Chapter 2

*Molecular Imprinted Polymers: A Review*
MOLECULAR IMPRINTED POLYMERS: A REVIEW

2.1. Introduction

Molecular imprinting is a generic technology that allows the introduction of sites of specific molecular affinity into three-dimensional crosslinked polymeric matrices. Specific molecular recognition is the fundamental process governing control of biological form and function. In the past decade, great interest in this new class of materials - molecular imprinted polymers (MIPs) - has been expressed by specialists in various branches of chemistry\textsuperscript{1}. This can be explained by the presence of highly specific binding sites in MIPs, which are complementary to various organic molecules in size, shape, structure, and physicochemical properties. The potentially high selectivity of MIPs for organic compounds, along with other useful properties opens up wide opportunities for the use of these materials in analytical chemistry\textsuperscript{2-4}. Molecular imprinted polymers (MIPs) are a group of compounds synthesised by a process where functional and crosslinking monomers are co-polymerised in the presence of a target analyte, which acts as a molecular template. The functional monomers initially form a complex with the imprint molecule, and following polymerisation, their functional groups are held in position by the highly crosslinked polymeric structure. Subsequent removal of the imprint molecule reveals binding sites that are complementary in size and shape to the analyte, introducing a molecular memory into the polymer, capable of rebinding the analyte (Scheme II. 1). The MIPs possess two of the most important features of the biological receptors: the ability to recognise and to bind specific target molecules. Because of the super crosslinked nature, MIPs are stable to physical and chemical treatment including
heating, organic solvents, acids and bases. Thus molecular imprinting is a powerful method for preparing synthetic recognition sites with predetermined selectivity. An important prerequisite for the preparation of these polymers is the formation of a stable pre-polymerisation complex between the monomer and the template molecules. The stability and low cost of molecular imprinted polymers make them advantageous for use in analysis, industrial scale production and application\textsuperscript{5-7}.

\textbf{Scheme II. 1.} Principle of molecular imprinting

The properties of the polymer obtained by this technology are different from the properties of the parent polymer because after the removal of the template, its three-dimensional imprint remains in the MIP.

Molecular recognition between a molecular receptor (host) and a substrate (guest) in matrix containing structurally related molecules requires discrimination and binding. This can happen only if the binding sites of the
host and guest molecules complement each other in size, shape and chemical functionality. Biological systems such as enzyme-substrate, antigen-antibody and hormone receptor systems demonstrate molecular recognition properties originating from natural selection\(^8\). The working hypothesis of the binding site structure in molecular imprinted polymers is based on the idea that the pre-polymer complex is locked into place by polymerisation. This assumption postulates the formation of a cavity with functional groups in complementary array for the convergent interactions with the template. The relationship of the template to the imprinted cavity corresponds to the lock and key principle proposed by Emil Fisher for enzyme catalysis\(^9\).

2.2. Approaches in molecular imprinting

MIPs are synthesised in the presence of specially introduced target template molecules, which are intended for imprinting. Because of the formation of a pre-polymerisation complex, the molecules of a functional monomer are particularly arranged and fixed around the template molecule in the course of the entire process of polymerisation. Generally molecular imprinting can be approached in two ways: the self-assembly approach developed by Mosbach and coworkers\(^10\) and the pre-organised approach developed by Wulff and coworkers\(^11\). These two approaches, which differ with respect to the interaction mechanism, follow common molecular recognition terminology\(^12,13\).

The pre-organised molecular imprinting approach (covalent imprinting) involves formation of strong reversible, covalent arrangements of the monomers with the print molecule before polymerisation. Thus the print molecule needs to be derivatised with the monomers before the actual imprinting is performed. After cleaving the covalent bonds that hold the print molecules to the macroporous polymer matrix, recognition sites complementary to the analyte
remain in the polymer matrix (Scheme II. 2a). The interaction between the pairs like amine-aldehyde, diol-ketone and acid-amine are normally covalent in nature. Thus imprinting with Schiff’s base\textsuperscript{14}, boronic acid esters\textsuperscript{15} and metal co-ordination bonds\textsuperscript{16} follow covalent method. Since the bond between the functional monomer and the template is covalent, polar solvents can be used as porogen during the polymerisation. However, slow binding kinetics restricts the analytical application of covalently imprinted polymers. Furthermore, covalent binding tends to be very limited since it is very specific for particular functional groups and is generally directional.

In non-covalent imprinting a very important property of polymer molecules, which forms the basis for the organisation of complex biological structures is used. It is the capacity of macromolecules for self-organisation or self-assembly. This method involves host-guest complexes produced from weak inter molecular interactions such as ionic or hydrophobic interaction, hydrogen bonding, $\pi-\pi$ interactions, induction, dispersion and metal co-ordinations between the analyte and the monomer precursors. These self-assembled complexes are spontaneously established in the liquid phase and are then sterically fixed by polymerisation with a high degree of crosslinking. After removal of the print molecule from the resulting macroporous matrix, vacant recognition sites that are specific to the print molecule are established (Scheme II. 2b). The shape of the sites maintained by the polymer backbone and the arrangement of the functional groups in the recognition sites results in affinity for the analyte. In non-covalent imprinting the bonds are less specific, non-directional and the binding sites are heterogeneous in nature. Although hydrophobic forces are potentially more difficult to master because they are less specific and have less direction, combinations of polar and hydrophobic interactions may be used to generate strong and selective binding. The non-
covalent technique is more versatile in terms of the range of template molecules. It is also easier to implement because simply mixing a template with a functional monomer in an appropriate solvent performs complexation and the template is removed by repeated washing of the polymer with a solvent or a solvent mixture. The non-covalent approach was used in the synthesis of polymers with the molecular imprints of dyes\textsuperscript{17-19}, aminoacids and their derivatives\textsuperscript{20-22}, pharmaceuticals\textsuperscript{23-26}, pesticides\textsuperscript{27-29} and other components\textsuperscript{30,31,32}. The great majority of currently known MIPs that are found in analytical chemistry are synthesised by the method of non-covalent molecular imprinting.

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(a) & (b) \\
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\textbf{Scheme II. 2.} Schematic representation of (a) covalent imprinting, and (b) non-covalent imprinting

Another technique is the intermediate semi-covalent technique, in which a template is covalently bound to a functional monomer in the course of polymerisation, whereas only non-covalent interactions are involved in the secondary binding\textsuperscript{31,32}. 

\textit{Molecular Imprinted Polymers: A Review}
2.3. Synthesis of imprinted polymers

Molecular imprinting provides a way to synthesise new materials containing artificial receptors that can be employed in a variety of applications such as in separation, sensors or catalysis. The latest developments in the technique of molecular imprinting have made polymers that can be used in the detection of drugs, toxins, peptides, food components and other molecules that would be difficult to isolate otherwise. Research on MIPs is focused on two main areas: at a molecular level on the improvement in the composition and interactions of the polymer, and at a macromolecular level on the improvement and development of new morphologies, processes of polymerisation and their applications in sensors, assay technology and separation. Different uses and potential applications of the MIP demand different properties from the polymers. Factors such as specificity, capacity or environment of the sample require particular characteristics. In response to this demand, different methods to produce imprinted polymers have been developed. So far MIPs have been prepared by bulk, suspension, two-step swelling, precipitation and emulsion core-shell polymerisations. Other methods employed are film synthesis, aerosol polymerisation and polymerisation on silica particles.

The first polymerisation method employed to synthesise MIP was based on “bulk” polymerisation and is widely used for imprinting because of its simplicity and universality. It is used exclusively with organic solvents and consists of mixing all the components (template, monomer, solvent and initiator) and polymerising them, resulting in a polymeric block which is to be crushed and ground to obtain particles of irregular shape and size between 20 and 50 µm. It has the disadvantage that a lot of the polymer produced is wasted in the process of grinding. It may also produce areas of heterogeneity...
in the polymeric matrix resulting from the lack of control of the process during polymerisation, particularly when UV initiation is used.

A fast and reliable methodology that synthesises particles by UV irradiation in less than 2 h is suspension polymerisation, first described by Mayes and Mosbach\textsuperscript{46}. The beads obtained have a diameter that can vary between 5 and 50 µm depending on the stirring speed and the amount of surfactant. It uses a perfluorocarbon solvent in the continuous phase, which allows the establishment of the same interactions that occur in bulk polymerisation. The fluorocarbon suspending medium can be easily recycled by distillation.

Another method that can provide particles in the submicron scale (0.3-10 µm) is precipitation polymerisation\textsuperscript{47,48}. It is based on the precipitation of the polymeric chains out of the solvent in the form of particles, as they grow more and more insoluble in an organic continuous medium. Here the particles are prevented from coalescence by the rigidity obtained from the crosslinking of the polymer and so it does not need any additional stabiliser. Precipitation is one of the most simple and well suited methods to obtain imprinted beads with the desired characteristics. The advantages of this polymerisation strategy in terms of capacity and homogeneity associated to binding sites have been demonstrated\textsuperscript{49,50}.

Monodisperse particles in the micron size range (2-50 µm) with good control of the final size and number of the particles were synthesised by a two-step polymerisation developed by Hosoya in 1994\textsuperscript{51}. It required several swelling steps on the initial particles with the imprinting mixture before polymerisation proceeds and the continuous phase of the polymerisation medium was water.
Core-shell particles are obtained by emulsion polymerisation$^{52-54}$. They have a structured morphology that allows the incorporation of any added property into the core of the particle without interfering with the imprinted shell. The continuous medium during polymerisation is water. Particles obtained by this method are monodisperse and can be produced in colloidal size with the range of 0.05 - 2 $\mu$m.

In situ polymerisation can be done for certain applications inside the chromatographic column$^{55}$ or in a capillary$^{56}$. In sensor applications usually imprinted polymer membranes were prepared as thin crosslinked films by precipitation from solutions in the presence of analyte$^{57}$ or by casting polymer in the pores of an inert support membrane$^{58}$. They can also be synthesised in situ at the electrode surface by electropolymerisation$^{37}$ or at a non-conducting surface by chemical grafting$^{59}$. Novel imaging strategies using fluorescent-labelled template was also reported$^{60}$. Metal ion imprinted polymeric beads are successfully applied in analytical applications$^{61}$. One of the first reports of imprinting of bacterial cells on the polymer surface was also observed$^{62}$. Computational design and synthesis of imprinted polymers with high binding capacity for pharmaceutical applications was demonstrated as the model case. Special protocols have been proposed for the imprinting of proteins$^{63}$.

Molecular imprinting in aqueous environments are in the preliminary stage. It was noted that most imprints worked best in the solvent, which was used for polymerisation$^{13}$. Therefore the use of water as a solvent has been studied for several targets with the aim of using the resultant imprinted polymers in aqueous systems. Hydrophobic interactions, caused by the exclusion of water, are frequently employed for imprinting in aqueous systems and hydrophobically driven molecular imprints have proven successful with a variety of small molecules in a range of polymer matrices.
Kugiyama relied on hydrophobic interactions between pyridine monomers and the chosen template sialic acid to produce a MIP capable of selective recognition in aqueous media\textsuperscript{64}. Molecular imprinting of the hydrophobic herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5 trichlorophenoxyacetic acid (2,4,5-T) in a mixture of methanol and water was already reported\textsuperscript{65-70}. This polar medium elicited hydrophobic binding site within a 4-vinylpyridine (4-VP) functionalised imprinted polymer. Perez-Moral produced core-shell particles with surface imprints that used shape-specific hydrophobic interactions to recognise the hydrophobic molecule cholesterol\textsuperscript{52,53}. Similarly, Carter formed core-shell particles prepared in water using emulsion polymerisation\textsuperscript{71,72}. These particles were non-covalently imprinted with caffeine and theophylline, using both hydrophobic and electrostatic interactions. In a different approach to the same templates, Han demonstrated a novel method of aqueous imprinting using an oil/water emulsion\textsuperscript{73}. The selected monomer and template sequestered into water droplets within the oil and formed complexes. These complexes were then transferred to a microporous membrane substrate and photopolymerised. Dirion used a high-throughput synthesis and evaluation of MIP sorbents to optimize a MIP for the local anaesthetic bupivacaine in aqueous systems\textsuperscript{74}. The resultant MIPs showed high imprinting factors in water, attributed to reduced non-specific binding to the imprinted polymer. Batches of these sorbents were successfully employed for the extraction of the template from blood plasma samples.

The templates are classified as convex and concave. Metal ion involving cyclisation is an example for convex template and enzyme is an example for concave template. The substrate has to fit in the cavity created by the enzyme to undergo chemical transformation. Polymers are also categorised
as reactive (catalytic) or non-reactive (non-catalytic) where the reaction rate is accelerated or unaffected.

The binding site consists of a functional group attached, capable of interacting with the template molecule and ideally the functional groups exist on the surface of the cavity left by the template, readily available for rebinding. The best results are obtained when the templates get attached to more than one binding site.

The bond between the template and the binding group should be as strong as possible during the polymerisation to enable the binding group to be fixed by the template in a definite orientation on the polymer chain during crosslinking. The template should be able to be removed as completely as possible. The interaction of the binding site with the template should be very fast and reversible.

2.4. Factors influencing the characteristics of imprinted polymers

A good success of imprinting method is predominantly dependent on the polymer structure and on the type of binding site interaction. Binding site interactions are necessary during the polymerisation procedure and are later used for binding of the substrate to the polymer. The characteristics of the polymer matrix like the matrix structure, matrix configuration, matrix nature, nature of template and extent of crosslinking have a significant effect on binding. Hence the optimization of the polymer structure is extremely important. Random incorporation of the functional monomer in the polymer matrix reduces its specificity. So the interactions between the monomer and the template should be as strong as possible during polymerisation and ideally they should be covalent. However systems utilising reversible covalent bonds exhibit slow kinetics during rebinding and often necessitate severe conditions
for desorption\textsuperscript{15}. This renders them unsuitable for many applications. Ideal imprinted polymers require mild conditions for desorption and rebinding which can be attained only through non-covalent method. Functional groups are satisfactorily distributed over a macromolecule so that both the more favourable and less favourable conformations will occur. The binding process depends on the appropriate geometric organisation of functional groups, including hydrophilic domains, within the host molecule that match or fit reciprocal functionality on the guest\textsuperscript{76}.

(i) **Rigidity/Flexibility of the polymer support**

An important prerequisite for the preparation of MIP is a high degree of crosslinking. The rigidity of the highly crosslinked polymer maintains the binding sites that feature the required functional groups in fixed three-dimensional orientations necessary for rebinding of the template\textsuperscript{77}. Yilmaz\textsuperscript{78} demonstrated that a molecular memory can be introduced by molecular imprinting even in low crosslinked polymers (19%). The selectivity is mainly influenced by the kind and amount of crosslinking agent used in the synthesis of MIP\textsuperscript{79}. Below a certain amount of crosslinking in the polymer (∼10%), no selectivity can be observed because the cavities are not sufficiently stabilised. Above 10% crosslinking selectivity increases steadily. Between 50 and 60% a surprisingly high increase in selectivity takes place, especially in the case of EGDMA crosslinking. Crosslinking with DVB has the advantage of less interaction with the functional groups\textsuperscript{80}.

Some flexibility is also necessary to allow a fast binding and splitting of the template within the cavities. Cavities with accurate shape but without flexibility will show kinetic hindrance to reversible binding. The studies on macroporous styrene-divinylbenzene\textsuperscript{81,82}, and styrene-diisopropyl benzene\textsuperscript{83,84}
copolymer by Shea revealed that relatively rigid, hydrophobic and highly
crosslinked materials provide chemical inertness required to withstand
subsequent chemical transformations. Thus the molecular imprinted polymers
should have optimum stiffness/flexibility to attain rapid equilibrium with the
template, to make imprinted cavities accessible for the template, to provide
mechanical stability to withstand stress in certain applications and thermal
stability to use at high temperature. Therefore crosslinking provides a
structural design that improves selective molecular recognition by MIPs.\(^{85}\)
Thus there should be a compromise between rigidity and flexibility to ensure
favourable kinetics for substrate binding.

(ii) Functional and crosslinking monomers

Several reports on molecular imprinting describe organic polymers
synthesised by radical polymerisation of functional and crosslinking monomers
having vinyl or acrylic groups. This can be attributed to the fairly straightforward
synthesis of these materials and to the vast choice of available monomers. Other
approaches are the polystyrene-based and polysiloxane-based systems, but to a
lesser extent. The functional monomers can be basic, acidic, permanently
charged, hydrogen bonding, hydrophobic and others (Fig. II. 1). Methacrylic acid
(MAA), acrylamide (AA) and 4-vinylpyridine (4-VP) were the most
frequently used functional monomers in non-covalent imprinting. Acrylamide
could be a promising functional monomer to form strong hydrogen bonds with
template molecule in polar solvents. In deciding a functional monomer, the
nature of the constituent donor atoms and the possibility of forming a stable
monomer-template associate are taken into consideration. Generally MAA is
used as a functional monomer in the synthesis of polymers with the molecular
imprints of organic compounds containing basic groups such as triazines\(^{86,87}\),
whereas 4-VP is used in the case of compounds containing acidic groups. 

![Monomers](image)

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

**Fig. II. 1.** Functional monomers commonly used in molecular imprinting

While the amount of crosslinking agent affects the rigidity of MIPs, the nature of this agent significantly affects the physicochemical properties of a polymer matrix. The choice of a crosslinking agent depends on the nature of the functional monomer. For efficient imprinting, the reactivity of the crosslinking agent should be similar to that of the functional monomer. DVB isomers are most frequently used as crosslinking agents in the synthesis of MIPs based on polystyrene, whereas EGDMA is used in the synthesis of MIPs based on acrylic
or methacrylic acids\textsuperscript{3} in organic solvents. N,N’-methylene-bis-acrylamide (NNMBA) is a typical water soluble crosslinking agent. The fundamental role of these agents is to fix the guest binding sites firmly in the desired structure and make the polymer insoluble in solvents. By the use of proper crosslinking agent, random copolymerisation occurs successfully and the functional residues are uniformly distributed in the polymer network.

The mole ratios of crosslinking agent to functional monomer are also important. If the ratios are too small, the guest binding sites are located so closely to each other that they cannot work independently. In extreme cases, the guest binding by one site completely inhibits the guest binding by neighbouring sites. At extremely large ratios the imprinting efficiency is damaged, especially when the crosslinking agents show non-covalent interactions with functional monomers and/or templates. Usually a crosslinking agent is taken in 20-fold excess to the functional monomer and its concentration in the reaction mixture is 70-90\%.\textsuperscript{91} At low crosslinking, the selectivity is poor since the polymer matrix is not stable enough to retain the shape of the cavity. In deciding on crosslinking agents, their good solubility in a pre-polymerisation mixture is also taken into account. For large templates such as proteins, the crosslinking agents having optimal length such as TTEGDMA or PEG400 DMA are more suitable.\textsuperscript{92} Thus the crosslinking monomers are an active part of imprinting, rather than merely ‘inert’ scaffolding for functional monomers. The most frequently used crosslinking agents\textsuperscript{30} are depicted in Fig. II. 2.
A simple method of molecular imprinting using a single crosslinking monomer N,O-bis-methacryloyl ethanolamine (NOBE) along with template, initiator and solvent was recently reported\textsuperscript{93}. This formulation eliminates the
need for additional functional monomers and empirical optimization of relative ratio of functional monomers, crosslinking monomer and template. Utilisation of NOBE alone often provides MIPs with better performance than MIPs incorporating functional monomer.

(iii) **Monomer-template ratio**

The molar relationship between the functional monomer and template had been found to be important with respect to the number and quality of MIP recognition sites\(^9^4\). Low M/T ratios afford less than optimal complexation on account of insufficient functional monomer. Too high an M/T ratio, the extreme case being a non-imprinted polymer, yields non-selective binding\(^9^5\). Studies\(^7^0\) on the column capacity, selectivity factor and imprinting effect of 2,4,5-T imprinted polymer with different molar ratios between template and functional monomer revealed that the retention of 2,4,5-T was proportional to the molar ratio between the functional monomer and template. When the molar ratio is low, the pyridine rings are mostly included in high affinity binding sites generated by the imprinting mechanism, where the imprinting effect should be very significant. When the molar ratio is high, the pyridine rings are mostly located outside the imprinted cavities, scattered in regions of the polymer where the imprinting effect is absent or almost negligible. A well designed imprinted polymer shows marked specific molecular recognition effects and negligible non-specific partition effects, while a non-imprinted polymer shows only partition effects without any sort of molecular recognition effects. Thus a parameter called “imprinting effect” can be considered as a good estimate of how much and how well the polymer was imprinted by a template\(^9^5\). Thus imprinting effect is only a measure of the net molecular recognition effect due to the imprinting process in defined experimental conditions. The molar ratio between template and monomer could be
approximately set from 1+2 to 1+4 for many of the polymers described in the literature\textsuperscript{96-98}. It has been demonstrated that for equilibrium constants between 10 and 1000 M\textsuperscript{-1}, a significant fraction of the template is complexed, provided that functional monomers will be in considerable excess\textsuperscript{99,100}. Anyway it may not be considered as a general criterion, since imprinting effects with a 1+1 molar ratio between template and functional monomer was reported\textsuperscript{101-103}. One problem with increasing the functional monomer is that the amount of crosslinker becomes too low, and the MIP loses its recognition properties from random motion of non-crosslinked polymer domains\textsuperscript{104}.

(iv) **Porogen/Rebinding solvent**

Molecular imprinted polymers utilise a solvent both as a porogen in polymerisation and as a medium for rebinding studies. The trivial role of solvents is to dissolve the agents for polymerisation and also to disperse the heat of reaction generated during polymerisation. The solvent also serves to facilitate mass transfer of the analytes to and from the binding sites and to create pores by phase separating into channels during polymerisation\textsuperscript{105}. It also affects adsorbent characteristics such as specific surface area and pore size\textsuperscript{106}. Thus porogen parameters such as polarity and hydrogen bonding have an important impact on the final morphology of the network structure, porosity and recognition properties of MIPs.

It is believed that solvents with low permitivities (toluene, dichloromethane, chloroform) are best suited for molecular imprinting\textsuperscript{107,108} because the monomer-template non-covalent interactions are stronger than in polar solvents. Chloroform is one of the most widely used solvents, since it satisfactorily dissolves many monomers and templates and hardly suppresses hydrogen bonding.
Choice of solvent depends on the kind of imprinting. In covalent imprinting many kinds of solvents are employable as long as they satisfactorily dissolve all the components. In non-covalent imprinting the choice of solvent is more critical to the promotion of the formation of non-covalent adducts between the functional monomer and the template enhancing imprinting efficiency. Polar solvents will interfere with the hydrogen bonding interaction between substrate and MIP and the substrate will tend to remain in solution rather than binding to the polymer due to strong solvation of the substrate.

The solvent specific behavior of the imprinted polymers has been explained in terms of the solvating properties of the solvents. The solvating ability of the porogen for polymer chains during polymerisation has been suggested to adjust the shape of the binding sites and the distance between the functional groups in the binding site. Thus the ability of the binding medium to recreate the binding site dimensions determines the binding performance of the polymer. Generally best recognition of imprinted polymers occurs when the rebinding medium and the porogen are the same.

(v) Polymerisation temperature

The position of equilibrium between free template-monomer and their corresponding complex is a product of both temperature and pressure. Sellergren group showed that high pressure (1000 bar) polymerisation could be used to enhance the selectivity of the resultant imprinted polymers. Previous studies have shown that lower temperature is favourable for the preparation of MIPs based on electrostatic interaction due to the greater strength of electrostatic interaction at lower temperature. At low temperature, the polymerisation process is slow and the chain formation does
not interfere with the template-monomer interactions. An optimum temperature should be found for each combination of template and monomer\textsuperscript{117}. Synthesis of MIPs could also be performed at reduced temperatures (15 to 20°C), while initiating the polymerisation reaction by UV-irradiation\textsuperscript{118-120}. In this case MIPs with a greater capacity for molecular recognition were obtained because the monomer-template complex in the polymerisation mixture is more stable at low temperatures.

The radical polymerisation of corresponding functional monomers is the main method for preparing MIPs. The rate of radical polymerisation depends on the nature and concentration of the initiator\textsuperscript{121}. Polymerisation in early applications was initiated by thermal decomposition of AIBN\textsuperscript{122,123}, an effective initiator, which undergoes homolytic cleavage to form two isobutynitrile radicals with the release of a nitrogen molecule upon heating the reaction mixture to 60°C.

2.5 Characterisation of molecular imprinted polymers

In the non-covalent approach the stability of the template-monomer pre-polymerisation complex will govern the resulting binding site distribution and the binding properties of the imprinted polymer matrix. Close analysis of the pre-polymerisation solution can provide fundamental insights into the various interactions occurring during imprinting. Consequently, spectroscopic studies of the pre-polymerisation mixtures provide prevalent information on the imprinting process. Since reorganisation of functional groups at the binding sites is required during rebinding, spectral studies before and after rebinding can also put light into the binding process.
(i) **FT-IR**

FT-IR spectra provide the fundamental analytical basis for rationalising the mechanism of interactions for selective binding site formation at the molecular level\(^\text{124}\). The interaction between the functional monomer and template during pre-polymerisation complex formation and the template incorporation into the imprinted polymer during rebinding can be confirmed by the characteristic FT-IR absorption analysis\(^\text{125}\).

(ii) **\(^1\)H NMR**

Proton NMR titration experiments facilitate observation of hydrogen bond formation between bases and carboxylic acid through hydrogen bonding. These studies have been introduced in molecular imprinting for investigating the extent of complex formation in pre-polymerisation solutions and to identify the specific sites in interacting structures that engage in complexation. Thus evaluating the shift of proton signals due to participation in hydrogen bonding and other interactions were used as the criteria for complex formation, M/T ratio and interacting forces\(^\text{126,81}\).

(iii) **\(^{13}\)C CP-MAS-NMR**

Being rigid solids, neither usual NMR spectroscopy nor X-ray diffraction methods can be applied successfully to follow rebinding with imprinted polymers\(^\text{103}\). Therefore it is not possible to obtain reliable structural information of the interactions occurring in the cavities between binding site and template. The polymer analysis using \(^{13}\)C CP-MAS-NMR techniques can give information about the polymer backbone.
(iv) **Scanning electron microscopy (SEM)**

SEM can be used in distinct ways to probe imprinted polymers on a variety of length scales. Scanning electron microscopy is the most widely used technique to study the shape, size, morphology and porosity of polymers\textsuperscript{127,128}.

2.6. **Swelling studies**

The efficiency of a functional monomer is governed by the accessibility of the reactive functional groups anchored on it, which, in turn, depends upon the extent of swelling and solvation\textsuperscript{129}. The rate of diffusion of a reagent into the polymer matrix mainly depends on the extent of swelling\textsuperscript{130}. Thus swelling is an important parameter, which controls the success of rebinding. The most effective solvent can carry out the rebinding reaction very effectively. The extent of swelling can be determined in terms of change in weight\textsuperscript{131,132}. Alternatively, by packing a definite weight of the polymer in a capillary tube and measuring the volume before and after incubation in the solvent, the swelling ratio can be determined in terms of change in volume\textsuperscript{133}.

2.7. **Selectivity parameters of molecular imprinted polymers**

The binding parameters of the MIPs are usually estimated from adsorption isotherms\textsuperscript{103,109} using mathematical models. One strategy to perform binding performance is based on saturation experiments and subsequent Scatchard analysis\textsuperscript{134-137}. The binding data were transformed into linear form and analysed to create Scatchard plots based on Scatchard equation:

\[
\frac{[S]_b}{[S]_f} = \frac{(S_{\text{max}} - [S]_b)}{K_D}
\]

where, $K_D$ is the equilibrium dissociation constant, $S_{\text{max}}$ an apparent maximum number of binding sites and $[S]_b$ is the amount of template bound to
MIP at equilibrium. From the plot of bound concentration ([S]_b), against the ratio between bound and free template concentration ([S]_b/[S]_f), it is possible to estimate the S_max and K_D where S_max is the X intercept and K_D is the negative reciprocal of the slope. For non-covalently synthesised MIPs the Scatchard plots result in a curve with the degree of curvature containing the information on the heterogeneity of the binding sites within the MIP matrix. The random arrangement of the templates at the binding sites and the incomplete complexation between the template and functional monomer led to the heterogeneity in the binding sites typical of the non-covalent imprinting.

The effectiveness of imprinting was verified by comparison of the binding of template with structural analogues\textsuperscript{138-141}. A complete secondary screen for binding and selectivity was performed for all the polymers in terms of separation factor\textsuperscript{142}.

\[
\text{Separation factor (} \alpha_{\text{template}} \text{)} = \frac{K_{\text{MIP}}}{K_{\text{NIP}}}
\]

MIPs possessing high separation factors should be capable of completely recovering the target molecule by the simple process of stirring the imprinted polymers with template solution. In general, the value of separation factor depends on the type of interactions between the template and the functional monomers. However, when non-covalent interactions are employed, comparatively less values in the range 1-2 is obtained. The selectivity of the imprinted polymer can also be expressed as selectivity factor\textsuperscript{143,70}.

\[
\text{Selectivity factor} = \frac{\alpha_{\text{template}}}{\alpha_{\text{analogue}}}
\]

It provides a good indication for the molecular recognition of the comparative molecule to the template.
2.8. Applications of molecular imprinted polymers

The initial imprinting structures of Wulff in the 1970s using sugar and amino acid derivatives inspired the application of molecular imprinted polymers to a wide range of substrates and functions. Investigations have involved the development of MIPs for separating mixtures of racemates of sugars\textsuperscript{144} and amino acid\textsuperscript{126} derivatives. But within the past few years the variety of substrates to which MIPs has been developed has increased to include pesticides\textsuperscript{145}, metal ions\textsuperscript{146-148}, steroids\textsuperscript{149}, drugs\textsuperscript{150}, peptides\textsuperscript{151} and antibiotics\textsuperscript{152}.

(i) Enantioseparation of racemic mixtures

The application of MIPs that has received significant attention is that of molecular imprinted chromatography (MIC). Numerous templates had been examined in imprinting protocols for separation science, ranging from small molecules such as amino acids to much larger species such as proteins\textsuperscript{153}. Since most commercially available chiral drugs are administered as racemate mixtures, the chiral resolution of drugs is a major potential application of MIPs\textsuperscript{154}. Molecular imprinted polymers had been prepared against chiral compounds and applied to enantiomeric separation\textsuperscript{24}. One major advantage of the use of MIPs in enantiomeric separations is that this technique allows the preparation of ‘custom-made’ supports\textsuperscript{155}. Optimized MIPs exhibit predictable elution orders with the imprinted enantiomer always retaining in the column for a long time\textsuperscript{156}.

In MIC, template selectivity is dependent upon the differences in specific and non–specific interactions between the analytes and the template in question\textsuperscript{157}. MIPs possessing separation factors of greater than 10 should be capable of completely resolving compounds by the simple process of stirring the racemate with the imprinted polymer and filtering\textsuperscript{158}. In general, the values
exhibited by MIP stationary phases range between 4-8\textsuperscript{157}, when ionic interactions are utilised between the template and the functional monomers. Yu and Mosbach\textsuperscript{159} have carried out extensive optimization of the chromatographic resolution of racemic mixtures of $\alpha$-amino acids using amide groups as the key recognition element within imprinted polymer networks.

A very pronounced stereo selectivity has been observed with an MIP specific for the cinchona alkaloids cinchonidine and cinchonine, resulting in chromatographic values up to 31\textsuperscript{160}. It is even possible to obtain chromatographic supports selective for compounds containing several chiral centers\textsuperscript{151}. If the molecule of interest contains more than two chiral centers, as is the case with carbohydrates, these properties of molecular imprinted materials become even more relevant. When polymers were imprinted against a glucose derivative, very high selectivity between the various stereoisomers and anomers were recorded\textsuperscript{161}.

Capillary electrochromatography (CEC) might be one of the more promising chromatographic techniques to be used in combination with MIPs, in particular for chiral separations\textsuperscript{162,163}. Enantioseparation of the $\beta$–blockers propranolol and metoprolol was achieved with MIP-CEC. The polymer was cast in situ in the capillary in the form of a microporous monolith attached to the inner wall, and the capillary could be prepared and conditioned within a few hours\textsuperscript{56}. The racemate of propranolol was resolved within only 120 s and when non-racemic samples were injected containing mainly the R-enantiomer, very small amounts (1%) of the S-enantiomer could be distinguished. Other possibilities of using MIPs in combination with capillary electrophoresis is in the form of continuous polymer rods\textsuperscript{164}, particles included in a gel matrix\textsuperscript{165} and small particles suspended in the carrier electrolyte\textsuperscript{166}.
(ii) Solid phase extraction

The separation technique that has been most intensively studied in the past few years with respect to the possible use of imprinted materials is solid phase extraction (SPE)\textsuperscript{167-170}. MIPs are not only more selective than common sample treatment methods using C\textsubscript{18} or ion exchange materials, but are at the same time more stable than immuno extraction\textsuperscript{171} matrices. Since MIPs are compatible with organic solvents, MIP-SPE can be applied directly after a solvent pre-extraction step. As SPE works in the adsorption-desorption mode, the low-resolution factors are not an issue. Thus SPE seems to be one of the most promising application niches for MIPs today. MIP-SPE has been used to extract the target analyte from blood plasma and serum\textsuperscript{172}, urine\textsuperscript{173}, bile\textsuperscript{168}, liver extract\textsuperscript{86}, chewing gum\textsuperscript{174}, environmental water and sediment\textsuperscript{119} and plant tissue\textsuperscript{170}. The SPE procedures were also applied to the clean up of phenyl urea herbicides in carrot, potato, corn and pea sample extracts\textsuperscript{149}. The quantification of the herbicide atrazine in beef liver is a good demonstrative example of the utility of imprinted polymers in SPE\textsuperscript{86}.

When MIPs are to be used as SPE materials, one of their more troublesome features is template leakage leading to inaccuracies in the quantitative measurement in the levels of the analyte of interest in the sample and it is a source of concern in the application of MIPs in the pharmaceutical industry. A possible method of circumventing the bleeding problem entirely is to use a template analogue during the imprinting step rather than the template itself. This approach was described by several researchers\textsuperscript{175,176}.

Barzana and co-workers\textsuperscript{177} have utilised MIPs for the selective adsorption of organosulfur compounds from fuel, with the ultimate aim of desulfurising diesel. The cross-reactivity of MIPs was exploited by Hui and
Qin in the determination of the degradation products of nerve agents in human serum via MIP-SPE\textsuperscript{178}. MIPs for the solid phase extraction of propranolol\textsuperscript{168}, pentamidine\textsuperscript{167}, sameridine\textsuperscript{148}, tamoxifen\textsuperscript{179} and atenolol\textsuperscript{180} were also reported. MIPS have also been used in other separation techniques such as thin layer chromatography\textsuperscript{181-183}, membrane based separations\textsuperscript{184-187} and adsorptive bubble flotation fractionation\textsuperscript{188}.

(iii) **Binding assays**

For analytes lacking physicochemical properties that allow selective and sensitive quantitation in a given matrix, ligand-binding assays can be used. The wide variety of techniques developed for the determination of analytes by immunoassay includes radio immunoassays (RIA) and enzyme immunoassays\textsuperscript{189-191}(ELISA). Since MIPs can bind a target molecule selectively like antibodies, they could conceivably be employed in immunoassay type binding assays in place of antibodies. This was first demonstrated by Mosbach’s group, who developed MIP based assays for the bronchodilator theophylline and the tranquilizer diazepam\textsuperscript{25}, analogous to the first solid-phase immunoassay for human growth hormone\textsuperscript{192}. This showed not only a very good correlation with an antibody-based enzyme immunoassay but even yielded a cross reactivity profile very similar to that of the natural antibodies. In MIA the most commonly used label is a radioactive isotope\textsuperscript{25}, but also detection systems based on fluorescence have been suggested\textsuperscript{68}. An innovative technical improvement is the use of magnetic MIP beads to facilitate separation of free and bound radiolabelled marker\textsuperscript{193}. Also sub-micron beads, more resistant to precipitation and aggregation, requiring less agitation during incubation may simplify the assay procedure\textsuperscript{194}. Use of MIA for analysis of drug compounds in blood-derived biofluids has already been presented\textsuperscript{195}. Some analyte MIP systems
can be employed equally well for organic solvent or aqueous buffer based assays. An MIA method by which plasma samples could be assayed directly was also demonstrated. Recently other approaches have been explored to improve enzyme-mimicking polymers. Thus antibodies prepared by imprinting the transition state analogue p-nitrophenyl methyl phosphonate against a phosphonic ester for alkaline ester hydrolysis enhanced the rate of hydrolysis by $10^3$-$10^4$ fold. The enhancement is due to the preferred binding of the transition state of the reaction.

(iv) Polymeric sensors

An area in which MIPs have considerable potential is as the recognition element in biosensors. In sensor systems, the MIPs replace the naturally occurring sensing element, and therefore provide mechanical stability to the system. Many sensors for environmental monitoring, biomedical and food analyses rely on biomolecules such as antibodies or enzymes as the specific recognition elements. Because of the poor chemical and physical stability of biomolecules, artificial receptors are therefore gaining increasing interest. The first reported integrated sensor based on an MIP was a capacitance sensor. Recently capacitive detection was employed in conjunction with imprinted electropolymerised polyphenol layers on gold electrodes. In another report, thin films of TiO$_2$ were imprinted with chloroaromatic acids such as 2,4-D and used as recognition layers in sensors based on ion-sensitive field-effect transistors. During the last few years there has been a big boost in the use of mass-sensitive acoustic transducers such as the surface acoustic wave (SAW) oscillator, the Love-wave oscillator (LWO) and the quartz crystal microbalance (QCM) for the design of MIP based sensors. If the target analyte exhibits a special property such as fluorescence or electrochemical activity that can be exploited
for the design of MIP based sensors. A very sensitive sensor for the detection of the hydrolysis product of a chemical warfare agent Soman has been described based on a polymer-coated fiber optic probe and a luminescent europium complex\textsuperscript{214}. The selectivity of a sensor is dependent on differences in interaction between analytes and sorbent to the same extent as in ligand binding assays. The sensitivity of a sensor is dependent on the affinity of the analyte or of the labelled ligand and on the detection principle. In conclusion due to their stability molecular imprints can be readily used as the sensing surface of a chemical sensor.

Usually the template selected for molecular imprinting studies were of either biological or environmental significance. This is the reason for the release of enormous imprinted polymers of various types mentioned.

\textit{(v) Herbicide selective polymers}

Maximising the world’s agricultural efficiency depends largely on controlling a variety of diseases and pests, especially weeds. Herbicides are the chemicals that are widely used in agricultural field for controlling the growth of herbs, weeds and bushes. The herbicide industry was built on the success of 2,4-D and 2,4,5-T and weed control research over the last 50 years had been focused almost exclusively on synthetic herbicides\textsuperscript{215}. The use of pesticides is accompanied by a variety of undesirable environmental effects. Current concerns about potential health hazards connected with pesticide use have focused on 2,4-D and 2,4,5-T as suspected cancer-causing agents. 2,4-D is extensively used as a broad leaf weed killer on field crops, turf, and non-crop lands. The wide spread use of 2,4-D and associated health concerns have made monitoring of environmental samples for the presence of 2,4-D. 2,4,5-T also is a broad range herbicide whose use has been banned in Europe and the
USA owing to the danger of dioxin contamination connected with the commercial product\textsuperscript{216,217}. Thus it may represent an interesting environmental contaminant of anthropic origin. These compounds are polar chlorinated herbicides with a carboxylic group attached to the benzene ring by means of an oxygen atom.

\[
\begin{array}{ccc}
\text{POA} & \text{2,4-D} & \text{2,4,5-T} \\
\end{array}
\]

With human’s increasing concern of environmental protection, the requirement of a sensitive and convenient method for environmental assay is strongly required. The classical analytical methods for herbicide analysis include HPLC, GC, GC-MS, ELISA and spectrophotometric methods\textsuperscript{218,219}. These conventional methods are still tedious and time consuming and are mostly laboratory-bound. An alternative modern method that eliminates some of these difficulties of conventional methods is the use of molecular imprinted polymers.

Attempts were made previously to imprint herbicides in polymers and to use them in various applications. The 2,4-D imprinted polymer coated bulk acoustic wave (BAW) sensor was fabricated in liquid phases\textsuperscript{29}, polymer layers on the surface of zinc selenide attenuated total reflection elements\textsuperscript{38}, immnosensing methods\textsuperscript{220}, differential-pulse voltammetry method\textsuperscript{66}, competitive fluoroimmunoassays\textsuperscript{68}, radioligand binding assay\textsuperscript{65}, biosensors\textsuperscript{221}, and solid phase extraction by means of polymeric resins\textsuperscript{222} were reported for the selective binding of the herbicide 2,4-D.
Chromatographic characterisation of 2,4,5-T imprinted polymers was reported\textsuperscript{69}. HPLC columns packed with 2,4,5-T imprinted polymers in hydrophilic solvent show column capacity, selectivity and imprinting effects controlled by ion-pair and hydrophobic interactions between the analyte and the stationary phase\textsuperscript{70}. Molecular imprinting of phenoxyacetic acid is not yet reported to our knowledge.

Many phenolic compounds are toxic for living beings, easily penetrating through natural membranes, causing a broad spectrum of genotoxic, mutagenic and hepatotoxic effects, and also modulating biocatalysed reaction in respiration and photosynthesis\textsuperscript{223,224}. Because of their toxicity, as well as their unpleasant organoleptic properties, phenols have been included in the priority pollutant list of the EEC and US-EPA\textsuperscript{225-229}. Molecular imprinting of such a phenolic compound \(p\)-hydroxybenzoic acid (\(p\)-HB) was reported\textsuperscript{230}, but the study was based on HPLC.

Most of the reported techniques especially that employed for the studies of 2,4-D, 2,4,5-T and \(p\)-HB imprinted polymers were complex, costly, time consuming, require sophisticated instruments and skilled technicians. Thus there is the need of developing faster screening methods that should be technically simple and useful for routine analysis of a large number of samples. Here an attempt is made to design imprinted polymers of the selected templates and to tailor the conditions for maximum specificity and selectivity employing the simple UV-spectrophotometric technique.
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