STUDIES OF RECOGNITION AND SELECTIVITY BY FLUORESCENT POLYMERS IMPRINTED WITH N^1-BENZYLIDENE PYRIDINE-2-CARBOXAMIDRAZONES

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Studies of recognition and selectivity by fluorescent polymers imprinted with N^1-benzylidene pyridine-2-carboxamidrazones

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Thesis Summary

As part of a programme of automated synthesis and biological screening, new fluorescent polymers imprinted with various N^1-benzylidene pyridine-2-carboxamidrazones have been successfully constructed and evaluated for their recognition of the original template and cross-reactivity to similar test molecules. Dramatic quenching of fluorescence approaching background levels was observed for most cases where the "empty" molecularly imprinted polymer was re-exposed to its template. Molecules too large to enter the imprinted cavities gave no reduction of fluorescence. Other compounds were found to quench the fluorescence and are assumed to have entered the imprinted cavities. There is also evidence for partial responses which may give some measure of partial binding. The fluorescence response profiles of substrates containing polycyclic aromatics were found to be quite different from those containing flexible substituents.

Keywords: Molecular imprinted polymer; Fluorescence; Selectivity; Recognition; High-throughout screening.

Dedication

To my parents, with love and respect for all of their selfless support, kind solicitude and great love.

Also to Miss Dan Fei for her endless love and encouragements.

Acknowledgements

I would like to express my gratitude to my supervisors Dr. D. L. Rathbone and Dr. Y. F. Wang for their guidance, patience and help. Thank you very much!

Also many thanks Mr. Mike Davis and everyone in the labs.

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Abbreviations

ABDV	2,2'-Azobis(2,4-dimethylvalernitrile)
AIBN	Azobisisobutyronitrile
BDPMP	Boronate diester phenyl-α-D-manno-pyranoside
cAMP	3',5'-Cyclic monophosphate
9EA	9-Ethyladenine
EDMA	Ethylene glycol dimethacrylate
HPLC	High-pressure liquid chromatography
IR	Infrared
MAA	Methacrylic acid
MIP	Molecularly imprinted polymer
MIPs	Molecularly imprinted polymers
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PETEA	Pentaerythritol tetraacrylate
PETRA	Pentaerythritol triacrylate
PMP	Phenyl-α-D-manno-pyranoside
T	Template
THF	Tetrahydrofuran
TRIM	Trimethylolpropane trimethacrylate
UV	Ultraviolet

VPBA	4-Vinylphenylboronic acid
VPD	2-(P-vinylphenyl)-1,3-propannediol
2VPy	2-Vinyl pyridine

1.1 Molecular recognition

The concept of molecular interactions is quite old. In the later half of the 19th century, modern ideas about these interactions began to emerge, *e.g.* through the work of Dutch physicist J. D. van der Waals [1] in his studies of interactions between atoms in the gaseous state, and in 1894 Emil Fischer presented his famous "lock-and-key"-analogy of the way a substrate interacts with an enzyme [2] (***Figure 1.1***). In this prophetic statement, an enzyme, which is large compared with the substrate, has clefts and depressions on its surface complementary to the shape of the substrate. Thus, the substrate fits like a key into the lock of the enzyme's active site.

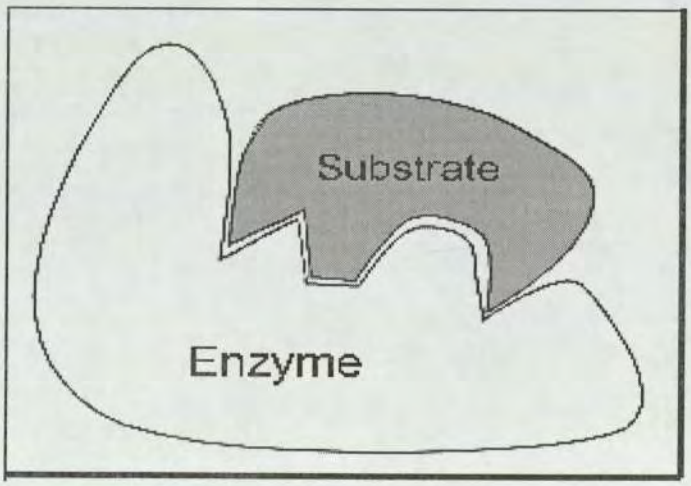


Figure 1.1. Schematic representation of Fischer's lock-and-key concept in an enzyme-substrate complex

This theoretical consideration had been developed over the centuries and aroused scientists towards a new approach for molecular recognition.

Molecular recognition is fundamental to the function of biological systems and of great practical importance in chemical, pharmaceutical sciences. Enzyme activity, receptor binding systems and the immune response require that the presence of one molecular species be perceived by another. Although biological systems are often very specific, the capacity for adaptation and apparent non-specificity is inherent. The polymeric system that provides the basis for these recognition events is the protein and nature's creation has been put to good use within the laboratory. However, protein based assays, immune-affinity chromatography columns, protein-based high-pressure liquid chromatography (HPLC) columns and enzyme catalyzed chemical reactions are extremely sensitive to changes in conditions, intolerant to misuse and expensive. It is therefore not surprising that alternative, biomimetic systems have been sought which are capable of specific molecule recognition but have the characteristics of durability, reliability and cost-effectiveness.

Considerable chemical efforts have being made to discover how to develop more and more selective recognition systems that are specific for a given molecule. Extremely high selectivity is obtained if, as in nature, a cavity exists that has a shape to match that of the substance to be embedded in it and rebinding sites in a definite spatial arrangement. To form the cavity, firstly low molecular weight ring or cage systems [3] such as crown ethers [4], cryptates [5], cyclophanes [6], or concave molecules [7] were synthesized as the 'host' structures which selectively bind 'guest' molecules. However, a major drawback with this approach is that it is far from generic, recognition problem requiring a novel solution. This

often requires complex and expensive chemistry and long development times. Other successful approaches utilized naturally occurring macromolecules, such as cyclodextrins [8,9] and cellulose derivatives [10] which can act as general recognition sites. In conclusion, each of these methodologies relies upon either a design/synthesis or a 'coincidental' fit approach.

1.2 Molecular imprinting technology

1.2.1 Historical backgrounds

However, only recently, a new, vigorous approach named '*molecular imprinting*' has been sufficiently developed which made the chemical work towards recognition easier and more efficient. And importantly, it provides applications in several fields besides chemistry.

The emergence of molecular imprinting has its original source in the development of immunology for the first time the notion of antigen-as-template became explicit. In the early 1930s Breinl and Haurowitz [11], followed by Mudd [12] and Alexander [13], who proposed that an antigen would be carried in the body to the site of protein formation, where it would serve as a template upon which the nascent antibody molecule might be constructed. Since the antibody molecule was to be synthesized upon the surface of the antigen, it seemed reasonable to propose a mechanism whereby the stereochemical structure of the antigenic site would determine a unique amino acid sequence on the antibody, thus accounting for the complementary and specific fit of antibody for antigen. Implicit in this

theory, of course, was the requirement that antigen persist throughout the course of antibody formation.

These theoretical considerations and the key/lock principle of Fischer as mentioned at the beginning were further arranged and elaborated by Linus Pauling, who recognized the great importance of shape selectivity for the action of enzymes and antibodies. In 1940, he postulated that the great diversity in antibody formation was due to the formation of different three-dimensional configurations of the antibody polypeptide chain induced by the interaction with the antigens [14]. Following these "instructive" theories on antibody diversity, the antibodies would be able to change their three dimensional structures in order to form as many interaction points as possible with the epitopes of the antigens. Thus, the antibody combining sites were "moulded" with the antigen as a template in a casting procedure, i.e. they were molecularly imprinted with the antigens (***Figure 1.2***).

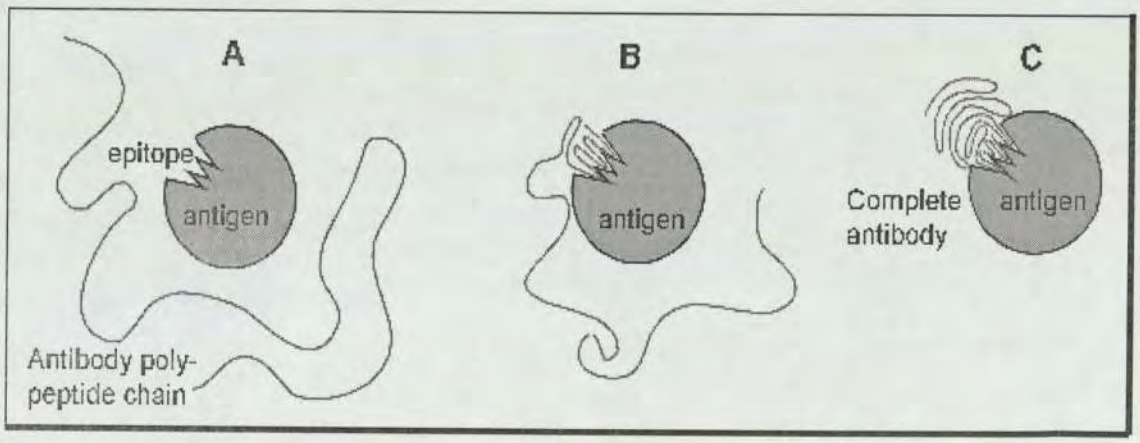


Figure 1.2. Antibody formation according to L. Pauling [15]

(A) The nascent antibody polypeptide chain encounters the antigen.

(B) The polypeptide chain starts to fold, directed by the structural prerequisites of the epitope.

(C) The guided antibody formation is complete.

Later, unfortunately, these theories were found to be incorrect and their instructive models abandoned in favour of the more appropriate "clonal selective" theory of antibody formation. Nevertheless, their models laid the foundations for the area of molecular imprinting.

Interestingly, in 1949, it was F. H. Dickey, Pauling's student, who prepared an 'imprinted' absorbent [16,17] for the first time. He precipitated silica gel in the presence of dyes (methyl orange and homologues). After drying the gel and eluting the dye, a material was obtained which adsorbed more than twice as much methyl orange as ethyl orange in a subsequent rebinding experiment. This binding preference could be revered by using ethyl orange as the print molecule as well. It is obvious that during the formation of the rigid silica gel matrix around the dye molecules, silanol groups are apparently so arranged that interactions with the dyes can occur.

1.2.2 The principle of molecular imprinting technology

Since those very early days the concept received little attention until seminal investigations by Klause Mosbach [18,19] who led to what is now described as *Molecular imprinting*. The process of it can be shown as a way of making artificial "Locks" for "molecule keys" *(Figure 1.3)*.

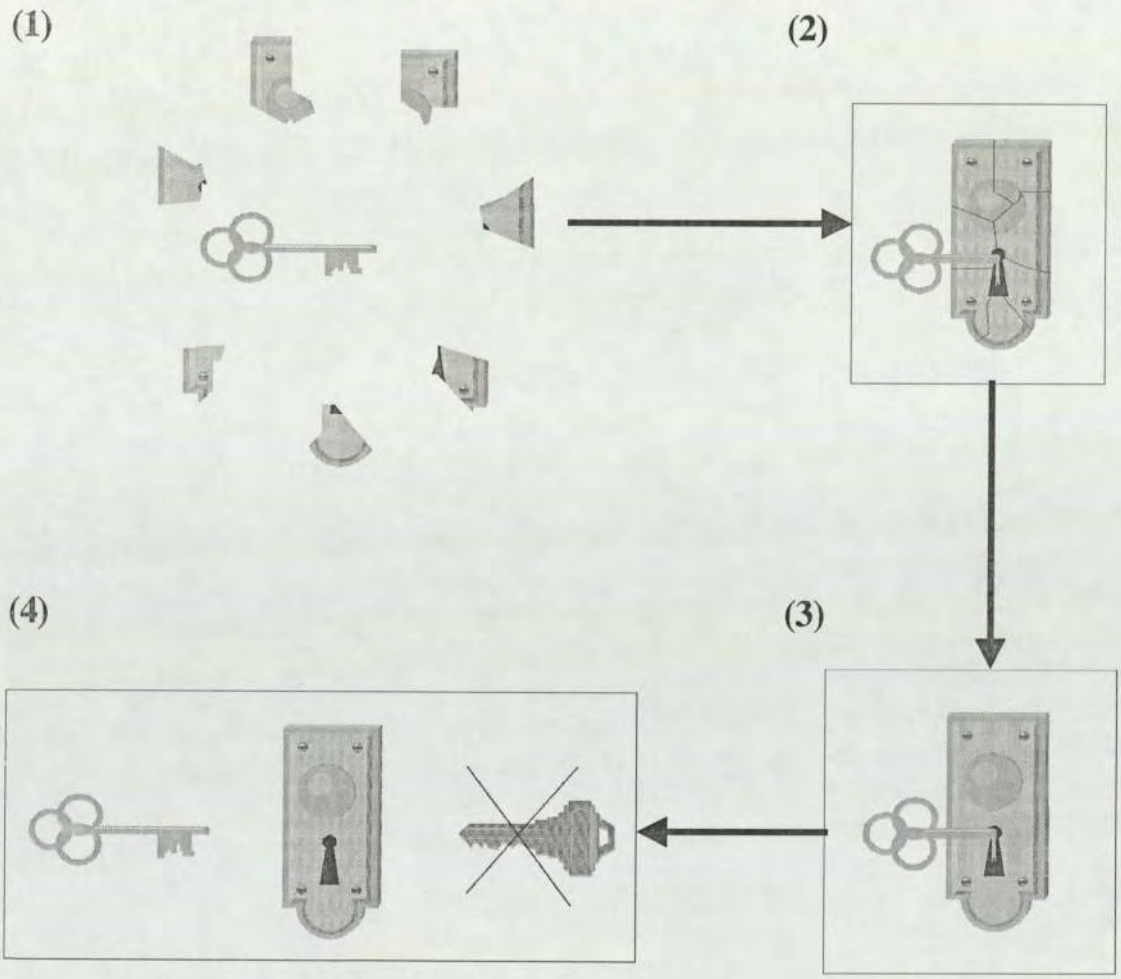


Figure 1.3. Cartoon showing of the principle of molecular imprinting [15]

(1) These selected molecule key is mixed with a variety of lock building blocks.

(2) The key and the building blocks are allowed to attach each other loosely or firmly.

(3) Then the complex of the key and building blocks is subsequently formed.

(4) The particular shape lock hole is given after the key is removed and it will not recognise any other shape key except itself.

The "molecule key" may, in principle, be any type of molecule - ranging from small molecules such as drug substances, amino acids, carbonhydrates, or steroid hormones to large molecules such as nucleic acids, co-enzyme, pesticides and proteins, etc. Larger molecular assemblies such as cells and viruses may also be perceived. Quite a lot of them have already been successfully used in molecular imprinting [20-23].

In general terms, *molecular imprinting* can be described as the 'specific organization of polymerisable units about a template molecular that, on subsequent polymerisation, results in the formation of a template specific three dimensional cavity'.

1.2.3 The approaches to molecular imprinting technology

Currently, three approaches to molecular imprinting may be distinguished in terms of the relationship between the template molecule and the functional monomers. They are non-covalent molecular imprinting, metal coordination molecular imprinting and covalent molecular imprinting (***Figure 1.4***).

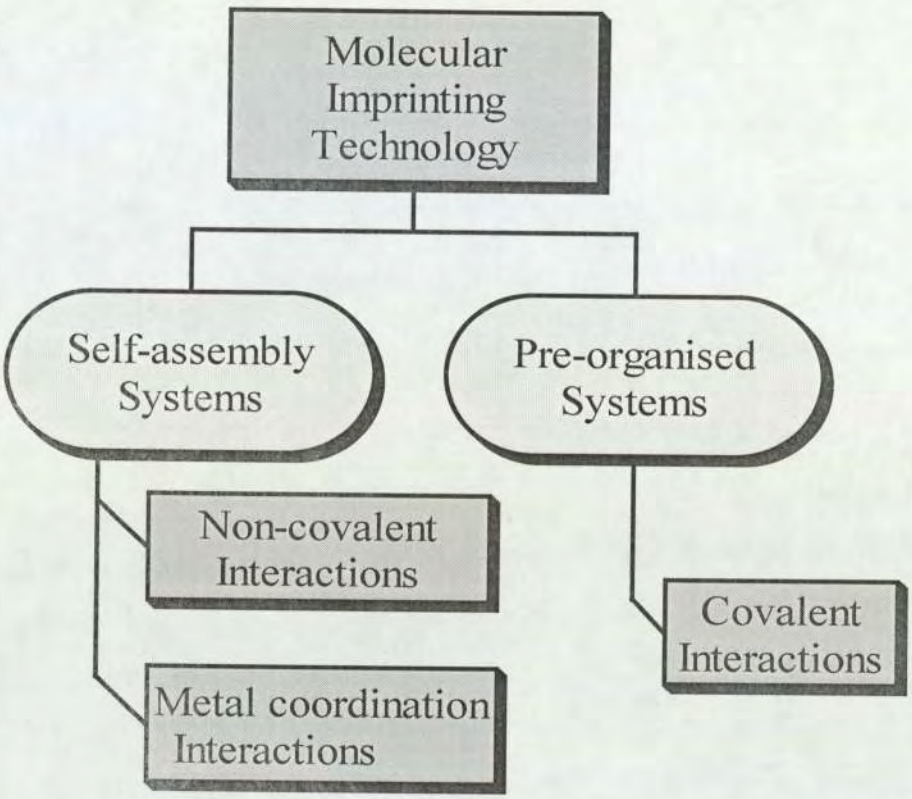


Figure 1.4. Schematic representation of the approaches to molecular imprinting technology

1.3 Molecularly imprinted polymers (*MIPs*)

1.3.1 The principle of molecularly imprinted polymers

The product of molecular imprinting is termed a *molecularly imprinted polymer* (MIP) subsequently (***Figure 1.5***).

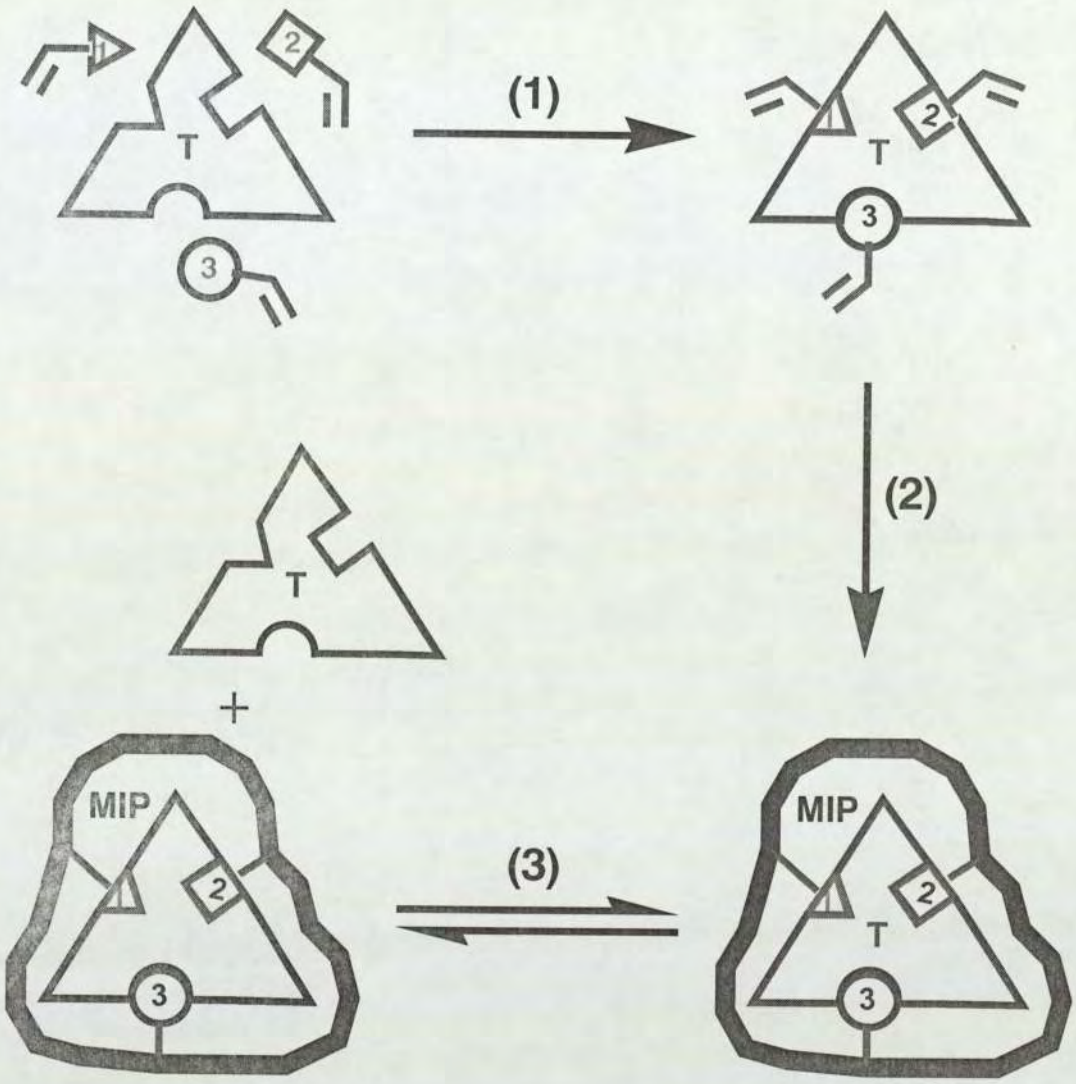


Figure 1.5. Schematic representation of molecularly imprinted polymer

(1) Formation of a pre-polymerisable complex with template (T) and functional monomers (1,2,3).

(2) Polymerisation with excess cross-linking agent to give the molecularly imprinted polymer (MIP).

(3) Template (T) removal/rebinding

Firstly the pre-polymerisation complex which has the template (print molecule) and functional monomers is formed. Then after polymerisation of the monomers in the presence of cross-linking reagents, imprinting is achieved by interactions, either non-covalent or covalent, which occur between complementary functionalities in the template molecule and functional monomer units. Thus when the template is extracted from the polymer, it leaves micro-cavities with a three-dimensional structure and "specific 'guest/host' sites" that are complementary to the templates or print molecules in terms of size, shape, chemical functionality orientations, thus enabling subsequent recognition of the templates.

1.3.2 The approaches to molecularly imprinted polymers

In terms of three different approaches to molecular imprinting technology, the approach to molecularly imprinted polymers can be classified into covalent imprinted polymers, non-covalent imprinted polymers, metal coordination imprinted polymers subsequently. In all of them, non-covalent polymers has become the method of choice in the majority of recently reported work with regards to its great advantages.

1.3.2.1 Covalent imprinted polymers

The covalent imprinted polymers were mainly started and developed by G. Wulff [24-26] in Germany, K. J. Shea [27,28] in California and their coworkers, where the aggregates in solution prior to polymerisation are held together mainly by (reversible) covalent bonds. Usually functional monomers are bound together with templates by covalent forces, then subsequent *co*-polymerisation in the presence of a cross-linking reagent results in the

incorporation of the template-functional monomers complex within a polymer matrix. An important requirement is that the bond between the template and the functional monomers is readily reversed as, to remove the template molecules, the covalent bonds between the template and functional monomers must be cleaved. This cleavage step is quite important and must be performed under conditions that will not profoundly alter the functionality or spatial arrangement at the imprinted site. Finally, the polymer is washed out to leave the vacant imprinted sites.

Rebinding of the template to the binding site is achieved either by the reestablishment of the original bonds or by the non-covalent interactions [29] at the precisely positioned functional groups of the imprinted site. The pioneering working in this area done by G. Wulff utilised the rapid and reversible nature of the boronic acid/diol reaction [30] (***Figure 1.6***).

Figure1. 6. The reversible boronic acid/diol reaction

Wulff synthesized the boronate di-ester of phenyl-α-D-manno-pyranoside (BDPMP) using one molecule of phenyl-α-D-mannopyranoside (PMP) and two molecules of 4-vinylphenylboronic acid (VPBA) by esterification with two OH groups of the sugar [31,32].

The boronate sugar was then co-polymerised with a large amount of acrylic cross-linking monomer to give a macroporous polymeric product (***Figure 1.7***).

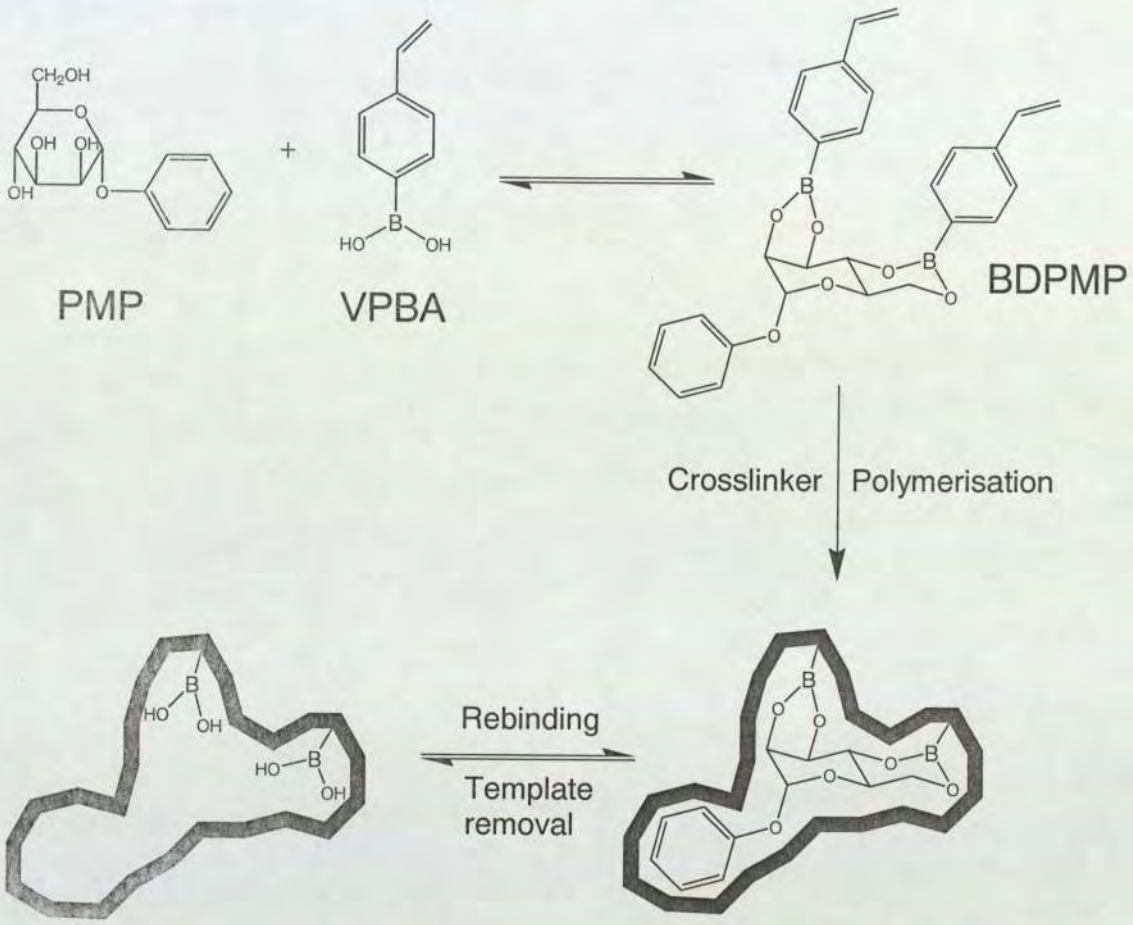


Figure 1.7. Schematic representation of the boronic ester approach to molecular imprinting of PMP using VPMA

In this process, boronic acid was chosen as the binding group (binding site) as this undergoes a rapid and reversible reaction with diols. So the template BDPMP is subjected to radical co-polymerisation with a large amount of crosslinker such as ethylene dimethylacrylate in the presence of an inert solvent (which act as a porogen to give a porous structure). This yields macroporous polymers with large inner surface area and a permanent

structure. Up to 95% of the template can be split off again from the polymers of this type by treatment with water or methanol. Rebinding the template to the imprint site require a change in conditions to those favoring the right side of the equilibrium.

Since the template BDPMP is optically active, the shape of the cavity can be verified by racemate resolution experiments. After the template has been removed, the polymer is equilibrated in a batch process with the racemate of it, whereupon the enantiomer used as the template is preferentially taken up. The selectivity is expressed by the separation factor α which is well known for chromatography (ratios between the distribution coefficients in solution and in the polymer for the D and L forms, K_D/K_L). Depending on the polymer structure and the equilibration conditions, values of α between 1.20 and 6.0 are obtained. With the highest values of α, D-BDPMP can be enriched to 60%-70% *ee* on the polymer in a batch process.

An alternative approach to covalent imprinting was used by Shea and his coworkers [27,28]. Their tactic was similar, in that a template was used to pre-position reciprocal functional groups within a polymeric cavity. However, these studies used the reversibility of the reaction of aromatic ketones with 2-(p-vinylphenyl)-1,3-propanediol (VPD) to give ketals as a means of positioning and removing the template (***Figure 1.8***).

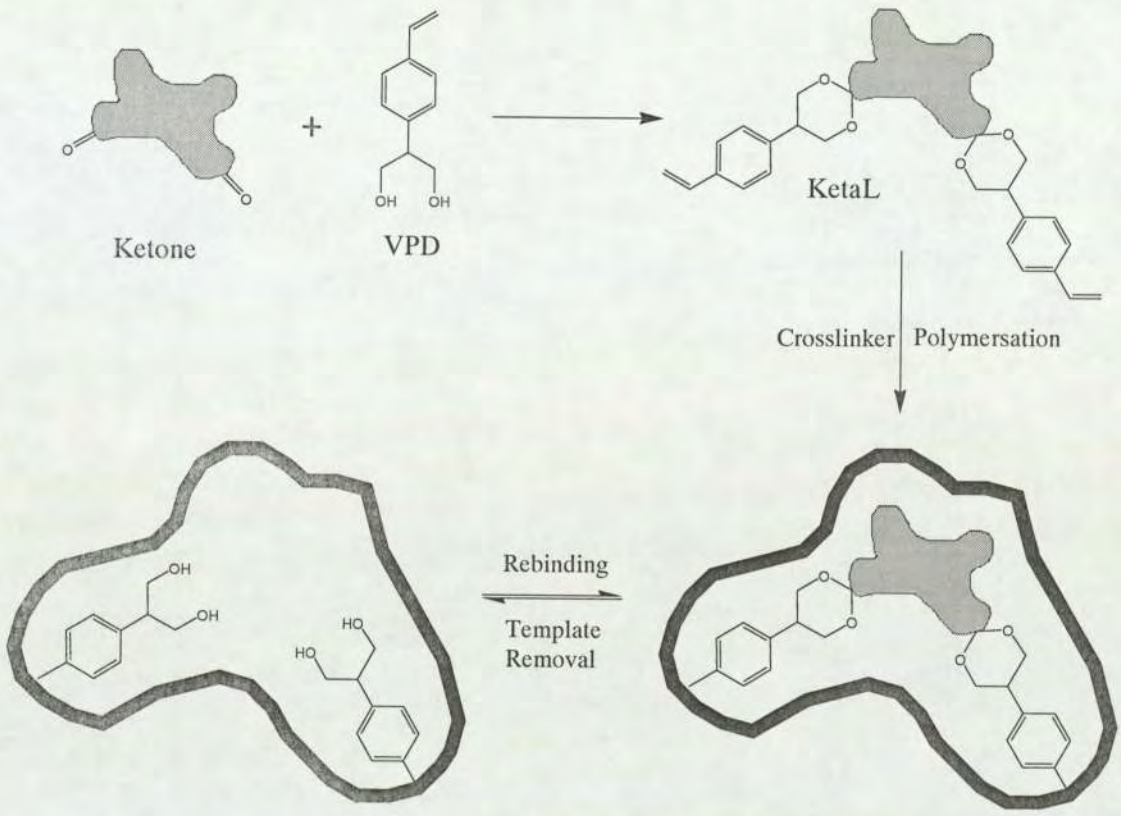


Figure 1.8. Ketone/ketal approach to covalent molecularly imprinted polymer using VPD

By selecting ketones of differing inter-carbonyl distances (***Figure 1.9***), and rebinding them to a single template ketal imprinted polymer, a notable result was demonstrated. That is, significant changes in selectivity can result from either small changes in carbonyl separation or from the initial positioning of the 1,3-diol groups.

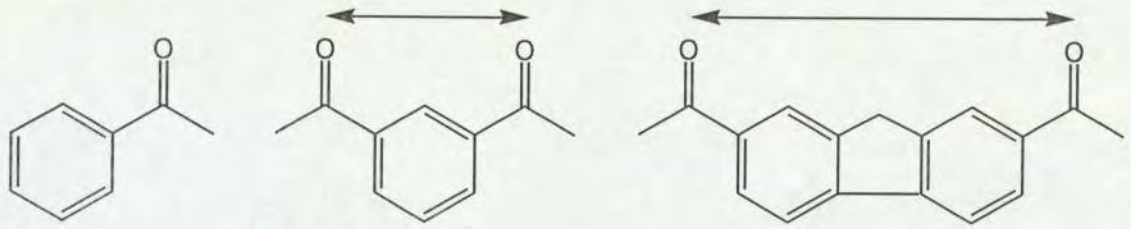


Figure 1.9. Ketones with different carbonyl separations

Furthermore, it was also shown, however, that other factors are at play in determing the kinetic selectivity. The sites may be viewed as hydrophilic regions on a hydrocarbon surface. Substrate rebinding will be depending not only upon spacing of the 1,3-diol groups but also on a hydrophilic attraction.

Schiff bases have also been extensively investigated with regard to their suitability in covalent imprinting [33-37]. Schiff bases are, in principle, suitable for imprinting and subsequent binding, since the position of the equilibrium is favorable. The rate of equilibration is, however, is too slow for chromatographic applications [34]. This has proved to be a significant limitation for this approach, since much of the technology relies on chromatography as a means of assessing the selectivity of the imprinting.

Nevertheless, covalent imprinting has been used to successfully produce selective sorbents for sugars, diketones, dialdehydes, aromatic ketones, transferrin, mandelic acid, amino acids and their derivatives [38].

1.3.2.2 Non-covalent imprinted polymers

Although optimization of the covalent imprinted polymers extended their properties very considerably, only when K Mosbach et al [39] achieved an effective imprinting effect by using exclusively non-covalent interactions could the usefulness of the process be considerably widened.

The fundamental process in the formation of all non-covalent imprinted polymers is the formation of a stable non-convent pre-polymerisation complex between the template molecule and functional monomers. In this approach, the template is non-covalently bound to the polymerisable moiety. Thus, conceptually, the template and functional monomer are allowed to self assemble by non-covalent interaction and the complex is co-polymerized in the presence of a crosslinker to yield a rigid, macroporous product. The integrity of the pre-polymerisation complex is maintained entirely by non-covalent interactions. Typically, proton transfer events, giving rise to ionic species, and hydrogen bonds are central to the formation of the complex and its persistence.

Many investigations in this direction were carried out with L-phenyalanine anilide as the template molecule. This was imprinted with methacrylic acid (MAA) as the functional monomer (***Figure 1.10***). As shown in ***Figure 10***, one methacrylic acid unit forms an electrostatic bond with the template L-phenyalanine anilide and others form the hydrogen bonds. The research groups of K. Mosbach extensively optimized the process, and achieve high selectivities for its template and the other analogues of it.

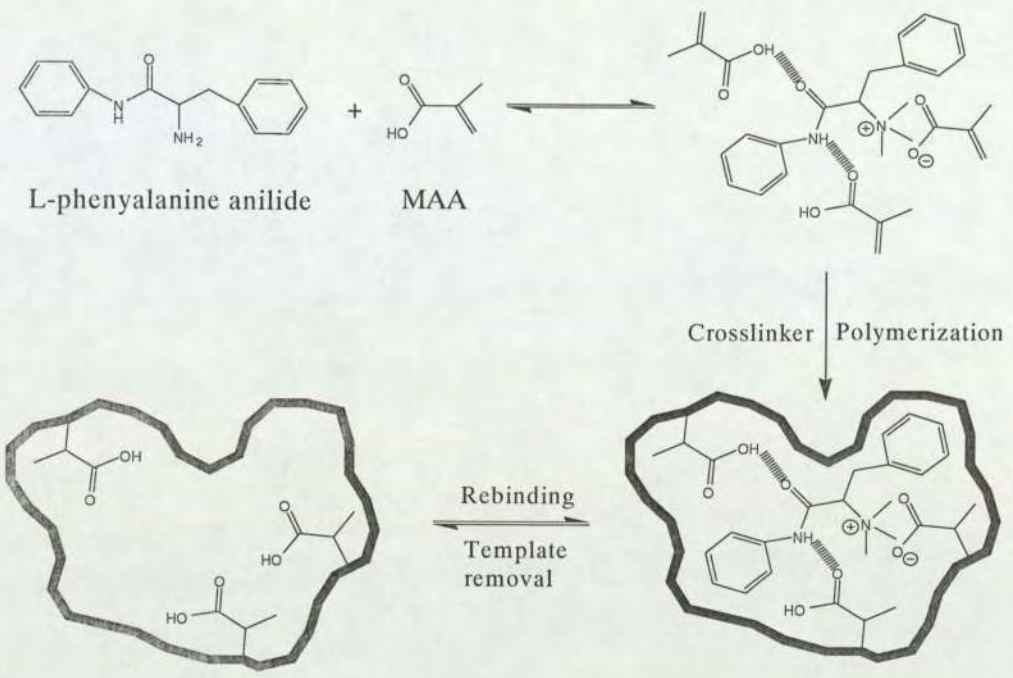


Figure 1.10. Schematic representation of a non-covalent imprinted polymer using anilide as the template

Another example shows the non-covalent process for an amphiphilic template where a pre-polymerisation complex is formed between the template two functional monomers, MAA and the basic 2-vinyl pyridine (2VPy) (***Figure 1.11***). The interactions between the template and functional monomers are hydrogen bonds and ionic forces.

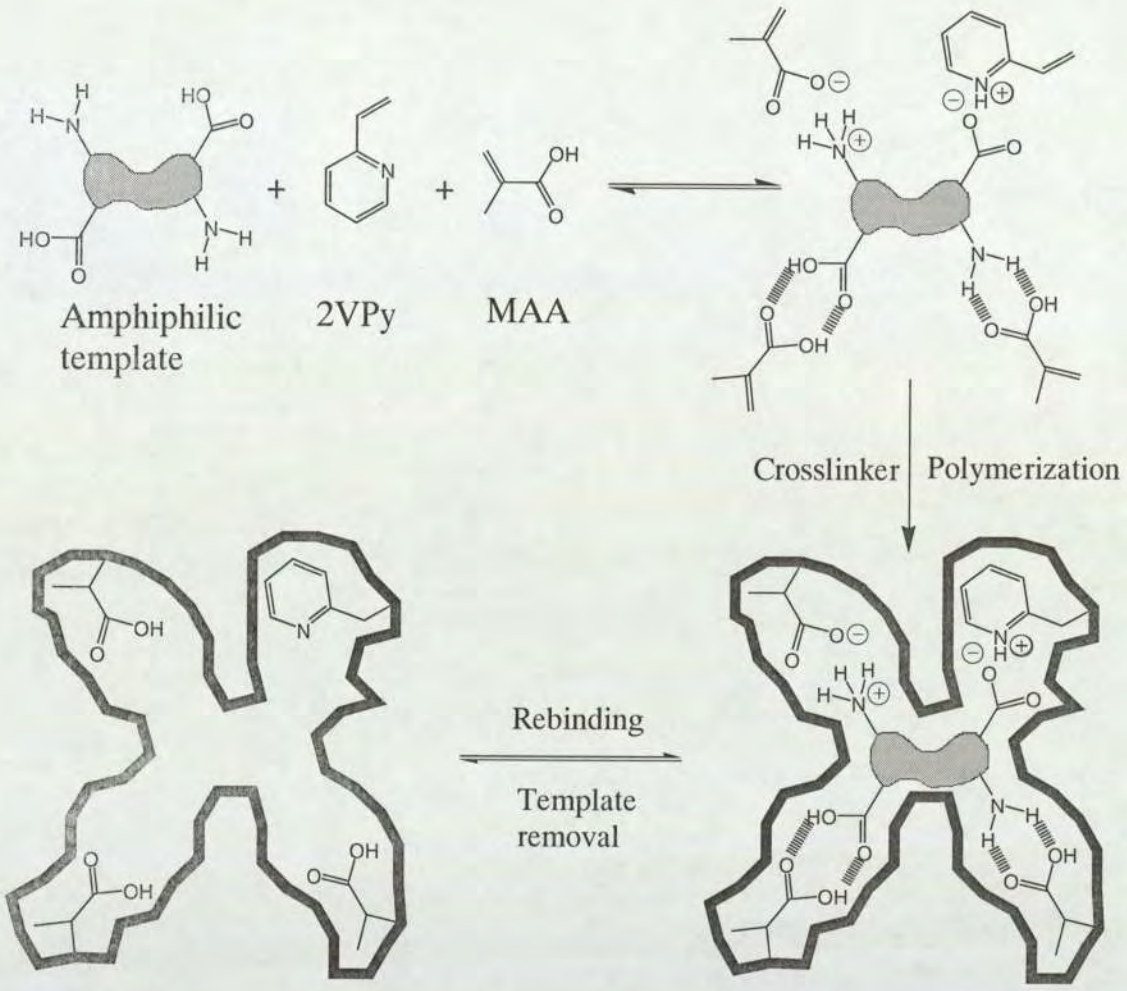


Figure 1.11. Schematic representation of a non-covalent imprinted polymer in the presence of amphiphilic template and using 2VPy and MAA as the functional monomers

Although other role of other interactions, such as hydrophobic, dipole-dipole, π-π coulombic and π-π Van der Waals forces, are not portrayed, it is thought that they can also play an significant role in the template polymerisation [40,41].

Recent advances in the area of non-covalent molecular imprinting having been largely associated with Mosbach's group in Lund, Sweden. Mosbach reported a macromolecular 'entrapment' process [18] and this led them to consider that void left in the acrylamide polymers when the entrapped species was removed had the potential for molecular rebinding.

In the early 1980s interest in non-covalent molecular imprinting increased world-wide after it was shown that non-covalent imprinting was capable of producing comparable results with those obtained using the covalent imprinting approach [42-45]. In addition, the versatility and practical ease of the non-covalent approach made it an attractive technology which gave it a definite advantage over covalent methods. This technology has already become a simple and straightforward method for preparing synthetic polymers of predetermined selectivity.

1.3.2.3 Non-covalent vs. Covalent imprinted polymers

1.3.2.3.1 Advantages of non-covalent imprinted polymers over covalent imprinted polymers

As mentioned before, the majority of recently reported work involves the non-covalent imprinted polymers for their apparent advantages. The advantages of non-covalent imprinted polymers over covalent imprinted polymers have been discussed [46] and summarized below in ***Table 1.1***.

Non-covalent imprinted polymers	*Covalent imprinted polymers*
No chemical modification of the template molecule is required	*Chemical modification of the template molecule is required*
A general strategy can be identified	*Templates must be individually considered*
Good kinetics	*Kinetics can be very poor*
Protocols are simple	*Chemistry can be complex*
Simple washing to remove the template	*Chemical cleavage to remove the template*
Broad distributions of binding affinities	*Narrow distributions of binding affinities*

Table 1.1 Advantages of non-covalent imprinted polymers over covalent imprinted polymers

The most obvious advantage is that no specific covalent modification of the template molecule is required because no chemical cleavage of the binding forces is needed. Although non-covalent interaction is weak, it could be enhanced by allowing a multitude of interaction points simultaneously. Additionally, the use of non-covalent interactions in the process of imprinting closely resembles the recognition pattern observed in nature. Furthermore, it may be difficult to find suitable covalent system for every molecule given in which the bonds should be able to easily cleaved and rebound.

In conclusion, the simplicity and elegance of non-covalent imprinted polymers makes it an attractive technology. Indeed, it allows specific molecular recognition approaches to be considered in areas where previously complexity has prevented their use.

1.3.2.3.2 Advantages of covalent imprinted polymers over non-covalent imprinted polymers

Although the approach to non-covalent imprinted polymers has been widely investigated and applied, it has inherent disadvantages. Because the non-covalent interactions between the template and functional monomers are weak, therefore, the population of the complex species is governed by equilibrium. Thus, an excess of functional monomers is necessary in order to complete the template-functional monomer pre-polymerisation complex and to maintain the complex stable under the polymerisation conditions. As a result, a fraction of functional monomers is randomly grafted in the polymer matrix, causing the formation of non-specific binding sites. Furthermore, several species of template-functional monomers pre-polymerisation complexes could be present and possibly imprinted in the case that a template possess more than one functional group capable of interaction with functional monomers. This would cause a heterogeneous property of the binding sites in term of affinity.

In contrast, in covalent imprinted polymers, the above shortcomings are overcome. Stronger binding between the template and functional monomers than a non-covalent approach is obtained and the functional monomers will strictly bind the favorable sites of the template and high affinity of the template itself may be achieved after rebinding.

As can be seen, both approaches have advantages, therefore, an appropriate approach need to be carefully chosen depending upon the template to be imprinted. The combination of these two approaches may find a new application, and in practice M Whitcombe et al. [29] proposed an excellent approach to cholesterol imprinting in which cholesterol 4-

vinylphenylcarbonate ester was imprinted by a covalent method and after the ester cleavage the binding sites capable of hydrogen bond formation with cholesterol remained in the polymer. Thus, cholesterol is bound into the empty cavity through a non-covalent hydrogen bond (***Figure 1.12***).

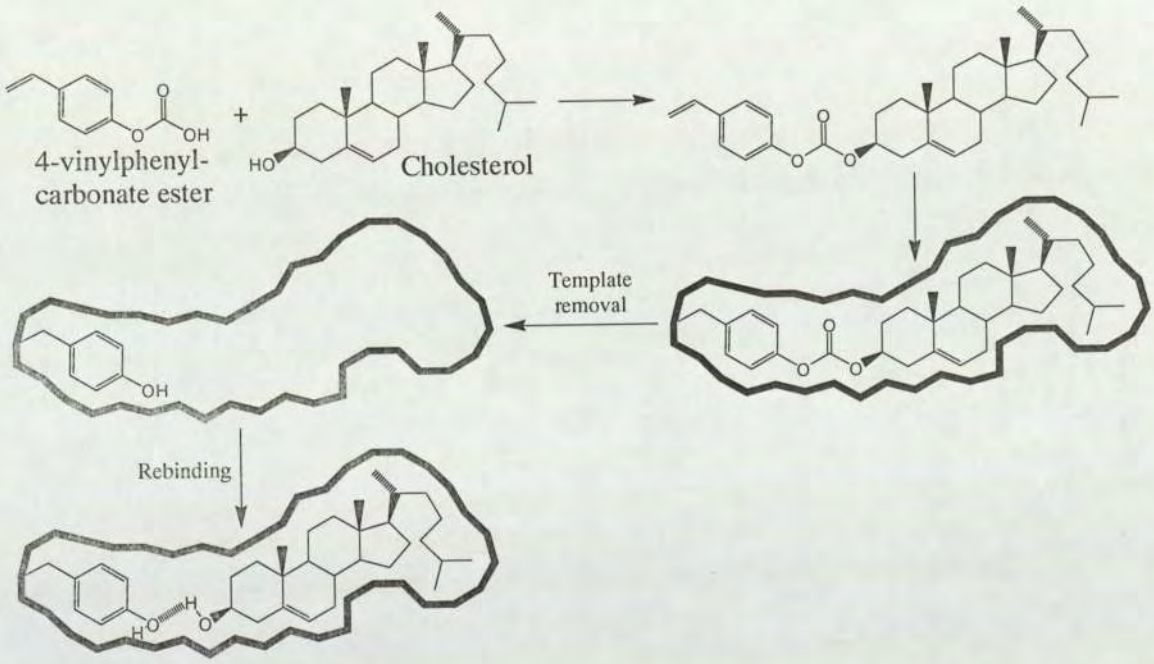


Figure 1.12. Schematic representation of the cholesterol imprinting

1.3.2.4 Metal coordination imprinted polymers

A very promising type of binding during the process of polymerisation and later in the final polymer can be obtained by coordinative bonds to metals in a self-assembly system.

This kind of polymer has big advantages that the strength of coordination bond can be controlled by experimental conditions, an excess of binding groups are not necessary because definite interactions occur during the polymerisation. Moreover, as the subsequent rebinding of the template to the polymer matrix is so rapid, even rapid chromatography is possible [42,43].

From 1991, F. H. Arnold and P. K. Dhal et al. investigated the bonding of imidazole-containting compounds with Cu^{2+} complexes (***Figure 1.13***) in great detail [49-52]. Model experiments were first carried out in which bisimidazoles with various distances between the imidazole groups were used as templates to position polymerisable iminodiacetate groups in the polymer [51,52]. These experiments were aimed at developing an effective recognition for proteins that depends on the correct spatial arrangement of a few binding sites on a polymer.

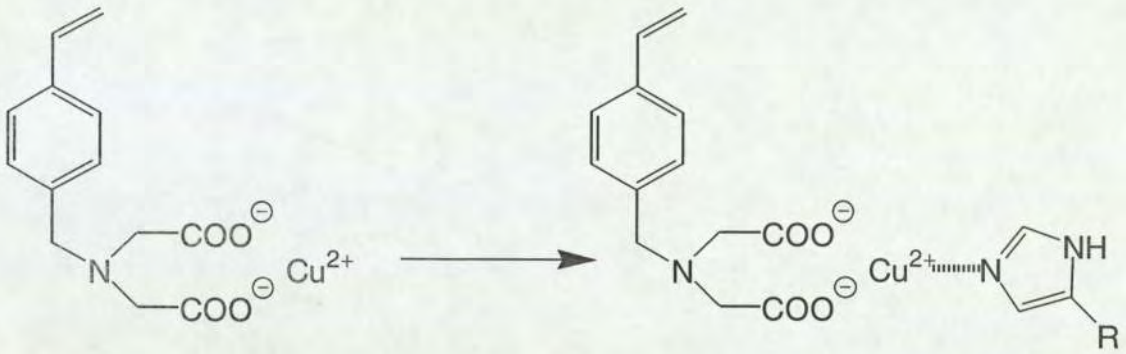


Figure 1.13. Representation of an imidazole-containing compound with Cu^{2+} complex

Recently, J. Matsui prepared a metal coordination imprinted polymer having a porphyrin-based center [53]. The imprinted polymer was conducted by modifying a porphyrin zinc complex to construct a three dimensional binding site on a porphyrin plane, in which a template molecule, 9-ethyladenine (9EA), is bound via coordination by a porphyrin center and hydrogen bonding by MAA (***Figure 1.14***). The result of the template rebinding showed a great character of recognition for the multi-point interactions between the template and functional monomers.

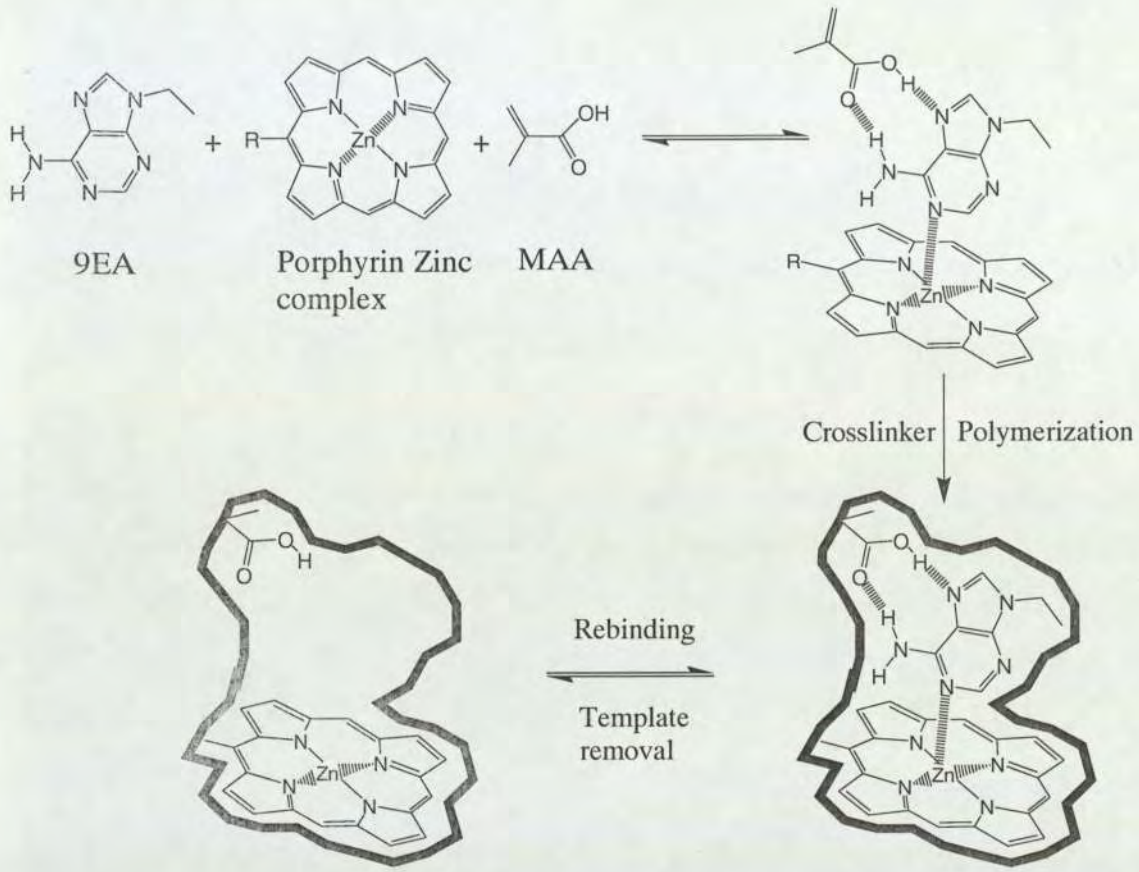


Figure 1.14. Schematic representation of molecular imprinting of a metal coordination imprinted polymer using 9EA as the template and a porphyrin zinc complex, MAA as the functional monomers

For the reason that the metal coordination binding forces between the template and functional monomers are stronger than is the case with non-covalent imprinting, it is difficult to wash the template out of the polymer matrix using extraction sometimes. Therefore, the use of chemical cleavage of the metal coordination binding forces might have to be considered.

Thus, depending on the strength of the binding forces, metal coordination imprinted polymers are somewhere between covalent imprinted polymers and non-covalent imprinted polymers.

1.4 Preparation of molecularly imprinted polymers

1.4.1 Template molecules

To be successfully imprinted, a template must contain level of functionality that can be paired with reciprocating moieties present on a polymerisable unit. Bowman *et al.* [54] observed that, in general, small, multi-functional, templates give rise to highly specific imprints whilst larger, mono-functional, templates produce imprinted sited which are less specific. In addition, a complex molecular backbone results in an imprint of more specific three-dimensional structure, which is likely to lead to a more highly specific imprint than a template with simple linear geometry [46].

For small, uncharged, solute molecules the strength of intermolecular non-covalent interactions is depend upon the local environment which is, in turn, defined by the properties of solvent [55-57]. In general terms, the stability of the pre-polymerisation complex is favored in non-polar solvents. Therefore solubility in potential porogens must be considered when assessing the suitability of a compound for molecular imprinting. For polar template molecules, there is usually a trade off between imprint specificity and solubility. If the solubility of the template is such that a polar solvent is required, then the complex will be less stable and the imprint less specific [58]. However, this is suitable for covalent imprinted polymers due to the strength of covalent bond not affected by solvents.

1.4.2 Functional monomers

The purpose of the functional monomers is twofold. Firstly, it undergoes regiospecific, weak, complementary, interaction with a particular moiety of the template molecule. Secondly, it contains a polymerisable unit. The choice of functional monomer should therefore be based upon the functionality of the molecule to be imprinted. The functional monomers used in covalent and metal coordination imprintings are general, compared with the functional monomers used in covalent imprinting that interaction sites with the template molecule need to be considered and designed. By far the most accessible are either polyacrylate-based or polyacryamide-based systems. Another approach is the polystyrene-based system, used to a lesser extent.

In self-assembly imprinting systems, MAA has been used extensively as a functional monomer as it fulfils the essential criteria of pocessing a polymerisable moiety and functionalities that can interact with opposite moieties in a reciprocating arrangement. The carboxylic acid group is capable of acting as both a hydrogen bond donor and acceptor. Although liquid phase dimerisation of MAA might be anticipated, it does not appear to be a significant practical problem [59]. Other typical functional monomers usually used are listed carboxylic acids (acrylic acid, vinylbenzoic acid), sulphonic acids (acrylamido-methylpropanesulphonic acid), and heteroaromatic (weak) bases (vinylpyridine, vinylimidazole) (***Figure 1.15***).

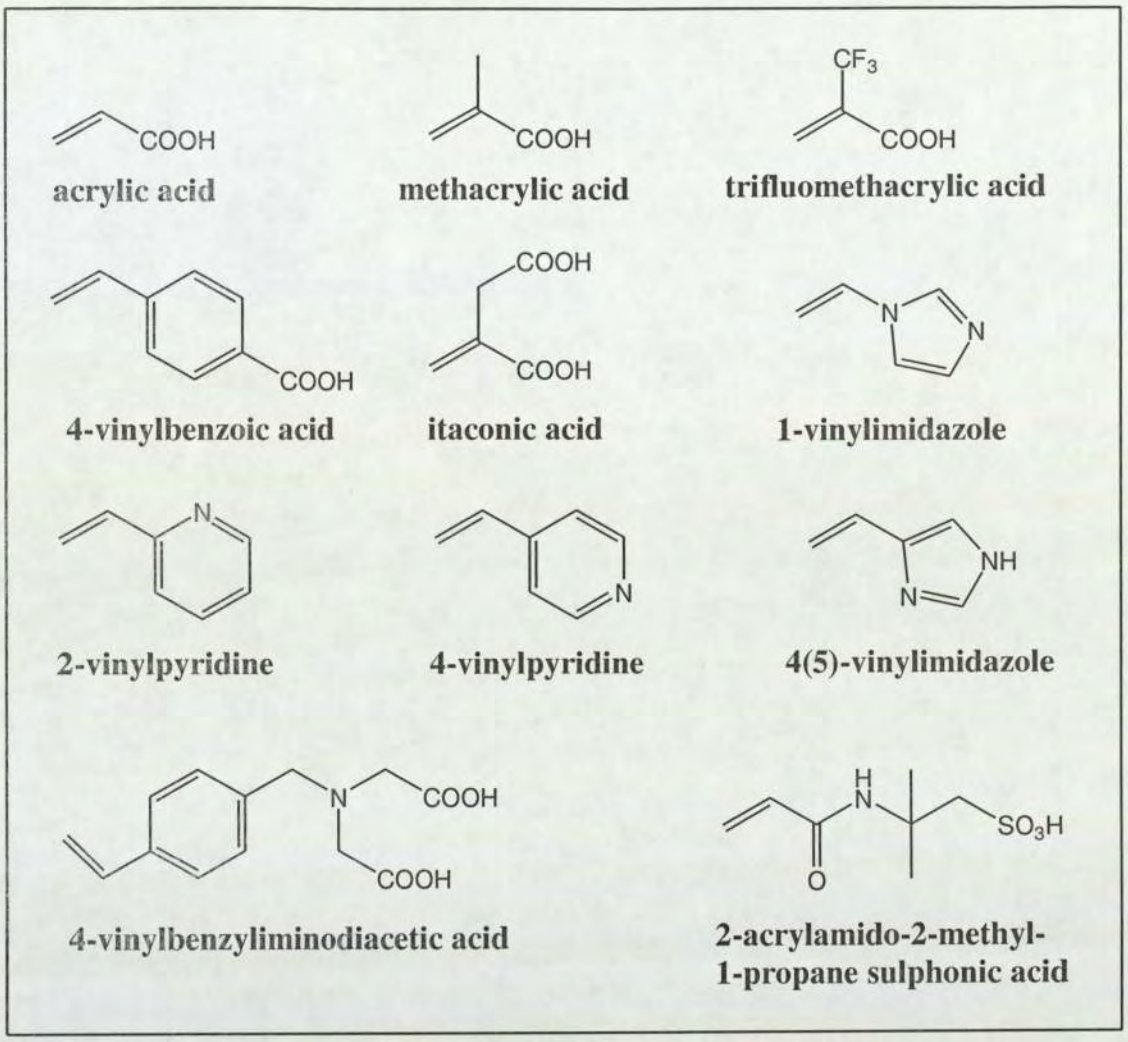


Figure 1.15. Selection of functional monomers used in self-assembly imprinting system

1.4.3 Crosslinkers

Since ligand accessibility and template definition are maximal in stiff polymers, and good kinetics and equilibration are favoured in flexible polymers, the choice of crosslinkers must be inevitably be a compromise [60]. It should be point out that, in addition of the type and proportion of the crosslinkers involved, the porogenic solvent and method of polymerisation both also affect the macro structure of the polymer in terms of porosity and internal surface area. Furthermore, the solubility of the crosslinker itself in the pre-polymeric solution and the solubility of the monomerised template species reduce the number of possible alternatives. Nevertheless, several different crosslinkers have been tried with different degrees of success. Originally, isomers of divinylbenzene were used for crosslinking of styrene and other functional monomers into polystyrenes. Later, it was found that acrylic or methacrylic acid based systems could be prepared with much higher specificity. Ethylene glycol dimethacrylate (EDMA) and trimethylolpropane trimethacrylate (TRIM) are presently commonly employed in several systems.

Wulff *et al.* [61] observed that, for EDMA, tetramethylene dimethacrylate and divinylbenzene, selectivity was not observed with crosslinker proportions from 0% to ~10%. An increased towards 50% gave a gradual rise in selectivity and, between 50% and 70%, a more rapid improvement was noted. Further increase in the content of crosslinker to 95% causes a further increase in selectivity. In general, the higher the proportion of crosslinker the great the selectivity. In terms of structural variation of the crossinker, they concluded that 'although many different crosslinkers were evaluated, including some synthesized for the first time, EDMA was the most suitable for most imprinting processes'

[30]. He also stated that EDMA gave better selectivity and resolution than some new optically active crosslinkers. More importantly, EDMA was also the cheapest and most easily purified.

Due to the very high degree of crosslinking is necessary for achieving specificity, only a limited number of crosslinkers have been utilized (***Figure 1.16***).

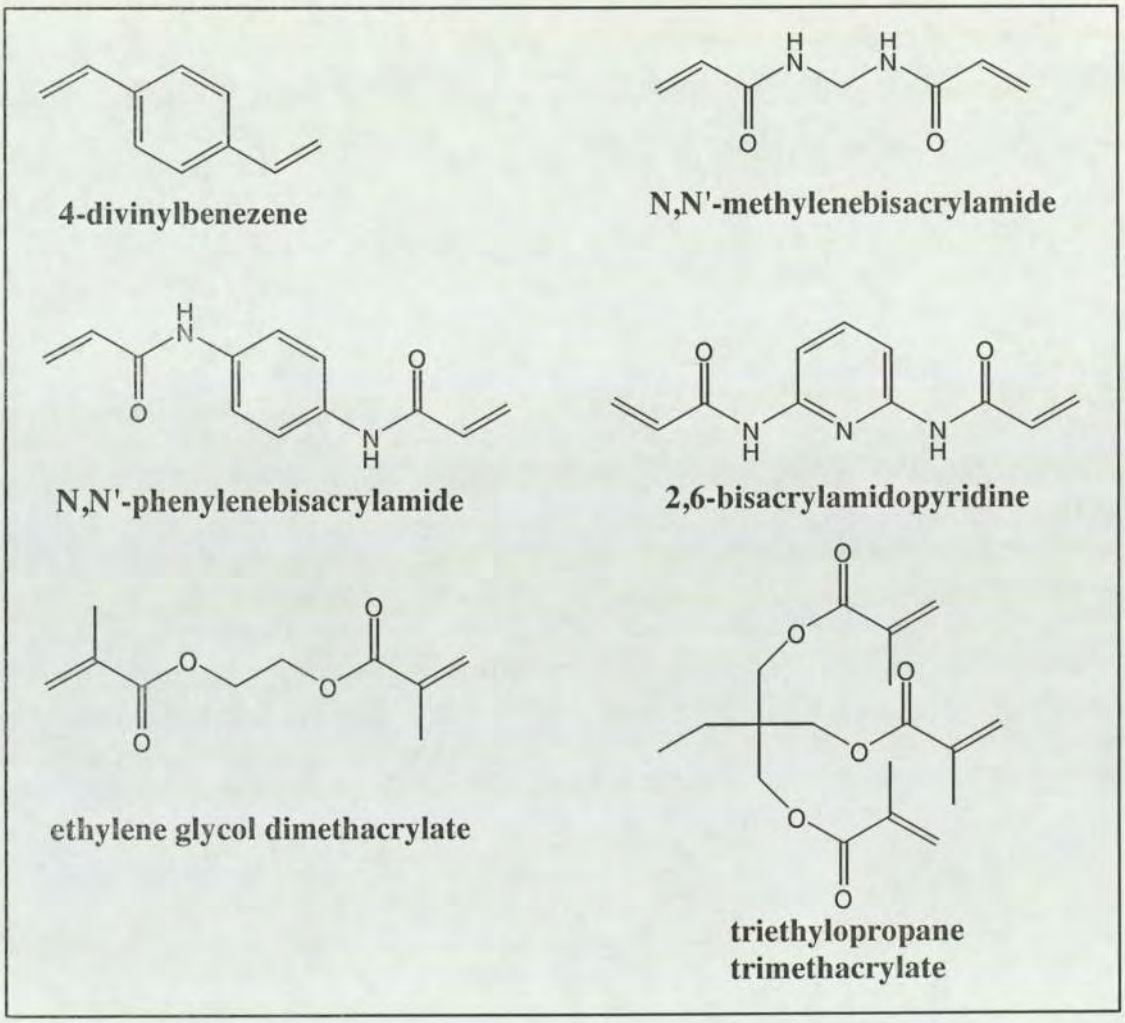


Figure 1.16. Selection of crosslikers

Recently, alternative crosslinkers have been investigated. Tri- and tetrafunctional acrylate crosslinkers, such as pentaerythritol triacrylate (PETRA) and pentaerythritol tetraacrylate (PETEA), have been used for the preparation of peptide-selective molecularly imprinted polymers. In addition to crosslinkers which are non-polar or weakly polar in nature, crosslinkers containing functional groups have been studied. As an example, a crosslinking monomer containing two amide functionalities flanking a pyridinyl moiety has been used in an imprinting protocol against barbiturates [29].

1.4.4 Solvents/Porogens

The solvent plays an important role in the outcome of a molecular imprinting process. Macroporous polymers are formed if polymerisation is carried out with a high proportion of crosslinker in the presence of a solvent. It is formation of the polymer around these solvent molecules that produces the pores. The solvent is therefore termed a *porogen*. Sellergren and Shea [62] showed that the porogen has a large influence on polymer morphology, but concluded that there was no obvious relationship between morphology and MIP selectivity. They concluded that it was the hydrogen bonding capacity of the porogen that was the main influence on selectivity.

The porogen effect is particularly pronounced in self-assembly systems. In general, it is thought that the significant interactions associated with the pre-polymerisation complex which contains the template and functional monomer are polar and, since the strengths of polar interactions are maximal in apolar solvents, the more apolar the solvent the more stable the pre-polymerisable complex [41, 45, 63]. Due to a positive correlation between

stability of the pre-polymerisation complex and recognition properties of the resultant MIP, generally, the more polar the porogen, the weaker the resulting recognition effect becomes.

On the other hand, the porogen influence on the structure of the prepared polymers may compensate for this apparent drawback on the specific surface area and the mean pore diameter is dramatically dependent on the type of porogen used. Thus, acetonitrile - a fairly polar solvent (dielectric constant, e = 36) - leads to more macroporous polymers than chloroform (e = 5) [62]. A lower surface area and a lower macroporosity may lead to diminished recognition, because of lower accessibility to the sites.

In the recognition step similar questions about the choice of solvent arise. Since all non-covalent forces are influenced by the properties of the solvent, non-polar solvents normally lead to the best recognition. When applying the polymers to gradually more polar solvents, the recognition is diminished. Also, in practical terms the choice of solvent that is as non-polar as possible yet still provides a system in which templates and functional monomers are freely soluble.

1.5 The imprinting process

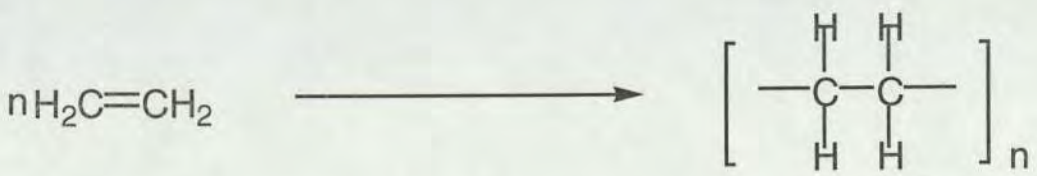
1.5.1 Pre-polymerisation complexation

It is generally accepted that hydrogen bonding, metal coordination force, steric effects and acid/base proton transfer equilibria are the prime interactions of self-assembled systems in the formation of stable pre-polymerisation complexes. In pre-organized systems, covalent interactions make the stabilities of pre-polymerisation complexes.

Furthermore, as the crosslinkers commonly used also possess functionality, they may also be involved in the formation of the pre-polymerisation complexes. It should be noticed that for self-assembly systems, the magnitude of a non-covalent or metal coordination interaction between reciprocal groups is not fixed and the stability of a pre-polymerisation complex is affected by solvent/porogen as well.

1.5.2 Polymerisation

Reported MIP systems have been random *co-* or *ter-* acrylic addition polymers based upon the classification of Carothers [64]. An addition polymer has a structural unit with the same molecular formula as the monomers (***Figure 1.17***).



Figurre 1.17. Representation of the cross-linking unit of monomer for MIPs

The polymerisation process consists of three stages: initiation, propagation and termination. During the initiation stage a reactive species is formed that starts the polymerisation of the relatively unreactive vinyl compounds. Propagation results in the formation of a high molecular weight polymer. The termination stage is a deactivation process which results in the formation of the stable polymeric product. This arises when radicals combine either by combination or by disproportionation (***Figure 1.18***).

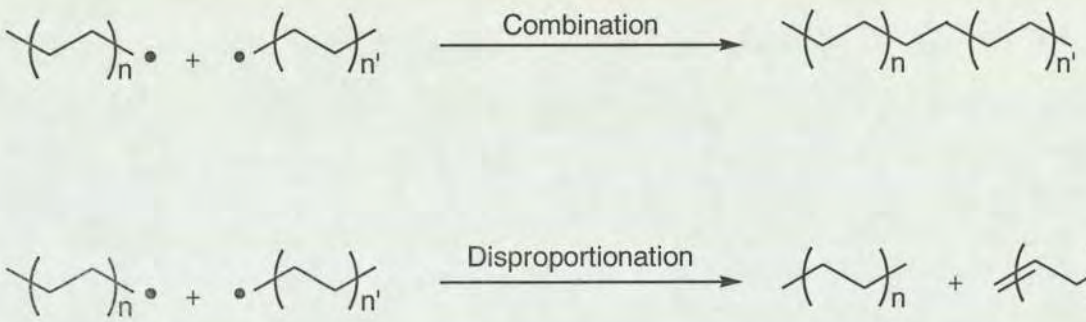


Figure 1.18. Representation of two different formations during the polymerisation of MIPs

Only free radical polymerisation, which requires the formation of reactive free radical species to initiate polymerisation, appears to have been used to form MIPs. Free radicals are produced by the decomposition of an initiator species by the action of heat or light. Commonly used initiators are benzoyl peroxide and azobis compounds such as AIBN or ABDV (***Figure 1.19***).

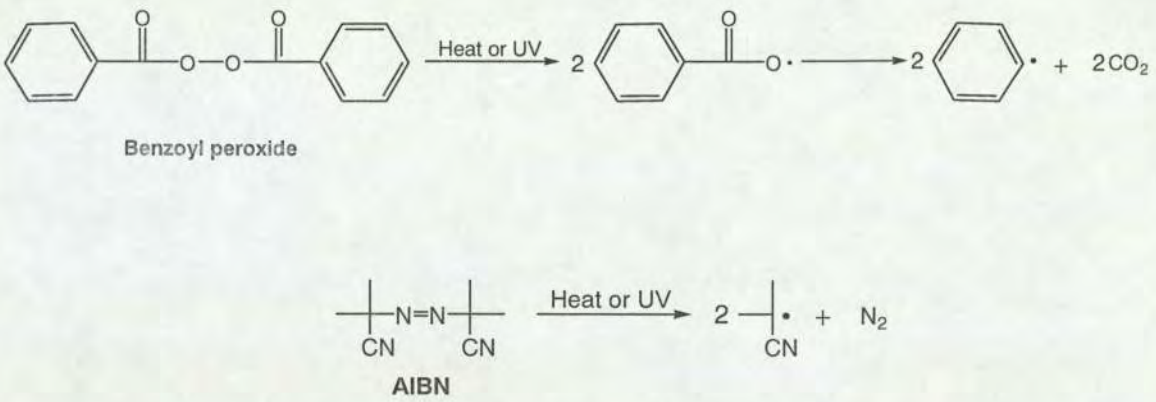


Figure 1.19. Representation of the formations of reactive free radical species of Benzoyl peroxide and AIBN

Once formed, the free radical species (R˙) attack the double bonds of the monomer to give rise to an initiated monomer radical. This, in turn, rapidly attacks further monomer to form a polymer chain (***Figure 1.20***).

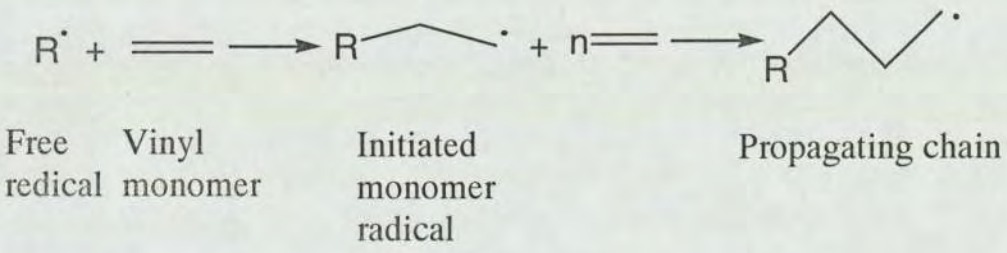


Figure 1.20. Representation of radical initiation and chain propagation

AIBN can be decomposed to produce free radicals by the action of heat (~60°C) or light (UV 366nm). It has been shown that polymerisation temperature has a large influence on the selectivity and binding affinity of MIPs [65]. For some MIPs UV initiation is not

available, *e.g* when the template is UV sensitive, and thermal initiation is required. In this situation ABDV is favoured since it thermally decomposes at lower temperature (45°C) than AIBN.

1.5.3 Post-polymerisation processing

MIPs are generally produced as opaque, vitreous, brittle and plastics. To be of practical use MIPs need to be reduced to a fine particulate material of uniform particle size. Typically, this is achieved by grinding processes, either by hand in a mortar and pestle or by mechanical means. The material is then sieved to give a powder of fixed upper particles size. Depending on the final application, this material is then precipitated over a pre-determined time period to remove material that is too fine. Recovery of MIP particles of controlled particle size after sedimentation can be as low as 40%.

1.5.4 Template recovery

This step is achieved by washing the polymer with a sufficiently polar solvent system to remove the template from the imprinted site. For non-covalent imprinted polymers, methanol or acetonitrile with the addition of acetic acid is commonly used. This has been carried out using soxhlet exhaustive hot extraction or by simple stirring, filtering and resuspending protocols. For metal coordination imprinted polymers and covalent imprinted polymers, binding break or cleavage need to be done.

1.6 Physical and chemical characteristics of MIPs

The end product of the MIP production process, in the vast majority of cases, is a fine powder containing a population of vacant binding sites with a small proportion of non-recoverable template trapped deep within the particle matrix. For monolithic polymers binding sites are two basic types: internal and external. External sites are the product of random cleavage of the cage structure of the total three-dimensional site. Their geometry depends upon the orientation of the cavity constituents relative to the fracture plane. These are believed to contribute only a small amount of the total binding sites, but may be of great significance in terms of site heterogeneity. Of more importance are the internal binding sites which are both entire and in much higher abundance.

Since many MIPs are acrylic *co*-polymers they possess many of the attributes that make this type of polymer such a useful material. They are highly resistant to physical factors such as mechanical stress, high pressures and elevated temperature and they are chemically inert, coping with acids, bases, various organic solvent and metal ions without loss of selectivity. Moreover, shelf life at room temperature is measured in years and with minimal care MIPs can cope with long periods of continual use [18].

1.7 Applications of MIPs

The fact that MIPs have the power to differentiate between structural and spatial minutiae has been instrumental in bringing MIP methodology to the attention of the scientific community. There are lots of significant works which impact upon the chemistry industry, research community, health care and environmental control areas. A non-exhaustive list of

highlighting applications for such technology includes downstream processing, biochemical monitoring, affinity chromatography phases [18], environmental monitoring [22], sample clean up [66], specific drug/analyte selection [67], biosensor [68], antibody/receptor binding mimics [69,70] and enzyme mimics/catalysis [71,72], *etc* (***Figure 1.21***).

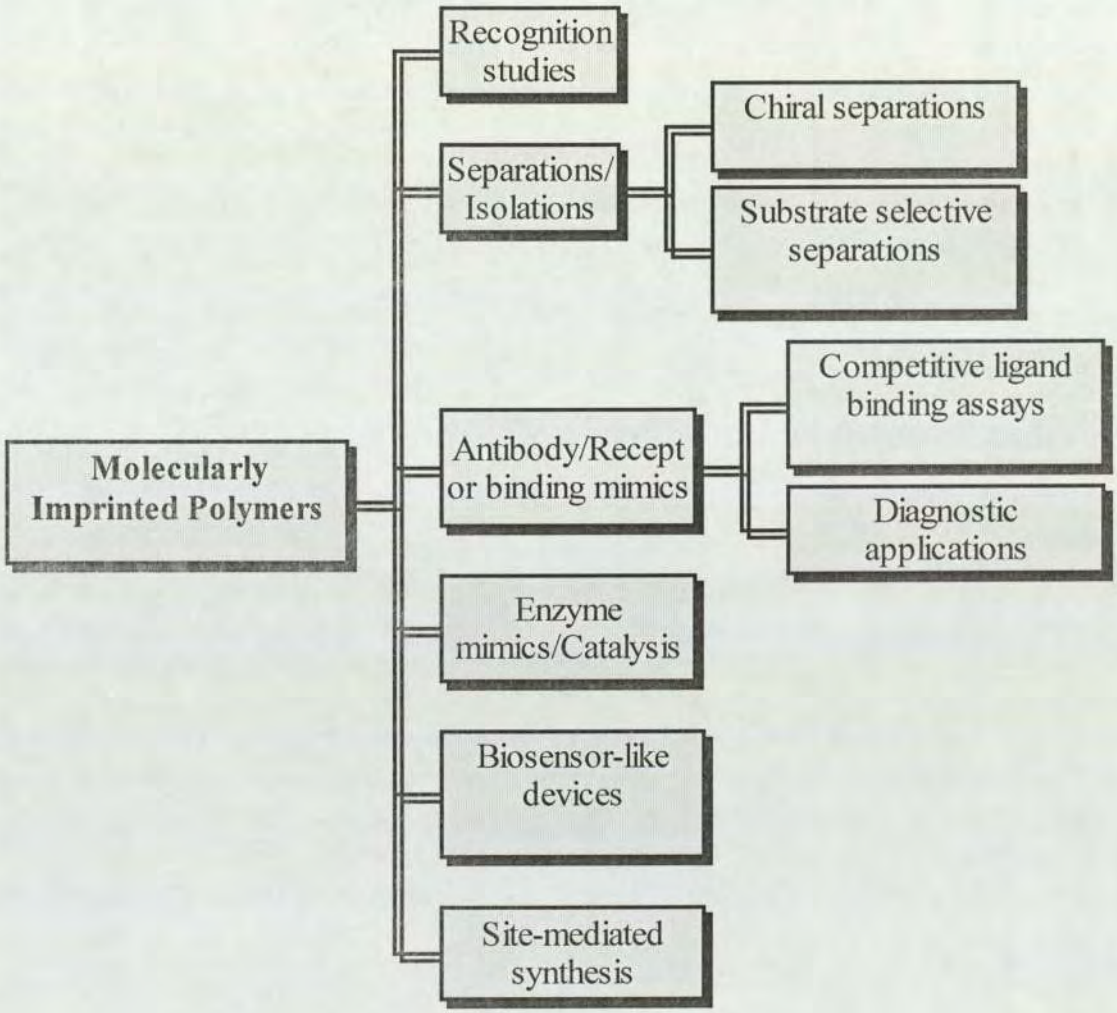


Figure 1.21. Some applications of MIPs

One attractive application is combinatorial screening. The phenomenon of cross-reactivity inherent in the current generation of MIPs was the basis of a novel method currently under investigation for the high throughout screening of complex mixtures, particularly combinatorial libraries. Bowman *et al.* [54], who constructed a HPLC column containing a mixture of MIPs each imprinted with a different active compound, found that it was possible to identify β-blocking drugs from a 'bookshelf' of diverse compounds. A potential advantage of such a system is it can be self-optimizing. Berglund *et al.* have recently described selection of phage display combinatorial library peptides with affinity for a yohimbine imprinted methacrylate polymer [73].

As part of drug discovery programme involving automated synthesis targeting new treatments for tuberculosis, we tried to investigate the phenomenon of molecular recognition by the molecular imprinting technology with a view to constructing a high-throughout fluorescence-based assay. This technique can optimistically be used as a pre-screening MIP-based process to reduce the large numbers of robotically-prepared anti-tubercular N^1-benzylidene pyridine-2-carboxamidrazones compounds (***Figure 1.22***) discovered in this laboratory [74,75] to a more manageable set for biological screening or to discover other new potential drug candidates which are similar in size, shape and hydrogen-bonding profiles to existing drugs.

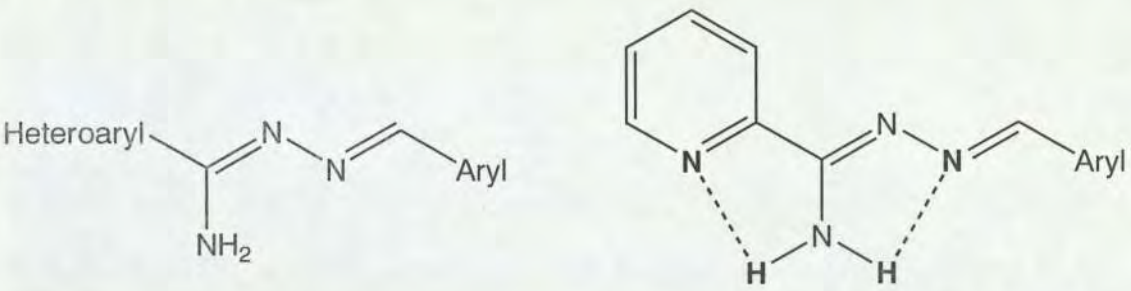


Figure 1.22. Generic structure of the N^1-benzylidene pyridine-2-carboxamidrazones

1.8 Fluorescent MIPs

The MIPs combined with fluorescence are termed *fluorescent MIPs*. The unique key of fluorescent imprinted polymers is that they were prepared using functional monomers with the fluorescent probes attached. Fluorescence is an attractive detection methodology for sensors on account of its high sensitivity and non-destructive nature. After extracting the template from the MIPs, the fluorescently-labelled cavities remain. Changes in fluorescence can be detected by fluorimetry compared with its background levels (the fluorescence of non-imprinted polymers) due to the rebinding of their templates and different test imprint compounds. There is no specific requirement of certain structural features, or for the template itself to be fluorescent.

Previous work in the field of fluorescent MIP focused on developing fluorescent receptors, such as the specific sensors for cAMP [68], D-fructose [76] and L-tryptophan [77]. Liao *et al.* [77] reported that the sensor also exhibited enantioselcetivity for the template L-tryptophan and showed advantages that no *de novo* design of the complementary binding

site is required and this technique does not rely on any specific structural features of the template molecule or prior knowledge of its three-dimensional structure.

In contrast, what we did is try to develop a pre-screening MIP-based process by monitoring the fluorescence. We now have synthesized several hundred members of N^1-benzylidene pyridine-2-carboxamidrazones compounds. The site of action is not determined. Nevertheless, by making the assumption that the members of this chemical class all act at the same receptor in the same way, and by appropriate superposition of the active compounds, one may effectively map out the volume of the receptor. Some previous work was done in our lab [78] to construct a number of fluorescent MIPs. The bonds between the reciprocal functionalities on template/test compounds and the fluorescent monomer units are hydrogen bonds. Furthermore, it was suggested that test molecules which are apparently too big to enter the empty imprinted cavities gave almost no reduction of fluorescence. Other "suitable" test molecules rebound which can quench the fluorescence are supposed to have entered the imprinting cavities due to the same reduction can be obtained when the templates themselves are rebound. This, on the other hand, may give an indication of how well the test molecules may bind into the cavities and interact with the putative receptor. This approach is especially amenable to high-throughput automation commensurate with the original automated synthesis.

Towards the overall goal of a fluorescent receptor mimic, initial recognition and selectivity studies from fluorescent MIPs imprinted with various N^1-benzylidene pyridine-2-carboxamidrazones are now presented.

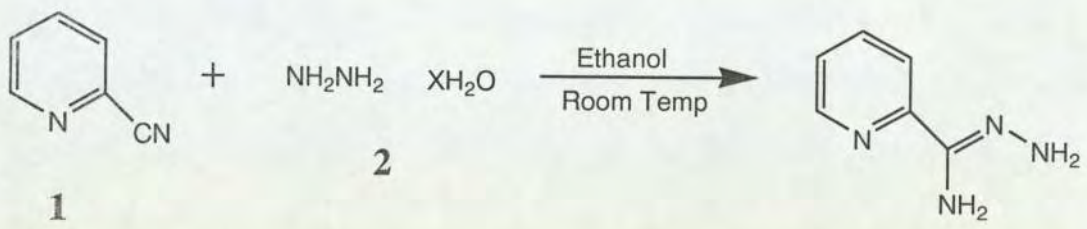
Experimental

2.1 General Methods

Unless otherwise noted chemicals were obtained from Aldrich (Sigma-Aldrich Company Ltd, Dorset, UK) and Lancaster (Lancaster Synthesis Ltd, Morecambe, UK) and used without further purification. Proton NMR spectra were obtained on a Bruker AC250 instrument operating at 250MHz as solution in $CDCl_3$ and referenced from δ 7.27 ppm. Infrared spectra were recorded as KBr discs on a Mattson 3000 FTIR spectrophotometer. Atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) was carried out on a Hewlett-Packard 5989B quadrupole instrument connected to an electrospray 59987A unit with an APCI accessory and automatic injection using a Hewlett-Packard 1100 series autosampler. Melting points were obtained using a Reichert-Jung Thermo Galen hot stage microscope and are corrected.

2.2 Preparation of the templates (print molecules)

2.2.1 Synthesis of pyridine-2-carboxamidrazone



1 **2**

2-Cyanopyridine **1** (59 g, 567.31 mmol) dissolved in ethanol (90 ml) was treated with hydrazine hydrate **2** (70 ml) at room temperature. The mixture was stirred at room temperature for three days after which a pale yellow precipitate was collected by filtration. The solid was washed with hexane (6×100 ml) and dried under vacuum overnight.

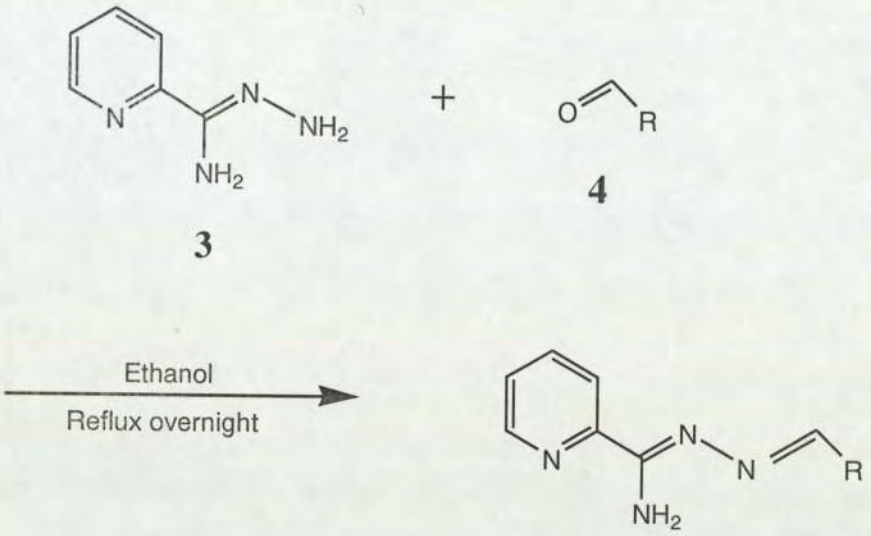
Yield: 69.44g, 510.58mmol, 90%. Pale yellow solid.

^{1}H NMR ($CDCl_3$): δ 4.18 (bs, 2H, NNH_2), 5.73 (bs, 2H, CNH_2), 7.33 (t, 1H, J = 6.62 Hz, Py-H), 7.74(t, 1H, J = 9.57 Hz, Py-H), 8.15(d, 1H, J = 8.28 Hz, Py-H), 8.54(d, 1H, J = 5.95 Hz, Py-H) ppm.

IR (KBr): ν3512, 3444, 3394, 3006,1529, 1465, 924, 785cm^{-1}.

MS (+ ve APCI): m/z = 137 $(M + H)^+$, Mp: 135.5-137.6 °C.

2.2.2 Synthesis of N^1-benzylidene pyridine-2-carboxamidrazones



A solution of pyridine-2-carboxamindrazone **3** (0.544 g, 4 mmol) and an aldehyde **4** (4.4 mmol) (***Table 2.1***) in ethanol (20 ml) was heated at reflux for 18 h. A solid was collected by filtration from the reaction mixture upon cooling to ambient temperature or by recrystallization after being extracted from petroleum ether (40-60 °C) (4×10 ml). The crude product was washed with dichloromethane quickly (3×5 ml) and dried under vacuum to remove the solvent. The information of the products see ***Table 2.2-2.5***.

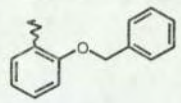
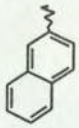
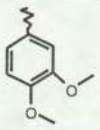
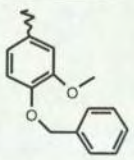
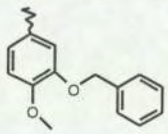
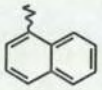
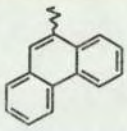
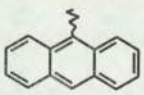
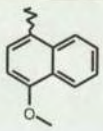
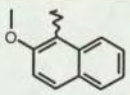
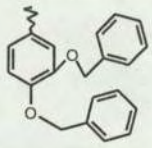
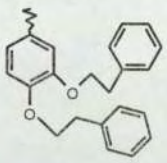
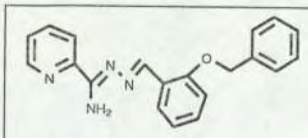
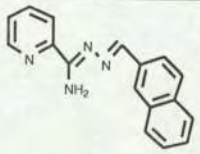
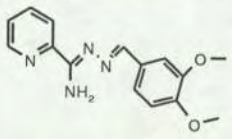
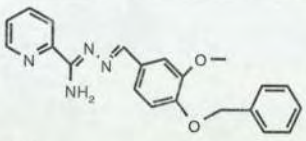
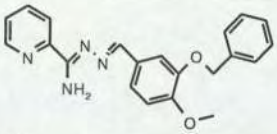
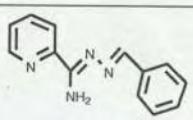
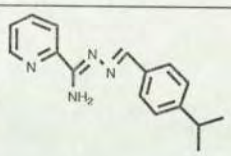
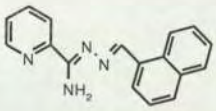
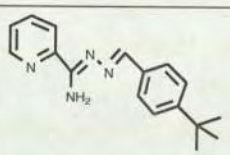
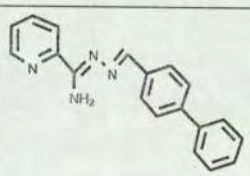
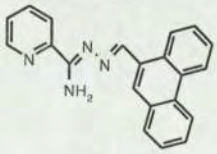
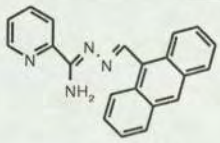
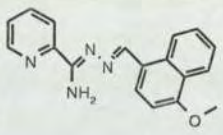
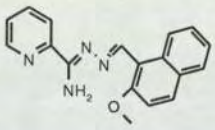
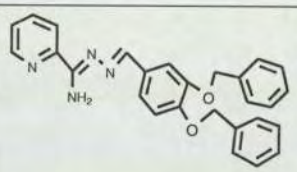
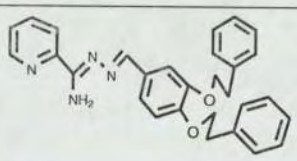
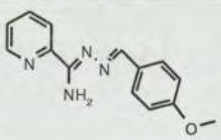
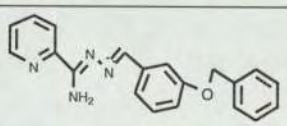
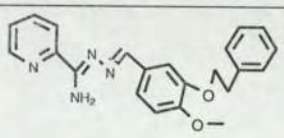
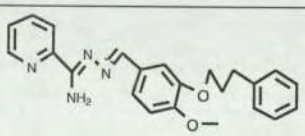
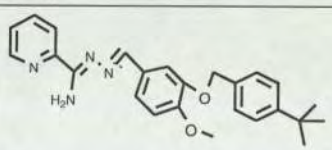
 R1	 R2	 R3	 R4	 R5
 R6	 R7	 R8	 R9	 R10
 R11	 R12	 R13	 R14	 R15
 R16				

Table 2.1. Aldehyde-derived substituents (R Groups)

 **C1** [75]	 **C2**	 **C3**
 **C4**	 **C5**	 **C6**
 **C7**	 **C8** [75]	 **C9** [79]
 **C10**	 **C11**	 **C12**
 **C13**	 **C14**	 **C15**
 **C16**	 **C17**	 **C18**
 **C19**	 **C20**	 **C21**

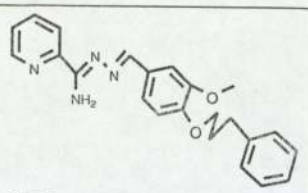
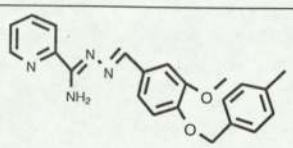
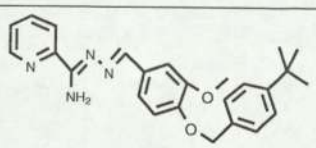
 C22	 C23	 C24

Table 2.2. N^1-benzylidene pyridine-2-carboxamidrazones used as templates and test compounds

Compounds **C17-C24** were kindly donated by Miss Katy Tims (Aston University).

Compound Number	*Yield (%)*	*Mp (°C)*
C1	1.19g, 3.61 mmol, 90%	109.3-111.3 (Lit.[75] for mp: 110.7-112.4°C)
C2	0.95g, 3.47 mmol, 87%	128.0-130.2
C3	0.96g, 3.38 mmol, 85%	176.6-178.9
C4	1.36g, 3.78 mmol, 94%	140.8-142.9
C5	1.38g, 3.83 mmol, 96%	153.4-155.7
C6	0.72g, 3.21 mmol, 80%	116.1-119.0
C7	1.03g, 3.87 mmol, 97%	75.3-76.8
C8	0.98g, 3.58 mmol, 89%	113.3-116.7 (Lit.[75] for mp: 112.7-115.9°C)
C9	0.92g, 3.29 mmol, 82%	120.6-124.8 (Lit.[79] for mp: 119.9-122.4°C)
C10	1.10g, 3.67 mmol, 92%	147.3-149.9
C11	1.27g, 3.92 mmol, 98%	148.3-150.2
C12	1.26g, 3.89 mmol, 97%	170.4-173.6
C13	1.20g, 3.95 mmol, 99%	98.2-100.2
C14	1.18g, 3.72 mmol, 93%	168.4-171.8
C15	0.16g, 3.67 mmol, 92%	173.6-176.7
C16	1.29g, 2.78 mmol, 70%	120.3-122.8

Table 2.3. Yields and melting points data for N^1-benzylidene pyridine-2-carboxamidrazones used as templates and test compounds

Compound Number	*^1H NMR (δ/ppm, $\delta CDCl_3$=7.27ppm)*
C1	5.21 (s, 2H, CH_2), 6.54 (bs, 2H, NH_2), 6.97-7.07 (m, 2H, Ar-H), 7.32-7.49 (overlapping m, 7H, Ar-H and Py-H), 7.80 (m, 1H, Py-H), 8.11-8.17 (m, 1H, Py-H), 8.38 (bs, 1H, Ar-H), 8.61 (m, 1H, Py-H), 9.07 (s, 1H, =CHAr)
C2	6.72 (bs, 2H, NH_2), 7.40-7.45 (m, 1H, Ar-H), 7.51-7.55 (overlapping m, 2H, Ar-H and Py-H), 7.82-7.93 (overlapping m, 4H, Ar-H and Py-H), 8.10-8.16 (overlapping m, 2H, Ar-H and Py-H), 8.49 (bs, 1H, Ar-H), 8.65 (d, 1H, J = 8.3 Hz, Py-H), 8.84 (s,1H, =CHAr)
C3	3.98, 3.95 (2s, 2H, -OCH_3), 6.89 (bs, 2H, NH_2), 6.69 (s, 1H, Ar-H), 6.92 (s, 1H, Ar-H), 7.28 (s, 1H, Ar-H), 7.42 (m, 1H, Py-H), 7.49-7.59 (m, 1H, Py-H), 7.69-7.72 (m, 1H, Py-H), 7.83 (m, 1H, Py-H), 8.63 (s, 1H, =CHAr)
C4	3.97 (s, 3H, -OCH_3), 5.22 (s, 2H, -OCH_2Ar), 5.71 ((bs, 2H, NH_2), 6.91 (d, 1H, J = 8.28, Ar-H), 7.17 (d, 1H, J = 8.28, Ar-H), 7.29-7.49 (overlapping m, 3H, Ar-H and Py-H), 7.71-7.74 (m, 1H, Py-H), 8.48 (s, 1H, =CHAr), 8.71- 8.74 (m, 1H, Py-H)
C5	3.94 (s, 3H, -OCH_3), 5.23 (s, 2H, -OCH_2Ar), 6.62 (bs, 2H, NH_2), 6.92 (d, 1H, J = 8.28, Ar-H), 7.28-7.51 (overlapping m, 4H, Ar-H and Py-H), 7.84 (m, 1H, Py-H), 8.55-8.63 (overlapping m, 2H, Py-H and =CHAr)
C6	6.59 (bs, 2H, NH_2), 7.37-7.48 (overlapping m, 4H, Ar-H and Py-H), 7.77-7.86 (overlapping m, 3H, Ar-H and Py-H), 8.37 (d, 1H, J = 7.24, Py-H), 8.60-8.65 (overlapping m, 2H, Py-H and =CHAr)
C7	1.27, 1.30 (2s, 6H, -CH($C\underline{H}_3$)$_2$), 2.96 (m, 1H, -$C\underline{H}$(CH_3)$_2$), 6.62 (bs, 2H, NH_2), 7.29 (d, 2H, J = 8.54, Ar-H), 7.38-7.43 (m, 1H, Py-H), 7.75-7.85 (overlapping m, 3H, Ar-H and Py-H), 8.42 (m, 1H, Py-H),8.61-8.64 (overlapping m, 2H, Py-H and =CHAr)
C8	6.71 (bs, 2H, NH_2), 7.40-7.47 (m, 1H, Ar-H), 7.51-7.66 (m, 3H, Ar-H), 7.82-7.96 (overlapping m, 3H, Ar-H and Py-H), 8.12 (d, 1H, J = 6.98 Hz, Py-H), 8.46 (bs, 1H, Ar-H), 8.65 (m, 1H, Py-H), 8.81 (d, 1H, J = 8.3 Hz, Py-H), 9.41 (s,1H, =CHAr)

Compound Number	*^{1}H NMR (δ/ppm, $\delta CDCL_3$=7.27ppm)*
C9	1.35 (s, 9H, -C(CH_3)$_3$), 6.35 (bs, 2H, NH_2), 7.38-7.47 (overlapping m, 3H, Ar-H and Py-H), 7.75-7.86 (overlapping m, 3H, Ar-H and Py-H), 8.47 (bs,1H,Py-H), 8.61-6.63 (m, 2H, Py-H and =CHAr)
C10	6.35 (bs, 2H, NH_2), 7.38-7.50 (overlapping m, 4H, Ar-H and Py-H), 7.63-7.69 (overlapping m, 4H, Ar-H), 7.80-7.93 (overlapping m, 3H, Ar-H and Py-H), 8.44 (bs, 1H, Py-H), 8.63 (d, 1H, J = 5.17, Py-H), 8.70 (bs, 1H, =CHAr)
C11	6.71 (bs, 2H, NH_2), 6.27-6.45 (m, 1H, Py-H),7.61-7.80 (overlapping m, 3H, Ar-H), 7.82-7.89 (m, 1H, Ar-H), 7.79-8.00 (m, 1H, Py-H), 8.33 (s, 1H, Ar-H), 8.48 (d, 1H, J = 7.76, Ar-H), 8.65-8.80 (overlapping m, 4H, Ar-H and Py-H), 8.95-8.99 (m, 1H, Py-H), 9.48 (s,1H, =CHAr)
C12	6.81 (bs, 2H, NH_2), 7.52-7.58 (overlapping m, 5H, Ar-H and Py-H),7.95-8.07 (overlapping m, 4H, Ar-H and Py-H), 8.54 (s,1H,Ar-H), 8.69-8.77 (overlapping m, 4H, Ar-H, Py-H and =CHAr)
C13	4.07 (s, 3H, -OCH_3), 6.68 (bs, 2H, NH_2), 6.89 (d, 1H, J = 8.28, Ar-H), 7.39-7.44 (m, 1H, Py-H), 7.52-7.68 (overlapping m, 2H, Ar-H), 7.81-8.03(overlapping m, 2H, Ar-H and Py-H), 8.34 (d, 1H, J = 8.54, Py-H), 8.52 (bs, 1H, Ar-H), 8.63 (d,1H, J = 4.66, Ar-H), 8.90 (d, 1H, J = 8.54, Py-H), 9.29 (bs,1H, =CHAr)
C14	4.01 (s, 3H, -OCH_3), 6.60 (bs, 2H, NH_2), 7.28 (m, 1H, Ar-H), 7.37-7.44 (m, 2H, Ar-H), 7.54-7.61 (m, 1H, Ar-H), 7.79-7.92 (overlapping m, 3H, Ar-H and Py-H), 8.49 (d, 1H, J = 5.69, Py-H), 8.62-8.65 (m, 1H, Py-H), 9.27 (d,1H, J = 8.54, Py-H), 9.42 (bs,1H, =CHAr)
C15	5.22, 5.24 (2s, 4H, -OCH_2Ar), 6.51 (bs, 2H, NH_2), 6.95 (d, 1H, J = 8.28, Ar-H), 7.22-7.56 (overlapping m, 13H, Ar-H and Py-H), 7.75-7.82 (m, 1H, Py-H), 8.37 (d, 1H, J = 8.02, Py-H), 8.48 (s,1H, =CHAr), 8.60 (m, 1H, Py-H)
C16	3.18 (m, 4H, -O-CH_2-C$\underline{H_2}$-), 4.26 (m, 4H, -O-C$\underline{H_2}$-CH_2-), 6.60 (bs, 2H, NH_2), 6.67 (d, 1H, J = 8.54, Ar-H), 7.21-7.49 (overlapping m, 13H, Ar-H and Py-H), 7.80 (m, 1H, Py-H), 8.44-8.62 (overlapping m, 3H, Py-H and =CHAr)

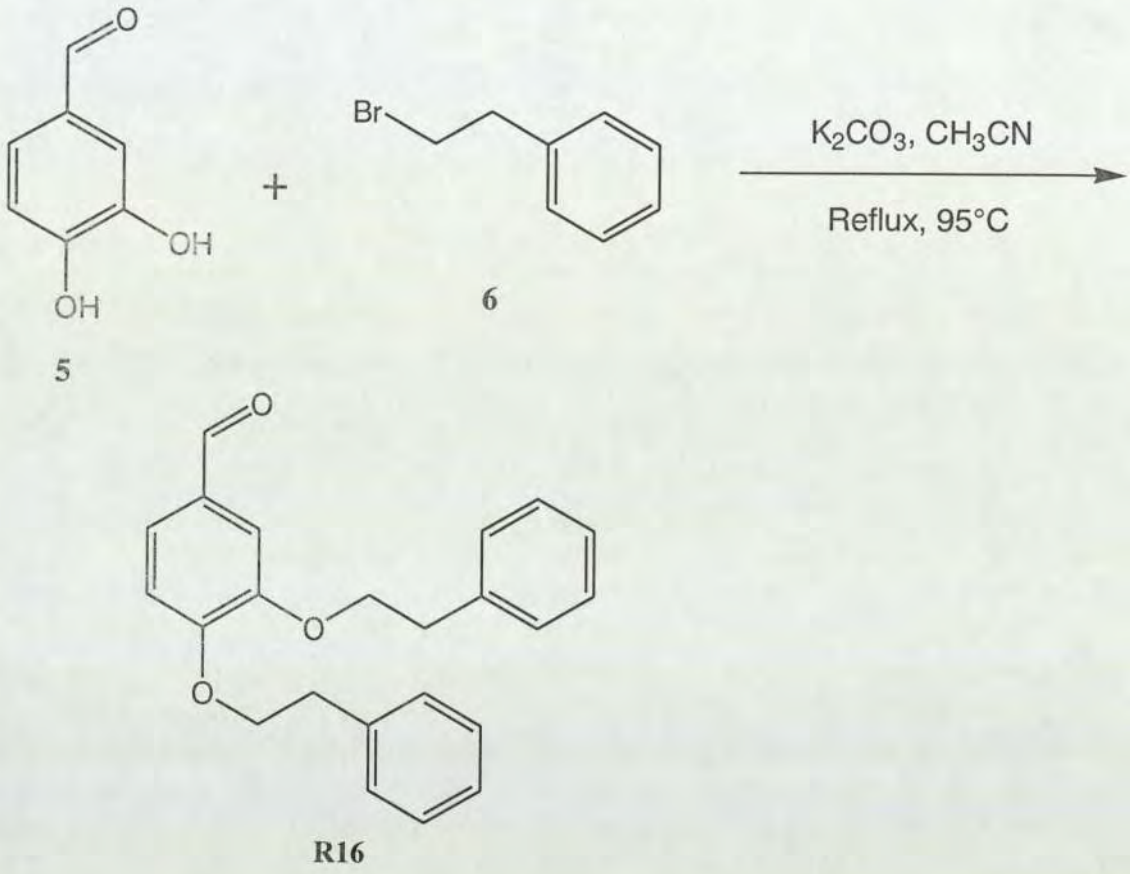
Table 2.4. ^{1}H NMR data for N^1-benzylidene pyridine-2-carboxamidrazones used as templates and test compounds

Compound Number	*IR* (ν/cm^{-1})	*MS APCI, m/z= $(M+H)^+$*
C1	3550, 3031, 1613, 1578, 1451, 1100, 963, 744	331
C2	3550, 3006, 1618, 1577, 1473, 1390, 1045, 825	275
C3	3550, 3066, 1618, 1583, 1463, 1348, 1010, 877	285
C4	3552, 3020, 1598, 1473, 1442, 1413, 1032, 848	361
C5	3552, 3033, 1598, 1427, 1371, 1253, 1004, 844	361
C6	3550, 3054, 1581, 1467, 1334, 1290, 1076, 798	225
C7	3553, 3055, 1558, 1415, 1396, 1049, 999, 796	267
C8	3550, 3254, 1474, 1386, 1166, 1125, 1089, 801	275

Compound Number	*IR* (ν/cm^{-1})	*MS* *APCI, m/z= $(M+H)^+$*
C 9	3550, 3024, 1519, 1467, 1398 1109, 1039, 894	281
C10	3550, 3027, 1571, 1483, 1332, 1155, 1007, 869	301
C11	3550, 3049, 1589, 1473, 1342, 1248, 1020, 893	325
C12	3550, 3016, 1583, 1469, 1388, 1282, 1045, 889	325
C13	3550, 3068, 1573, 1459, 1396, 1275, 1097, 804	305
C14	3550, 3072, 1583, 1435, 1328, 1250, 1074, 872	305
C15	3550, 3091, 1583, 1473, 1380, 1277, 1020, 898	437
C16	3550, 3081, 1583, 1475, 1384, 1261, 1026, 875	465

Table 2.5. IR and MS data for N^1-benzylidene pyridine-2-carboxamidrazones used as templates and test compounds

2.2.3 Synthesis of 3,4-benzyloxymethyl-benzaldehyde (aldehyde-derived substituent R16, See *Table 2.1*)



A mixture of 3,4-dihydroxybenzaldehyde **5** (5.70 g, 41.26 mmol), 2-phenylethyl bromide **6** (18.53 g, 100.16 mmol) and potassium carbonate (16.91 g, 122.38 mmol) in acetonitrile (80 ml) was heated at refluxed for 15 h under a flow of argon. The mixture was filtered and the solvent was evaporated from the filtrate under reduced pressure using a rotary evaporator. The residue was dissolved in chloroform (100 ml) and washed with distilled water (3×150 ml) using separation funnel. The organic layer was collected and stirred with magnesium sulphate (20 g) for 30 min. Then the mixture was filtered and the filtrate was evaporated to give a pale yellow oil. The remaining solvent

was removed under vacuum overnight. The crude product R16 (10.81 g) was thus purified by flash chromatography with the eluent dichloromethane : petroleum ether = 4 : 1 using silica gel (500 g, Fluka, 220-440 Mesh, Sigma-Aldrich Company Ltd, Dorset, UK).

Yield : 2.20 g, 6.36 mmol, 15.41%. Pale yellow oil.

^{1}H NMR ($CDCl_3$): δ 3.18 (m, 4H, -O-CH_2-C$\underline{H}_2$-), 4.26 (m, 4H, -O-C$\underline{H}_2$-CH_2-), 6.94 (d, 2H, J = 8.02, Ar-H), 7.26-7.43 (m, 12H, Ar-H), 9.81 (s, 1H, O=CHAr) ppm.

IR (KBr): ν 3034, 2987, 2890, 1710, 1600, 1513, 1465, 835 cm^{-1}.

MS (+ ve APCI): m/z = 465 $(M + H)^+$.

2.3 Preparation of fluorescently imprinted polymers : General preparation

2.3.1 Materials

(i) Template/Print molecule :

N^1-benzylidene pyridine-2-carboxamidrazones (***Table 2.2***)

(ii) Fluorescent monomer :

3-acrylamido-5-(2-methoxy-1-naphthylidene)-rhodanine [78] (***Figure 2.1***)

This compound was kindly donated by Danqing Su (Aston University).

(iii) Crosslinker : TRIM (***Figure 2.2***)

(iv) Porogen/Sovlent : toluene

(v) Initiator : AIBN

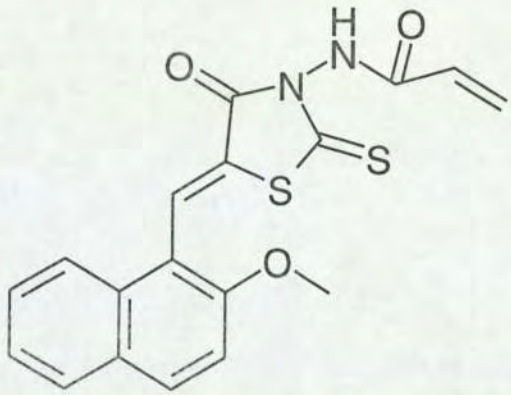


Figure 2.1. Fluorescent monomer

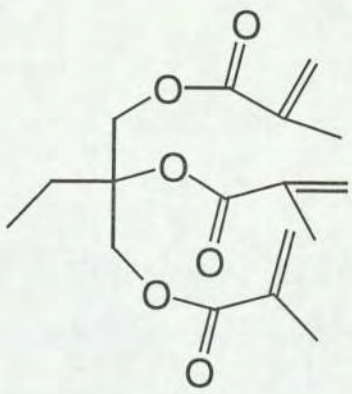


Figure 2.2. Crosslinker

2.3.2 General polymerisation procedure of MIPs

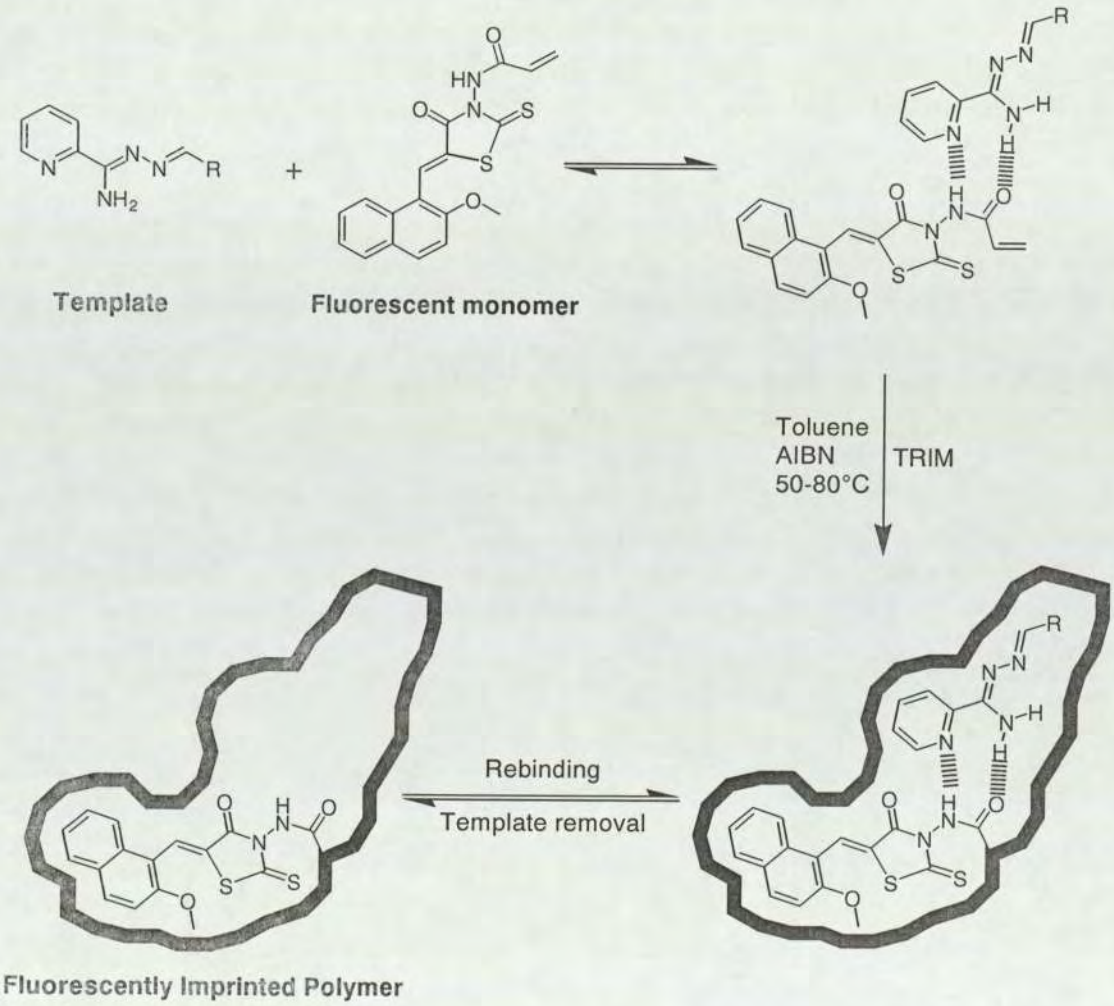


Figure 2.3. Schematic representation of general polymerisation procedure of MIPs

A mixture of the print molecule (0.05 mmol), fluorescent monomer (19.22 mg, 0.05 mmol), TRIM (9.09 ml, 32.44 mmol), toluene (30 ml) and AIBN (0.26 g, 1.59 mmol) was deoxygenated by alternate application of vacuum and argon (5 ×) and stirred at 50°C for the first day, then the temperature was increased to 80°C for a further two days. The yellow rigid crude polymer solid was collected by filtration and washed with chloroform (3×5 ml) under vacuum. Then it was ground carefully to a powder using mortar and pestle. The powder was extracted with chloroform (300 ml) using a soxhlet apparatus for two days. The powder was dried under high vacuum. The dry extracted powder was sieved using a sieve and shaker. The particles with a size of 250-390 μm were used for further studies.

2.3.3 Template Rebinding / Test compound rebinding

Each MIP was exposed to its template and a series of other test compounds. A solution of the template/test compound (50mg) (one of N^1-benzylidene pyridine-2-carboxamidrazones, ***Table 2.2***) in chloroform (5ml) was firstly prepared. Then the unbound MIP powder (100mg) was suspended in this solution (2ml, 10mg/ml) in a small sealed vial (5ml) and shaken for 3 h at room temperature. The MIP was collected by filtration and washed quickly with dichloromethane (3×5ml) under vacuum. It was then dried under high vacuum to remove the solvent.

2.4 Fluorescence measurements

2.4.1 Preparation of the measurements

MIP (approximately 5.0mg) (bound or unbound) were weighed accurately and loaded in triplicate into a 96-well black cliniplate carefully as the ***Test Group***. The chloroform solution containing the relevant template/test compound (10μl, 10mg/ml) (one of N^1-benzylidene pyridine-2-carboxamidrazones, ***Table 2.2***) was loaded in double as the ***Control Group***. The ***Blank Group*** comprised three empty wells only.

2.4.2 Measurement protocol

Methanol (350 μl) was added into each of the ***Test, Control, Blank Group*** wells and the fluorescence outputs were measured immediately in quintuplet using a Wallac Victor 1420 multilabel counter in standard 96-well format ($\lambda_{excitation}$=355, $\lambda_{emission}$=460 nm). The data presented are the averages of the results and are quoted as fluorescent counts per milligram of MIP.

3.1 Preparation of fluorescent imprinted polymers

3.1.1 Numbering of fluorescent imprinted polymers

The numbering of fluorescent imprinted polymers was depend on their own templates' numbers. For example, the **MIP** which used template **C5** in its polymerisation was named **MIP 5**. The information of templates and test compounds are given in ***Table 2.2***.

3.1.2 The concentrations of template and fluorescent monomer in polymerisation

A series of fluorescent MIPs was constructed and reported [78] using TRIM containing 5 μmol/g each of the template and the fluorescent monomer. These relatively low loadings of template and fluorescent monomer were used because firstly, the fluorescence responses were very easily measured at this loading, and secondly, using such small amounts of template opens the door to imprinting with molecules which are in short supply, for example, complex natural products. Thus, high concentrations of template and fluorescent monomer have less sense in practice.

3.1.3 Studies in the design of fluorescent monomer

From X-ray crystallographic studies [80], it is known that N^1-benzylidene heteroarylcarboxamidrazones are relatively planar by virtue of intermolecular hydrogen bonding as shown schematically for a 2-pyridyl example in ***Figure 1.22***. The same donor and acceptor atoms were also found simultaneously to form intermolecular hydrogen bonds. Therefore, we required a fluorescent monomer capable of adopting a conformation such that it might both donate to, and accept hydrogen bonds from, the planar N^1-benzylidene heteroarylcarboxamidrazones.

3-Aminorhodanine (***Figure 3.1***) was chosen as a suitable scaffold for the monomer due to its obvious hydrogen bond donor and acceptor properties [20] (partial atomic charges calculated by Dr. D. L. Rathbone, Aston University). The amino group is available for conversion to the polymerisable acrylamide moiety and the methylene may be coupled with various fluorescent aldehydes so that the fluorescence response may be tuned and the overall shape of the monomer may be adjusted. Thus, in this study, the fluorescent monomer employed was 3-acrylamido-5-(2-methoxy-1-naphthylidene)-rhodanine (***Figure 2.1***).

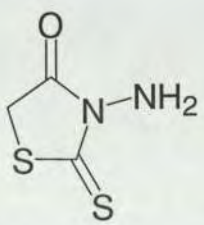


Figure 3.1. 3-Aminorhodanine

3.1.4 Choice of initiator

As shown in the introduction part, the initiator AIBN can be decomposed to produce free radicals by the action of heat (~60 °C) or light (UV 366 nm). However, owing to the UV absorption profiles of both the template and the fluorescent monomer, polymerisation initiation in our research was by thermal decomposition of AIBN rather than by UV irradiation at low temperature.

3.1.5 Choice of porogen

The solvent plays a very important role in the outcome of a molecular imprinting process. A role which is particularly pronounced in self-assembly system. In our research, chloroform was initially used as the porogen. The **MIP 5** thereby obtained template-unbound/bound fluorescence ratios in the region of 2/1 under the excitation/emission wavelength pairs 355/460 nm and 355/535 nm. However, when a larger test compound **15** was tried to rebind **MIP 5**, it showed lower fluorescenct counts (***Figure 3.2***). It could be assumed that the larger test compound **15** fitted the imprinted cavities of **MIP 5** even better than its template **5**. It is contrary to the fact of their molecular sizes and this MIP made in chloroform doesn't has the ability of molecule recognition.

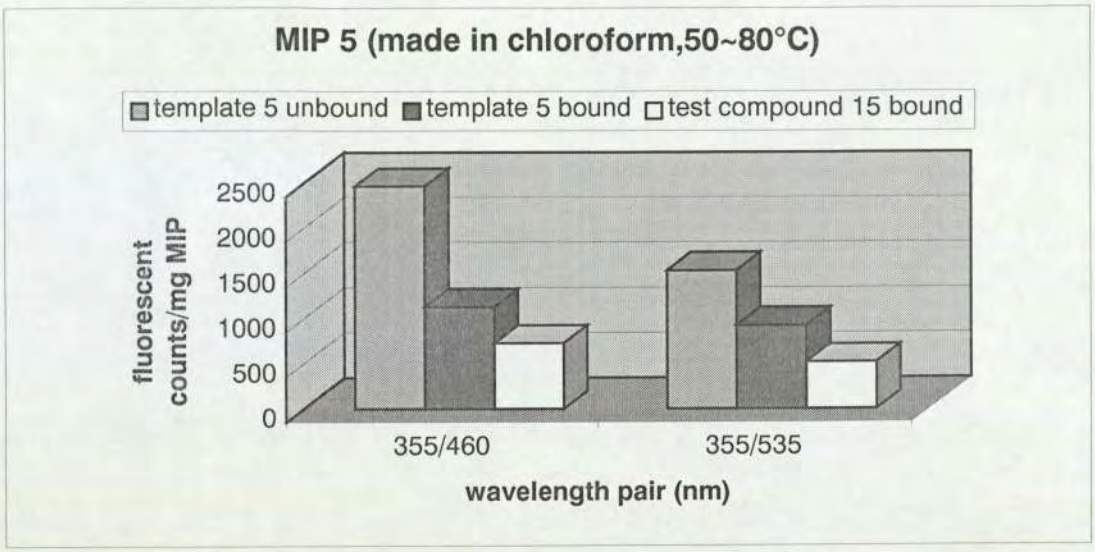


Figure 3.2. Fluorescence response of **MIP 5** which was made in chloroform

Later, THF was used. Though the template-unbound/bound fluorescence ratios under the excitation/emission wavelength pairs 355/460 nm and 355/535 nm were much lower than that of chloroform, it still showed poor recognition with the larger test compound **15**. The fluorescent response of test compound **15** bound MIP was twice of that of template **5** bound MIP under the same wavelength pair (***Figure 3.3***).

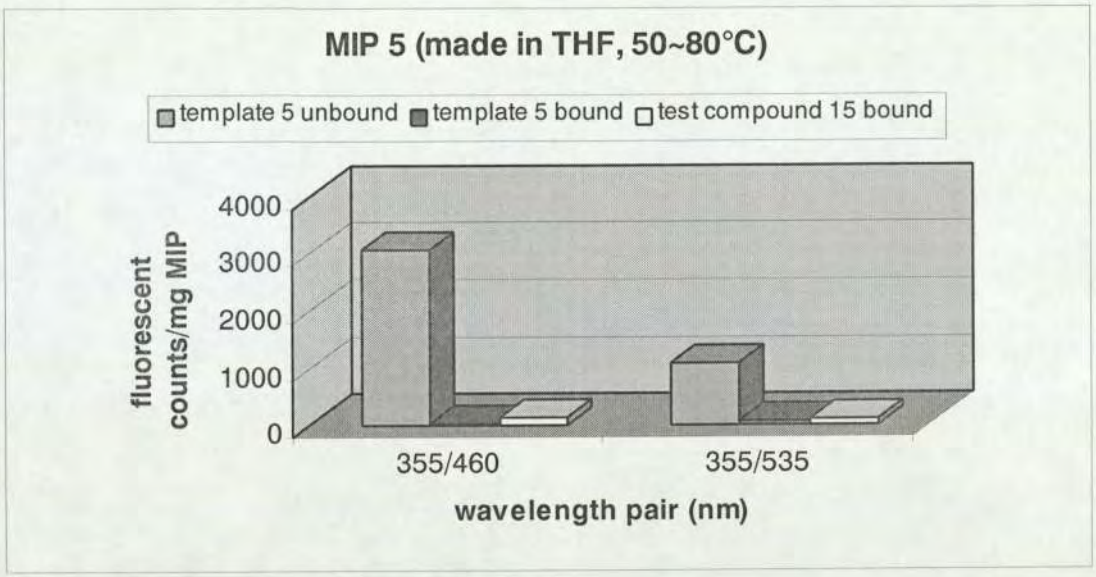


Figure 3.3. Fluorescence response of **MIP 5** which was made in THF

Finally, toluene was used as the porogen due to it being less polar. This time both the ratios of fluorescent response in template-unbound/bound group and template bound/test compound **15** bound group were dramatic (***Figure 3.4***). The selectivity was improved 10-fold when toluene was used, so the **MIP 5** made in toluene has the selectivity between its template and larger test compound. Thus, all data presented herein use toluene as the porogen.

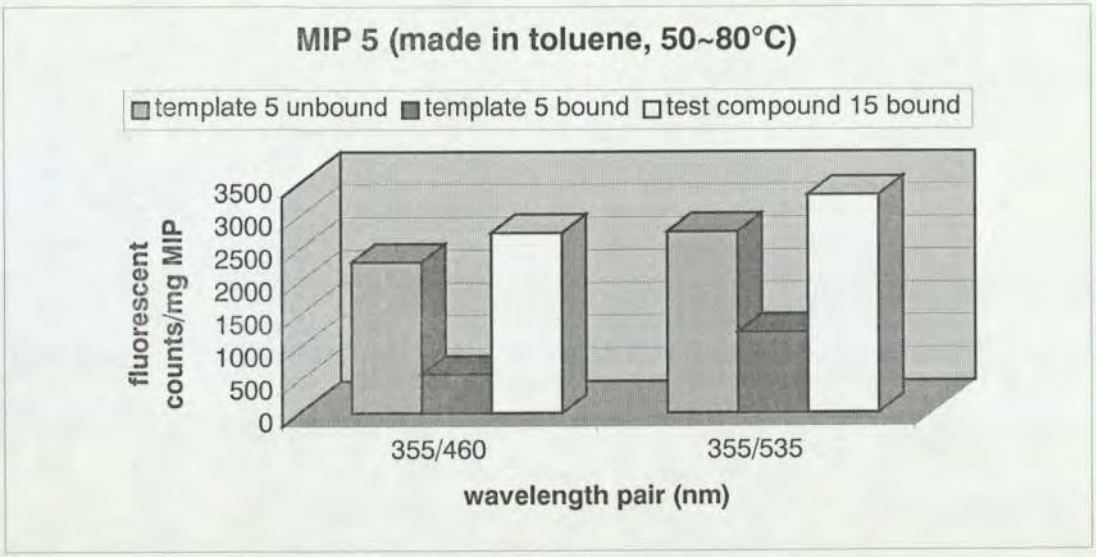


Figure 3.4. Fluorescence response of **MIP 5** which was made in toluene at 50~80°C

These results above confirmed that when applying the polymers to relatively more polar solvents, the recognition is diminished. The more polar the porogen, the weaker the resulting recognition effect becomes, as a consequence of the influence of the solvent polarity on the non-covalent interaction which is also the main interaction force in polymerisation of fluorescent imprinted polymer. The best imprinting porogens, for accentuating the binding strengths, are solvents of very low dielectric constant. The use of more polar solvents will inevitably weaken the interaction forces formed between the templates and the functional monomers resulting in poorer recognition.

Also, the morphology was affected since the swelling of the polymers is dependent on the surrounding medium. The swelling was more pronounced in chlorinated solvents, such as chloroform, as compared to THF and toluene. The swelling in THF is approximately the same as for Toluene. As a rule of thumb, the best choice of recognition solvent should be more or less identical to the imprinting porogen in order

to avoid any swelling problems. The polymer swelling taking place when polymers are prepared in organic porogen, is however not necessarily a gross obstacle.

3.1.6 Temperature in polymerisation

Usually the temperature in polymerisation is raised to 60°C, thus starting the homolytic cleavage of AIBN to nitrogen an isobutyronitrile radicals.At the beginning, **MIP 5** was polymerised in toluene at 50°C for one day then changed to 60°C for a further day. Comparing with the result (***Figure 3.4***) of **MIP 5** which polymerised in toluene at 50°C for one day and at 80°C for another two days, it showed more molecular selectivity at the later temperature (50~80°C) (***Figure 3.5***). For the reason that template, monomer and crosslinker could dissolve and pre-organise well at 50°C and then make the cross-linking as complete as possible at 80°C for further two days rather than 60°C, better result was achieved.

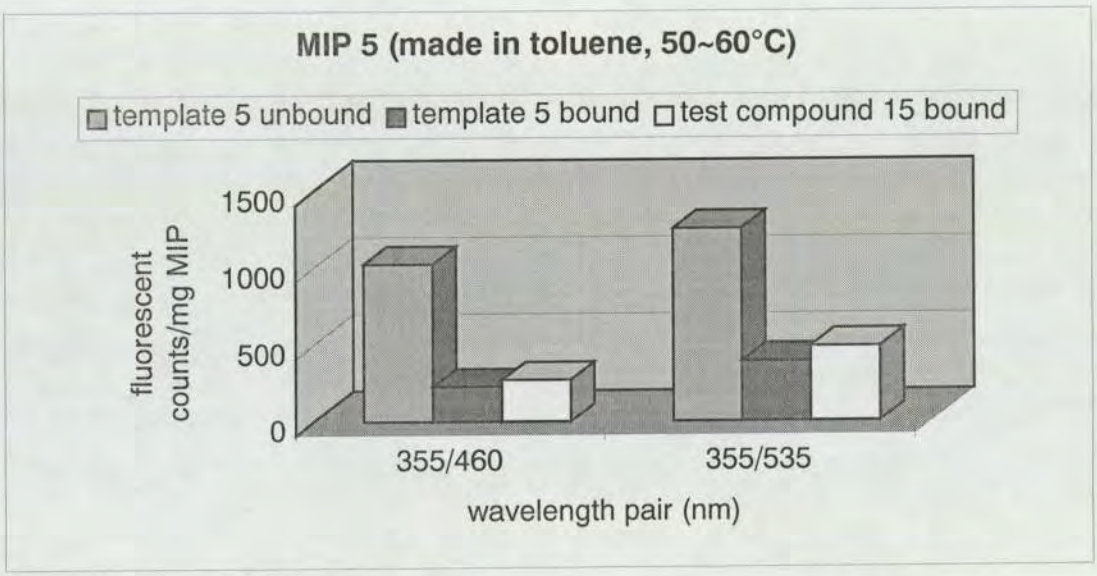


Figure 3.5. Fluorescence response of **MIP 5** which was made in toluene at 50~60°C

3.1.7 The particle sizes of MIPs

After grinding and exhaustive extraction with chloroform, the crude polymers were fractioned by sieving. Four different size particles from fluorescent **MIP 5** were obtained: >425 μm, 425~355 μm, 355~300 μm, <300 μm and this **MIP 5** was polymerised in chloroform at 50~80°C. The particles which are bigger than 425 μm are too big to be prepared for measuring. Except for this particle size, however, it was found that particle size had a negligible effect on selectivity or fluoresce response (*Figure 3.6, 3.7*).

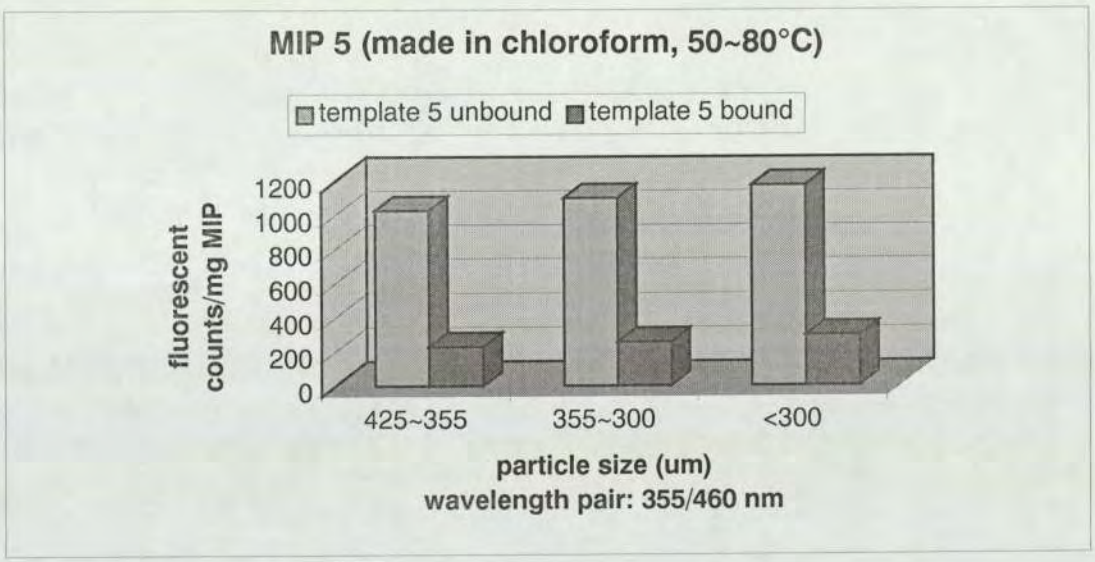


Figure 3.6. Fluorescence response of **MIP 5** at $\lambda_{ex}355/\lambda_{em}460$ nm

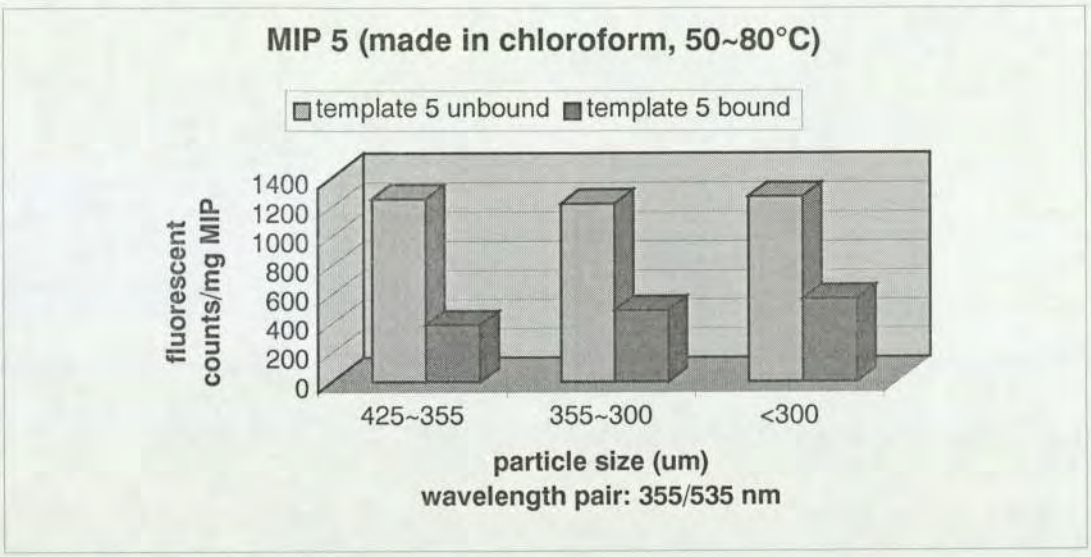


Figure 3.7. Fluorescence response of **MIP 5** at $\lambda_{ex}355/\lambda_{em}535$ nm

It is obvious that there are only small differences in fluorescence among different particle sizes at the same wavelength pair. Although the **MIP 5** used was polymerised in chloroform, not the ideal porogen toluene, it showed only a very small effect on the template's selectivity by the comparison of template-unbound and bound fluorescence response using different particles. Nevertheless, our studies arise from polymers with particle sizes in the range 250~329 μm.

3.2 Fluorescent wavelength pairs in the measurements

Thirty-three pairs of excitation/emission wavelengths were firstly examined using appropriate combinations from λ_{ex}=355, 390, 450, 485, 490, 530 and 544 and λ_{em}=450,460, 510, 535, 545, 570 and 590 nm. They were:

355/450, 355/460, 355/510, 355/535, 355/545, 355/570 and 355/590 nm

390/450, 390/460, 390/510, 390/535, 390/545, 390/570 and 390/590 nm

450/510, 450/535, 450/545, 450/570 and 450/590 nm

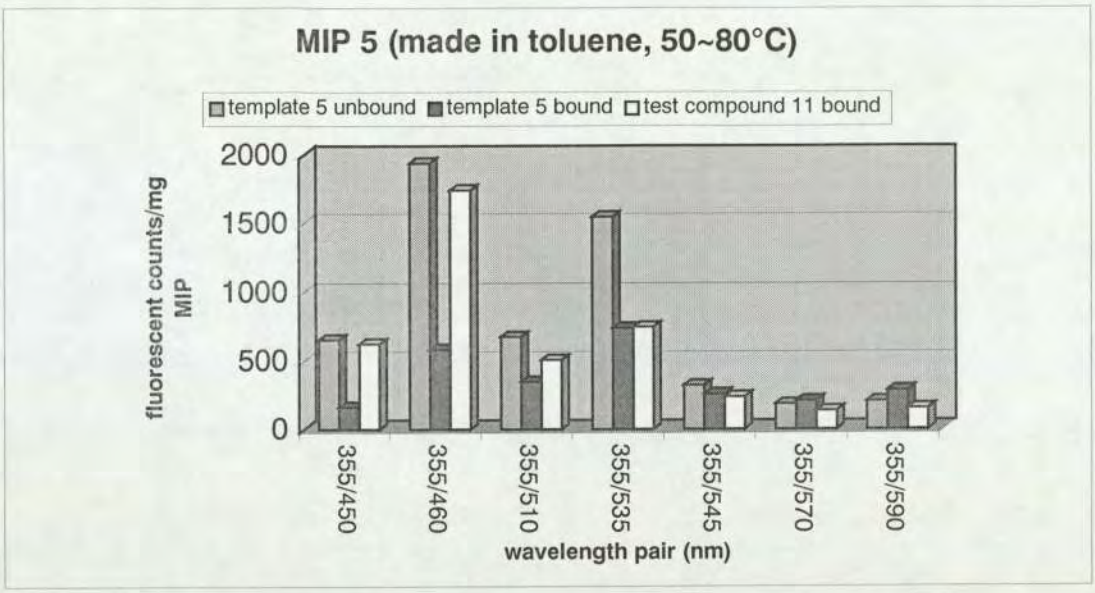
485/510, 485/535, 485/545, 450/570 and 450/590 nm

490/510, 490/535, 490/545, 490/570 and 490/590 nm

530/570 and 530/590 nm

544/570 and 544/590 nm

However, for MIP 5, only excitation at 355 or 390 nm combined with emission at 450, 460, 510 or 535 nm was found to give the best compromise between sensitivity and selectivity. Some results are given in ***Figure 3.8***.



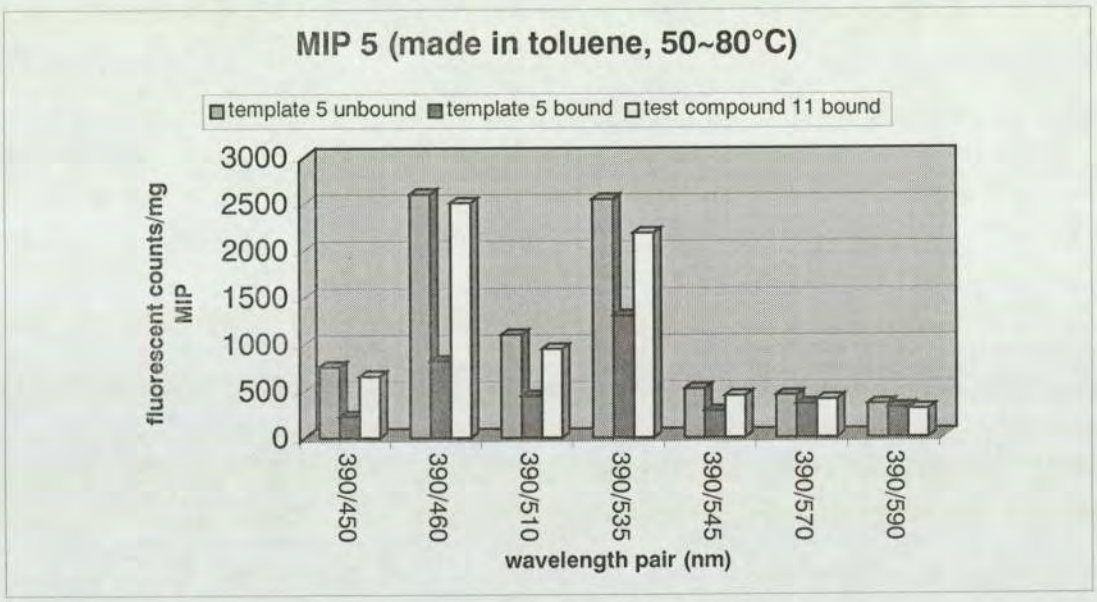


Figure 3.8. Fluorescence response of **MIP 5** at different pairs of excitation/emission wavelengths

Therefore, our further fluorescent results were based on wavelength pairs: $\lambda_{ex}/\lambda_{em}$ = 355/460 and 355/535 nm.

3.3 Studies of selectivity

Dramatic quenching of fluorescent response approaching background levels was gained from most cases where the "empty" (template unbound) MIP was re-exposed to its template.

As expected, **MIP 6**, derived from the smallest template, discriminated against all other test compounds in that no test compound gave a change in fluorescence response as great as that of the template itself (***Figure 3.9***). At $\lambda_{ex}355/\lambda_{em}535$ nm, binding of template **6** gave a 79.7-fold reduction in fluorescence response whilst the closest response (test compound **8**) gave a 3.1-fold quenching.

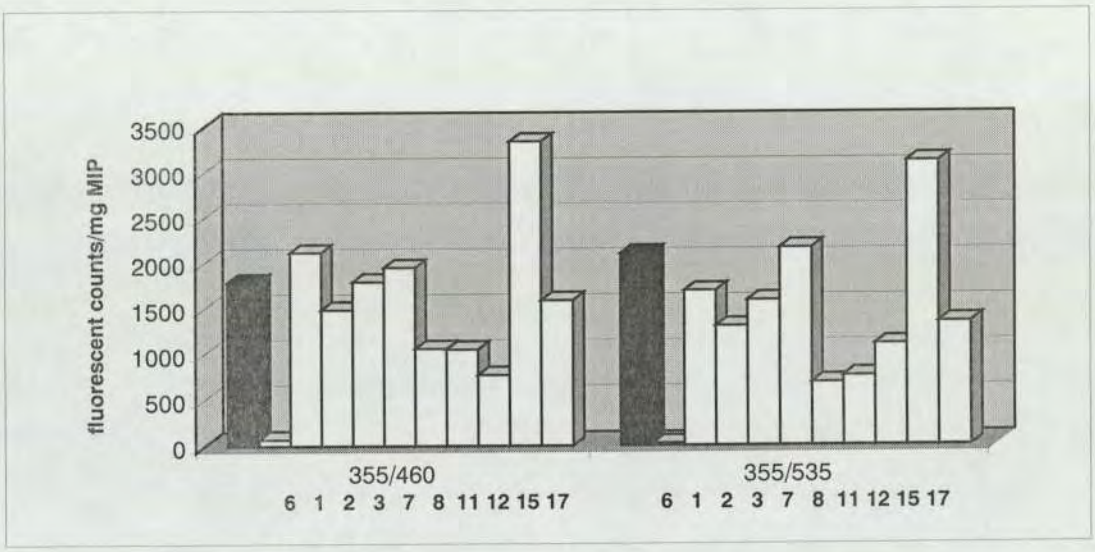


Figure 3.9. Fluorescence response of **MIP 6** in the absence (black bar)or presence (white bars) of substrates at $\lambda_{ex}355/\lambda_{em}460$ nm nd $\lambda_{ex}355/\lambda_{em}535$ nm

MIP 16 which derived from the biggest template, on the contrary, lost its discrimination against all the other test compounds since their fluorescence responses were as low as that of template **16**. All the test compounds were supposed to fully go into the cavities of **MIP 16** and thus dramatically quench the fluorescence (***Figure 3.10***).

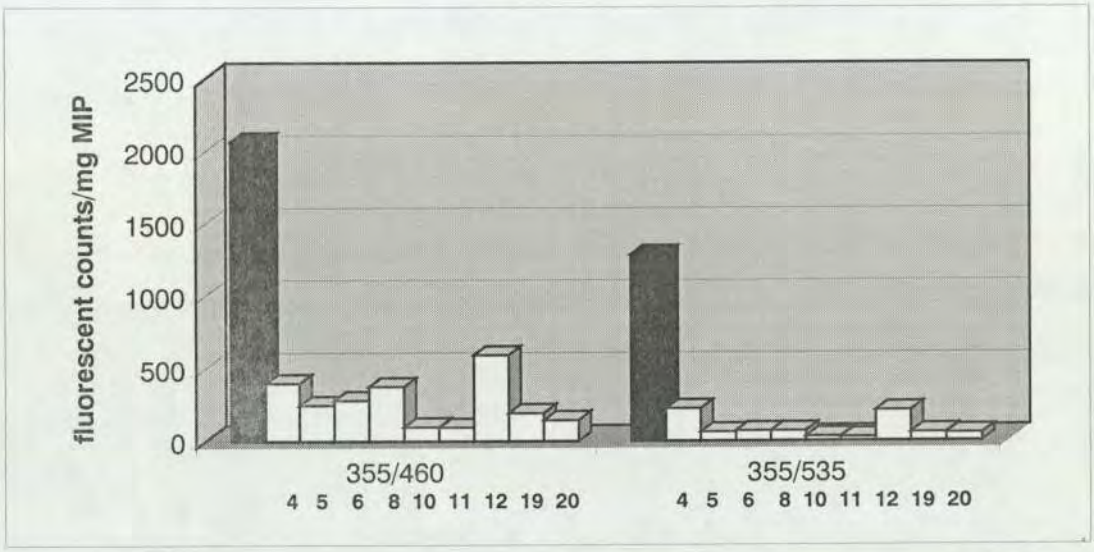


Figure 3.10. Fluorescence response of **MIP 16** in the absence (black bar) or presence (white bars and columns) of substrates at $\lambda_{ex}355/\lambda_{em}460$ nm and $\lambda_{ex}355/\lambda_{em}535$ nm

By comparing with the results of the other MIPs, it was found that large and relatively inflexible molecules such as the polycyclic aromatics **2,8,10,11,12,13,14** were more easily discriminated against than the more flexible alkoxy-substituted benzylidene compounds **1,4,5,15,16,18,19,20**. Thus **MIP 8** successfully discriminated against inflexible compounds **11-14**. In contrast, the more flexible compounds **1,4,5,15** were able to bind and quench the fluorescence (***Figure 3.11***). In addition, the cases of test compounds **2,17** represent the possibility of a partial response, that is, test compounds **2** and **17** being able to partially fit into the cavities of **MIP 8** due to their three-

dimensional structures. The same example, which might also be true, was **MIP 6** imprinted with test compounds **8,11,12** (***Figure 3.9***).

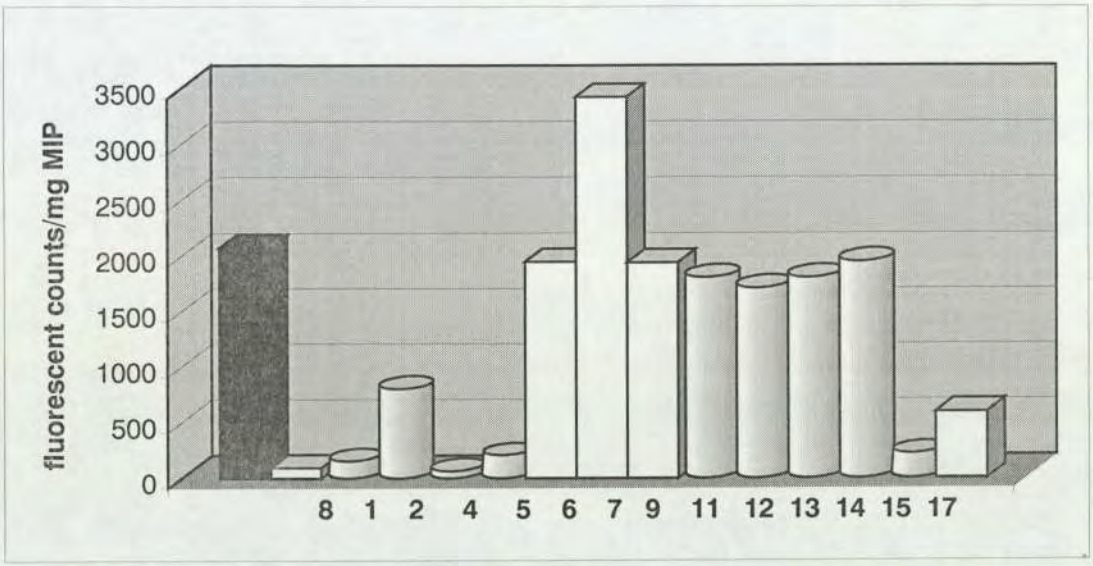


Figure 3.11. Fluorescence response of **MIP 8** in the absence (black bar) or presence (white bars and columns) of substrates at $\lambda_{ex}355/\lambda_{em}460$ nm

Another convincing example was **MIP 15** that could discriminate against inflexible compounds **8,10,11,12**, what was contrary to the more flexible compounds **1,5,16** (***Figure 3.12***). Especially, the biggest molecule **16** which was able to quench the fluorescence indicated that the c-o bond in alkoxy-substituted benzylidene compounds might rotate, thus changed their three-dimensional structures and make them fit into the empty miro-cavities in MIPs.

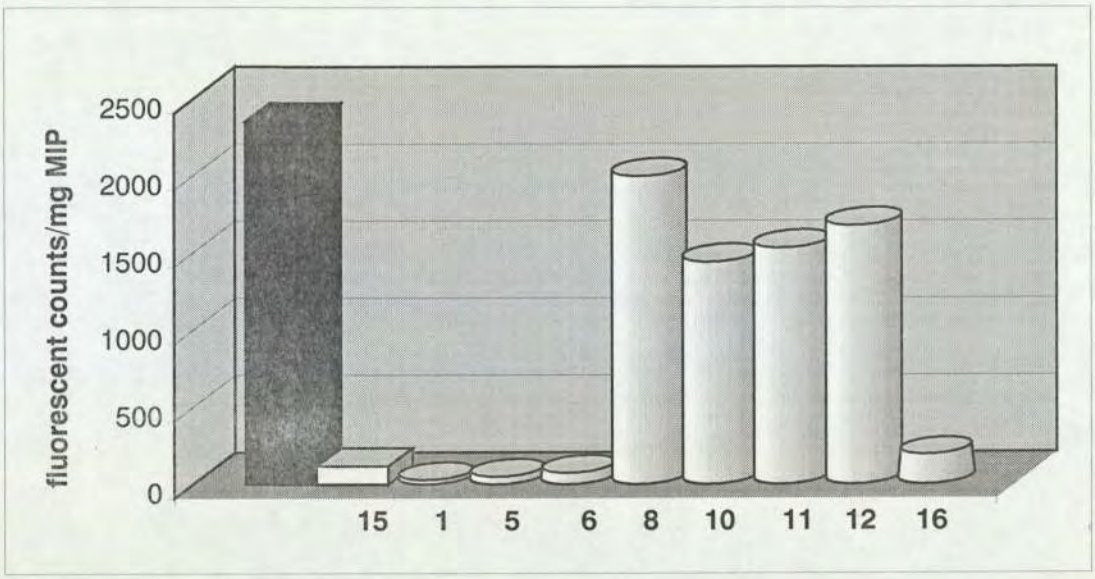


Figure 3.12. Fluorescence response of **MIP 15** in the absence (black bar) or presence (white bars and columns) of substrates at $\lambda_{ex}355/\lambda_{em}535$ nm

The MIPs **1**,**4**,**5** all each bound the other's templates. **MIP 5** did not bind the relatively inflexible compounds **10**,**11**. In contrast, the similar **MIP 15** turned down test compounds **10**,**11** too (***Figure 3.12***). Furthermore, a confusing result was the fact that the large and flexible molecule **15** could at least been partially bound by **MIP 4** and fully bound by **MIP 1** but not recognised by **MIP 5**, even though the chemical structures between compounds **4** and **5** are similar.

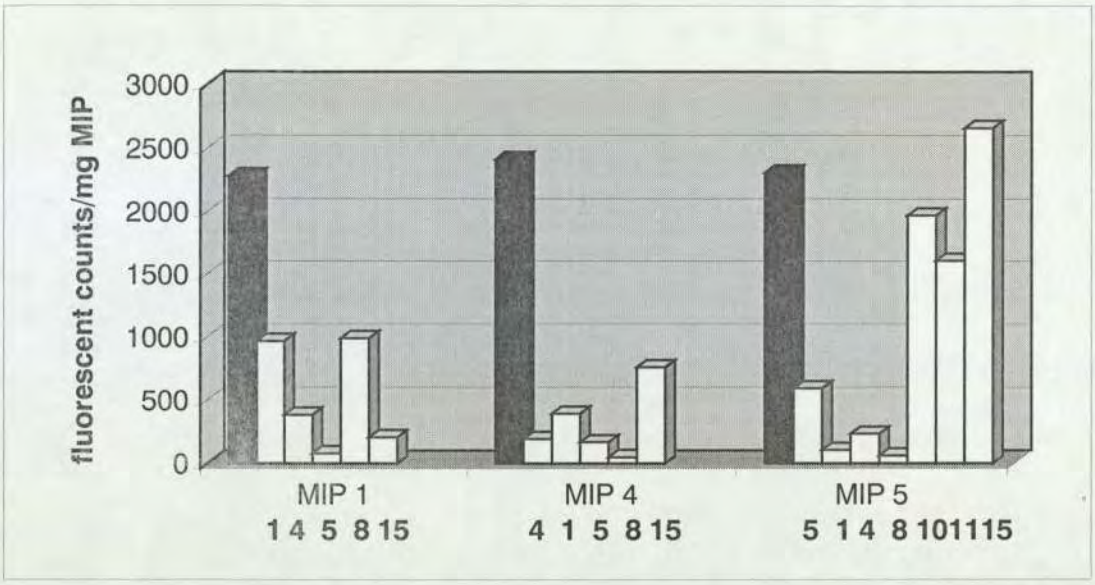


Figure 3.13. Fluorescence response of **MIP 1,4,5** in the absence (black bar) or presence (white bars) of substrates at $\lambda_{ex}355/\lambda_{em}460$ nm

The results above verified the intuitively obvious assumption that the fewer the degree of freedom of a test compound presented to a MIP, the higher the likely selectivity that can be achieved. In our hands the more flexible substrates have been able to accommodate themselves to a variety of cavity shapes and sizes, an extreme example of this being the binding of test compound **15** in **MIP 6** (***Figure 3.9***). On the contrary, the more inflexible substrates were rejected by MIPs even though the substrates are small or similar with MIPs' templates. A typical instance is the fluorescent response of **MIP 8** bound with test compound **7**(***Figure 3.11***).

Conclusion

In conclusion, being constructed methodically and successfully, these fluorescent MIPs have demonstrated considerable selective recognition of test compounds which are very similar in structure to their templates. Better selectivity was observed for the test compounds with fewer degrees of freedom presented to MIPs. Moreover, large, relatively inflexible test molecules and flexible test molecules had a big difference between them in the fluorescence response when they were presented to the same MIP. More discrimination against the other test compounds can be obtained using large, inflexible molecules rather than flexible molecules.

Thus, if a suitable template molecule maybe designed, this attractive approach could optimistically be used as a pre-screening MIP-based process to reduce the large numbers of robotically-prepared anti-tubercular compounds to a more manageable set for biological screening or to discover other new potentially useful compounds which are similar in size, shape and hydrogen-bonding profiles to existing drugs. Of course, it still needs further improvements in its selectivity and the straightforward way of measurement for the future possible automation.

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