Chapter 2

Molecular Imprinted Polymers: A Review

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MOLECULAR IMPRINTED POLYMERS: A REVIEW

2.1 Introduction

Molecular recognition involves both the selection and recognition of a ligand by a given receptor structure. It is the basis for most biological processes such as ligand-receptor binding, enzyme-substrate interaction, translation and transcription of the genetic code and is therefore of universal interest. Design and synthesis of artificial receptor molecules have been a focal research area for understanding the molecular recognition phenomena in biological systems and for developing novel materials mimicking biological functions usable in analytical applications.[1] Molecular imprinting is one of the most promising technologies that allow the introduction of sites of specific molecular recognition capabilities to synthetic polymers[2] similar to that of natural receptors. The synthesis of biomimetic recognition elements featuring synthetic receptor sites capable of selective target binding/rebinding with comparable efficiency to substrate/enzyme or antibody/antigen interaction is among the main goals of molecular imprinting. Cameron Alexander defined molecular imprinting as: “The construction of ligand selective recognition sites in synthetic polymers where a template (atom, ion, molecule, complex or a molecular, ionic or macromolecular assembly, including microorganisms) is employed in order to facilitate recognition site formation during the covalent assembly of the bulk phase by a

polymerization or polycondensation process, with subsequent removal of some or all of the template being necessary for recognition to occur in the spaces vacated by the templating species."[3]

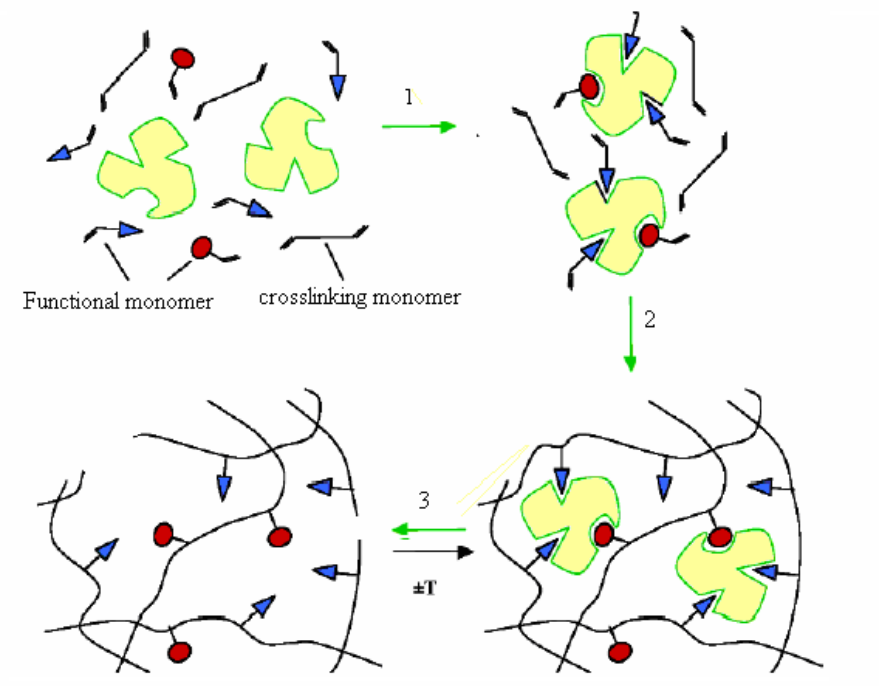
Molecular imprinting sometimes referred to as 'template polymerization'[4] has a long history dating back to 1931. This methodology is based on the principle of using the functionality of a target molecule (template) to assemble its own recognition site by forming interactions with "complementary" functional groups of appropriate polymerizable monomers. These interactions are then "frozen in" by polymerization in the presence of a high concentration of crosslinking agent. The template may be reversibly removed from the polymer network while the functional monomers remain covalently bound to the polymer itself. Three-dimensional cavities are left in the polymer matrix that is complementary in shape to the template with desired functionality in a specific arrangement. The highly rigid structure of the polymer favors selectivity[5] by enabling the cavities to retain their shape even after the removal of the template. This technique allows the formation of tailor made recognition sites exhibiting enantioselectivity, substrate selectivity and catalytic activity[6] in synthetic polymers. Molecular imprinted polymers (MIPs) can therefore be used in applications relying on specific molecular binding events.

The architectural elements[7] that have been shown to contribute to the substrate binding strength and specificity of imprinted polymers are: (i) the number of interactions between the functional monomer(s) and the template; (ii) the innate binding strength of the functional monomer(s) for the template; (iii) the nature of the non-covalent interactions employed,

(iv) the co-operativity of template substructures towards binding (v) the size and shape of the template, and (vi) the spatial relationships between template substructures.

In all molecular imprinting processes the template is of central importance which serves two functions. The first is as a space-filling three-dimensional object around which a complementary polymer cavity can be formed. The second is to organize complementary interactions between groups on the template and functional monomers during polymerization. One of the most predominant advantages of MIPs is their versatility in terms of the analytes. A wide range of molecules can serve as templates, ranging from small molecules like metal ions, drugs, amino acids, nucleotide bases, steroids and sugars to large entities such as proteins, cells, and crystals[8]. The structure of the template molecule is one of the key factors to affect the stability of pre-polymerisation complex, which is decisive for the strength and positioning of the monomer-template interactions. Zhang et al[9] by comparing the recognition ability of three molecularly imprinted polymers of 4-hydroxybenzoic acid, gentisic acid and salicylic acid using acrylamide as a functional monomer, demonstrated that the molecular recognition ability decreases when the template itself forms intramolecular hydrogen bond in the molecular imprinted process. Commonly this technique has been shown to be effective when targeting small molecules of molecular weight <1500, while the imprinting of larger molecules such as polypeptides and proteins needs specially adapted protocols. The wide range of imprintable analytes along with the comparative straight forwardness of MIP preparation, and the chemical, mechanical, and

thermal stability of the obtained functionalized materials owing to the super crosslinked nature are the advantages of the prepared synthetic receptors[10].



Scheme II. 1. Schematic representation of molecular imprinting
(1) formation of pre-polymerization complex,
(2) polymerization and (3) template removal/rebinding

An outline of the generally accepted mechanism for MIP binding site formation is illustrated[11] in Scheme II.1. Step 1 is the formation of pre-polymerisation complex (PPC), functional monomers are organized in the vicinity of the template molecule resulting in the formation of PPC. Step 2 is the copolymerization of the monomer with crosslinking monomers to form a polymer network around the template molecule. In step 3, the template molecules are extracted from the polymer matrix

providing functionalized cavities which have remembered the spatial features and bonding preferences of the template. Thus a molecular memory is introduced into the polymer.

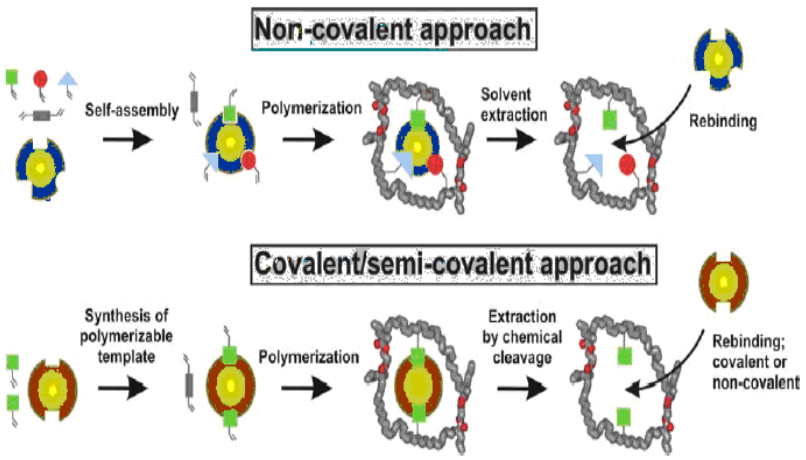
The binding process arises as a result of attractive forces that exist between complimentary site on MIP and template molecule. The conceptionally simple "lock and key" hypothesis proposed by Emil Fisher for enzyme catalysis is perhaps the most useful illustration of the principle[12].

2.2 Molecular imprinting strategies

Various methods have been developed to synthesize MIPs[13]. Depending on the chemical nature of the interactions between the templating molecule and the interactive functional groups in the monomer, there are essentially two strategies for molecular imprinting **(**Scheme II.2**)**. Wulff[14,] developed the covalent or the preorganized approach where print molecule needs to be derivatised before the actual imprinting is performed through reversible covalent bonding such as boronate ester, acetal/ketal, Schiffs-base, or metal coordination. Copolymerization with crosslinking agents in porogenic solvents impart structural rigidity to these template-monomer complexes and is become integrated into a macro porous network. Template removal is subsequently accomplished by chemical cleavage of the supporting covalent bonds liberating the corresponding functional groups located within polymer-embedded cavities. The non-covalent or the self-assembly approach introduced by Arshady and Mosbach[15] is based on the formation of relatively weak non-covalent interactions between selected monomers and template molecule before polymerization**.** It has been demonstrated

that in non-covalent imprinting a significant contribution in the interaction between template molecules and functional groups on the surface of the binding sites is due to the hydrogen bond[16], ionic interaction [17] and the environment around the binding site.[18] In aqueous media hydrophobic interactions play an important role[19]. In addition to Vander Waals interactions and hydrophobic effects comparatively weak interactions such as π-π stacking may occur between aromatic rings in the polar porogenic solvent like methanol and water.

Semi-covalent imprinting[20] attempts to combine the advantages of the covalent and the non-covalent approach. The target molecule is imprinted as a stable complex with the functional monomers via covalent interactions, whereas rebinding occurs via non-covalent interactions taking advantage of faster rebinding kinetics. Two variations in the semi-covalent approach are: (i) the template and the monomer are connected directly or (ii) the template and the monomer are connected using a spacer group[21].



Scheme. II.2. Approaches in molecular imprinting

The simple preparation procedures, the wide range of imprintable compounds and reversible host-guest binding based on non-covalent interactions (biomimetic binding) render the non-covalent imprinting approach the most widespread method for MIP preparation. Since the non-covalent interactions between the functional monomers and the templates are usually too weak, to form stable complexes an excess of functional monomers needs to be added to shift the equilibrium towards complex formation and to maintain the complex stable under the polymerization conditions. The result of using the excess amount of functional monomer is that the majority of functional monomer exists randomly oriented in the mixture without any association with the template. Thus, various structures of the pre-polymerisation complex would be expected including non-associated functional monomer. The different structures of the complexes and non associated functional monomer will form binding sites with varied association constants. Thus the binding site will be heterogeneous[22] and not well defined.

Covalently imprinted polymers are characterized by thermodynamically rather homogeneous binding characteristics, reduced non-specific adsorption and high yields of stable and functional template binding sites[6a,14]. However, despite the high affinities of the polymeric material the range of functional groups which can be targeted is restricted and removal of the template molecules by chemical cleavage is tedious and these limits the application of covalently prepared MIPs. Furthermore slow binding kinetics due to the necessary formation of the covalent bond between template and MIP restrict the analytical application of covalently imprinted MIPs if rebinding is based on reversible covalent bonds. Metal

coordination[23] however would be good alternative for further development of covalent systems since the stability of bonding is not too weak and ligand exchange kinetics would be fairly fast compared to other covalent bonding which could be controlled by the conditions employed. Covalently prepared MIPs will be most useful for applications where a high percentage of "good" sites are required such as chromatography whereas non-covalently prepared MIPs might effectively be applied in cases where high affinities are needed and low concentrations can be tolerated such as in biosensors.

MIPs are typically prepared in bulk by the free radical copolymerization of functional monomers and crosslinkers using azo-bis-isobutyronitrile (AIBN) as radical initiator and in the presence of a template molecule and a porogenic solvent. The azo initiator AIBN can be conveniently decomposed by photolysis (UV) when irradiated at 366 nm or thermolysis at 60^{o}C to give stabilized, carbon-centered isobutyronitrile radicals capable of initiating the growth of a number of vinyl monomers. In situations where the print molecule is thermally unstable the use of AIBN at 60^{o}C for the preparation of polymers is not advisable. The initiator 2,2'-azo-bis-(2,4-dimethylvaleronitrile) (ABDV) has a thermal decomposition temperature lower than that of AIBN and allows thermal polymerization to be initiated at 40^{o}C. Azobis nitriles also undergo photolytic decomposition and it was demonstrated that molecular imprints could be made using this type of initiator at a temperature as low as 0^{o}C[24]. Free radical polymerizations can be performed under mild reaction conditions (e.g. ambient temperatures and atmospheric pressures) in bulk or in solution, and are very tolerant of functional groups in the monomers

and impurities in the system. It is for these reasons, as well as the fact that many vinyl monomers are available commercially at low cost that free radical polymerization is usually the method of choice for preparing molecularly imprinted polymers[25].

2. 3 Advantages of molecular imprinted polymers

Molecular imprinted polymers (MIPs) possess two of the most important features of biological receptors- the ability to recognize and bind specific target molecules. In comparison to their biological counterparts other than possessing antibody-like molecular selectivity the major advantages of using molecularly imprinted polymers are (i) because of the three dimensional polymeric structure they exhibit high physical resistance against external degrading factors and are stable against mechanical stress, high temperature and pressure, resistant against treatment with acids, bases or metal ions and are stable in a wide range of solvents,[26](ii) they can be stored in the dry state at ambient temperatures for several years and can be regenerated and reused many times without loss of their molecular memory, (iii) polymers can be imprinted with substances against which natural antibodies are difficult to raise. Therefore, artificial receptors prepared by molecular imprinting can provide an attractive alternative or complement to natural antibodies and receptors in many applications.

2. 4 Disadvantages of molecular imprinted polymers

MIPs possess many disadvantages. Traditional polymer monoliths tend to be relatively dense leading to difficulty in the accessibility of the binding site sculpt in the three dimensional polymer networks. Such poor

mass transport and permanent entrapment result in inadequate recognition properties. The heterogeneity in binding affinities, slow mass transfer in and out of the polymer matrix, overall low binding affinity, lack of a read-out for complexation and trapped template slowly leaching out[27] are the drawbacks most often mentioned for these synthetic polymers

2.5 Factors favoring the formation of stable and high affinity binding sites

1. There should be one or more functional monomers capable of forming stable complexes with template molecule during polymerisation.

2. A high nominal crosslinking level as lower levels are insufficient for preserving the template sites for longer period of time.

3. The use of an apolar aprotic solvent as porogen as this favors the electrostatic interaction most commonly utilized between the functional monomer and the template. There are also many other factors such as the ratios of the components involved, the solvents, the reaction temperature and the pressure that can have a significant effect on the imprinting process and thus on the final properties of the MIPs obtained.

2.6 Polymer formats, polymerization methods and morphology

The physical form of the imprinted polymers has a significant effect on their performance and should thus be tailor-made for specific applications. MIPs in thin-film format are preferred for sensor applications[28]. Particles used as stationary phase in HPLC should preferably be spherical and of uniform size between 5 and 25 μm in

diameter to provide optimal chromatographic efficiency[4b]. At the same time uniform MIP particles smaller than those used in chromatography (1µm and smaller) are possibly more suitable for immunoassays because of their stable suspension in solvents[2b]. Selecting the appropriate polymerization method allows preparation of MIPs in different formats ranging from monolithic block polymers to micro and nano spheres and polymer films[29].

The most common methodology for molecular imprinting is the monolithic approach[30] where MIPs are prepared in bulk and subsequently ground and sieved to the desired size. Due to the tedious and time-consuming experimental steps it normally takes several days to complete the whole procedure to prepare and evaluate molecularly imprinted polymers (MIPs). In addition the crude post-treatment tends to produce sharp edged, irregular polymeric bits which compromise the quality of the material thus limiting its application in areas like chromatography and solid-phase extraction. In order to avoid the grinding and packing of HPLC columns a number of reports describe the use of *in situ* polymerization methods i.e. the polymer is formed inside a column as a porous monolith[31] or agglomerate[32]. Similar *in situ* methods have been used within capillaries to prepare monoliths or coatings for electrochromatographic separations[33] .

There have been reports of the grafting of an imprinted layer on the surface of preformed particles of materials such as silica. In one of the approaches first described by Mosbach and coworkers[34] and adapted by others[35] a polymerizable group was first attached to the silica surface and polymerization was then carried out using template, crosslinker and

functional monomer. Imprinted materials with binding sites situated at or close to the surface of the imprinting matrix have many advantages like easy accessibility of the binding site, fast mass transfer and binding kinetics and target molecules conjugated with bulky labels can still bind.

Template molecules can also be immobilized on the surface of porous silica particles[36]. The pores are then filled with the monomer mixture and the polymerization is initiated. The silica is removed by chemical dissolution leaving behind a porous polymeric structure which is the negative image of the original bead. The potential advantages of this approach include the possibility of imprinting templates insoluble in polymerization solvents and the generation of binding sites that are more homogeneous and accessible through their proximity to the surface of the MIPs. The surface imprinting approach[37] has been one of the most effective strategies for the imprinting of proteins.

An alternative approach to surface imprinting that is currently being studied is the water-in-oil (w/o) emulsion method which is very similar to conventional bulk imprinting. In this novel technique the organic-aqueous interface in w/o emulsions is utilized as a recognition field toward a target molecule. The target molecule forms a complex with the functional host molecule and the orientation of the functional host molecule is fixed at the oil-water interface. This provides after polymerization the complementary recognition sites to the imprint molecule at the inner cavity surfaces of the imprinted bulk polymer. The bulk polymer obtained is ground to appropriate particle size in order to interact with the target molecules in an aqueous solution. To date this

technique has been successfully applied to imprint amino acids[38], metal ions and nucleotides[39].

When a polymer is needed in the form of a thin film at a surface, standard technique such as spin or spray coating can be chosen to synthesize it *in situ*. One elegant fabrication method is soft lithography[40] which can be used to create patterned surfaces for multi analyte sensors and high-throughput screening systems. Thin imprinted polymer films have been used by many authors in particular for sensors, they can be *in situ* synthesized at an electrode surface by electropolymerisation[41] or at a non-conducting surface by chemical grafting[42].

The most common procedure for polymerizing water insoluble monomers involves suspension or emulsion techniques. Both employ surfactants or emulsifying agents that partition the monomer in the suspending medium usually water. Since the presence of bulk water phase can interfere with the chemistry of non-covalent imprinting, liquid per fluorocarbons have been used as a more compatible suspension medium[43]. Polymer beads produced using this method showed excellent chromatographic performance and good selectivity even at high flow rates.

Two-stage swelling polymerization method have been successfully used to prepare enantioselective, uniformly sized spherical MIP particles for a range of pharmaceutically relevant chiral compounds and are well suited for chromatographic applications by the step-wise swelling of a seed polymer with a mixture of fresh monomer and solvent. Non crosslinked seed particles prepared by emulsion polymerization can be swollen with porogen, template, functional monomer and crosslinker in

the second stage which resembles a suspension polymerization for the preparation of uniform-sized spherical MIP particles[44].

MIP nanobeads can be synthesized by precipitation polymerization or emulsion polymerization. Precipitation polymerization is performed with pre-polymerization mixtures similar to those for bulk polymers except that the relative amount of solvent present in the mixture is much higher. Ye and co-workers have successfully used this method to prepare imprinted particles for binding assays[45] and in applications where binding site accessibility was important[46].

In the preparation of surface imprinted MIP beads one of the most common strategies is to use preformed support beads for the deposition of imprinted polymers. This imparts a coreshell structure to the final imprinted polymer. Coreshell particles[47] are formed in a two-stage process from seed latex. However, unlike the two-stage swelling the seed particle (which may be crosslinked) is surrounded by a shell of new polymer in a second emulsion polymerization. The prepared core shell MIPs exhibited excellent recognizing, separating, catalyzing and biosensing properties. The most common type of material used as support beads is silica, mainly due to its stability, favorable physical properties and its ease of derivatization. Other support materials include polystyrene, chitosan or other types of polymers depending on the requirements of the application.

2.7 Parameters affecting the characteristics of the imprinted polymers

Studies have shown that nature of the functional monomers, crosslinkers, solvents and polymerisation conditions affects the

recognition performance of imprinted polymers[48,4b]. Enantiomeric and substrate selective recognition properties of molecularly imprinted polymers are largely dependent on the size, shape, the physical/chemical properties and relative position of the functional groups of the recognition sites and the template molecule. The best results for MIP formulation can only be determined by empirical optimization via synthesis and evaluation of several polymers. Although the methodology of MIP formation is relatively easy, optimization of MIP formulation components is complicated by variables such as which are the functional monomers (FM) to be used, how many functional monomers are to be used, what are the type of crosslinker (CL), the optimum ratio of functional monomer/crosslinker (FM/CL), the optimum ratio of template/functional monomer (T/FM/) and various other parameters like concentration of the template solution, mass of the polymer, time, and solvent.

1 Flexibility/rigidity of the crosslinked polymer

Crosslinking concentration is a critical factor in creating synthetic receptors with high affinity for their target molecules.[49] Crosslinking provides a structural design feature that improves selective molecular recognition by functional groups. One reason for this is that crosslinking bind the functional group covalently to the matrix greatly reducing conformational entropy of monomer motion. Consequently this reduces the conformational flexibility[50] of the pendent functional groups which creates a more stable interaction with the template. The amount of crosslinker should be high enough to maintain the stability of the recognition sites. When the percentage of crosslinker was less than the optimum level the MIP showed weak recognition ability. Below optimum

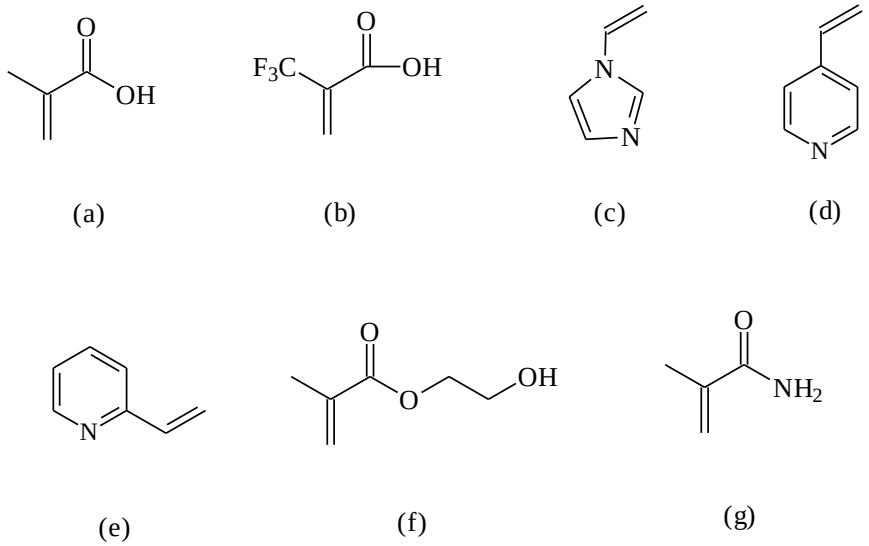
percentage it cannot hold the template due to the lack of stability of the network to maintain the binding site in the required geometry[51]. High degree of crosslinking enables the micro cavities to maintain three dimensional structure and thus the functional groups are held in an optimal configuration for rebinding the template allowing the receptor to 'recognize' the original substrate. The ideal properties of the crosslinked polymer matrix with embedded binding sites are a compromise between structural rigidity of the matrix maintaining the integrity of the binding sites and sufficient flexibility facilitating access to the binding pockets. Therefore the type and amount of crosslinker in the polymeric matrix are very important. Thus the molecular imprinted polymers should have optimum stiffness/flexibility to attain the rapid equilibrium with the template, easy accessibility for the imprinted sites, mechanical stability to withstand stress in certain applications and thermal stability to use at high temperature.

2 Functional and crosslinking monomers

Functional monomer is the polymerisable entity which interacts with the print molecule and is responsible for the binding interactions in the imprinted binding sites. It is clearly very important to match the functionality of the template with the functionality of the functional monomer in a complementary fashion[52] (e.g. H-bond donor with H-bond acceptor) in order to maximize complex formation and thus the imprinting effect. The functional monomer can be basic, acidic, permanently charged, hydrogen bonding, hydrophobic and others.

Number of functional monomers with chemically diverse structures and polarities are commercially available and many more can

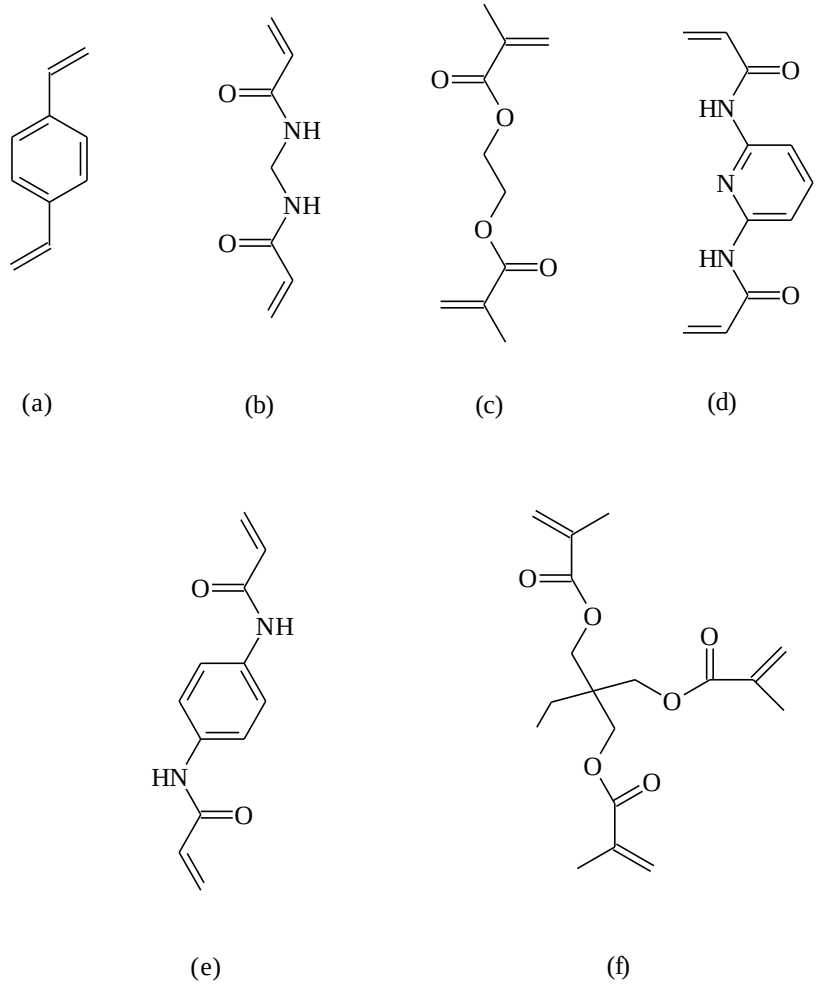
be prepared by rational design **(**Fig. II.1). The widely used acidic and basic functional monomers are methacrylic acid (MAA) and 4-vinylpyridine (4-VP) respectively[53]. For achieving stronger ionic interaction 2-or 4-vinylpyridine is normally used for a template with carboxyl functionality[54] whereas MAA was used as a functional monomer in the synthesis of imprinted polymers of organic compounds containing basic group such as triazine.[55] The combined use of vinyl pyridine and methacrylic acid has been found to be particularly useful for the preparation of MIPs against carboxylic acids resulting in improved recognition capabilities as compared with MIPs prepared by use of only one of the functional monomers.[56,] A report published by Yu and Mosbach describes the use of acrylamide instead of methacrylic acid as the functional monomer for imprinting[19b]. With the amide functional monomer imprinting can be performed in more polar solvents which may in some cases avoid template solubility problems and because the amide group is uncharged non-specific binding due to ionic interactions is reduced. When two or more functional monomers are used simultaneously in "cocktail" polymerisation it is important to bear in mind the reactivity ratios of the monomers to ensure that copolymerization is feasible. Free radical initiated vinyl polymerization remains the most commonly applied method for the production of molecularly imprinted materials due to the ease of preparation and the commercial availability of a broad range of monomers and crosslinkers. Other approaches are polystyrene based or polysiloxane based systems used to a lesser extent. The selection of appropriate functional monomers and the determination of their stoichiometry applied are most important in the design of a molecularly imprinting system for given target molecules.



(a) methacrylic acid, (b) trifluoromethacrylic acid, (c) N-vinylimidazole, (d) 4-vinylpyridine, (e) 2-vinylpyridine, (f) hydroxyethyl methacrylate, (g) acrylamide

Fig. II.1. Functional monomer commonly used in the imprinting technology

Crosslinking agents are unit with two or more attachment possibilities with the functional monomers. While the amount of crosslinking agent affects the rigidity of MIPs the nature of it significantly affects the physicochemical properties of a polymer matrix[57]. A number of crosslinkers compatible with molecular imprinting are known many of which are commercially available and a few of which are capable of simultaneously complexing with the template and thus acting as functional monomers[58]. The chemical structures of the well known crosslinkers are shown in Fig. II.2**.** Ethylene glycol dimethacrylate (EGDMA) and 2,2-bis(hydroxymethyl) butanol trimethacrylate (TRIM) are the most commonly used crosslinking agents employed in several systems.



(a) (b) (c) (d)

(e) (f)

(a) Divinylbenzene (DVB), (b) N,N'-Methylene-bis-acrylamide (NNMBA),
(c) Ethylene glycol dimethacrylate (EGDMA), (d) 2,6-Bisacrylamidopyridine
(e) N,N'-Phenylene-bis-acrylamide (NNPBA), (f) Trimethylolpropane trimethacrylate (TRIM)

Fig. II. 2. Crosslinking agents used in the imprinting technology

It has been shown that polymers prepared using TRIM as crosslinker were partially macro porous and when these MIPs were used for chromatographic separation they provided superior separation

factors and loading capacities[59]. Another trifunctional crosslinker pentaerythritol triacrylate has also been utilized producing MIPs of higher capacity, selectivity, and resolving capabilities.[60,4b] A typical water soluble crosslinking agent is N,N'-methylene-bis-acrylamide (NNMBA). The divinylbenzene (DVB) crosslinking imparts rigidity and hydrophobicity to the polymer support which increases with increase in crosslinking. By using different kinds of crosslinking agents we can control both the structure of the guest binding sites and the chemical environment around them.

3 Template/Functional monomer ratio

As the recognition sites are considered to arise from the self assembly of monomers and template prior to polymerization the relative ratios of these entities must be of significant importance[61] for the recognition characteristics of the product polymers. The degree of complexation between the templates and monomers greatly influences the imprinting efficiency (a measure of the degree of complexation). Andersson et al[62] studied the effect of monomer-template molar ratio on the selectivity of the imprinted polymer. They found that low ratios result in less than optimal complexation due to insufficient amounts of functional monomers and selectivity is thus reduced. On the other hand excess monomer yields a high number of noncomplexed, randomly distributed monomers which contribute to nonspecific binding. Typically with non-covalent imprinting an excess of functional monomer is used to increase the complexation between functional monomer and the template molecule. In the cases where an excess of functional monomer is used this has been shown to lead to more non-specific binding with a loss of

selectivity[63]. The capacity of the polymer is smaller but the relative yield of high affinity sites is higher. Of course this is expected due to functional monomer that is left in an uncomplexed state and its subsequent incorporation in the growing polymer chains. The molar ratio between template and functional monomer could be approximately set from 1:2 to 1:4 for many of the polymers described in the literature.[64] It may not be considered as a general criterion since imprinting effect with a 1:1 molar ratio between template and functional monomer was reported.[65]

4 Porogen/rebinding solvent

The choice and quantity of porogenic solvent used in a polymerization affects both the imprinting process and the physical state (pore structure, pore size distribution, swellability, toughness and morphology) of the MIP[48b]. In molecular imprinting the solvent acts both as a porogen and as a rebinding medium. The solvent serves to bring all the components in the polymerisation i.e. template, functional monomer(s), crosslinker and initiator into one phase. It serves a second important function in that it is also responsible for creating the pores in macro porous polymers. For this reason it is quite common to refer to the solvent as the "porogen". Use of a thermodynamically good solvent tends to lead to polymers with well developed pore structures and high specific surface areas and facilitates access to the binding pockets. Use of a thermodynamically poor solvent leads to polymers with poorly developed pore structures and low specific surface areas. The large pores provide permeability through the monolith and also facilitate convection thus greatly enhancing mass transport. Increasing the volume of porogen increases the pore volume. Besides its dual roles as a solvent and as a pore

forming agent the solvent in a non-covalent imprinting polymerisation must also be judiciously chosen such that it simultaneously maximizes the likelihood of template, functional monomer complex formation. The porogenic solvents should be of relatively low polarity in order to reduce the interferences during complex formation between the imprinted molecule and the monomer. The actual interactions contributing to the selectivity of the recognition sites mainly hydrogen bonding and ionic interactions are much stronger in apolar, non-protic solvents like toluene, chloroform, dichloromethane[66]. Hence the recognition specificities observed are higher when these artificial antibodies are used in nonaqueous media. Chloroform is one of the most widely used solvents as it dissolves many monomers and templates satisfactorily and hardly suppresses hydrogen bonding. The presence of water however is usually regarded as incompatible with non-covalent molecular imprinting techniques because its polar protic nature readily disrupts crucial template-functional monomer interactions. An investigation of the contribution of the hydrophobic effect on recognition in aqueous solvents is presented by Yu et al[19a] The best recognition seems to take place when the rebinding medium is the solvent used in the polymerization ("porogen effect")[67]. Polymer immersed in a solvent different from the one used at polymerisation swells or shrinks losing the ability of the imprinting site to bind the template molecule. Consequently the solvent used as a porogen in the polymerisation should be more or less similar to the recognition solvent[68]. Based on analytical data, the porogen effect was explained by the solvating properties of the solvent used. It was suggested that the ability of the porogen to solvate the polymer chains during polymerization permits a better adjustment of the shape of the binding sites and of the

distance between the functional groups in the binding sites. Thus using the porogen as the rebinding medium allows the formation of binding sites that have the very same dimensions as those of the sites formed during the polymerization improving the binding performance of the MIP. Different solvents can be readily used for covalent imprinting as long as they satisfactorily dissolve all the components.

5 Polymerisation temperature

The effect of polymerization temperature on MIP performance has been the subject of several studies[69]. Stability of the pre-polymerisation complex as well as the polymerisation reaction itself plays a very important role in determining the recognition performance of the imprinted polymers. The nature of the exothermic polymerization reaction and the subsequent increase in temperature will inherently change the association of template-monomer complexes[70]. The higher temperature is expected to drive the equilibrium[71] away from the template-functional monomer complex toward the unassociated species resulting in a decrease in the number of strongly binding cavities. Usually at lower temperature there should be a stronger pre-polymer complex which is ultimately responsible for the formation of the specific binding sites within the polymer[72.] Where complexation is driven by hydrogen bonding then lower polymerisation temperatures are preferred and under such circumstances photo chemically active initiators may well be preferred as these can operate efficiently at low temperature. In particular photo initiation at low temperature[24] should promote high sensitivity and strong binding by materials that rely on temperature sensitive interaction for recognition.

2.8 Characterization methods

The structural analysis of the active sites in the imprinted polymers is problematic and is not amenable to X-ray crystallography or microscopic techniques due to their amorphous and heterogeneous nature. This renders the use of spectroscopic or diffraction techniques informative in only a few isolated cases. A number of innovative characterization techniques are therefore being assessed for characterizing the imprinted sites. In the non-covalent approach the efficient formation and stability of template/functional monomer complexes formed in the pre-polymerization solution will govern the resulting binding site distribution and the recognition properties of the imprinted polymer matrix[73]. Hence, analytically characterizing the molecular mechanisms occurring in the pre-polymerization solution is

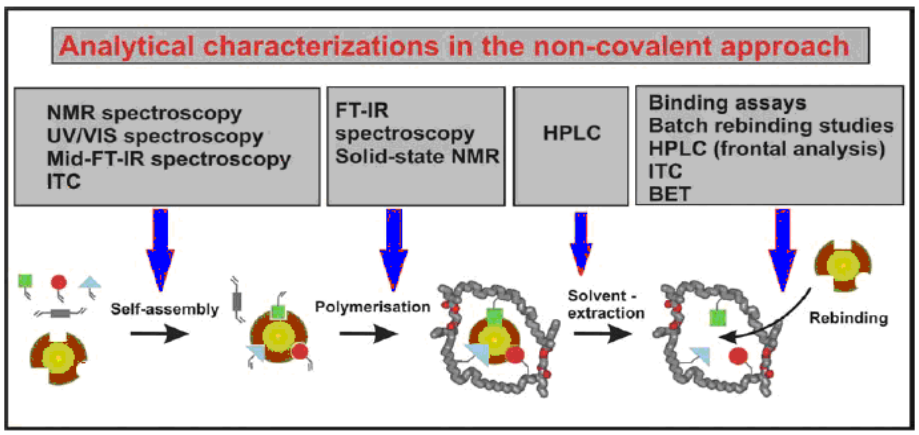


Fig. II.3. Analytical methods for the characterization of different processing steps during the preparation of non-covalently imprinted polymers

probably the most important step for rational understanding of self-assembly based imprinting. Direct observation of complexation between monomers and template molecules or their analogues has been observed by changes in spectroscopic properties of the mixtures. NMR[74] methods and UV spectrophotometry are the most popular; however, FT-IR has also been applied to the problem[13b].

1. ^{1}H NMR

It is one of the best tools to detect the formation of the non-covalent adduct formed in the pre-polymerization mixture and for identifying the specific sites in interacting structures that engage in complexation[75]. Evaluating the shift of a proton signal due to participation in a hydrogen bond was used as selection criterion for a suitable monomer deriving data on complex formation, ratios, and interacting forces[76] Furthermore the investigation of chemical shifts with NMR enables the calculation of association constants[77]. Chemically non-equivalent nuclei in a molecule are differently shielded leading to separate signals in the NMR spectrum.

2. UV-vis

UV spectroscopic method was also used for the analysis of the molecular imprinting 'pre-polymerization phase'. This is a rapid and valuable tool to evaluate the interaction between the functional monomer and the template in solution[62]. Through monitoring changes in UV spectra of the template upon titration with functional monomer plots of monomer concentration as a function of change in absorbance yield saturation

isotherms from which dissociation constants may be calculated using binding plot analysis.

3. Fourier-transform infra-red spectroscopy (FT-IR)

The FTIR spectra of imprinted polymers can be used to extract quantitative information on the composition of the polymer. IR spectra also reflects the structure of the pre-organized monomer–template adduct and the template incorporation into the imprinted polymer during rebinding. Duffy et al. employed infrared spectroscopy to monitor hydrogen bond formation[78] in the pre-polymerisation complex.

4 Fluorescence

Fluorescence is the most sensitive of all existing methods to detect intermolecular interactions[79] and is inexpensive and easy to implement. The changes in fluorescence emission spectra of fluorescent template like Boc-L-Trp-OH and Boc-L-Phe-OH were recorded using spectroscopic titration by adding 4-VP into these template solutions in chloroform.

5 ^{13}C-CP-MAS-NMR spectroscopy

Owing to their insoluble, intractable nature, imprinted polymers are generally not amenable to characterization methods involving the solution state[80] e.g. solution state NMR. Thus although ^{1}H NMR is quite useful to analyze liquid samples the signals for solid samples are very broad and not very informative. Solid state NMR (NMR signal of $^{13.}$C, ^{31}P and ^{29}Si) techniques circumvent the need to work in solution and therefore enable the NMR spectra of insoluble species to be acquired. Their signals sufficiently sharp even for solid samples and provide precise information.

The polymer analysis using ^{13}C-CP-MAS-NMR technique can give information on the polymer backbone

6 Morphological characterization by Scanning Electron Microscopy (SEM)

It is possible to probe the morphology of imprinted polymers in much the same way as one is able to do with most porous solids. Scanning electron microscopy (SEM) can often be used to image macropores to study the shape, size, morphology and porosity of polymers[81].

2.9 Swelling studies

Macro porous polymers are permanently porous even in the dry state and solvent can be used to access the pore network. The rate of diffusion of a reagent into the polymer matrix mainly depends on the extent of swelling[82]. Thus swelling is an important parameter which controls the success of rebinding. By measuring the amount of solvent intake by a polymer an estimate can be made of the swelling ratio.

The swelling ratio (S_R) of the polymer was calculated from the following equation

$$S_R = (m_s\text{-}m_{o)}/m_o$$

where m_s is the weight of the swollen polymer and m_o is the weight of the dry polymer

2.10 Measurement of binding characteristics.

Estimations of the binding performances (i.e. K_D and S_{max},) and the selectivities of the artificial antibodies are easily determined by simple Scatchard-type analysis[83]. The free template concentration after

incubation with a known amount of polymer is measured and plotted in a Scatchard plot based on Scatchard equation

$$[S]_b/[S]_f = [(Smax - [S]_b)]/ K_D$$

where, K_D is the equilibrium dissociation constant, $[S]_{b,}$ amount of template bound to the MIP at equilibrium, $[S]_{f,}$ molar concentration of free substrate in solution containing the imprinted polymer, Smax is the number of accessible sites. In the linear relationship between $[S]_b$ /$[S]_f$ verses $[S]_{b,}$ negative reciprocal of the slope and X intercept gave the value of K_D and S_{max}. The polyclonal nature of the artificial antibodies obtained through the imprinting protocol results in non-linear Scatchard-plots.

1 Separation factor

Separation factor[84] is a measure of the binding characteristics of the polymer. In the rebinding step it is calculated based on a reference which can be an internal standard or the rebinding to a non-imprinted polymer. It compares the binding of the template by the imprinted and nonimprinted polymers. It expresses the ratio of the specific to nonspecific binding for each polymer system and serves as a useful yardstick for comparing the relative effectiveness of the imprinting processes

$$\text{Separation factor } (\alpha_{\text{Template}}) = K_{MIP} / K_{NIP}$$

$$K = \frac{Template_{Bound}}{Template_{Free}}$$

MIP possessing high separation factor should be capable of completely recovering the target molecule by the simple process of mixing the

imprinted polymers with template solution and its value depends on the type of interaction between the template and functional monomer. However when non-covalent interactions are employed comparatively less value in the range 1-2 is obtained.

2 Selectivity factor

The selectivity factor[85] is defined as the ratio of the separation factors of the two species, template and the analogue respectively and is an index of polymer selectivity towards analogues as of the template molecule. It is calculated as

$$\text{Selectivity factor} = \alpha_{\text{Template}} / \alpha_{\text{Aanalogue}}$$

2.11 Application of molecular imprinted polymers

Molecularly imprinted polymers (MIPs) with tailor made cavities and engineered chemical functionalities are increasingly appreciated as highly target specific polymeric molecular recognition materials with a broad range of potential applications such as chromatographic stationary phase for separation and isolation, antibody and receptor mimicking, enzyme mimetic catalysis, organic synthesis and drug delivery

1 Substance-specific absorber materials

To date, the application that has been most extensively explored is the use of molecularly imprinted materials in separation and isolation owing to their high selectivity, stability as well as their compatibility with both aqueous and organic solvents and affinity for the target molecules[86]. MIPs are now intensively evaluated as tailored substrate specific sorbents for solid-phase extraction (SPE)[87]. Molecularly imprinted solid phase

extraction (MISPE) facilitating selective sample clean-up and pre-concentration offers the potential of reducing multiple and frequently tedious sample preparation steps[88] **(**Fig. II. 4). This is of particular interest in food and beverage analysis, environmental analysis and biomedical analysis where the introduction of highly selective sorbent materials reduces time-consuming sample preparation. Since imprinted polymers can be used in organic solvents and often also in aqueous buffer they might have advantages over other extraction procedures e.g. those based on immunoaffinity and more so if the corresponding antibody is difficult to obtain. The use of molecularly imprinted solid phase extraction improved the accuracy and precision of the HPLC method and lowered the limit of detection (0.005 ppm). The feasibility of MISPE was demonstrated for liver extract[55] blood serum[89],red wine[66b],chewing gum[90], vegetable extracts[91] and river water samples[92].

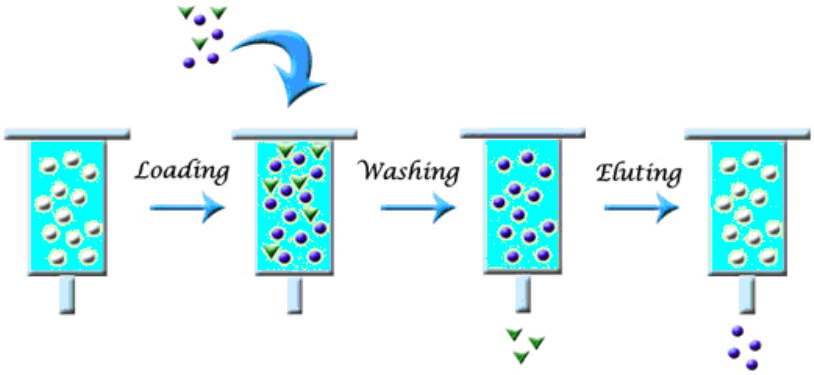


Fig. II. 4. Molecularly imprinted solid phase extraction

Drawbacks encountered in MIP based chromatography such as peak broadening is of minor importance in SPE, while template-bleeding at trace and ultra-trace levels remains a critical issue addressed by 'dummy imprinting' with structural analogues[93].

2 Sensors

One of the areas where specific recognition phenomena play a key role is in sensor technology. A sensor is characterized by two key components a recognition element, which has a specific interaction with an analyte or environmental condition, and a transducing element, which converts this interaction into a measurable effect. Many sensors for environmental monitoring, biomedical and food analysis etc rely on natural receptors, such as antibody/antigen, enzymes, nucleic acids/DNA, cellular structures/cells, as the specific recognition elements due to their evolved high affinity and specificity[94]. Molecular imprinting technology (MIT) offers an alternative means to produce materials that are able to mimic natural binding entities. Taking into account the very high specificity that can be obtained as well as the chemical and physical stability of imprinted polymers there have been a number of attempts to construct chemical sensors based on these materials as the recognition elements[95,1a]. The analytical techniques by which binding is transduced into a readout are varied and include measuring changes in the MIP's optical (*e.g.*, fluorescence)[96], electrochemical properties[97], mass (QCM and SAW)[98] and refractive index (SPR)[99]. The potential use of the molecular imprinting technique for sensing applications has been realized in several reviews[100].

3 Catalysis - Enzyme mimics

One strong driving force in the development of MIPs has been the mimicking of enzyme action. Creation of enzyme mimics or artificial enzymes as novel catalysts[6a] has been a dream of chemists for a long time. Imprinted polymer catalysts are yet to match enzymes or even catalytic

antibodies in terms of rate enhancements[101]. This is partly due to the involvement of just a single functional monomer in the catalytic sites of polymers prepared so far, compared with the highly cooperative interactions of numerous amino acid residues in the active sites of biological catalysts. However the true potential of 'printzymes' lies not in their ability to compete with proteins but to complement them as robust catalysts made for particular reactions for which no enzymes can be found. As an example of this new kind of catalyst, researchers have used a rhodium-containing polyurethane imprinted with a chiral ligand to catalyze the reduction of ketones to alcohols yielding an enantiomer ratio consistent with a 'memory' for the chiral template[102].

4 Template assisted synthesis

MIPs have also been used for synthetic applications[103] in controlling or inducing certain chemical reactions. Even though the polymers are not catalytic in this approach, they facilitate the assembly of the reactants through their specific binding properties. By using a specific reaction component as a print molecule, whether it is a product or a reactant, the reaction equilibrium can be shifted in a desired direction using molecularly imprinted polymers. Thus imprinted polymers are used to shift thermodynamically unfavorable equilibria of enzymatic reactions. The principle is that reaction product is constantly removed through adsorption on an imprinted polymer which has been prepared using reaction product as a template. As model system evaluated the enzymatic synthesis of aspartame[104] from *Z*-L aspartic acid and L-phenylalanine methyl ester. Addition of polymer imprinted against *Z*-aspartame resulted in considerable increase (40%) in product yield. Continuous isolation of

the product would also be possible in the same way simply by physically separating the adsorbent. In view of the attractive physical features displayed by molecularly imprinted polymers, such as high pressure and temperature stability, allowing MIPs to withstand sterilization conditions, this methodology may find use in various synthetic applications.

The use of the imprinted recognition sites as potential chiral cavities for enantioselective synthesis was first described by the research groups of Shea[105] and Neckers[106] and synthetic transformations including enantioselective protonation-deprotonation[107] and selective hydrolysis[108] have also been carried out in imprinted sites. However, perhaps the most elegant early work in this area was due to Wulff and co-workers who targeted their initial studies to C-C bond formation in a biomimetic synthesis of α- amino acids from glycine[109].

5 Molecular imprinted sorbent assay - antibody mimics

A very attractive application of molecularly imprinted polymers is in immunoassay-type binding assays[110] instead of antibodies. Molecularly imprinted sorbent assay[111] (MIA) means radiogland assay using molecularly imprinted polymers as recognition elements. In clinical and research laboratories the most common applications of antibodies are in sandwich-type or competitive immunoassays[112] and immunoaffinity chromatography. They are also used in immunosensors in combination with some form of transducer that detects the binding of antigen and antibody directly.[113] The binding utilises the recognition properties of an antibody for the antigen, in which the antigen fits exactly into the antibody's binding site. Molecularly imprinted materials appear to offer a potential recognition element alternative to natural antibodies. MIPs have the following

advantages: they are more stable than their biological counterparts; they are applicable to both aqueous and non-aqueous assays; there is no need for conjugation of the template to an immunogenic carrier as haptens for antibody production and the need to use laboratory animals for antibody production is avoided. Molecularly imprinted polymers cannot compete in their present forms with natural antibodies for use in techniques in which they are used in their soluble form (e.g., in immunodiffusion, immunoelectrophoresis, immunoblotting, and tissue immunofluorescence). However for techniques such as immunoassay, immunoaffinity chromatography, and immunosensors, which utilize antibodies bound to a solid support molecularly imprinted polymers appear to offer a potential recognition element alternative to natural antibodies[114].

6 Drug Analysis

The behavior of the enantiomer of a chiral drug may show striking differences in terms of biological activity, potency, toxicity, transport mechanism and routes of metabolism:- one may be therapeutic while the other causes disastrous side effects. This has led to intense activity directed towards the development of new and improved means for the separation of optical isomers[56b]. Modern drug discovery and demand for quick diagnostics require development of highly sensitive and efficient analytical methods to detect trace amounts of analytes. Chiral pharmaceutical compounds such as timolol[115], naproxen[54b] and ephedrine[116] have been separated by use of MIPs. The controlled delivery of therapeutics or removal of detrimental compounds ‘on-demand’ by polymeric systems is highly desirable and this can be achieved by biomimetic networks. Only in the past few years[117] researchers have begun to discuss and address the

applicability of MIPs in controlled drug delivery systems and there has been limited actual demonstration of the possibilities.

7 Separation of enantiomers[118]

Molecular imprinting technology offers the unique opportunity to tailor chiral stationary phases with predetermined enantioselective binding properties by employing the enantiomers of interest as binding-site forming templates[119].The operational simplicity of this chirality transfer from a templating enantiomer to the polymer network also obviates the need for sophisticated receptor designs, lengthy synthetic routes, and elaborate immobilization procedures. Unlike the traditional chiral stationary phases which separate mixtures mainly on the basis of non-specific interactions MIPs can show a very high specificity towards the print molecule allowing even compounds differing for instance in only one single methyl group within the template to be effectively distinguished[111d]. Another characteristic feature of molecularly imprinted chiral stationary phases is that the elution order of the enantiomers can easily be predicted. Chiral separation of racemic mixtures has attracted strong interest because of the increasing demand for optically pure compounds. Polymers prepared by the molecular imprinting technique with chiral template result in chiral stationary phases (CSPs) and those often show quite good chiral recognition ability[115,54b]

The first example[120] of molecularly imprinted chiral stationary phase prepared using a racemic template is the chiral discrimination of *N*-(3,5-dinitrobenzoyl)-α-ethylbenzylamine (DNB) on the molecular imprinted stationary phase prepared using racemic DNB as the template. Molecular imprinting technology has been extensively exploited to produce target-

specific CSPs for broad range of chiral compounds[121,1b] for example amino acid derivatives[122,54a], peptides[5] natural compounds, and a variety of drugs. MIP type CSPs have excellent chiral recognition properties for the templating chiral species which are manifested in high enantioselectivity, pronounced substrate-specificity, and predictable order of elution, with the enantiomers employed as templates being the more strongly retained species. A particularly attractive feature of MIP-type CSPs is their capability of discriminating not only between enantiomers but also between stereoisomers of close structural similarity.

In the early 1950's chiral selectivity for mandelic acid and camphor sulfonic acid enantiomers had been demonstrated by Curti et al. using imprinted silicas as stationary phases in column chromatography[123]. Optical resolutions of amino acids or amino acid derivatives[124], direct enantioseparation of drugs such as β-adrenergic blockers[48c,115] and regio- and enantioseparation of sugar or sugar derivatives[125] have been reported. The applications of molecular imprinted polymers in HPLC and CEC chiral separations have been extensively studied, and numerous recent detailed reviews have been published [126, 1b,29c]

Chirally imprinted MIPs provide interesting prospects for the development of membrane-based enantiomer separation processes. Dzgoev and Haupt[127] reported the preparation and evaluation of Z-L-tyrosine-imprinted composite membranes, Donato et al[128] created enantioselective composite membranes for the anti-inflammatory drug naproxen and vice versa. The membranes which were imprinted by a given amino acid print molecule recognized not only the print molecule analogue but also other

racemic α-amino acids, whose absolute configuration is the same as that of the print molecule[129].

Separation approach combining thin-layer chromatography and molecular imprinting is reported.[130] Thin-layer chromatography plates were made based on the molecular imprinting technique. Rapid chiral separation of L- and D phenylalanine anilide as model compounds was effected. The obtained chiral separation factor α was 3.5.

A highly enantioselective polymer was reported[131] for the separation of optically active tryptophan methyl ester by a surface molecular imprinting technique. A synthetic host molecule phenyl phosphonic acid monododecyl ester was proved to be effective for recognizing the chirality of amino acid esters. The L- or D-tryptophan methyl ester (TrpOMe)-imprinted polymer containing the functional host molecules revealed high enantioselectivity toward the corresponding imprinted isomer while the racemic TrpOMe-imprinted and non-imprinted polymers did not show the enantioselectivity at all. These results mean that the complementary binding sites such as 'template-fit pockets' in which the position and the alignment of the functional group in the functional host molecule are optimally adjusted for binding the corresponding imprinted isomer are a principal factor to recognize the target molecule. These enantioselectivities were quantitatively supported by high binding constants for the corresponding imprinted isomer.

Molecularly imprinted polymeric membranes were prepared[132] from various oligopeptide tweezers by the adoption of N-tert-butoxycarbonyl-D-tryptophan (Boc-D-Trp-OH) or N-tert-butoxycarbonyl-L-tryptophan (Boc-L-Trp-OH) as a print molecule. The chiral recognition ability of the formed molecular recognition sites was dependent on the absolute configuration of

the print molecule adopted in the membrane preparation (molecular imprinting) process. In other words the membranes imprinted by the D-isomer recognized the D-isomer in preference to the corresponding L-isomer and vice versa.

A novel method for capillary electrochromatographic separations of amino acid enantiomers using molecularly imprinted polymers is reported[133]. The substrate selective polymers were prepared using L-phenylalanine anilide as print molecule and methacrylic acid as the functional monomer.

The present work is aimed at the preparation of molecular imprints with selectivity towards amino acid derivatives and to exploit the specificity and selectivity of the successful system for the selective binding of the enantiomers of amino acid derivatives. Here an attempt is made to design imprinted polymers of Boc-L-tryptophan, Boc-L-phenylalanine and D-mandelic acid with optimum specificity and selectivity employing the simple UV-vis spectroscopic technique. L-mandelic acid and its derivatives are multifunctional precursors for synthesis of many optically pure amino acids, angiotensin converting enzyme inhibitors, and coenzyme A[134]. For this reason, a facile and effective method for control of optical purity of this class of compounds was highly desirable. Many studies aimed at optimizing non-covalent imprinting have been reported in the literature[135,51]. The majority of these have been chromatographic studies the goal of which has been to produce stationary phase with enhanced performance[136]. Here we present an alternative simple UV-vis spectrometric technique for the designing of molecular imprints for the specific and selective recognition of amino acid derivatives.

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