Molecularly Imprinted Polymers as Stereoselectors in Diels- Alder Reactions

A thesis submitted in accordance with the conditions Governing candidates for the degree of Philosophiae Doctor in the University of Wales

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Abstract

The long term goal of this project was to use the high selectivity, but low binding capacity of MIPs, with the high efficiency of microreactors to increase the yield of a minor isomer. In this initial study, the aim was to develop a polymer system capable of increasing the minor product (exo 6) of a simple Diels- Alder reaction between cyclopentadiene and benzyl acrylate.

In the first instance the endo (major) product isomer (endo 6) was used as the MIP template (as more of the isomer was available) and 3 MIPs with different functional monomers- Methacrylic acid, 4- viny pyridine and acrylamide were produced and evaluated. The imprinting effect was observed for both the 4-vinyl pyridine based MIP and the acrylamide based MIP with endo 6 though the imprinting effect for the 4- vinyl pyridine based MIP dropped sharper with increasing endo 6 concentration (difference in MIP and NIP binding dropped from 35 % to 5 % when endo 6 concentration was increased from 10 μM to 50 μM) compared to the acrylamide based MIP (30 % to 15 % for the same respective endo 6 concentrations). The reaction between cyclopentadiene and benzyl acrylate was conducted at initial reactant concentrations of 1, 2 and 4 mM and an initial concentration of 2 mM for both reactants would generate a suitable concentration of endo and exo 6 isomers to observe the imprinting effect as the amount of endo 6 generated (67 μM) was low enough to observe specific MIP binding and the amount of exo 6 generated (17 μM) was greater than its detection limit.

These results demonstrated that a suitable MIP system was identified and the MIPs produced were able to bind specifically to the product isomer used as the template (endo 6) in MIP production. Furthermore, the ability for the product isomers to be generated in concentrations used in the binding studies was proven. Hence it is reasonable to suggest that MIPs can be used to influence the stereo outcome of the model reaction using the MIP system and reaction conditions identified though more work is required to understand the effect of scaling up MIP and NIP systems and batch variations.

Acknowledgements

Firstly, I would like to thank my supervisors Dr. Chris Allender and Professor Thomas Wirth for their guidance and support both during my 3 years in the lab and during writing up.

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1 Introduction

1.1 Project Overview

Many organic reactions result in the formation of products with chiral centres, hence forming stereoisomers. One stereoisomer will usually form more predominantly than the other. This proves to be a problem if only the minor product is useful, for example in the synthesis of drugs.[1] Extensive research had been carried out to enhance the stereoselectivity of reactions using various catalysts, supports and solvents.

The long term vision of this project intends to provide an alternative solution to this problem using a molecularly imprinted polymer (MIP) imprinted with the disfavoured minor product (i.e. a polymer which has cavities designated to give a lock and key fit for the minor product in a similar manner to that of enzymes), in a segmented flow microreactor (Figure 1.1).

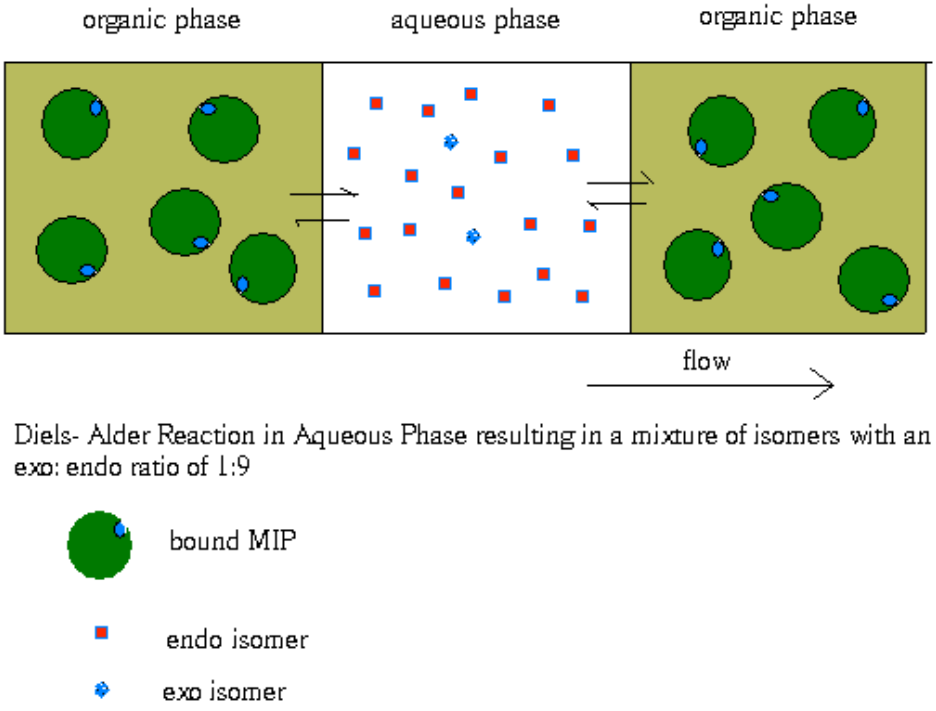
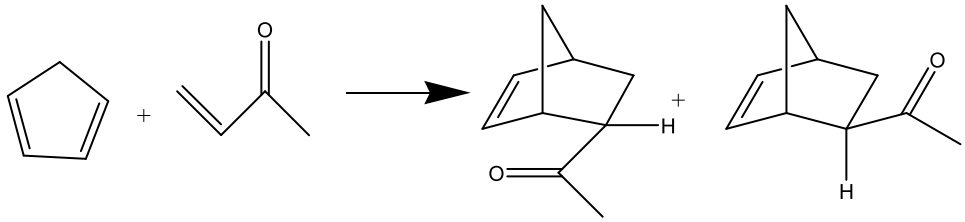


Figure 1.1 Segmented Flow Microreactor. The increase in the amount of exo isomer produced is achieved by using an exo imprinted MIP to selectively remove the exo isomer as it was formed in the aqueous phase to the organic phase.

A MIP is used to selectively remove the minor product from the aqueous phase to the organic phase hence shifting the equilibrium in the aqueous phase to favour the minor product. Initially, the Diels- Alder reaction between cyclopentadiene and methyl vinyl ketone would be used as a model reaction to demonstrate this (Figure 1.2) as Breslow[2] reported an enhancement in stereoselectivity for this reaction in water.



endo exo

Figure 1.2 Proposed Diels- Alder Reaction. Diels- Alder reaction between cyclopentadiene and methyl vinyl ketone to form endo 1 and exo 1.

This project combines several proven and well documented concepts such as the ability of MIPs used as selectors in chemical reactions,[3] the use of MIPs in Diels- Alder reactions,[4,5,6] and the efficiency of using a microreactor in chemical reactions in segmented flow[7]. Furthermore, the enhancement in stereoselectivity of the Diels- Alder Reaction between cyclopentadiene and methyl vinyl ketone in water[2] have also been demonstrated and reported in literature. In Section 1.2, the background of MIPs and microreactors and their uses in organic synthesis will be discussed.

1.2 Molecular Imprinted Polymers

Molecular Imprinted Polymers are polymers that have an engineered 3D shape, which give a 'Lock and Key' fit to specific target molecules. They are inspired by natural receptors such as antibodies and enzymes that are highly specific due to their unique 3D shapes created by intramolecular attractions of different functional groups within the protein chain.

Molecular imprinting of inorganic polymers was first demonstrated by Wulff[8] (who used a MIP to separate a racemic mixture of D, L- glyceric acid) and Klotz[9] (who reported that a MIP imprinted with methyl orange has a greater number of binding sites and exhibit stronger binding to methyl orange

than a NIP) independently in 1972. Since then, MIPs had been widely developed and used in a variety of applications such as stationary phases in liquid chromatography[10-11], solid phase extraction[12], biosensors[13] and synthesis[14].

1.2.1.1 Different Approaches to Making a MIP

1.2.1.1.1 The Basic Concept

The process of making a MIP can be summarised in 3 steps. Firstly, the functional monomers and template molecules are allowed to form complexes in solution. Then, the monomer template with addition of a crosslinking monomer is polymerised, effectively freezing the functional groups at the specific location. Lastly, the template molecule is removed, leaving an empty cavity, which will be the binding site of the MIP.[15] Figure 1.3 shows the flow chart of making a MIP.

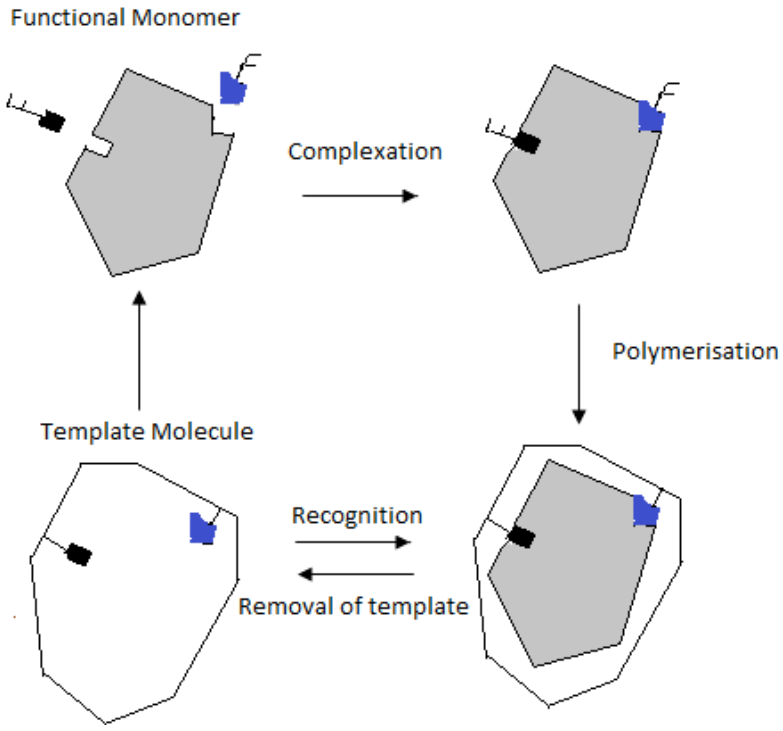


Figure 1.3 Schematic of making a MIP (adapted from ref. 15). Firstly, functional monomers and the template form complexes in solution. Then, the functional monomer and the cross- linker undergo polymerisation and hence freezing the functional monomers at specific locations. Lastly, the template is removed, hence leaving an empty cavity which will be the binding site of the MIP.

Free radical polymerisation is used in making MIPs. There are 3 main stages: initiation, propagation and termination. In initiation, the initiators break down when exposed to UV, microwaves or heat to form radicals by homolytic cleavage (Figure 1.4). Thermal initiation of MIPs which involves heating a pre-polymerisation mixture at 60 °C for 18- 24 hours, is the most common method though the main disadvantage is that the reactants are exposed to excess thermal energy which results in a higher system temperature.[16] Photochemical initiation of MIPs lowers the temperature of the polymerisation mixture and results in faster reaction times, more rigid MIPs and an improvement in specificity as demonstrated by Piletsky.[17] More recently, Turner and co-workers compared the binding performance and surface area of microwave induced and thermal induced caffeine imprinted MIPs and reported that thermally induced MIPs had a 30- 50 % higher binding capacity compared to microwave induced MIPs due to shorter reaction times. Furthermore, the non-specific binding for the microwave induced NIP was lower compared to the thermally induced NIP.[18]

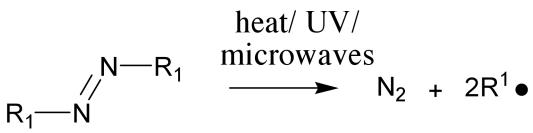


Figure 1.4 The initiation step in free radical polymerisation. An initiator is broken down by heat, UV or microwaves to form radicals.

Then, these radicals attack the carbon-carbon double bonds in either the monomer or the cross- linker (Figure 1.5).

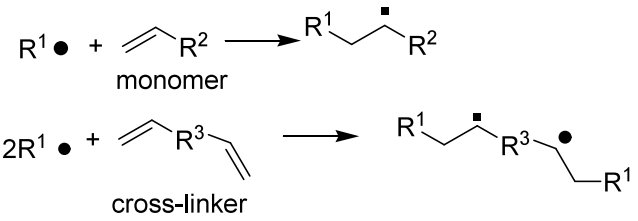


Figure 1.5 The propagation stage in free radical polymerisation- radicals react with the monomer or cross- linker.

The polymer chain then continues to grow as the radicals attack more monomers or cross-linkers in the same way (Figure 1.6).

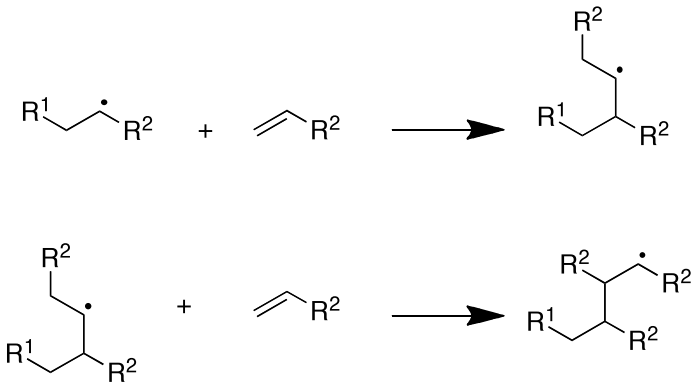


Figure 1.6 Continued propagation in free radical polymerisation

Lastly, the polymer chain stops growing when 2 radicals meet and react with each other (Figure 1.7).

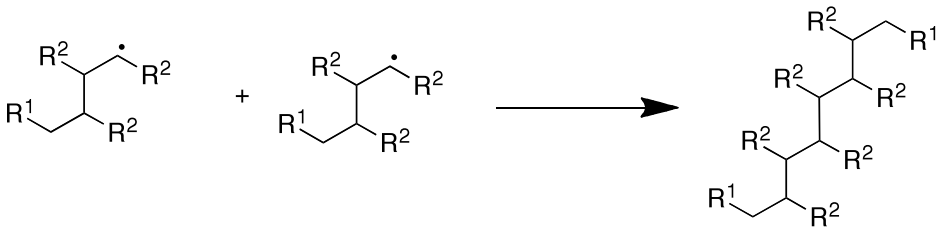


Figure 1.7 The termination step in free radical polymerisation. The polymer chains stop growing when two radicals react with each other.

The imprinted cavity is created using three main approaches: covalent, non-covalent and semi- covalent approach.

1.2.1.1.2 Covalent Imprinting

In this approach, reversible covalent bonds were used to join the monomer and the template in the complexation stage (refer to step 2 in figure 1.3). The covalent conjugate was then polymerised in conditions where the covalent link will remain intact. Lastly, the template is removed by cleaving the covalent bond. The same covalent linkage will form when the template binds to the MIP. This will be illustrated using the first covalent imprinting reported[19] as an example.

4- Nitrophenyl-α-*D*-mannopyranoside (template **2a** and **2b**) undergoes esterification in the presence of 4-vinylphenylboronic acid (functional monomer **1**) to form the covalent conjugate (**3a** and **3b**) (Figure 1.8).

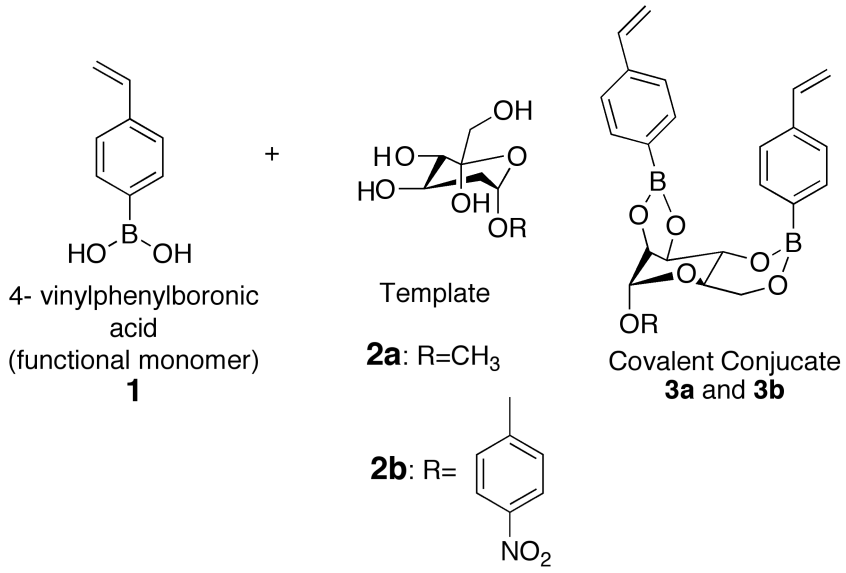


Figure 1.8 Forming the covalent conjugate 3a and 3b by the esterification of 2a and 2b respectively in the presence of the functional monomer 1. (Adapted from ref. 19)

The covalent conjugate then undergoes polymerisation in the presence of cross-linker and the relevant solvent (Figure 1.9).

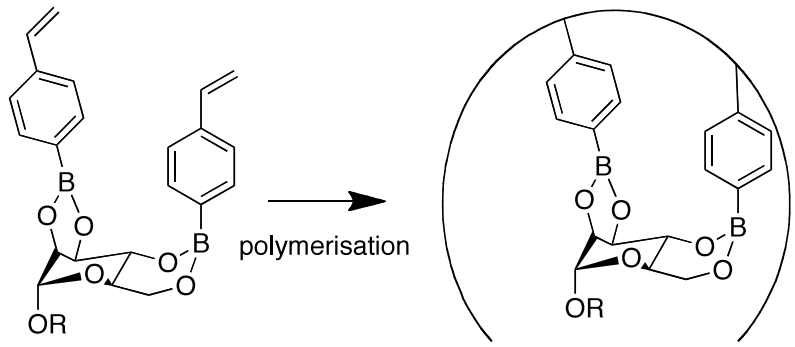


Figure 1.9 The covalent conjugate undergoes polymerisation (adapted from ref. 19)

The template was subsequently removed by hydrolysis, hence leaving a cavity which would be the specific binding site during rebinding (Figure 1.10).

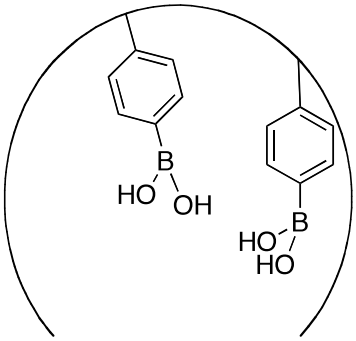


Figure 1.10 Cavity left after Template was removed by hydrolysis (adapted from ref. 19)

1.2.1.1.3 Noncovalent Imprinting

This approach was first demonstrated by Mosbach et. al.[20]where rhodanile blue and safranine were used as templates. Non- covalent interactions such as hydrogen bonding, electrostatic interaction etc. was utilised to align the template and the monomer before polymerisation. The template was then removed after polymerisation by washing. No chemical bond was formed between the monomer and the template involved at any point. The imprinting of theophylline (a drug; **4**) as shown by Vlatakis et al[21] is used as an illustration. When theophylline (**4**) and methacrylic acid (**5**) were mixed together, they would align themselves as shown below due to hydrogen bonding between the amine groups in the template and the carbonyl group in the monomer and also between the carbonyl group of the template and the hydroxyl group. The polymer system mixture was then polymerised and then the template was removed as shown in Figure 1.3.

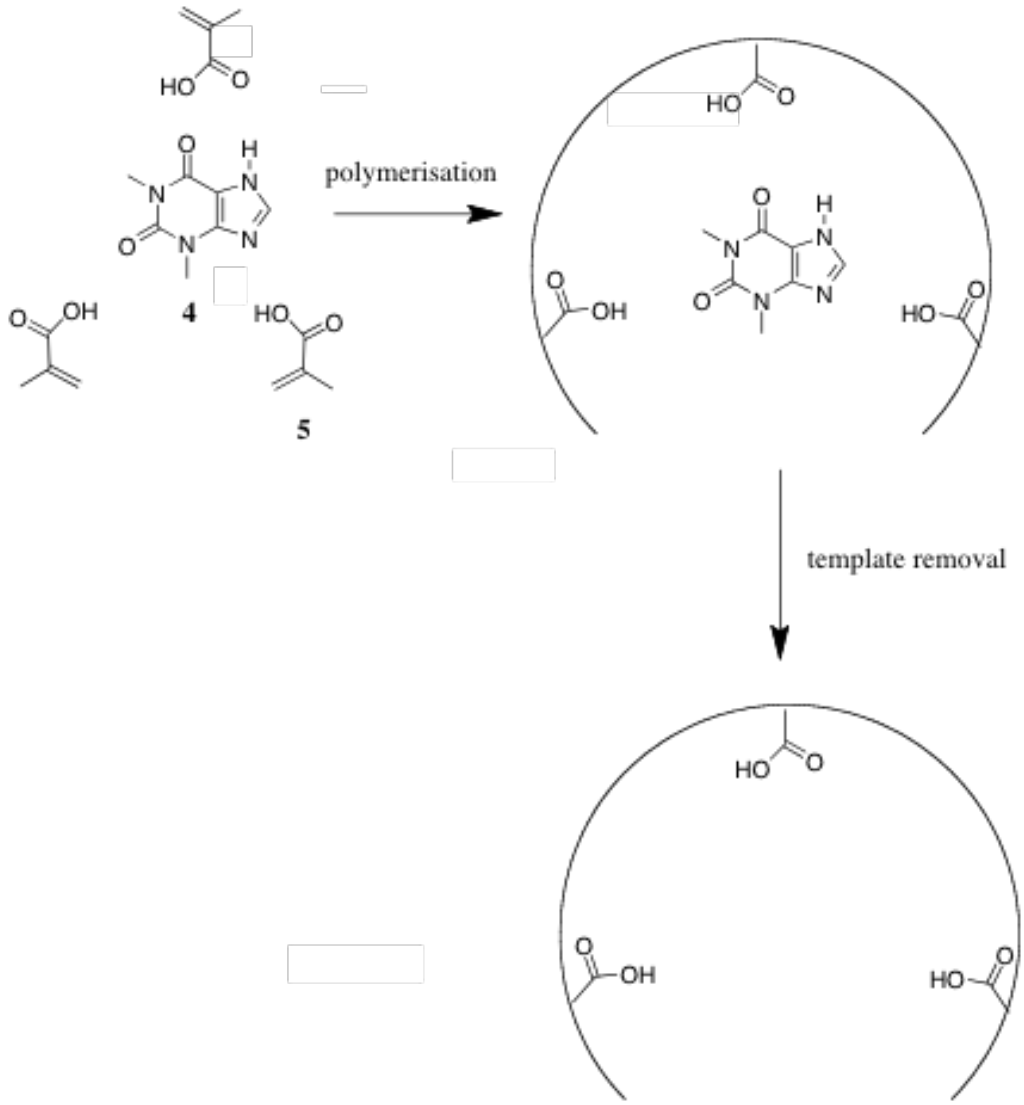


Figure 1.11 Non- covalent imprinting of theophylline 4 using methacrylic acid 5 as the functional monomer (adapted from ref. 21)

1.2.1.1.4 Advantages and Disadvantages of the Covalent and Non- Covalent Approach

Both covalent and non- covalent approaches have their own advantages and disadvantages.[15] The non- covalent approach is used widely as it is an easier process (no template- monomer conjugate needed and template can be removed by washing the MIP with solvent) and can be used for many template structures. The guest binding and release is also fast compared to the covalent approach. However, an excess amount of monomer is needed. This leads to the formation of cavities with a range of different shapes as polymerisation may occur as a result of other intermolecular interactions (e.g. between two

templates to form a dimer, between cross- linker and template etc.). Lastly, polymerisation conditions need to be selected carefully in order to maximise the amount of product formed.

The covalent approach does not have the issues of excess monomers as stoichiometric amounts of monomers are used and hence the imprinted sites are more uniform. A variety of polymerisation conditions such as high temperatures, very high or low pH, highly polar solvents etc. can be used as the monomer and the template are joined by covalent bonding and are sufficiently stable. However, templates which can form reversible complexes are limited. It is also more complicated as synthesis of the monomer- template conjugate is needed. Lastly, there is slow guest binding and release as a covalent link needs to be formed.[15]

1.2.1.1.5 The Semi- Covalent Approach

The semi- covalent approach attempts to combine the advantages of both methods by creating the cavities covalently and rebinding in a non-covalent manner.[22] There are two main variations to this approach: 1) the template and functional monomer are connected directly; 2) the template and functional monomer are connected via a spacer. Sellegren and Andersen[23] reported the first truly semi- covalent approach by imprinting L-2-amino-3-(4-hydroxy-phenyl-)-1-propanol (**7**). Firstly, monomer- template conjugate (**6**) was synthesised by reacting the template (**7**) with acryloyl chloride. Next, **6** was polymerised using an excess of DVB in acetonitrile to form the MIP. Then, **7** was removed using a concentrated NaOH/ MeOH solution and the selectivity of the cavities created were evaluated by batch rebinding of radioactively labelled D and L- p- aminophenylalanine ethyl ester (p-NH_2PheOEt) (Figure 1.12). In the binding studies however, the polymers preferred binding to the D- form of the substrate even though L- form print molecules (**6**) were used in the polymerisation step due to stereospecific electrostatic and hydrogen bonding interactions between the aliphatic and aromatic amide groups of the substrate with the carboxyl groups at the binding site of the MIP.

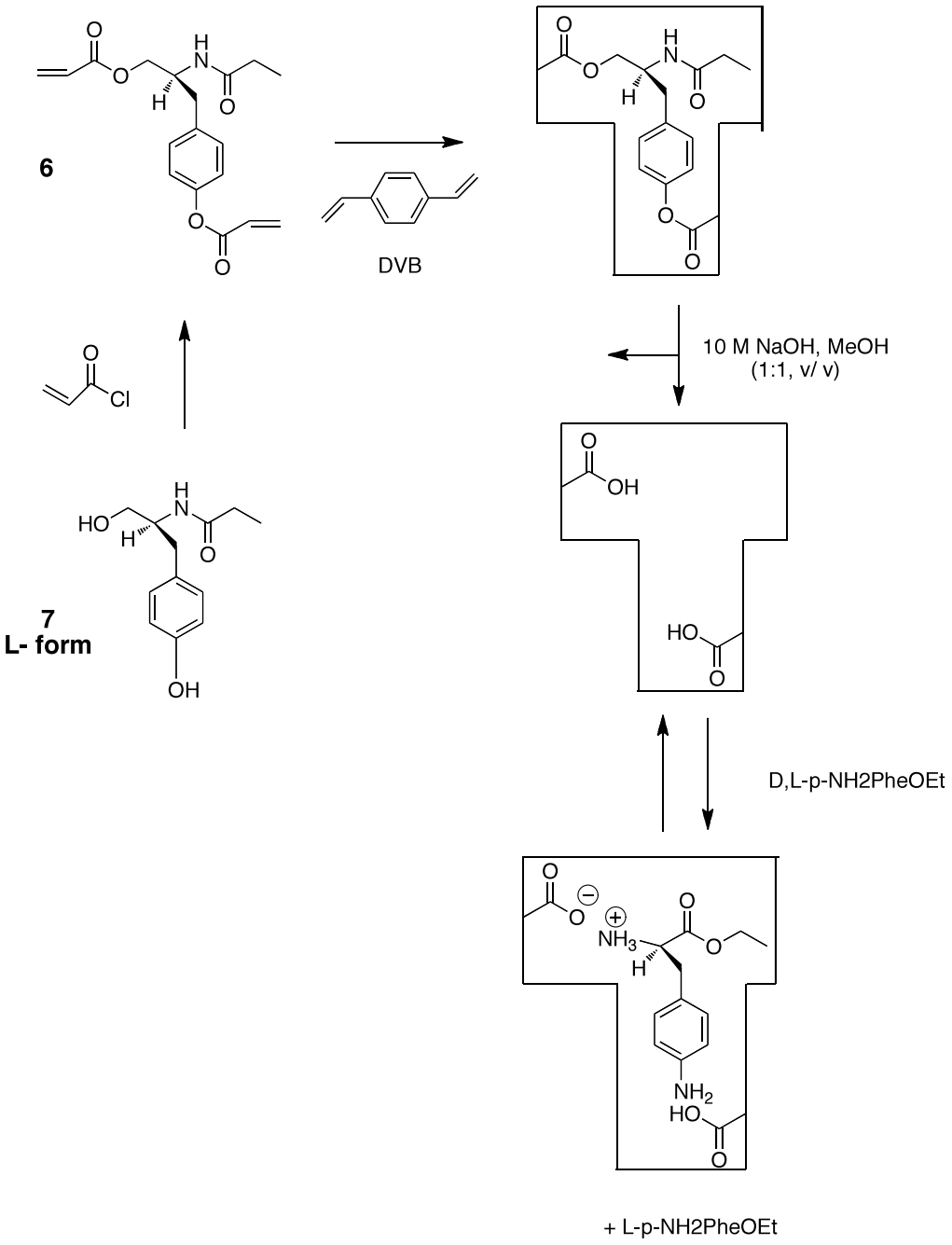


Figure 1.12 Production of a MIP to selectively recognise L- p- aminophenylalanine using the Semi- Covalent Approach. A MIP was made using 6 which was a derivative of 7 and was polymerised using DVB. 7 was subsequently removed using 10M NaOH/ MeOH (1:1) and the rebinding was assessed by batch rebinding of radioactively labelled D and L forms of p- aminophenylalanine. (Adapted from ref. 23)

The second variation of the semi- covalent approach in which the template and the functional monomer are connected using a sacrificial spacer was developed by Whitcombe et.al.[24] in order to avoid overcrowding in the

binding site and allow easier access for the template during rebinding. The method used the 4- vinylphenyl carbinate ester (**8**) which acted as a covalently bound functional monomer where the template (red part of **8**) could easily be cleaved off by hydrolysis. The carbonyl group in **8** (as shown in blue in Figure 1.13) acted as a sacrificial spacer and was lost as CO_2 during the removal of the template. Hence adequate space was left as a result of CO_2 removal to establish hydrogen bonding between the functional groups of the imprinting cavity and the template.

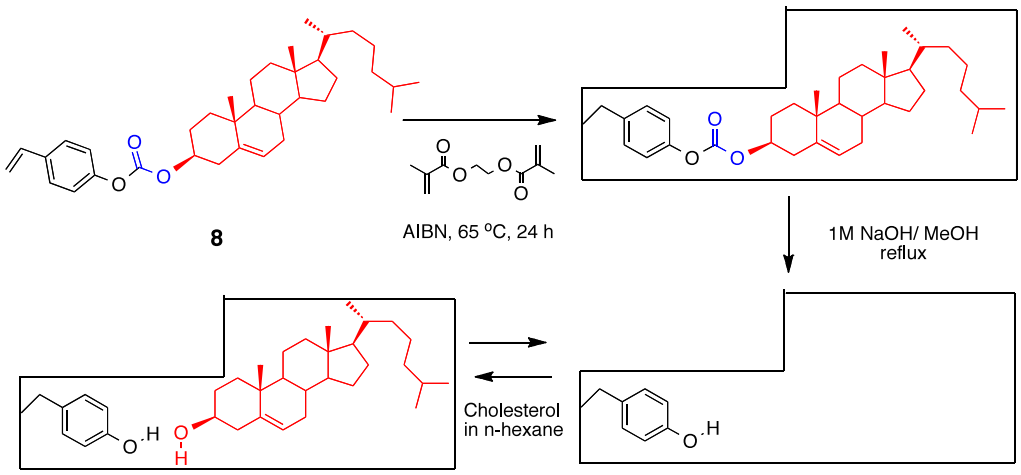


Figure 1.13 Production of a MIP capable of recognising cholesterol using the sacrificial spacer (blue part in 8) approach. Functional monomer 8 was polymerised using AIBN as the initiator and at 65 °C for 24 h. The template (red part in 8) was subsequently removed by hydrolysis resulting in the formation of a non- covalent binding site. (Adapted from ref. 24)

The physical characteristics and selectivity of MIPs for phenols prepared via the semi- covalent spacer method and the non-covalent method was compared by Qi et al.[25] 4-chlorophenyl (4-vinyl)phenylcarbonate (**11**) was used as the monomer- template conjugate for the semi- covalent spacer method and was synthesised by reacting 4- vinylphenol (**9**) and 4- chlorophenyl chloroformate (**10**) (Figure 1.14 a). Then, the template was polymerised using EGMA in chloroform. Lastly the template was removed using 1 M NaOH/ CH_3OH solution (Figure 1.14 b). 4- chlorophenol and 4- vinyl pyridine were used as the template and functional monomer respectively for the non- covalent method. In terms of physical characteristics, the MIP made via the semi-covalent method had a larger surface area and a smaller pore size compared to

the non- covalently synthesised MIP. In terms of selectivity, the MIP made via the semi- covalent spacer method had a higher imprinting factor with chlorophenyl in equilibrium binding studies (imprinting factor of 1.5 for semi-covalent produced MIP and imprinting factor of 1.4 for non- covalently imprinted MIP) and narrower and sharper peaks when evaluated chromatographically using HPLC than the MIP made using the non- covalent method as the semi- covalent spacer method MIP had a more uniform distribution of binding sites. Furthermore, no leaching of template for the MIP made using the semi- covalent spacer approach under normal conditions were reported as the template remained covalently attached to the MIP.

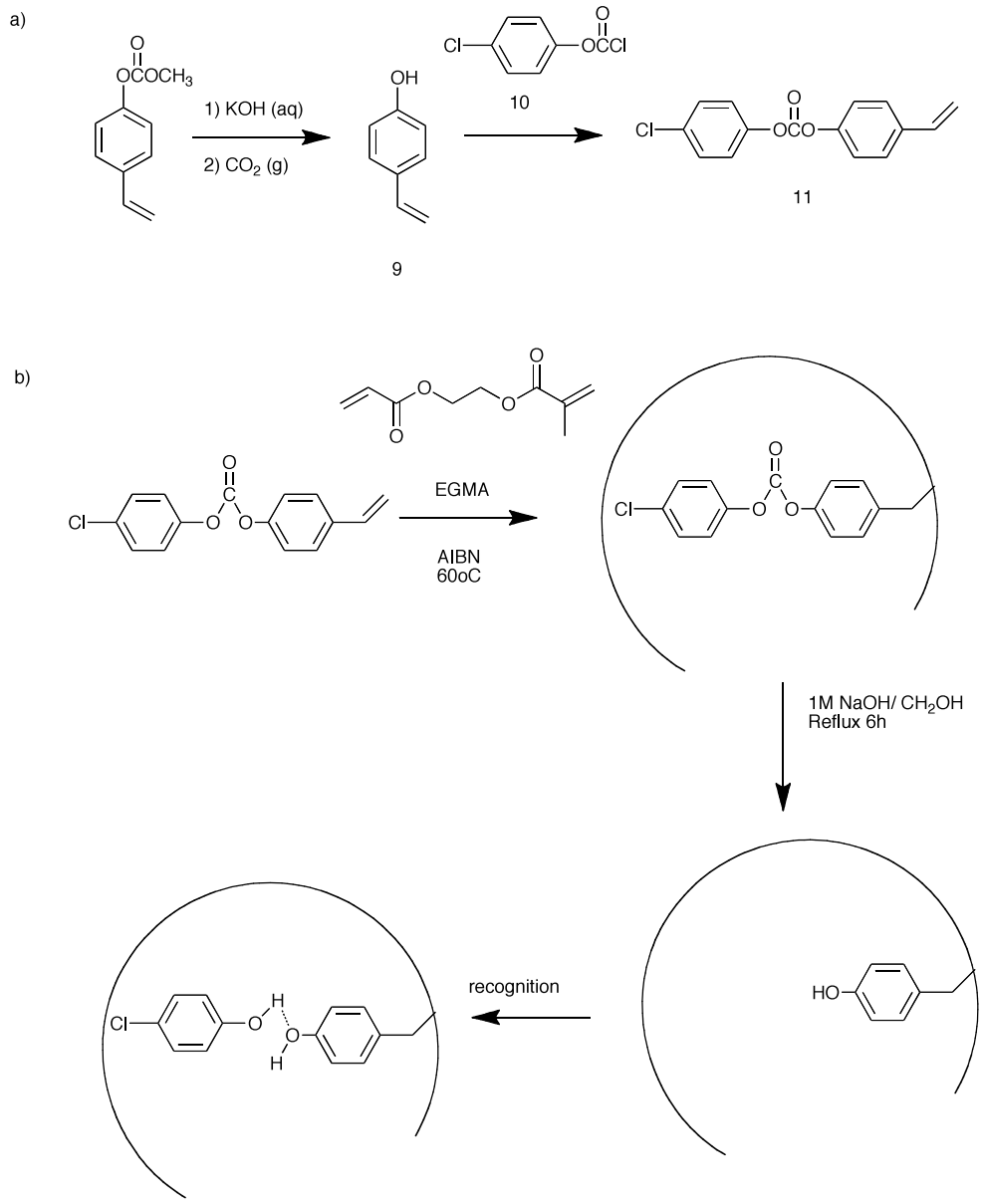


Figure 1.14 Production of a MIP for recognising phenols using the semi- covalent spacer approach. a) Template- conjugate synthesis- The template- conjugate (11) was produced by reacting 4- vinylphenol (9) and 4- chlorophenyl chloroformate (10). b) MIP was produced by polymerising template- conjugate (11) and EGMA. Template removal was achieved using 1 M NaOH/ CH_2OH and reflux for 6 h. (Adapted from Ref. 25)

1.2.1.2 General Factors which Need to be Considered when Making a Non- Covalent MIP

1.2.1.2.1 Monomer

There are many possible monomers which can be polymerised to form a MIP. The monomers have at least one C=C bond as this bond is prone to be attacked by free radicals and hence easy to initiate. The choice of monomer depends on the functional groups of the target molecule. They are divided into 3 groups: basic, neutral and acidic. Figure 1.15 shows some of the monomers which can be used. Methacrylic acid (MAA) is most commonly used and it is known to form strong interactions with amines, amides, urethanes and carboxylic acids.

In addition to the choice of functional monomer, the ratio of functional monomer to template to cross- linker in the polymer system is also important. The typical functional monomer to template to cross- linker ratio used in MIP production is 1: 4: 20 and studies of how this ratio affects imprinting specificity were carried out by Yilmaz et. al[26] who investigated the variation in specific binding for different functional monomer (2-(trifluoromethyl) acrylic acid (TFMAA)) to template (^{3}H-theophylline) ratios ranging from 4:1 to 5000:1. At functional monomer to template ratios as high as 500:1, the ^{3}H-theophylline imprinted MIP still bound more ^{3}H-theophylline than the respective NIP. At low functional monomer to template ratios, a larger number of low affinity sites are created as association and disassociation processes takes place simultaneously at equilibrium. At high functional monomer to template ratios, a smaller number of high affinity sites were created as the equilibrium is shifted to favour complex formation due to an excess of functional monomers. Yilmaz et. al. also investigated the effect of imprinting specificity with varying amounts of cross- linker (19, 36 and 69 % DVB) and found that there was no change in specificity with different amounts of cross-linkers.

1.2.1.2.2 Cross- Linker

The position of the binding sites of the MIP can only be preserved if the polymer is rigid. As a result, cross- linkers are introduced to the polymer to increase its mechanical stability. The concentration of the cross- linker also influence the size of the pores formed in the polymer. Furthermore, the reactivity of the cross- linker should be similar to the functional monomer in order to achieve an even distribution of binding sites. There is a wide range of cross- linkers which can be used and some examples are also shown in Figure 1.15. Ethylene glycol dimethacrylate (EDMA) is used most often though some cross- linkers such as trimethylolpropane trimethacrylate (TRIM) have been shown to be more efficient and leads to a more porous polymer than using EDMA.[15] TRIM cross-linked MIP will be much more rigid than an EDMA cross- linked MIP as each TRIM molecule has 3 carbon- carbon double bonds while EDMA only has 2 carbon- carbon double bonds. Furthermore, EDMA is a linear molecule while TRIM is not a linear molecule (Figure 1.15) which leads to functional monomers being closer together and in turn more closely packed binding sites hence resulting in a decrease in specificity due to overcrowding when imprinting large templates.

1.2.1.2.3 Solvent

In order to maximise the number of binding sites, the polymer should also be porous. Hence, the MIP should have low polarity and low hydrogen bonding capacity when used in non- covalent imprinting. Theta Potential (θ) is a parameter which describes the conformation of the polymer chain due to the interaction between the chain segments and solvent molecules. In good solvents, the polymer chains expand in order to maximise segment- solvent contacts. In poor solvents, polymer chains collapse to minimise the contact between polymer segments and the solvent. Competing with this effect is the excluded volume (volume that is not accessible to other molecules or parts of molecules due to the presence of the 1st molecule[27]) effect. A solvent in which these two effects are balanced are called a theta solvent.

The solvent used in MIP production plays an important role in the pore structure of the resulting MIP. Firstly, a porogenic solvent is needed to form a

macroporous polymer as it cause phase separation of the polymer matrix. If there is full conversion, there will be a polymer matrix phase and a liquid or solvent phase. The liquid phase acts as a template for the permanent pore structure of the polymer. The point at which phase separation occurs depends on the nature of the porogen (solvent), the compatibility with the MIP/ NIP polymer matrix and the amount of porogen used.[28] Secondly, increasing the amount of solvent decreases the particle size of the resulting polymer as the growing polymer chains can not take up the whole reactor volume and a dispersion of particles are formed.[29]

In addition to being soluble to the template, monomers and cross-linkers, there are two criteria that need to be satisfied. Firstly, it needs to be aprotic (does not have any dissociable hydrogen). Secondly, it should not compete with the polymer to bind to the target molecule.

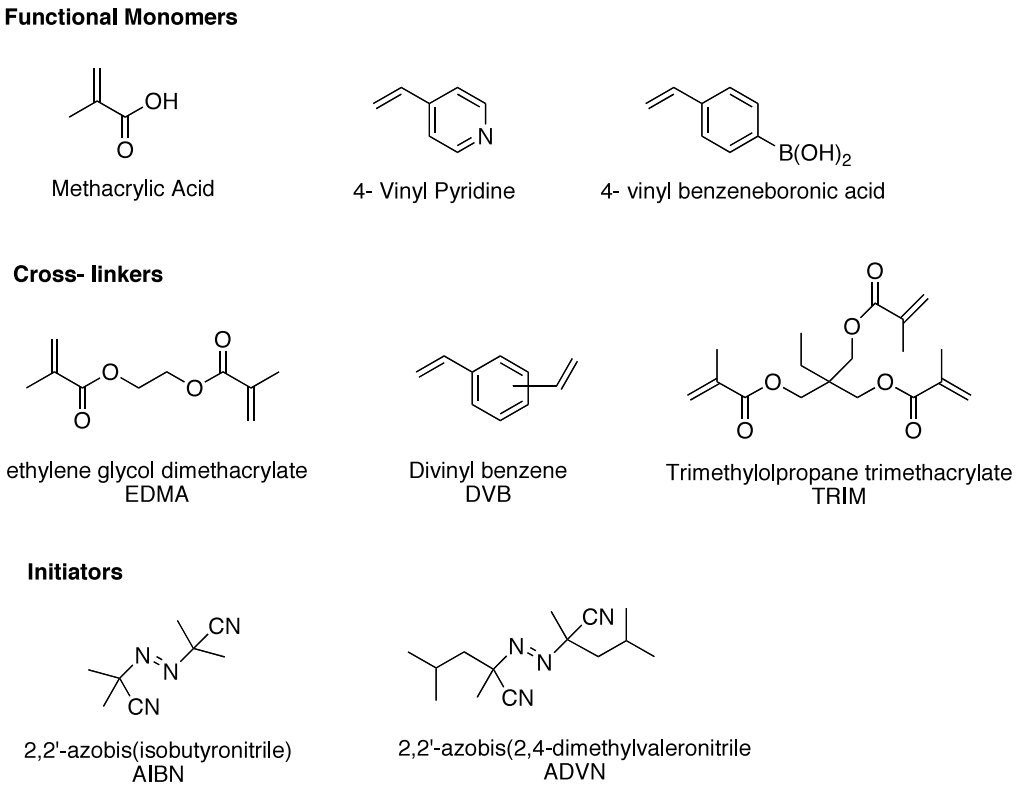


Figure 1.15 Reagents for Making MIPs (adapted from ref. 15)

1.2.1.2.4 Initiator

An initiator is a molecule that undergoes homolytic fission and reacts with the monomer when exposed to electromagnetic radiation. This results in

the formation of an active centre. In making MIPs, azonitrile group initiators are used as the decomposition kinetics and rate are not affected by different solvents. Some initiators are shown in Figure 1.15. AIBN is most commonly used in the production of MIPs. However, there are also reports of using Fenton reagents such as Iron (II) Chloride and Hydrogen Peroxide as initiation systems as demonstrated by Cirillo et. al.[30] who reported that MIPs using redox initiation systems has comparable selectivity to MIPs synthesised using traditional initiator systems such as AIBN (58 % for redox intiation system and 61 % for traditional initiators) and has additional advantages such as shorter reaction times and lower polymerisation temperatures.

1.2.1.3 Strategies to Make Polymer Microspheres

Traditionally, most MIPs were made using bulk polymerisation where a porogenic solvent was used to produce a block of macroporous polymer. This block was then crushed, ground and sieved to give particles around 25 μm. However, these particles were irregular and the process was labour intensive. Furthermore, the yield was low[31]. Hence, it would be desirable if polymer microspheres can be formed during polymerisation as opposed to obtaining it from grinding after polymerisation. Other strategies have been attempted such as suspension polymerisation[17] and a 2- step swelling technique[32], but they require special dispersing phase and tedious swelling processes respectively.

Precipitation polymerisation is ideal in preparing polymer microspheres as the spheres formed are uniform and no stabilisers or complicated procedures are needed. Furthermore, the particle size of the resulting polymer decreases as the amount of solvent added increases. Precipitation polymerisation uses an excess (usually greater than 95 % volume) of solvent to give uniform MIP microspheres with a good yield (at least 85 %) with a particle size of around 0.1- 5 μm.[16]

1.2.1.4 Application of MIPs

MIPs had a wide range of applications such as liquid chromatography, solid phase extractions and as biosensors and had been discussed in various

reviews[33] and book chapters. The subsections which follow would give an example[12,34,35] in which MIPs had been used in these areas.

1.2.1.4.1 Stationary Phases in Liquid chromatography

Matsui et. al. imprinted cinchona alkaloids (**12- 15**, Figure 1.16) using methacrylic acid (MAA) and 2- (trifluromethyl)acrylic acid (TFMAA) by in situ molecular imprinting, i.e. filling an empty chromatographic column with the MIP polymerisation mixture and incubating the column resulting in MIP formation within the column.[34] The performance of the MIPs in the columns were then evaluated by chromatographic studies. Using TFMAA as the functional monomer resulted in a higher retention factor (k') for the respective analyte (**12- 15**) (migration rate of an analyte on a column) which meant a longer analyte elution time and hence indicating an increase in selectivity of the TFMAA MIP. This increase in selectivity of the TFMAA MIP was due to a greater acidity of the strongly electron withdrawing trifluoromethyl group which shifted the equilibrium of the monomer- template interactions to favour complex formation (ion pairing or hydrogen bonding) between the acid group of the monomer (TFMAA) and the nitrogen of the templates.

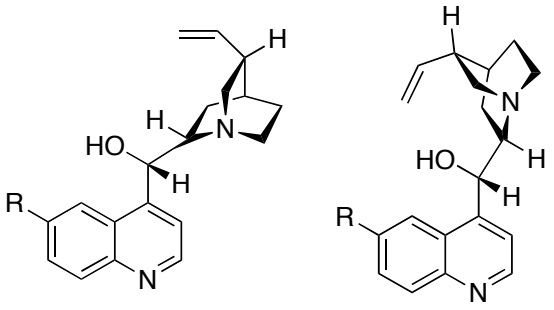


Figure 1.16 Structure of Cinchona Alkaloids (12-15) used to produce MIPs via in situ molecular imprinting for Chiral Stationary Phases in Liquid chromatography. (Adapted from ref. 34.)

1.2.1.4.2 Solid Phase extraction

Liu et. al. produced a nicotine imprinted methacrylic acid (MAA) based MIP (Figure 1.17) to remove nicotine from tobacco smoke.[12] The ionic interaction and hydrogen bonding between the nitro groups of nicotine and the hydroxyl group of MAA (Figure 1.17) was observed by the change of UV

absorption spectrum of nicotine from 260 nm (no MAA present) to 238 nm (4.0 equivalent MAA in chloroform).

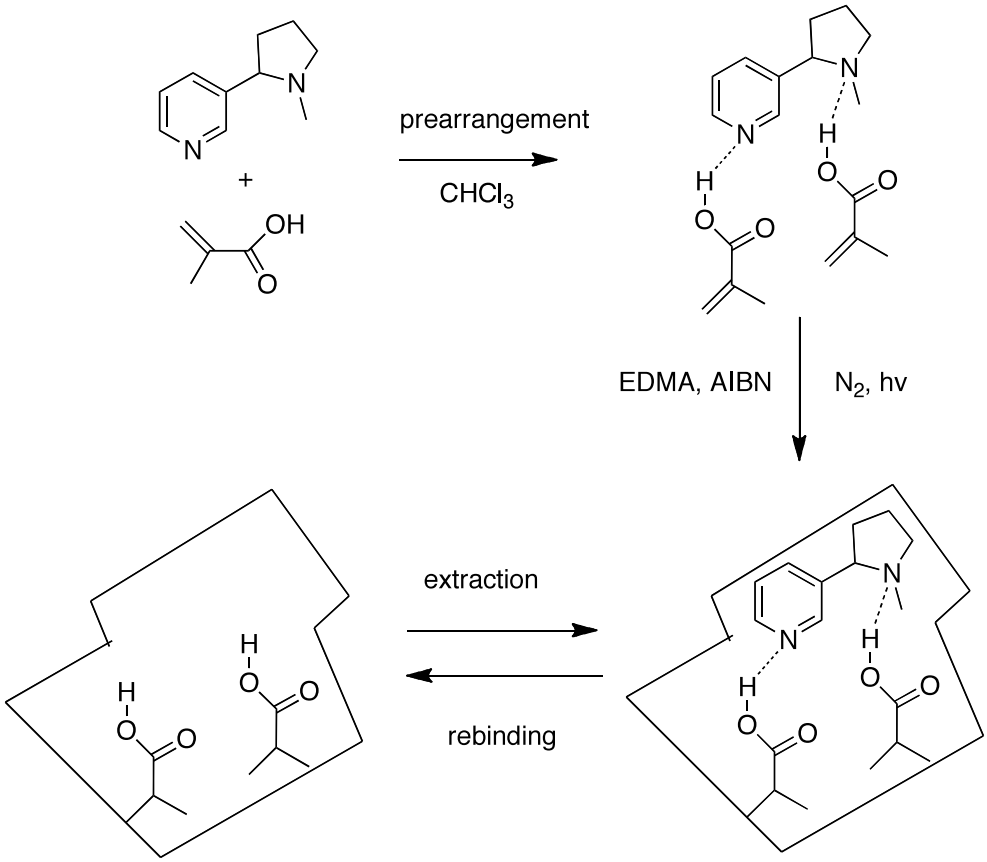


Figure 1.17 The production of a nicotine imprinted methacrylic acid (MAA) based MIP by utilising ionic and hydrogen bonding interactions between the nitro group of nicotine and the hydroxl group of methacrylic acid. (Adapted from ref. 12).

1.2.1.4.3 Biosensors

Ye et. al.[35] incorporated a fluorescent functional monomer (**16**) into the tritium labelled (S)- propanolol imprinted methacrylic acid based MIP capable of changing the fluorescent intensity when a target analyte bound to any of the MIP cavities (both non- specific and specific binding). According to the authors, this approach was useful in 1) a direct quantification of the template in a mixture of radiolabelled compounds and 2) in a competitive assay where the labelled compound act as a tracer and the unlabelled compound act as an inhibitor to the binding of the labelled compound (Figure 1.18).

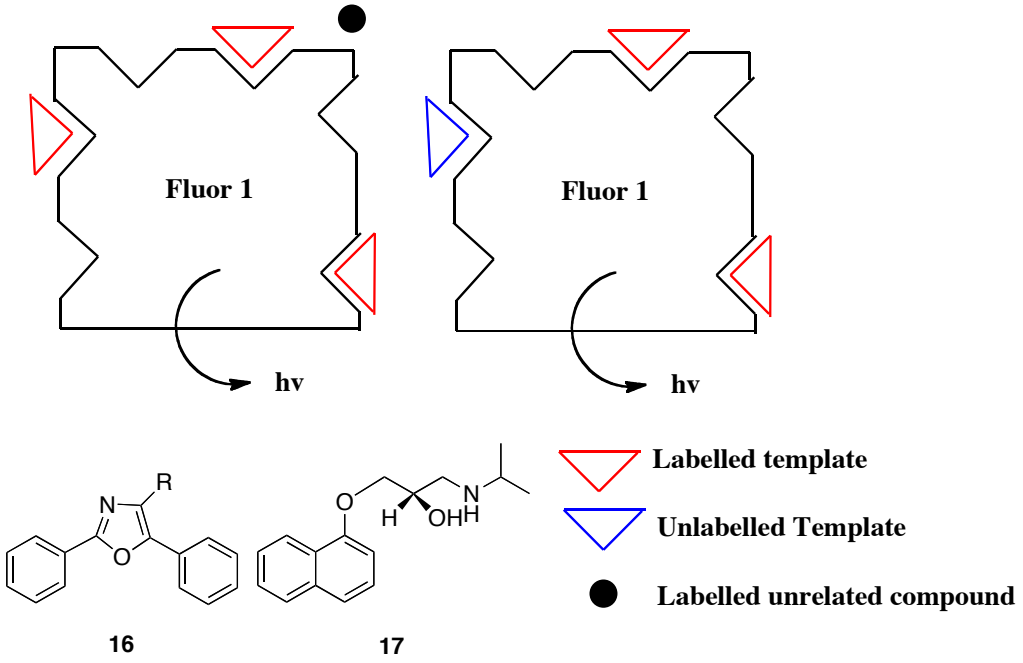


Figure 1.18 Diagram to illustrate a chemical sensing MIP. Fluorescent compound 16 was incorporated into a (S)- propanolol (17) imprinted MIP which converts β radiation from the bound tritium labelled template to a fluorescent signal. The MIP was useful in 2 ways: a) in a direct quantification of the template in a mixture of radiolabelled compounds and b) in a competitive assay where the labelled template acted as a tracer and the unlabelled template acted as an inhibitor to the binding of the labelled template (adapted from ref. 35)

In order to assess the MIP response to the tritium labelled template (**17**), increasing amounts of MIP and NIP were incubated in 500 μL toluene with 0.5 % volume of acetic acid with a fixed amount of [H^3](S)- propanolol. Samples were then counted without the removal of [H^3](S)- propanolol. The MIPs count (half the maximum counts) was two times higher than the NIPs when 0.2 mg of polymers were used. This was due to the specific binding of the labelled template with the MIP. In the competitive assay, the binding of the tritium labelled (S)-propanolol MIP was inhibited by increasing the amount of unlabelled template which resulted in the decrease in fluorescent counts. The fluorescent counts of the NIP did not change significantly when increasing the amount of unlabelled template.

1.2.2 MIPs in Chemical Synthesis and Catalysis

Early reports of MIPs being used in synthesis and catalysis were from Damen and Neckers.[36] They attempted to use MIPs to influence the formation of a metastable product (β-truxinic acid) in the solid- state photodimerisation of trans- cinnamic acid. A monomer- template conjugate (**19**) was formed by reacting β-truxinic acid (**17**) with vinyl- benzyl chloride (**18**) in the presence of triethylamine (Figure 1.19). It was then copolymerised with styrene and divinylbenzene. Afterwards, the template was removed by acid hydrolysis in methanol, leaving 2 benzyl alcohol groups in the cavity (Figure 1.19).[37]

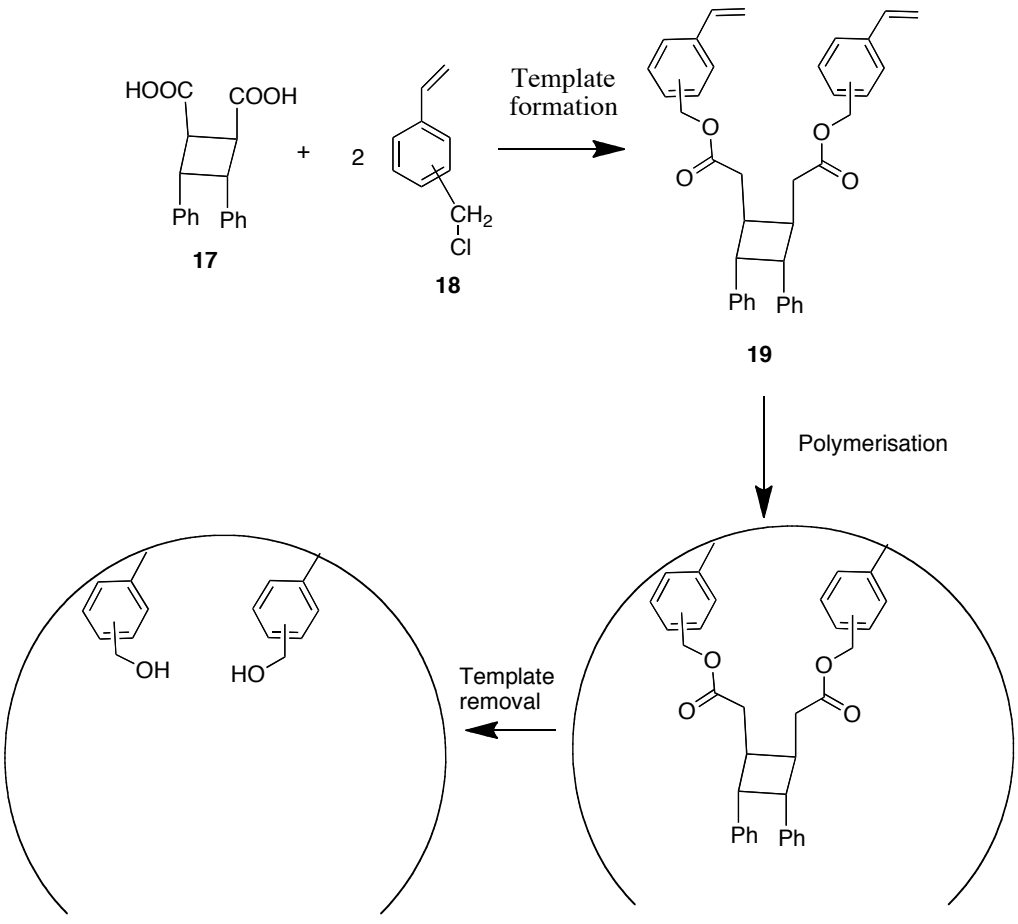


Figure 1.19 Formation of MIP for Use in Synthesis of β- truxinate. *β-truxinic acid (17) was reacted with vinyl- benzyl chloride (18) in the presence of triethylamine to form monomer- template conjugate (19). 19 was then copolymerised and the template was removed by acid hydrolysis. (Adapted from ref. 37).*

The polymer was then reacted with transcinnamoyl chloride (**20**) (reagent to produce the template) followed by irradiation from mixtures of photodimers.[37] β- truxinate was formed in 53 % of the MIP cavities (Figure 1.20) while the stable α form was the only product in the uncatalysed reaction.

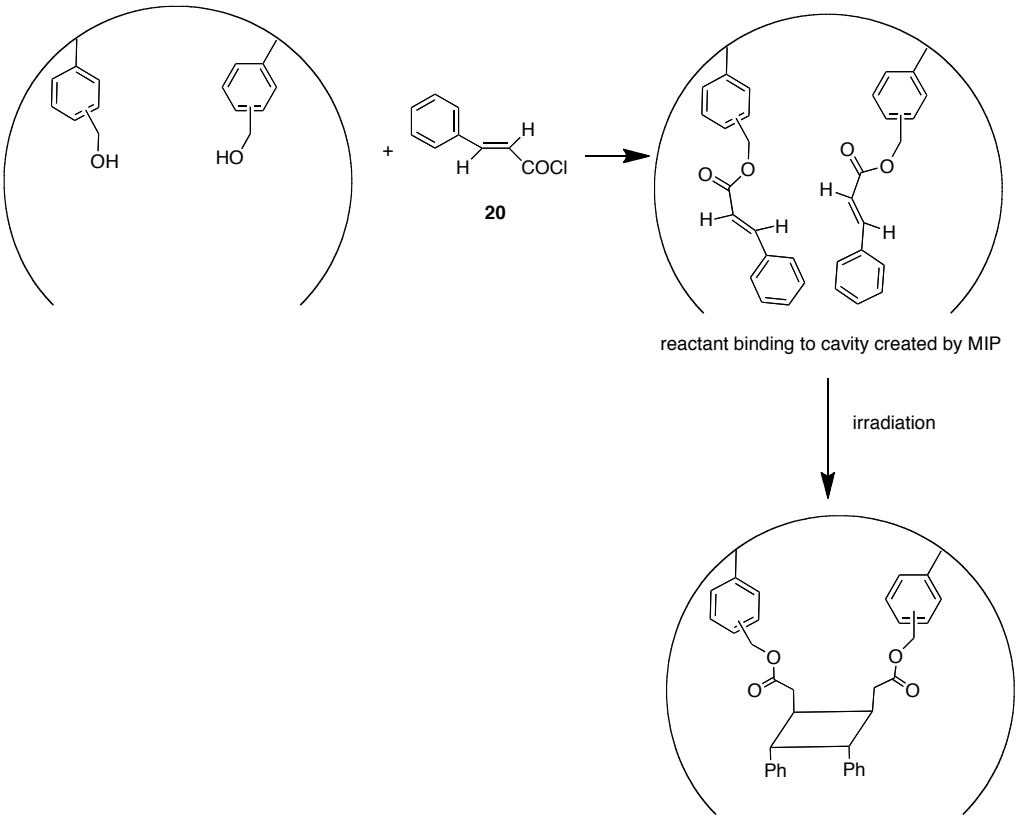


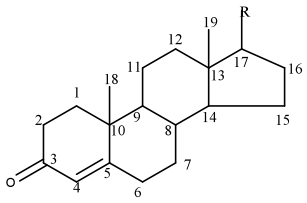
Figure 1.20 Formation of β- truxinate using MIPs as a steroselective catalyst. The 19 imprinted MIP was reacted with transcinnamoyl chloride (20) to form β-truxinate. (Adapted from ref. 37)

Since then, this area had drawn an increasing amount of interest as the high selectivity of MIPs could be exploited and used as screening aids, imprinted polymer reagents, protecting groups and catalysts. Some examples[38-43] will be discussed in the following section.

1.2.2.1 Use of MIPs as Screening Aids

The use of MIPs in screening combinatorial libraries was first reported by Ramstrom et al in 1997[38] who used MIPs as a recognition matrice in the screening of a library of closely related Δ^4-androsten-3-one structures. A MIP was made using 11-α-hydroxyprogesterone (**21**) as a template and another MIP

was made using corticosterone (**22**) as a template [(**21**) MIP and (**22**) MIP respectively in Figure 1.21].



21: R= $COCH_3$, 11α- OH

22: R= $COCH_2OH$

Figure 1.21 Steroid General Structure and Structure of Templates (adapted from ref. 38)

After the MIP particles were made using bulk polymerisation using the noncovalent approach, they were packed into HPLC columns. Both MIPs had high specific binding with their respective templates as the respective templates were not eluted.

1.2.2.2 Imprinted Polymer Reagents

Bystrom et al[39] attempted to control the regio and stereochemistry of hydride reductions of steroidal ketones (**23** in Figure 1.22) using MIPs. Templates (**24** and **25** in Figure 1.22 and 1.23 respectively) with similar chemical structure to the templates were polymerised covalently. The templates were removed by reacting the polymer with $LiAlH_4$. $LiAl_4$ in THF was used to attach the hydride groups to the hydroxyl cavities and the excess was then removed before the polymer was suspended in THF at room temperature. The original ketone (**23**) was added and reduced.

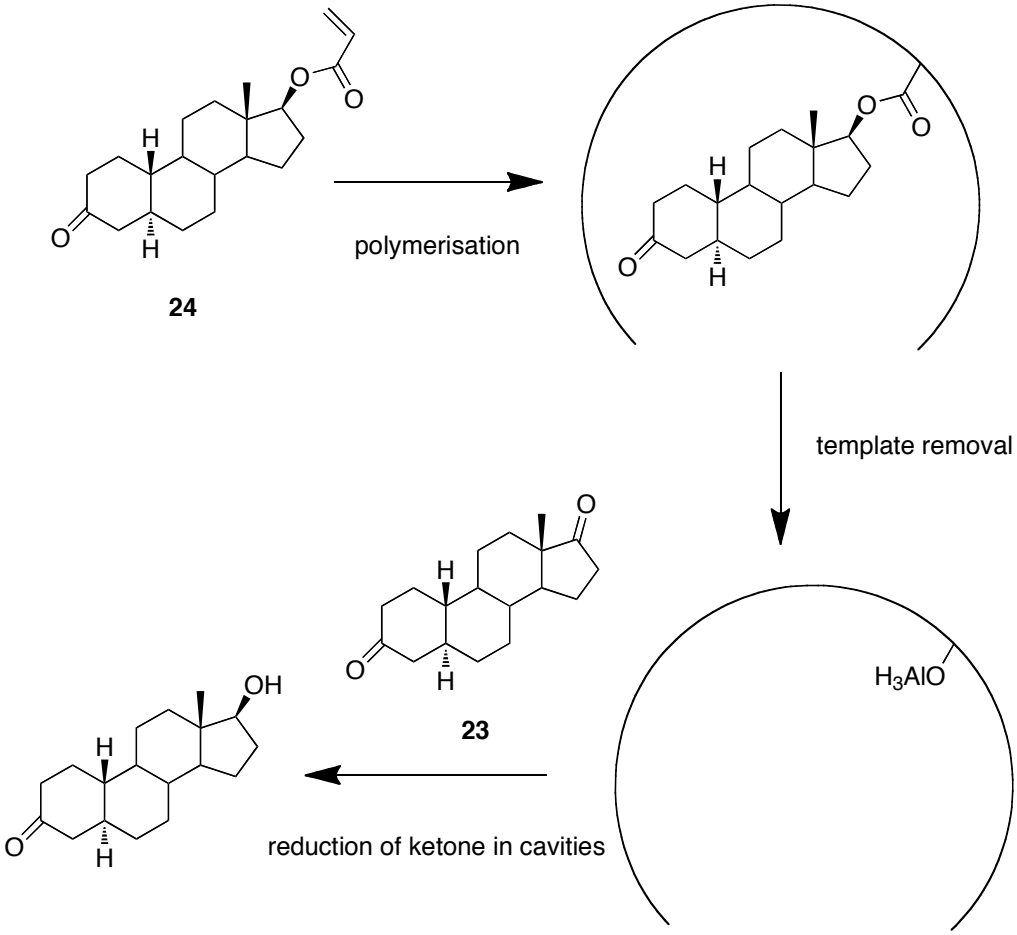


Figure 1.22 Reduction of 23 in the cavity of 24 imprinted MIP resulting in a predominant reduction of 23 at position 17 (adapted from ref. 39)

The **24** imprinted polymers only reduces **23** at the 17- position (17- position to 3-position ratio: 85:15) while compound **25** imprinted polymers reduces **23** at the 3- position (17- position to 3- position ratio 5: 95). This demonstrates the high specificity of MIPs and its potential as a stereo and regioselector.

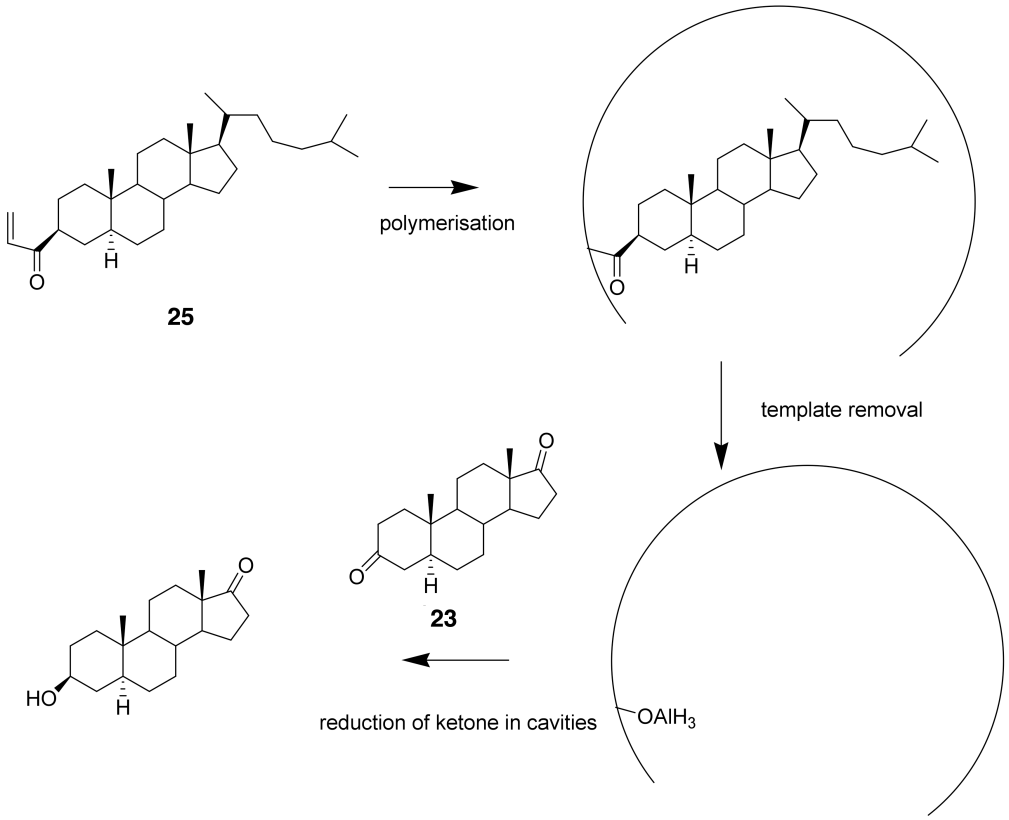
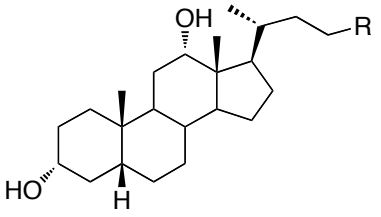
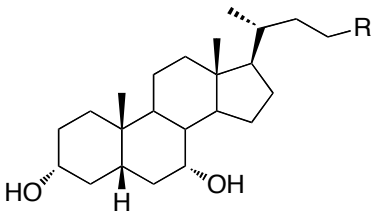


Figure 1.23 Reduction of 23 in the cavity of 25 imprinted MIP resulting in a predominant reduction of 23 at position 3 (adapted from ref. 39)

1.2.2.3 Using MIPs as Protecting Groups

Alexander and coworkers[40] investigated the use of MIPs as protecting groups in regioselective acylation of trihydroxysteroids. **26** and **27** (Figure 1.24) were used as templates to form MIPs. The polymer was then washed with a mixture of THF/ methanol/ water to remove the template. Two sets of acylation experiments were then carried out: modification of **28** and **29** (Figure 1.24) in **26** imprinted MIP and **28** and **29** in **27** imprinted MIP.





26: R= $CO_2C(CH_3)_3$

27: R= $CO_2C(CH_3)_3$

28: R=CH_2OH

29: R=CH_2OH

Figure 1.24 Structures of 26, 27, 28 and 29. 26 imprinted MIPs and 27 imprinted MIPs were made and 28 and 29 were used for rebinding experiments (adapted from ref. 40)

The polymer- template complexes were then classed as 'match' and 'unmatched', depending on whether the template fits the polymer cavity in the theoretical way. This is illustrated in Figure 1.25 for **26** imprinted MIP.

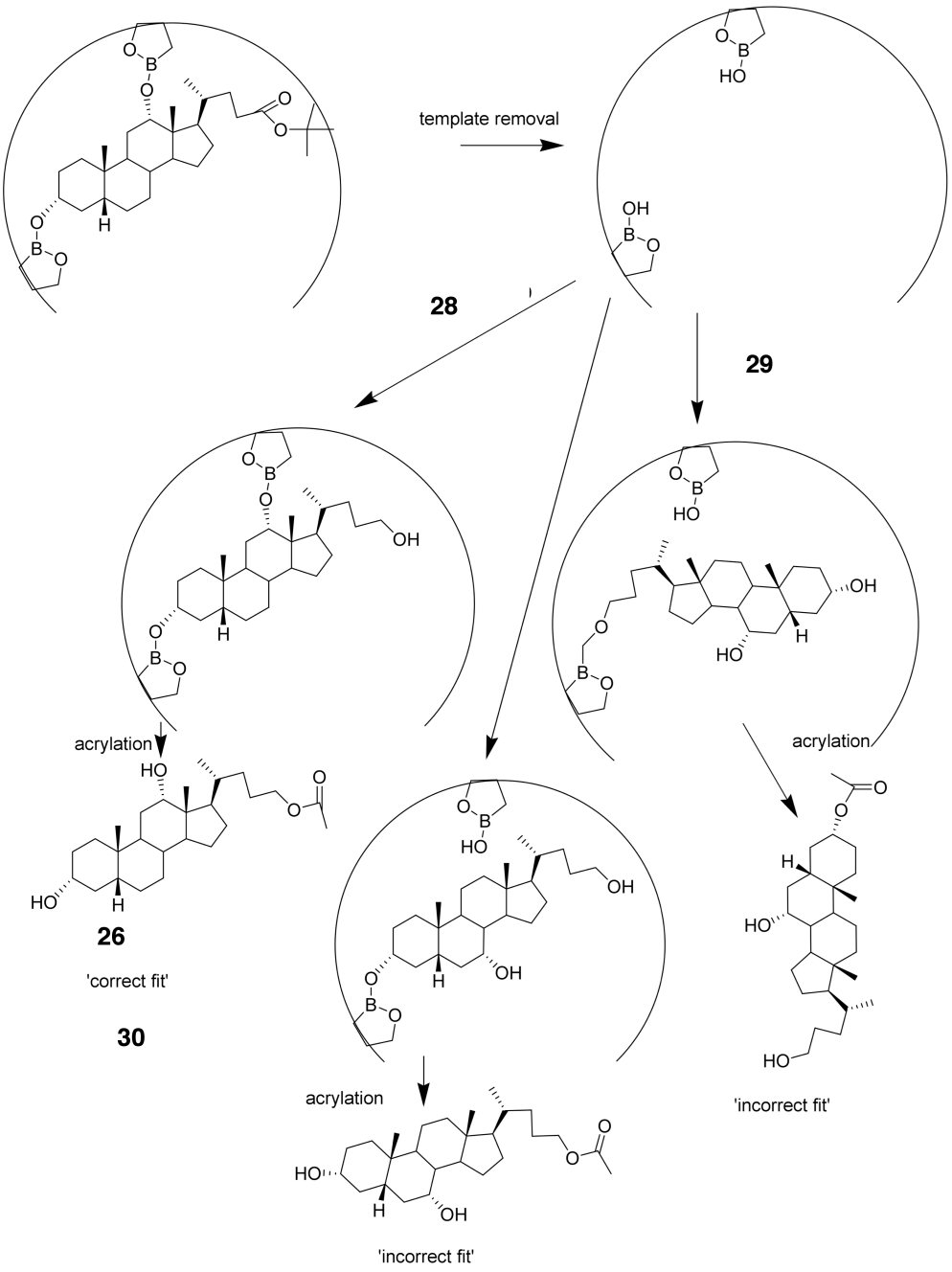


Figure 1.25 Modification of 28 and 29 using a 26 imprinted MIP. 2 ester bonds were formed between hydroxyl group at C3 and C12 of 28 and the boronophthalide residue in the 26 imprinted MIP which leaves the remaining hydroxyl group at C24 free for modification to form 30 only. Only 1 ester bond was formed between 29 and 26 imprinted MIP which resulted in a mixture of mono and diesters. (Adapted from ref. 40)

As seen from Figure 1.25, the 'correct' fit would result in two ester bonds forming between the boronophthalide residues and the hydroxyl groups

at C3 and C12, or C3 and C7 in the case of the **27** imprinted MIP. This leaves the hydroxyl group at C24 free for modification which gives one product- **30**. As a result, only 1 product (**26**- acetoxysterol) was formed. The 'incorrect' fit gave a mixture of mono- esters and di-esters.

1.2.2.4 MIPs in Catalysis

Many of the early reports of using MIPs in catalysis were hydrolysis reactions, though with a few exceptions, there had been little success.[41,42] One of the early examples of using MIPs in catalysis for the formation of carbon-carbon bonds was reported by Matsui et. al.[43] They prepared a dibenzoylmethane (**31**) and cobalt (II) ion imprinted MIP using 4- vinyl pyridine as the functional monomer and copolymerised with divinylbenzene and styrene (Figure 1.26).

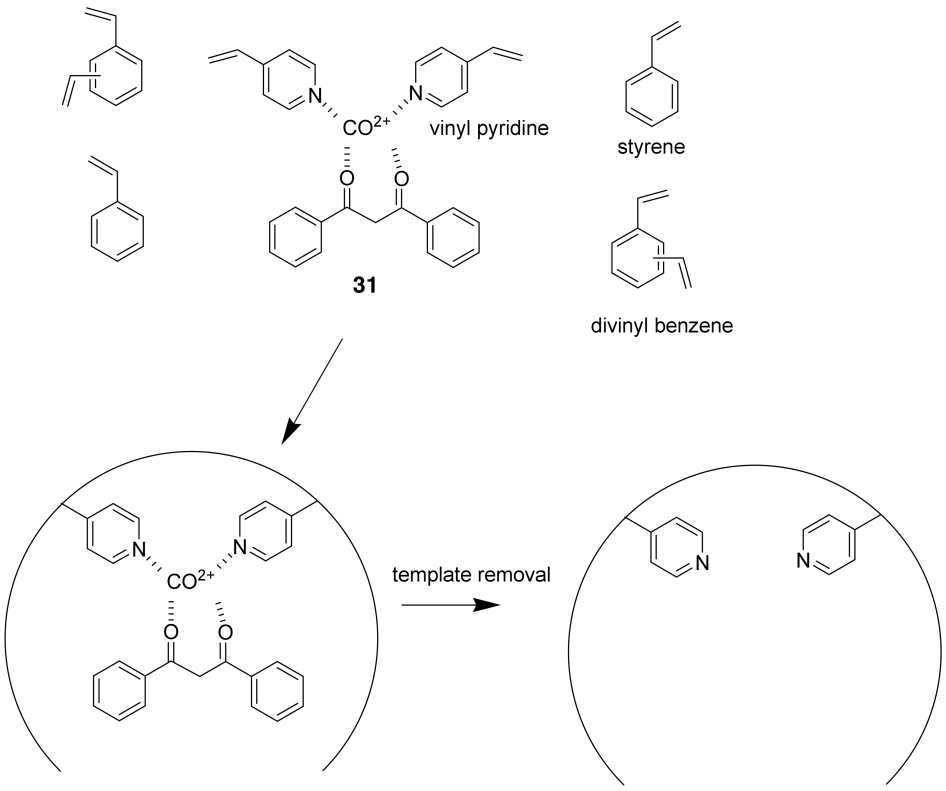


Figure 1.26 Figure to show the process of making the dibenzylmethane (31) imprinted MIP. A cobalt (II) ion was used to coordinate 2 4- vinyl pyridine molecules and 31. The 4- vinyl pyridine was copolymerised to form the MIP and 31 was removed. (Adapted from ref. 43)

This MIP was then used to catalyse the aldol condensation of acetophenone (**32**) and benzaldehyde (**33**) and resulted in the formation of chalcone (**34**) (Figure 1.27).

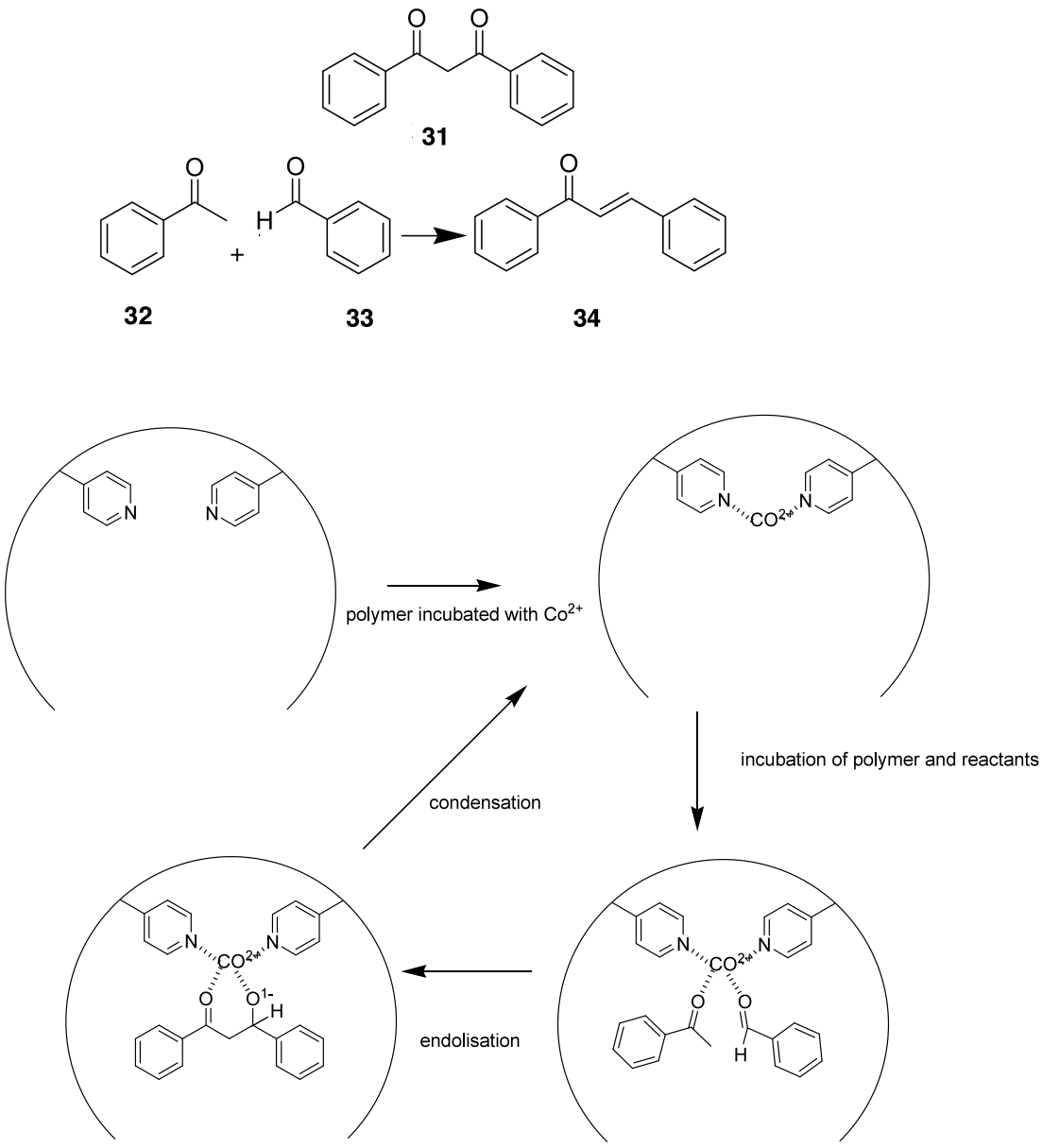


Figure 1.27 The dibenzylmethane (31) imprinted MIP was used to catalyse the aldol reaction between acetophenone (32) and benzaldehyde (33) to form chalcone (34) resulting in an eight- fold increase in reaction rate. (Adapted from ref. 43)

The reaction rate was increased by eight times when MIP was used and it was able to withstand vigorous reaction conditions without losing a substantial amount of its initial activity (loss of 5- 20 % of initial activity after being in DMF at 100 °C for a few weeks).

1.2.3 MIPs in cycloadditions

MIPs can be used in cycloaddition reactions as catalysts and stereoselectors. Two main approaches are used to achieve this: using transitions state analogues (TSAs)[44, 45, 46, 47] and by anto- idiotypic imprinting.[48] In the first case, a MIP imprinted with a transition state analogue (TSA) of the desired product is used to encourage the reactants to adopt the specific confirmation hence forming the desired product. For example, Kirsch et al imprinted MIPs with transition state analogues (**39** and **40**) and reported an increase in the rate of reaction of **35** and **36** to obtain **37** and **38** (Figure 1.28). However, they noted that the sites in the MIP are not able to influence the stereochemical outcome of the reaction as the interaction points between the functional monomer and the templates in both pathways are too similar.[44]

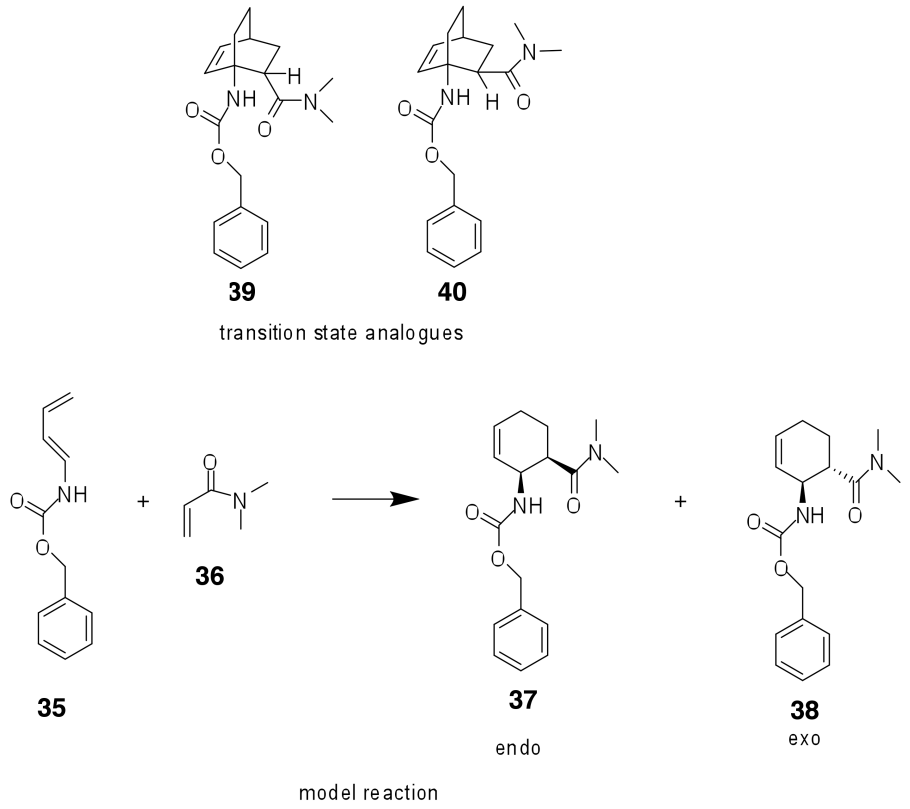


Figure 1.28 An example of MIPs used as catalysts using Transition State Analogues (TSA). An increase in reaction rate between 33 and 34 was observed when MIPs imprinted with 37 and 38 (transition state analogues of 35 and 36 respectively) were used. However these MIPs were not able to control the stereooutcome of the reaction. (Adapted from ref. 44)

In the second case, a polymer imprinted with the desired isomer was used to bind one of the reactants hence fixing it in an orientation which would increase the probability of a reaction of the desired product isomer when attacked by the other reactant.[49] For example, Zhang et al. used a MIP to increase the reaction rate and yield of **43a** (Figure 1.29). By aligning the two nitrogen containing groups in **42a** to the carboxylic acid functional group in the MIP cavity, **42a** was fixed in the MIP cavity hence making it easier for **41a** to react with it and form **43a**.[48]

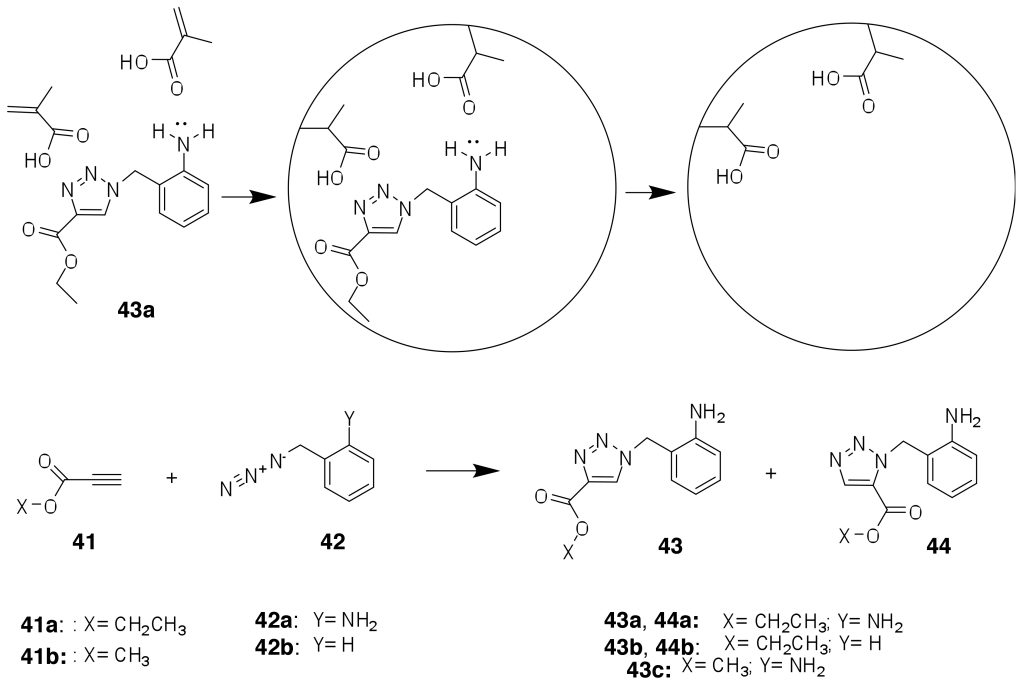


Figure 1.29 Templates used and model reaction for Anti- idiotypic imprinting. A 43a imprinted MIP was used bind and hold 42a in position such that 41a was more likely to approach and react with 42a to favour the formation of 31a. (Adapted from ref. 48)

1.2.4 Microreactors

Microreactors are devices in which a chemical reaction takes place in channels which have a width between 10- 100 microns (microchannels, Figure 1.30).[50, 51]

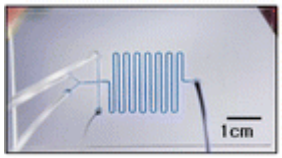
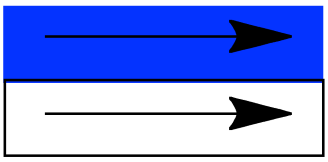


Figure 1.30 A microreactor chip (adapted from ref. 50)

Performing reactions in microreactors have several advantages over the traditional 'round bottom flask' approach, including good mixing and temperature controls due to laminar flows within the channels and the channel's high surface area to volume ratio.[51]

1.2.4.1 Laminar Flow and Segmented Flow

There are 2 types of flow patterns in a microreactor- parallel flow and segmented flow. Parallel flow is where 2 immiscible liquids flow along each other in a side- by- side manner (Figure 1.31). Segmented flow is where regular liquid segments of one phase are separated by segments of the other phase (Figure 1.31).



Parallel flow: 2 phases flow along each other in a side- by- side manner.

Segmented flow: Regular segments of one phase is separated by the other.

Figure 1.31 Parallel flow and segmented flow in a microreactor

1.2.4.2 Microreactors in Organic Synthesis

Many reactions had been performed in a microreactor such as peptide synthesis[52,53], Wittig reactions[54,55], nitrations[56], Suzuki reactions[57], natural product synthesis[58], radical reactions[59], deuteration[60], fluorination[61] and Swern oxidations[62]. Multi- component reactions have also been attempted.[63,64]

For example, Riva et. al[65] used microreactors to convert esters to their corresponding hydroxamic acids (Figure 1.32). They started with methylbenzoate (R= C_6H_5 and R'= CH_3) to optimise the reaction conditions and then compared it to doing the reaction in batch and in a sealed tube with microwave irradiation. They observed a higher conversion rate of 80 % in the microreactor than in batch (58 %) and in a sealed tube with microwave irradiation (72 %).

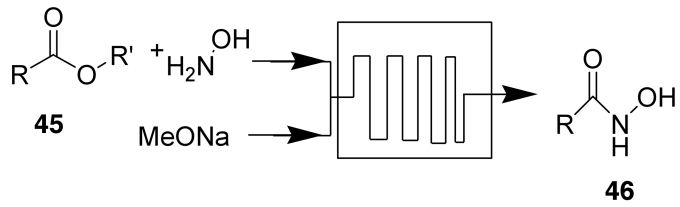


Figure 1.32 Synthesis of Hydroxamic Acids using a microreactor (adapted from ref. 65)

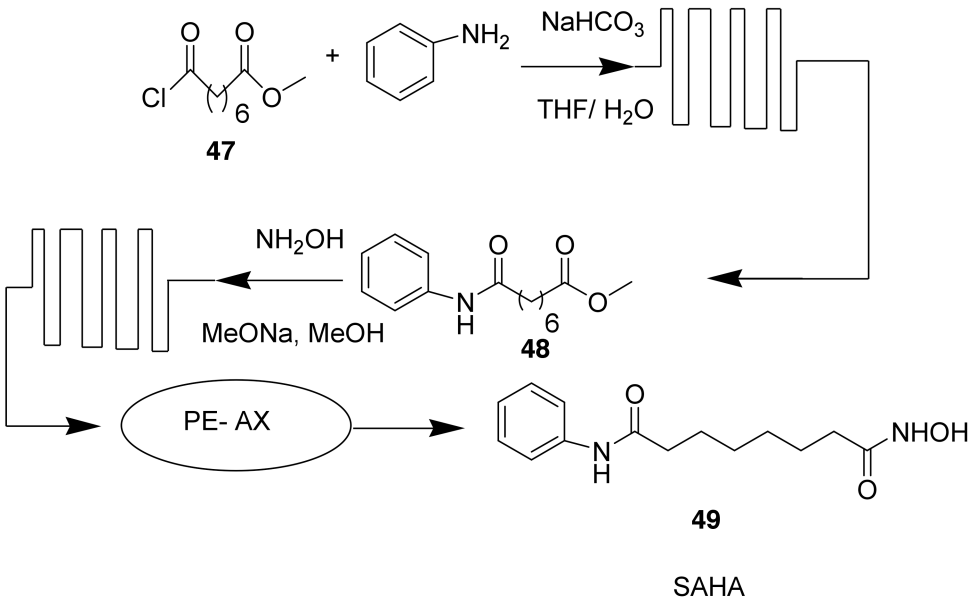


Figure 1.33 Multistep preparation of suberoylanilide hydroxamic acid (SAHA) (adapted from ref. 65)

Next, they investigated the ability to scale up the reaction. By doubling the concentration of **45** 4.3g of product were obtained after 1.5 h (production rate of 2.9 g/ h). Yield and purity were comparable to the initial scaled down version. Lastly, microreactors were used in a multistep synthesis of suberoylanilide hydroxamic acid (SAHA) which is an inhibitor for cancer thearapy. Firstly, 1 M of suberoyl chloride in

THF and 1M aniline and sodium carbonate in THF/ H_2O were pumped simultaneously into the first microreactor. The ester was then isolated and redissolved in methanol and used in the second reaction. Lastly, the final product was flowed through a silica-supported quarternary amine packed column which selectively removed the carboxylic acid by product (8- oxo- 8-(phemylamino)octanoic acid) (Figure 1.33). Quantitative yield was obtained in less than 2 mins and no product isolation after the first reaction was needed. SAHA was obtained in 84 % yield and 99 % purity or 80 % yield in 2 steps after further optimisation.

1.3 Aim and Objectives

1.3.1 Aim

The aim of the project was to demonstrate that the stereo- selectivity of a chemical reaction could be influenced by using a MIP to selectively sequester one of a pair of product stereoisomers thus steering the equilibrium in favour of its formation.

1.3.2 Objectives

The objectives of this project are:

1) Identify a suitable Diels- Alder reaction and obtain sufficient amounts of both product isomers (endo and exo).

2) Compare and evaluate different polymer systems on MIP performance.

3) Develop suitable HPLC methods for the analysis of batch rebinding experiments.

4) Performing the chosen Diels- Alder reaction at low concentrations.

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2 Preliminary Studies

2.1 Synthesis and Isolation of Template

2.1.1 Introduction

2.1.1.1 Diels Alder Reaction

The Diels- Alder reaction is a cycloaddition between a conjugated diene and a dienophile (alkene) to form a 6- membered ring with 1 double bond (Figure 2.1). This type of reaction is regularly used in synthesis due to its versatility and high steroselectivity. A wide range of dienes and dienophiles with a variety of attached functional groups can be used in Diels- Alder reactions.[66,67]

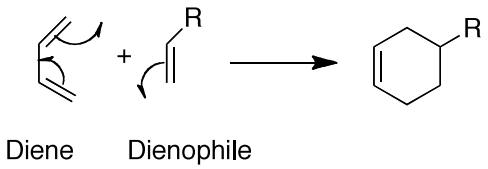

Figure 2.1 Diels–Alder Reaction between a Diene and a Dienophile

One of the main reasons for the widespread application of Diels- Alder reactions in for example natural product synthesis is the high stereoselectivity.[68] Up to 4 chiral centres can form in each reaction and some isomers occur either exclusively or predominantly in the product mixture. Figure 2.2 shows how the endo and exo isomer arise.[69] The dienophile can align itself with the diene in two ways: either resulting in the side group facing out of the ring (a) and result in the formation of the exo isomer or with the side group directly under the diene (b) and result in the formation of the endo isomer.

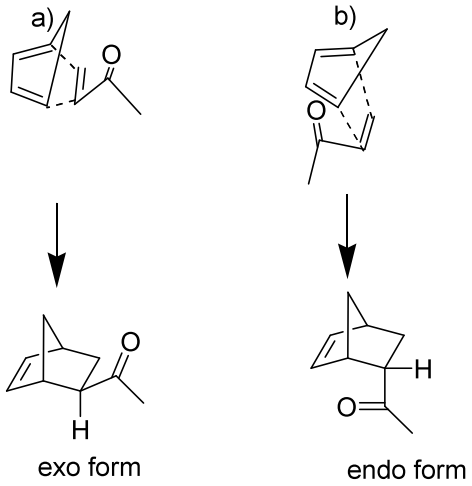

Figure 2.2 The formation of endo and exo isomers (adapted from ref. 4)

The stereoselectivity can be enhanced by utilising various catalysts and solvents. There are many reports of using catalysts such as Lewis acids (compounds which are able to accept a pair of electrons from an electron pair donor)[70], thiourea[71], (CSN_2H_4) and inorganic solid supports[72] to enhance the stereoselectivity.

However, a solvent is not needed for the reaction to take place and in general, most hydrocarbons are suitable. However, Breslow and co-workers[73] reported an enhancement in endo/ exo ratio and a 200 fold increase in the rate of reaction when water was used as the solvent instead of acetonitrile in the reaction of *N*-ethylmaleimide and hydroxymethylanthracene (Figure 2.3). This is because both reagents are hydrophobic and are thought to clump and hence forced close to each other when in water.

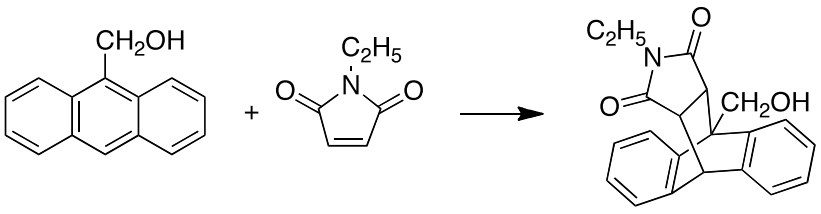

Figure 2.3 Reaction between N-ethylmaleimide and hydroxymethylanthracene (adapted from ref. 8)

However, the methods above increase the amount of endo formed (which is the major isomer of the reaction when no catalysts are used) and not the amount of exo.

Dinwiddie et al.[74] treated the racemic mixture of the endo and exo adduct of the proposed model reaction (Figure 2.2) with sodium methoxide in absolute methanol at reflux. This resulted in an improvement of the exo: endo ratio (from 10.0: 90.0 to 69.8: 30.2) as the exo isomer is the thermodynamic product. However, this is a lengthy process as 44 hours of reflux is needed in order for equilibration of the reaction to occur.

2.1.1.2 Medium Pressure Liquid Chromatography (MPLC)

Since the endo and exo isomers had similar polarities and could not be separated by column chromatography, a more refined form of chromatography called Medium Pressure Liquid Chromatography (MPLC) was used. This technique uses the same principle as standard column chromatography, but the equipment is different in 3 aspects: 1) silica gel in the column has a smaller particle size (15- 40 μm); 2) the product mixture is injected into a machine and is pumped through the column at an elution pressure of 20 mbar and 3) each fraction is collected automatically.[75]

2.1.1.3 Aim and Objectives

A confinement of the reactants and products of the model reaction in the aqueous phase and the MIPs in the organic phase was essential in order to observe the benefits of a 2 phase segmented flow system. Hence, the Diels-Alder reaction between cyclopentadiene and methyl vinyl ketone was chosen as the initial model reaction (Figure 2.2) as Breslow[76] reported an enhancement in stereoselectivity for this reaction in water.

The product isomers (endo and exo products) would then be purified and separated using MPLC. The endo isomer would be used as a template initially as the endo product was predominantly formed. ^{1}H NMR titrations would be performed to identify the functional monomer and solvent which would give the highest amount of specific binding. MIPs and NIPs would then be made using the most suitable functional monomer and solvent pair. The MIP and NIP would be washed once polymerisation was complete to remove template in the MIP and excess unreacted reactants in both the NIP and MIP. The NIP was used as a control to the MIP in the binding studies. Hence, the possible differences in particle size between the MIP and NIP need to be

determined and recognised before drawing any conclusions in the binding studies as the amount of non- specific binding (binding on any site that is not created by the polymerisation of the MIP) is dependent on the surface area of the polymers. Next, equilibrium binding studies would be performed to determine the amount of specific binding. Lastly, the ability of the model reaction to react at low concentration would be determined. MIPs have low binding capacities; hence low concentrations of template need to be generated in order for the imprinting effect to be observed. The suitability of the template and model reaction would then be evaluated on the amount of specific binding, the ease of obtaining the template and the ability of performing the reaction at low concentrations.

2.1.2 Chemicals and Materials

Dicyclopentadiene (90 %) was obtained from Alfa Aesar (Ward Hill, MA) and methyl vinyl ketone (99 %) was obtained from Aldrich (St. Louis, MO). Hexane (lab reagent grade) and ethyl acetate (lab reagent grade) were both obtained from Fisher (Pittsburgh, PA). For Medium Pressure Liquid Chromatography (MPLC), a BÜCHI (Oldham, United Kingdom) Model 681 Chromatography Pump was used and a BIO- RAP (Hercules, CA) Model 2128 Fraction Collector was used. The silica gel used in the MPLC column was LiChroprep® Si 60 from Merck (Feltham, United Kingdom). A Bruker 500 NMR (Coventry, United Kingdom) was used. Thin Layer Chromatography Plates (Aluminium TLC sheets coated with Silica Gel 60 F254) were obtained from Merck (Feltham, United Kingdom).

2.1.3 Methods

Approximately 2 g of dicyclopentadiene was cracked via fractional distillation to obtain cyclopentadiene. 28 mmol (1.87 g) of cyclopentadiene and 28 mmol (1.98 g) of methyl vinyl ketone were added to a 50 mL round-bottomed flask and was stirred at room temperature for 26 h. TLC (Solid phase: silica plates. Mobile phase: hexane/ ethyl acetate 90/10) was used to confirm the formation of products.

Medium Pressure Liquid Chromatography was used to separate the crude product into its isomers. The product mixture was injected in 1 mL

batches into the machine using a plastic syringe and pumped through the column in a mobile phase of hexane/ ethyl acetate (90/10) and was eluted over 30 minutes. The separated products were collected automatically in 20 mL fractions using test tubes. Thin layer chromatography was used to identify the product containing fractions. Fractions with the same R_f value were mixed together and the solvent was evaporated using a Rotary Evaporator to obtain the desired isomer (Figure 2.2).

Endo 1 (Refer to Fig. 2.2 for chemical structure and Appendix 5 for NMR spectra.)

^{1}H NMR (500 MHz, $CDCl_3$): δ= 1.33(m, 1H), 1.44-1.51(m, 2H), 1.72-1.78(m, 1H), 2.13(s, 3H), 2.91(brs, 1H), 3.00-3.04(m, 1H), 3.25(brs, 1H), 5.86(dd, 1H, J=2.8, 5.7 Hz), 6.2 (dd, 1H, J=3.1, 5.6 Hz) ppm

Exo 1 (Refer to Fig. 2.2 for chemical structure and Appendix 5 for NMR spectra.)

^{1}H NMR (500 MHz, $CDCl_3$): δ= 0.9(m, 1H), 1.17-1.23 (m, 2H), 1.79-1.85 (m, 1H), 2.15 (s, 3H), 2.30-2.35 (m, 1H), 2.83 (s, 1H), 2.92 (s, 1H), 6.05-6.10 (m, 2H) ppm

2.1.4 Results and Discussion

A crude yield of 99 % by mass was obtained for the reaction of 28 mmol of cyclopentadiene with 28 mmol of methyl vinyl ketone (Figure 2.2). The R_f values for the reactants and products were shown in Table 2.1.

	Rf
cyclopentadiene	**0.865**
Product 1	**0.486**
Product 2	**0.324**
Methyl vinyl ketone	**0.514**

Table 2.1 R_f Values (distance travelled by component/ distance travelled by solvent) for 1st reaction 1st attempt, in ethyl acetate/ hexane (90/10)

TLC of the reaction was compared to TLC of the starting materials. The disappearance of the starting material (cyclopentadiene R_f- 0.865 and methyl vinyl ketone R_f- 0.514) and the appearance of Products 1 and 2 (R_f- 0.486 and 0.324 respectively) was observed (Table 2.1). As explained in Section 2.1.3, a maximum of 1 mL of the crude mixture could be separated by the MPLC at a time, hence MPLC was performed 3 times (Batch 1- 3 in Table 2.2) to separate all the crude products obtained in the reaction. The yield from the separation of 1 g of crude product was 0.63 g endo 1 and 0.03 g exo 1 for the second batch and 0.42 g endo 1 and 0.005 g exo 1 for the third batch (Table 2.2). The endo to exo ratio was obtained by finding the proportion of endo 1 and exo 1 in the racemate, i.e by dividing the amount of endo 1 with the sum of the amount of endo 1 and exo 1 and by dividing the amount of exo 1 with the sum of the amount of endo 1 and exo 1 respectively.

The next step would be producing sufficient amounts of both isomers for subsequent experiments in this chapter, i.e. spectroscopic analysis on HPLC, ^{1}H NMR titrations, MIP production and binding studies. Although endo 1 was initially chosen as the template because the isomer was available more readily, a sufficient quantity of exo 1 was required for use in binding studies. Hence, it was necessary to repeat the separation of the crude product 10- 15 times. The endo: exo product ratio for each separation was 95: 5 (Table 2.2).

Batch	Fraction	Amount/ g	Endo: Exo
1[a]	-	-	-
2	Impurity	0.0195	95:05
	Exo	0.0341	
	Endo	0.6292	
3	Impurity	-	99:01
	Exo	0.0053	
	Endo	0.4159	

Table 2.2 Amount of Impurity, Endo and Exo formed and the Endo: Exo ratio for Reaction 1 [a]no data as product came out before collection started; [b]the weight of the impurity is less than the minimum measureable value on the balance (0.001 g)

2.2 Spectroscopic analysis of Reactants and Products of Reaction 1 by HPLC

2.2.1 Introduction

MIP imprinting effect would be evaluated via a batch equilibrium approach by using HPLC to determine the free equilibrium concentration of analyte. In addition, the progress of reaction 1 (Figure 2.2) would also be monitored using HPLC. Hence, this section describes the evaluation and optimisation of an appropriate HPLC method required for MIP binding and the monitoring of reaction 1 (Section 2.6 and 2.7 respectively).

2.2.2 Chemicals and Material

Acetonitrile (HPLC Grade) was obtained from Fisher (Pittsburgh, PA) and was used as bought. Deionised water was filtered through a 0.20 μm GNWP nylon membrane (Millipore, Ireland) prior to use. Cyclopentadiene was obtained by the fractional distillation of dicyclopentadiene at 40 °C. A Perkin-Elmer (Waltham, MA) Lambda 5 UV spectrometer was used. For HPLC, a Kromasil (Reading, United Kingdom) C18, 250 mm x 4.6 mm reverse phase column was used. Syringe filters were from Chromacoal (Herts, United Kingdom). The HPLC pump (P4000), auto sampler (AS3000) and UV detector (UV2000) were all obtained from Thermo (Waltham, MA).

2.2.3 Method

100 μM solutions of endo 1 and exo 1 were prepared in acetonitrile. A UV spectrophotometer was used to determine the wavelength at which UV absorbance was maximal. The retention time of cyclopentadiene, methyl vinyl ketone, endo 1 and exo 1 were analysed by HPLC (acetonitrile/ water (50/50); 1 mL/ min; 215 nm; 20 μL).

2.2.4 Results and Discussion

The HPLC retention times and the wavelength used to detect the isomers are shown in Table 2.3. The wavelength for maximum UV absorbance was found to be 215 nm and the detection limit was around 1 mAu (the amount

of background noise x 3). 215 nm was quite close to the UV cut off of acetonitrile (190 nm) which was used in the mobile phase. The UV cutoff wavelength is the point where the solvent stops being transparent to UV (does not shadow the isomer peaks in the chromatogram) to totally absorbing UV. At this point, the detection limit would increase and would make the calculation of the peak area less accurate (Fig 2.4). This was not an issue at this stage as the concentration of cyclopentadiene, methyl vinyl ketone, endo 1 and exo 1 used (100 μM) were relatively high. Hence the peak heights observed were much higher than the detection limit (100 mAu for 100 μM cyclopentadiene compared to a detection limit of 1 mAu).

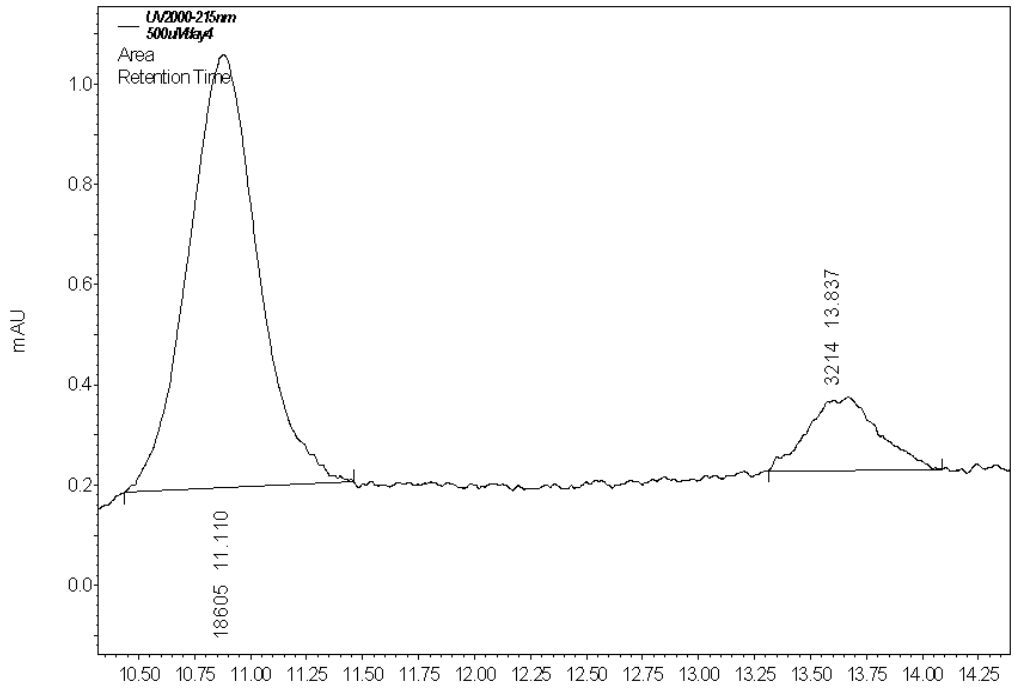

Figure 2.4 Chromatogram showing Endo 1 and Exo 1 at low concentrations. The area of a peak with a small peak height is greatly affected by the background noise which makes the determination of concentration more inaccurate.

However, monitoring the formation of endo 1 and exo 1 at 215 nm in the latter section of this chapter (Section 2.7) would be more challenging as the amount of endo 1 and exo 1 formed would be low, hence resulting in the formation of small peaks that were close to the detection limit (Fig 2.4). Furthermore, side products were produced and overlapped with the peaks of the reactants and products (Fig. 2.10- 2.13).

Sample	Wavelength/ nm	Retention time/ mins
Cyclopentadiene	215	2.9
Methyl vinyl ketone	215	2.3
Endo 1	215	5.4
Exo 1	215	6.6

Table 2.3 Retention Time and wavelength used for analysis for endo 1 and exo 1

2.3 NMR Titrations

2.3.1 Introduction

NMR can be used to determine which monomer or solvent is most likely to strongly interact with a particular template.[77] Intermolecular attraction between the functional groups of different monomers and the template leads to the chemical shift of NMR peak/ peaks of neighbouring proton/ protons of that functional group. The amount of shift increases with the strength of the intermolecular interaction.

2.3.2 Chemicals and Materials

Acetamide (> 99 %) was obtained from Fluka (Buchs, Switzerland). Acetic- d_3 Acid- d (99 % atom), acetonitrile- d_3 (99.8 % atom), chloroform- d (99.8 % atom), pyridine- d_5 (99.5 % atom) and toluene- d_8 (99.6 % atom) were obtained from Aldrich (St. Louis, MO). Model of NMR used for analysis was described in Section 2.1.2.

2.3.3 Methods

1.25 mg (1.04×10^{-5} mol) endo 1 and 500 μL of the relevant deuterated solvent were added to a 2 mL HPLC vial (Solution A). 250 μL of this solution was transferred to a NMR tube to which an additional 250 μL of deuterated solvent (acetonitrile, chloroform or toluene) was also added to give an endo 1 concentration of 10.4 mM (Solution B). 250 μL of monomer analogue (acetamide, deuterated acetic acid and deuterated pyridine) was added to the template solution left in the HPLC vial (Solution C). ^{1}H NMR spectra was obtained for the sample in the NMR tube. The relevant amount of solution of

Solution C (first column in Table 2.4) was then added and another ^{1}H NMR spectra was recorded. This process was repeated for each titration (Figure 2.5).

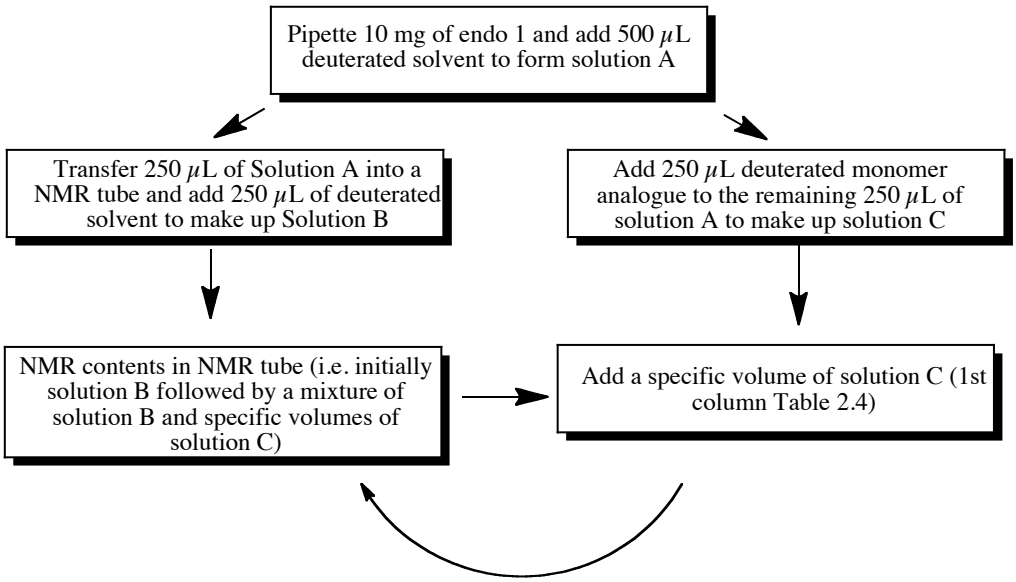

Fig. 2.5 A flow chart to explain the procedure for NMR titrations

The amount of monomer added and the concentrations of both endo 1 and the monomer at each titration for acetic acid are shown in Table 2.4. The concentration of solvents used was not taken into account and hence the values shown in Table 2.4 vary only by the monomer analogue used. The number of moles, the concentration of the other two monomer analogues used and the resulting monomer to template ratio (M/ T ratio) were shown in Table 2.5.

Amount HPLC vial/ uL[1]	$V_{accumulated/}$ ml [2]	$V_{TOT/}$ ml [3]	Endo 1/ mol [4]	$n_{AcOD/}$ mol[5]	[Endo 1]/ M[6]	[AcOD]/ M[7]	M/T ratio[8]
0	0	500	1.042E-05	0	2.083E-02	0	0
1	1	501	1.044E-05	8.731E-06	2.083E-02	1.743E-02	0.837
1	2	502	1.046E-05	1.746E-06	2.083E-02	3.479E-02	1.670
1	3	503	1.048E-05	2.619E-05	2.083E-02	5.208E-02	2.500
1	4	504	1.050E-05	3.493E-05	2.083E-02	6.930E-02	3.326
1	5	505	1.052E-05	4.366E-05	2.083E-02	8.645E-02	4.150
2	7	507	1.056E-05	6.112E-05	2.083E-02	0.1206	5.786
3	10	510	1.063E-05	8.731E-05	2.083E-02	0.1712	8.217
4	14	514	1.071E-05	1.222E-04	2.083E-02	0.2378	11.41
5	19	519	1.081E-05	1.659E-04	2.083E-02	0.3196	15.34
6	25	525	1.094E-05	2.183E-04	2.083E-02	0.4158	19.95
10	35	535	1.115E-05	3.056E-04	2.083E-02	0.5712	27.42
30	65	565	1.177E-05	5.675E-04	2.083E-02	1.004	48.22
60	125	625	1.302E-05	1.091E-03	2.083E-02	1.746	83.82
75	200	700	1.458E-05	1.746E-03	2.083E-02	2.495	119.7

Table 2.4 Acetic acid and template concentration at each [1]H NMR titration. [1]Amount of endo 1 and deuterated monomer analogue mixture added from the HPLC vial at each NMR titration; [2]Volume of endo and deuterated monomer mixture added from the HPLC vial accumulated in the NMR tube for each NMR titration; [3]Total volume of the amount of solution in the NMR tube at each titration; [4]The number of moles of endo 1 in the NMR tube at each titration; [5]The number of moles of deuterated acetic acid at each titration; [6]Concentration of endo 1 at each titration; [7]Concentration of deuterated acetic acid at each titration; [8]M/ T- Monomer to template ratio

N (pyridine)/ mol	[pyridine], M	M/T ratio	N (acetamide)/ mol	[acetamide], M	M/T ratio[1]
0	0	0	0	0	0
6.240E-06	0.012	1	2.201E-07	0.000	0
1.248E-05	0.025	1	4.402E-07	0.001	0
1.872E-05	0.037	2	6.602E-07	0.001	0
2.496E-05	0.050	2	8.803E-07	0.002	0
3.120E-05	0.062	3	1.100E-06	0.002	0
4.368E-05	0.086	4	1.541E-05	0.030	1
6.240E-05	0.122	6	2.201E-05	0.043	2
8.735E-05	0.170	8	3.081E-05	0.060	3
1.186E-04	0.228	11	4.181E-05	0.081	4
1.560E-04	0.297	14	5.502E-05	0.105	5
2.184E-04	0.408	20	7.703E-05	0.144	7
4.056E-04	0.718	34	1.431E-04	0.253	12
7.800E-04	1.248	60	2.751E-04	0.440	21
1.342E-03	1.876	90	4.732E-04	0.662	32
2.090E-03	2.503	120	7.373E-04	0.883	42
2.652E-03	2.867	138	1.001E-03	1.049	50

Table 2.5 Concentrations of pyridine and acetamide at each ^{1}H NMR titration. ^{1}M/T- Monomer to template ratio

The process was repeated for all deuterated monomer analogue and deuterated solvent combinations. The chemical shifts in all the hydrogen peaks of endo 1 were noted and the concentration of the monomer was plotted against the

amount of chemical shift (Figure 2.7) using GraphPad Prism (Version 5.00, GraphPad Software, USA).

2.3.4 Results and Discussion

Deuterated acetic acid, pyridine (analogue for the functional monomer methylacrylic acid, 4- vinyl pyridine respectively) and acetamide (which was used as an analogue for acrylamide) were used in the titrations and deuterated acetonitrile, chloroform and toluene were used as solvents. An analogue was used because the signals of the vinyl groups of the deuterated monomers, such as the vinyl group of 4- vinyl pyridine may result in complex coupling due to the restricted rotation around the C=C bond.[78] Both 4- vinyl pyridine and methacrylic acid were asymmetrical alkenes which meant that the alkenyl H atoms were in different environments and would hence couple with each other and result in many signals over a broad range (4.5- 6.5 ppm) which would overlap with some signals from the template. Deuterated analogues were used in order to avoid the overlap of signals between the analogue and the template on the NMR spectra.

The monomer- solvent combination that gave the largest chemical shift was pyridine and acetonitrile respectively for hydrogen 10- 12 in Figure 2.6. These protons correspond to the CH_3 next to the C=O bond. There was very little chemical shift (0.005- 0.008 at a pyridine concentration of 3 M) for all other protons.

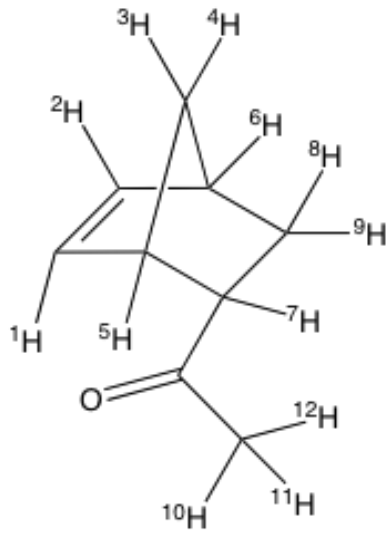

Figure 2.6 Figure to show the hydrogen atoms in endo 1

An increase in chemical shift of the tree equivalent protons 10- 12 in endo 1 was observed with increasing pyridine concentration (Figure 2.7). This suggested that the strongest interaction point between pyridine and endo 1 in acetonitrile was between the δ+ carbon atom of the C=O of endo 1 and the δ- nitrogen atom in the conjugated system of pyridine. This suggested that this same interaction could be used to recognise endo 1 specifically using a vinyl pyridine based MIP. 4- vinyl pyridine was used as the functional monomer instead of 2- vinyl pyridine as the nitrogen was further away from the C=C bond. This meant that a MIP made using 4- vinyl pyridine would have more sterically exposed nitrogen atoms to interact with endo 1 more easily. Hence, the endo and exo imprinted MIP and the NIP were made using 4- vinyl pyridine as the functional monomer.

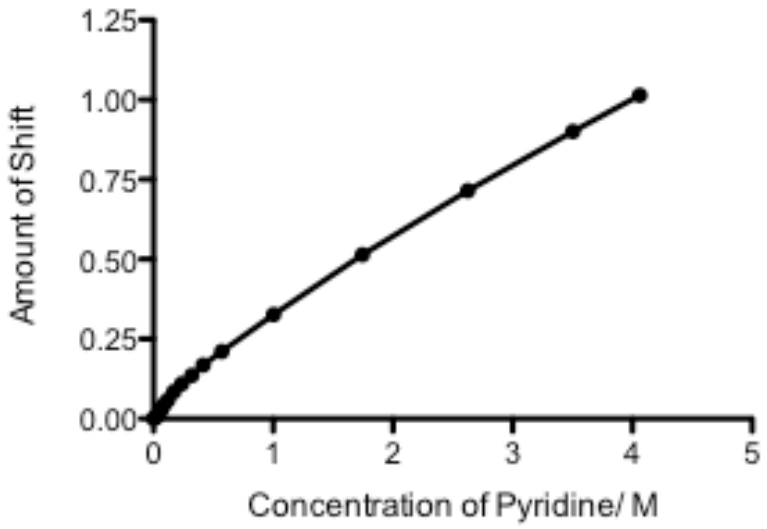

Figure 2.7 A graph to show the change in chemical shift of hydrogens 10- 12 in endo 1 against the concentration of pyridine. Deuterated acetonitrile was used as the solvent.

2.4 Polymer Synthesis

2.4.1 Introduction

In this study precipitation polymerisation was used to prepare endo 1 imprinted polymers. Precipitation polymerisation uses an excess (usually greater than 95 % volume) of solvent to give uniform MIP microspheres with a

good yield (at least 85 %) with a particle size of around 0.1- 5 μm.[79] The advantages of using this process are that uniform spheres are formed and no stabilisers or complicated procedures are needed.

2.4.2 Chemicals and Materials

4- Vinyl Pyridine (95 %) and Divinyl benzene (80 % tech. grade) were obtained from Aldrich (St. Louis, MO). Azobisisobutyronitrile (AIBN) was obtained from Acros (Geel, Belgium). Acetic Acid (Glacial), methanol (HPLC grade) and toluene (lab reagent grade) were obtained from Fisher (Pittsburgh, PA). Acetontrile was obtained as described in Section 2.2.2. A Büchi (Oldham, UK) Re111 Rotary Evaporator and a Büchi (Oldham, UK) B- 480 water bath were used. The vacuum oven was obtained from Gallenkamp (Loughborough, UK).

2.4.3 Methods

2.4.3.1 Polymerisation

To prepare the NIP (non- imprinted polymer- a polymer made in the same way as the MIP but in the absence of template), 6.0 mmol (0.631 mL) of 4- vinyl pyridine (this monomer showed the greatest amount of intermolecular interaction with endo 1 in the NMR titrations (Section 2.3)), 28.8 mmol (4.103 ml) of DVB, 1.9 mmol (312.0 mg) of AIBN and 128 ml of acetonitrile/ toluene (90/10 or 115.2 mL/12.8 mL) mixture were added to a 250 mL round bottomed flask. The solution was bubbled with oxygen- free nitrogen for 10 mins at 0 °C. The flask was then sealed and put onto the Rotorary Evaporator. Next, the flask was purged with nitrogen for another 2 mins (to ensure the solution was free from oxygen) before rotated at 8 rpm in a water bath at 60 °C for 19 h. The MIPs were prepared in the same way but using 128 mL acetonitrile/ toluene (82.5/17.5 or 103.125 mL/22.4 mL) and using 1.5 mmol (13.6 mg) of the product isomer (endo 1 or exo 1) as template.

2.4.3.2 Washing and Drying of NIPs and MIPs

The polymer was recovered from the suspension by filtration and washing with 60 mL of acetonitrile, 60 mL of toluene, 120 mL of methanol/ acetic acid (70/30) mixture, 40 mL methanol, 40 mL acetonitrile, 40 mL

toluene, 40 mL acetonitrile, and 40 mL methanol. They were then dried in a vacuum oven at 60 °C for 19 h. To confirm template removal, a suspension of MIP in acetonitrile (1 mg/ mL) was shaken overnight before the MIP was removed by filtration. HPLC analysis was then performed on the supernatent (Section 2.2.3).

2.4.4 Results and discussion

Polymer spheres of the NIP and MIP were white in colour after polymerisation.

2.5 Particle Size Analysis of NIPs and MIPs

2.5.1 Introduction

In this thesis, equilibrium binding studies would be used to evaluate the specificity of the MIP cavity for all the MIPs (Section 2.6 and 3.2). In these binding studies, the specificity of the MIP was evaluated by comparing the amount of MIP binding with the amount of NIP binding with the same ligand. NIPs were made in the same way as MIPs but in the absence of the template and was hence regarded as a suitable control such that any differences between the MIP and NIP binding was due to the specificity of the MIP cavity. However, some physical differences such as particle size and surface area were possible due to the presence of the template when the MIP was made. Hence, the particle size of the NIP and MIP would be determined and compared.

2.5.2 Chemicals and Materials

Acetontrile was obtained as described in Section 2.2.2. A Beckman Coulter (High Wycombe, UK) N4 Particle size Analyser was used.

2.5.3 Methods

Approximately 5 mg of polymer was suspended in 2 mL of acetonitrile in a cuvette and was analysed using a Particle Size Analyser.

2.5.4 Results

The NIPs made were 1 micron (with a coefficient variation of 56 %) in diameter and the MIPs were found to be 2 microns (with a coefficient variation of 16 %) in diameter (refer to appendix 6 for particle size distribution graphs). MIPs had a larger diameter than NIPs as the MIPs had imprinting cavities which suggested that the template had an important impact on the solvating conditions such as Theta condition and phase separation (Section 1.1.2.3) during polymerisation.[80] This difference in size and the surface area between the MIP and NIP could result in difference in specific binding due to the difference in surface morphology. As a result, equilibrium binding study experiments between the endo 1 imprinted MIP with exo 1, which was a stereoisomer of endo 1, were performed in addition to binding studies between the endo 1 imprinted MIP with endo 1. This would demonstrate the specificity of the MIP cavities and would also demonstrate that the difference in binding between the MIP and the NIP was not purely due to the physical differences such as the particle size or surface area of the MIP and NIP.

2.6 Binding studies

2.6.1 Introduction

Equilibrium binding studies were used to evaluate the affinity of a 'receptor' for a 'ligand'. Commonly this involved incubating a known amount of receptor in a solution of known ligand concentration and then determining the amount of ligand 'free' and the amount 'bound' to the receptor at equilibrium. The ligand could bound to the receptor either specifically or non-specifically. Specific binding is the binding between the guest and the host at the intended site created when making the MIP. Non- specific binding is binding at any site other than the intended one. This can be caused by the difference in surface charge or the difference in hydrophobicity between the two molecules. An optimised polymer system would have low non- specific binding and high specific binding.

In this study such an approach will be used to compare the relative template affinities of MIP and NIP. A possible interpretation is that the amount

of ligand binding to the NIP represents non- specific binding whilst that biniding to the MIP is the sum of the amount of non- specific and specific binding induced during the imprinting process. Hence, the difference in binding between a MIP and a NIP corresponds to the percentage that is bound specifically.

2.6.2 Chemicals and Materials

Chloroform (HPLC Grade) was obtained from Fisher (Pittsburgh, PA). Hexane was obtained as described in Section 2.1.2. Acetonitrile was obtained as described in Section 2.2.2. Methanol and toluene were obtained as described in Section 2.4.2. The HPLC was obtained as described in Section 2.2.2. 0.2 μm PTFE syringe filters were obtained from Chromacoal (Herts, UK).

2.6.3 Methods

The samples were prepared (using either acetonitrile, chloroform, toluene and hexane) in 2 mL Eppendorf tubes. The composition of each tube is shown in Table 2.6 in the following page.

The Eppendorf tubes were then wrapped in Parafilm and mechanically shaken overnight. The contents of the Eppendorf tubes were then filtered (0.2 μm PTFE syringe filters) into 2 mL vials and analysis by HPLC (Section 2.2.3). Glass syringes were used instead of plastic syringes since lubricant in the plastic syringes was known to dissolve in the solvents used hence giving rise to interfering peaks in the chromatogram.

A graph of concentration against absorption was plotted for a range of standard solution (samples 1-12) and the concentration of the test 'free' samples was determined from its slope. The percentage of template that was bound was calculated (the raw data and calculations for the binding study between endo 1 imprinted MIP and endo 1 in acetonitrile is shown in Appendix 1) and a bar chart showing the concentration and the percentage binding was plotted.

Sample no.[a]	MIP con./ mg/ mL	MIP vol./ mL	NIP con./ mg/ mL	NIP vol./ mL	template con./ μM	template vol./ mL
1-3	0	0	0	0	0	2
4-6	0	0	0	0	4.4	2
7-9	0	0	0	0	22	2
10-12	0	0	0	0	44	2
13-16	0	0	0	1	0	1
17-20	0	0	1	1	4.4	1
21-24	0	0	1	1	22	1
25-28	0	0	1	1	44	1
29-32	0	1	0	0	0	1
33-36	1	1	0	0	4.4	1
37-40	1	1	0	0	22	1
41-44	1	1	0	0	44	1

Table 2.6 Table to show the contents of each Eppendorf used in the binding study a- Sample 1- 12 were used to create a standard curve; samples 13-28 were used to find the amount of template after incubation with NIP and samples 29-44 were used to find the amount of template after incubation with MIP.

2.6.4 Results and Discussion

15.3 % and 16.1 % of specific binding, i.e. difference between MIP and NIP binding (Section 2.6.1) was observed for control concentrations at 22 and 44 μM respectively in the binding studies for endo 1 in an endo 1 MIP in acetonitrile (Figure 2.8 and Table 2.7).

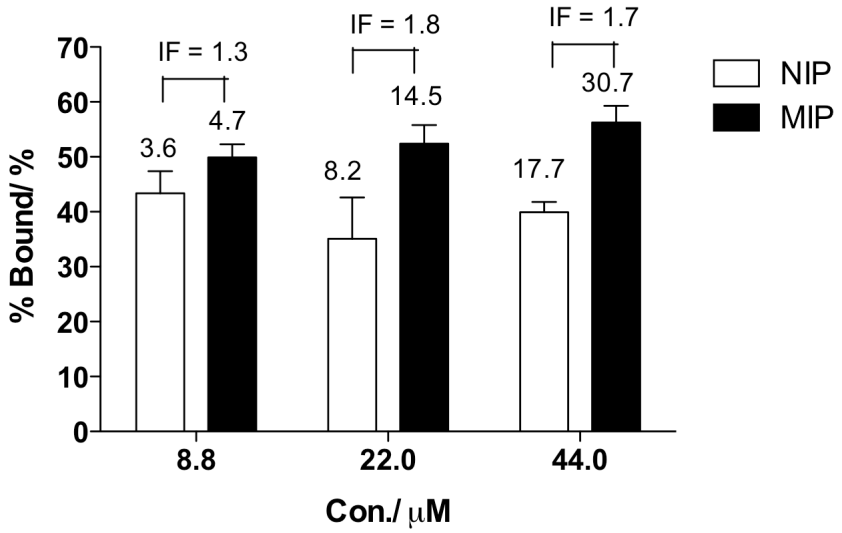

Figure 2.8 Bar chart to show the percentage bound of endo 1 for NIP and MIP in acetonitrile with varying endo 1 concentrations. The amount of specific binding is the difference in percentage binding between the MIP and the NIP. The amount of endo 1 per milligram of polymer was given at the top of each bar in nmol / mg. IF (Imprinting Factor): Ratio between the amount of endo 1 bound to MIP and the amount of endo 1 bound to NIP.

Endo 1 Con./ μM	Average NIP percentage bound (non-specific and specific binding)/ %	Standard Deviation for bound NIP	Average MIP percentage bound (specific binding)/ %	Standard Deviation for bound MIP	Percentage specific binding/ %
8.8	43.2	4.44	50.8	3.26	0
22	34.4	7.51	52.4	3.38	15.3
44	40.1	1.91	56.2	3.03	16.1

Table 2.7 Table to show the amount of specific and non- specific binding

In order to demonstrate the endo/ exo selectivity of the endo 1 imprinted MIP, the equilibrium binding of exo 1 to the endo 1 imprinted MIP was evaluated under the same conditions and no specific binding was observed (Figure 2.9).

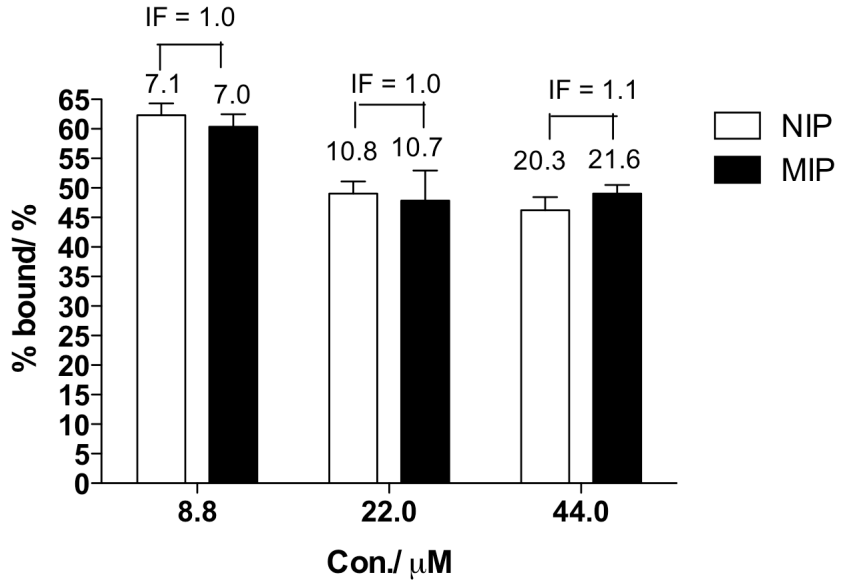

Figure 2.9 Binding Studies for endo 1 MIP in exo 1 in acetonitrile. The amount of exo 1 per milligram of polymer was given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of exo 1 bound to MIP and the amount of exo 1 bound to NIP.

The binding study was then repeated using chloroform and toluene instead of acetonitrile. However both chloroform and toluene absorb UV light strongly at the same wavelength as the endo 1 peak (215 nm) and hence overshadow the endo 1 peak. In order to overcome this issue, the chloroform/ toluene in the Eppendorf tubes were evaporated after overnight shaking by purging the samples in a stream of nitrogen. The samples were then reconstituted with the mobile phase. However, endo 1 left in the samples were also evaporated along with the chloroform/ toluene and hence the amount of 'free' endo 1 could not be determined. Vacuum filtration was also attempted, but endo 1 was again evaporated along with the solvent. The evaporation of endo 1 was not surprising considering the small amount of endo 1 in each Eppendorf tube (0- 88 nmol). Hence, solvents which do not absorb UV light at the same wavelength as endo 1 (215 nm) should be used in equilibrium binding studies. A binding study was performed in methanol, but low levels of specific binding was observed. This was because methanol was a very polar solvent and therefore would interact with endo 1 by hydrogen bonding. There may be much more sites for hydrogen bonding compared to the specific binding sites. Hence,

a more non- polar solvent such as hexane may improve the amount of specific binding and as a result, a binding study using hexane as the solvent was attempted. However, no specific binding was observed. This was because hexane was a very non- polar solvent and recognition between MIP and the template was hindered by hydrophobic interactions between the template and the polymer with the solvent. As a result, acetonitrile was selected as the solvent used in the model reaction as the highest specific binding was demonstrated.

2.7 Reaction 1 at Low Concentrations

2.7.1 Introduction

The specific binding in MIPs was only observed in low concentrations (8.8- 44 μM; refer to Figure 2.8 and Table 2.7) as MIPs have a low number of specific binding sites.[81] Hence the template concentration produced in the reaction needs to be similar to those used in the binding studies (Figure 2.8) for specific binding to be observed. The objectives of this experiment were: 1) to investigate whether the reaction would occur at 100 μM concentrations; 2) to find out if the products formed were the same as those formed when the reaction was performed neat (i.e. endo and exo 1) or if there were other side products and 3) to investigate whether the reaction could be monitored quantitatively using HPLC.

2.7.2 Chemicals and Material

Cyclopentadiene and methyl vinyl ketone were obtained and prepared as described in Section 2.1.2. Acetontrile and deionised water were obtained as described in Section 2.2.2.

2.7.3 Method

Six reactions were set up in 10 mL vials. The volume and concentrations of reagents in each vial were shown in Table 2.8, experiments were conducted at 50 °C for 10 days. The progress of the reactions was monitored using HPLC (1 mL/ min; acetonitrile/ water (50 /50)) (Section 2.2.3).

Vial	Reagents
1	2 mL acetonitrile
2	2 mL 200 μM cyclopentadiene
3	2 mL 200 μM methyl vinyl ketone as obtained.
4	2 mL 200 μM distilled methyl vinyl ketone
5	1 mL 100 μM cyclopentadiene and 1 mL 100 μM methyl vinyl ketone as obtained
6	1 mL 100 μM cyclopentadiene and 1 mL 100 μM distilled methyl vinyl ketone

Table 2.8 Table to show the contents in each vial in an experiment to investigate the ability of conducting reaction 1 in low concentrations. Acetonitrile was used as a solvent for all solutions

2.7.4 Results and Discussion

After 10 days the reaction mixtures were analysed by HPLC. For Vial 1 which only contained 2 mL of acetonitrile, no peaks were observed as expected. For Vial 2 which contained 200 μM cyclopentadiene in acetonitrile, a few more side products (retention time at 0.97 mins, 1.43 mins, 1.97 mins, 6.22 mins in Figure 2.10) which probably belonged to dicyclopentadiene were observed in addition to the cyclopentadiene peak at 2.60 mins.

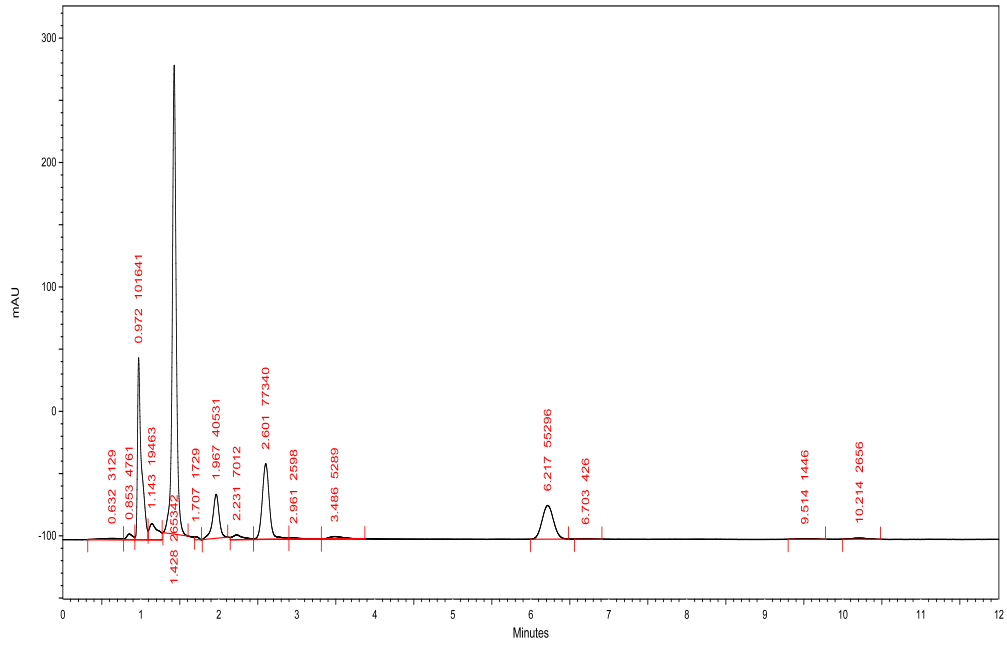

Figure 2.10 Chromatogram of Vial 2 after 10 days. In addition to the cyclopentadiene peak at 2.60 mins, a few more peaks which corresponded to dicyclopentadiene were observed at 0.97 mins, 1.43 mins, 1.97 mins, 6.22 mins respectively.

For Vials 3 and 4 which contained 200 μM as obtained and distilled methyl vinyl ketone respectively, there was no formation of other side products after 10 days. There was a peak which corresponded to the impurity in the methyl vinyl ketone at 4.52 mins and the area of this peak was around 10 times greater in Vial 3 (as obtained) than in Vial 4 where the methyl vinyl ketone was distilled. However, the impurity peak was much smaller (100 times less) compared to the methyl vinyl peak at 2.10 mins. The resulting chromatogram of Vial 5 contained several peaks which suggested the formation of many side products (retention time at 0.98 and 10 mins in Figure 2.11). However, cyclopentadiene, methyl ketone, endo 1 and exo 1 could still be seen at 2.08, 2.60, 4.52 and 6.71 mins respectively.

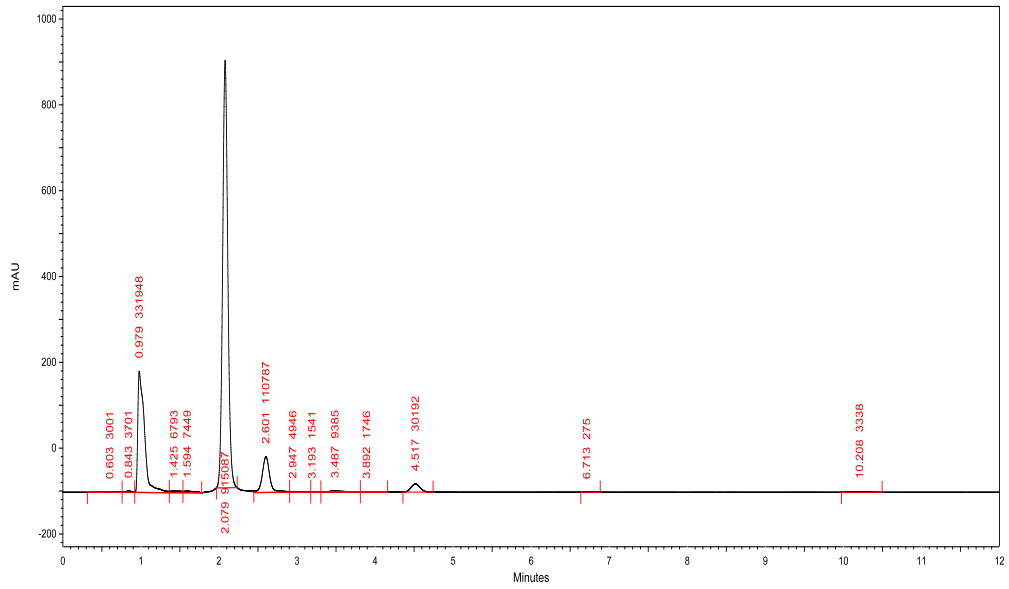

Figure 2.11 Chromatogram of Vial 5 after 10 days. The formation of endo 1 and exo 1 could be seen at 4.5 mins and 6.7 mins respectively. However a few unidentified side products (0.98 mins and 10 mins) were also formed. Furthermore, an impurity of methyl vinyl ketone overlaps with the endo 1 peak at 4.5 mins.

The peaks observed 0.97 mins and 10.21 mins were also observed in Vial 2 (Figure 2.10) which suggested that the peaks were dimers or trimers of cyclopentadiene. Unfortunately, the chemical structures of these side products could not be determined by NMR as the concentrations were too low even though they could be observed on HPLC. However, these side products may be identified by LC/ MS if necessary. As mentioned in the previous paragraph, the impurity present in methyl vinyl ketone was observed in Vial 3 and 4 after 10 days at 4.52 mins which overlaps with the endo 1 peak. These 2 peaks were separated successfully by changing the mobile phase to acetonitrile/ water (25/ 75) (Figure 2.12).

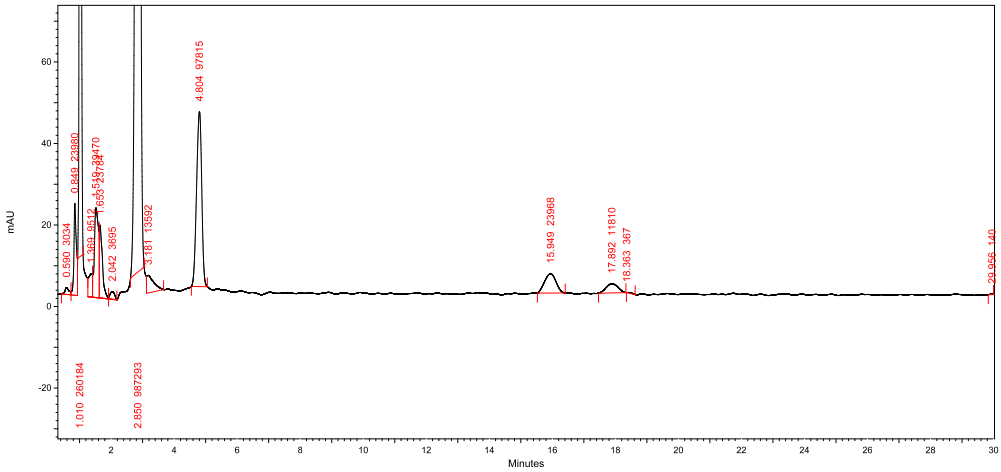

Figure 2.12 Chromatogram of Vial 5 after 10 days using acetonitrile/ water (25/75) as the mobile phase to separate the endo 1 peak (16 mins) and the impurity peak of methyl vinyl ketone (18 mins).

Endo 1 eluted at 16 mins (identified by running known endo 1 standard solutions under the same mobile phase and setup) and the impurity of methyl vinyl ketone eluted at 18 mins. The endo 1 formed was less than 10 μM and no exo 1 formation was observed after 30 mins. Using distilled methyl vinyl ketone for the Diels- Alder reaction decreased the formation of impurity at 0.98 mins and the endo 1- methyl vinyl ketone impurity peak at 4.52 mins. The decrease of the peak at 4.52 mins was expected as there was less methyl vinyl ketone impurity in the reaction as distilled methyl vinyl ketone was used. However, more endo 1 was formed in Vial 5 (16 mins in Figure 2.12) than in Vial 6 (4.5 mins in Figure 2.13) even though distilled methyl vinyl ketone was used in Vial 6.

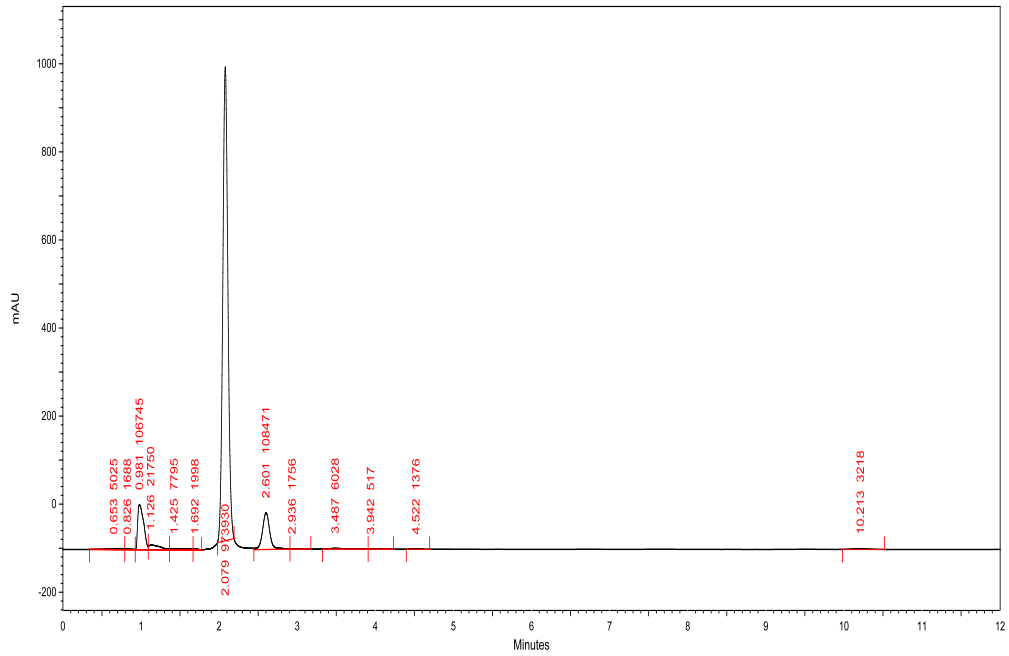

Figure 2.13 Chromatogram of Vial 6 after 10 days. The formation of endo 1 was observed at 4.5 mins but no exo 1 was observed. However a few unidentified side products (0.98 mins and 10 mins) were also formed. The peak at 4.5 mins which was due to endo 1 and the impurity of methyl vinyl ketone was much smaller than in Vial 5 as the impurity of methyl vinyl ketone was 100 times less as the methyl vinyl ketone used was distilled prior to use.

2.8 Conclusions

The reaction between cyclopentadiene and methyl vinyl ketone was performed and the product isomers- endo 1 and exo 1 were separated and characterised. HPLC studies were performed for the reagents and products and standard curves were plotted. NMR titrations were performed successfully and the most suitable monomer analogue- solvent combination was found to be pyridine and acetonitrile. A NIP and an endo 1 MIP were then made by precipitation polymerisation. The particle size of the polymers made was then determined and binding experiments in various solvents were performed and analysed by HPLC using acetonitrile. Acetonitrile was found to be the most suitable solvent to study binding between endo 1 and the 4- Vinyl Pyridine based MIP compared to methanol and hexane. A Diels- Alder reaction between cyclopentadiene and methyl vinyl ketone was attempted at lower concentrations resulting in a yield of 10 % for the endo isomer (determined by

HPLC). However no exo isomer was observed. Many unidentified side products were also formed. As a result, another model reaction needed to be used as both product isomers could not be detected and hence the effect of MIPs changing the amount of each isomer formed could not be demonstrated.

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3. Reaction 6

3.1 Selecting a New Model Reaction

3.1.1 Introduction

It was concluded in the conclusion of Chapter 2 that a new model reaction was required and needed to satisfy a few criteria. Firstly, the dienophile should have a good chromophore to allow easier detection using HPLC. Secondly, it should give rise to a similar molar ratio of isomers so that the effect of a MIP, in favouring the formation of one of the isomers, would be more apparent. Lastly, the reaction should be amenable to be carried out in a biphasic flow system where products and reactants are separable on the basis of their relative solubilities in two immiscible solvents. In addition, it would also be beneficial if the dienophiles were commercially available. Hayashi et. al.[82] performed a reaction between cyclopentadiene and benzyl acrylate in a two-phase system (water and organic phase) by centrifugation with a yield of 81 % and an endo to exo ratio of 80:20. Hence, four chemically similar dienophiles (phenyl methacrylate, benzyl methacrylate, furfuryl methacrylate and benzyl acrylate) (Figure 3.1) were identified as suitable candidates.

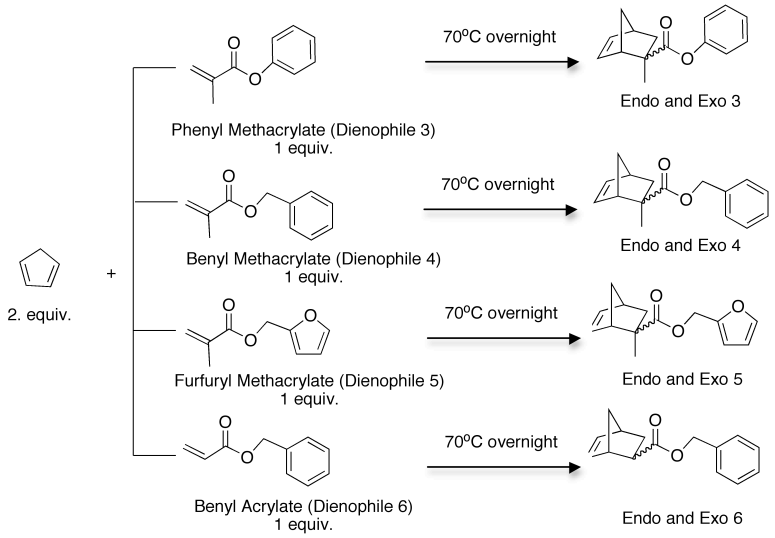


Figure 3.1 Reagents and reaction conditions of 4 Diels- Alder reactions that were performed to select another model reaction.

3.1.2 Chemicals and Materials

Cyclopentadiene, hexane (Lab Reagent Grade), ethyl acetate (Lab Reagent Grade) and the MPLC were obtained and prepared as described in Section 2.1.2. The HPLC was used as described in Section 2.2.2. Methanol was obtained as described in Section 2.4.2. Phenyl Methacrylate, Benzyl Methacrylate and Furfuryl Methacrylate were purchased from Sigma Aldrich (Dorset, United Kingdom) and were used as received. Benzyl Acrylate was obtained from Alfa Aesar (Heysham, United Kingdom).

3.1.3 Method

3.1.3.1 Diels- Alder Reactions and Separation of Isomers

All reactions were performed by heating 2 molar equivalents of cyclopentadiene and 1 molar equivalent of the respective dienophile at 70 °C for 17 h (Figure 3.1). TLC was used to optimise the Medium Pressure Liquid Chromatography (MPLC) mobile phase that was used to separate the product isomers on a preparative scale.

3.1.3.2 Analysis of Adducts

^{1}H, ^{13}C NMR and mass spectrometry were used to confirm the product structures.

Exo 6 (The exo product of reaction 6 (Figure 3.1 for structure and Appendix 5 for NMR spectra))

^{1}H NMR (500 MHz, $CDCl_3$): δ=1.30 (m, 1H), 1.47 (m, 1H), 1.85 (m, 1H), 2.22 (m, 1H), 2.85 (m, 1H), 3.00 (m, 1H), 3.15 (bs, 1H), 5.07 (m, 2H), 6.02 (m, 1H), 6.06 (m, 1H), 7.27 (m, 5H)

^{13}C NMR (125 MHz, $CDCl_3$): δ= 30.6 (CH_2), 42.0 (CH), 44.3 (CH), 46.4 (CH_2), 46.7 (CH), 66.0 (CH_2), 128.09 (CH), 128.14 (CH), 128.57 (CH), 135.7 (CH), 136.2 (C), 138.1 (CH), 175 (C)

Endo 6 (The endo product of reaction 6 (Figure 3.1 for structure and Appendix 5 for NMR spectra))

^{1}H NMR (500 MHz, $CDCl_3$): δ= 1.20 (m, 1H), 1.37 (m, 2H), 1.83 (m, 1H), 2.83 (bs, 1H), 2.93 (m, 1H), 3.17 (bs, 1H), 5.00 (dd, 1H, J= 2.8, 5.6 Hz), 5.80 (dd, 1H, J= 3.1, 5.6 Hz), 7.26 (m, 5H)

^{13}C NMR (125 MHz, $CDCl_3$): δ= 29.7 (CH_2), 42.6 (CH), 44.0 (CH), 45.8 (CH_2), 49.6 (CH), 66.0 (CH_2), 128.06 (CH), 128.14 (CH), 128.50 (CH), 132.7 (CH), 136.2 (C), 138.1 (CH), 175 (C)

3.1.3.3 HPLC Optimisation of Adducts

The HPLC method used was the same as in Section 2.2.3, but endo and exo products of reaction 3- 6 were used instead of endo and exo 1 and methanol was used instead of acetonitrile.

3.1.4 Results and Discussion

3.1.4.1 Diels- Alder Reactions and Separation of Products

All the reactions in Figure 3.1 were successfully completed and NMR showed the disappearance of reactants and formation of products. For product mixtures 3-5, hexane / ethyl acetate (3/2) was used as the mobile phase for TLC initially but the spots of the two products were overlapped and were close to the solvent front (R_f= 0.91) on TLC. Decreasing the polarity by increasing the ratio of hexane was not possible as higher amounts of hexane resulted in the products forming an emulsion and not wetting the TLC plate. Hence, MPLC was performed using hexane / ethyl acetate (3/2) as mobile phase. However, the isomer co-eluted and separation was not successful. For product mixture 6, the mobile phase used was hexane / ethyl acetate (4/1). Although both isomers were also close to the solvent front, the bands did not overlap on the TLC plate (R_f= 0.71 for endo 6 and 0.76 for exo 6). Hence MPLC was carried out using hexane / ethyl acetate (4/1) and the two isomers were obtained with an endo to exo ratio of 3:1.

3.1.4.2 Analysis of Adducts

Since ^{1}H NMRs for endo and exo 6 were not found in literature, mass spectrometry was also used to confirm that the products obtained were in fact isomers. Samples of both isomers were sent to the EPSRC Mass Spectrometry Facility at Swansea University. The mass spectrometry results were obtained by chemical ionisation (ammonia was used as a reagent gas). Hence the mass to charge ratio was the sum of the mass of fragment and a proton (M+ H) or the sum of the mass of fragment and an ammonium ion (M+ NH_4^+). Endo 6 obtained by MPLC was shown to have the expected mass molecular peak of 246 and was hence pure. However, the spot observed on TLC that corresponded to exo 6 (R_f = 0.76) also contained a few side products (295.3 and 312.4 in Figure 3.2) other than exo 6 (246.3 in Figure 3.2). These side products had the same R_f value and hence not separated by MPLC. The formation of the side products probably resulted from an excess of cyclopentadiene in the reactants. Hence the products were a result of a Diels-Alder reaction between endo 6 / exo 6 with cyclopentadiene (Structures shown in Figure 3.2). The reaction was performed in 2/1 cyclopentadiene to benzyl acrylate to increase the rate of reaction and hence the formation of the side products observed could be avoided by using equimolar amounts of reactants.

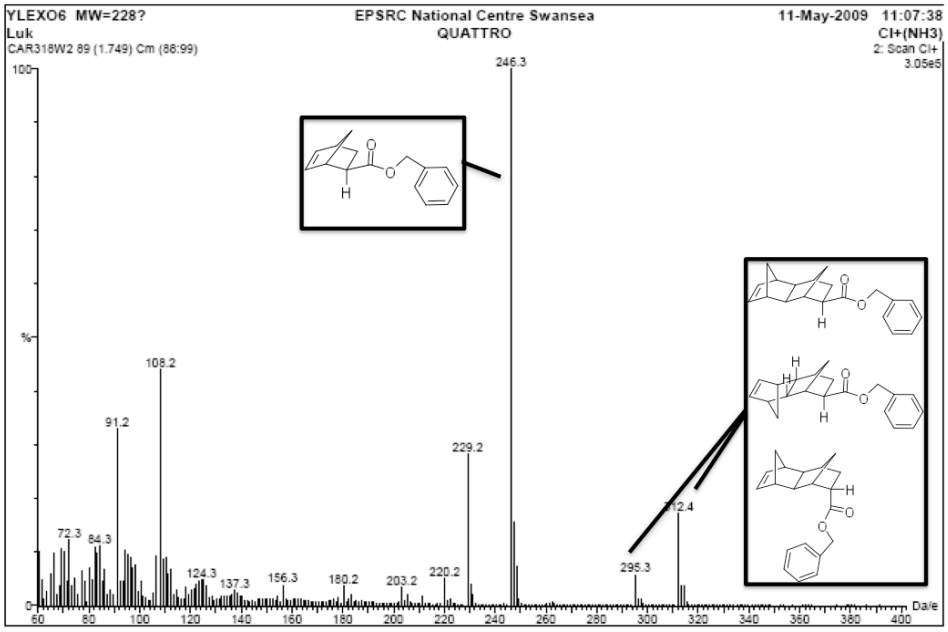


Figure 3.2 Mass Spectrometry Results for Exo 6. The peaks at M_w 229.2 and 246.3 corresponded to exo 6. Impurities at M_w 295.4 and 312.4 were observed and corresponded to the product of a further Diels- Alder reaction between endo 6 and exo 6 with cyclopentadiene.

3.1.4.3 HPLC Optimisation of Adducts

Products 3-5 did not elute in 100 % methanol and hence non-aqueous reverse phase chromatography[83, 84, 85] was considered. Non- aqueous reverse phase chromatography uses solvents of low polarity such as methylene chloride or tetrahydrofuran to separate water insoluble, non- polar compounds. Product mixture 6 was found to be soluble in 100 % methanol, but the retention time of endo and exo 6 being too close to the solvent front when 100 % methanol was used as the mobile phase. Furthermore, there was little separation between endo 6 and exo 6. Sufficient separation was achieved using methanol/ water (90/10) as mobile phase and the retention times were 5.3 mins and 5.8 mins for endo 6 and exo 6 respectively. Hence, reaction 6 was selected as the next model reaction as the product isomers could be physically separated by MPLC and could be easily identified as separate peaks on analytical HPLC.

3.2 Polymer Screening for Endo and Exo 6

3.2.1 Introduction

As concluded in Section 3.1, reaction 6 was selected as the model reaction for demonstrating the ability of molecularly imprints to influence the outcome of a Diels-Alder reaction. The next challenge was to prepare a suitably selective molecularly imprinted polymer. In order to identify a suitable polymer system, ^{1}H NMR titrations were used to identify the most suitable polymer system in the preliminary studies (Section 2.3). Although this method was a good starting point to making a MIP with high specificity, the possible changes in orientation of the functional groups in the monomer due to polymerisation were not taken into account.

An alternative approach was to make small amounts of non-imprinted and imprinted polymers (Mini MIPs) with different functional monomer and solvent combinations via bulk polymerisation and then screen their binding ability by HPLC as demonstrated by Sellergren and co workers.[86] A similar procedure was used in this study to find the binding ability of each functional monomer combination.

3.2.2 Chemicals and Materials

Methacrylic Acid (99 %) was obtained from Sigma Aldrich (Dorset, United Kingdom). Acrylamide (ultrapure MB Grade) and *N, N'*-Methylenebisacrylamide (ultra pure MB Grade) were obtained from USB Cooperation (Cleveland, OH). Acetonitrile was obtained as described in Section 2.2.2. Di-vinyl benzene (80 % tech. grade), 4- vinyl pyridine (95 %), azobisisobutyronitrile (AIBN), acetic acid, methanol and toluene were obtained as described in Section 2.4.2. The model Rotary evaporator and the vacuum oven were described as in Section 2.4.2.

3.2.3 Method

3.2.3.1 Production of NIPs and MIPs by Bulk Polymerisation

The reagents used to prepare each of the polymers are shown in Table 3.1. The molar ratio of cross- linker to functional monomer to initiator was 4:1:0.01. The template to monomer ratio was 1:4. The volume of solvent used was approximately the volume of the cross- linker and the functional monomer (Appendix 2). **P2-8** and **P3-8** were made in the same way, but half as much moles of template was used (0.022 g). Non- imprinted polymers were made in the same way but in the absence of template.

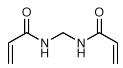
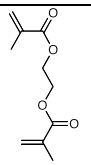
P1	**P2**	**P3**	**P4**	**P5**[b]
				—
				
4 equiv.	4 equiv.	4equiv.	4 equiv.	
$CHCl_3$[c] (0.5 mL)	$CHCl_3$[c] (0.515 mL)	$CHCl_3$[c] (0.434 mL)	$CHCl_3$[c] (0.434 mL)	$CHCl_3$[c] (0.5 mL)

Table 3.1 Compositions of MIPs P1 –P5. NIPs were made in the same manner but in the absence of endo 6.[a] [a]All polymers also contain 0.01 equiv. of azobisisobutyronitrile (AIBN). MIPs also contain 0.25 equiv. of endo 6.

The vials were tightly capped and purged with oxygen free nitrogen in the vials for reasons explained in Section 2.4.3.1. The vials were then heated in an oven at 60 °C for 17 h. 4 monolithic blocks of polymer were obtained by breaking the vials. Next, the polymers were ground into a fine powder and sedimented in acetonitrile in order to generate a polymer particle of an appropriate size. This was achieved by suspending the ground polymers in 250 ml of acetonitrile and leaving them to settle through a solvent height of 30 cm. Particles still in suspension after 60 mins were discarded whilst those that had settled were washed (methanol, toluene and acetic acid) and dried overnight prior to use.

3.2.3.2 Binding Studies

The experimental procedure used was the same as described in Section 2.6.3, but with a differing range of concentrations (0, 10, 25, 50, 100, 250 and 500 μM) and templates (endo and exo 6 instead of endo and exo 1).

3.2.4 Results and Discussion

3.2.4.1 Production of NIPs and MIPs by Bulk Polymerisation

All NIPs and MIPs were transparent post polymerisation. Polymer 2 MIP and NIP were tinted a light red. Polymer 3 and 4 NIP and MIP were slightly yellow. Polymer 1 NIP and MIP were colourless. An opaque white powder was formed for all polymers after grinding and washing.

3.2.4.2 Binding Studies

P1 (methacrylic acid as monomer) showed no difference in the amount of template bound to the MIP compared to the control (Figure 3.3) while **P2** (4-vinyl-pyridine as functional monomer) and **P3** (acrylamide as functional monomer) showed 6.6 % (Figure 3.4) and 7.7 % (Figure 3.5) more specific binding compared to their controls respectively. This was taken as an indication of an imprinting effect. Although it is speculative to hypothesise as to the type and number of interactions responsible for binding between template and polymer, it is interesting to note that there is no suggestion of an imprinting effect for the methacrylic acid containing **P1** while binding to the MIP appeared to be favoured for both the 4- vinyl pyridine and acrylamide containing polymers (**P2** and **P3**). Therefore neither acid nor basic group appears to be pre- requisite for producing an imprinting affect. The most likely point of interaction for **P2** is a hydrogen bond between the nitrogen of 4- vinyl pyridine and the carbonyl carbon of the template whilst for **P3** a number of different hydrogen bonds are conceivable between the amide group of acrylamide and the template carbonyl group (Figure 3.6). Given the nature of the environment it is also possible that interfacial hydrogen bonding, between the acrylamide amide and template benzyl group, might also make a contribution.[87, 88] ^{1}H NMR titrations could be conducted to further investigate the interaction point if necessary.

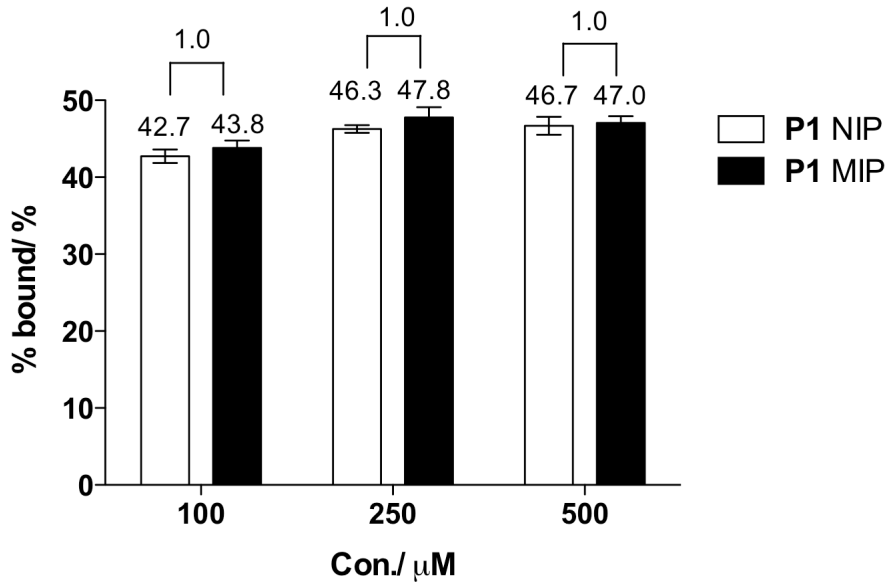


Figure 3.3 Binding Studies for P1 endo 6 MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

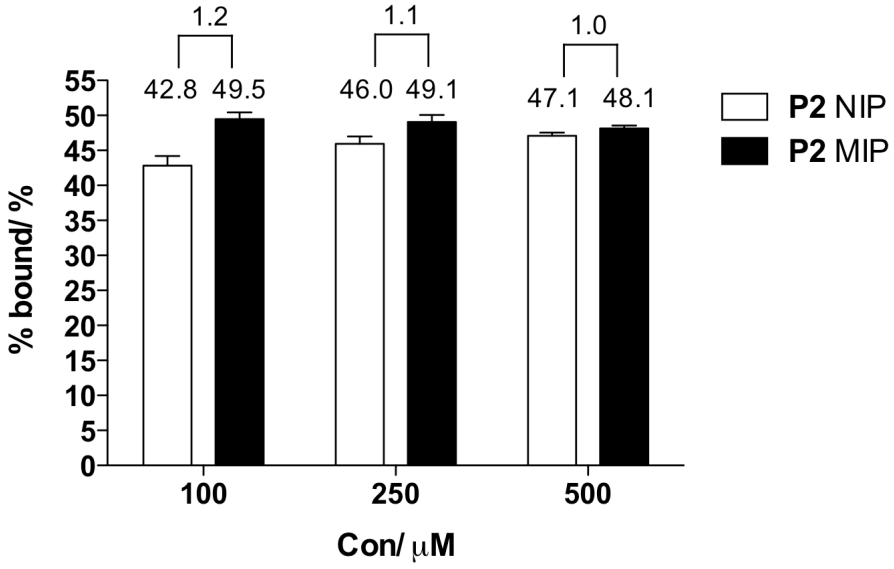


Figure 3.4 Binding Studies for P2 endo 6 MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

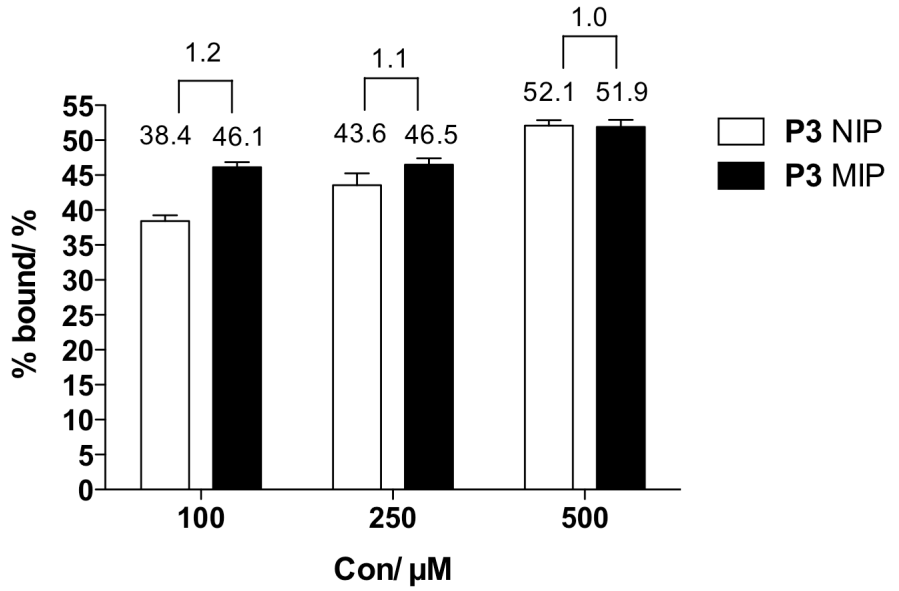
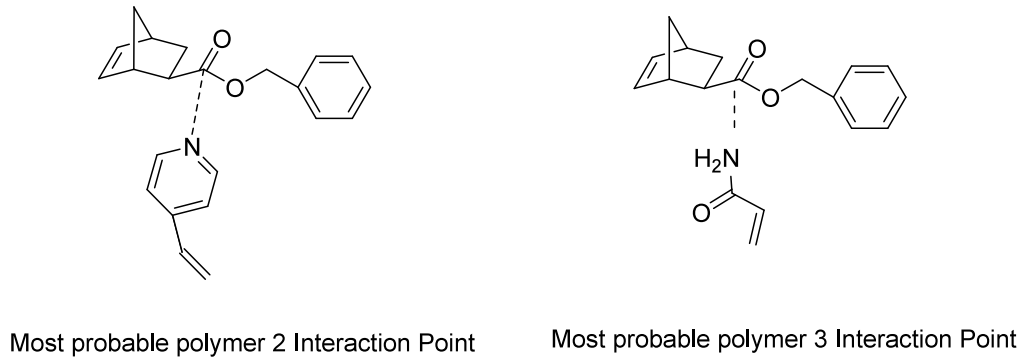


Figure 3.5 Binding Studies for P3 endo 6 MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol /mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.



Most probable polymer 2 Interaction Point

Most probable polymer 3 Interaction Point

Figure 3.6 Polymer 2 and Polymer 3 Interaction Points with Template

Following these initial sightings, more extensive binding studies were undertaken in order to construct binding isotherms for the binding of endo 6 to **P2** and **P3** (MIP and control polymers) (Appendix 3). At starting concentrations >100 μM apparent specific binding (nmoles bound per mg MIP – nmoles bound per mg control polymer) was low suggesting saturation of available MIP binding sites whilst at template concentrations <100 μM significant apparent specific binding was observed. A predictable decrease in

apparent specific binding was observed as initial template concentration increased (Figure 3.7).

A number of interesting observations arose when the percentage bound of **P2** and **P3** NIP and MIP were plotted against the initial template concentrations of 10, 25 and 50 μM (Figure 3.7). Firstly, **P2** MIP bound a very similar percentage (~50 %) of template over the range 10 μM to 50 μM whilst for the **P2** NIP there was a gradual increase in the percentage bound (11 % to 42 %) over the same template concentration range. For **P3** MIP, over the same concentration range, the percentage bound falls from 50 % to ~40 % whilst for the control polymer the percentage bound remained relatively consistent. It could be misleading to over interpret such binding data since MIP binding site affinity is extremely polyclonal and estimations of available binding site concentration are fraught with difficulties. However, it was surprising, yet reassuring, that given the difference in monomer composition, **P2** and **P3** MIPs and control polymers behaved in a similar manner.

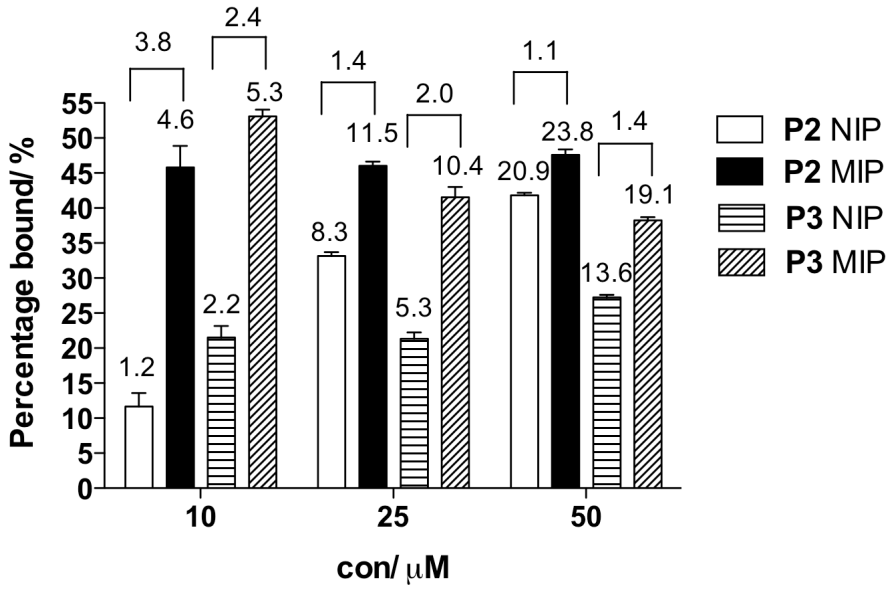


Figure 3.7 Binding Studies for P2 and P3 endo 6 MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer is given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

In an attempt to further reduce non- specific interaction between template and polymer, an alternative cross- linking monomer, ethylene glycol dimethacrylate (EGMA), was evaluated. Previously it had been reported that EGMA could result in reduced non- specific binding in apolar solvents and could provide the additional benefit of improving polymer flexibility and accessibility.[89] However, when the binding of endo 6 to a non-imprinted EGMA polymer **P5** was evaluated under the same conditions, non- specific binding was found to be greater than an equivalent divinylbenzene (DVB) cross- linked polymer. As a result, DVB was favoured as the cross-linker. A further polymer modification was evaluated where the acrylamide in **P3** was replaced with an equimolar amount of *N,N'*-methylene bisacrylamide (MBA) in **P4**. The reason for this modification was to create conformation dependence between adjacent amide groups and increased rigidity in and around the imprinted site. In previous studies this had shown to give rise to improved MIP performance. However, by maintaining equimolar amounts of acrylamide and MBA, the number of amide groups in **P4** was doubled compared with **P3**. Therefore in order to make valid comparisons between acrylamide and MBA containing polymers a further polymer (**P3-8**) was prepared containing 8 equivalents of acrylamide. For completeness a polymer containing 8 equivalents of 4-vinylpyridine was also synthesised (**P2-8**).

Interestingly the substitution of acrylamide in **P3** with an equimolar amount of MBA in **P4** resulted in a slight increase in MIP and NIP binding for low template concentrations (10 μM) and a slight decrease for higher template concentrations (25 and 50 μM). This was despite the fact that **P4** contained twice the number of amide residues compared to **P3** (Figure 3.8). However, the difference between MIP and NIP binding was similar (~14 % at 25 μM) for **P3-8** and **P4** (Appendix 4).

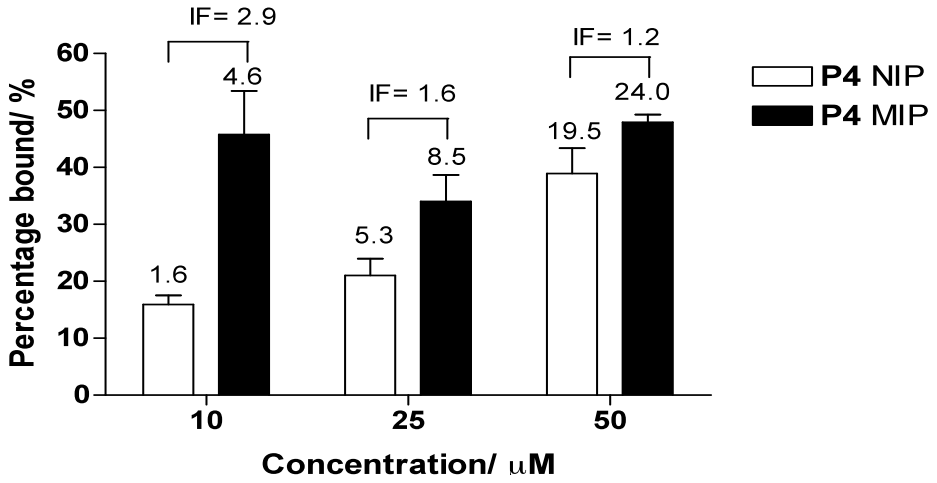


Figure 3.8 Binding Studies for P4 endo 6 imprinted MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer is given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

At 25 μM, the imprinting factor (IF) for **P3**, **P4** and **P3-8** were 2, 1.6 and 1.7 respectively. This indicated that the imprinting effect was not affected by the number of acrylamide groups. The amount of non- specific binding for **P3** and **P4** were the same (5.3 nmol/ mg) even though **P4** had twice as many acrylamide groups. However, **P3-8** had lower non- specific binding compared to **P4** (3.2 nmol/ mg for **P3-8** and 5.3 nmol/ mg for **P4**) even though both polymers contained the same number of acrylamides.

From the different polymers, **P3** gave the largest amount of specific binding (Figure 3.7). In order to evaluate the *endo*/ *exo* selectivity, the equilibrium binding of exo 6 to **P3** (MIP imprinted with endo 6 and NIP) was evaluated. Figure 3.9 clearly shows that there was no difference between the amount of exo 6 binding to MIP as compared to the NIP. It is interesting to note that, particularly at lower ligand concentrations, non- specific binding was significantly greater for the *exo* compound as compared to the endo compound [10 μM endo 6 to NIP = 1.2 nmol/ mg; exo 6 to NIP = 3.3 nmol/ mg].

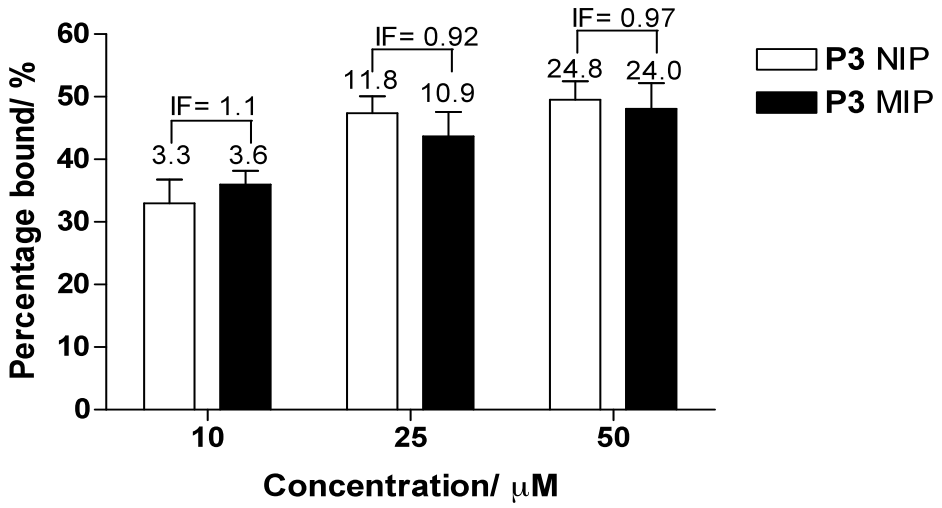


Figure 3.9 Binding Studies for P3 endo 6 imprinted MIP in exo 6 in acetonitrile. The amount of exo 6 per milligram of polymer is given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of exo 6 bound to MIP and the amount of exo 6 bound to NIP.

3.3 Reaction 6 at Low Concentrations

3.3.1 Introduction

As discussed in Section 2.7.1, MIP specificity was only observed at low template concentrations due to the low number of specific binding sites. The objective of this experiment was to evaluate whether reaction 6 could take place in low concentrations and to monitor the formation of products.

3.3.2 Chemicals and Materials

Cyclopentadiene was obtained and prepared as described in Section 3.1.2. Acetonitrile and HPLC setup were obtained and setup as discussed in Section 3.2.2. Benzyl acrylate was obtained as described in Section 4.1.2.

3.3.3 Method

3.3.3.1 Determining the Retention Times of the Products and Reactants of Reaction 6

The method to determine the retention times and detector wavelength was the same as described in Section 3.2.3, but cyclopentadiene, benzyl

acrylate, endo 6 and exo 6 were used instead of methyl vinyl ketone, endo 1 and exo 1 and the mobile phase used was methanol/ water (80/20).

3.3.3.2 Performing Reaction 6 at Low Concentrations

Initially, the reaction was performed by adding 10 mmol of each reactant (0.825 mL cyclopentadiene and 0.830 mL benzyl acrylate) to a 10 mL round bottomed flask to give a concentration of 6.06 M for both reactants. The contents in the flask were stirred at 70 °C for 18 h in absence of a solvent. HPLC (acetonitrile/ water (80/20); 1 mL/ min; 215 nm and 235 nm; 20 μL) was performed on the sample and the area under curve for both reactants and products were obtained. The sample for HPLC analysis was prepared by diluting 1 μL of the reaction mixture at specific time points (0, 15, 30, 45, 60, 120 mins) with 1.8 mL of acetonitrile to give a concentration of 3.36 mM (1 μL of a 6.06 M solution contains 6.06 μmol of reactants. When 1.8 mL acetonitrile is added, the concentration of reactant= (6.06 x 10^{-6}) x 0.001801= 3.36 mM). Next, the solubility of cyclopentadiene and benzyl acrylate in acetonitrile was investigated. 1 mL of acetonitrile was added to 12 mmol (1 mL/ 0.8 g) of cyclopentadiene to form a 6 M solution (Total volume of solution is 2 mL- 1 mL cyclopentadiene and 1 mL acetonitrile). The solution was then subsequently diluted. The solubility of benzyl acrylate was also performed in the same way. The experiment was then repeated using 1, 2 and 4 mM reactant solutions made up in acetontrile.

3.3.4 Results and Discussion

3.3.4.1 Determining the Retention Times of the Products and Reactants of Reaction 6

The HPLC retention times and the wavelength used to detect the isomers are shown in Table 3.2.

Sample	Retention Time/ mins
Cyclopentadiene	5.3
Benzyl Acrylate	4.9
Endo 6	10.5
Exo 6	13.0

Table 3.2 Table to show the HPLC retention times for cyclopentadiene, benzyl acrylate, endo 6 and exo 6 (acetonitrile/ water (50/50); 1 mL/ min; 215 nm and 235 nm; 20 μL).

The λ_{max} for cyclopentadiene, benzyl acrylate, endo 6 and exo 6 was 215 nm. As discussed in Section 2.7.4, most compounds absorb at low wavelengths and hence other impurities (such as stabilisers for the mobile phase and benzyl acrylate) also show up in the chromatogram. The impurity peaks can overlap with reactant or product peaks and hinder analysis. Furthermore, the retention times of cyclopentadiene and benzyl acrylate are close and benzyl acrylate absorbs 15 times more strongly than cyclopentadiene at 215 nm. As a result, cyclopentadiene would be hard to detect in low concentrations. Hence the sample was rerun with a mobile phase of methanol/ water (70/30) in order to achieve a better separation. However, the separation between the two peaks only increased slightly (from 0.4 mins to 0.6 mins) and the endo 6 isomer did not elute till after 27 mins. In an attempt to improve the analytical method, detection wavelength was changed to 235 nm. Although sensitivity was reduced at this wavelength the absorbances for cyclopentadiene and benzyl acrylate peaks were similar. Hence, the mobile phase was kept at methanol/ water (80/20) with a detection wavelength of 235 nm.

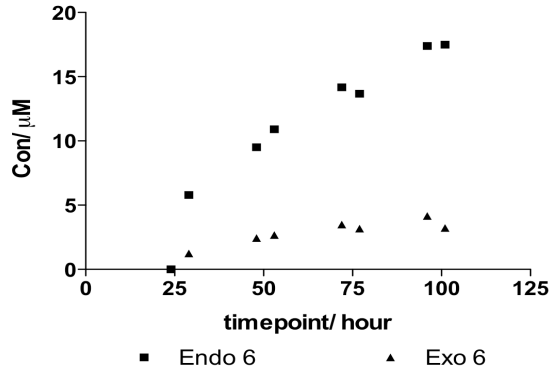
3.3.4.2 Performing Reaction 6 at Low Concentrations

Initially, the reaction between cyclopentadiene and benzyl acrylate was performed at 70 °C and in the absence of solvent to check that cyclopentadiene, benzyl acrylate, endo 6 and exo 6 could simultaneously be determined by the HPLC methods described in Section 3.3.4.1 at the retention times in Table 3.2. The reaction was complete after 1 hour with a crude yield

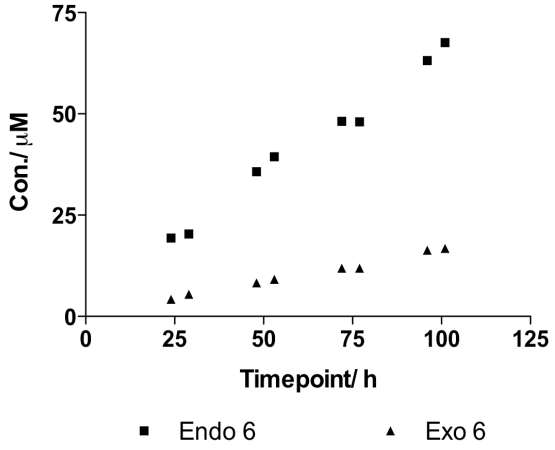
of 96 % and the reactants and products appeared at the retention times stated in Section 3.3.4.1. with no overlapping of peaks.

The same reaction was repeated using acetonitrile as solvent since previous MIP binding studies concluded that higher specific binding was observed in acetonitrile than in methanol or hexane (Section 2.6.4). The solubility of cyclopentadiene and benzyl acrylate in acetonitrile was investigated before the reaction was performed. Cyclopentadiene was found to form an emulsion at concentrations below 4 M and benzyl acrylate dissolved in acetonitrile at all concentrations attempted (1- 6 M). The cyclopentadiene emulsion formed however became a clear solution when an equimolar solution of benzyl acrylate was added.

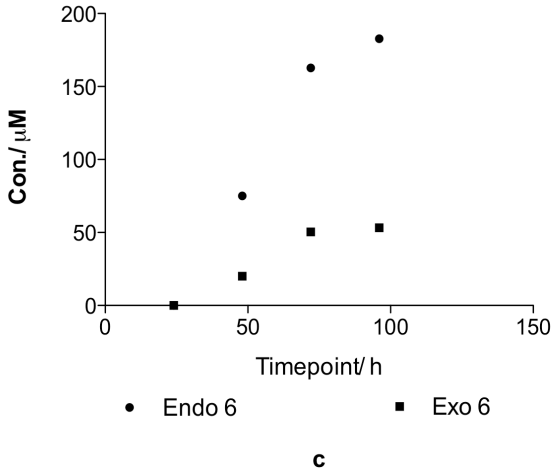
The next step was to find the concentration of cyclopentadiene and benzyl acrylate required to form endo and exo 6 in concentrations similar to those used in binding studies in Section 3.2.4.2 and in a reasonable time frame (i.e. slow enough to allow HPLC analysis at each time point but fast enough to prevent secondary reactions as observed in Section 2.7.4). In order to demonstrate the imprinting effect of the endo 6 imprinted MIP, at least 10 μM of exo 6 needed to be generated after 5 days. Template concentrations of 1, 2 and 4 mM were attempted.



a



b



c

Figure 3.10 Reaction 6 performed in different cyclopentadiene and benzyl acrylate concentrations: a) 1mM; b) 2 mM and c) 4 mM

When an initial cyclopentadiene and benzyl acrylate concentration of 1 mM were used, 17 μM endo 6 and 3 μM exo 6 were formed after 101 hours (Figure 3.10a). The amount of exo 6 generated was at the detection limit of the HPLC. Hence, if MIPs were added to the reaction it might be expected that the amount of free exo 6 would fall below the detection limit of the system making quantification impossible. Hence, a higher initial concentration of cyclopentadiene and benzyl acrylate was needed in order to generate higher concentrations of product. When the initial concentration of cyclopentadiene and benzyl acrylate was increased to 4 mM, 183 μM of endo 6 and 53 μM of exo 6 were formed after 96 hours (Figure 3.10c). Previous binding studies showed that specific binding decreased dramatically when template concentrations were higher than 100 μM due to saturation of available MIP binding sites (Figure 3.5). In order to observe the influence of an endo MIP on the endo: exo product ratio, it was necessary to target endo concentration of <100 μM for a large proportion of the reaction and also that the exo concentration would be achieve a concentration greater than the analytical detection limit. In order to achieve this starting concentrations in the range 1-4 mM of cyclopentadiene and benzyl acrylate were identified. An initial evaluation was carried out at a starting concentration of 2 mM. At this concentration product yield was 67 μM of endo 6 and 17 μM of exo 6. As a result, 2 mM starting concentration was used in subsequent experiments.

3.4 Conclusion

Following the detection and reaction rate issues described in Chapter 4, the Diels- Alder reaction between cyclopentadiene and 4 other dienophiles (phenyl methacrylate, phenyl acrylate, furfuryl methacrylate and benzyl acrylate (Figure 3.1)) were screened to identify a new model reaction. The reaction between cyclopentadiene and benzyl acrylate was chosen as the model reaction as the endo and exo product could be separated easily by MPLC to give an endo: exo ratio of 3: 1.

The endo product was selected initially as the template and a number of MIPs, containing different functional polymers (methacrylic acid, 4- vinyl pyridine and acrylamide) were prepared and screened. Non- imprinted

polymers were also made in the same way, but in the absence of endo 6. The MIP in which acrylamide was used as the functional monomer (**P3**) gave the highest amount of specific binding in equilibrium binding assays. A non-imprinted polymer using EGMA as cross- linker (**P5**) was prepared and was found to demonstrate higher non- specific binding than NIPs prepared with DVB as a cross- linker. Another MIP was made by replacing the acrylamide in **P3** with equimolar amounts of *N,N'*-methylene bisacrylamide (MBA) in **P4** but no improvement of specific binding was observed.

Lastly, the reaction between cyclopentadiene and benzyl acrylate was performed at low concentrations. HPLC retention times, using a mobile phase of methanol/ water (50/50), were: 5.3 mins for cyclopentadiene; 4.9 mins for benzyl acrylate; 10.5 mins for endo 6 and 13.0 mins for exo 6. Reaction 6 was performed in equimolar reactant concentrations at 70 °C and at three different initial reactant concentrations (1, 2 and 4 mM). 2 mM was selected as the ideal initial reactant concentration as the amount of endo 6 generated (67 μM) was low enough to observe specific MIP binding and the amount of exo 6 generated (17 μM) was greater than its detection limit.

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4 Scaling Up the Production of MIP and NIP

4.1 Producing P3 (4:1) MIP and NIP in a Large Scale

4.1.1 Introduction

In binding studies described in Chapter 3, endo 6 imprinted MIPs bound endo 6 to a greater extent than exo 6. The next step in achieving the aim (Section 1.2.1) was to introduce the endo 6 MIP into a model reaction in order to demonstrate increased formation of the template. However, the selective capacity of the MIPs (difference between MIP and NIP binding) was found to be low (3.1 nmol/ mg for **P3 (4:1)** for a template concentration of 10 μM) (Figure 3.7). Hence, it was necessary to increase the MIP concentration (1 mg/ mL used in binding studies in Chapter 2 and 3) in order to improve the stereoselective effect. Furthermore, it was also observed that even when prepared using the same starting material and reaction conditions, the binding capacities of NIP and MIP varied between batches. Since a difference in polymer friability was noted during grinding it was thought that variation in capacity may have arisen due to differences in morphology arising from variations in polymerisation conditions, in particular the rate of heating. Therefore in order to reduce this source of variability, large batches of NIP and MIP **P3 (4:1)** were produced in order to negate the effect of batch-to-batch variability.

4.1.2 Chemicals and Materials

Acrylamide (ultrapure MB Grade) was obtained as described in Section 3.2.2. Acetonitrile was obtained as described in Section 2.2.2. Di-vinyl benzene (80 % tech. grade), azobisisobutyronitrile (AIBN), acetic acid,

methanol and toluene were obtained as described in Section 2.4.2. The model Rotary evaporator and the vacuum oven were described as in Section 2.4.2.

4.1.3 Method

4.1.3.1 Scaled- up Production of NIP and MIP by Bulk Polymerisation

NIPs and MIPs **P3 (4:1)** were prepared as described in Section 3.2.3.1. Initially, the reagents were increased by 20 fold (10 g yield of NIP/ MIP per vial instead of 0.5 g), but there was not enough room for the nitrogen produced in the decomposition of AIBN to dissipate and this resulted in the bursting of the vial cap during polymerisation. As a result, the reagents were increased by 10 fold (5 g of NIP/ MIP per vial) and the vial number increased to two as shown in Table 4.1. The NIPs were produced in the same way but in the absence of the template. Hence, a total of 4 vials (2 NIPs and 2 MIPs) were purged with nitrogen, put into an oven (60 °C for 17 h) to be polymerised and washed as described in Section 3.2.3.1.

	P3 (4:1)
Divinylbenzene (DVB technical grade 80 % mix of isomers)	4.34 mL (30 mmol)
Monomer	Acrylamide 0.533 g (7.5 mmol)
Template (Endo 6)	0.856 g (1.875 mmol)
Azobisisobutyronitrile (AIBN)	0.007 g (45 µmol)
Chloroform	4.34 mL

Table 4.1 Table to show the starting materials required to make 20 g of P3 (4:1) MIP. The NIP was prepared in the same way but in the absence of template.

4.1.3.2 Binding Studies

The experimental procedure was as described in Section 2.6.3, using endo and exo 6 concentrations of 0, 10, 25 and 50 μM.

4.1.4 Results and Discussion

4.1.4.1 Scaled- up Production of NIP and MIP by Bulk Polymerisation

Both **P3 (4:1)** NIP and MIP had the same colour post polymerisation and post grinding and washing as discussed in Section 3.2.4.1. The NIP was suspended in acetonitrile twice and the supernatant was analysed on HPLC in order to confirm the removal of unreacted reactants. However, the chromatogram showed a number of unidentified peaks suggesting that the NIP required further washing. These peaks were not observed in the smaller scale polymerisations. However, since the peaks were small (peak height 5 times that of baseline noise), it was not unreasonable that the same contaminants were present in the small- scale polymers (Section 2.2.3.1) but at concentrations below the HPLC detection level. Hence, the NIP was washed in the same way as the MIP in order to remove the contaminants observed on HPLC. No peaks were observed on the HPLC chromatogram of the supernatant of the NIP after washing in methanol/ acetic acid (4:1).

4.1.4.2 Binding Studies

The specific binding observed for small- scale production of **P3 (4:1)** in Figure 3.7 (~ 20% and ~ 10% for endo 6 MIP with 25 μM and 50 μM endo 6 respectively) was not observed when the production of NIP and MIP were scaled up (Figure 4.1). Furthermore, there was no difference in the imprinting factors (both at 1.3 for a template concentration of 25 μM and 1.2 for a template concentration of 50 μM) observed for the binding of an endo 6 imprinted MIP to endo 6 and an endo 6 imprinted MIP to exo 6 (Figure 4.1 and 4.2).

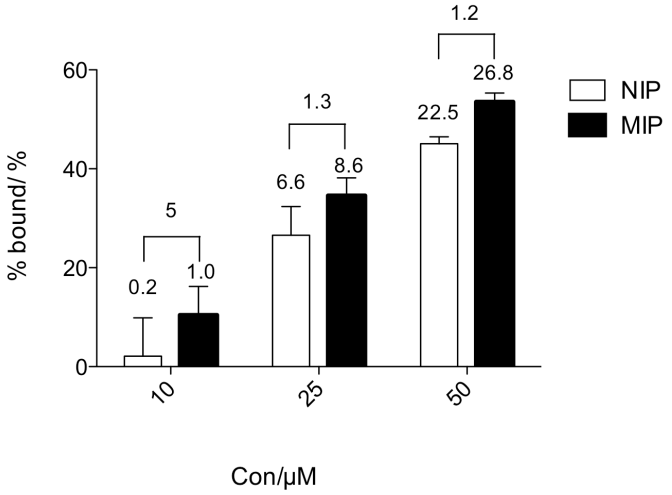

Figure 4.1 Binding Studies for P3 (4:1) endo 6 MIP in endo 6 in acetonitrile (10 g per vial scale). The amount of endo 6 per milligram of polymer is given at the top of each bar in nmol/ mg. The value above the horizontal bracket is the Imprinting Factor (IF) which is the ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

As a result, a binding study using an endo 6 concentration of 50 μM was performed to compare the difference in binding between the **P3 (4:1)** MIP used to obtain the results in Figure 3.7 (Old MIP) and the **P3 (4:1)** NIP and MIP which was used to obtain the results in Figure 4.1 (New MIP and NIP in Figure 4.1 represent the new batch of MIP and NIP which were prepared for this experiment). Unfortunately, there was not enough of the Old NIP (NIP used to obtain the results in Figure 3.7) for this binding study hence the non-specific binding between the Old NIP and New NIP could not be compared. So, the possibility that the difference in the performance of Old MIP and New MIP was due to the difference in non- specific binding due to surface morphology and not specific binding could not be verified.

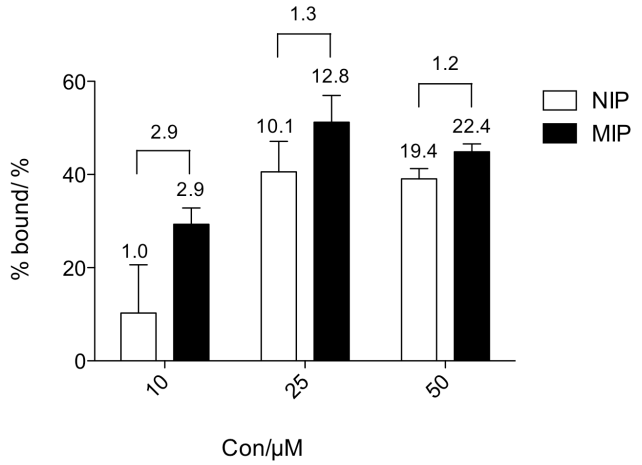

Figure 4.2 Binding Studies for P3 (4:1) endo 6 MIP in exo 6 in acetonitrile. The amount of exo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. The value above the horizontal bracket is the Imprinting Factor (IF) which is the ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

The amount of specific and non- specific binding to the Old MIP (40 % for 50 μM endo 6; Figure 4.3) was comparable to the result obtained previously (38 % for 50 μM endo 6; Figure 3.7) and to the New MIP (46 % for 50 μM endo 6; Figure 4.3). However, the amount of binding to the NIP used in Figure 4.1 was much higher (45 % for 50 μM endo 6) compared to the value obtained for the NIP used in Figure 3.7 (27 % for 50 μM endo 6). The rebinding studies were repeated (for 5 g per vial scale polymerisations) using freshly synthesised endo 6 and exo 6 (using newly purchased reagents) and a number of new batches of endo 6 imprinted MIP and NIP, but the results each time were consistent to those described in Figure 4.1. As a result, a number of experiments were carried out in order to determine the reason for the change in selectivity.

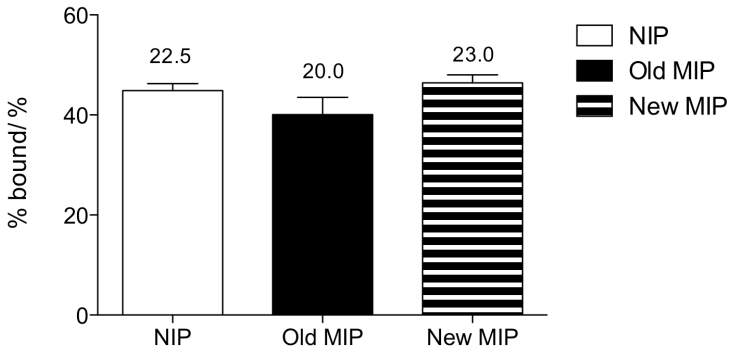

Figure 4.3 Binding Studies to compare the difference between the P3 (4:1) Endo 6 MIP used to produce the data in Figure 3.7 (Old MIP) and the P3 (4:1) Endo 6 MIP used to produce the data in Figure 4.1 (New MIP). A template (endo 6) concentration of 50 μM was used and the binding study was performed in acetonitrile. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. The value above the horizontal bracket is the Imprinting Factor (IF) which is the ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

4.2 Effect of AIBN on Specific Binding

4.2.1 Introduction

After reviewing the methodologies for the bulk polymerisation reactions at both 0.5 g per vial and the 5 g per vial scale (Appendix 2) it was noted that an error had been made in the scaling of reagents and that the concentration of AIBN in the 5 g polymerisation was 1/ 10 that used in the 0.5 g scale. Hence an experiment was carried out to investigate whether the difference in binding observed in Figure 4.1 was due to the amount of AIBN used to initiate polymerisation.

Polymers made with more initiator would theoretically be more rigid resulting in the formation of better- defined imprinting cavities whilst reactions performed at a reduced initiator concentration would yield less heat energy thus reducing any temperature rise occurring during polymerisation. Either of

these could influence the performance of a resulting MIP. Piletsky et. al. investigated the effect of different initiators and different quantities of the same initiator on specific binding. (-)- ephedrine was used as the template and 2 initiators- 1,1'-azo-bis(cyclohexane-1-carbonitrile) (ACC) and 2,2-dimethoxy-2-phenylacetophenone (DPMA) were evaluated.[90] Changing from one initiator to another had a relatively small influence on MIP performance compared to other factors such as polymerisation temperature, amount of initiator and polymerisation time. MIP performance was determined by separation factors when the MIPs were used as stationary phases in HPLC. MIPs prepared with higher amounts of initiators produced more rigid polymer networks containing fewer unreacted double- bonds but were less selective than MIPs prepared with lower amounts of initiator. This reduction in performance was attributed to the disruption of the monomer- template complex brought about by an increase in polymerisation temperature.

4.2.2 Chemicals and Materials

The chemicals and equipment used were described in Section 4.1.2.

4.2.3 Method

4.2.3.1 Production of P3 (4:1) NIP and MIP by Bulk Polymerisation

P3 (4:1) NIP and MIP with the original AIBN to functional monomer ratio were made in the same way as described in Section 3.2.3.1. **P3 (4:1)** NIP and MIP were also made the same way but only one tenth of AIBN was used.

4.2.3.2 Binding Studies

The experimental procedure used was the same as Section 4.1.3.2.

4.2.4 Results and Discussion

4.2.4.1 Production of P3 (4:1) NIP and MIP by Bulk Polymerisation

All NIPs and MIPs made in Section 4.2.3.1. had the same post polymerisation, grinding and washing colour as described in Section 3.2.4.1.

4.2.4.2 Binding Studies

In contrast to the observation by Pilesky et. al.[90] as described in Section 4.2.1, no difference was observed in the imprinting factors of **P3 (4:1)** NIP and MIP prepared using the AIBN- functional monomer ratio used in chapters 2 and 3 and the **P3 (4:1)** NIP and MIP prepared using 10 times less AIBN (Figure 4.4). Furthermore, the **P3 (4:1)** NIP and MIP described in Section 4.2.3.1 were prepared using the original scale (0.5 g per vial batch) but the amount of binding observed was similar to the **P3 (4:1)** NIP and MIP made in the scaled up version (~ 40 % using the original batch size (Figure 4.4) and 45 % using the scaled up batch size (Figure 4.3)). Hence, the difference in NIP and MIP binding between Figure 4.1 and Figure 3.7 was not due to the scale up in the production of the polymers.

Piletsky's explanation for the increase in separation factor with increase in initiator concentration was that using more initiator resulted in higher temperatures during polymerisation that outweighed the positive effect of producing a polymer with increased rigidity. As a result, Piletsky concluded that using smaller amounts of initator along with other factors such as longer polymerisation times and lower temperatures would give rise to a MIP with the optimal enantioseparation performance. However, in the Piletsky et. al. study polymers were evaluated chromatographically (non- equilibrium) rather than under equilibrium conditions. Furthermore, the Piletsky study did not use control NIPs instead in relying on the changes in MIP function to gauge effects of different parameters and therefore the effect of changes such as the porosity and surface area were not considered. When polymerisation was initiated with a high concentration of initiators, the polymer growth tends to give rise to polymers with high microporosity due to high concentration of initiation/ propagation sites favouring early termination due to combination of growing chains (Figure 1.5). This process gives rise to a high number of nucleation sites resulting in high micro- porosity. Piletsky analysed the surface area of the MIPs using nitrogen adsorption isotherms (BET) and found that increasing the initiator concentration from 1 % to 5 % resulted in an increase of surface area of the MIPs produced from 56.94 m^2/ g to 72.59 m^2/ g. Hence the slight increase in separation factor (from 1.10 to 1.30 for a 12 h polymerisation time

in a oil bath at 80 °C using ACC (1,1'-azo-bis(cyclohexane-1-carbonitrile)) as initiator) observed when the initiator concentration was increased from 1 % to 5 % could be due to an increase in non- specific binding (e.g. more template molecules binding to the surface of the MIP) and not due to an improvement in the imprinting effect where more template molecules were bound to the MIP cavities in a 'Lock- and- Key' manner. Results in Figure 4.4 showed that although the imprinting factor (i.e. ratio between the amount of endo 6 bound to the MIP and the amount of endo 6 bound to the NIP) was the same regardless of the amount of AIBN used, the non- specific binding decreased from 22.0 % to 19.2 % when the amount of AIBN used was increased 10 fold. Although this observation did not support the hypothesis that a higher quantity of initiator would result in an increased amount of non- specific binding due to a larger surface area, it does however suggest that other morphology changes occur when different quantities of AIBN were used.

Furthermore, although the increase in separation factors observed in Piletsky's results may have indicate an enhanced imprinting effect, it is difficult to predict changes in equilibrium binding parameters based on changes in separation factors in non- equilibrium binding. Hence, making quantitative comparisons between the results shown in Figure 4.4 with the results Piletsky obtained is not reasonable.

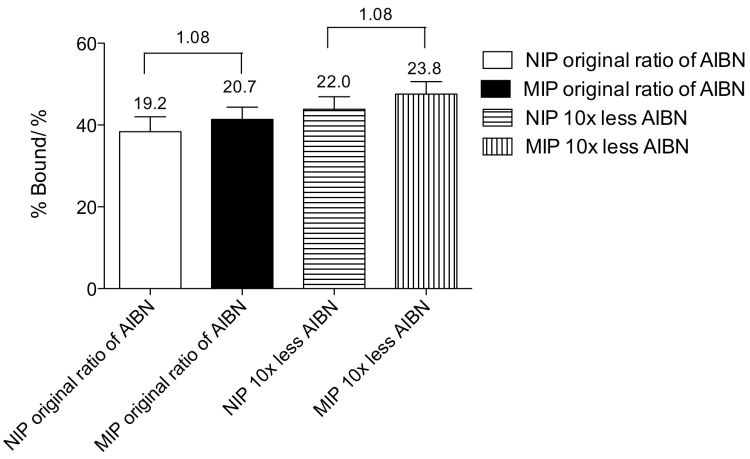

Figure 4.4 Binding Studies to compare the effect of the amount of AIBN on Specfic binding. The binding study was performed using a P3 (4:1) endo 6 imprinted MIP and endo 6 as template. Acetonitrile was used as the solvent. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. The value above the horizontal brackets is the Imprinting Factor (IF) which is the ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

4.3 Washing of NIP and MIP

4.3.1 Introduction

In the preliminary studies, the 4- vinyl pyridine based NIP and endo 1 imprinted MIP were washed in the same way (Section 2.4.3.2). HPLC analysis of the supernatants of the washing steps suggested that non- polymerised material had been removed from the NIP after being suspended in acetonitrile. Hence in the Mini- MIP experiments in Chapter 3, the only washing step that the NIPs were subjected to was suspension in acetonitrile. HPLC analysis of the supernatants of the NIP washes and the 'blank' binding study (same setup as samples 13-16 in Table 2.6 in Section 2.6.3.) suggested that no material was leaching out from the NIP. Hence, the amount of washing was deemed sufficient. As a result, the NIP was dried and was used for binding studies. This decision was taken in order to reduce the time (and solvent use) to clean the

polymers for the Mini- MIP experiments. It should be noted that the method used for washing the MIPs was not changed. When the NIP and MIP were scaled up as described in Section 4.1, the NIP was washed in the same way as the MIP (i.e. using the same method of washing as the MIP) as peaks were present in the supernatant of the 2nd acetonitrile wash. As a result, the difference in binding observed may have been due to the fact that the NIP made in Section 4.1 was washed more thoroughly. Hence a study to evaluate the effect of washing on NIP binding was performed.

4.3.2 Chemicals and Materials

The chemicals and equipment used were described in Section 4.1.2.

4.3.3 Method

4.3.3.1 Production of P3 (4:1) NIP and MIP by Bulk Polymerisation

P3 (4:1) NIP and MIP were prepared as described in Section 3.2.3.1. Both NIP and MIP were ground and recovered from suspension in acetonitrile by filtration as described in Section 2.4.3.2. The NIPs were then split into 2 batches- Batch 1 was not washed further (i.e. dried in the oven overnight after being suspended in acetonitrile) and Batch 2 was washed in the same way as the MIPs as described in Section 4.4.3.2. HPLC analysis was performed on all supernatants to check that no detectable unreacted reactants or templates were present.

4.3.3.2 Binding Studies

The experimental procedure used was the same as Section 4.1.3.2.

4.3.4 Results and Discussion

The non- specific binding for **P3 (4:1)** Batch 1 NIP (freshly made as described in Section 4.1.3.2 and suspended in acetonitrile) was much lower than **P3 (4:1)** Batch 2 NIP (also freshly made as described in Section 4.1.3.2 and suspended in acetonitrile and washed with acetic acid, methanol and toluene in the same way as the MIP was washed). A possible explanation for this observation was that **P3 (4:1)** Batch 1 NIP was not washed sufficiently

(Figure 4.5). The amount of binding for **P3 (4:1)** Batch 2 NIP was 40 % which was comparable to the results observed in Figure 4.1 (45 % binding for NIP) though the binding for **P3 (4:1)** Batch 1 NIP were much lower than what was observed in Figure 3.7 (14 % for **P3 (4:1)** Batch 1 NIP (Figure 4.5)).

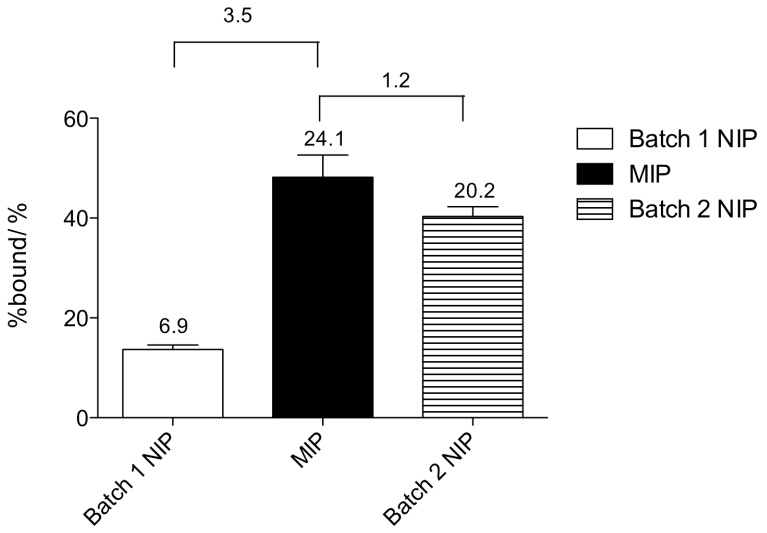

Figure 4.5 Binding Studies to compare the effect of the extend of washing of NIPs in non- specific binding. Batch 1 NIP was only suspended in acetonitrile and not washed further. Batch 2 NIP was suspended in acetonitrile and also washed with the same solvents as the MIP. The binding study was performed using a P3 (4:1) endo 6 imprinted MIP and endo 6 as template. Acetonitrile was used as the solvent. The amount of endo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. The value above the horizontal brackets is the Imprinting Factor (IF) which is the ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

Peaks were observed in the HPLC chromatograph of the acetonitrile supernatant that was used to disperse both NIPs. This indicated that this step was important in removing unreacted material from the NIP. However, no peaks were observed in the supernatant of the acetic acid, methanol and toluene washes that were used for the Batch 2 NIP. This suggested that no other material had leached from the NIP. This observation was comparable to the observation made in the washing of the 4- vinyl pyridine NIP in Section 2.4.4.

Furthermore, there was no difference in binding between **P3 (4:1)** Batch 1 NIP and **P3 (4:1)** Batch 2 NIP with exo 6 (Figure 4.6) even though the NIP used was prepared in the same batch and was washed as one batch as the NIPs used in Figure 4.5.

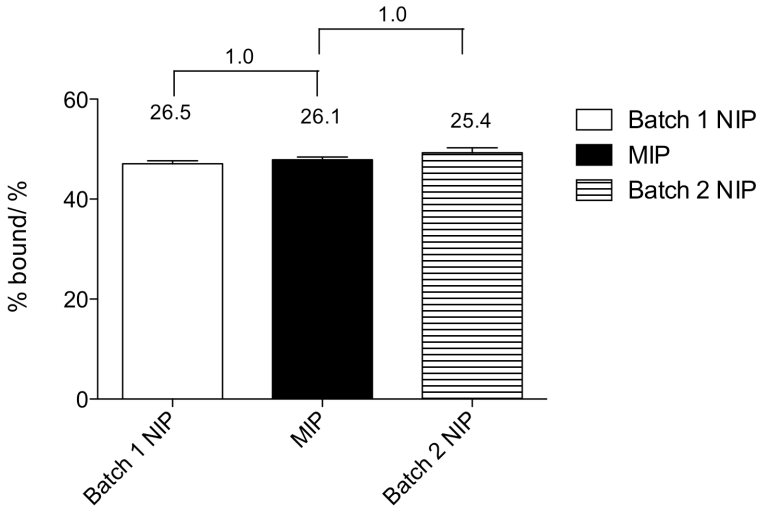

Figure 4.6 Binding Studies to compare the effect of the extend of washing of NIPs in the amount of binding. Batch 1 NIP was only suspended in acetonitrile and not washed further. Batch 2 NIP was suspended in acetonitrile and also washed with the same solvents as the MIP. The binding study was performed using a P3 (4:1) endo 6 imprinted MIP and exo 6 as template. Acetonitrile was used as the solvent. The amount of exo 6 per milligram of polymer was given at the top of each bar in nmol/ mg. The value above the horizontal brackets is the Imprinting Factor (IF) which is the ratio between the amount of exo 6 bound to MIP and the amount of exo 6 bound to NIP.

4.4 Conclusion

The production of P3 (4:1) NIP and MIP was scaled up in order to reduce the effect of inter batch variability in studies to evaluate polymer performance. However, the high levels of specific binding observed previously (**P3 (4:1)** Figure 3.7) was not seen when the polymer synthesis was scaled up

(Figure 4.1 and 4.2). Hence a few experiments were conducted to determine the reason for the change in specific binding.

Firstly, a binding study was set up to compare the **P3 (4:1)** MIP (corresponding NIP not used due to insufficient amounts) used to produce Figure 3.7 (Old MIP) with the **P3 (4:1)** MIP and NIP used to produce Figure 4.1 and 4.2 (New MIP and New NIP respectively). The binding of both MIPs was similar (40 % for the old MIP and 46 % for the new MIP at 50 μM endo 6; Figure 4.3) and the binding of the old MIP was comparable to what was observed previously (38 % for 50 μM endo 6). The binding of the new NIP was much higher (45 % for 50 μM endo 6; Figure 4.3) compared to the binding of the old NIP observed in Figure 3.7 (27 % for 50 μM endo 6).

Next, the effect of the amount of AIBN on binding was investigated. No difference in binding was observed between NIPs and MIPs made with the AIBN to monomer ratio used in section 3.2.3.1 and the NIP and MIP made with 10 times less AIBN in section 4.1.3.1.

Lastly, the washing of NIPs was investigated. In the binding study between the endo 6 imprinted MIP and endo 6, the non- specific binding for the NIP which was only suspended in acetonitrile (Batch 1) was much lower than the NIP which was washed the same way as the MIPs (Batch 2) (14 % for P3 (4:1) Batch 1 NIP and 42 % for P3 (4:1) Batch 2 NIP (Figure 4.5). However, HPLC analysis of the supernatent of the washes did not indicate any material being washed out from Batch 2 NIP. Furthermore, in the binding study between the endo 6 imprinted MIP and exo 6, no difference in binding was observed between the NIP suspended only in acetonitrile (Batch 1 NIP) and the NIP that was washed in the same way as the MIP (Batch 2 NIP) (Figure 4.6).

[90] Mijangos, I.; Navarro- Villoslada, F.; Guerreiro, A.; Piletska, E.; Chianella, I.; Karim, K.; Turner, A.; Piletsky, S Biosens. Bioelectron. 2006, **22**, 381-387

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5 General Discussion and Conclusion

5.1 General Discussion

The original aim of the project was to increase minor product formation by using a polymer to selectively remove the minor product hence shifting the chemical equilibrium of the reaction to favour the formation of more minor product. Initially, the Diels- Alder reaction between cyclopentadiene and methyl vinyl ketone was used as the model reaction. Although the imprinting effect was observed between the endo 1 imprinted **P2** (4- vinyl pyridine based) MIP and endo 1 (imprinting factor of 1.7 for 44 μM endo 1 in acetonitrile; Figure 2.7) but not with exo 1 (imprinting factor of 1.1 for 44 μM exo 1 in acetonitrile; Figure 2.8) in equilibrium binding studies, introducing the MIP to the Diels- Alder reaction to demonstrate the imprinting effect remained a challenge as the imprinting effect was only observed at low concentrations (4.4- 44 μM endo 6 used in binding studies; Figure 2.8) due to their low MIP binding capacity (30.7 nmol/ mg for **P2** MIP and 17.7 nmol/ mg for **P2** NIP at an endo 1 concentration of 44 μM; Figure 2.8). The yield of the reaction between 200 nmol cyclopentadiene and 200 nmol methyl vinyl ketone was only 10 % and no exo 1 was detected by HPLC after 10 days (Figure 2.11 and 2.13). Hence, a new model reaction between cyclopentadiene and benzyl acrylate was investigated. Although the product isomers were detected easier due to the benzyl side group and that a more similar molar ratio of isomers were formed (an endo 6/ exo 6 ratio of 3:1 as opposed to an endo 1/ exo 1 ratio of 95:5), the binding capacities of the endo 6 imprinted MIPs (19.1 nmol/ mg for **P3 (4:1)** MIP and 13.6 nmol/ mg for the corresponding NIP for 50 μM endo 6; Figure 3.7) made were similar to the endo 1 imprinted MIP. Binding capacities describe the maximum amount of template which can bind to the MIP selectively.[91] Sellegren et. al. imprinted MIPs with L- phenylalanine anilide and analysed the MIP performance by using the MIP as a stationary phase in PLC experiments and observed that only 15 % of the theoretical maximum number of binding sites created during MIP polymerisation (i.e. the

amount of template added to the monomer mixture) were available for the rebinding of template due to the heterogeneous distribution of binding sites in the MIP as a result of two main effects.[92] Firstly, the binding sites are not identical due to the amorphous nature of polymer, e.g. there may be more binding sites in areas with different cross- linking densities and accessibilities. Secondly, the monomer- template association in the complexation stage is incomplete, i.e. the functional monomer exists in a free and dimerised form and does not associate with the template. As a result, only some of the template in the monomer mixture is used to form the selective binding sites. The binding capacities in non- covalent binding can be increased by lowering polymerisation temperatures, increasing the amount of functional monomers used and by matching the functional groups between template and functional monomers which interacts strongly in solution.

Acetonitrile was the solvent which gave the highest amount of specific binding in the binding studies for both endo 1 and endo 6 which at the current stage of the project was not an issue. However, the long term aim of this project was to implement the polymers and the reaction to a two phase microreactor in segmented flow. Acetonitrile is a solvent with medium polarity and will be miscible in wide range of solvents. Furthermore, the MIPs need to preferentially partition in that solvent. However, acetonitrile is immiscible with some hydrocarbons such as cyclohexane and heptane. Furthermore, liquid perfluorocarbons are immicible with most organic solvents. The MIPs made used divinyl benzene as the cross- linker hence would be quite non- polar. Using ethyl glycol methacrylate (EGMA) as a cross- linker results in a more polar MIP. So, the polarity of the MIPs could be adjusted by varying the ratios of DVB and EGMA. Finding two immicible solvents in which the reactants (cyclopentadiene and benzyl acrylate) and the products (endo 6 and exo 6) partition mainly to one phase and the MIP mainly to the other is not easy. However, segmented flow can be used to perform single phase reactions where the MIPs and the reaction takes place in one phase while the other immicible phase is used to form segments. Ahmed- Omer et. al.[93] compared the reaction rate of segmented flow and parallel flow for the arylation reaction of aniline (**50**) with styrene (**51**) (Figure 5.1). It was reported that segmented flow gives a

yield of 66 % while a yield of 52 % was achieved for the same reaction in laminar flow after a reaction time of 20 mins at room temperature. The same reaction was also performed in bulk (i.e. in a flask) and a yield of 83 % was obtained after a reaction time of 20 h. The increase in yield was due to rapid mixing in each segment due to the generation of internal fluid vortex during the formation of the segments. Hence it might be possible to perform the reaction between cyclopentadiene and benzyl acrylate in acetonitrile and use another immicible solvent such as cyclohexane, heptane or perflurocarbons to form segments. Furthermore, the MIP can be in the acetonitrile phase so both the yield and the stereoselectivity can be enhanced by using the segmented flow microreactor system.

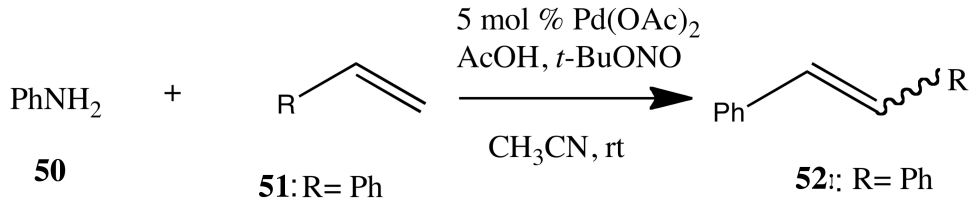


Figure 5.1 The arylation reaction of aniline (50) with styrene 519) (Adapted from ref. 93).

After determining the ideal polymer system (acrylamide as functional monomer and a functional monomer to template ratio of 4:1) and reaction conditions (2 mM cyclopentadiene and 2 mM benzyl acrylate in acetonitrile at 60 °C), the next step was to introduce the MIP into the reaction to demonstrate the increased formation of endo 6. The production of **P3 (4:1)** NIP and MIP was scaled up in order to predict the amount of MIP needed to demonstrate the imprinting effect in the model reaction based on the results of the binding studies as discussed in Chapter 4. However, high levels of specific binding observed in Chapter 3 (Figure 3.7) was not observed when the same polymer systems was scaled up. A few experiments were carried out to understand the reasons for the different observations such as repeating the MIP and NIP production in the scale in Chapter 3, the effect of the quantity of AIBN and the effect of different washing methods. However, as discussed in Chapter 4, the

experiments did not provide a conclusive answer to the difference in specific binding.

In order to understand the reasons for the difference in specific binding between the results in Figure 3.7 and the results in Figure 4.1, several investigations could be carried out. The equilibrium binding studies assumed that the amount of non- specific binding between **P3 (4:1)** NIP and endo 6 would be similar to that of **P3 (4:1)** MIP and endo 6 as the NIP was made in the same way as the MIP but in the absence of endo 6. However, differences in morphology (such as the surface area and porosity) of the NIP and MIP produced could result in a difference in non- specific binding. Hence determining the surface area and porosity by BET analysis would confirm and quantify the morphological differences between the NIP and MIP. Performing BET analysis on NIPs and MIPs of the same polymer system but polymerised in different batches would help to understand the variation in morphology in supposedly identical polymerisation conditions. Piletsky performed BET analysis on MIPs made with different initiator concentrations and found that the surface area of the MIPs increased with increasing initiator concentration (Section 4.2.4.2). As a result, BET analysis could be performed on the polymers made in Section 4.2.3.1 and compare the results with Piletsky's observations.

From Figure 3.7 in Chapter 3, it could be seen that the non- specific binding between the NIP and exo 6 was higher than the non- specific binding between the same NIP and endo 6. Hence in addition to performing BET analysis on **P3 (4:1)** NIP and MIP, a MIP imprinted with exo 6 instead of endo 6 could be made under the same polymerisation conditions and using the same reagents as Section 3.2.3.1. The NIP would only be suspended in acetonitrile after grinding and dried overnight in order to repeat the results obtained in Figure 3.7 and 3.9. Binding studies between **P3 (4:1)** exo 6 MIP and exo 6 using the same experimental procedure as described in section 3.2.3.2 could be carried out. A further binding study between **P3 (4:1)** MIP and endo 6 could be evaluated to investigate and compare the difference of endo/ exo selectivity with the **P3 (4:1)** endo 6 MIP made in Section 3.2.3.1. There could be three possible outcomes in the experiment.

Firstly, if **P3 (4:1)** exo 6 MIP had a similar amount of specific binding (difference between the amount of MIP binding and NIP binding) with exo 6 as **P3 (4:1)** endo 6 MIP and that both isomers (endo 6 and exo 6) bound to the **P3 (4:1)** NIP by a similar amount to previous results (13.6 nmol/ mg (Figure 3.7) and 24.8 nmol/ mg (Figure 3.9) for 50 μM endo 6 and exo 6 respectively, it would suggest that exo 6 has a higher non- specific binding with **P3 (4:1)** NIP than endo 6.

On the other hand, the binding of both endo 6 and exo 6 with **P3 (4:1)** exo 6 MIP could increase and the binding of both endo 6 and exo 6 were similar to values obtained in Figure 3.7 and 3.9 respectively (13.6 nmol/ mg and 24.8 nmol/ mg). The imprinting factors (i.e. ratio between the amount of exo 6/ endo 6 bound to MIP and the amount of exo 6/ endo 6 bound to NIP) of both sets of binding studies could be the same or the imprinting factor for exo 6 could be greater than endo 6 which led to the second and third scenario respectively. If the imprinting factors were the same in both sets of binding studies, it would indicate that exo 6 and endo 6 bind equally well to the **P3 (4:1)** exo 6 MIP cavities which meant that the MIP cavities were not specific enough to distinguish between endo 6 and exo 6. If the imprinting factor of exo 6 was greater than endo 6, it would indicate that both exo 6 and endo 6 would bind to the **P3 (4:1)** MIP cavities, but exo 6 would bind to the cavities preferentially.

5.2 Conclusions

The long term aim of the project was to introduce reaction 6 and MIP in a biphasic segmented flow system to enhance the stereoselectivity of the minor product (exo 6). The findings described in Chapter 2- 4 showed some starting points in achieving this long term aim. Firstly, acetonitrile should be used as one of the two solvent phases as the highest amount of specific binding was achieved using that solvent. Secondly, acrylaimde was the most suitable functional monomer in demonstrating the imprinting effect of endo 6. Thirdly, the initial concentration of cyclopentadiene and benzyl acrylate should be at 2 mM each in order to generate sufficient quantities of both isomers (i.e. high

enough to observe the imprinting effect on HPLC but below the binding capacities of the MIP).

[91] Mirsky, V.; Yatsimirsky, A. Artificial Receptors for chemical Sensors; Wiley: Weinham Germany **2010**, pp 414.

[92] Sellegren, B.; Shea, K. J. J. Chromatogr. **1993**, *635*, 31-49

[93] Ahmed- Omer, B. ; Barrow, D.; Wirth, T. Chem. Eng. J. **135**, 280-283

Appendix 1

Endo 1 (con)

Mw/ g	120	
NMR starting- 1.25 mg of Endo 1 in 500uL d-acetonitrile		
no of moles of endo 1	1.04167E-05	mol
	1.04167E-05	mol of endo 1 in 500uL d-acetonitrile
concentration= n/ v=	0.020833333	M
HPLC vial starting		
	0.020833333	M of endo 1 in 50:50 acetic acid: acetonitrile

new no of moles of endo1=NMR starting+No. of moles of endo1 in n uL from HPLC vial
4.167 *10^-5+ ((4.167*10^-5)n)/500

Pyridine (con)

density/ g/ ml	1.05	
Mw/ g	84.14	
volume/ ml	0.25	
mass of acetic acid= d * v	0.2625	g
no of moles of acetic acid:	0.0031198	mol
con/ M	6.239600666	

for n uL of solution from HPLc vial, no. of mol of acetic acid is
6.207* (n * 10^-6)

EXPNO	amount HPLc vial/ ul	$V_{accumulated/}$ µl	$V_{TOT/}$ µl	endo1$_{/}$ mol	n(pyridine)$_{/}$ mol	[Endo 1], M	[pyridine], M	M/T ratio
1	0	0	500	1.042E-05	0	0.02083	0	0
2	1	1	501	1.044E-05	6.240E-06	0.02083	0.012	1
3	1	2	502	1.046E-05	1.248E-05	0.02083	0.025	1
4	1	3	503	1.048E-05	1.872E-05	0.02083	0.037	2
5	1	4	504	1.050E-05	2.496E-05	0.02083	0.050	2
6	1	5	505	1.052E-05	3.120E-05	0.02083	0.062	3
7	2	7	507	1.056E-05	4.368E-05	0.02083	0.086	4
8	3	10	510	1.063E-05	6.240E-05	0.02083	0.122	6
9	4	14	514	1.071E-05	8.735E-05	0.02083	0.170	8
10	5	19	519	1.081E-05	1.186E-04	0.02083	0.228	11
11	6	25	525	1.094E-05	1.560E-04	0.02083	0.297	14
12	10	35	535	1.115E-05	2.184E-04	0.02083	0.408	20
13	30	65	565	1.177E-05	4.056E-04	0.02083	0.718	34
14	60	125	625	1.302E-05	7.800E-04	0.02083	1.248	60
15	90	215	715	1.490E-05	1.342E-03	0.02083	1.876	90
16	120	335	835	1.740E-05	2.090E-03	0.02083	2.503	120
17	100	435	935	1.948E-05	2.714E-03	0.02083	2.903	139

Appendix 2

Amount of polymer to be made: 10g

4:1:0.01 cross-linker : functional monomer: initiator ratio required

DVB needed:	8	g
Mw DVB:	130.19	g/ mol
no. of mole needed:	0.061448652	
	around 0.06	mol
actual DVB weight:	7.8114	g
relative density DVB:	0.9	
DVB volume needed:	8.679333333	mL
approximately	8.68	mL
acrylamide needed/ mol:	0.015	mol
Mw:	71.08	g/ mol
weight needed:	1.0662	g
Template (Endo 6) needed	0.00375	mol
Mw	228.15	g
Mass needed	0.8555625	g

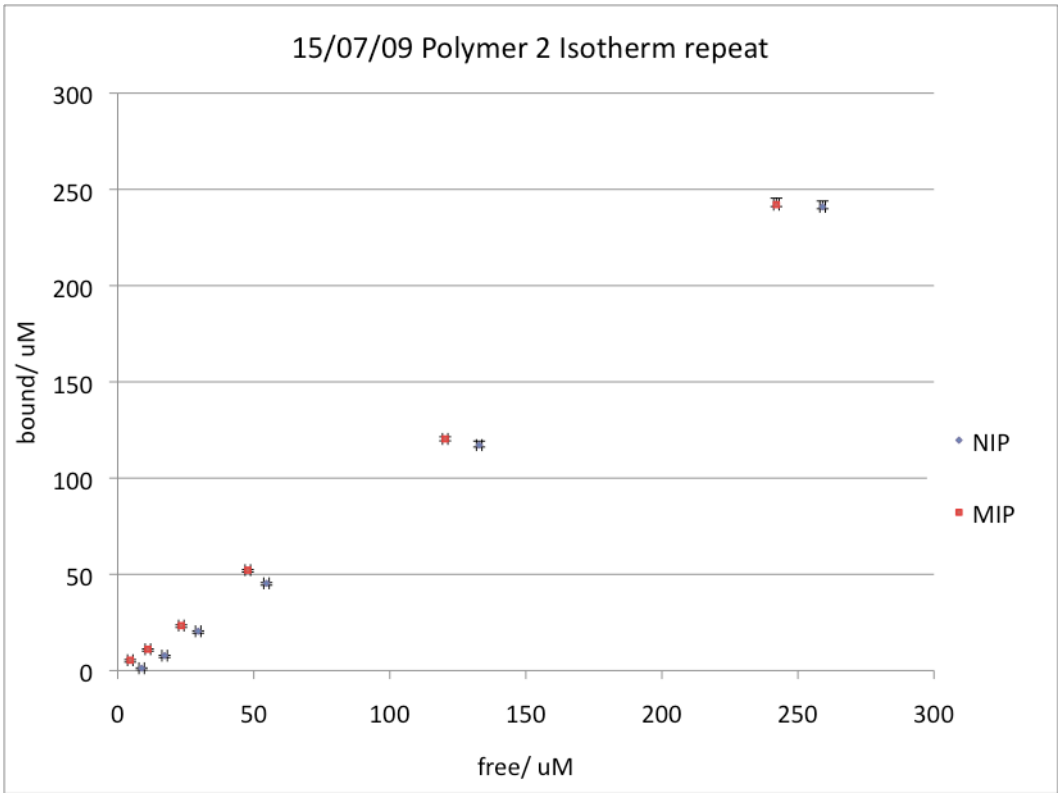
volume of solvent is the same as volume of DVB + acrylamide

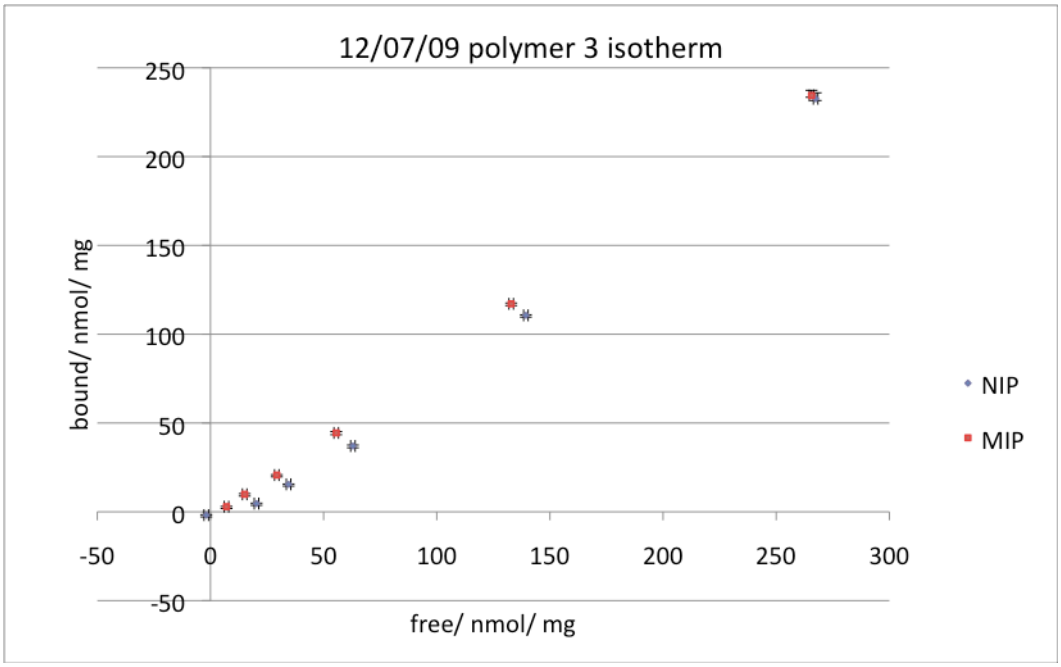
	8.679333333	mL
approximately	8.68	mL

paper

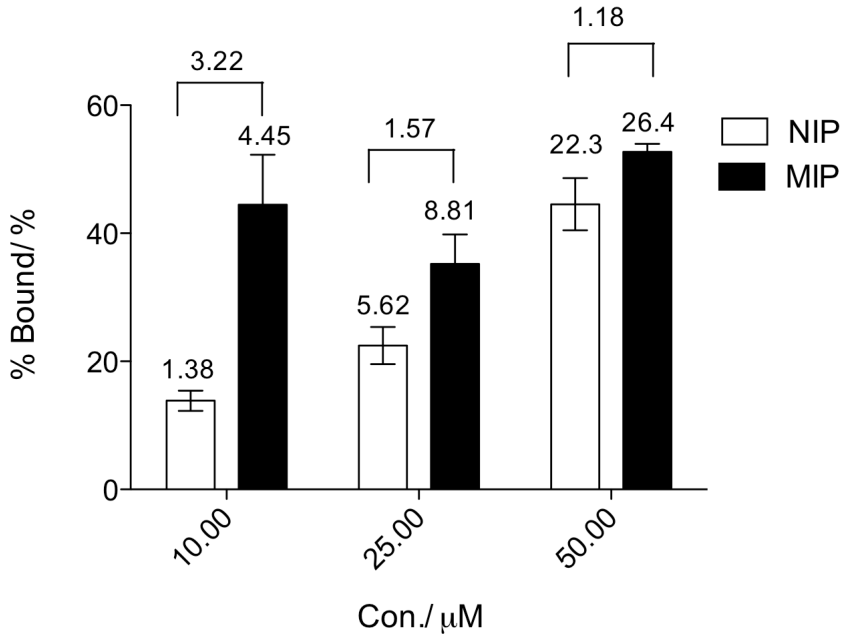
mol ratio of AIBN and monomer	3 to 50	
no. of moles of AIBN used	0.0009	mol
Mw	164.21	g/ mol
amount needed	0.147789	g

Appendix 3


15/07/09 Polymer 2 Isotherm repeat
bound/ uM
free/ uM
NIP
MIP
0
50
100
150
200
250
300


12/07/09 polymer 3 isotherm
bound/ nmol/ mg
free/ nmol/ mg
NIP
MIP
-50
0
50
100
150
200
250
300

Appendix 4



Binding Studies for P3(8:1) endo 6 MIP in endo 6 in acetonitrile. The amount of endo 6 per milligram of polymer is given at the top of each bar in nmol/ mg. IF (Imprinting Factor): Ratio between the amount of endo 6 bound to MIP and the amount of endo 6 bound to NIP.

Appendix 5

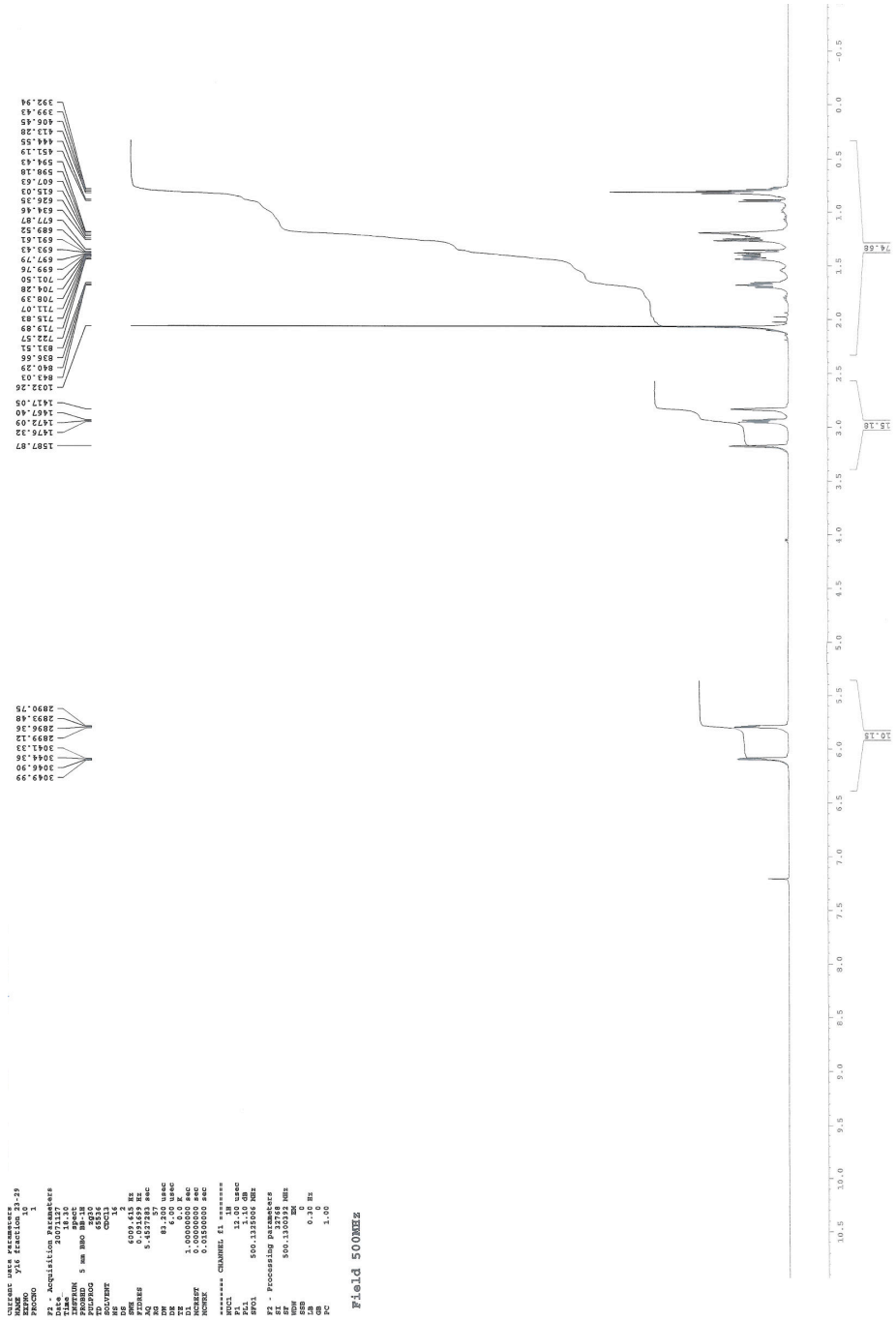


Figure a. ^{1}H NMR of Endo 1

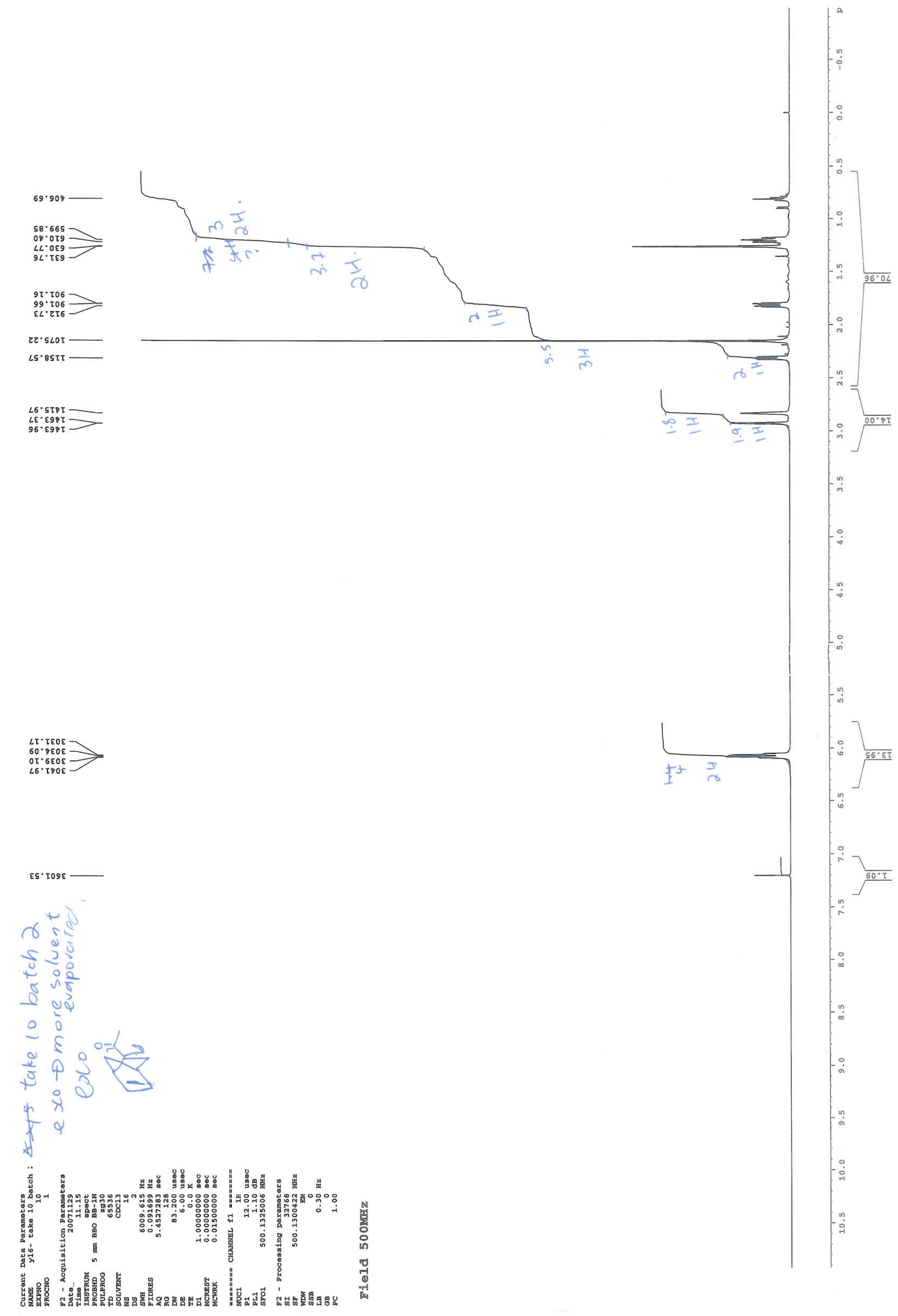


Figure b. ¹H NMR of Exo 1

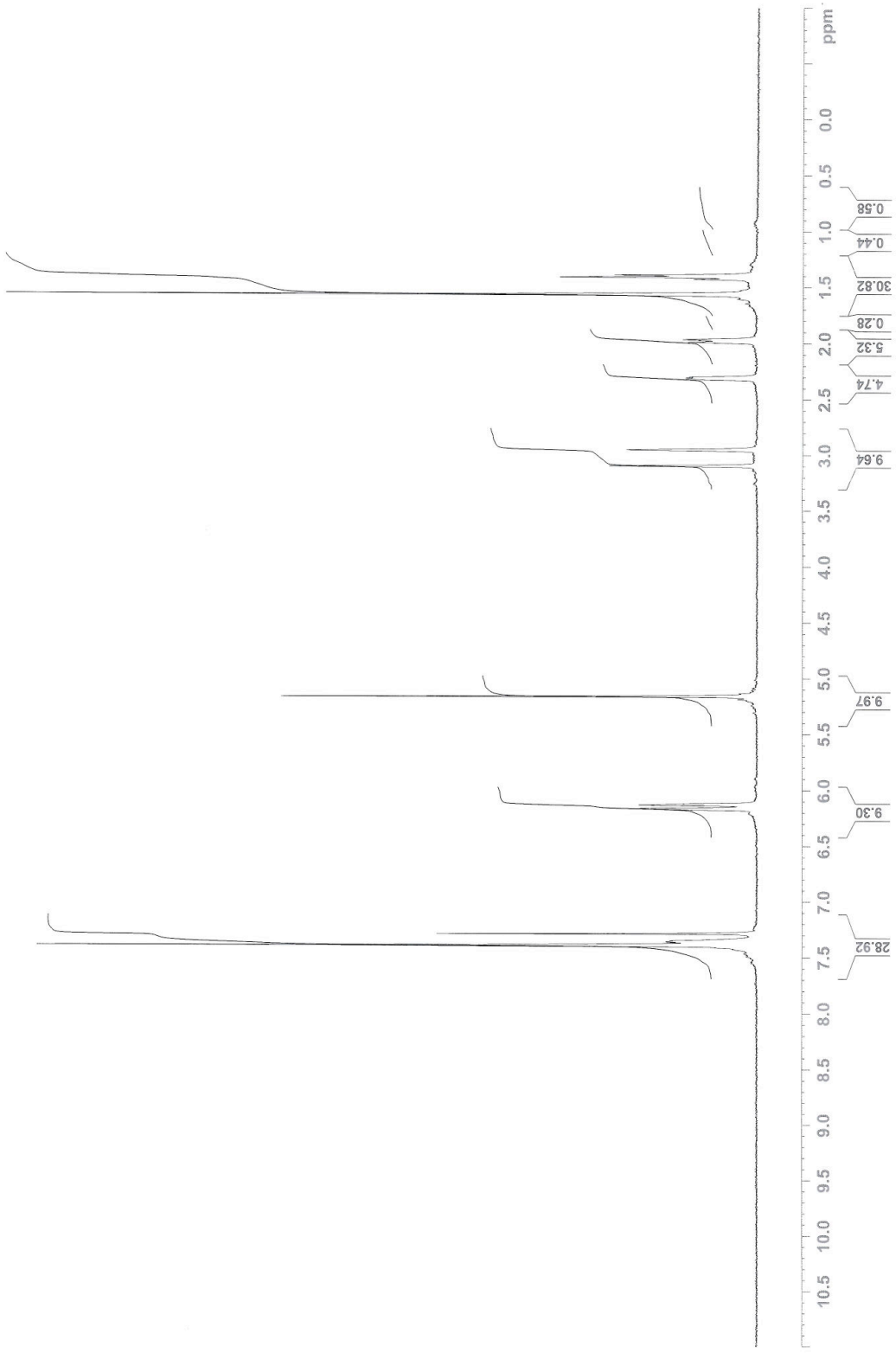


Figure c. ^{1}H NMR of Exo 6

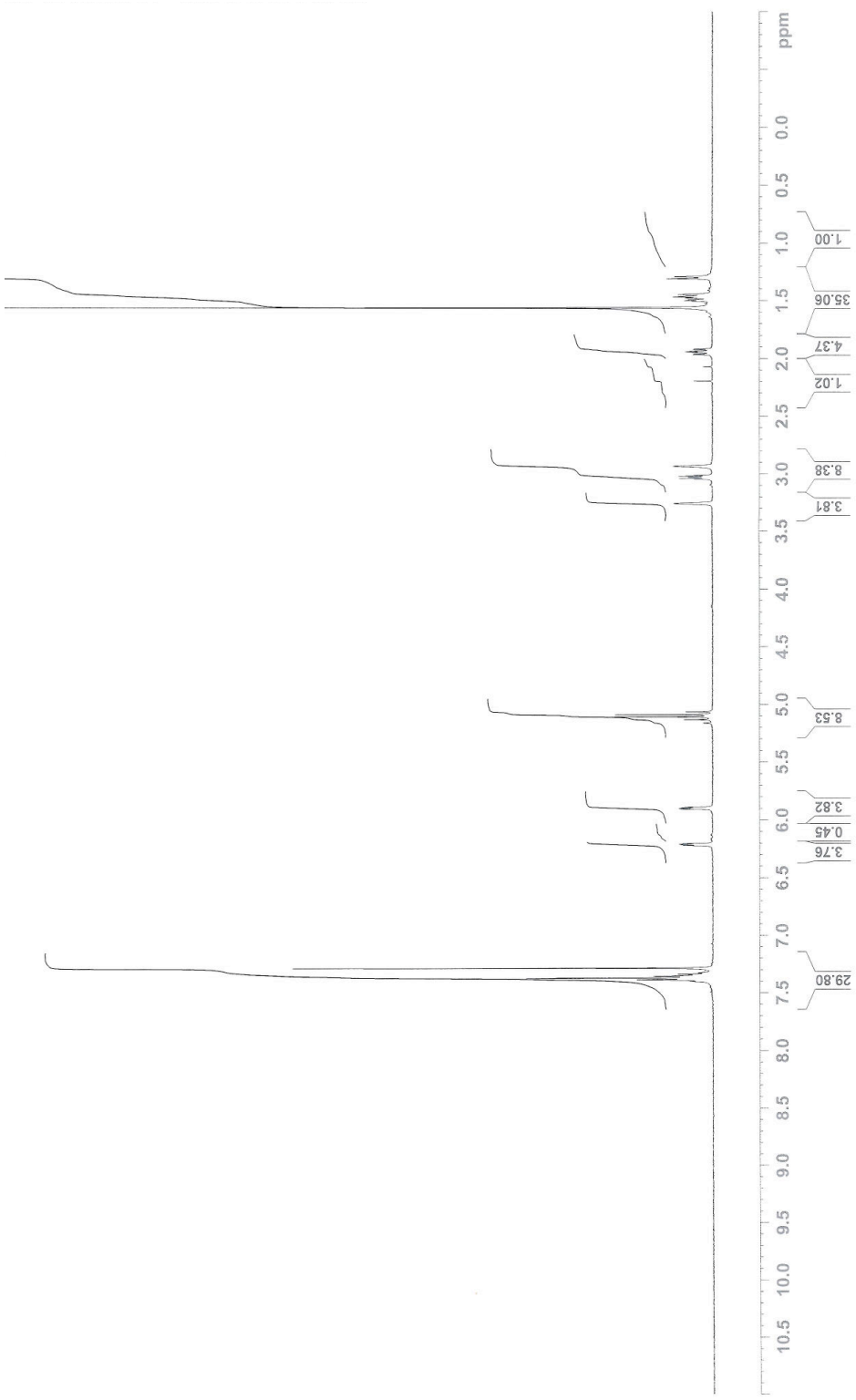


Figure d. ^{1}H NMR of Endo 6

Figure e. ^{1}H NMR of Racemic of reaction 3

Figure f. ^{1}H NMR of Racemic of reaction 4

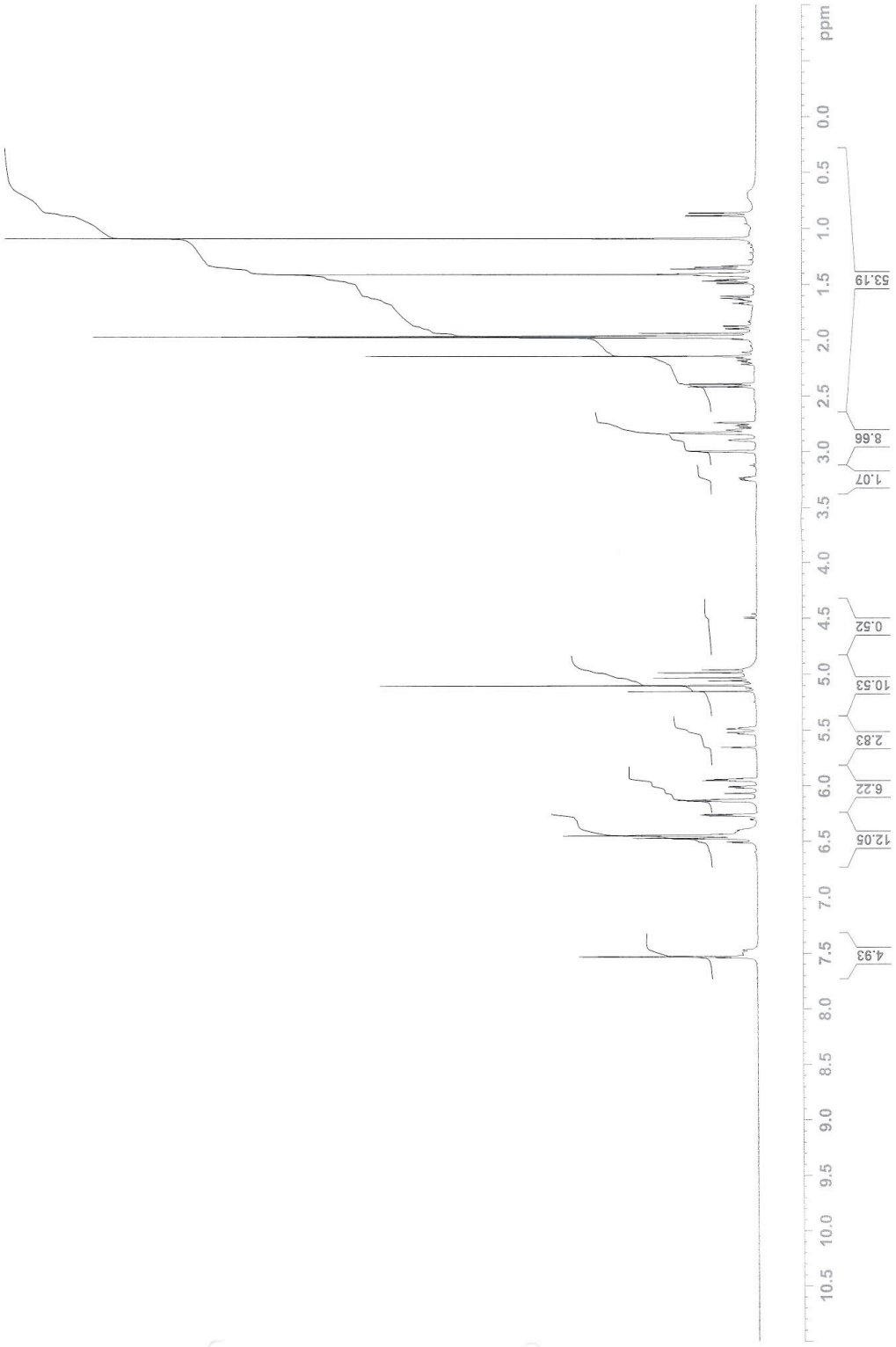


Figure g. ^{1}H NMR of Racemic of reaction 5

Appendix 6

COULTER — Yvonne NIP — N4 Plus

Sample: Yvonne NIP

Comment:	
Lot Number:	Yvonne
Company:	
Operator:	
Estimated Size:	Unknown

Preparation: NIP 1

Comment:	
Diluent:	Chloroform

Run: Run Started 1-Apr-08 at 11:31:56

Comment:	
Profile:	Jenna
Angles Selected:	90.0
Run Times:	180
Sample Time:	Automatic
Prescale:	Automatic
Equilibration Time:	1.0 minutes
Repetitions:	Repeat 3 times for each angle
Automatic SDP:	3.0nm-10000.0nm, 31 Bins
Temperature:	20.0°C
Alpha:	1.000e+000
Beta:	1.000e+000
Viscosity:	0.570 centipoise
Refractive Index:	1.443

N4 Plus Run, 90.0 degrees, 180 seconds

Temperature:	20.0 C
Run Time:	180 seconds (Manual)
Sample Time:	8.5 µs (Automatic)

COULTER

Yvonne NIP

N4 Plus

Sample: Yvonne NIP

Comment:
Lot Number: Yvonne
Company:
Operator:
Estimated Size: Unknown

Preparation: NIP 1

Comment:
Diluent: Chloroform

Run: Run Started 1-Apr-08 at 11:31:56

Comment:
Profile: Jenna
Angles Selected: 90.0
Run Times: 180
Sample Time: Automatic
Prescale: Automatic
Equilibration Time: 1.0 minutes
Repetitions: Repeat 3 times for each angle
Automatic SDP: 3.0nm-10000.0nm, 31 Bins
Temperature: 20.0°C
Alpha: 1.000e+000
Beta: 1.000e+000
Viscosity: 0.570 centipoise
Refractive Index: 1.443

Unimodal Summary

Angle	Run Time (sec)	Sample Time (µs)	Prescale	Counts per Sample Time	Overflows	Baseline Error	Intensity (counts/sec)	Unimodal Mean (nm)	Unimodal S.D. (nm)	Polydispersity Index
90.0°	180	8.5	16	4.721	0	4.48%	5.554e+005	948.1	Broad	0.962
	180	8.0	16	4.399	0	0.63%	5.498e+005	871.3	Broad	0.811
	180	8.0	16	4.558	0	10.89%	5.697e+005	1323.3	Broad	1.416
Average:								1047.6	471.1	1.064
Run Average:								1047.6	471.1	1.064

COULTER

Yvonne NIP

N4 Plus

Sample: Yvonne NIP

Comment:
Lot Number: Yvonne
Company:
Operator:
Estimated Size: Unknown

Preparation: NIP 1

Comment:
Diluent: Chloroform

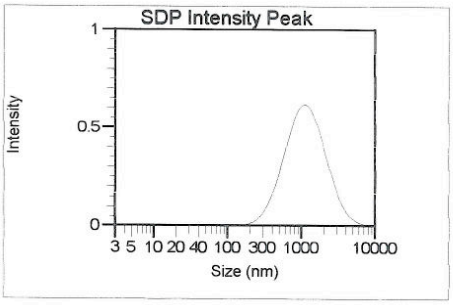
Run: Run Started 1-Apr-08 at 11:31:56

Comment:
Profile: Jenna
Angles Selected: 90.0
Run Times: 180
Sample Time: Automatic
Prescale: Automatic
Equilibration Time: 1.0 minutes
Repetitions: Repeat 3 times for each angle
Automatic SDP: 3.0nm-10000.0nm, 31 Bins
Temperature: 20.0°C
Alpha: 1.000e+000
Beta: 1.000e+000
Viscosity: 0.570 centipoise
Refractive Index: 1.443

Intensity Peak

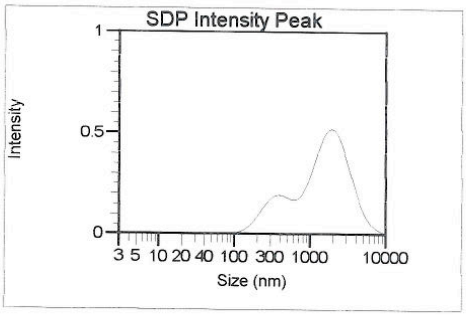
Amount	Size (nm)	SD (nm)
99.6%	1327.4	738.7
0.4%	7.7	1.1

Coefficient variation = 56%.



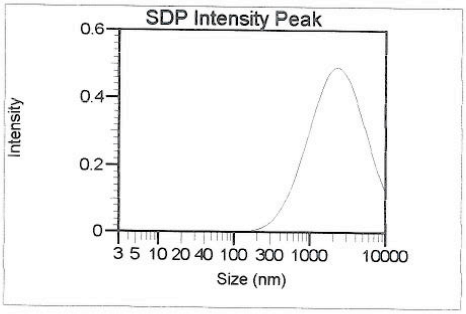
Intensity Peak

Amount	Size (nm)	SD (nm)
75.8%	2143.4	1036.1
24.2%	389.2	160.5



Intensity Peak

Amount	Size (nm)	SD (nm)
100.0%	3617.3	3075.8



Sample: Yvonne MIP Endo 1

Comment:
Lot Number: Yvonne MIP Endo 1
Company: Yvonne
Operator:
Estimated Size: Unknown

Preparation: MIP endo 1

Comment:
Diluent: Chloroform

Run: Run Started 1-Apr-08 at 12:06:42

Comment:
Profile: Jenna
Angles Selected: 90.0
Run Times: 180
Sample Time: Automatic
Prescale: Automatic
Equilibration Time: 1.0 minutes
Repetitions: Repeat 3 times for each angle
Automatic SDP: 3.0nm-10000.0nm, 31 Bins
Temperature: 20.0°C
Alpha: 1.000e+000
Beta: 1.000e+000
Viscosity: 0.570 centipoise
Refractive Index: 1.443

Unimodal Summary

Angle	Run Time (sec)	Sample Time (µs)	Prescale	Counts per Sample Time	Overflows	Baseline Error	Intensity (counts/sec)	Unimodal Mean (nm)	Unimodal S.D. (nm)	Polydispersity Index
90.0°	180	18.5	8	2.605	0	1.70%	1.408e+005	2241.2	753.9	0.207
	180	17.5	8	2.327	0	0.99%	1.330e+005	2070.3	Narrow	0.025
	180	15.5	4	1.980	0	0.39%	1.277e+005	1660.7	Broad	0.409
Average:								2057.4	600.2	0.214
Run Average:								2057.4	600.2	0.214

COULTER

Yvonne MIP Endo 1

N4 Plus

Sample: Yvonne MIP Endo 1

Comment:	
Lot Number:	Yvonne MIP Endo 1
Company:	Yvonne
Operator:	
Estimated Size:	Unknown

Preparation: MIP endo 1

Comment:	
Diluent:	Chloroform

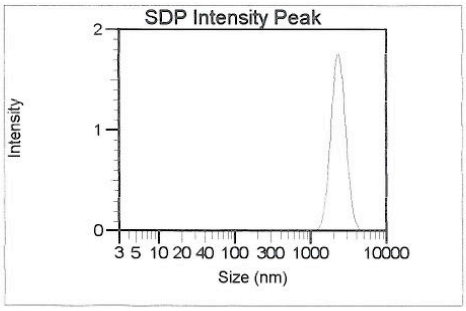
Run: Run Started 1-Apr-08 at 12:06:42

Comment:	
Profile:	Jenna
Angles Selected:	90.0
Run Times:	180
Sample Time:	Automatic
Prescale:	Automatic
Equilibration Time:	1.0 minutes
Repetitions:	Repeat 3 times for each angle
Automatic SDP:	3.0nm-10000.0nm, 31 Bins
Temperature:	20.0°C
Alpha:	1.000e+000
Beta:	1.000e+000
Viscosity:	0.570 centipoise
Refractive Index:	1.443

Intensity Peak

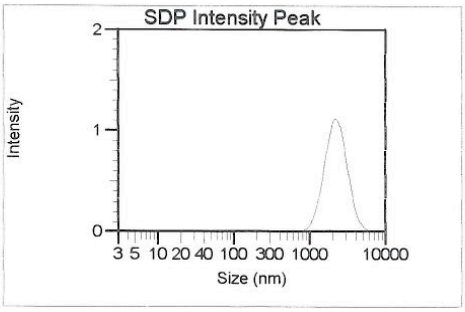
Amount	Size (nm)	SD (nm)
100.0%	2261.7	370.1

efficient variation = ~~16.4%~~ 16%.



Intensity Peak

Amount	Size (nm)	SD (nm)
100.0%	2185.8	584.3



Intensity Peak

Amount	Size (nm)	SD (nm)
99.9%	2582.5	1518.1
0.1%	6.8	0.5