Co^{2+} ions. These beads can easily be regenerated by using 0.1 M EDTA solution.

MIPs have been prepared in the bead form through suspension polymerization techniques [140, 141], core-shell emulsion polymerization [142], or dispersion/precipitation polymerization techniques [137, 143]. Sometimes the spherical particles produced possess narrow size distribution with improved recognition or kinetic properties. These methods make it difficult to obtain the desired particle morphologies, and tedious trial and effort is often needed to arrive at an acceptable compromise. To overcome these problems MIPs have been prepared as grafted coatings on silica supports [144], organic polymer supports [145], and on the walls of fused silica capillaries [146]. In these methods, prior to polymerization, the polymerizable double bonds must first be formed, which can add to the growing polymer chains in solution, thus linking them to the surface. However, it is difficult to control the thickness of the polymer layer, and capillary forces upon evaporation of the solvent may cause incomplete wetting of the surface. Moreover, the maximum density of grafted polymer chains is limited here due to kinetic and steric factors. To obtain the grafting thin films of MIPs Sulitzky et al. [147] immobilized surface-bound free radical initiators on silica particles though covalent and noncovalent methods prior to the polymerization. This method provided the fine-tuning of layer thickness with improved kinetic properties or enhanced capacity in chromatographic or sensor applications. By using this method, thin films for L-phenylalanine anilide, with, ca. 0.8-nm average film thickness and 10-nm average pore diameter on silica were prepared. These particles resulted in high column efficiencies with plate numbers (N) for the imprinted enantiomer of ca. 700 m^{-1} and for the antipode of ca. 24,000 m^{-1}. In other studies [148, 149], MIP films grafted on porous and/or nonporous silica particles were used as stationary phases in capillary electrochromatography for the enantioseparation of L-phenylalaninie anilide. The capillaries packed with silica particles containing grafted films of imprinted polymer were stable and could be reproducibly prepared with acceptable inter-capillary and inter-run reproducibility.

Separation of bioactive compounds using MIPs

Molecularly imprinted polymers have been used for the direct extraction of pharmacophoric compounds from herbs [150]. Molecular imprints were prepared using quercetin, a highly active compound in the falvonoid family, and used for the extraction of structural analogous from the hydrolyzate of ginko leaves (Chinese traditional herb). By using these MIP cartridges, quercetin and kaempferol were successfully separated. With the same quercetin MIP, Zhu and Xu [151] and Zhu et al. [152] selectively separated inhibitors of epidermal growth factor receptor (EGFR) from *Caragana jubata* extracts. Quercetin is a natural protein kinase inhibitor even at micromolar concentration. The EGFR belongs to the class of trans-membrane growth factor receptor protein tyrosin kinases and can be inhibited by quercetin. Generally EGFR is overexpressed in a large percentage of clinical cancers and is associated with poor prognosis. In this study MIPs were used as stationary phases to extract two main anti-EGFR inhibitors, piceatannol and butein, from the plant extract with methanol as a mobile phase. These studies indicated that

MIP could be successfully used for drug discovery and screening, and also could avoid the tediousness and inefficiencies of traditional isolation.

Screening of bioactive molecules using MIPs

MIPs have also been employed for the screening of drugs and inhibitors and for other biomedical applications instead of highly sensitive and costly biomacromolecules. Mosbach et al. [153] developed an ''anti-idiotypic'' approach using imprinted polymer for the template-guided synthesis of bioactive molecules. These authors prepared an imprinted polymer using a Kallikrein inhibitor as a model target and used this MIP to facilitate the synthesis of new inhibitors (mimics). Kallikrein inhibitors possess the common feature of a positively charged amino or guanidine group connected to a hydrophobic moiety; the two residues resemble the side chains of the peptide sequence Phe-Arg, which binds to the S2–S1 pocket of the enzyme's active site. A representative nonpeptidic inhibitor is 2-(4-amidinophenylamino)-4-chloro-6-phenylethylamino-triazine, which has been chosen as a template for MIP preparation. For the imprint development (2-trifluoromethyl)acrylic acid (TFMAA) and divinylbenzene were employed as a functional monomer and cross-linker, respectively. The TFMAA formed strong ionic interactions with the amidine group, while DVB provided the possibility of π–π stacking with the aromatic moieties in the template. The resultant imprints resembled the active center of tissue kallikrein enzyme and specifically bind 2-(4-amidinophenylamino)-4-chloro-6-phenylethylamino-triazine. This imprint was further challenged with other artificially synthesized inhibitors for the drug screening. In another study, a steroid library composed of 22 closely related compounds was screened for estrogen specificity [154]. Further isolation, identification, and drug screening can be done in the future by designing more inhibitors of active MIP sites.

Sensors

Biological recognition molecules such as enzymes, antibodies, and receptors have been widely employed as affinity recognition molecules for the preparation of biosensors. Artificial receptors such as MIPs are currently under investigation in numerous applications owing to their stability and advantageous features [155]. Some reports on the design and preparation of atrazine MIPs are available [156–160]. Shoji et al. [161] reported an atrazine sensor based on a MIP-modified gold electrode. The atrazine sensor was successfully fabricated by directly polymerizing the atrazine-imprinted polymer using MAA and EDMA onto the surface of a gold electrode. By introducing LiCl into the MIP, atrazine was reduced at −800 mV vs. Ag/AgCl at pH 3.0. This electrode exhibited a good sensitivity for atrazine compared to other triazines. Piletsky et al. [162] reported a conductometric sensor for the analysis of atrazine using MIP as a recognition agent. The MIP was prepared by using MAA and EDMA in the presence of atrazine as a template. This sensor was able to analyze atrazine in the range of 0.01–0.5 mg L^{-1}, and the membrane was highly stable for up to 4 months without loss of sensitivity. A piezoelectric sensor has been reported by Chianella et al. [163] for the analysis of microsystin LR, a highly toxic compound produced by freshwater cyanobacteria, present in aqueous samples. Microsystin MIs were used for solid-phase extraction from the water samples and recognition receptors in piezoelectric sensor. The use of MIP-SPE provided up to 1,000-fold preconcentration, which was sufficient for achieving the required detection limit (1 nM) for microcystin LR in drinking water. The minimum detectable amount of toxin for this sensor was 0.35 nm.

Online clean-up and determination of chloramphenicol from opthalmic and spiked milk samples were reported by Mena et al. [164]. Chloramphenicol is an antibiotic with a wide range of bioactivity. Chloramphenicol was eluted from the MIP microcolumn by using methanol; a good recovery of 96.4% from an initial concentration of 9.7 g L^{-1} was obtained. Detection of chloramphenicol was carried out by square-wave voltammetry at electrochemically activated carbon fiber microelectrodes. Good linearity for chloramphenicol was obtained between the concentrations of 3.0×10^{-8} and 1.0×10^{-5} mol L^{-1}. Rodríguez et al. [165] reported a competitive flow-through assay for the analysis of chloramphenicol using a MIP as recognition phase. Chloramphenicol-imprinted polymers were designed using a mixture of diethylaminoethylmethacrylate and vinylpyridine as functional monomers, EDMA as cross-linking agent, and tetrahydrofuran as the porogen. The sample throughput of this system was 5–6 samples per hour with a detection of limit down to 100 μg mL^{-1}.

Kröger et al. [166] reported a molecular imprinting screen-printed electrode-based biomimitic sensor for the detection of 2,4-dichlorophenoxyacetic acid (2,4-D) using differential pulse voltammetry. The screen-printed electrodes were coated with a homogeneous thin layer of polymer and covered with an agarose layer to protect the molecular imprint layer from the washing steps. The analysis of competitive binding strategy was performed with an irrelevant electrochemically active probe. The screen-printed biomimitic sensor showed a good calibration curve for the analysis of 2,4-D over the μM range. Thin films of a molecularly imprinted sol–gel polymer for parathion have been developed by Marx et al. [65]. The films were cast onto glass substrates or glassy carbon electrodes and were used to detect parathion in aqueous solutions. Gas-phase binding measurements were performed on coated QCM resonators, and the liquid-phase experiments were carried out using GC-FPD and cyclic voltammetry. To develop parathion MIs, functional silanes containing phenyl and primary amine groups have been employed owing to their ability to interact non-

covalently with parathion within the polymer film and provide the chemical anchors for rebinding. The imprinted films showed good selectivity towards the parathion from the mixture of organophosphorus pesticides (OPs). However, in the gas phase relatively high nonspecific binding was reported compared to the liquid phase as a result of the difference in the nature of hydrogen bonds in the gas and liquid phases. Water molecules assist the binding of the solvated parathion molecule in the liquid phase, which stabilizes the complex formed between parathion and the matrix [65]. This type of MIP could be very useful for the construction of chemosensors for the quantification of OPs.

The use of molecular imprints as the recognition agent in an ELISA plate assay format has been reported for epinephrine analysis [167]. MIs against epinephrine were coated on microplate wells by polymerization of 3-aminophenylboronic acid as a functional monomer in the presence of ammonium persulfate. After polymerization, brown transparent films were formed on the wells and washed thoroughly with 10 mM HCl solution and then deionized water. The resultant polymer-binding properties were tested in an enzyme-linked assay using competition between free ligand and HRP-N conjugate. The obtained MI exhibited high specificity towards the epinephrine. The pH and concentration of buffer showed considerable effects on the polymer affinity and on the strength of the electrostatic and reversible covalent interactions [167]. It is anticipated that MIP-coated microplates could be particularly useful for the development of diagnostic assays and drug screening. Corticosteroids are involved in metabolism of anti-inflammatory action, electrolyte and water balance, and various functions of the nervous system. Radioimmunoassay and enzyme-linked immunosorbent assays are widely employed for the selective recognition of a particular corticosteroid. There are a multitude of steroids with very similar structures. Hence, for the specific quantification of steroids, highly selective, sensitive, and stable molecular recognition agents are necessary. Ramstrom and Mosbach [168] reported a MI method for the detection of corticosteroids at sensitive levels. The polymer was prepared using MAA as functional monomer, EDMA as cross-linker, and tetrahydrofuran or acetone as porogen. Azobisisobutyronitrile was used as initiator (Fig. 5). The resultant polymers exhibited high selectivities with detection limits for cortisol and corticosterone in the range of 10^{-7}–10^{-8} M using anti-cortisol and anti-corticosterone polymers, respectively.

Surugiu et al. [169] reported the first 2,4-D imaging assay analogous to competitive enzyme immunoassay. Microtiter plates were coated with polymer microspheres imprinted with 2,4-D using poly(vinyl alcohol) (PVA) as glue. In a competitive mode the analyte–peroxidase conjugate was incubated with the free analyte in the microtiter plate; the bound fraction of the conjugate was then quantified using luminol as the chemiluminescent substrate. The light emission was measured/imaged using a cooled CCD camera. At higher concentrations of PVA, the binding of the conjugate decreased substantially due to the masking of a large number of the PVA binding sites. By using this method, 2,4-D could be measured up to 34 nM with a useful range from 68 nM to 680 μM, and it was possible to measure large number samples at a time. Surugiu et al. [170] also reported an imprinted polymer-based capillary assay using chemiluminescence and a PMT for 2,4-D detection. A glass capillary was modified by covalently attaching 2,4-D-imprinted polymer to the inner capillary wall. 2,4-D was labeled with the tobacco peroxidase and in a competitive format the bound fraction of the conjugate was quantified in flow injection detection mode. The MIP capillary could be regenerated after each measurement, thereby allowing for consecutive measurements of large numbers of samples. Owing to the easy regeneration facility, this assay could be easily transformed into a FIA system and automated. A detection limit two orders of magnitude lower was reported when detection was done in a discontinuous mode and the chemiluminescence light was conducted inside the photomultiplier

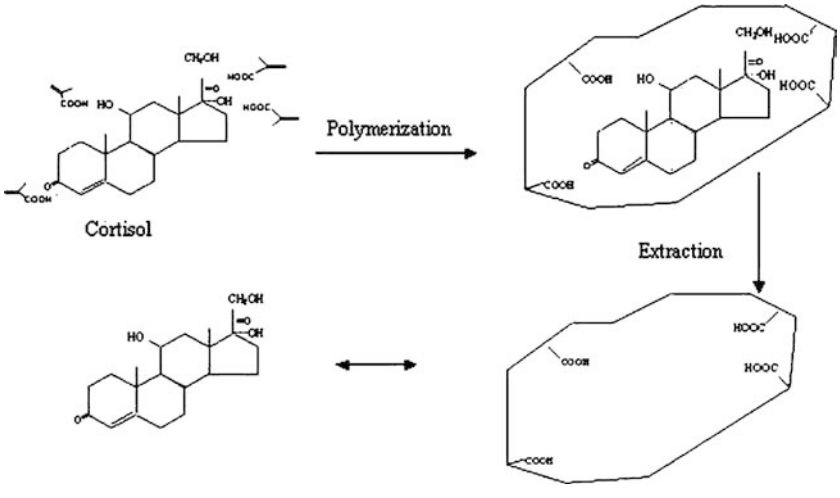

Fig. 5 A schematic drawing of the molecular imprinting procedure for cortisol (adapted from ref. [168]): porogen, tetrahydrofuran or acetone; functional monomer, MAA; cross-linker, ethylene glycol dimethacrylate; initiator, azobisisobutyronitirile

tube by an optical fiber bundle. A dynamic range of detection from 5 pg mL^{-1}–100 ng mL^{-1} (22.5 pM–450 nM) was obtained in this manner.

A highly sensitive piezoelectric sensor incorporating a MIP as the recognition element has been reported for the detection of 2-methylisoborneol (MIB) [171]. MIB and geosmin are off-flavor compounds produced by a variety of microorganisms, which cause odor problems in drinking water and in fish [172, 173]. Highly sensitive detection limits down to 10 ng mL^{-1} were obtained due to the intercalation of a nylon layer between the QCM and the MI. The sensitivity of these devices has been improved by 20-fold while maintaining selectivity in contrast to a previous report [174]. For the design of MIP-based sensors during the past few years, mass-sensitive acoustic transducers, such as the surface-acoustic wave oscillator [175, 176], the QCM [174, 177], or the Love-wave oscillator [178], have been employed. Ultrathin films of titanium oxide gel imprinted with 4-(4-propyloxyphenylazo)benzoic acid (C3AzoCO2H) were prepared by repeated immersion of a gold-coated quartz crystal microbalance (QCM) electrode or a quartz plate in solutions of C3AzoCO2H and titanium butoxide ($Ti(O\text{-}nBu)_4$) in toluene/ethanol [179]. The templating azobenzene carbodylic acid reacted with titanium butoxide though covalent bonding in the film. The template molecules were completely removed from the thin film using diluted aqueous ammonia. Binding and desorption studies were done by measuring frequency changes in the presence of template molecules. Rebinding of template molecules is fast (< 1 min).

Dickert et al. reported some MIPs for the detection of microbial cells in aqueous samples using QCMs [180–182] and this author also recently published a review on sensor strategies for microorganism detection [183]. Biological recognition materials like antibodies showed promising sensitivity towards the detection of cells. But the main limitation was the limited stability of the resulting sensor layers. Dickert and Hayden [184] resolved this problem by employing artificial recognition elements such as MIPs. Based on this strategy, yeast imprints were made on QCM electrode surfaces using the titania oxide sol–gel stamping method. The yeast-imprinted sensor coatings showed a regular honeycomb-like surface with 1-μm depth. The yeast cells can be measured up to 21 g L^{-1} in growth media [182]. This kind of detection system is very useful for the fermentation and biotechnological industries for cell counting and contamination detection. Viruses are smaller than bacterial and yeast cells and cannot be observed or enumerated with light microscopes. Recently Dickert et al. [185] reported a bioimprinted QCM for the detection tobacco mosaic virus (TMV) in aqueous media (pnat sap). By using this sensor, TMV was detected quickly in the range of 100 ng mL^{-1}–1 mg mL^{-1}, and imprints can be easily regenerated and reused. In the future, MIPs for the specific detection of cell surface markers of microbial cells may be developed along these lines.

Leung et al. [186] reported a MI for 3-chloro-1,2-propanediol (3-MCPD). The presence of chloropropanol in foods is carcinogenic. According to the European Community (EC) the limit on 3-MCPD in acid-hydrolyzed vegetable proteins and soya sauces is 0.02 mg kg^{-1} (dried weight). GC is widely employed for the analysis of chloropropanols; however, this procedure requires derivatization schemes, which is a time-consuming process and requires the use of further solvents. Sometimes incomplete derivatization leads to false results. Hence, for industrial use, chemosensors are highly desirable. 3-MCPD imprints have been successfully developed using 4-vinylphenylboronic acid as the functional monomer [186]. By using this imprint as a recognition element, potentiometric response of the glass electrode is reasonably linear over the range 0–3.2 mM for 3-MCPD. However, in the case of real samples some problems like blocking of sites by the interferences present in the real sample (soya sauce) were encountered. A few reviews have been published on applications of molecular imprinting technology in food analysis [58, 187].

Fluorescence-based sensors are highly sensitive and convenient for analysis. The use of external fluorescence (conjugates) is presently a practiced method. In this kind of approach where direct analyses of target molecules are possible, changes of fluorescence intensity appear in the presence of target molecules without any use of external reagents or conjugates. Brune et al. [188] reported that a mutant of *Escherichia Coli* phosphate binding protein labeled with a fluorescent dye adjacent to its binding site exhibited a large increase in fluorescence upon binding of inorganic phosphate. The same kind of labeling studies were conducted for the determination of maltose [189] and glucose [190]. However, the design of fluorescent MIs has been hampered by the lack of suitable fluorescent tags, which should respond to the binding event with significant fluorescence intensity changes. Gao et al. [191] synthesized a fluorescent monomer, which allowed for the preparation of fluorescent sensors for *cis*-diols using molecular imprinting method. Using this monomer an imprint polymer for D-fructose was prepared. The resultant polymer showed significant enhancement of fluorescence intensity upon binding with D-fructose. The enhancement was dependent on template concentration. It was also reported that the fluorescent monomer developed could be used for the preparation of fluorescent sensors of other sugars and biologically important catecholamines, which also have a *cis*-diol moiety. In another study, a fluorescent adenosine 3′,5′-cyclic monophosphate (cAMP)-imprinted polymer was prepared by polymerization of trimethylolpropane trimethacrylate, 2-hydroxyethyl methacrylate (HEMA), and the fluorescent functional monomer in the presence of cAMP. Methanol was used as the solvent porogen because of its high solubility of cAMP and all monomers [192]. The recognition properties of the cAMP-imprinted polymer were studied in aqueous media by fluorescence spectroscopy. This fluorescent molecular imprinted polymer displayed a

quenching of fluorescence in the presence of aqueous cAMP, whereas almost no effect was observed in the presence of the structurally similar molecule, cGMP. The association constant for the binding of cAMP to the imprinted polymer was determined to be in the order of 10^5 M^{-1}. These selective fluorescent polymer imprints will be very useful for the development of fluorescent chemosensors for the aqueous detection of cAMP. Homocysteine is a nonessential thiol-containing amino acid formed as an intermediate during the metabolism of methionine. Blood plasma of a normal adult contains 5–15 μM of total homocysteine. Homocysteine is toxic to endothelial cells of blood vessels, and an elevated plasma level of homocysteine can induce vascular dysfunctions that may lead to arteriosclerosis, stroke, and cerebrovascular, peripheral vascular, and coronary heart diseases. The clinical determination of homocysteine relies mostly on chromatography, GC-MS and HPLC, capillary electrophoresis, and immunoassay. These methods are time-consuming and expensive. Chow et al. [193] reported a fluorescence-based method for the determination of homocysteine present in blood plasma. In their work, MIP was developed using *N*-(1-pyrenyl)maleimidyl-*dl*-homocysteine (PM-H) or *N*-(1-pyrenyl)maleimidyl-*dl*-cysteine (PM-C) as template and MAA, trimethylolpropane trimetharcylate (TRIM), and 1-azobiscyclohexanecarbonitrile (ABCN) as recognition matrices.

A molecularly imprinted sol–gel material for the detection and quantification of DDT was reported by Grahm et al. [194]. Both covalent and noncovalent strategies were employed; however, good selectivity and binding were obtained though covalent imprints as a result of the lack of strong intermolecular interactions between the sol–gel polymer and DDT. A covalent imprinting strategy was employed by generating a sacrificial spacer through the reaction of two 3-isocyanatopropyltriethoxysilanes with one of two different template molecules: 4,4-ethylenedianiline (EDA) or 4,4-ethylidenebisphenol (EBP). After formation of the sol–gel, the bonds linking the spacer template to the matrix were cleaved in a manner that generated a pocket of the appropriate size bordered by amine groups. The bonds were aided in the binding of DDT through weak hydrogen-bonding interactions. These imprints were able to bind selectively with DDT. For construction of the chemosensor, an environmentally sensitive fluorescent probe, 7-nitrobenz-2-oxa-1,3-diazole (NBD), was located adjacent to the DDT binding site. Fluorescence fluctuations occurred at the binding site in response to the presence or absence of DDT. By using this fluorescent MI, 50 pg mL^{-1} of DDT could be detected with a response time of < 60 s. Repeated measurements could also be made with the same sensing films after regeneration with acetone. However, this fluorescent MI showed significant problems such as minimal increase in fluorescence intensity and limited dynamic range of the sensor as a result of the improper positioning of the NBD fluorophore in close proximity to the binding sites.

A conductometric chemosensor based on MIPs has also been reported. In this study benzyltriphenylphosphonium was taken as a model analyte (template). Molecular imprinted polymer against the template molecule was prepared by photoinitiated polymerization at 366 nm with template, MAA, EDMA, and AIBN initiator in acetonitrile. The binding ability of the MIP was checked by measuring the absorbance at 268 nm [195]. The imprinted polymer exhibited improved conductivity compared to nonimprinted polymer on the electrode surface. Hedborg et al. [62] reported an integrated sensor based on a MIP, a capacitance sensor consisting of a field-effect capacitor covered with a thin phenylalanine anilide imprinted polymer membrane [24]. Panasyuk et al. [196] reported a capacitative detection method in conjunction with imprinted electropolymerized polyphenol layers on gold electrodes. Lin and Yamada [197] reported a chemiluminescence flow-through sensor for 1,10-phenanthroline based on the combination of molecular imprinting and chemiluminescence. A ternary complex 4-vinylpyridine–Cu(II)–1,10-phenanthraline was synthesized and used as functional monomer for the 1,10-phenanthroline imprint development in combination with styrene and divinylbenzene and packed into the column. The polymer containing ternary complex is an efficient catalyst for the decomposition of H_2O_2. When analyte and H_2O_2 passed into the column with the buffer stream, these were complexed by the pyridine–Cu(II) binding sites and encountered H_2O_2 molecules. During the H_2O_2 decomposition, superoxide radical ion is formed which reacts with 1,10-phenanthraline and liberates a chemiluminescence signal. The 1,10-phenanthraline is destroyed during the chemiluminescent reaction, liberating the binding site for another analyte molecule.

Kugimiya and Takeuchi [61] reported a MIP for the detection of sialic acid and ganglioside. Various sequences of oligosaccharides, which contain sialic acid at the non-reducing end in glycoproteins and glycolipids, play important roles as cellular adhesion receptors and in molecular interaction. These oligosaccharides have been used as markers of biological information. In this study, an SPR-based sensing system for detection of ganglioside (GM1), a glycolipid containing sialic acid at the non-reducing end, was developed. On the surface of an SPR chip, sialic acid MIP was coated through UV irradiation initiation and used for the detection of ganglioside (GM1). The performance of MIP-SPR chips in the presence of other structurally related compounds was also investigated. The resonance angle changes as a function of concentration and was linear from 0.1 to 1.0 mg mL^{-1} of GM1. The estimation of creatinine levels is very important in the clinical diagnosis of renal, thyroid, and muscular malfunctions. Higher levels may indicate diabetic nephropathy, eclampsia, muscular dystophy, pre-eclampsia, pyelonephritis, reduced renal blood flow, urinary tract obstruction etc. The currently available spectrophotometic and enzymatic detection techniques suffer from interference with compounds

present in the biological fluids. Recently [198] a capacitative creatinine sensor was reported based on a photografted MIP using a gold electrode. Creatinine binding was detected by a decrease in the electrode capacitance. The sensor response is reversible and highly selective. No response to the addition of sodium chloride, creatine, urea, or glucose was detected. The detection limit for creatinine is 10 μM, which is optimal for medical applications.

Catalysis

In many cases, enzymes exhibit low catalytic activities due to the presence of organic solvents, inhibitors, and/or complex mixtures and perturbations in the temperature and solution pH. These problems may be avoided by employing synthetic biomimitic catalytic counterparts [199] instead of biomolecules such as enzymes and catalytic antibodies [200]. The catalytic counterparts can be synthesized by tuning the enzyme active site through molecular imprinting with substrates or their transition state analogues (TSAs) [201–203]. For the preparation of catalytically active MIs, a cavity has to first be made with a defined shape corresponding to the shape of the substrate or, even better, to the shape of the transition state of the reaction. At the same time, functional groups are incorporated that act as binding sites, coenzyme analogs, or catalytic sites within the cavity and in a defined stereochemical manner [26]. These artificial polymeric catalysts are more durable and more resistant to harsh environments than biomolecules [204, 205], thus they may be highly advantageous for industrial continuous transformation and/or conversion reactions. Beach and Shea [206] reported a molecular imprinted catalyst (MIC) that could catalyze the dehydrofluorination of 4-fluoro-4-(*p*-nitrophenyl)butan-2-one [206]. 2-Benzylmalonic acid was used as a template to orient the *N*-(2-aminoethyl)methacrylamide monomers prior to polymerization. Polymerization was carried out in DMF solvent in the presence of EDMA and 2,2′-azobisisobutyronitrile. These polymers are insoluble, open-cell macroporous solids with internal surface areas. Finally the template molecules were removed from the cavities with dilute NaOH solution. The recovery of templates was 78–85% [150]. In order to direct proper positioning of two amino groups in the catalytic cavity, different dicarboxylic acid template molecules were employed. The catalytic activity of the imprint was greatly influenced by the position of carboxylic groups on the template. The most active imprinted polymer was achieved with benzylmalonic acid as a template (Fig. 6). This approach highlighted the possibility of changing the position of functional groups in the active sites as a strategy for developing high-activity MIP catalysts. Sellergren and Shea [207] and Sellergren et al. [208] reported a highly cross-linked polymer catalyst for the hydrolysis of *N-tert*-butoxycarbonyl phenyl alanine-*p*-nitrophenyl ester. The cross-linked polymer had appropriately placed hydroxyl, imidazole, and carboxyl functional groups to mimic the amino acid residues of chymotrypsin. One catalyst was constructed in which a chiral phosphonate analog of D-phenylalanine was used as the template. The two other catalysts were control polymers, one in which the carboxylic acid groups were randomized using an achiral template and without the

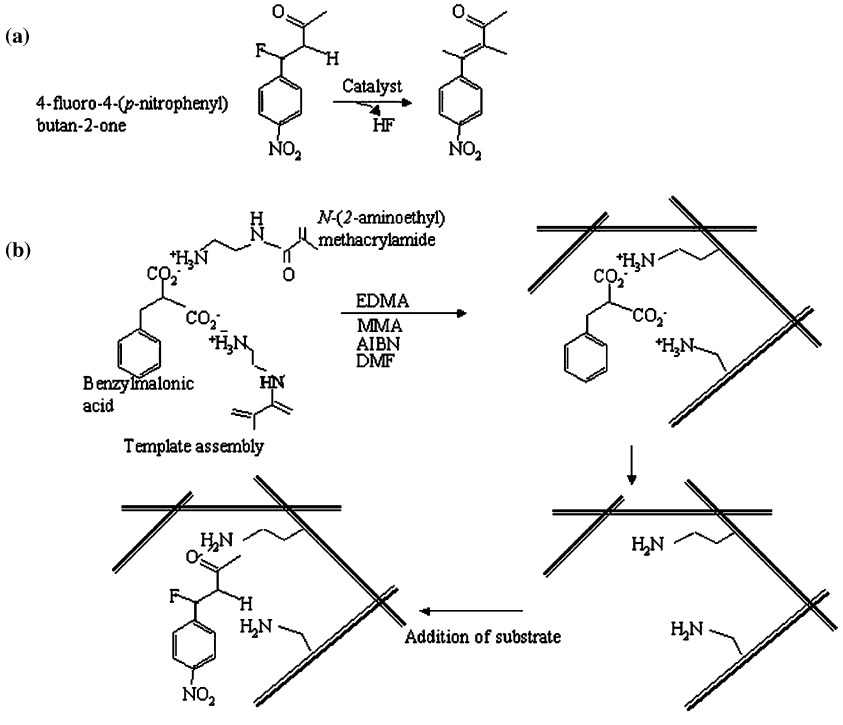

Fig. 6 Catalyst design for the dehydrofluorination prepared by imprinting (adapted from ref. [206]): **a** reaction mechanism, **b** catalyst preparation through MIP

tetrahedral phosphonate, and the other in which the phenolimidazole functionality was removed. The maximum rate enhancement for the hydrolysis of Boc-D-PheONP observed with the polymers was tenfold over reaction in solution without polymers. The control polymers showed less activity, approximately 5.7-fold or less over the reaction in solution and complete loss of enantioselectivity. The polymer catalyst showed a 1.85-fold rate enhancement of the D-isomer over the L-isomer; the control polymers showed no preference for one isomer over the other.

Leonhardt and Mosbach [209] reported an imprinted matrix with esterolytic activity, in which cobalt(II) ions were used to coordinate catalytically active vinylimidazole groups and the template during the imprinting process. The introduction of the substrates (*p*-nitrophenyl esters of methionine or leucine) to the sites resulted in an accelerated and substrate-specific hydrolysis of activated amino acid analogues [209]. Wulff et al. [210] used *N*,*N*′-diethyl(4-vinylphenyl)amidine (DEVPA) as a functional monomer to bind to phosphonate TSA-imprint molecules. The authors reasoned that a complementary shape to the transition state analogue itself might not be sufficient for catalysis, and that appropriately positioned amidine functionality similar to that of arginine had to be introduced for efficient catalysis to occur. Furthermore, the efficiency could be improved if the noncovalent bonds had sufficiently high association constants to give 1:1 complexes of template and binding site, an interaction termed the ''stoichiometric noncovalent interaction.'' The double salt bridge of the DEVPA–phosphonate TSA complex provided an 'oxyanionic hole' for TS stabilization similar to that found in serine proteases. Basic hydrolysis of an aryl ester was shown to be accelerated > 100-fold in the presence of the MIP catalyst. The catalyst exhibited Michaelis–Menton kinetics ($K_m = 0.60$ mM; $k_{cat} = 0.8\times10^{-4}$ min^{-1}). The same group also studied in the possibility of using DEVPA to catalyze the hydrolysis of carbonates and carbamates. Imprinted polymers were prepared in bulk and in suspension using cross-linker EDMA, functional monomers MMA and DEVPA, and diphenyl phosphate as template molecule in the presence of acetonitrile (MeCN), cyclohexanol-*n*-dodecanol, or toluene as porogen. Under pseudo-first-order conditions, the hydrolysis of diphenyl carbonate and diphenyl carbamate showed rate enhancements of 588 and 1,435 times, respectively, compared to the rates of the uncatalyzed reactions. Relative to that of the control polymer, the MIP catalyst showed rate enhancements of 10 and 24 times, respectively [211]. Thus, MIP catalysts showed larger rate accelerations than catalytic antibodies.

There is significant importance in developing materials that effectively adsorb and/or degrade toxic and pesticide compounds [212–214]. Phosphotriesterase is capable to hydrolyze a variety of phosphodiester bonds in the detoxification of pesticides and chemical warfare agents [215, 216]. In addition to enzymatic methods, nonenzymatic phosphoester bond-cleaving methods have also been reported [217–219]. Meng et al. [212] constructed an artificial phosphotriesterase by the copolymerization of 4(5)-vinylimidazole–Zn^{2+}–MAA cluster with a divinylbenzene polymer. Compared with the spontaneous hydrolysis, the resulting polymer catalyst showed 105-fold rate acceleration towards the hydrolysis of diethyl *p*-nitrophenyl phosphate (paraoxon). The catalytic activity of the MIC prepared in this study was fivefold higher than the polymer catalyst copolymerized with Co^{2+}–imidazole cluster [220, 221]. The MIC showed a turnover rate of 7.4×10^{-2} s^{-1} towards the hydrolysis of paraoxon [212]. By employing the MIC as catalyst, an amperomertic sensor was constructed, which showed a detection limit of 0.1 mM for paraoxon with an excellent stability even at higher temperatures. Morihara's group reported extensive studies on molecular imprinting with TSA on acidically hydrolyzed silica gel surfaces doped with Al^{3+}. These Al^{3+} ions provided Lewis acid centers for catalysis, and imprinting created binding sites complementary to the template on silica gel surface [222–226]. The selectivity and catalytic activity relied on a combination of imprint molecule shape and Lewis base activity of the aluminate components of the particles. Markowitz et al. [227] reported surface imprinted silica particles for the catalytic activity. A surfactant derivative of a α-chymotrypsin transition state analogue (TSA) was molecularly imprinted on surfaces using organosilanes *N*-(3-aminoethylaminomethyl)-phenyltrimethoxysilane (PEDA), carboxyethylsilanetriol (CTES), and *N*-(3-triethoxysilylpropyl)-4,5-dihydroimidazole (IPTES) added along with tetraethoxysilane (TEOS) to imprint the chemical functionality. Surfaces imprinted with *N*-α-decyl-D-phenylalanine-2-aminopyridine enantioselectively catalyzed the hydrolysis of benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA). No hydrolysis of D-BAPNA was observed. The catalytic activity of surface-imprinted particles formed with TEOS and PEDA was significantly greater than that of surface-imprinted particles formed with TEOS and IPTES. Recently Wulff [26] published an excellent review on MICs. Although MICs were highly stable under different harsh conditions, their catalytic activities were low compared to biocatalysts (e.g., enzymes). The exact mimic of enzyme catalytic sites was quite difficult due to its complexity of structure and interactions. For the improvement of catalytic activity of MICs, intense research, new polymers systems, highly selective substrates and/or TSAs, and new strategies are necessary. An overview of various applications of MIPs are listed in Table 1.

Conclusions

The increased demand for medical, environmental, and industrial analyses during the last decade has created a need for quick and specific devices/receptors for the separations and analyses of various compounds in aqueous and non-aqueous phases. Molecular recogni-

Table 1 List of some applications of molecular imprinting technology

Template/target	Application	References
Nicotine	Extraction	[228, 229]
Caffeine	Assay and sensor	[138, 230]
Glucose	Sensor	[231, 232]
Fructose and galactose	Separation	[87]
Aspartame	Detection and catalyst	[233]
Fructose	Separation	[86]
Monosodium glutamate	Separation	[234]
Amino acids	Separation	[235]
Vitamin K_1	Sensor	[236]
OP pesticide	Detection and sensor	[220, 221, 223]
Triazine	Sensor	[237]
Atrazine	Sensor and extraction	[112, 162]
DDT	Detection and sensor	[194]
Penicillin G	Separation	[238]
Ampicillin	Analysis	[239]
Ephedrine	Separation	[240]
Anesthetics	Separation	[241]
Cephalexin	Extraction	[242]
Chloramphenicol	Analysis	[165]
Tylosin	Detection	[243]
Cholesterol	Sensor	[244]
Cholesterol	Catalyst	[245]
Steroid	Catalyst	[246]
Morphine	Sensor	[247]
Menthol	Analysis	[248]
Peptides and proteins	Recognition	[13]
Bovine serum albumin	Catalyst	[249, 250]
Bentazone	Extraction	[251]
Cu^{2+}	Detection	[252]
Zn^{2+}	Detection	[253, 254]
RNase A	Separation	[18]
Nucleotide bases	Separation	[255]
4-Nitrophenyl acetate	Catalyst	[256]
Benzamidine, pentamidine	Separation	[257]
L-Phenylanilide	Separation	[258, 259]
Propranolol	Separation	[146, 260]
Amino acid phenylesters	Catalyst	[139]
Sulfonamides	Separation	[261]
Biotin	Assay	[262]
Fructosyl valine	Catalyst	[263]
Hydroxycoumarin	Extraction	[264]
Polycyclic aromatic hydrocarbons	Sensor	[265, 266]
Fenvalerate	Sensor	[267]

tion agents with high affinity, selectivity, and stability are therefore needed to meet this demand. Biological recognition agents such as antibodies and enzymes play a pivotal role in analytical and diagnostic applications; however, their instability and irreversible deactivation limit their application and long-term use in biosensors and separation processes. The high selectivity, affinity, stability, and integrity of MIPs provide good alternatives to the biological recognition agents. Most molecular imprinted polymers are applied mainly for chromatographic uses because they are rigid gel matrices. However, the utilization of MIP potentials in separation and purification technology is still at a juvenile stage. Furthermore, efforts are needed to develop new MIP methods towards high molecular weight targets such as biopolymers and microorganisms. There is a great demand for new functional monomers and cross-linkers to aid the design of high affinity imprints for a wide variety of compounds. Easy-to-use instruments and techniques suitable for onsite use by unskilled persons are required. Hence, for the construction of real-time and highly sensitive chemosensors, self-signaling polymer imprints and new mechanisms are also highly necessary. One more obstacle in the development of biosensors is the lack of suitable interface between the MIP and the sensor element; hence further work has to be intensified in this direction for the effective utilization of MIPs.

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