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The Technique of Molecular Imprinting – Principle, State of the Art, and Future Aspects

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Abstract

Molecular imprinting has gained increasing research interest during the past few years. In this overview we would like to explain to the readers how a seemingly simple concept can eventually lead to useful applications in several areas. The main focus will be on the present state of the art of molecular imprinting, as several breakthroughs have occurred since most reviews by us and other groups have been written. In the last part of this article we would like to discuss future developments in the area of molecular imprinting.

Introduction

Molecular recognition underpins the structure and function of the entire biological world. Living processes rely on specific interactions at the molecular level, which include as well-known examples DNA replication, transcription, and translation; antibody-antigen, enzyme-substrate/inhibitor recognition; and many other systems. Because of its fundamental importance in deciphering biological functions, extensive research efforts have focused on understanding the basic mechanisms behind specific molecular host–guest interactions. Besides research for the sake of understanding, there is also enormous interest in translating such findings to practical applications. The rational design of artificial receptors such as macrocyclic compounds depends on a thorough knowledge of the structure of the guest molecules. Successful host systems are constantly being created involving often complex, highly advanced and beautiful organic chemistry (early efforts in this area were, as known, bestowed upon with the Nobel Prize to Cram, Lehn and Pedersen in 1987). Formation of host–guest complexes is driven by intermolecular interactions involving ionic pairing, hydrogen bonding, van der Waals forces, hydrophobic effects and others. These interactions are also exploited in a related research area, i.e., molecular imprinting.

The seemingly simple technique of molecular imprinting can be characterized as a synthetic approach towards a molecular host via template-guided synthesis in a self-assembly mode. Mainly, until now, largely polymerizable monomers such as acrylic compounds have been used leading to molecularly imprinted polymers (MIPs). In the process of molecular imprinting, a molecular template (print molecule) is used to direct the arrangement of the functional monomers around the template, which are then chemically fixed by co-polymerization with a cross-linking monomer. This results in a rigid polymer matrix embedding the template. Removal of the template reveals recognition sites specific to the template and its close analogues (Figure 1). A typical imprinting system consists of a template molecule, at least one type of functional monomer and cross-linker, and a porogenic solvent. To induce radical polymerization, an appropriate initiator is included as well. Formation of the initial template-functional monomer complex (imprinting), as well as of the template-MIP complex (re-binding) may be driven by non-covalent interactions or reversible covalent bonds. The non-covalent approach [1] that was first realized by Mosbach and co-workers is easier to proceed, although it may generate heterogeneous binding sites due to the relat-

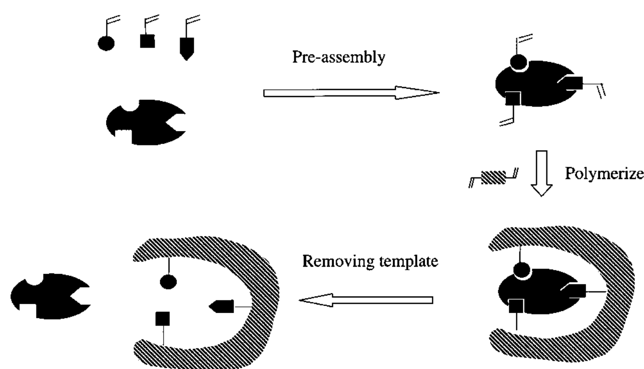

Figure 1. Schematic representation of a molecular imprinting process. Pre-assembly of functional monomers is driven by their complementary interactions with the template (print molecule). Co-polymerization with a cross-linker 'freezes' binding groups to form a template-defined 'cavity'. Removal of the template by solvent extraction or chemical cleavage affords binding sites specific to the original template based on the position of the complementary groups and shape of the cavity.

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ively weak interactions utilized. The covalent approach [2] pioneered by Wulff and co-workers should provide more homogeneous binding sites, however the re-binding is slower due to the necessary formation of the covalent bond between template and MIP, and is more cumbersome requiring prior derivatization of the template. Attempts have been made to combine the advantages of both the covalent and non-covalent approach, whereby imprinting is carried out using polymerization of the functional monomer being covalently coupled to a template, and selective rebinding utilizing non-covalent interactions [3].

As antibody and receptor binding mimics, MIPs have displayed very high affinity and specificity for many systems. The favorable physical and chemical robustness of MIPs allow these artificial 'antibodies' to be used under harsher conditions such as in organic solvents, at pH extremes, high pressures and elevated temperatures, where biological macromolecules are often denatured. MIPs therefore have potential applications in the areas of separation, trace analysis, assays, biomimetic sensors, (bio)chemical synthesis and others.

Present status

In their most common format, MIPs are prepared as a macroporous monolith that is ground to appropriate particle sizes. Novel physical configurations of MIPs have been obtained in the past few years in our and other research groups. Molecular imprinting using novel polymer systems and inorganic materials has also been increasingly reported.

New configurations of molecularly imprinted polymers

In addition to the conventional MIP particles obtained by grinding the macroporous monolith, new physical configurations of MIPs have been obtained using different preparation methods (Table 1). Advances in this direction have brought better use of MIPs in affinity separations, assays and biomimetic sensors. Compared with irregular shaped MIP particles, uniform beads prepared by suspension polymerization in perfluorocarbon gave much better chromatographic separation of chiral compounds even at higher flow rates [4]. We have used a precipitation polymerization method to prepare imprinted microspheres against various target molecules [15]. This method is easy to carry out, since no time-consuming grinding and fractionation operations are needed. The reaction condition is compatible with both covalent and non-covalent imprinting, as there is no interfering surfactant or stabilizer present in the reaction system. The small MIP microspheres can be easily suspended in assay solvents and dispensed, and easily separated by simple centrifugation. These characteristics are ideal for binding assays using MIPs instead of immobilized antibodies. Because binding sites on these MIP microspheres are made accessible to the large template-enzyme conjugate, we have successfully used imprinted microspheres in ELISA type assays to replace the biological antibodies [26, 27]. MIP microspheres were also used as chiral selector suspended in carrier electrolyte to separate (*R*)/(*S*)-propranolol using capillary electrophoresis [28], which would have been difficult to realize with the larger ground particles. With a MIP layer *in situ* coated on a quartz crystal microbalance (QCM), (S)-propranolol was selectively detected in the presence of the *R*-enantiomer, a selectivity coefficient as high as 5 was obtained [29].

Novel functional monomers

In the non-covalent imprinting approach, methacrylic acid has been largely used as a 'universal' functional monomer due to its hydrogen bond donor and acceptor characteristics, and suitability for ionic interactions. A first report on antibody binding mimics [30] based on this approach has been followed by many studies leading to a large number of MIPs expressing high binding affinity and specificity to various target analytes [31–33]. In addition to methacrylic acid and vinylpyridine, other functional monomers capable of forming strong interactions with different templates have been utilized [34–40]. Wulff and co-workers synthesized a polymerizable benzamidine derivative that can form a stable complex with the carboxyl group of a phosphonic acid monoester. This was used to prepare an enzyme-analog, a catalytically active MIP for the alkaline hydrolysis of 4-carboxybenzenacetic acid esters [41]. Lübke *et al.* have recently synthesized a bis(boronate-amide) monomer (carboxylate receptor, **1**) and a polymerizable chlorinated quinone (amine receptor, **2**), and used stoichiometric amounts of the two functional monomers in preparing MIPs capable of efficient binding of ampicillin from aqueous solutions (Figure 2) [42]. Analogous to the binding groups involved in antibodies and enzymes, researchers have derivatized various amino acids with polymerizable moieties and incorporated them as part of MIPs' binding sites [43, 44]. In this way a wide range of molecular interactions such as ionic pairing, hydrogen bonding and hydrophobic forces may be simultaneously exploited applying non-covalent imprinting. In addition, MIPs based on functionalized sugar monomers have also been reported [45].

Usually radical polymerization is used for making imprinted polymers. The free radicals generated during the imprinting reaction do not destroy the template-functional monomer complex, a prior requisite for generating high affinity and specific MIPs. However, condensation polymerization has also been utilized recently to prepare MIPs based on diisocyanate monomers [46]. The past few years also witnessed increased use of molecularly imprinted inorganic materials. This was partially attributed to the availability of new organosilane functional monomers [47–49].

Target templates

For low molecular weight templates, imprinting has proven to be efficient in giving recognition materials suitable for different analytical applications. Although binding cavities were complementary only to the small templates, by a modification in the process of making MIPs, the latter can be made to take up template-enzyme conjugates as well for,

Table 1. New configurations of molecularly imprinted polymers for low-molecular-weight templates

Configuration	Templates/print molecules	Preparation method	Reference
Polymer beads	Boc-L-Phe Z-L-Asp(L-Phe-OMe)-OH	Suspension polymerization in perfluorocarbon liquid	[4, 5] [6]
	Atrazine	Suspension polymerization in water	[7]
	Metal ions	Cross-linking functional surfactants using suspension polymerization in aqueous continuous phase	[8]
Magnetic beads	(S)-Propranolol	Suspension polymerisation in perfluorocarbon liquid	[9]
Composite beads	Boc-L-Phe	Polymerization on the surface of supporting poly(TRIM) particles	[10]
	1,4-(Bisimidazol-1-ylmethyl)benzene	Polymerization on the surface of silica particles	[11]
	(S)-(+)-N-(3,5-dinitrobenzoyl)-α-methylbenzylamine	Polymerization following multi-step swelling of seed particles	[12]
	(S)-Naproxen		[13]
	17β-Estradiol		[14]
Microspheres	Theophylline 17β-Estradiol	*in situ* precipitation polymerization	[15]
	Metal ions	Cross-linking swollen microspheres consisting of linear functional polymers	[16]
	Cholesterol	Polymerization of a template surfactant over a cross-linked polystyrene core	[17]
Membranes	Boc-L-Trp	Casting membranes from a polymer solution containing oligopeptide residues	[18]
	Theophylline	Phase inversion of acrylonitrile-acrylic acid copolymer	[19]
Continuous polymer rods	1,8-Diaminonaphthalene	*in situ* polymerization inside HPLC column	[20]
Superporous monolith	(R)-Propranolol	*in situ* polymerization inside capillaries	[21]
Polymer coating	S(+)-2-Phenylpropionic acid	*in situ* polymerization on the inner surface of capillaries	[22]
	Epinephrine	Grafting of an oxidized aminophenylboronic acid layer in microplate wells	[23]
Monolayer	6-[(4-Carboxymethyl)phenoxy]-5,12-naphthacene quinone	Photochemical imprinting of recognition sites in monolayers on gold electrodes	[24]
	Cholesterol	Self-assembly of hexadecyl mercaptan in the presence of cholesterol on gold surfaces	[25]

e.g., competitive ELISA assays for the target analytes [26, 27]. A similar principle was used in an ‘epitope’ imprinting of the neurohypophyseal hormone, oxytosin [50]. As large templates such as proteins cannot diffuse through the highly cross-linked polymer matrix, we used an alternative method to generate binding sites on the surface of a supporting material. Specific binding sites were created as demonstrated for the enzyme ribonuclease A (RNase A). Pre-assembly of the enzyme through its two exposed histidine residues with polymerizable chelators was allowed to take place, followed by fixing the complex to the surface of silica particles [51]. After removing the template protein, lysozyme and RNase A could successfully be separated using the adsorbent as the chromatographic stationary phase. Another example of protein surface-imprinting was described by the group of Ratner, in which radio-frequency glow-discharge plasma de-

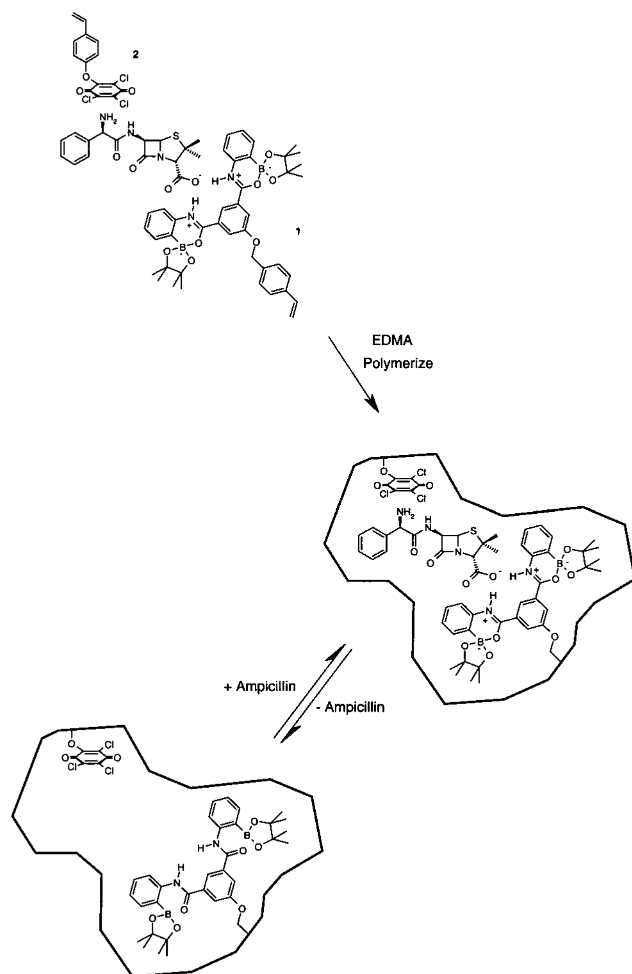

Figure 2. Non-covalent imprinting of ampicillin using a carboxylate receptor (**1**) and an amine receptor (**2**) as the functional monomers. EDMA: Ethylene glycol dimethacrylate. Adapted from Ref. 42 with permission.

position was used to prepare thin polymer films bearing covalently attached disaccharide molecules that were preassembled on the surface of the target proteins [52]. The authors showed that a surface imprinted with bovine serum albumin (BSA) preferentially adsorbed the template protein from a binary mixture with immunoglobulin G (IgG). The possibility of using loosely cross-linked, protein-imprinted polyacrylamide gel for tailored separation of protein mixtures has also been demonstrated [53].

Imprinted cavities specific for whole cells have been prepared using a bacteria-mediated lithographic procedure, where the cells acted as temporary protecting groups and structural templates during the multi-step imprinting process. It was shown that the resultant polymeric beads exhibited on their surface functionally anisotropic patches of dimensions defined by the template, and could be further modified in the sites to introduce further recognition elements for example antibodies [54]. The same group also developed a protocol for imprinting of synthetic polymers with motifs of inorganic crystals. The calcite-imprinted polymer matrix had a surface functionality mirroring the crystal face, which was able to promote the growth of specific crystal phases [55].

Applications

Although MIPs capable of chiral resolution [56–59] may find future use in the pharmaceutical industry, the loading capacity of present MIP-based stationary phases still needs to be increased. The majority of publications on molecular imprinting to date have been for analytical uses. These include solid phase extraction (SPE) for sample preparation in trace analysis [60–65]. To prevent the problem of template leakage from the MIP adsorbents that made accurate quantification of target analyte difficult, Andersson *et al.* described use of a target analogue as the template at the imprinting step. Although leakage of the latter was observed, it could be readily resolved from the target analyte using gas chromatography and thus the concentration of the target analyte accurately determined after SPE treatment [66].

The feasibility of using MIPs as artificial receptors for screening of combinatorial libraries has been demonstrated [67–69]. The advantage of MIP-based screening is that MIPs are inexpensive, more stable and relatively easy to produce, especially when the target receptors are difficult to obtain. Although no immediate bioactivity data are obtainable from the polymeric receptor, the compounds that are identified to be most similar to the original template, e.g., by binding strength can be evaluated in subsequent bioassays.

MIPs have been used as recognition elements to build up biomimetic sensors. In most cases imprinted polymers were put in physical contact with a transducer. The physicochemical response (change in mass, resistance, capacitance, refractive index, etc.) from binding a target analyte was translated into a sensor signal [29, 46, 70–73]. This simple method however often leads to MIP sensors showing low sensitivity and specificity. In a more sophisticated manner, a reporter such as a fluorescent monomer can be incorporated into the MIP's binding cavity. The fluorescence intensity is then changed on binding a target analyte to the cavity [74–76]. In order to increase the signal, the reporter monomer should be able to bind the template of interest in the cavity, and has to be specifically synthesized for different templates. To circumvent this drawback, we have utilized the principle of proximity scintillation, and developed a 'universal' reporter for signal generation (Figure 3) [77]. The scintillation fluor (**3**) incorporated by its copolymerisation into the polymer converts β-radiation from the bound tritium-labelled template into a fluorescent signal. The labelled template free in solution is too far away from **3** for effective energy transfer, therefore no fluorescent light could be generated. The imprinted scintillation polymers were used in competitive assay for the chiral drug, (S)-propranolol.

Similar to the generation of catalytic antibodies, catalytic MIPs have been generated using appropriately selected templates. These include compounds mimicking the reaction substrates [78–80], transition states [41, 47, 81–85], and product [86]. In addition to the required substrate selectivity of the binding sites, an important issue is to place correctly oriented catalytic groups within the active site mimic [87]. In one example a metal ion cofactor was introduced in the preparation of a class II aldolase mimic. The complex of an intermediate analogue with cobalt (II) ions was imprinted

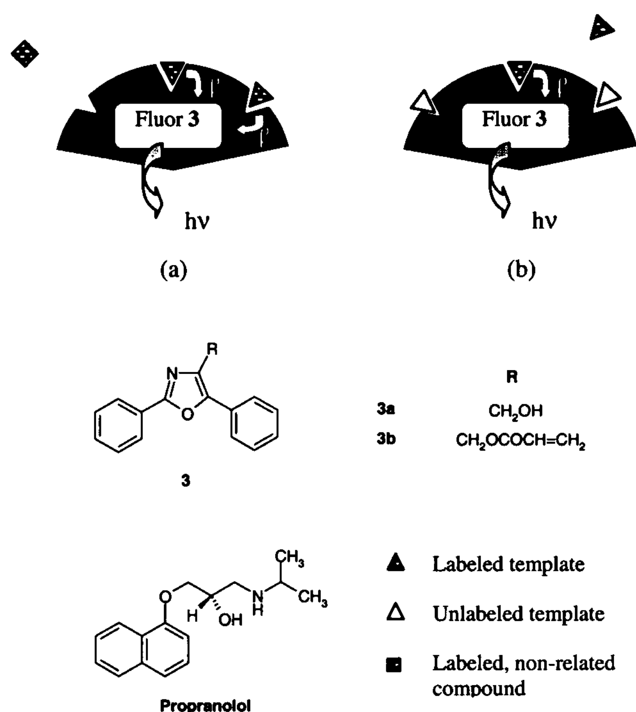

Figure 3. Schematic representation of chemical sensing with an imprinted polymer. Scintillation fluor (3) incorporated by its co-polymerization into the polymer converts β-radiation from the bound tritium-labeled template into a fluorescent signal. In contrast unbound labeled compound is too far away from **3** for effective energy transfer, therefore no fluorescent light can be generated. Reproduced from Ref. 77 with permission.

using 4-vinylpyridine as the functional monomer. The MIP was capable of catalysing the condensation of acetophenone and benzaldehyde to produce chalcone. Both substrate selectivity and true turnover were observed [88].

In other synthetic applications, MIPs have been used as microreactors for regioselective and stereoselective reduction of steroids [89], and stereoselective amino acid synthesis [90]. MIPs were also used as specific adsorbents for *in situ* product removal in an enzymatic reaction [91], and to remove low concentration of a side product in the chemical synthesis of a dipeptide [92].

New methods for MIP preparation

For imprinting against small target molecules, the templates are commonly allowed to form complexes with functional monomers free in solution. An alternative route demonstrated by our group was to use a template immobilized on a solid support. Following imprinting polymerization, the template and the carrier support was removed by chemical dissolution leaving surface imprinted sites on the obtained MIP (Figure 4) [93]. In this manner we could control not only the orientation of the binding cavities, but also the surface shape of the resulting MIPs, using for example silica beads with different porous structures as the carrier. It should be noted that our approach of using an analyte-carrier as template is analogous to the generation of biological antibodies using hapten-protein conjugates. In both cases specific binding sites for small target molecules are generated.

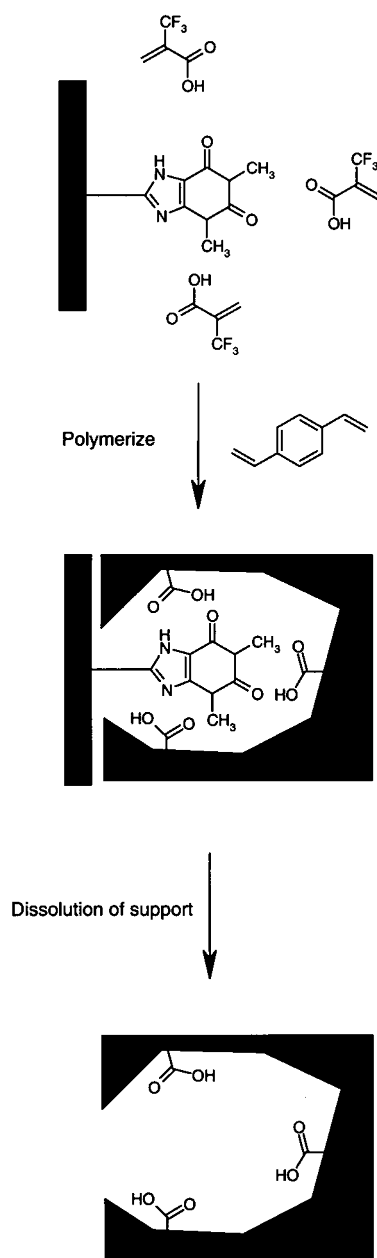

Figure 4. Molecular imprinting of theophylline immobilized on a solid support. The immobilized theophylline is allowed to form a complex with the functional monomer, trifluoromethylacrylic acid, followed by co-polymerization with the cross-linker, divinylbenzene. After polymerization, the silica support is dissolved by HF treatment to give surface-imprinted binding sites for theophylline. Adapted from Ref. 93 with permission.

In a recent publication, Hiratani *et al.* described use of a reversible cross-linker (N, N'-bis(acryloyl)cystamine) to prepare imprinted gels selective for calcium ions. In the absence of Ca^{2+}, opening up and subsequent reconnecting the S–S bonds distorted the polymer network, which resulted in a lowered Ca^{2+} binding affinity. However, on loading again with Ca^{2+}, followed by formation of the S–S bonds, the Ca^{2+} binding affinity of the resulting gel was increased [94]. Research along this line may eventually lead to MIPs with target affinities that may be influenced by environmental stimuli.

Shinkai and co-workers have synthesized saccharide-imprinted fullerene-bisadducts using the double addition reaction between fullerene and D-threitol- or L-threitol-boronic acid complex [95]. Their imprinted adducts diastereoselectively rebound the original saccharide templates. Both imprinting and re-binding were carried out in a homogeneous solution system utilizing fullerene as a soluble nano-size matrix.

Combinatorial methodology has also been used to optimize the synthetic conditions for the preparation of MIPs [96–98]. For a given target template, a number of MIPs were synthesized on a small scale by changing the reaction components (monomers) and their relative ratios. Rebinding tests of these MIPs was easily carried out with an automated liquid delivery system. The best imprinting protocol was then used for preparing the MIP suitable for the real application.

Outlook

One of the advantages of using molecular imprinting is the ease with which highly specific, tailored polymer hosts can be prepared from simple building blocks, e.g., functional monomers and cross-linkers. In the near future we can expect more 'epitope'-specific oligomers to be developed carrying several functional groups, which then would be specific for patches of a template. Various combinations of these monomers may be used to address a large variety of target molecules.

Imprinting in aqueous solution is still a challenging task. Development of potent functional monomers useful in an aqueous environment will facilitate imprinting of biomacromolecules such as proteins. For imprinting against small molecules, more homogeneous binding sites are desired for practical applications. The loading capacity has to be improved for MIPs designed for preparative separation purposes. Micro-fabrication of MIPs on chip may find its place in the area of sensor and microanalysis [99].

Although catalytic MIPs have shown high selectivity, the rate of MIP catalyzed reactions and turnover rate have been rather modest. One way to improve the catalytic performance would be to mimic the induced fit phenomenon often encountered in enzymatic systems. This might be achieved by studies towards making the conformation of MIPs more flexible.

Finally, using biological targets as templates may lead to new drug candidates as presented in connection with the 1st International Workshop on Molecularly Imprinted Polymers held in Cardiff, U.K. [100].

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