Molecularly Imprinted Polymers as Nucleotide Receptors

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Abstract: Molecularly imprinted polymers (MIPs) are highly cross-linked porous polymers with a predetermined selectivity for a given analyte or structurally related compounds, that make them ideal materials to be used in a wide range of areas including chemical sensing, separation, drug delivery and catalysis. The aim of this short review is to outline the molecular imprinting technology with particular reference to the development of MIPs as artificial receptors for the recognition of nucleotide bases and their derivatives.

Keywords: MIPs, nucleotide bases, molecular imprinting, artificial receptors, molecular recognition, polymerization.

1. INTRODUCTION

The recognition and subsequent complementary binding between a receptor and a target molecule is the first step in many natural processes. Enzyme-substrate binding, protein-receptor interactions and complementary RNA or DNA hybridization are some examples of natural recognition processes found in living systems. The design of synthetic materials which are able to mimic the recognition processes found in nature has become an important and active area of research in recent years. Molecular imprinting is one of the strategies followed to create materials having recognition ability comparable to the natural systems [1, 2]. The molecular imprinting process involves the formation of a template-monomer complex mediated by specific interactions, followed by polymerization. After the removal of the template molecules, complementary recognition sites are left in the polymer matrix. Intermolecular interactions like hydrogen bonds, dipole-dipole and ionic interactions between the target molecule and the functional groups of the polymer matrix drive the molecular recognition phenomena.

The main advantages of molecularly imprinted polymers (MIPs) are the high selectivity and affinity for the target analyte used in the imprinting procedure. Furthermore, MIPs are characterized by high mechanical and thermal stability, and inertness towards acids, bases, metal ions and organic solvents compared to biological systems. In addition, the molecular imprinting is a very inexpensive procedure for the development of artificial receptors. In the majority of the cases, the price of a MIP depends entirely on the price of the template used. If the template is expensive, it is possible to recover the template and use it again. Alternatively, inexpensive template analogues can be used for the preparation of MIPs. In addition, depending on their size, MIPs can have thousands or millions of binding sites, whereas biological receptors have a few or even just one site.

In this era of genomics and proteinomics, there is a huge demand for affinity matrices which are able to recognize and to bind complex biological entities such as proteins, peptides, nucleic acids and their fragments, and the development of synthetic receptors that recognize nucleotide bases and their derivatives has become an important area of research. Therefore, artificial receptors for each of the common nucleotide bases have been prepared recently and applications are envisioned in the fields of biosensors, drug therapy, separation science and genetic engineering.

This short review aims to outline the molecular imprinting technology with particular reference to MIP synthesis, characterization and applications and to summarize the development of MIPs as nucleotide receptors in recent years.

2. MOLECULAR IMPRINTING TECHNOLOGY

Molecular imprinting is a process where a functional monomer and a cross-linker are copolymerized in the presence of a template molecule [1-4]. First, prior to polymerization the template and the monomer form a stable template-monomer complex. Then, the complex formed is polymerized in the presence of the cross-linking agent. The resulting polymer is a macroporous matrix possessing microcavities with a three-dimensional structure complementary to that of the template. Thus, the removal of the template molecules from the cross-linked matrix leaves binding sites that are complementary in shape and chemical functionality to the template. Consequently, the resultant polymer recognizes and binds selectively the template molecules.

The binding sites obtained by molecular imprinting show different characteristics, depending on the interactions established during the polymerization. Actually, two strategies for molecular imprinting have been developed based on covalent or non-covalent interactions between template and functional monomers. A semi-covalent approach which is a hybrid of the two previous strategies has been also developed [1, 3, 4]. In all these approaches, the template molecule interacts with an appropriate functional monomer to establish specific interactions. The removal of the template results in a material that contains imprinted cavities with a favourable size, shape and chemical environment to selectively rebind target molecules.

2.1. MIP Synthesis

MIPs can be synthesized by three different imprinting approaches: the non-covalent, the covalent and the semi-covalent. The non-covalent approach [1, 3-5] is based on the

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formation of relatively weak non-covalent interactions between the monomer and the template molecule before polymerization. The affinity of binding sites prepared using this approach is generally weaker than those prepared using covalent methods because the non-covalent forces, such as hydrogen bonds, ion pairs, dipole-dipole interactions and van der Waals forces, between template and functional monomers, are used exclusively in forming the molecular assemblies. However, the non-covalent approach is still the most frequently used method to prepare MIPs because of the relatively simple experimental and complexation steps, the simple processes to remove the template and the resulting greater numbers of higher affinity sites. Moreover, the non-covalent approach can be used for a vast number of compounds, including the biological ones, which are capable of non-covalent interactions with polymerizable monomers. Furthermore, the use of non-covalent interactions in the imprinting step closely resembles the recognition pattern observed in nature.

On the other hand, the covalent approach [3, 4] is based on the formation of reversible covalent bonds between the template and the functional monomer before polymerization. This method gives rise to better define and more homogeneous binding sites compared to the non-covalent approach, since the interactions between template and monomer are more stable and defined during the imprinting process. Nevertheless, to remove the template from the polymer matrix after the synthesis via covalent approach, it is necessary to cleave the covalent bonds by an acid hydrolysis procedure.

The semi-covalent approach is a hybrid of the non-covalent and the covalent methods [1, 4]. Covalent bonds are established between the template and the monomer before polymerization. Once the template has been removed from the polymer matrix, the subsequent rebinding of the analyte to the imprinted polymer exploits non-covalent interactions, as in the non-covalent imprinting approach. In general, the MIPs obtained by the semi-covalent approach present a more homogeneous binding site distribution and higher binding capacity compared to non-covalent polymers, owing to the better-defined complex in the pre-polymerization step.

Free radical polymerization is the most used synthetic method to obtain MIPs today. Usually, it is performed under mild reaction conditions (e.g. ambient temperature and atmospheric pressure) in bulk or in solution, and it is tolerant of functional groups in the monomers and impurities in the system (e.g. water). For these reasons, as well as the fact that many vinyl monomers are available commercially at low cost, the free radical polymerization is usually the method of choice for preparing MIPs. Many chemical initiators with different chemical properties can be used as the radical source in free radical polymerization. Normally they are used at low levels compared to the monomer, e.g. 1 mol.% respect to the total number of moles of polymerizable double bonds. The rate and mode of decomposition of an initiator to radicals can be triggered and controlled by light or by chemical/electrochemical means, depending upon its chemical nature. For example, azobisisobutyronitrile (AIBN), usually utilized as initiator, can be decomposed by photolysis (UV) or thermolysis to give stabilized, carbon-centred radicals capable to polymerize vinyl monomers. The presence of oxygen gas retards the free radical polymerization, thus it is necessary to remove the dissolved oxygen from monomer solutions prior to polymerization. The removal of the oxygen can be achieved by ultrasonication and/or by sparging the monomer solution with an inert gas, e.g. nitrogen or argon.

In all molecular imprinting processes the template is of central importance because it directs the organization of the functional groups pendent to the functional monomers. Unfortunately, not all the templates are amenable to templating since the template should possess functionalities that interact with the functional monomer and should be ideally stable and inert under the polymerization conditions. However, a wide range of template molecules such as drugs, amino-acids, carbohydrates, proteins, nucleotide bases, hormones, pesticides and co-enzymes have been successfully used in the development of MIPs. In the conventional imprinting protocol traces of template usually remain in the material even after exhaustive extraction protocols. This template fraction tends to leach out of the polymer upon changing the solvent, precluding accurate quantification analyses when the template corresponds to the analyte. Therefore, a close target analogue is often chosen as template, leading to binding sites that can capture also the target analyte.

The functional monomers are responsible for the binding interactions in the imprinted binding sites and in the non-covalent approach they are normally used in excess relative to the number of moles of template (template to functional monomer ratio of 1:4) to favour the formation of template-monomer assemblies. It is very important to match the functionality of the template with that of the functional monomer in a complementary manner (e.g. H-bond donor with H-bond acceptor) in order to maximize the formation of the template-monomer complex and thus the imprinting effect. Typical functional monomers used are carboxylic acids (acrylic acid, methacrylic acid, vinylbenzoic acid), sulphonic acids (acryloamidomethylpropanesulphonic acid), heteroaromatic bases (vinylpyridine, vinylimidazole), etc.

In an imprinted polymer also the cross-linker fulfils important functions. It is important in controlling the morphology of the polymer matrix, serves to stabilize the imprinted binding sites and imparts mechanical stability to the polymer matrix. High cross-link ratios are generally used in order to access permanently porous (macroporous) materials with adequate mechanical stability. Polymers with cross-link ratios in excess of 80% are often the norm. Quite a number of cross-linkers compatible with molecular imprinting are known, many of which are commercially available. Ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM) are the most commonly employed.

The solvent also plays an important role in the molecular imprinting process. It serves to bring all the components into one phase in the polymerization. In addition, it is responsible for creating the pores in macroporous polymers. For this reason, it is quite common to refer to the solvent as the "porogen". Consequently, the morphological properties of porosity and surface area of MIPs are determined by the type of solvent used in the polymerization. Another important role of the solvent is its effect on the complexation between template and functional monomers before, during and after polymerization. Before and during polymerization, the

extent of formation of the non-covalent pre-polymer complex is affected by the polarity of the solvent. Less polar solvents such as chloroform or benzene increase the complex formation facilitating polar non-covalent interactions such as hydrogen bonds. On the other hand, more polar solvents tend to dissociate the non-covalent interactions in the pre-polymer complex, especially protic solvents that afford a high degree of disruption of the hydrogen bonds. Moreover, it was observed that after the polymerization the rebinding performance is optimized when carried out in the same solvent used for the imprinting, suggesting that the optimum rebinding conditions require the same or very similar solvation conditions used for the polymerization.

MIPs can be prepared in a variety of physical forms depending on their final application. The conventional approach is to synthesize MIPs in bulk, grind the resulting polymer and sieve the resulting particles into the desired size ranges. This method is the most popular since it is simple. Nevertheless, crushing, grinding and sieving to obtain the appropriate particle size is tedious and time-consuming and often produces particles that are irregular in size and shape. In addition, some interaction sites are destroyed during grinding reducing the MIP loading capacity and, since only a portion of the original polymer is used, this method suffers from high consumption of the template molecules.

In order to overcome these problems, alternative methods to prepare MIPs have been developed in recent years, such as suspension polymerization and precipitation polymerization, that allow to obtain directly uniformly sized and monodispersed particles [6, 7]. Surface grafting of MIP layers onto preformed beads has been also proposed as a technique to obtain chromatography-grade imprinted polymers. In this method, thin imprinted layers have been used as coatings on chromatography-grade porous silica or spherical polymers using several techniques to retain the radical polymerisation at the surface of the beads [6, 7]. Monolitich MIPs have been also prepared by a simple, one-step, *in situ* free-radical polymerization process directly within the confines of a chromatographic column without the need of grinding, sieving and column packing [7, 8].

2.2. MIP Characterization

Imprinted polymers are notoriously difficult to characterize because of their insoluble nature. However, some analytical techniques can be utilized for their chemical and morphological characterization [2, 3], including solid-state NMR techniques, elemental micro-analysis and Fourier-transform infrared spectroscopy (FT-IR), which can applied to obtain qualitative and quantitative information on the composition of the polymer. In particular, FT-IR and solid state NMR methods are useful for the measurement of functional group incorporation, especially for the quantification of the degree of polymerization and of reactivity for each type of polymerizable group on the monomer.

It is also possible to investigate the morphology of MIPs and, depending on the analytical method utilized, useful information can be obtained on the specific pore volumes, pore sizes, pore size distributions and specific surface area of the materials. Specific pore volumes (ml/g) can be estimated by means of solvent uptake experiments measuring the amount of solvent uptaken by a macroporous polymer in a dry state. Nitrogen sorption porosimetry and mercury intrusion porosimetry can be used to obtain information on the specific surface area, specific pore volume, average pore diameter and pore size distribution of the polymer. Nitrogen sorption porosimetry is a method particularly useful for analyzing medium- (meso-) and small-size (micro-) pores, while mercury intrusion porosimetry is generally more sensitive at probing larger (macro-) pores. In contrast to the last two methods which analyze polymers in the dry state, inverse size exclusion chromatography (ISEC) enables to probe the porous structure of polymers in the wet-state.

The morphological characteristics of MIPs can be also investigated by microscopy techniques, such as light microscopy to verify the natural integrity of polymer beads and scanning electron microscopy (SEM) to image polymer macro-pores.

A very important level of characterization for MIPs concerns the molecular recognition behaviour. One of the best methods for evaluating binding capacity and selectivity in MIPs is batch rebinding [3]. MIPs can also be used directly as chromatographic stationary phases which generally provide a quicker and easier method for analyzing the binding properties of MIPs [3, 8].

3. APPLICATION OF MIPs

The peculiar properties of MIPs have made them a highly interesting tool for different scientific fields, including separation sciences, purification, chemical sensors and catalysis [10-22].

Molecular imprinting chromatography (MIC) has been the most extensively studied application of MIPs [7, 10]. Imprinting is highly suitable for chromatographic separation, allowing the preparation of tailor-made supports with predetermined selectivity. When used as sorbents in separation techniques, such as high-pressure liquid chromatography (HPLC), the imprinted polymers are usually synthesized in bulk, ground and sieved to remove fine particles and packed in the chromatographic column [11]. More recently, monolithic molecularly imprinted columns have been also developed. These monolithic materials are typically prepared directly inside stainless steel columns or capillary columns without the tedious procedures of grinding, sieving and column packing [12]. Of special interest are the chiral separations and, since there are presently about 500 optically active drugs on the market, the racemic resolution of drugs is the major potential application [13, 14].

The use of MIP sorbents in capillary electrochromatography (CEC) is also attractive since it combines the selectivity of the molecular recognition process with the enhanced flow dynamic of CEC, that can result in higher efficiency and shorter analysis times [15, 16].

In the last years, MIPs have also emerged as new selective sorbents for solid phase extraction (SPE) procedures, allowing not only the pre-concentration and cleaning of samples but also the selective extraction of target analytes from complex matrices [17-19]. As in conventional SPE procedures, a small amount of imprinted polymers (50-200

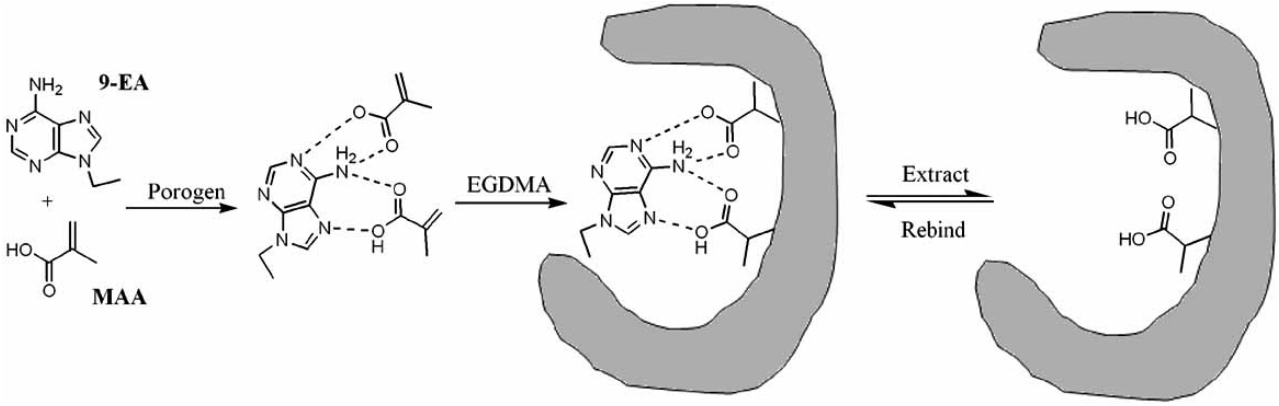

Fig. (1). Schematic representation of molecular imprinting of 9-ethyladenine (9-EA) using methacrylic acid (MAA) as functional monomer.

mg) is packed into a cartridge. Subsequently, the steps of conditioning, sample loading and elution are carried out. The use of MIPs for sample concentration and cleanup by SPE is attractive owing to their high specificity and stability and also to their compatibility with both aqueous and organic solvents.

Over the last few years, several studies have demonstrated that MIPs can serve also as artificial binding mimics of natural antibodies and can be used as recognition elements in immunoassay-type analyses [7]. MIPs, owing to their specificity, ease of preparation, low price, and high chemical and physical stability, provide an useful complement or alternative to biological receptors for the use as recognition elements in such assays. This is especially true in cases where a natural receptor does not exist or is difficult to obtain in large quantities. For the same reasons, MIPs can be successfully utilized as recognition elements in biosensor-like devices [20] substituting natural sensing elements, such as enzymes, antibodies or receptors.

4. MIPs AS NUCLEOTIDE RECEPTORS

In recent years, the design of artificial receptors that can recognize selectively nucleotide bases and their derivatives has gained importance from the theoretical as well as the application point of view. Adenine has been well studied in this regard, especially in organic solvents [23, 24]. Shea *et al.*, have prepared MIPs containing adenine recognition sites by mixing of MAA and 9-ethyladenine (9-EA) in chloroform followed by photoinitiated copolymerization with EGDMA and N,N'-1,3-phenylenebis(2-methyl-2-propenamide) (PDB MP) (Fig. **1**) [23]. 9-EA was removed from the polymer network by Soxhlet extraction and batch rebinding studies in chloroform were carried out to quantitatively evaluate the affinity of adenine and related derivatives to the polymer; an association constant (K_a) of 7,6 x 10^4 M^{-1} and a number of selective binding sites of 20 μmol/g were calculated for the 9-EA imprinted polymers. The binding capacity and selectivity of this kind of MIPs were also evaluated from its performance as chromatographic support and it was shown that the polymers imprinted with 9-EA retain adenine bases over other purine or pyrimidine bases. The specificity is due to the 9-EA imprinting since similar polymers imprinted with benzylamine (BA) did not show preference for the adenine derivatives over other purine or pyrimidine bases [23].

Successively, Spivak *et al.* have explored the factors that influence the binding capacity and selectivity of polymers imprinted with 9-EA [24]. First, the effect of the temperature on the free-radical polymerization was examined. Photoinitiated polymerization at 5 °C afforded polymers with greater binding capacity and selectivity for 9-EA than polymers that were polymerized thermally at 60 °C. NMR studies have shown that lower temperatures result in an increase in the concentration of the pre-polymerization complex [25], that could be responsible for an increase in the number of specific binding sites created during the polymerization and, consequently, for a better binding behaviour of the polymers. Next, the influence of the porogen on the binding behaviour of MIPs was investigated. It was observed that 9-EA imprinted polymers have an enhanced binding capacity when immersed in the same solvent in which they were polymerized, suggesting that the solvent affects the microenvironment of the binding sites created in the polymer. Consequently, the ideal rebinding conditions for a given template should include the solvent used as porogen. Finally, the retention of the 9-EA template on its imprinted polymers was found to be pH dependent, suggesting that rebinding interactions of 9-EA in imprinted polymers are strongly influenced by electrostatic interactions. In addition, quantitative structure-binding relationship studies were carried out to determine which sub-structural elements of 9-EA contribute to the binding capacity and specificity of the 9-EA imprinted polymers. It was found that the two nitrogen atoms positioned α or β to each other, one being part of an aromatic ring, are mainly responsible for the binding capacity and specificity of 9-EA to its imprinted polymers [24].

Interest has been also focused on receptors for adenine in aqueous media [26-28]. Mathew and Buchardt have developed imprinted polymers capable of recognizing adenine in aqueous medium using MAA and vinylimidazole as co-monomers, EGDMA as cross-linking agent and a mixture methanol-water as solvent; after adding AIBN the mixture was polymerized at 65°C for 24 h [26]. The affinity of these polymers for adenine was evaluated; a number of accessible sites of 1.86 μmol/g and a K_a of 4.3 x 10^3 M^{-1} were calculated. The value of K_a is significantly lower than that calculated for a similar system in non-aqueous medium [23]. However, considering the fact that water effectively competes for the hydrogen bonding interactions, the valued K_a indicates an appreciable degree of receptor-ligand interac-

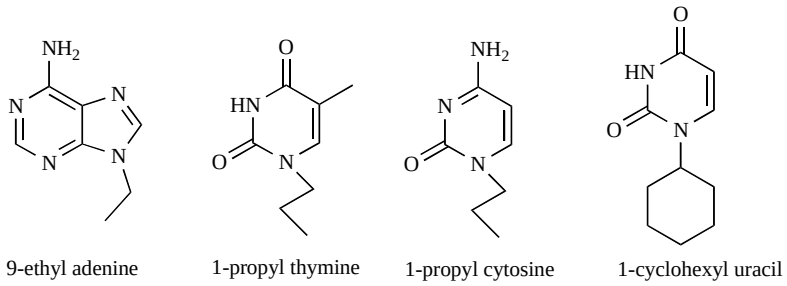

Fig. (2). Alkylated derivatives of nucleotide bases used for imprinting.

tion. In addition, the binding of adenosine triphosphate (ATP) to the adenine imprinted polymers was evaluated and an enhanced binding capacity compared to adenine was observed [26]. The binding of ATP by the polymers was pH dependent (with the maximum at about pH 3) indicating hydrogen bonding and ionic interactions between ATP and the polymer. A similar pH dependence was found for triphosphate derivatives of thymidine (TTP), cytidine (CTP) and guanosine (GTP). The catalytic effect of these polymers for the hydrolysis of ATP to adenosine diphosphate (ADP) and adenosine monophosphate (AMP) was also observed, suggesting that the imidazole groups of the polymer favour the hydrolysis of ATP either by a nucleophilic mechanism or by activating the attack by a water molecule [26].

Recently, ATP, ADP and AMP have been also utilized by Sreenivasan [27] as template molecules in the preparation of polymers based on aniline. This study showed that affinity sites for more than one component can be created in a polymer matrix (polyaniline) through molecular imprinting in aqueous media.

Sreenivasan has also demonstrated the feasibility of the formation of MIPs in water using aniline as monomer and the simple four nucleic acid bases (thymine, cytosine, adenine and uracile) as templates [28]. Polyaniline formed in the presence of the template molecules showed affinity towards the respective bases. However, the equilibrium adsorption of the templates on the MIPs was found to vary from base to base although the polymers were prepared under similar conditions. The extent of adsorption of thymine and uracile by the MIPs was found much higher than the amount of cytosine or adenine adsorbed by the corresponding polymers. Infrared analysis showed strong hydrogen bonding between the C=O groups of the template and NH groups of the polymer, suggesting that the hydrogen bonding is responsible for the base to base variation in the equilibrium adsorption. In fact, thymine and uracile contain two C=O groups enabling stronger hydrogen bonding with the polymer matrix. This results in the formation of more affinity sites in the polymer and, consequently, in an higher adsorption. The uptake of adenine was found to be less since it does not contain C=O groups.

The creation of MIPs in aqueous media continues to be a significant challenge, as aqueous solutions interfere in hydrogen bonding interactions which are the most crucial factor governing the recognition phenomena particularly in non-covalent MIPs. In order to overcome the problems resulting from the use of water-soluble biological or biomedical molecules in the molecular imprinting, Tsunemori *et al*. have proposed a molecular imprinting technique called "surface molecular imprinting method" using a water-in-oil (W/O) emulsion [29]. The solid polymer is prepared by polymerizing the W/O emulsion consisting of a water-soluble imprint molecule, a functional molecule which has to interact with the imprint molecule, an emulsion stabilizer and a cross-linking agent. The imprint molecule forms a complex with the functional molecule and the orientation of the functional molecules is fixed at the oil-water interface. This leaves, after the polymerization, the recognition sites in the inner cavity of the imprinted bulk polymer. This imprinting technique was adapted for the preparation of nucleotide recognition polymers, using dimethyldistearyl ammonium chloride as the functional molecule, inosine-5'-monophosphoric acid (IMP) as the imprint molecule and the structural analogue guanosine-5'-monophosphoric acid (GMP) as the competitive molecule. The IMP-imprinted polymers showed an high selectivity toward the imprinted molecule IMP. The binding constants for the IMP-imprinted polymers prepared at pH 9.0 and 8.5 were calculated to be 2.8×10^4 M^{-1} and 1.7×10^4 M^{-1}, respectively.

In a manner similar to the polymers imprinted with 9-EA, organic solvent soluble alkylated derivatives of cytosine, thymine and uracile (Fig. **2**) were subjected to the imprinting process [30]. Each nucleotide base derivative was dissolved in a mixture of MAA, EGDMA, AIBN and the porogen acetonitrile. The polymers were obtained by photo-polymerization below ambient temperature, followed by Soxhlet extraction to remove the template. Binding affinity and selectivity of the MIPs were evaluated chromatographically. In addition, the binding and selectivity of nucleoside derivatives were investigated using organic soluble derivatives of all five nucleotides, specifically those with a fully acetylated ribose unit (Fig. **3**) allowing to study also the influence on the selectivity of ribose substituents on the bases [30, 31]. The nucleoside derivatives were imprinted in the same fashion as the nucleotide base derivatives. Specific binding was found for adenine, cytosine and guanosine derivatives. These three bases contain a 2-aminopyridine substructure previously found to be important for polymer imprinting with MAA as the functional monomer [24]. In contrast, thymine and uracile derivatives do not contain this moiety and their imprinted polymers exhibited little or no affinity. The results obtained using the single nucleotide base derivatives have formed the bases of designed materials for the binding of sequences of oligonucleotides and imprinted polymers were prepared using oligothiophosphate adenine dimer and

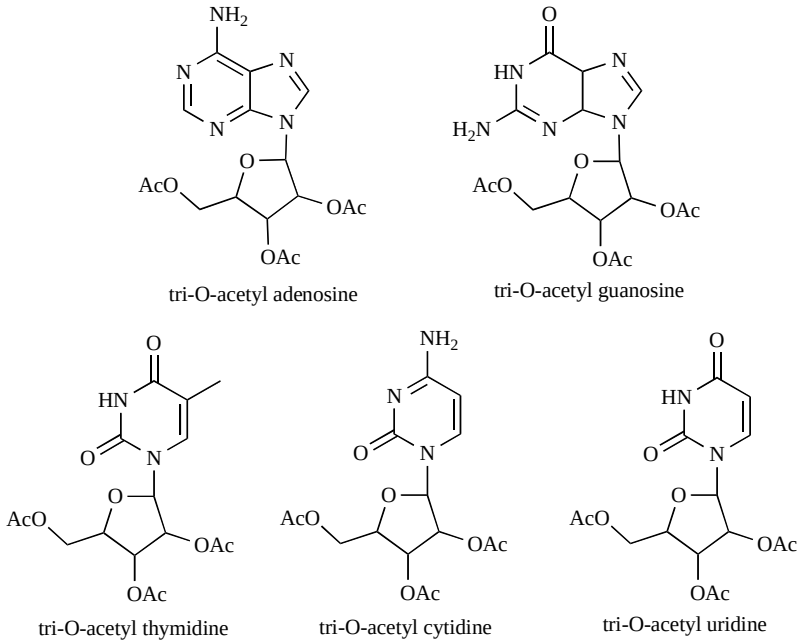

Fig. (3). Nucleosides with acetylated sugar units used for imprinting.

tetramer [31]. They showed selectivity for the dimer while the tetramer exhibited little affinity for the corresponding imprinted polymers, probably due to size effects.

As reported above, MIPs have shown promising features as novel stationary phases in liquid chromatography and, recently, also in capillary electrochromatography. A monolith MIP comprising 9-EA was prepared *in situ* inside the capillary for the electrochromatographic separation of nucleotide bases by Huang *et al.* [32]. The capillary wall was first functionalized with 3-trimethoxysilylpropyl methacrylate and 1,1-diphenyl-2-picrylhydrazyl in toluene. The capillary was then filled with acetonitrile containing 9-EA, MAA, EGDMA and AIBN. After the polymerization started by a thermal initiation, a syringe pump was applied to the capillary to shrink the polymer into a thin film against the capillary wall, then the template was removed with methanol. The feasibility of the MIP column for the separation of nucleotide bases (adenine, cytosine, guanine and thymine) was evaluated; the results showed that the MIP column demonstrates better recognition properties at a pH range of 6-8.

Recently, Li *et al.* have reported a new process for the preparation of uniform molecularly imprinted polymeric nanopheres (MIPNs) for the recognition of alkylated derivatives of nucleotide bases combining both molecular imprinting and block copolymer self-assembly techniques [33]. A diblock copolymer was synthesized with one block containing functional groups for both hydrogen bond formation and cross-linking. After interacting with the template molecules to form hydrogen-bonding complexes, this block copolymer was allowed to self-assemble to form spherical micelles in a selective solvent. This structure was then locked in by cross-linking, and the cross-linked nanospheres were extracted to remove the template molecules. Alkylated derivatives of uracile and thymine were synthesized and utilized as template molecules. The resulting MIPNs have demonstrated higher rebinding capacity than traditional bulk MIPs and comparable size and shape selectivity in rebinding the target molecules.

Of particular interest is the work of Wandelt *et al.* about the monitoring of nucleotide-imprinted polymers by fluorescence spectroscopy [34]. Conventional functional monomers together with a fluorescent monomer were copolymerized in the presence of adenosine 3',5'-cyclic monophosphate (cAMP) as template. The polymer was copolymerized in thin-layer film directly on quarts plates. After removal of the template molecules the functionalized cavities existing in the fluorescent material are able to specifically bind the template with consequent quenching of fluorescence of the polymer. This fluorescent MIP chemosensor contains both the recognition element (the imprinted cavity) and the measuring element (receptor incorporated in the cavity) able to transform the recognition information for fluorescence signal.

Molecularly selective field-effect transistors have been also developed for determining oxidized and reduced forms of nicotinamide adenine dinucleosides (NAD^+ and NADH) and their phosphates $NAD(P)^+$ and NAD(P)H [35]. Copolymers of acrylamide and acrylamidophenylboric acid imprinted with the nucleotides were used as sensing elements of the sensors. After removal of the nucleotide, a cavity corresponding to the surface of the imprinted nucleotide was retained and ensured the selectivity of the sensor.

In the most studies about the preparation of MIPs as nucleotide receptors, MAA was used as functional monomer making the polymer preparation a simple and facile process. MAA can form hydrogen bonds with the template molecule in porogen prior to polymerization. A more deliberate approach using synthetically designed functional monomers could enable a better control in the formation of high-affinity binding sites for each corresponding template minimizing, at the same time, the inherent non-specific binding properties common in non-covalent imprinted polymers.

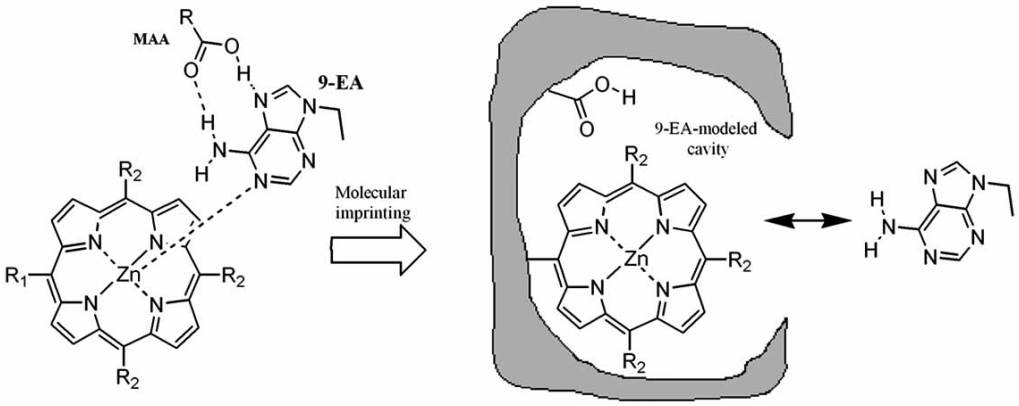

Fig. (4). Schematic representation of molecular imprinting of 9-ethyladenine (9-EA) using Zn(II) 5,10,15-tris(4-isopropylphenyl)-20-(4-methacryloyloxyphenyl)porphyrin (1) and methacrylic acid (MAA) as functional monomers.

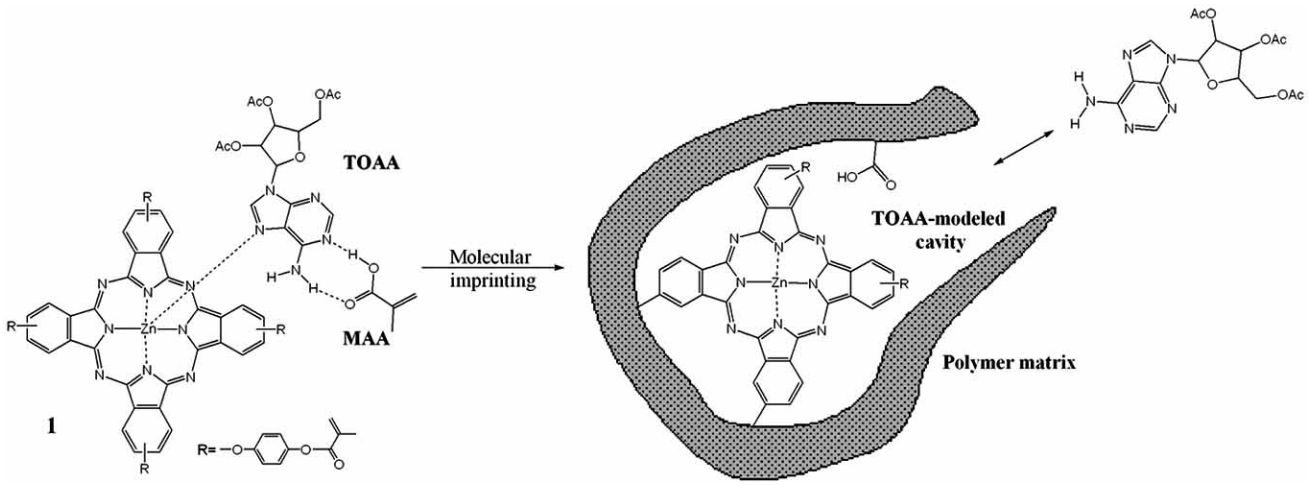

Fig. (5). Schematic representation of molecular imprinting of tri-*O*-acetyladenosine (TOAA) using Zn(II) tetra(4'-methacryloyloxyphenoxy)phthalocyanine (1) and methacrylic acid (MAA) as functional monomers.

Yano *et al.* have synthesized a polymerizable 2,6-bis(acrylamido)pyridine for the use as a new functional monomer in molecular imprinting applications [36]. This monomer forms multiple hydrogen bonds with a specific target molecule by mimicking multiple hydrogen bonds between nucleotide bases, thus allowing a specific molecular memory in MIPs.

Successively, Matsui *et al.* have proposed a new strategy involving the use of a polymerizable Zn(II) porphyrin as functional monomer in the imprinting of 9-EA (Fig. **4**) [37]. A 9-EA imprinted polymer, called PPM(9-EA), was prepared using both Zn(II) 5,10,15-tris(4-isopropylphenyl)-20-(4-methacryloyloxyphenyl)porphyrin and MAA as functional monomers. Reference imprinted polymers were also prepared using either the Zn(II) porphyrin or MAA, namely PP(9-EA) and PM(9-EA), respectively. It was observed that PPM(9-EA) marked a larger K_a (7.5×10^5 M^{-1}) than PP(9-EA) (3.8×10^4 M^{-1}) and PM(9-EA) (1.36×10^5 M^{-1}). This suggested that the Zn(II) porphyrin and MAA are cooperatively arranged by the single template molecule to form the high-affinity binding sites. It is also notable that the binding constant estimated for PPM(9-EA) was higher compared to artificial 9-EA receptors previously reported [23, 24, 26].

Recently, we have used such a cooperative-interaction approach in MIP formation to prepare MIPs as nucleoside receptors using both MAA and a Zn(II) phthalocyanine peripherally substituted with methacrylic groups as functional monomers [38, 39]. The receptor site is a three-dimensional cavity around the phthalocyanine plane in cross-linked polymers to which the analyte molecule can be specifically bound via coordination through the metal of the phthalocyanine and hydrogen bonding/electrostatic interaction with MAA and the modifiers linked to the phthalocyanine (Fig. **5**). An organic soluble nucleoside derivative, tri-*O*-acetyladenosine (TOAA), was utilized as template and the phthalocyanine-based MIP was prepared by adding to a solution of TOAA in dichloromethane the phthalocyanine and MAA as functional monomers, EGDMA as cross-linker and AIBN as initiator, and then polymerizing by heating at 60 °C. The MIP prepared exhibited higher binding affinities for TOAA (K_a 2.96×10^4 M^{-1}) compared to the polymer prepared solely with MAA (K_a 1.48×10^4 M^{-1}). On the other hand, the MIP prepared using only the phthalocyanine, did not show any binding capacity for TOAA. This means that the phthalocyanine contributes to higher binding affinities, although itself barely interacts with TOAA. The effects of the simultaneous use of the two functional monomers sug-

gests the effective cooperation of the phthalocyanine-based and carboxylic residues rather than independent operation for retaining of TOAA.

5. CONCLUSION AND OUTLOOK

Important progresses in the synthesis and application of MIPs are clearly evident within recent years. Low cost, excellent stability and continuously advancing performance of MIPs render these materials the most promising synthetic materials for molecular recognition in different scientific fields.

Particular attention has been directed to the development of MIPs as selective synthetic receptors for nucleotide bases. Artificial receptors for each of the common nucleotide bases and their derivatives have been prepared and applications are envisioned in the fields of biosensors, drug therapy, separation science and genetic engineering.

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