# Development of Molecularly Imprinted Polymers for Corticosteroids

By

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Based on research carried out under the supervision of

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## Declaration

I hereby declare that this thesis is my own work, in fulfilment of the requirements of Doctor of Philosophy degree. It is based on research carried out in the Department of Chemical and Life Sciences, Waterford Institute of Technology, Ireland.

_____
Laurence Fitzhenry

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Date

# INTO MY OWN

One of my wishes is that those dark trees,
So old and firm they scarcely show the breeze,
Were not, as ‘twere, the merest mask of gloom,
But stretched away unto the edge of doom.

I should not be withheld but that some day
Into their vastness I should steal away,
Fearless of ever finding open land,
Or highway where the slow wheel pours the sand.

I do not see why I should e’er turn back,
Or those should not set forth upon my track
To overtake me, who should miss me here
And long to know if still I held them dear.

They would not find me changed from him they knew—
Only more sure of all I thought was true.

*Robert Frost*

## *For My Children*

# *Acknowledgements*

I would like to thank my supervisors, Pat and Peter, for all of their help and support over the last four years. I am grateful to have had the freedom to try the things I wanted to try, and to have had their experience and ability to help me get it right when it went wrong. I feel privileged to have learned from you both.

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# *Abstract*

The aim of this research was to prepare a series of molecularly imprinted polymers (MIPs) for corticosteroid templates using bulk and precipitation polymerisation strategies. Initially, a systematic study was undertaken, investigating the factors affecting the synthesis of MIPs by precipitation polymerisation. Careful optimisation of polymerisation parameters, including the monomer concentration, initiator type and solvent, allowed for a high degree of control over particle size and morphology. Pre-equilibrium interactions between the primary target budesonide (BDN) and functional monomers were investigated using NMR studies and showed weak interactions with dissociation constants, $k_{diss}$, ranging from 0.534-1.47 M

Due to this weak functional monomer-template interaction, polymers were prepared in bulk format using a target analogue approach utilising hydrocortisone-17-butyrate (HCB) as a low-cost alternative to BDN. The HCB imprinted ground bulk polymers demonstrated imprinting factors (*IF*) as high as 7.5 for the template. Selectivity studies on six steroidal analogues showed that recognition was driven by shape and size selectivity as well as hydrophobic interactions, with *IF* values ranging from 1.2-9.5 in both organic and aqueous based systems.

Applications of the imprinted polymers were demonstrated by solid-phase extraction (SPE) and analytical column chromatography. SPE studies utilising HCB imprinted polymers displayed a seven-fold increase in the specific retention of BDN. Precipitated prednisolone imprinted polymers achieved chromatographic separation between the template and hydrocortisone, a close structural analogue. Selectivity factors as high as 1.4 and a resolution value of 0.75 were achieved.

Two custom functional monomer strategies were utilised towards enhancing MIP performance. The synthesis of tailor-made monomers based on derivatised cyclodextrins (CD) achieved a substitution of 2.5 polymerisable vinyl groups per CD. This derivatised CD was used to prepare precipitated polymers (0.3-7.2 μm) in a 40% aqueous acetonitrile porogen. A polymerisable hydrocortisone methacrylate monomer was successfully synthesised for use in semi-covalent imprinting which produced discrete microspherical beads (1.2-5.1 μm) using divinylbenzene as the crosslinker.

## List of abbreviations

MIP: Molecularly imprinted polymer

NIP: Non-imprinted polymer

MAA: Methacrylic acid

HEMA: 2-(hydroxyethyl)methacrylate

EGDMA: Ethylene glycol dimethacrylate

DVB: Divinylbenzene

TRIM: Trimethylolpropane trimethacrylate

AIBN: Azobisisobutyronitrile

ACCN: 1,1'-azobis(cyclohexanecarbonitrile)

CVA: 4,4'-azobis(4-cyanovaleric acid)

HCB: hydrocortisone-17-butyrate

BDN: budesonide

PRD: prednisolone

HYD: hydrocortisone

HAC: hydrocortisone-21-acetate

PAC: prednisolone-21-acetate

AcOD: acetic acid-$d_6$

CD: cyclodextrin

α-CD: α-cyclodextrin

β-CD: β-cyclodextrin

γ-CD: γ-cyclodextrin

MAC: methacryloyl chloride

NMA: *N*-(hydroxymethyl) acrylamide

CDM: cyclodextrin methacrylate

β-CDA: acrylamidomethyl β-cyclodextrin

γ-CDA: acrylamidomethyl γ-cyclodextrin

CDMP: cyclodextrin molecularly imprinted polymer

HMAMP: hydrocortisone methacrylate molecularly imprinted polymer

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# Chapter 1

# Introduction

# 1. Introduction

The field of molecular imprinting in synthetic polymers is one of the most exciting areas in chemistry and material science today. The imprinting of polymers on the molecular level may be described as the formation of sites or cavities within the polymer that are receptive to a specific molecule or group of related molecules. The formation of these cavities is achieved by the synthesis of the polymer in the presence of a specific molecule. The molecule, around which the polymer is synthesised, is known as the template and the subsequent removal of the template, after polymer formation, results in the creation of the selective sites. These sites are then available for rebinding of the template based on shape, size and perhaps most importantly, functionality, all combining to promote a high degree of molecular recognition. The polymer thus formed is known as a molecularly imprinted polymer (MIP).

## *1.1 The History of Molecular Imprinting*

The history and development of the field of molecularly imprinted polymers has been well documented in a number of excellent and comprehensive reviews [1, 2]. With the aid of such reviews and the MIP database [3] it is possible to present a brief account of the most notable stages in this, by now, rapidly growing field.

It is now generally accepted [1, 4] that the first recorded instance of molecular imprinting was carried out in the 1930s by the Soviet chemist M.V. Polyakov who reported interesting adsorption properties while investigating silica gel matrices [5]. In this work benzene, toluene or xylene was added to the silica matrix prior to drying. Adsorption studies were carried out on dried silica, from which the additives had been removed by extensive washing. These studies showed selective adsorption for the additive present during the formation of the gel. Despite the fact that Polyakov published a number of other papers [6, 7] in that decade his work appeared to go largely unnoticed within the wider scientific community [1].

It was not until the work of a graduate student of Linus Pauling, Frank Dickey, in the late forties, detailing the then termed, ‘specific adsorption’ of methyl orange dye in silica gels, that this area of research received more attention [8]. Dickey stated that the

investigation itself was not so much a quantitative one as a qualitative one and he attributed the adsorption to the formation of "pockets that fit closely enough to the foreign molecule to hold it in by Van der Waal's forces, hydrogen bonds, ionic attraction and other types of intermolecular interaction". Dickey published a more detailed paper six years later investigating such topics as the amount of attractive surface, the formation of the cavities and problems surrounding what in contemporary scientific literature is now termed the 'un-extracted template' [9]. Around that time there were a number of other works published correlating the work of Dickey as well as an early use of 'specific adsorption' for the separation of stereoisomers in the work of Curti and Colombo [10-12]. It is worth noting that many of the uses investigated and perhaps more importantly, the problems encountered, are much the same as the uses and problems being investigated today.

An article by Morrison *et al*. published in 1959, saw a challenge to Dickey's suggestions for the cause of selective adsorption, with the likely cause ascribed to association between un-extracted dye and dye present in the rebinding solution [13]. This fact notwithstanding, the 1960's saw a continuation in the research of "stereoselective adsorbents" where the range of applications began to become more diverse, with papers outlining their use in, for example, the 'imprinting' of bacteria to investigate the optical activity of receptors in dissymmetric organisms by Patrikeev *et al.* and early work on their use as drug receptor and enzyme active site models by Beckett and Youssef [14, 15].

It is generally stated, that it was the advent of molecular imprinting in organic polymers by Wulff and Sarhan in the seventies, and later the group of Arshady and Mosbach in the eighties [16, 17] that ushered in, as one commentator has termed it: "the new era of molecular imprinting" [18] and has led to the abundance of literature pertaining to molecular imprinting that is present in contemporary scientific research. Before discussion of this wealth of literature, however, it is necessary to first discuss the underlying principles of molecular imprinting.

## *1.2 Principle of molecular imprinting*

Molecular imprinting has two major scientific areas at its core: molecular recognition and polymer chemistry. While molecular recognition often takes precedence in the design of imprinted polymers, the fact that this recognition must take place in a synthetic polymer can not be overlooked. With this in mind it is considered necessary to give a brief overview of the basics of polymer chemistry.

### 1.2.1 Polymers

Polymer molecules are large molecules with high molecular weights made of simple repeating units [19], known as monomers. The number of monomeric units per polymer is the degree of polymerisation [20]. Polymers prepared from a single monomer type are known as homopolymers, while copolymer is the term used for those prepared with more than one monomer. The incorporation of these different monomers into the polymeric matrix can occur in a number of ways [19]. Alternating copolymers occur when the monomeric units alternate in a linear fashion, random copolymers, as the name suggests, involve randomly incorporated monomers, while block copolymers involve blocks of one monomer connected to blocks of the other monomer. Some examples of monomers and their corresponding polymers can be seen in Figure 1.1 [21].

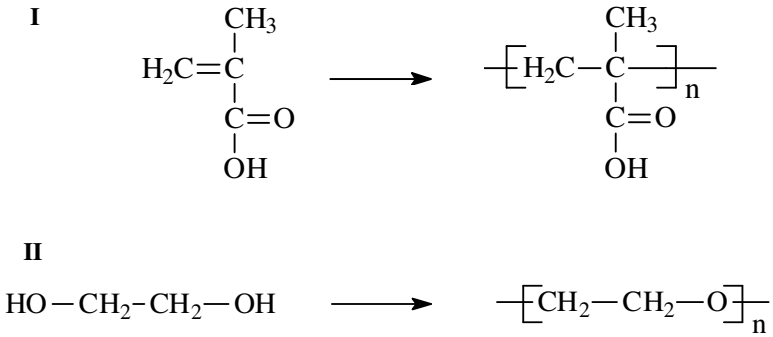



**Figure 1.1: I Methacrylic acid and its corresponding polymer, polymethacrylic acid; II ethylene glycol and its corresponding polymer, polyethylene glycol [21].**

Polymers possess characteristic chain structures and the monomers of which they are composed are related chemically *via* a primary bond, usually covalent in nature [21]. When these chemically bonded monomers form polymers possessing no side chains the

result is termed a *linear polymer*. Where side chains are present a *branched polymer* is formed while *network polymers* possess a three-dimensional structure arising from the linking together of polymer chains in a process known as *crosslinking* [19, 21]. The crosslinking of polymer networks can effect major changes in the physical properties of polymers. Crosslinked polymers, for example, despite swelling considerably in appropriate solvents, are not soluble in any solvents [22].

Whether polymers are prepared as linear, branched or networked polymers, two general mechanisms are responsible: Step growth (condensation) and chain growth (addition) polymerisations [23]. These were first classified by Carothers in 1929, using the terms condensation and addition, and this theory was expanded by Flory in the 1930s [24, 25].

#### *1.2.1.1 Step Growth Polymerisations (Condensation).*

Step growth polymers are generally formed by a reaction between two different functional groups with the loss of some small molecule, such as water, in the course of the formation of the new bond. Examples of polymers formed by condensation reactions are nylon and polyester. Different types of condensation reactions include:

- Activated polycondensations
- Polyadditions
- Branching polyadditions

#### *1.2.1.2 Chain growth polymerisations (addition).*

Chain growth polymerisations are usually formed by the addition of one monomer unit to another in a repetitive manner. Here the addition of the monomer molecule will result in transference of the reactive site to the new chain end [23]. Addition polymerisation is a straight or linear chain process, which continues until the process is terminated in some way. The typical steps in the reaction are as follows:

- Initiation
- Propagation
- Termination

These polymerisation reactions can be initiated by a variety of methods and the resultant polymerisation reaction types are named in accordance with the mechanism of initiation. These include [19, 20]:

- Anionic polymerisations: In anionic polymerisation the propagating chain is a carbanion.
- Cationic polymerisations: The propagating chain is a carbocation in cationic polymerisations.
- Free radical polymerisations: Free radical polymerisation involves the addition of monomers to a free-radical bearing reactive site.

As the majority of molecular imprinting reactions are free radical initiated polymeric reactions, this topic will be discussed in more detail in Section 1.4.4.

### 1.2.2. Molecular recognition

Molecular recognition may be described as a set of phenomena under the control of specific non-covalent interactions [26]. These interactions, and the subsequent molecular level 'recognition' events, are often necessary in order to perform or induce specific functions or effects. Nobel laureate, J.M. Lehn states that binding alone is not recognition but that recognition can be considered to be "binding with a purpose" [27].

#### *1.2.2.1 Molecular recognition in biological systems*

These molecular recognition processes are of paramount importance in nature and thus, life itself. This can be seen in DNA where molecular recognition is responsible not only for the structure of DNA molecules but also in ligand-DNA or protein-DNA interactions [28]. The formation of base pairs occurs by means of specific hydrogen bonds, for example the adenine:thymine base pair has two hydrogen bonds while the guanine:cytosine base pair has three. This is represented in Figure 1.2.

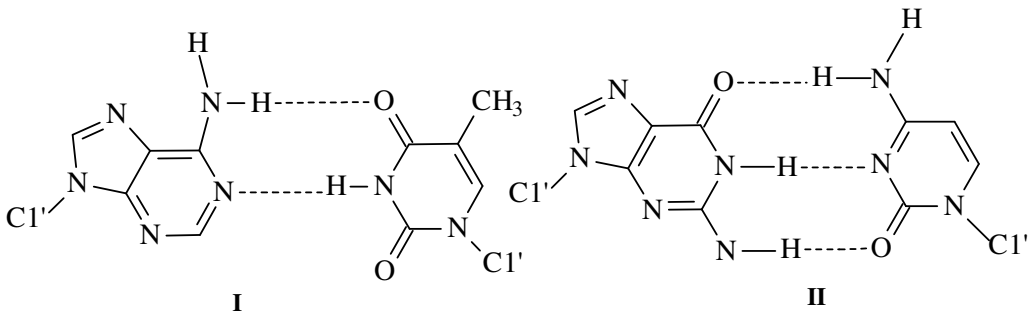



**Figure 1.2: I Adenine:thymine base pair and II guanine:cytosine base pairs showing hydrogen bonding**

In double-helical DNA it is the occurrence of these hydrogen bonds between bases on separate strands that hold the double helix together. For ligand-DNA recognition, such as DNA-drug recognition, the incoming ligand must have a hydrogen bonding (H-bonding) capability complementary to the H-bonding potential of the base pairs, which remains after base pairing itself takes place. There are a number of categories of DNA-binding proteins, a particular example being regulatory proteins that generally bind to highly specific regions of DNA and control the processing of a gene [28].

The specificity of enzymes is well documented and the stereochemical specificity of the active site is effected by the presence of at least three different points of interaction between enzyme and substrate [29]. Binding sites interact with specific groups on the substrate which determines the specific orientation, in which the substrate and enzyme are held with respect to each other. The binding site is three-dimensional and bonds formed are generally non-covalent. In 1894 Emil Fischer suggested that enzyme specificity was a result of sites bearing functionality complementary to that of the substrate and the substrate would fit into the active site in a manner analogous to a key and a lock [30]. While this lock-and-key model was later determined to be insufficient to account for the flexibility of proteins it is often cited as the inspiration for synthetic replication of biological specificity.

Antibodies too, are proteins that exhibit a high degree of specificity. In this case, the proteins are antigen-specific immunoglobins (antibodies) that are part of the immune system's response to attack by an antigen [31]. The aim of this response is the specific elimination of the antigen utilising the antigen-specific antibodies. These antibodies

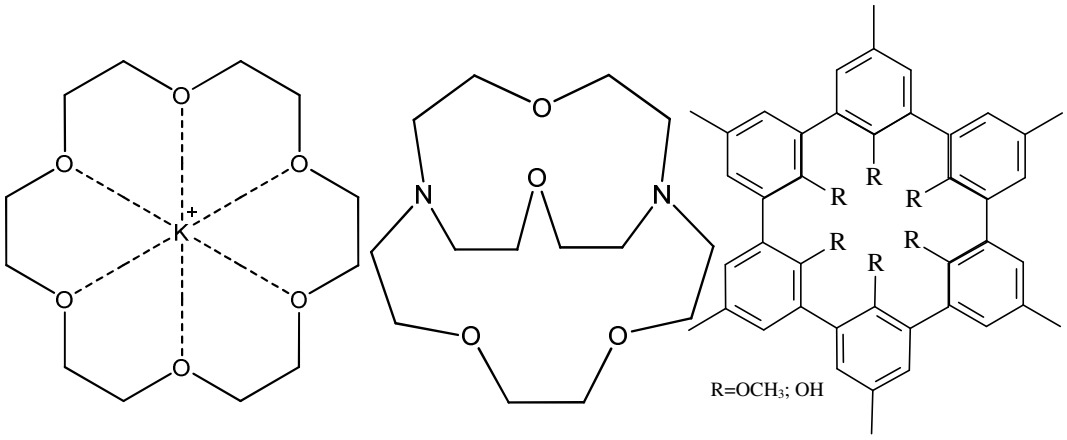
have affinity and binding complementarities for the antigen. The response is polyclonal, where a mixture of antibodies for the antigen is made and upon specific binding to the antigen, it is destroyed. Again, these interactions are based mainly on non-covalent interactions in three-dimensional domains where non-polar interactions can also play a role [31].

*1.2.2.2 Molecular recognition within synthetic materials*

Given the high degree of specificity involved in the molecular recognition that takes place in biological systems, coupled with the myriad functions of this recognition, it is little wonder that the incorporation of these properties into synthetic materials has been the focus of much scientific and technological research. This research has developed into the wide-ranging areas of supramolecular and host-guest chemistry.

Early work was carried out in this field by the groups of Nobel Laureates: Pedersen, Lehn and Cram. The crown ether was first observed 'by chance' by Pedersen in 1967 as a side product due to an impurity in one of the starting materials [32-34]. Once the metal-ion binding properties were observed a wide variety of compounds were developed, crown ethers being followed by cryptands, initially developed by Lehn. These have chemical bridges and also involved N atoms, giving a three-dimensional cavity [35] and spherands, developed by Cram where the donor centres point to the interior of a rigid ring [36].

These synthetic molecules have found many uses in synthetic chemistry where ions or ionic intermediates are involved. The synthesis can often be modified or improved [33] and they may also be used in chemical analysis or selective sensing. Examples of these are shown in Figure 1.3.




**I** **II** **III**

**Figure 1.3: I Crown ether of the type developed by Pederson with $K^+$ ion complexed within the molecule; II cryptand of the type developed by Lehn; III spherand of the type developed by Cram.**

Binding between target molecules and compounds of the type seen in Figure 1.3, as with those discussed in biological systems, is typically based on "simultaneous non-covalent interactions between single binding sites" [37]. The presence of a number of binding sites is necessitated by the inherently weak nature of non-covalent interactions and recognition is achieved by concurrent interaction between the individual binding sites. Lehn [27] states a number of factors necessary to induce the large difference in binding energies between those of a given substrate and other substrates that are required to achieve the desired level of recognition and they include the following:

- Steric complementarity between substrate and site
- Interactional complementarity within three-dimensional sites
- Large contact areas
- Multiple interaction sites
- Strong overall binding
- Solvent effects

With these considerations firmly in mind, plus the advantages of readily synthesised artificial receptors, the appeal and potential for utilising polymeric material in which these receptor sites could be created and maintained is readily seen.

### 1.2.3 Molecular imprinting

The process of molecular imprinting is often represented in a schematic fashion similar to that shown in Figure 1.4.

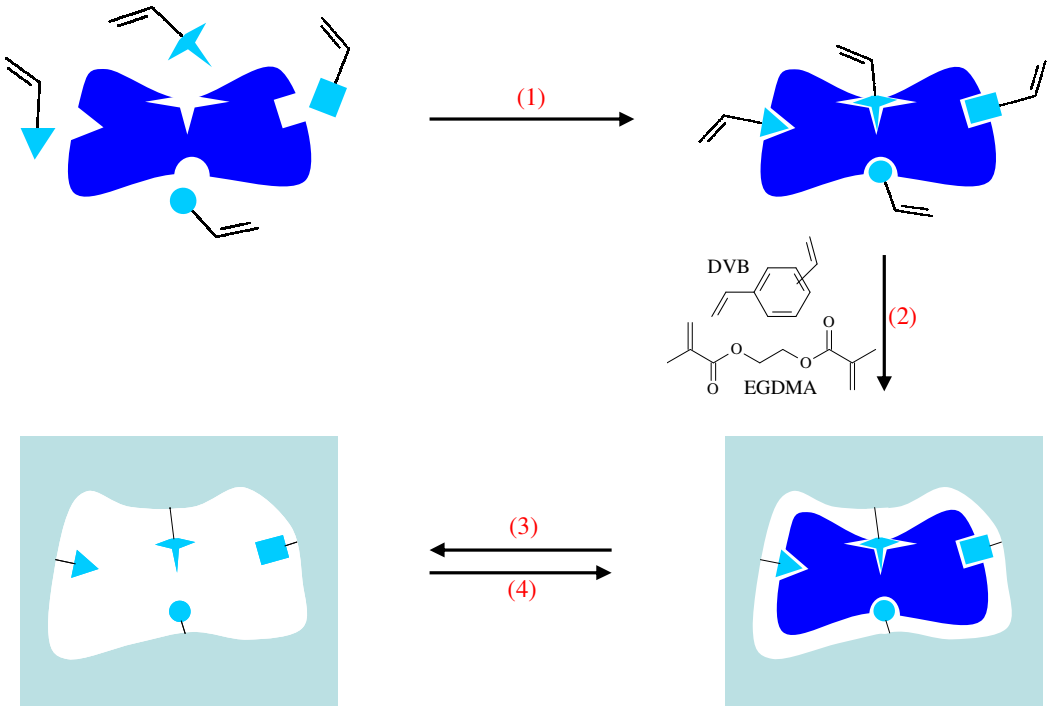



**Figure 1.4: Schematic representation of the imprinting process involving the following steps: (1) interaction of the template (blue) and functional monomers to form a template-monomer complex; (2) Reaction of template-monomer complex and crosslinking monomer to form a crosslinked polymer containing a template specific binding site; (3) Removal of the template; (4) Rebinding of the template.**

It must be stressed that Figure 1.4 is a highly schematic representation of the molecular imprinting process and does not adequately represent the three-dimensional nature of the site, the cavity nor the polymeric matrix. Although highly schematic, Figure 1.4 readily allows for a description of the fundamental steps that are considered necessary for molecular imprinting, as outlined in the following sections.

#### *1.2.3.1 Formation of the template-monomer complex*

The template molecule and the functional monomer are added to a solvent (or porogen) in conditions and at ratios that are designed to promote interaction between the two species. This interaction leads to the formation of the template-monomer complex and

may proceed via a number of processes, e.g. covalent or non-covalent, that will be discussed in more detail in Section 1.3.

*1.2.3.2 Polymerisation*

Polymerisation is carried out in the porogen, selection of which is carried out to facilitate the template-functional monomer interaction. This polymerisation of the functional monomers involved in the template-monomer complex is now carried out in order to 'lock' the complex within a porous, polymeric material. This is achieved by the addition of crosslinking monomers, which contain at least two polymerisable moieties. An organic initiator is typically used, which causes the polymerisation reaction to proceed. Upon completion, the result should be a rigid, porous polymer, containing sites in which the template-monomer complex is locked within the matrix of the polymer. The rigidity of the polymer is achieved using a high degree of crosslinking monomer, typically between 10 and 80 mol% [38]. Appropriate selection of the crosslinking monomer and the porogen imparts adequate porosity to the polymer, thus allowing the analyte access to and from the cavities [39].

*1.2.3.3 Extraction of the template*

This involves breaking the bonds between the functional moieties of the polymer and the template with subsequent removal of the template from the polymeric material. How this is achieved is dependent on the type of bond formed in the template-monomer complex. For example, if covalent bonds were formed then a step such as hydrolysis would be necessary, whereas if non-covalent bonds were formed then disruption of the bonds could be brought about by washing in an acidified or basified solvent. Whatever the method, the desired result is the creation of a site within the polymeric matrix that is complementary in shape, size and functionality to the template molecule. This is an important step in the preparation of molecularly imprinted polymers, as un-extracted template can result in a reduction of the number of binding sites available for rebinding or errors in analytical measurements due to template bleed [40].

*1.2.3.4 Rebinding of the template*

At this stage, it should be possible to rebind the template with the polymer using the empty imprinted sites or cavities. This is typically achieved with the greatest success under conditions similar to those used for the formation of the polymer, i.e. solvent, etc. [41, 42].

The overall success of molecularly imprinted polymers is, to varying degrees, dependent on how each of the four steps listed above is designed and implemented and a greater understanding of the design of molecularly imprinted polymers, requires a deeper understanding of the factors involved and the extent to which they affect this process. The following sections will attempt to outline some of these factors and discuss how they may be best adapted for the myriad tasks to which MIPs may be applied.

## *1.3 Methodologies for the creation of imprinted sites*

The interaction between the functional monomer and the template is responsible for the preparation of the template-monomer complex and thus is ultimately responsible for the complementary functionality of the binding site in the prepared polymer. This interaction is therefore of paramount importance to the performance of the resultant polymer and proper selection of the type of interaction must be tailored to suit not only the template but also the final application of the polymer. While there is an ever growing number of processes by which the functional or binding site monomer may interact with the template molecule, these can be separated into two main strategies: covalent and non-covalent. These strategies and a number of their variants will be discussed in the following sections.

### 1.3.1 Covalent imprinting

The covalent imprinting strategy involves a reversible covalent linkage between the template and the functional monomer. The template-monomer complex is then incorporated into the polymer matrix and requires chemical treatment, such as hydrolysis, to break the chemical bond between the template and the functional moieties

of the binding site monomers. Upon rebinding, the chemical bond is then re-formed between the monomers and the template.

As mentioned in Section 1.1 the first such imprinted organic polymers were prepared by Wulff and Sarhan [16] and were utilised in the resolution of D,L-glyceric acid. The template-monomer complex in this instance was a synthesised derivative of glyceric acid, a 2,3-*O*-*p*-vinylphenylboronic ester of *p*-vinylanilide. This method was also used for D-mannitol [43]. The polymers were synthesised using divinylbenzene (DVB) as the crosslinking monomer. The template, template-monomer complex and diagrammatic representation of the binding pocket for glyceric acid are shown in Figure 1.5.

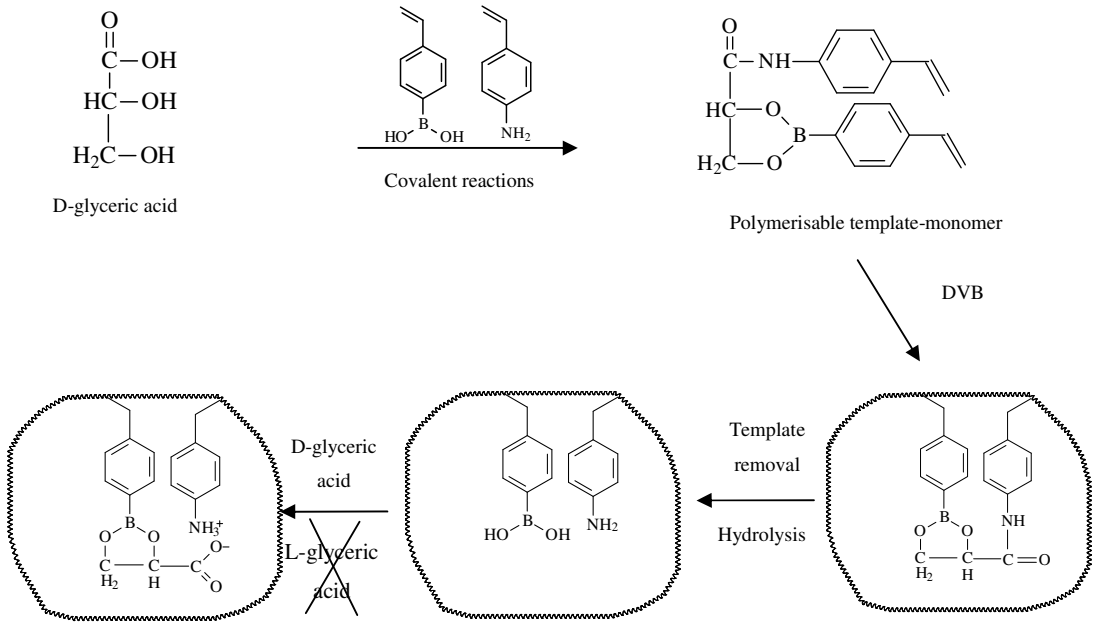



**Figure 1.5: Schematic representation of the imprinting protocol for D-glyceric acid developed by Wulff and Sarhan whereby a covalently bonded template-monomer complex was used to effect selectivity for D- over L-glyceric acid. (Adapted from references [16] and [43])**

Figure 1.5 demonstrates how the template-monomer complex is incorporated into the resultant polymer and how upon hydrolysis, the template is removed and the functional groups are maintained in the desired spatial arrangement for rebinding of D-glyceric acid. As such, the binding moieties would not be as suitably aligned for L-glyceric acid recognition. It was possible to remove 50% of the template and the polymer had the capacity to rebind this amount. In subsequent racemate resolution the polymer

preferentially bound D-glyceric acid allowing for enrichment of the L-glyceric form in solution. The α-value, the ratio of the distribution between the solution and the two forms of glyceric acid, was determined to be 1.034. Similar values were also obtained with this polymer for structural analogues of glyceric acid.

This boronate ester approach has often been utilised for the imprinting of carbohydrates, with recent use for the preparation of electrochemical sensors for monosaccharides by Willner *et al.* [44]. Here, 3-hydroxyphenylboronic acid was used to form a template-monomer complex incorporated into an electropolymerised polyphenol matrix. Ferrocene functionalised derivatives of D-mannose and D-glucose were prepared in order to calculate association constants, $K_{ass.}$, and values of 870 ± 40 $M^{-1}$ and 170 ± 30 $M^{-1}$ were found for D-mannose and D-glucose, respectively and enantioselectivity was also observed in these polymers.

More recently, Schumacher *et al*. have used a polymerisable benzoboroxole to improve the complexation of fructose at neutral pH [45]. Here two template-monomers were synthesised for fructose using both 5-methacrylamido-2-hydroxymethylphenylboronic acid and 3-vinylphenylboronic acid. At a basic pH of 11.4, where the high concentration of hydroxide ions would promote the complexation of fructose, the binding capacities of the polymers prepared from both boronic acids were comparable, attaining values of 135.6 nmol $g^{-1}$ for the benzoboroxole and 114.7 nmol $g^{-1}$ for the boronic acid. However, at pH 7.4, where the structure of the boron monomer would be expected to play a more significant role, the polymer prepared from the benzoboroxole had a three-fold increase in binding capacity over the polymers containing pendant vinylphenylboronic acid moieties, with values of 60 nmol $g^{-1}$ and 18.2 nmol $g^{-1}$ for the former and the latter, respectively.

Schiff's bases have also been used for the imprinting of amino acids by Wulff and Vietmeier [46] while ketal and acetal formation between diols and carbonyl compounds have been employed for imprinting of aromatic ketones by Shea and Dougherty [47] during investigations into the influence of functional group positioning on molecular recognition. Covalent imprinting has been applied to a number of different templates, such as bisphenol A, widely used in the production of epoxy resins [48], the tricyclic

antidepressant, nortryptiline [49] and steroids such as cholesterol [50] and castasterone [51].

#### *1.3.1.1 Advantages and disadvantages of covalent imprinting*

Despite the ability to prepare binding sites using the exact stoichiometric ratio of functional monomer to template, thereby reducing non-specific binding, as well as the increased stability of the template-monomer complex during polymerisation, a factor that lends itself to increased homogeneity of the binding sites, a number of problems arise in the case of covalent molecular imprinting. These problems include the relatively low number of templates and corresponding functional monomers that can be used to synthesise appropriate template-monomer complexes. Template removal has also been cited as a problem [2] as well as the slow kinetics of template release or rebinding, which would make this approach less desirable for applications requiring fast binding kinetics, such as chromatography. The impact of some of these negative factors can often be overcome, or at the very least decreased, by the use of non-covalent or semi-covalent binding strategies.

### 1.3.2 Non-covalent imprinting

The non-covalent imprinting strategy involves the self-assembly of functional monomers about the template molecule by way of non-covalent interactions. This self-assembly may be driven by forces similar to those in nature such as hydrogen bonds, hydrophobic interaction, the formation of ion-pairs, dipole-dipole forces or π-π interactions. While the template-monomer complexes, or adducts, formed by non-covalent interactions are not as inherently stable as those formed by covalent linkages, the range of possible interactions (and the monomers that can utilise them) allows for greater choice of template. This factor, plus the reduced need for synthetic chemistry when commercially available functional monomers are employed, has led to non-covalent imprinting becoming the most widely used strategy in molecular imprinting today [1, 2, 52].

The non-covalent strategy was pioneered by Mosbach and Arshady who prepared the then termed ‘substrate-selective polymers’ for two organic dyes, rhodanile blue and

safranine O [17]. These dyes and a number of the monomers used to provide complementary functionality are shown in Figure 1.6.

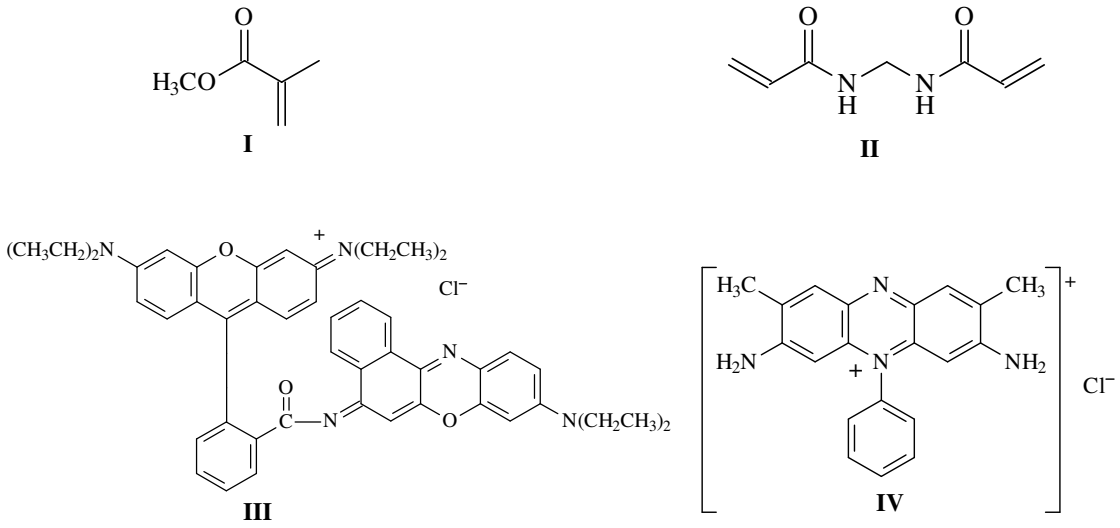



**Figure 1.6: Templates and monomers used by Mosbach and Arshady during initial non-covalent imprinting studies: I methyl methacrylate; II *N,N'*-methylenediacrylamide; III rhodanile blue; IV safranine O.**

The polymers prepared from the templates and monomers shown in Figure 1.6, were prepared in $H_2O$:DMF mixtures at 40 °C using ammonium persulfate as the initiator. The rebinding and selectivity capabilities of the resultant polymers were investigated by comparison to polymers prepared without the presence of the templates, which is now commonly referred to as a non-imprinted polymer or 'NIP'. The binding capacity was measured to be as high as 72 µmol/g of polymer with specific binding as high as 100%.

Since the advent of this early work in the 1980s, involving the generation of such promising results, the range of templates, the number of functional monomers and the applications for non-covalent molecular imprinting has grown exponentially. Some template examples include the widely used pharmaceutical actives propranolol, morphine and benzodiazepines [53, 54], proteins such as myoglobin [55], herbicides and fungicides [56, 57], chemical warfare agents [58], amino acids [59, 60] and steroids [42, 61-64], as demonstrated by the work of Ramstrom *et al.* for which the imprinting strategy is depicted in Figure 1.7.

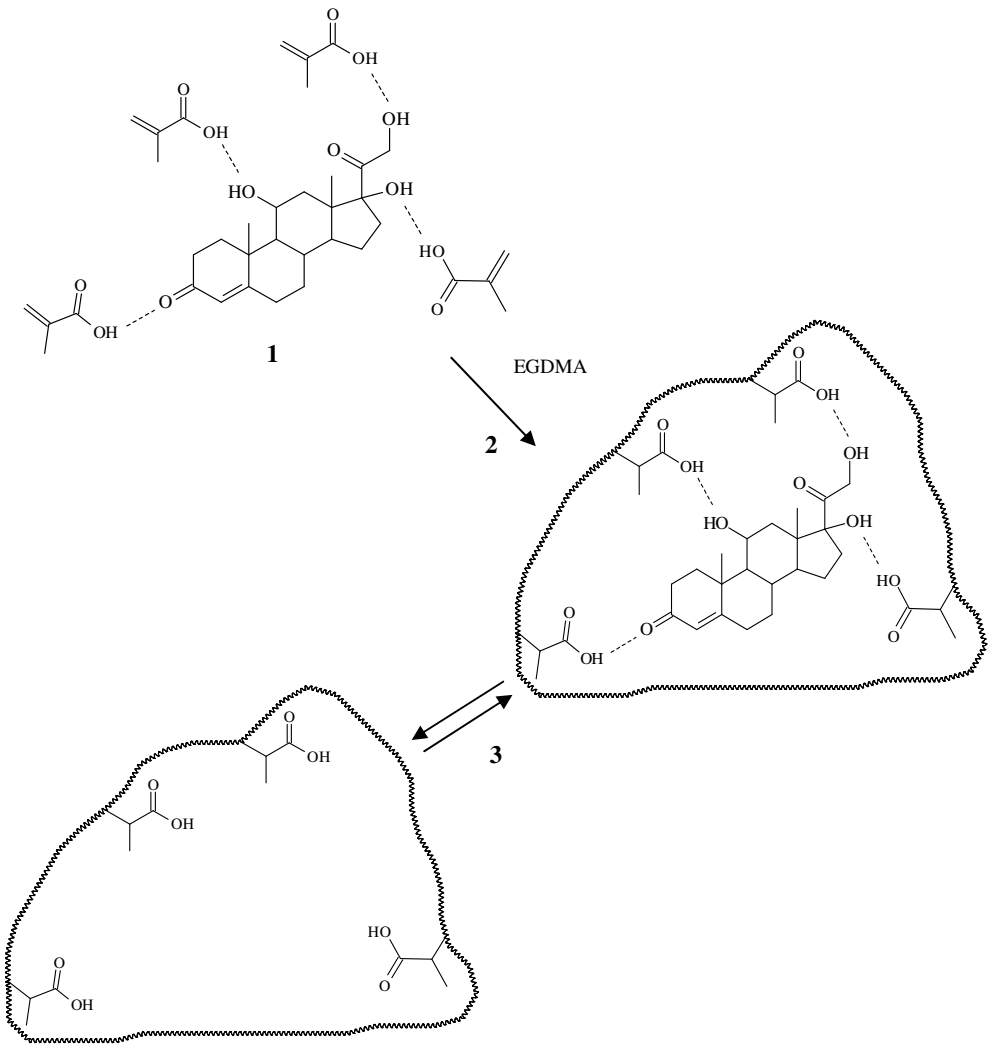



**Figure 1.7: Schematic for the non-covalent imprinting of hydrocortisone using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as the crosslinker. Adapted from reference [63].**

Figure 1.7 illustrates the non-covalent approach used by Ramström *et al.* for the preparation of polymers imprinted with hydrocortisone. At **1**, the functional monomers are shown assembling about the template at the proposed sites of interaction, based on their hydrogen bonding capabilities. Once this complex has been given sufficient time to form, the crosslinking monomer and initiator were added and the polymer was formed, as in **2**. The template was extracted and upon rebinding, the non-covalent interactions employed to form the initial template-monomer complex should then act to rebind the template within the template specific cavities, **3**.

In this study the NIP bound only 10-16% of the quantity of template that was successfully bound to the MIP. The cross-reactivity or selectivity of the polymers for the template over close structural analogues was shown to be comparable to monoclonal antibodies used for corticosteroids with values as low as 4% in the MIP, where the corresponding antibody value was 0.1%. There were, however, a number of problems cited, such as the heterogeneity of the binding sites. Two types of binding sites were observed: high affinity sites with a dissociation constant, $K_{diss.}$, of $0.57 \pm 0.16 \times 10^{-6}$ M and a binding site density of $0.21 \pm 0.05$ μmol $g^{-1}$ and low affinity binding sites with $K_{diss.}$ of $1.59 \pm 0.73 \times 10^{-3}$ M and a binding site density of $280 \pm 120$ μmol $g^{-1}$. This demonstrates that the quantity of low affinity sites was considerably larger than high affinity sites. This heterogeneity of the binding sites is one of a number of problems encountered within non-covalent imprinting.

Despite these problems, the ability to non-covalently interact with the template in a biomimetic fashion is an attractive attribute of this method. An example of this approach was carried out by Jiang *et al.* on the separation of racemic ketoprofen [65]. Here a tailor-made, chiral monomer was prepared from cinchonine and used as both functional and crosslinking monomer. The monomer was designed to have a proposed three-point interaction with the chiral target, (*S*)-ketoprofen, employing H-bonding, ionic, and $\pi$-$\pi$ interactions. The use of a number of binding interaction types such as these, demonstrates the biomimetic nature of the molecularly imprinting approach. When the resultant polymer was used as packing material for a HPLC column, (*R*)- and (*S*)-ketoprofen were separated with a resolution of 1.25.

*1.3.2.1 Advantages and disadvantages of non-covalent imprinting*

The advantages of the non-covalent strategies are many and include the myriad templates that may be imprinted, the vast array of functional monomers that may be employed and the fast rebinding kinetics possible. The potential to use a wide range of solvents and a number of initiation methods as well as the low risk of damage to the template during the polymerisation have also been cited as advantages of the non-covalent method [66]. Despite these obvious advantages, there are also a number of disadvantages inherent to this strategy, including, as stated in the preceding section, a lack of homogeneity of the binding sites. This is due in part because of the need to use

an excess of functional monomer to drive template-monomer complex formation as the interactions between monomer and template are generally weak [67]. This leads to a relatively large number of complexes that may be formed between the template and the functional monomers and can result in a relatively low yield of high-affinity receptor sites in comparison to low-affinity sites [2]. For these reasons, a number of strategies have been developed in order to combine the most desirable properties of both covalent and non-covalent methodologies.

### 1.3.3 Semi-covalent imprinting

Semi-covalent molecular imprinting attempts to couple the homogenous binding sites created by covalent imprinting with the fast rebinding kinetics achieved using non-covalent methods by utilising covalent linkages to create the binding site but employing non-covalent interactions to effect rebinding. Interestingly, the first covalent imprinting protocol, discussed earlier, involved additional rebinding capability due to electrostatic interaction (Figure 1.5) [16].

The first truly semi-covalent approach has been cited [1] as that used by Andersson and Sellergren, for the imprinting of *p*-aminophenylalanine ethyl ester [68]. In this instance, a structural analogue that had two covalently attached polymerisable groups containing ester linkages was used. Upon completion of polymerisation and removal of the imprint molecule two carboxylic acid groups remained in the binding sites, each having the capacity to rebind the amino acid via non-covalent interactions. This method is exemplified by Cacho *et al*. during the preparation of an MIP prepared for the herbicide, propazine, by semi-covalent methods [69]. An outline of the procedure used is given in Figure 1.8.

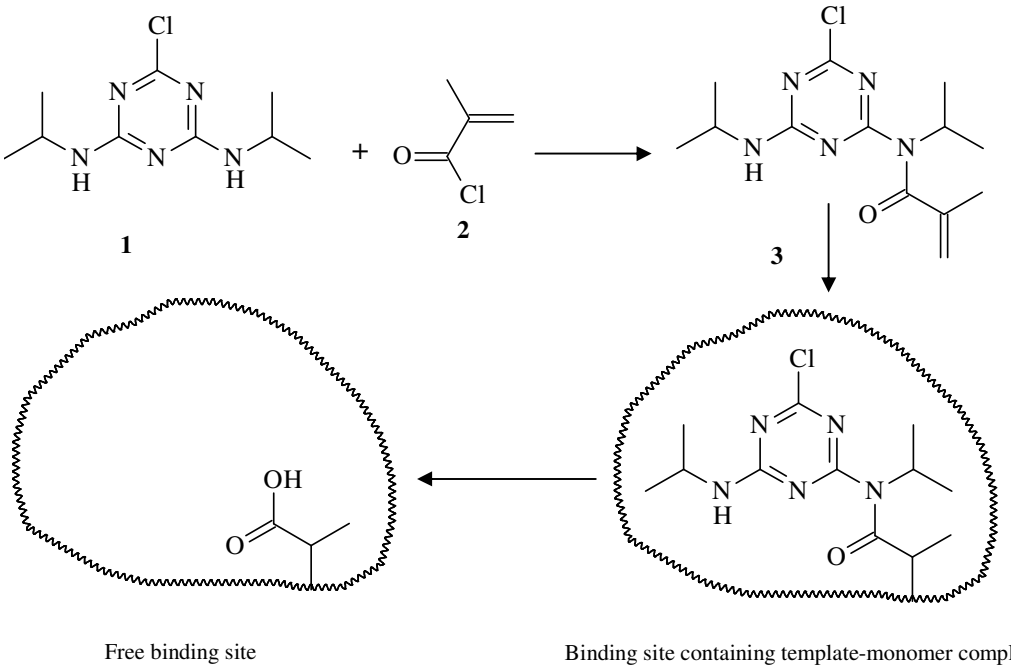



**Figure 1.8: Schematic for the procedure used by Cacho *et al.* for the semi-covalent imprinting of propazine. 1: propazine; 2: Methacryloyl chloride; 3: propazine methacrylate.**

Figure 1.8 depicts the procedure used for the semi-covalent imprinting of propazine. The template (**1**) was reacted with methacryloyl chloride (**2**) in dichloromethane, to prepare the vinyl amide template-monomer (**3**). After polymerisation, basic hydrolysis was used to remove the template, leaving the binding site free to non-covalently rebind propazine.

The method shown in Figure 1.8 was compared to earlier work carried out by Cacho *et al.* where propazine was imprinted using a non-covalent strategy [70]. It is interesting to note that the semi-covalent method produced a polymer containing 52% more binding sites (though this was still considerably lower than that expected by such methods) and binding isotherms revealed a heterogeneity index almost 20% higher than that seen in the non-covalent approach. However, the binding constant obtained for the semi-covalent polymer using isotherm data, at 151.7 $mM^{-1}$, was considerably lower than that observed for the non-covalent method, which had a value of 1484 $mM^{-1}$. This was attributed to the high affinity binding sites prepared in non-covalent polymers due to the greater molar ratio of monomer to template compared to the 1:1 stoichiometry used for this method.

In 2009, Curcio *et al*. applied the semi-covalent method in conjunction with one-stage mini-emulsion polymerisation to prepare molecularly imprinted polymers for glucopyranoside [71]. This method employed a polymerisable surfactant template to enhance the surface imprinting effect and improve binding kinetics. This was achieved using a one-step mini-emulsion polymerisation and resulted in a polymer that was designed to have four carboxylic acid residues per binding site after hydrolytic removal of the template from the polymer, as illustrated in Figure 1.9.

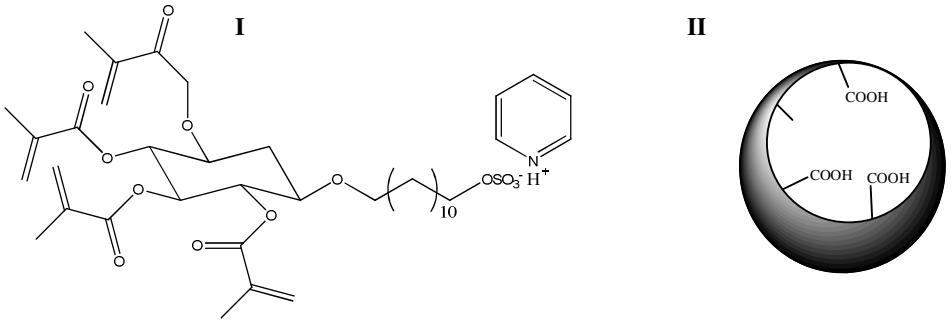



**Figure 1.9: I The polymerisable surfactant template monomer used for the preparation of II, the surface imprinted polymer bearing a proposed four carboxylic acid residues per binding site [72].**

As well as herbicides and amino acids, the semi-covalent approach has also been used for the imprinting of resin production intermediates such as bisphenol-A [73], testosterone [74] and 4-nitrophenol [75]. Interestingly, for bisphenol-A and 4-nitrophenol, comparisons were made to non-covalent polymers prepared in tandem and these outperformed the semi-covalent polymers in terms of selectivity. A variation of this method has been developed, which also uses covalently bound template-monomer complexes and rebinds the target *via* non-covalent means, and is known as the sacrificial spacer method.

*1.3.3.1 The sacrificial spacer method*

The sacrificial spacer method was introduced by Whitcombe *et al.* as a method to effectively imprint cholesterol, a molecule considered to have poor functionality for imprinting by non-covalent means [76]. Here the template used was a 4-vinylphenyl carbonate ester of cholesterol and upon removal of the template the 'sacrificial spacer', $CO_2$ was lost and a recognition site bearing a phenolic residue remained, as shown in Figure 1.10.

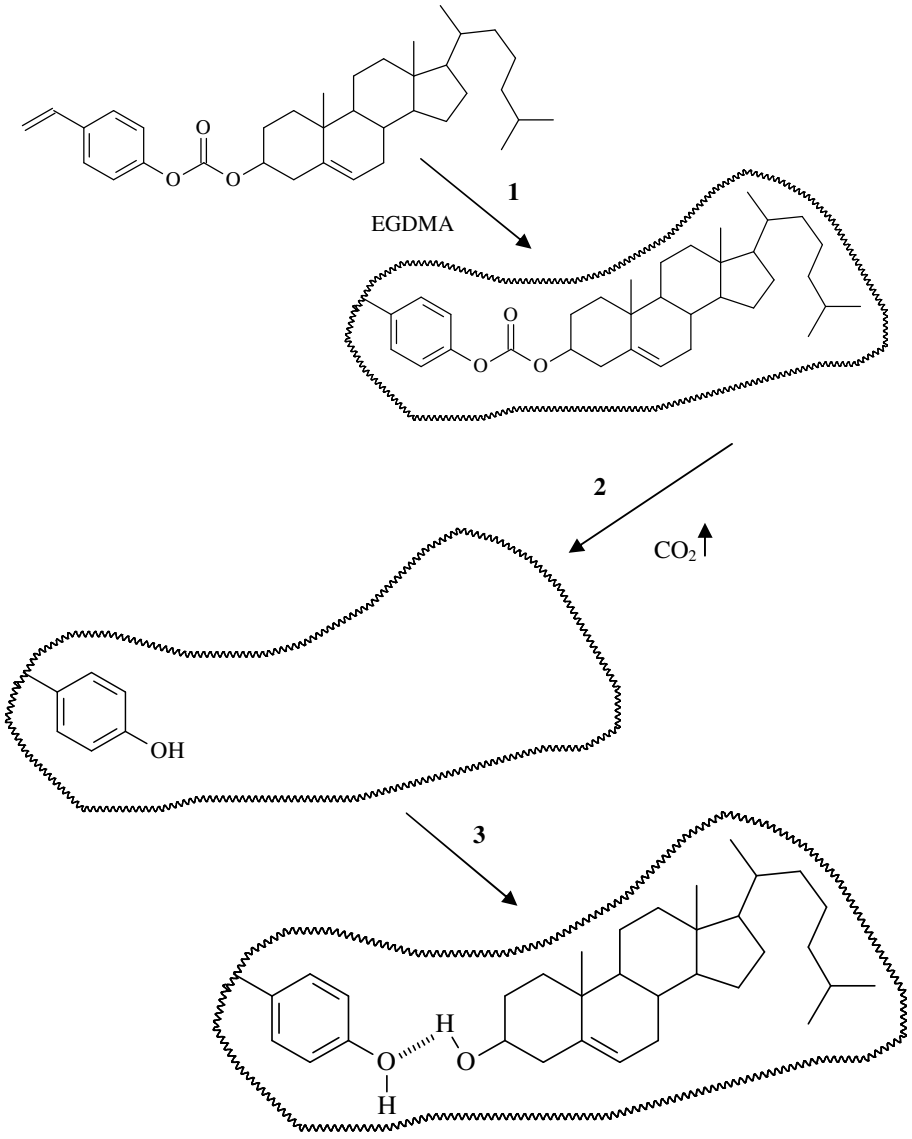



**Figure 1.10: Sacrificial spacer method of imprinting for cholesterol. 1: Polymerisation of template monomer, cholesteryl (4-vinyl) phenyl carbonate to form a polymer containing the template; 2: Removal of template leaving phenolic residue in the binding site; 3: Rebinding of template via non-covalent interaction. Adapted from [76].**

After polymerisation of the template-monomer with EGDMA, a polymer is formed containing the template-monomer within the binding sites. Upon removal of the 'sacrificial' $CO_2$ group by hydrolysis, the site is free to non-covalently bind the target. The loss of this spacer reduces problems caused by steric crowding during the non-covalent rebinding step. The binding affinities of these polymers were studied using Scatchard analysis, a method for quantifying the binding interaction between ligand and

receptor, and revealed affinities as high as $K_{diss}$ = 0.59 ± 0.12 mM at a capacity of 114 ± 6 µmol/g [76].

Cholesterol has also been the target for chitin-based molecularly imprinted polymers prepared using the semi-covalent method [77]. Here, Tong *et al*. prepared cholesteryl chitin carbonate, a novel chitin derivative, and cross-linked the polymer using toluene-2,4-diisocyanate. This resulted in a polymer, which was able to bind up to 30% more cholesterol than either the non-imprinted polymer or an imprinted polymer prepared *via* non-covalent means.

Other templates imprinted in this manner have included *N*-heterocycles such as pyridine, where the size selectivity of the imprinted sites was greater than for the conventional non-covalent approach [78], testosterone [79], methyl paraben [80] and paracetamol [81].

### 1.3.4 Stoichiometric non-covalent imprinting

Stoichiometric non-covalent imprinting involves the use of monomers that have such high affinity for the template species that the vast majority of the functional monomer molecules interact with the template prior to, and during polymerisation [2, 67]. This situation therefore negates the use of excess functional monomer to drive association, thereby reducing the occurrence of low affinity binding sites and increasing in strength and number, the high affinity sites. To achieve this, the association constants, $K_{ass}$, between the functional monomer and the template must be in the order of $10^3$ $M^{-1}$. To date, this has been achieved using specially designed functional monomers, a strategy pioneered by Wulff *et al.*, which utilises such monomers as shown in Figure 1.11

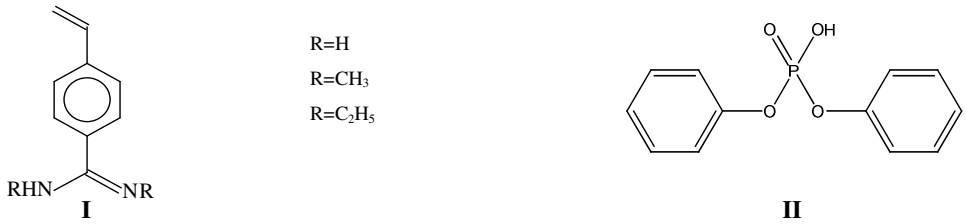



**Figure 1.11: I Amidinium monomers synthesised for use in stoichiometric non-covalent imprinting and II typical template for which these monomers have been used [82, 83].**

Figure 1.11 above presents an example of the type of monomers used in stoichiometric non-covalent imprinting. This type of monomer was first used to imprint a phosphonic monoester transition state analogue [82] but has since been used to prepare molecularly imprinted artificial models of cholesterol esterase [84] and carboxypeptidase A [83]. In the case of the latter example catalytic enhancement values, $k_{cat}/k_{uncat}$, as high as 410 000 were reported. Pasetto *et al*. have also utilised the stoichiometric non-covalent approach for the preparation of molecularly imprinted nanogels for catalysis of a carbonate hydrolysis reaction [85]. Here, apparent affinity constants between the phosphate template and the polymerisable arginine monomer were cited to range as high as $10^4$ to $10^5$ $M^{-1}$.

As can be deduced from the preceding examples, the stoichiometric non-covalent strategy for molecular imprinting is particularly well suited to catalytic applications, though it has also been used for polymers designed for use in the solid-phase extraction of the alkaloid huperzine A [86]. The advantages of this method have been ascribed to the combined attributes of non-covalent and covalent imprinting, i.e. the high splitting yields, fast rebinding kinetics and precise location of the functional groups.

## *1.4 Optimising the performance of molecularly imprinted polymers*

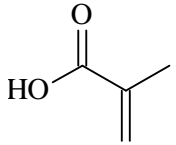
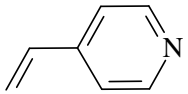
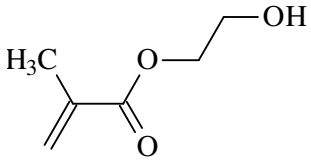
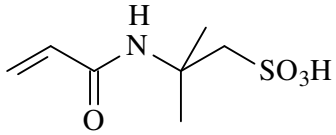
One of the most attractive features of molecular imprinting, and non-covalent molecular imprinting in particular, is the potential to adapt this technology to a wide range of target analytes. Such templates may vary greatly from each other in terms of polarity, functionality, solubility, availability, toxicity and potency, etc. As well as the wide range of templates, the applications and requirements for the resultant polymer may also vary greatly, with respect to factors such as the environment in which it will be used and the desired lifetime of the polymer. With such a degree of variation in both templates and polymerisation strategies, it is obvious that to achieve the greatest degree of success, it is necessary to optimise the composition of the polymers as well as their method of synthesis for each application.

### 1.4.1 The functional monomer

This brief introduction to the methodologies used for site creation within MIPs shows that the functional monomer is largely responsible for the formation of template specific binding sites within the polymer matrix, as well as subsequent rebinding of the template within these cavities. In both instances this is due to the presence of functional moieties on the monomer, which are complementary to the functionality of the target analyte in conjunction with the subsequent promotion of interaction between these functionalities. For this reason careful consideration of the functional monomer is of paramount importance when setting about the task of developing and optimising an MIP.

Karim *et al*. state that over 4000 polymerisable compounds with potential use as functional monomers have been reported in the literature [87]. Despite this, however, a relatively small number are regularly utilised for imprinting purposes. While, as discussed in section 1.3, imprinting strategies other than conventional non-covalent methods require tailor-made or custom functional monomers, the vast majority of functional monomers put to use are commercially available. These monomers can be broken down into the following general groups: acidic, basic, neutral and electrostatically charged [2, 88]. An example of each type of monomer and some applications are presented in Table 1.1 below.

**Table 1.1: Examples of functional monomer structure and applications of the different types of monomer.**

| Monomer | Structure | Functionality | Applications | Reference |
|---|---|---|---|---|
| Methacrylic acid (MAA) |  | Acidic | 17β-estradiol, Aspirin, melamine, clozapine | [89-92] |
| 4-vinyl pyridine (4-VP) |  | Basic | Chlormequat, 17β- estradiol, quercitin, fancy red | [93-96] |
| 2-Hydroxyethyl methacrylate (HEMA) |  | Neutral | Cholesterol, hydrocortisone, glycyrrhizic acid | [97-99] |
| 2-acrylamido-2-methylpropane sulfonic acid (AMPSA) |  | Charged | Bovine haemoglobin, creatinine | [100, 101] |

While the monomers presented in Table 1.1 represent a mere fraction of those that are regularly used for the synthesis of MIPs, they serve to show the variety of possible monomers that exist. The examples in Table 1.1 also serve to show that different types of monomers may be used for the same template, as shown by the reported use of both the acidic MAA and the basic 4-vinyl pyridine (4-VP) for the steroid 17β-estradiol by Gadzala-Kopciuch *et al.* and Watabe *et al.* [90, 93]. It is worth noting that Gadzala-Kopciuch *et al.* studied the use of MAA and 4-VP but in this particular example MAA based MIPs had improved binding ability over the 4-VP based MIPs. This was attributed to increased hydrophilicity of the polymers containing MAA. This example serves to demonstrate that for a particular template, careful choice will have to be made regarding the required functionality of the monomer.

Researchers have often found it beneficial to use a combination of two or more monomers, such as the use of the neutral 2-hydroxyethyl methacrylate (HEMA) combined with the basic dimethylaminoethyl methacrylate for the nitroimidazole drug, (*S*)-ornidazole [102]. Here, a monolithic polymer was prepared for use in capillary electrophoresis and was used to separate the template from its enantiomer and a range of structural analogues. Bovine haemoglobin has also been imprinted using a combination of two functional monomers [103]. In this work, Janiak *et al*. observed that the use of the negatively charged 2-acrylamido-2-methylpropane sulfonic acid combined with the positively charged 3-methacrylamidopropyl trimethylammonium chloride gave increased specificity due to the improved swelling properties brought about by the combination of monomers but decreased selectivity due to the presence of the negative charge, which interacted strongly with a competing protein.

When selecting a functional monomer, as well as selecting the appropriate functionality, it is also necessary to determine the possibility of monomer-monomer self-association and to match the reactivity ratios of the functional monomer with either a second functional monomer or a crosslinking monomer or both, to ensure that each monomer will be incorporated into the polymer matrix to the desired extent [88]. In order to promote the interactions utilised by non-covalent molecular imprinting, e.g. H-bonding, the formation of ion-pairs, dipole-dipole forces or π-π interactions, it is necessary to select polymerisation conditions conducive to such interactions, whether in the pre-

polymerisation complex or in subsequent rebinding of the template. In both instances, the role of the solvent must not be underestimated.

### 1.4.2 The role of the diluent

The role of the diluent, or solvent, is central to the synthesis, subsequent characteristics and performance of molecularly imprinted polymers. It is the solvent that brings all of the components, i.e. the monomers, the template and the initiator, into a single phase [88]. In addition to bringing the polymer components together at the pre-polymerisation stage it also plays a fundamental role in promoting interaction between the functional monomer and the template [104]. The solvent is also responsible for dictating the degree of porosity by affecting the timing of phase separation [88, 104, 105] and by occupying space growing polymer matrix. For these reasons, the solvent is commonly referred to as the porogen.

#### *1.4.2.1 Solvent promotion of interaction between the template and functional monomer*

It is generally considered that to impart maximum affinity in the resultant polymer, it is necessary to promote maximum interaction between the template and the functional monomer in both the pre-polymerisation complex and the rebinding solution [106]. These are interactions that the solvent may promote or interfere with. Of the non-covalent interactions that are commonly used for imprinting, H-bonding is considered to be one of the most important in non-aqueous solutions [107]. In order to promote H-bonding it is therefore necessary to select a less polar solvent, such as chloroform, which will facilitate such interactions rather than a polar, or worse, a protic polar solvent, which can cause maximum disruption and compete with H-bonding interactions [104].

This was shown in a comprehensive study by Sellergren and Shea which investigated, amongst other factors, the importance of the H-bonding capability of the solvent on subsequent rebinding of the template L-phenylalanine anilide, depicted in Figure 1.12 [108].

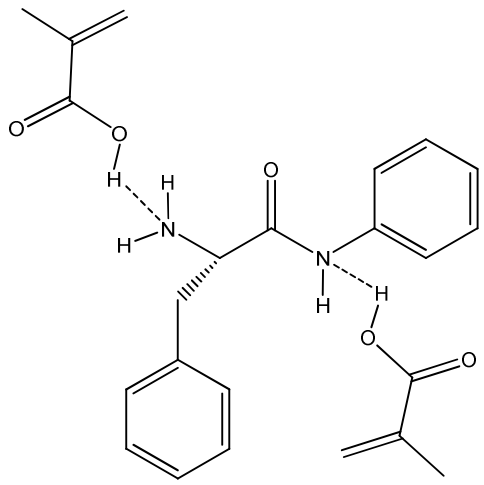


**Figure 1.12: L-phenylalanine anilide, the template molecule used by Sellergren and Shea for the investigation on the effect of solvent on rebinding [108].**

This work, carried out using the model template L-phenylalanine anilide showed, that in going from a solvent with very strong H-bonding capability, such as acetic acid, to one with weak H-bonding capability, such as benzene, the enantioseparation factor in subsequent chromatographic experiments increased from 1.9 to 6.8.

This was further corroborated by the work of Nomachi *et al.* where a pseudo-template, *N*-propionyl-5-methoxytryptamine was used to prepare polymers imprinted for melatonin in chloroform ($CHCl_3$), acetonitrile (MeCN) and tetrahydrofuran (THF) [109]. The chemical structures of the template and pseudo-template used in this study are given in Figure 1.13.

**I** **II**

**Figure 1.13: I target molecule, melatonin and II, the pseudo-template, *N*-propionyl-5-methoxytryptamine used by Nomachi *et al*. during investigations on the effect of solvent on molecular imprinting [109].**

Nomachi *et al*., using NMR spectroscopy, found that the H-bonding capability of the solvent was the most important factor in determining the degree of affinity in the final polymer. They found that in $CHCl_3$, the solvent of the three that showed the lowest degree of H-bonding with the template, the MIP proved to have the highest degree of selectivity over the corresponding non-imprinted polymer. It is worth noting that when aqueous solutions are required for rebinding, it is hydrophobic interactions that will promote rebinding, such as in the work of Puoci *et al.* where cholesterol, a relatively hydrophobic molecule, was rebound to the greatest extent on the most hydrophobic polymer in MeCN:$H_2O$ mixtures [98].

### *1.4.2.2 The solvent and physical characteristics of MIPs*

Another important contribution made by the solvent relates to the morphology of the resulting polymer, which ultimately affects how well the target molecule can access the binding sites within the polymer matrix [88, 104, 105]. The diluent achieves this by affecting the timing of the phase separation based on whether it is a thermodynamically good solvent or a thermodynamically poor solvent for the growing polymer [110]. The use of a good solvent, typically MeCN, where phase separation occurs later, leads to the formation of well developed pore structures and high surface areas while the use of a poor solvent, typically $CHCl_3$, where phase separation starts earlier, leads to the opposite [104].

While the use of a pore-forming agent, and the resultant porosity of the polymer, does affect accessibility to the binding sites, two related studies carried out by Spivak *et al*. have shown that this is not essential for selective and efficient rebinding [111, 112]. In this work, 9-ethyladenine was used as the template for a range of polymers prepared with different crosslinking ratios and in two solvents, $CHCl_3$ and MeCN. While the former is not considered a pore-forming agent and the latter is, successful rebinding was achieved in both cases. This suggested that for non-porous polymers the binding sites were not situated on the surface of the pores or the particle but rather in the non-porous gel of the polymer and that this non-porous section of the gel is swollen in the presence of the solvent, thereby allowing the template to diffuse in and out of the gel to the binding sites [112].

The most important finding from this work was that effective rebinding of the template was most successfully achieved when carried out in the solvent in which the polymers were prepared. This was the case for the polymers prepared in both $CHCl_3$ and MeCN and was attributed to the swelling effects of the solvent and that the exact arrangement and proximity of the functional groups responsible for binding the template was only achieved when the same or similar solvent conditions were observed during rebinding. This was due to the so-called 'solvent memory effect'. While the solvation of the template is often overlooked, the interaction between the template and the solvent both during the formation of cavities and rebinding to those cavities should be taken into consideration.

Studies by Haginaka *et al*. [113] and Liang *et al*. [114] have looked at the effect of the porogen on the resultant binding properties and enantioselectivity of polymers prepared using a range of porogens. In the former, Haginaka *et al*. imprinted *d*-chlorpheniramine using toluene, phenylacetonitrile, benzylacetonitrile or chloroform as the porogen. These imprinted polymers were prepared *via* a multi-step swelling procedure and the resultant uniform beads were tested as chromatographic stationary phases [113]. The morphologies and surface areas in the dry state of eight different polymers were seen to vary greatly. The highest surface area obtained was 323 $m^2/g$ for the polymer prepared using 2-(triflouromethyl)acrylic acid (TFMAA) as the functional monomer and phenylacetonitrile as the porogen and the lowest was 10 $m^2/g$ for the same monomer, using chloroform as the porogen. Despite these vast differences in the dry state, however, enantioselectivity of chlorpheniramine was 1.86 for both polymers. This result suggested that despite the low surface area of the latter polymer, when in the solvent-swollen state, the enantioselective sites were still present and readily accessed by the template. It was suggested, however, that the polymer prepared in chloroform, due to its limited pore structure, could have a lower number of binding sites for the template than the other polymers tested.

Liang *et al*. investigated the use of a range of liquid and solid porogens for their effects on the binding properties and enantioseparation of the enantiomers of ursodeoxycholic acid [114]. Porous core-shell molecularly imprinted polymers were prepared using acrylamide and ethylene glycol dimethacrylate as the functional and crosslinking monomers, respectively. Toluene, acetone and dodecyl alcohol/cyclohexanol mixtures

were used as liquid porogens while solid granules of $CaCO_3$ and $Na_2SO_4$ were used as solid particle porogens. Polymers without the use of porogens were also studied. As expected, the polymers using toluene and $CaCO_3$ as porogens, either separately or together, produced the polymers with the highest surface area, 158.83, 130.28 and 138.88 $m^2/g$, for toluene, $CaCO_3$ and the mixture, respectively. The latter polymer had the largest average pore diameter at 19.39 nm. Not surprisingly, the polymer prepared in the absence of any porogen had the lowest surface area, at 55.41 $m^2/g$. When the enantioselectivity of the polymers was tested, the three polymers with the largest surface area had the highest selectivity factor (α) values, ranging from 2.88 to 3.24, while that which was prepared with no porogen attained the considerably lower value of 1.73.

These results again suggest that the greater the defined pore structure of the polymer, the better the imprinting performance. Once a rigid pore structure has been achieved, appropriate selection of the rebinding solvent should swell the polymer to an extent that will allow for access to, at least, a number of the binding sites. Despite playing this defining role in the ultimate morphology of the polymer, the solvent alone does not dictate this characteristic. Indeed, the crosslinking monomer also has a vital part to play in this area and it is by careful optimisation of the two components that the most suitable morphology may be achieved. Characterisation of such physical parameters is an important aspect of molecular imprinting and will be discussed in more detail in Section 1.6.

### 1.4.3 The crosslinking monomer

The crosslinking monomer or 'crosslinker' can comprise between 70 and 98% of the total polymer composition and as such has a number of roles in the synthesis of molecularly imprinted polymers [88]. One of the major aspects of the polymer for which the crosslinker is partly or wholly responsible, is the morphology of the polymer, i.e. whether the polymer formed is gel-type, macroporous or microgel powder. It is also the crosslinking monomer which imparts rigidity to the imprinted polymer, thereby enhancing the fidelity of the binding site by 'locking' the functional moieties into the correct spatial and functional configuration for rebinding of the analyte. The final aspect in which the crosslinker plays a role is in the ultimate mechanical stability of the

polymer [2]. While far fewer crosslinking than functional monomers are put to regular use, there is still quite a considerable number and some examples of the most widely used are given in Figure 1.14.

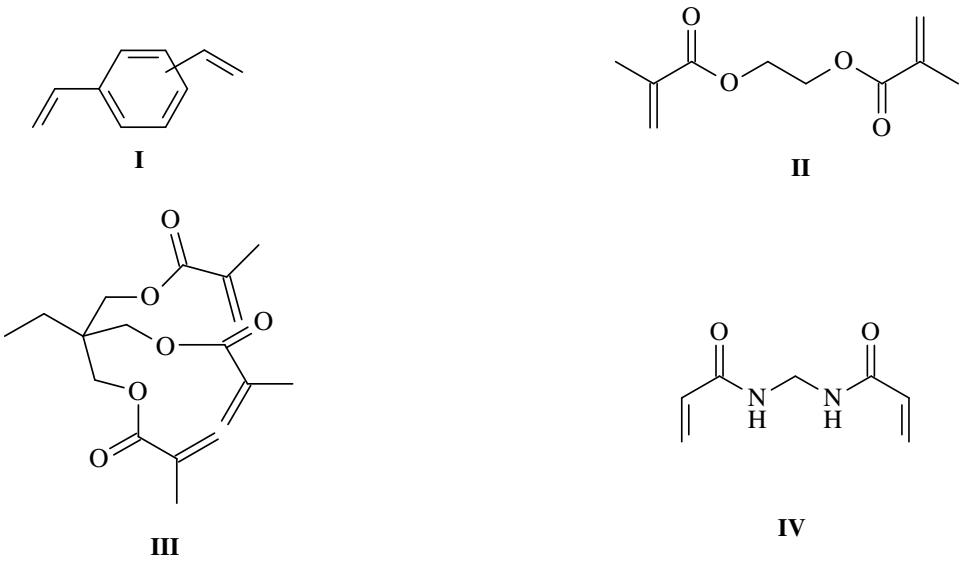



**Figure 1.14: Crosslinking monomers reported in the literature: I divinylbenzene (DVB); II ethylene glycol dimethacrylate (EGDMA); III trimethylolpropane trimethacrylate (TRIM); IV methylene-*bis*-acrylamide.**

The crosslinkers in Figure 1.14 are examples of a number of different classes of crosslinking monomers that have been put to use during the development of the molecular imprinting technology. Divinylbenzene (DVB), **I** above, was the first crosslinker employed for the purpose of modern molecular imprinting and this was in the work of Wulff and Sarhan discussed in Section 1.3.1 [16]. A later study by Wulff *et al*. compared the use of DVB with ethylene glycol dimethacrylate (EGDMA) (**II**) and three other crosslinkers, two other methacrylates and a novel optically active crosslinking monomer [115]. In that work it was found that EGDMA was the most effective crosslinker and polymers prepared with 70-95% crosslinking monomer were the most successful for racemic resolution.

EGDMA has since become the most widely used crosslinker [2] and a number of studies have been carried out that correlate the effect of the crosslinking ratio. In one such investigation, Sellergren observed that the separation factor of the polymer during chromatographic investigation reached a maximum at 75% EGDMA but below this

selectivity started to drop considerably and at 50%, almost all selectivity was lost [116]. More recent investigations have employed novel methodologies, which have also corroborated the importance of a high degree of crosslinker. Notably, the work of Holland *et al.* where GC-MS based thermal desorption studies and UV spectroscopy demonstrated that as the concentration of EGDMA in the polymer composition decreased, so too did the specificity of the polymer [117].

While a high proportion of crosslinking monomer is deemed necessary for selectivity in MIPs, a number of studies have shown that for some applications this may not be necessary. These include the work of Alvarez-Lorenzo *et al*. where soft contact lenses were prepared using molecular imprinting technology to impart selectivity for timolol and ratios of crosslinking (EGDMA) to functional monomer (MAA) as low as 1:10 demonstrated selectivity [118]. Work by Hillberg *et al*. succeeded in preparing theophylline selective flexible membranes with as little as 12.5% (molar) crosslinking monomer (tri-ethylene glycol dimethacrylate) [119].

As well as using divinyl crosslinkers such as DVB and EDGMA a number of trivinyl and tetravinyl monomers, such as the trivinyl monomer trimethylolpropane trimethacrylate (TRIM) (Figure 1.14 **III**) have been used, and, in a number of cases have proven to be more effective than EGDMA. This is exemplified by the work of Mosbach *et al.* for templates such as dipeptides [120] and steroids [121]. Water soluble monomers such as methylene-*bis*-acrylamide (Figure 1.14 **IV**) have also been shown to serve as effective crosslinking monomers for use in aqueous or aqueous based solutions, as seen in the work of Suedee *et al.* where dopamine imprinted polymers were prepared for use in the analysis of urine for adrenergic drugs [122]. Due to the polarity of dopamine it was not soluble in any organic solvents and synthesis of the polymer had to be carried out in aqueous methanol.

Similarly, Cirillo *et al*. employed ethylene-*bis*-acrylamide for imprinting the anti-oxidant, phytic acid, using a 3:1 water to ethanol mixture as the porogen [123]. The use of the relatively hydrophilic crosslinker was important due to the hydrophilic nature of the template, and thus the necessity to use aqueous solutions as the porogen, as well as the intended end use of the polymer as a controlled release platform. The release profiles of the polymers were studied in simulated gastrointestinal fluids and at acidic

pH the drug was completely released from the NIP after 2.5 hours, while only 40% had been released from the MIP at this stage.

An interesting study by Ates and Guven used positron annihilation lifetime (PAL) experiments to study the cavity sizes within radiation induced glucose imprinted polymers prepared with crosslinking monomers with increasing chain lengths [124]. The crosslinking monomers used were diethyleneglycol diacrylate (DEGDA), polypropylene glycol dimethacrylate (PPGDMA) and triethylene glycol dimethacrylate (TEGDMA), and these were studied over increasing molar concentrations relative to the functional monomer, HEMA. The PAL experiments showed that the free-volume radius, *R*, was only slightly affected by the increasing chain length when concentrations of the crosslinker were low, i.e. 10-30% but at higher concentrations, i.e. 70%, the increased chain length of the PPGDMA contributed to an increase in *R* of the order of 13%. Swelling studies corroborated the difference in the pore volumes and in terms of molecular recognition, DEGDA showed the highest affinity for glucose. This was attributed to the optimisation of the cavity size for glucose recognition.

Papaionnou *et al*. studied the use of three functional and three crosslinking monomers for imprinting the tripeptide, Arg-Gly-Asp, denoted as RGD [125]. They found that not just the functional monomer but also the crosslinking agent had a profound effect on the percentage net binding of the template, as well as the overall imprinting factor. They observed that for a poly(MAA-co-TRIM) imprinted polymer an imprinting factor of almost 2.5 was observed compared to approximately 1.5 for MAA and bisacrylamide or EGDMA. When acrylamide was used as the functional monomer, however, the polymer using EGDMA as a crosslinker outperformed the others.

Occasionally, researchers have found it useful to employ tailor made crosslinkers, as mentioned in section 1.3.2, where Jiang *et al*. synthesised a custom monomer from chinchonine and was used as both functional and crosslinking monomer for the separation of racemic ketoprofen [65]. Gavrilovic *et al.* synthesised a steroidal crosslinking monomer, which allowed for the separation of testosterone and its epimer, epitestosterone [126]. The use of this crosslinker allowed for postulated Van der Waal's stacking of the steroid and baseline resolution was achieved for the two epimers, which

co-eluted perfectly using the non-imprinted polymer. The steroidal crosslinker used is shown in Figure 1.15.

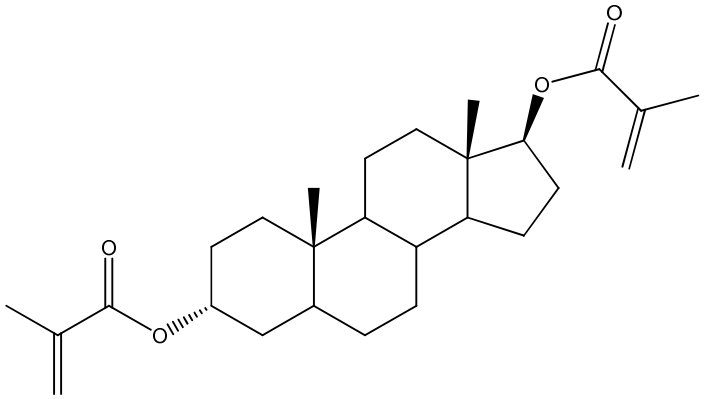


**Figure 1.15: Steroidal crosslinker used for the separation of testosterone and epitestosterone [126].**

As is the case with the functional monomer and the porogen, careful selection of the crosslinking monomer is also necessary and while it is generally stated that the crosslinker does not interact to any great extent with the template, it is necessary to match reactivity ratios with the functional monomer and take factors such as solubility, etc. into consideration.

### 1.4.4 Polymer initiation

While the methods of initiation applied to molecular imprinting technologies have been many and varied, including γ-irradiation [127], an ever-increasing use of iniferters [128, 129] and the production of radical species using peroxide based compounds [130], the most widely used strategy remains the use of azo-compounds and their decomposition to produce radical species [1]. Compounds such as 2,2'-azobis(2-methylpropionitrile) (or azobisisobutyronitrile - AIBN) [131], 1,1-azobis(cyclohexanecarbonitrile) [132] and 4, 4-azobis(4-cyanovaleric acid) [133] have all been utilised for MIP preparation.

These azo-compounds can undergo decomposition to form radicals using both thermal and photochemical means. The use of photochemical means allows for initiation at low temperatures, which could better promote interaction between template and functional monomer. Studies published in 2011 by Zhang *et al.* [134] and Dopico-Garcia *et al.* [135] have compared the two methods of initiation.

The former study used AIBN as the initiator and demonstrated that while the polymers prepared by both means had an almost identical molecular structure (as observed by FT-IR), transmission electron microscopy (TEM) demonstrated that differences in the pore structure of the MIP and NIP were more pronounced for photo-initiated than thermally initiated polymers. This difference between the imprinted and non-imprinted polymers for photo-initiated polymers was considered to be important for the performance of the polymers and results showed that imprinted polymers prepared in this fashion had higher adsorption capacities and selectivity than their thermally initiated counterparts.

Dopico-Garcia *et al.* also found that photo-initiated polymers outperformed thermally initiated polymers, with the former having the capability to rebind the template while the latter did not. It is worth noting, however, that Lu *et al*. [136] state that while low temperatures allow for the formation of a more stable template-monomer complex in the pre-polymerisation solution, higher temperatures can improve the quantity and quality of template recognition sites within the polymer by achieving more complete polymerisation reactions.

### 1.4.5 A rational design approach to molecular imprinting

Judicious selection of functional and crosslinking monomers, as well as solvent and initiating strategy is most often determined by empirical means and this method of determining the most appropriate composition is perhaps still the most popular and successful route for MIP synthesis. Due to the very often time consuming nature of this method, various approaches have been taken in an effort to reduce the time necessary to synthesise successfully imprinted polymers. This ever-increasing number of methods aimed at improving the speed and success with which MIPs are developed is often collectively termed as a 'rational design approach'.

It is widely accepted that the success of non-covalent molecular imprinting is due in no small part to suitably strong complexation of the template and functional monomer in the pre-polymerisation complex [106, 137, 138]. While it is recognised that these adducts are exposed to a much different environment during polymerisation (due to factors such as high temperatures and growing polymer chains), it has been shown that

polymers prepared in such a way as to maximise complex formation have achieved good success in terms of ultimate performance [139-141].

A variety of computational and spectroscopic methods have been developed (for use alone or in tandem with each other) to fully illuminate the processes taking place in the pre-polymerisation complex, thereby allowing for the most suitable selection of the components for use in the preparation of MIPs. The first of these to be discussed here are the computational approaches.

### *1.4.5.1 Computational approaches to the rational design of MIPs*

One of the earliest MIPs prepared using computational methods to predict interactions in the pre-polymerisation complex was a biotin imprinted polymer synthesised by Takeuchi *et al*. [142]. The molecular modelling studies carried out here were based on Monte Carlo simulations, a computational algorithm. These simulations predicted that the most stable complex structure for the functional monomer, MAA, and the biotin derivative being used as a template, involved the formation of bidentate hydrogen bonds between the two compounds. This was supported by NMR studies and the polymer prepared was capable of binding 6.81 μmol/g of polymer and Scatchard analysis gave association constants of $2.5 \times 10^5$ $M^{-1}$ and $6.3 \times 10^3$ $M^{-1}$ for the high and low affinity binding sites, respectively.

A range of molecular modelling software packages have been employed in the preparation of MIPs, including the Cerius$^2$ software utilised by Pavel and Lagowski [143], GaussView by Isarankura-Na-Ayudhya *et al*. [144] and Gaussian03 software by Diaz-Diaz *et al*., density functional theory [145], and the Hartree-Flock model by Li *et al*. [146] and Mojica *et al*. [147]. A number of packages have stood out in the literature, having demonstrated success for a variety of templates. One such software package is that developed and used over the last decade by the group of Piletsky *et al*., i.e. The Leapfrog algorithm.

The Leapfrog algorithm is used to screen a library of the most commonly used functional monomers against a variety of templates, in order to determine which monomer has the highest binding score, a value incorporating Van der Waal's forces,

hydrogen bonds, ionic interactions and spatial considerations [87]. Once the most suitable monomers have been selected, a simulated annealing process is employed whereby the template is placed inside a virtual box with the largest number of the chosen functional monomer possible. Energy is then added to the system in a series of energy minimisation and heat addition steps and the optimal template to monomer ratio is determined. This system has been used for the imprinting of abacavir, a HIV-1 reverse transcriptase inhibitor [148], the mycotoxin aflatoxin B1 [149], cholic acid [150] and atrazine [151]. In the last example, three monomers were identified as the most appropriate, MAA, itaconic acid (ITA) and acrylamide (AA). The software generated complexes for the three monomers and the template are shown in Figure 1.16.

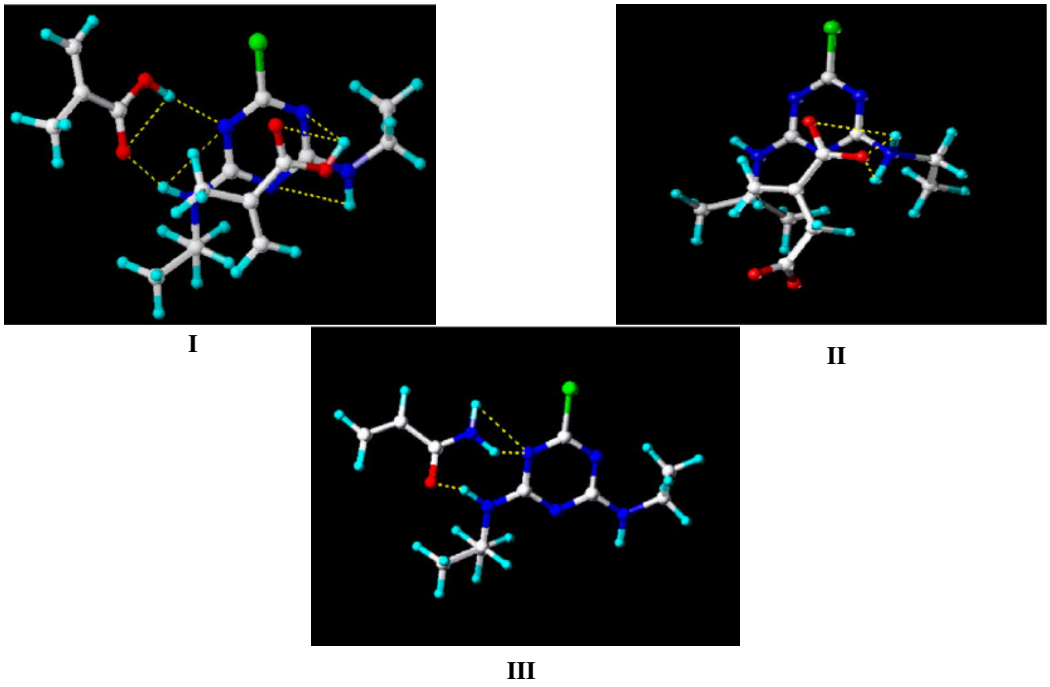



**Figure 1.16: Software images for the complexes between, atrazine and I MAA; II ITA; III AA. Yellow dotted lines represent H-bonding interaction [151].**

Figure 1.16 **I** shows that two molecules of MAA were predicted to interact with the template while only one of each of the other two monomers was seen to take part in complex formation. This proved to play a significant role in the resultant binding properties of the imprinted polymers and the MAA based polymers were shown to have the highest specificity (~100% analyte rebound in the MIP and <10% in the NIP) and capacity (12.5 mg/g of polymer) of the three monomers tested [151].

This software uses the dielectric constant of the solvent to account for the effect of the solvent and allows for the input of the crosslinking monomer into the software. However, as can be seen from Figure 1.16, this is generally not included, as the interaction between the crosslinker and the template is considered to be negligible.

Another notable software package is the AMBER programme, developed by Pearlman *et al*. to "simulate the structural and energetic properties of molecules" [152], which has been adapted by Mizaikoff *et al*. for use in the study of the pre-polymerisation complex in the preparation of molecularly imprinted polymers for 17β-estradiol [107, 153]. This software package places more focus on the hydrogen bonds formed as this is deemed to give more direct information regarding the strength of the bonds in the template-monomer complex in the pre-polymerisation solution. This package also allows the input of solvent molecules into a virtual box, analogous to the one used by Piletsky *et al*. and incorporates factors, such as the density of the solvent, in subsequent calculations.

This molecular dynamics simulation approach was retrospectively used to provide a more detailed insight into the pre-polymerisation solution of a successfully prepared 17β-estradiol imprinted polymer [107]. The polymer had been prepared in an earlier work using MAA and DVB as the functional and crosslinking monomers, respectively and yielded polymers with a mean affinity constant of $2.45 \times 10^{-11}$ $M^{-1}$ (obtained from Freundlich isotherm) [154]. An image of the pre-polymerisation complex is portrayed in Figure 1.17.

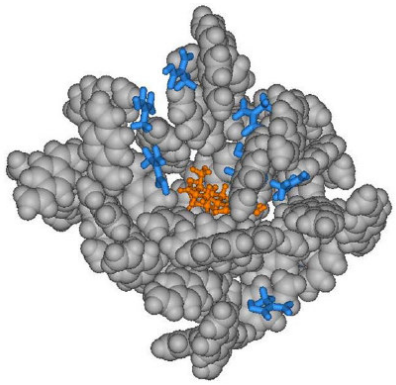


**Figure 1.17: Image of the pre-polymerisation complex for 17β-estradiol MIP, generated by the Amber software package. Colour scheme: 17β-estradiol: orange; MAA: blue; DVB: grey. (From reference [107]**

In later work on 17β-estradiol, results from modelling experiments suggested that the monomers MAA, 2-(diethylamino)ethyl methacrylate and methacrylamide were the most suitable monomers [153]. Since MAA had been shown to be successfully used for the preparation of 17β-estradiol imprinted polymers [154], this was chosen for further NMR investigations where the optimum 6:4 MAA:17β-estradiol complex formation predicted by the modelling study was confirmed by NMR analysis.

The more recent use of the Amber software suite, as applied by the group of Nicholls *et al*. for computational analysis that is stated to be "the simulation of a complete molecularly imprinted polymer pre-polymerisation system" [155] could, at present, be deemed the state of the art in computational modelling. The first study by this group used molecular dynamics software to look at the interactions of the template, bupivicaine, and the other components in the pre-polymerisation solution. In this work, two pre-polymerisation solutions were studied: the solution containing the template, functional monomer, crosslinker, initiator and solvent, and the solution with the crosslinker and initiator omitted. The presence of the initiator and the crosslinker was seen to have a clear impact on the interaction of MAA, the functional monomer, with the template. It was suggested that the interactions between the crosslinker, EGDMA, and the template, though weak, were present and indeed contributed to the presence of shape selective sites in the resultant polymer.

While interaction between the template and the initiator were not observed, it was noted that dimerisation of MAA was less favoured when both the crosslinker and the initiator were present. The porogen, chloroform, was seen to be mainly located in the non-polar regions of the template. This use of the Amber software has since been successfully applied to targets such as the anticoagulant warfarin [156] and the vitamin biotin [157].

Interestingly, in the preceding six examples spectroscopic analysis was used in conjunction with the computational systems in a bid to further elucidate the interaction events taking place in the pre-polymerisation mixture with good agreement between the two methods being demonstrated. The use of spectroscopic analysis either alone or in conjunction with molecular modelling software, has become a powerful tool in the rational design of molecularly imprinted polymers.

*1.4.5.2 Spectroscopic methods for the rational design of MIPs*

Spectroscopic methods, be they NMR, IR or UV, allow for a property of either the template or the functional monomer to be monitored as it changes due to complexation with the other in an environment chosen to mimic the pre-polymerisation solution [106, 158, 159]. Here Job plots, where the total molar concentration of two complex forming species is held constant while varying their respective mole fractions [160], can be used for determination of the complex ratio. The construction of titration curves by changing the concentration of one component while holding the other constant and monitoring the change in signal of either component [161], can be used to calculate association constants.

Early work carried out by Sellergren *et al.* employed NMR spectroscopy to fully elucidate the enantioselectivity and substrate selectivity of L-phenylalanine imprinted polymers [162]. In this study a number of polymers were prepared with the L-form of phenylalanine derivatives and their selectivity was demonstrated using chromatographic methods. For the L-phenylalanine anilide imprinted polymer a capacity factor, $k'_L$, of 4.3 and a separation factor, α, of 3.2 for the D- and L- forms were achieved. NMR studies were used to rationalise the behaviour of the imprinted polymers. This spectroscopic technique was used to monitor changes in the line widths of the protons of the MAA and the exchangeable protons of the amino and carboxyl groups of the template and revealed that this was due in part to the presence of 1:1, 1:2 and 1:3 template:monomer complexes in the pre-polymerisation solution.

Later work by Nicholls and Andersson used UV spectroscopy to investigate the pre-polymerisation complexes of MAA based polymers previously imprinted by Nicholls *et al.*, namely cinchonidine, yohimbine and *N*-acetyl-L-phenylalaninyl-L-tryptophanyl methyl ester imprinted polymers [163]. This work was presented as a suitable method for optimising the pre-polymerisation composition of new imprinted polymers and since then spectroscopic methods have been put to routine use in the rational design of molecularly imprinted polymers.

A notable example of the use of spectroscopic methods in the evaluation of pre-polymerisation complexes was a three part NMR study by Ansell *et al.* where the most

suitable monomers and solvents were chosen for the preparation of ephedrine MIPs by first fully probing the interactions between MAA and the template [164]. This was followed by determining the most suitable solvent for use with this monomer [165] and finally investigating the use of three other functional monomers [141]. This study yielded a polymer capable of achieving an α value of 2.46 for the separation of (±)-ephedrine. The effect of the crosslinker in the pre-polymerisation solution was also investigated in each case, demonstrating the extent to which the pre-polymerisation conditions may be examined.

The work of Osmani *et al*. has involved the use of two spectroscopic methods, where both NMR and IR were combined to elucidate the interactions between the template, 2-aminopyridine (2-apy), and the functional monomer, MAA [106]. The complementary correlation between the findings of the NMR and FT-IR studies is here exemplified by agreement of the experimentally derived complex stoichiometry represented in Figure 1.18.

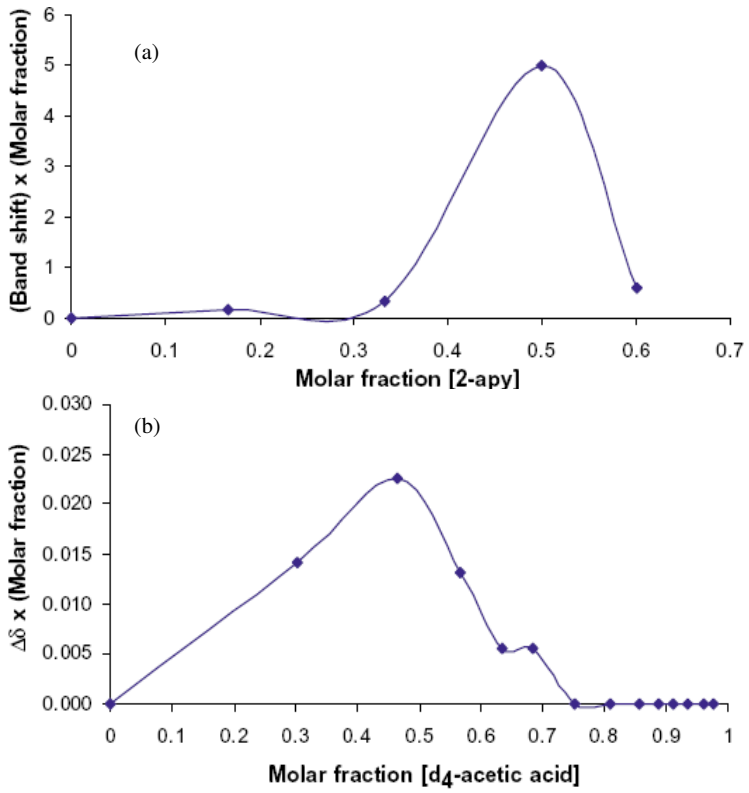



**Figure 1.18: Job plots obtained by (a) FT-IR and (b) NMR experiments both depicting a 1:1 stoichiometry for the 2-apy-MAA complex in the pre-polymerisation solution [106].**

Zhang *et al*. have quite cleverly used $^{1}$H NMR studies to study their theories on the effect of functional monomer aggregation on the presence of background sites in imprinted and non-imprinted systems [166, 167]. In their work on the use of a urea monomer, they have looked at monomer-monomer and monomer template aggregation [167]. Solution state NMR studies demonstrated that the monomer-monomer aggregation had a constant, $K_{agg}$, of 3.5 $M^{-1}$ in chloroform (the porogen) alone, but when DMSO was added, no aggregation took place. When the interaction between the template, tetrabutlyammonium diphenyl phosphate, and the monomer was studied in the presence of the porogen (chloroform), a value of 176 $M^{-1}$ was observed for the association constant.

It was postulated that when the template was present in the pre-polymerisation complex, the interaction between template and monomer would predominate, forming template-monomer complexes. When no template was present, however, the monomers would self-associate and limit the number of "background binding sites" and thus, significantly reduce the non-specific binding due to binding moieties, which would typically be indiscriminately spread throughout the polymer matrix. Indeed, the imprinted polymer had a capacity of 23 µmol $g^{-1}$, which was a four-fold increase over that of the non-imprinted polymer at only 5.4 µmol $g^{-1}$, demonstrating a strong imprinting effect.

As previously mentioned, studies were carried out on the aggregation of the functional monomers in increasingly polar solvents (by addition of DMSO) and when the resultant polymers were formed, the MIP had a modest decrease in its uptake capacity. What was more significant, however, was the large increase in the uptake capacity of the NIP, which rose from 5.4 to 15 µmol $g^{-1}$ upon addition of the DMSO to the pre-polymerisation mixture. This was attributed to the disruption of the self-association of the monomer in the pre-polymerisation of the binding sites by the presence of the competing polar solvent.

What can be seen from this and the preceding sections is that the use of molecular modelling and spectroscopic methods can aid in the careful selection of the best components for the preparation of MIPs. These methods are typically aimed towards the rational design of molecularly imprinted polymers and while there are still arguments

against this, what is important about these methods is that they contribute towards a deeper understanding of the processes that are involved in the synthesis of MIPs.

Whether using this approach, using tried and tested monomers or empirically testing a range of compositions, it is important that the most appropriate polymer format and method for polymer preparation be chosen, taking into account factors such as the final application of the polymer.

## *1.5 Methods of polymerisation*

As with all polymers, molecularly imprinted polymers can be prepared *via* a range of polymerisation methods and, since the earliest work on imprinted polymers a number of general polymerisation strategies have been adapted for use in molecular imprinting. Presented here is a brief outline of some of the common polymerisation methods that have been applied to the synthesis of MIPs.

### 1.5.1 Bulk polymerisation

The preparation of a polymer in bulk monolith format is achieved by mixing together all of the components, i.e. the template, the monomers and the initiator, in the solvent, where polymerisation takes place. All components must be soluble in the solvent and the monomer concentration in terms of total volume of pre-polymerisation solution is typically 40-60% [121]. The result is a polymeric block comprised of densely fused and interlinked microgels. The polymeric block is then crushed, ground and sieved to obtain particles of the required size.

Since the first organic imprinted polymer by Wulff and Sarhan [16], this has historically been the most widely used strategy for the preparation of MIPs [133]. The widespread use of this method can be ascribed to its synthetic simplicity and general applicability, although it also has a number of disadvantages, including the need for labour intensive grinding and sieving, the loss of a high percentage of polymer (up to 70%) during the sieving process and the possible destruction of binding sites during the grinding process. While this method is still regularly used [45, 141, 145, 168], these disadvantages, coupled with the possible advantages (and often times necessity), of using different

polymer formats appears to have brought about a shift in focus in terms of polymerisation strategies.

### 1.5.2 Molecularly imprinted beads

A number of techniques have been applied to the preparation of MIPs in bead form, as good control over particle size may be achieved and spherical particles are considered advantageous for a number of applications, such as chromatography [169] and drug delivery [170]. A range of approaches have been used, most notably suspension and precipitation techniques.

#### *1.5.2.1 Suspension polymerisation of MIPs*

Suspension polymerisation involves the dispersion of, typically water-insoluble, monomers as small droplets in the chosen solvent with each of the monomeric droplets behaving as a "mini-bulk reactor" [23, 133, 171]. Early work utilising suspension polymerisation for non-covalently imprinted polymers was carried out by Mayes and Mosbach, where a liquid perfluorocarbon was employed as the dispersing phase [172]. The reaction was UV initiated and depending on the amount of the stabilising polymer used, the bead size could be controlled to between 5 and 50 µm. While the fluorocarbon has the advantage of not disrupting the interactions between the template and the monomers, as would be the case with water, it has the disadvantage of being quite expensive.

An alternative to this method was presented by Kempe and Kempe, where mineral oil and acetonitrile were used as the dispersant and solvent, respectively, for the preparation of spherical propranolol imprinted beads prepared in roughly the same size range as those of Mayes and Mosbach [173]. This made the process cheaper and acetonitrile's lack of solubility in mineral oil negated the need for added stabiliser. Acetonitrile can, however, interfere with H-bonding interactions and the use of other porogens would not be suitable with mineral oil. Where mineral oil is not used, stabilisers are generally employed and can be responsible for unwanted functionality on the surface of the polymer.

While these novel methods of suspension polymerisation have been put to use, conventional suspension methods currently enjoy a large degree of popularity and have been used for templates such as the triazine, melamine [92], the peptide hepcidin [174] and Sudan dyes [175]. As discussed in section 1.3.3, Curcio *et al*. synthesised a surfactant template based on glucopyranoside (see figure 1.9) [71]. This surfactant template had the dual role of allowing for an emulsion to be formed, as well as ensuring that due to its surfactant nature, the imprinted sites would be at the surface of the imprinted bead, i.e. the interface of the two phases. In this case, a mini-emulsion was formed by sonication allowing for the synthesis of beads in the range of 150 nm.

*1.5.2.2 Precipitation techniques for MIPs*

Precipitation polymerisation is a technique that will be discussed in more detail in Section 2.1.4 but a brief introduction to the technique will be presented here. This strategy is based on the use of diluents in which the monomers (typically <5% of the total volume) are soluble but the growing polymer chain becomes increasingly insoluble, thus causing the polymeric beads to precipitate out of solution [20, 176, 177]. Beads are formed in the range of 0.3-10 μm, no added stabiliser is necessary and careful optimisation of the solvent composition allows good control over particle size and morphology.

Early work by Mosbach *et al*. employed this method for the synthesis of theophylline and 17β-estradiol imprinted beads, where the microspherical particles exhibited superior rebinding ability over the bulk monolith particles [121, 178]. This method has since been used for the preparation of beads imprinted for chemical warfare agents [58], as well as anticonvulsant [179], antimalarial [180] and antipsychotic drugs [89].

Recent work by Long *et al*. has provided a greater understanding of the processes taking place in MIPs prepared by precipitation polymerisation [181]. For this insightful study, NMR and dynamic light scattering techniques were utilised to investigate a range of factors including the amount of monomer incorporated into the polymer relative to time as well as the size and growth rate of the nanoparticles. Two model templates were used, propranolol and testosterone, and NMR studies were carried out on the polymerisation mixture at different time points. For the propranolol system,

approximately 60% of the free template was bound to the polymer after twenty four hours, with 70% of this bound template associated with the particles after the first three hours. This suggested that the majority of binding sites were already formed during the first three hours. When testosterone was studied, it was observed that only 20% of the template was associated with the polymer after the full twenty-four hours. This disparity in the amount of material associated with the polymer was predicted by pre-polymerisation binding studies where the only noticeable complex induced shift observed was a change in chemical shift of 0.041 ppm, related to a weak hydrogen bond to the 17β-OH of the steroid. This was in stark contrast to the changes in chemical shift observed for propranolol, where the change in shift values ranged from 0.224 to 0.505 ppm.

Rebinding studies were also carried out on the growing polymer particles at time points throughout the study and it was seen that after three hours, the amount of propranolol bound to the imprinted polymer remained relatively static. This was in good agreement with the suggestion that the majority of the binding sites were formed by this time. Dynamic light scattering studies showed that the hydrodynamic volume of the polymer particles changed to a small extent after only thirty minutes but the apparent swelling of the polymers suggested that the particles became more heavily crosslinked as reaction time increased.

This particular study, while being informative about the processes involved in precipitation polymerisation for MIPs, also serves to demonstrate an inherent problem in the method: when the degree of interaction between the template and the monomer is low, as was the case with testosterone, the final amount of template associated with the polymer prepared in such dilute solutions will also be considerably lower than is typically desired for molecularly imprinted polymers.

#### *1.5.2.3 Core-shell MIPs*

A method which has also been used for the preparation of spherical MIP beads, and which may to some extent counter the dilution problems of precipitated beads, is the use of core-shell polymerisation techniques. Carter *et al*. [182] have attributed the origins of this method for use in imprinted polymers to Yu *et al*. during their work on the selective

extraction of copper (II) and nickel (II) [183]. During work on the use of core-shell imprinting for cholesterol, Perez *et al*. [184] have outlined the process as having two distinct stages; firstly a seed (or core) is formed, often by emulsion polymerisation of any of a number of monomers. This seed is then added to another batch of monomers (or the monomers are fed to the seed solution) and the thin imprinted 'shell' is then formed on the surface of the core. This has the obvious advantage that the template should be more readily removed from this thin layer of polymer and conversely, binding kinetics should be faster. Other advantages include the degree of control over bead size and the ability to prepare larger beads.

The term 'core-shell imprinting' has come to encapsulate a number of different strategies. Perez *et al*. [184, 185], Carter *et al*. [186] and more recently Liang *et al*. [114, 187] have utilised monomers such as styrene, DVB and EGDMA to synthesise the core *via* conventional emulsion methods, while Ma *et al*. [188] and Jiang *et al*. [65] have employed silica nanoparticles as the core for beads selective for estradiol and ketoprofen, respectively.

Barahona *et al*. have applied to molecularly imprinted polymers, the method developed by Stover and Li [189] for the synthesis of core-shell beads *via* two-step precipitation [56, 190]. Here, the core-shell MIP beads are prepared by first synthesising the core using a simple precipitation method for crosslinked DVB beads. These DVB microsphere cores were then coated with the 'shell', which was composed of a mixture of DVB, MAA and the template, the fungicide thiabendazole. This resulted in the preparation of an inline solid-phase extraction method where it was possible to inject samples of citrus fruits and orange juices, from which accurate determination of the target as low as 0.8 mg $kg^{-1}$ was achieved.

### 1.5.3 Film and lens type MIPs

Applications such as sensors require the preparation of imprinted polymers in formats suitable for such use. In a number of cases the preparation of polymers as thin films has been used to prepare sensors for targeting analytes such as myoglobin [55], billirubin [191] and melamine [192]. Methods such as spin-coating [193, 194], surface-grafting polymerisation [195] and electropolymerisation [196] have been employed to

incorporate molecularly imprinted films of the requisite thickness onto the surface of sensors such as quartz crystal microbalances and potentiostats.

The preparation of lens type materials for application in drug delivery also requires the synthesis of polymers in formats suitable for such use. Hiratani *et al.* [197-199] have employed polypropylene and Teflon to construct moulds capable of forming gels of a given thickness to develop the use of molecularly imprinted contact lenses. Careful consideration must be given to the biocompatibility of the monomers and polymer when used for such applications.

Disadvantages of these methods can include low density of imprinted sites and slow diffusion of the analyte, as well as thickness control [44] [200].

### 1.5.4 Advantages and disadvantages of selected polymerisation strategies

There are a number of advantages and disadvantages associated with each polymerisation method and these are summarised in Table 1.2.

**Table 1.2: Advantages and disadvantages of some common methodologies employed for the preparation of MIPs**

| Technique | Advantages | Disadvantages |
|---|---|---|
| Bulk monolith | Easily optimised<br>Suitable for most polymer formulations | Irregular particle size and shape<br>Low yield of usable polymer<br>Time and labour intensive |
| Suspension | Good control over particle size<br>Spherical particles obtained | Need for surfactant or stabiliser<br>Issues surrounding component solubility in continuous phase |
| Precipitation | Good control over particle size and morphology<br>Spherical particles obtained<br>No stabiliser necessary | Needs optimisation for each set of functional monomers |
| Core-shell | Good control over particle size and morphology<br>Spherical particles obtained | Multiple steps in synthesis<br>Often stabiliser necessary |
| Thin film | Well suited for specific applications, e.g. sensors | Slow diffusion of analyte<br>Poor thickness control |

While the polymerisation strategies listed should not be considered exhaustive, the applications, as well as the advantages and disadvantages associated with each method serve to demonstrate that careful consideration of the final application is necessary before deciding which polymerisation methodology to pursue. Whatever the strategy used, physical characterisation of the resulting polymer is necessary to gain a full understanding of the ultimate behaviour of the polymers.

## *1.6 Characterisation of molecularly imprinted polymers*

The characterisation of molecularly imprinted polymers can be roughly divided into three areas: chemical characterisation, morphological characterisation and characterisation of binding site or molecular recognition properties [88].

### 1.6.1 Chemical characterisation

The inherent insolubility of crosslinked molecularly imprinted polymers means that chemical characterisation is most readily achieved using spectroscopic methods. Fourier-transform infrared spectroscopy (FTIR) is one such method. This has been applied to determine the incorporation of functional groups, as well as the extent of polymerisation. Wang *et al*. have employed this technique for surface-imprinted polymers for 3-chlorophenol where they monitored the successful modification of the core silica particle [201] while Zhang *et al*. have used FTIR to determine the differences in polymers initiated by either photochemical or thermal means [134].

Solid-state NMR is another technique that provides information on the chemical composition of molecularly imprinted polymers [104]. Analysis of fenitrothion imprinted polymers has been carried out by Pereira and Rath using this technique [202], while Weng *et al.* have characterised MIPs with catalytic activity towards *o*-fluoroacetophenone in the same manner [203].

### 1.6.2 Morphological characterisation

The morphological properties of molecularly imprinted polymers may be investigated by using any of a number of techniques. Solvent uptake studies are one example, where the amount of solvent taken up by the polymer, can be used to give an estimation of the specific pore volume of the polymer [88]. Solvent swell ratios were employed by Holland *et al*. as part of a study on the influence of morphology on the performance of 2-amino-pyridine imprinted polymers prepared using varying amounts of crosslinker and polymers with higher surface areas were shown to have lower swell ratios [204].

Microscopy has also been used to study the morphology of imprinted polymers with simple optical microscopy being employed by Wei *et al*. to obtain a rough estimation of the particle size and shape of estradiol imprinted polymers prepared by precipitation polymerisation [154]. This group then used scanning electron microscopy (SEM) to get a more detailed analysis of the surface morphology of the polymers, a technique also employed by Esfandyari-Manesh *et al*. when investigating the effect of the porogen on precipitated polymers [179]. Transmission electron microscopy (TEM) has also been employed for morphological analysis in imprinted polymer technologies [205].

Nitrogen physisorption experiments can reveal a great deal of information relating to the surface area, pore size and pore size distribution of polymers in the dry state [88, 104]. This technique involves the construction of adsorption-desorption isotherms by exposing a fixed mass of dry material to a gas at a constant temperature and monitoring the equilibrium pressures, as a series of known quantities of gas are added to the sealed chamber [88, 110, 206]. As it is relevant to this study, this topic will be dealt with in more detail in Section 3.1.3.

### 1.6.3 Binding site characterisation

Quantitation of the strength and number of binding sites present within the matrices of molecularly imprinted polymers is essential to a full understanding of the effect templating processes have on polymer synthesis. This is typically achieved by obtaining adsorption data for a polymer over a specified concentration range, where concentrations of bound (*B*) and free (*F*) analyte are measured. This data is then treated using a suitable mathematical model capable of calculating physical parameters such as the number (*N*) and affinity (*K*) of the sites present.

The construction of Scatchard plots has been employed to achieve this throughout the development of imprinting technology [76, 207, 208]. This is a method used to analyse the data for reversible ligand-receptor interactions and the plot should provide a straight line, the slope of which gives the binding constant, $K_a$ [104]. This method has typically produced a curve when applied to MIP binding data, which in turn has been used to construct two straight lines, suggesting the existence of two types of binding site [207]. In fact, Umpleby *et al*. discuss how the binding site heterogeneity that is practically inherent to MIPs can be problematic when applying the largely homogenous model of Scatchard analysis [207].

In the early 2000s a series of studies published by Shimizu and co-workers, investigated the application to imprinted polymers of binding models that may be more readily used for heterogeneous materials [207, 209-213]. These studies have focussed on application of the Freundlich and the Langmuir-Freundlich isotherms. Both of these models are power functions, which take into account the heterogeneity index, *m*, and *a*, a variable related to the median binding affinity as well as the concentrations *B* and *F* [211, 212].

This group applied the Freundlich isotherm to data obtained for twelve polymers previously reported in the literature and found that a "consistent mathematical relationship" was observed for the data [211]. This suggested that the Freundlich isotherm is generally appropriate for the calculation of binding parameters for imprinted polymers. They did note, however, that the Freundlich isotherm can only be reliably used for sub-saturation concentrations and its applicability for a particular polymer must be shown before the data obtained is to be considered valid.

The Langmuir-Freundlich, however, is free from the limitation of studying sub-saturation concentrations [212] and while it is considered to be more universally applicable, the Freundlich isotherm is considered to be more readily applied and sufficient in the majority of cases [213]. A more detailed discussion of the use and choice of these models will be presented in Chapter 3 but it is worth noting that these models are continuous distribution models that incorporate a distribution of the totality of binding site types.

## *1.7 Applications of molecularly imprinted polymers*

The high degree of selectivity and specificity reported for MIPs, coupled with their inherent robustness and stability under harsh conditions and in a variety of environments, make them attractive for use in any number of applications. The following sections demonstrate how the ability to prepare molecularly imprinted polymers for a wide range of templates has made them the focus of intense research in a number of areas where the specific recognition of an analyte is necessary. This is not an exhaustive list of the myriad applications to which MIPs have been put, rather a set of examples selected to demonstrate their wide-ranging applicability.

### 1.7.1 Solid phase extraction

Solid phase extraction (SPE) is a sample preparation technique used in complex matrices or where the target analyte is present in only trace amounts. Here, either the analyte of interest or the interfering components are adsorbed onto the sorbent allowing for subsequent elution and enrichment of the target analyte.

Since Sellergren first used molecularly imprinted polymers for the selective enrichment of pentamidine [214] SPE has become one of the most successful applications of MIPs where the technology, now known as molecularly imprinted solid phase extraction or MISPE, has found commercial success with examples such as SupelMIP [215] and POLYIntell [216]. Templates with a wide range of functionality have been the focus of research aimed at the preparation of molecularly imprinted sorbents for SPE and some examples are presented in Figure 1.19.

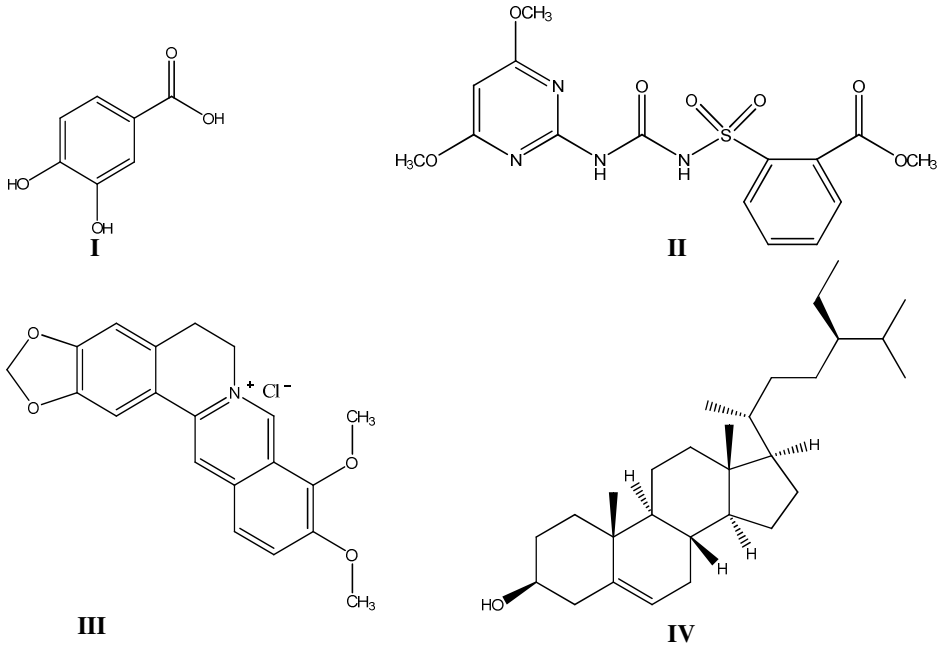



**Figure 1.19: Templates reported in the literature for use in the preparation of MISPE techniques: I Protocatechuic acid [217]; II Bensulfron-methyl (BSM) [57]; III Berberine chloride [218]; IV β-sitosterol [219].**

As discussed as far back as the fifties by Dickey [9], an inherent problem pervasive in the technology of molecular imprinting is that of un-extracted template. In few applications is this problem as relevant as it is in solid-phase extraction, where the predominant objective is to enrich analytes that are present in trace quantities. A method that has been used to counter this problem is the use of a template analogue, i.e., using a template that is similar in shape, size and functionality to the target analyte, but which will not interfere with analysis of the target analytes.

The use of this method for MISPE is exemplified by the work of Baggiani *et al*. for solid-phase extraction of a class of azo-dyes, known as Sudan dyes, in food samples

[175]. The template analogue and two of the nine analytes studied are shown in Figure 1.20.

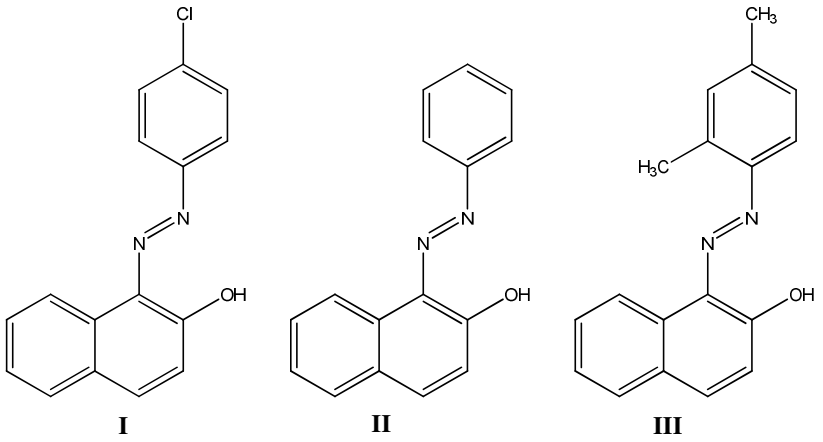



**Figure 1.20: I 1-(4-chlorphenyl)azonaphthalen-2-ol (chloro-Sudan) the template analogue used for analysis of Sudan dyes, including II Sudan I and III Sudan II [175].**

Preliminary investigations in this work showed that there was significant bleed when Sudan I was used. For this reason, **I** in Figure 1.20 was synthesised for use as a 'template mimic'. This approach allowed for preparation of an imprinted polymer capable of the selective extraction of several banned Sudan dyes from five different food samples. While template bleed was still observed at a low level, approximately 0.01-0.05 µg/mL, the retention time in the analytical method was different than all of the other analytes allowing for quantitation of the dyes to as low a level as 0.75 µg/mL in spiked food samples.

### 1.7.2 MIPs in catalysis

Catalysis is a process whereby the rate of a chemical reaction is altered by the use of another substance, which itself, is not used up in the reaction. The ability to use MIPs as catalysts in chemical reactions has considerable appeal due to their ability to selectively bind a target analyte under a variety of conditions. While many reports of using substrates for a particular catalytic or enzymatic reaction as templates exist, such as *p*-nitrophenyl palmitate as a lipase substrate [220] and glutathione as a glutathione peroxidase substrate [221], a good deal of the work involving catalytic MIPs has involved the preparation of an imprinted polymer, specific for a particular transition state analogue, TSA. The MIP stabilises the transition state of a particular reaction to a

greater extent than the ground state [222]. This is analogous to the use of catalytic enzymes and results in an overall reduction of the activation energy for the process, thus enhancing the rate of reaction [1]. The ability of MIPs to selectively bind a target analyte in the presence of structurally similar molecules is essential, as the TSA may be similar in structure to either the reactant or the product or both.

The TSA approach has been employed for ester hydrolysis [223], class I and class II aldolase reactions [224, 225], transamination [226] and Diels-Alder reactions [227]. A recently reported example of the use of the TSA approach by Li *et al*. has employed the use of 4-nitrophenyl phosphate imprinted sites to create a catalytically active and thermosensitive polymer for the hydrolysis of 4-nitrophenyl acetate [228]. This positively thermosensitive polymer used the interaction between poly(2-trifluoromethlyacrylic acid) and poly(1-vinylimidazole) to prepare a polymer that possessed catalytic capability at 40 °C but had a significantly reduced capability at 20 °C.

As discussed in section 1.3.4, stoichiometric non-covalent strategies are particularly well suited to catalytic applications due to the use of strong 1:1 template to functional monomer interactions, reducing the need for excess functional monomer thus allowing for precise positioning of the functional groups and reducing non-specific interactions. An example of the transition state analogues and substrates employed by Wulff *et al*. for the preparation of catalytic MIPs using stoichiometric non-covalent methods is shown in Figure 1.21.

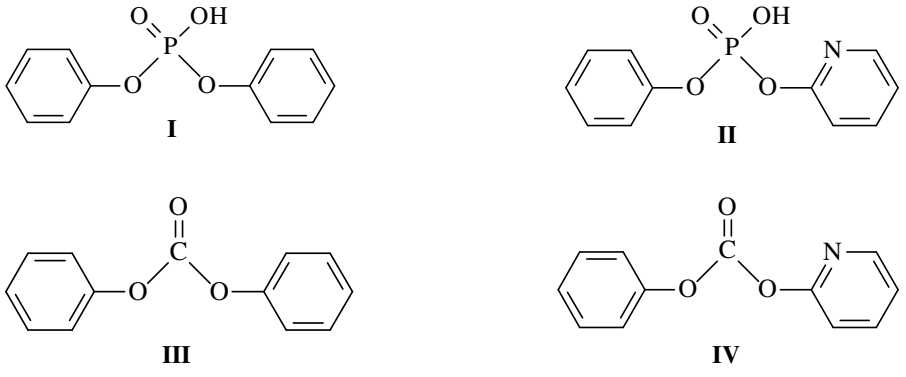



**Figure 1.21: Transition state analogues, I and II, and substrates, III and IV, used by Wulff *et al*. in the preparation of MIPs with carboxypeptidase A activity [83, 229].**

The tetrahedral templates presented in Figure 1.21 were used to mimic the tetrahedral transition state intermediate in the hydrolysis reactions of the substrates. This enabled the preparation of molecularly imprinted polymers that were capable of enhancing the rate of carbonate hydrolysis by a factor of up to 410 000 [83]. While this rate enhancement is atypically high for MIPs, with rate enhancements of 20-50 commonly reported [225, 226], it serves to show the capabilities of molecularly imprinted catalysts.

### 1.7.3 MIPs in drug delivery

The use of molecularly imprinted polymers in drug delivery has received considerable attention since early work by Sreenivasan proposed the use of MIPs as drug retaining matrices with controllable release properties [97] and Norell *et al.* investigated their use as sustained release platforms [230]. The drugs used as templates in both studies were, respectively, the corticosteroid, hydrocortisone and the methyl xanthine, theophylline.

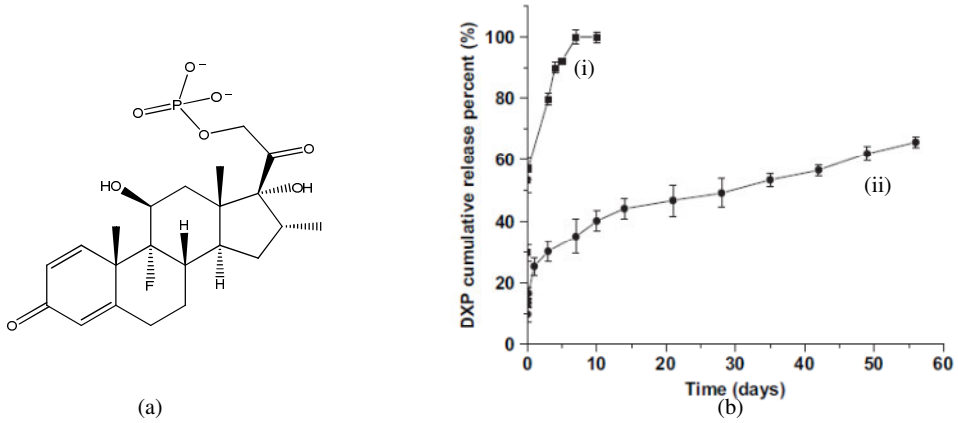
Norell *et al.* showed that at pH 7.0, the rate of release of theophylline was $4 \times 10^{-5}$ mmol $g^{-1}$ $min^{-1}$ for the NIP and $3 \times 10^{-5}$ mmol $g^{-1}$ $min^{-1}$ for the MIP [230]. While it was noted that the difference in behaviour between the imprinted and non-imprinted system was relatively small, it served to demonstrate the potential use of MIPs in sustained release drug delivery systems (DDS).

Sreenivasan showed that 95% of the rebound template, hydrocortisone, was retained in the imprinted polymer matrix after thirty days in a methanol/water mixture compared to almost 80% released after 1 day in the NIP [97]. Subsequent work on hydrocortisone imprinted polymers demonstrated the potential of MIPs as template responsive release systems [231]. In this study testosterone was loaded onto the hydrocortisone MIP and its rate of release was monitored both with and without the presence of hydrocortisone in the release solution. To 100 mg of MIP, 175 µg of testosterone was rebound and during release studies where no template was present only 79 µg was released after 24 hours. When hydrocortisone, the template, was present in the release solution, 171 µg of testosterone were released in 4 hours.

These early demonstrations of the potential of MIPs in drug delivery appear to have coincided with a change in focus in the development of DDS towards ‘intelligent

therapeutics', where drug delivery systems are now required to respond to the individual patient's needs by releasing drugs in response to a particular location, environment or the presence of biological triggers [232]. It has been proposed that it is in meeting these demands that the technology of molecular imprinting may have its greatest potential [233]. A number of strategies and approaches have been investigated in an effort to meet these demands.

One such strategy is the use of molecularly imprinted polymers as excipients in sustained delivery platforms, exemplified by the work of Wang *et al.* where polymers imprinted with dexamethasone-21 phosphate disodium (DXP) were used to release the anti-inflammatory drug, dexamethasone, for reduction of inflammation at the site of an implantable biosensor for glucose [234]. Indeed, the imprinted polymer was seen to release the template at lower pH, which would be typical of the site of inflammation. A composite was formed using a hydrogel, prepared from HEMA, and the imprinted nanospheres prepared by precipitation techniques. The use of the hydrogel/nanospheres composite prolonged the release of the template by about 50 days longer than the hydrogel on its own, as shown in Figure 1.22 (b).




(a) (b)

**Figure 1.22: (a) Structure of dexamethasone-21 phosphate disodium (DXP); (b) release profile of DXP from (i) hydrogel alone and (ii) hydrogel/imprinted nanosphere composite [234].**

The use of hydrogels in molecularly imprinting is an approach which allows for the direct synthesis of molecularly imprinted drug delivery platforms. Hydrogels are insoluble, cross-linked polymer network structures composed of hydrophilic co-polymers that have the ability to absorb significant amounts of water [235]. This ability to absorb significant quantities of water makes them suitable for use in biological

applications. The groups of Hiratani and Alvarez-Lorenzo have used hydrogels in the successful synthesis of soft contact lenses for the sustained delivery of drugs such as timolol and norfloxacin [118, 236, 237].

Biomimetic strategies, whereby an understanding of biological processes at the molecular level is exploited for use in synthetic systems [238], have been employed in the preparation of hydrogel based lenses and membranes [239, 240]. An example of this approach can be seen in the work of Ali *et al.* where hyaluronic acid, a polysaccharide used in the treatment of ocular discomfort and dry eye syndrome, was imprinted in soft contact lenses [241]. CD44 is a hyaluronic acid binding protein where the amino acids, asparagine, tyrosine and arginine are important for binding of the acid. The FDA approved monomers acrylamide, N-vinyl pyrrolidine and 2-(diethlyamino)ethyl methacrylate bear functional groups analogous to those present on the amino acids, as illustrated in Figure 1.23.

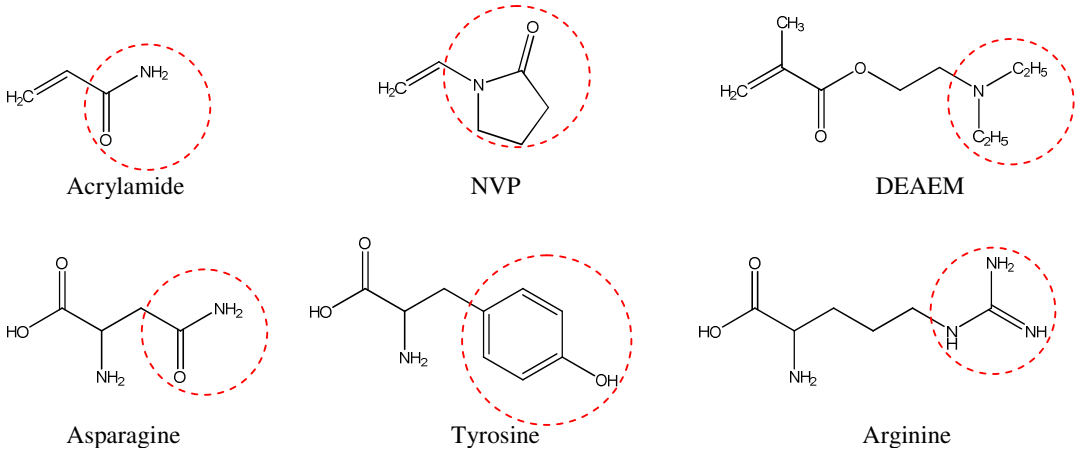



**Figure 1.23: Comparison of the functional groups present in both FDA approved functional monomers and amino acids important for the binding of hyaluronic acid (adapted from reference [241]).**

The use of a combination of the monomers shown in Figure 1.23 and careful optimisation of the polymer composition enabled the preparation of a contact lens capable of releasing hyaluronic acid at a therapeutic rate of 6 µg/h for 24 hours. The same group has also investigated the use of 'living/controlled' polymerisation (LCP) strategies to enhance the loading and delay the release of drugs from typically weakly crosslinked gels and showed that the release could be extended over a two-fold time period by using LCP compared to conventional free-radical methods [242].

The use of molecularly imprinted microspherical hydrogel beads has also been an important step in the development of MIP based drug delivery systems. Micro- and nanospheres are particularly suited to drug delivery as they typically have a large surface area to volume ratio and an interior network capable of incorporating drugs and biomolecules [243]. They can have a high degree of uptake into cells as well as causing minimal irritation if administered subcutaneously [244, 245]. These attributes make them likely candidates for the targeted drug delivery proposed by Sellergren and Allender whereby the MIP releases its drug load when it binds to its target on the cell wall or is internalised into the cell [233]. Precipitation polymerisation strategies are well suited for use in drug delivery applications due to the bead size obtained and the fact that, due to incompatibility between the final polymer and the solvent, the amount of residual organic solvent is drastically decreased [246].

Sustained release of drug molecules under biological conditions has been achieved by the group of Puoci *et al*. for the prodrug sulfasalizine [170], and the antineoplastic agent, 5-fluorouracil [72] while Javanbakht *et al*. have demonstrated the release of diypyridamole, which acts to inhibit thrombus formation [247]. The structures of these examples are presented in Figure 1.24.

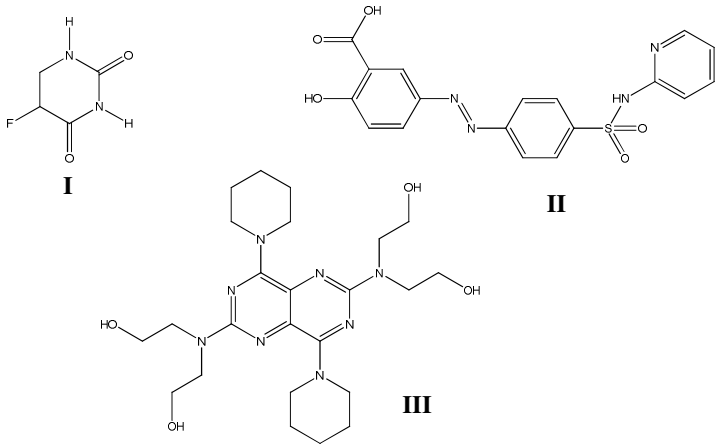



**Figure 1.24: Drug templates used in the preparation of beads by precipitation polymerisation. I 5-fluorouracil; II sulfasalizine; III dipyridamole [72, 170, 247]**

MAA-co-EGDMA polymers were used for *in vitro* release studies for 5-fluorouracil, where the same quantity of drug was reloaded onto both the MIP and the NIP. While all the drug was released from the NIP after 5 hours, the release of the drug from the MIP had still not been completed after 50 hours [72].

## *1.8 Scope of the thesis*

This study will investigate the preparation of molecularly imprinted polymers selective for corticosteroids, with a particular focus on budesonide and prednisolone as target compounds for recognition. The preparation of corticosteroid specific polymers in bulk format, as well as novel precipitated microspheres and microparticles will be outlined. A systematic study that investigated the factors affecting precipitation polymerisation will be described and the importance of these results in the achievement of highly controllable polymerisation strategies will be examined and discussed. An NMR investigation into the interaction between commercially available functional monomers and the weakly functionalised corticosteroids will be discussed. These studies will show that such interactions were weak and the significance of these findings and correlations between pre-polymerisation studies and chromatographic evaluation of the resultant polymer will be critically examined.

The ability to optimise these polymeric systems will be shown by their demonstrated applicability for solid-phase extraction and chromatographic separation of close structural analogues. The application of knowledge related to particle optimisation will be highlighted for the latter example, where control over particle size will be shown to be crucial for improving chromatographic resolution.

Results from this study will highlight the need for strategies to enhance the strength and number of corticosteroid selective binding sites within the matrix of molecularly imprinted polymers. Two approaches to achieve this will be investigated. The successful preparation of tailor-made monomers capable of increased interaction with the template in aqueous systems will be outlined and the potential to use these polymers in the preparation of corticosteroid MIPs prepared in aqueous based porogens will be shown. A semi-covalent strategy, with the potential to increase the number of steroid specific binding sites within the polymeric matrix by direct polymerisation of a template-monomer will be discussed and the application of morphology control to prepare uniform microspherical polymers with the successfully derivatised steroidal template-monomer, will be demonstrated.

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# Chapter 2

# Towards a budesonide specific molecularly imprinted polymer

# 2. Towards a budesonide specific molecularly imprinted polymer

## *2.1 Introduction*

### 2.1.1 Steroids

Steroids are an important class of biologically active compounds with a wide reaching range of biological functions [1, 2]. These compounds are based on a tetracyclic hydrocarbon structure with variation in the degree of saturation and substitution of the rings. The most widely recognised member of this class of compounds is cholesterol, a steroid which is metabolised to bile acids in the liver and functions as a precursor for the synthesis of steroid hormones. An example of the former is cholic acid, which is necessary for digestion to proceed in the intestine where this compound forms micelles with water-insoluble fats and fatty acids [1].

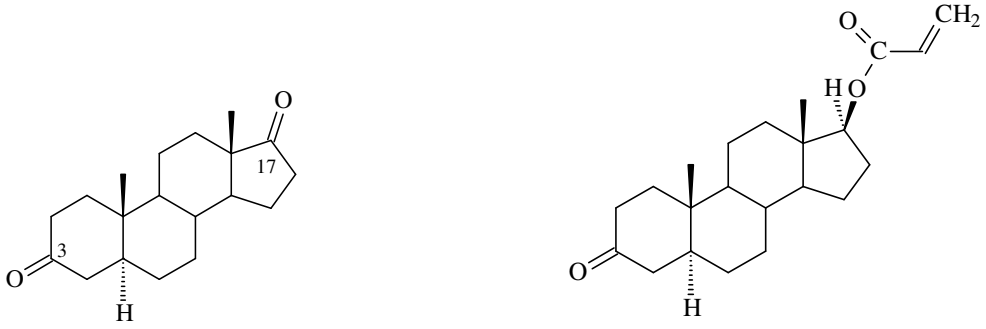
Steroid hormones are further divided based on their function [1]. Subdivisions include progestins such as progesterone, which are synthesised in the ovarian cells, androgens such as testosterone, important for the development of male characteristics and estrogens such as estradiol, which is responsible for the growth of female characteristics. Another such subdivision is the glucocorticoids, for example hydrocortisone, a steroid present in abundance during situations of stress and inflammation and which also has a suppressant effect on the immune system. Due to their obvious importance in biological functions, steroids are often an integral part of pharmacological formulations [2]. Steroids are used in the treatment of a diverse range of conditions, for example, asthma [3], Crohn's disease [4] and age-related macular degeneration [5]. While steroids have many beneficial properties, they can also be used as illegal growth promoters, as in the case of anabolic steroids [6] and estrogens can act as environmental endocrine disruptors, which are environmental pollutants that can have adverse affects on the health of humans and other organisms [7, 8].

### 2.1.2 Steroids in molecular imprinting

In molecular imprinting strategies the bulk of the polymer is typically hydrophobic, with specific binding sites created using functional monomers capable of interaction,

such as hydrogen bonding, with the template. Similarly, in biological molecules, such as the DB3 monoclonal antibody, the vast majority of the hydrophobic steroid structure is incorporated into hydrophobic pockets, where a small number of hydrogen bonding contacts effect further selectivity and interaction [1, 9]. The similarity of these situations and the dual positive and negative roles of steroids in areas such as medicine and environmental pollution, as well as an urgent need for the development of new analytical strategies for steroidal components of pharmaceutical formulations [10] serve to demonstrate why steroids are a suitable target for research into the preparation of biomimetic polymers within the MIP community.

Early imprinting work involving development of a polymer capable of the selective catalytic reduction of steroidal 3- and 17-ketones was carried out by Bystrom *et al.* [11]. The chemical structures of one of the template-monomers used in the covalent imprinting procedure and the corresponding target analyte, androstane-3,17-dione, are given in Figure 2.1.




**I** **II**

**Figure 2.1: I Androstane-3,17-dione, the steroid targeted for selective reduction using an imprinted polymer prepared with II, a polymerisable template-monomer [11].**

In this study polymerisable template monomers, such as **II** in Figure 2.1, were used to create imprinted polymers that allowed for the selective reduction of either the 3- or the 17-ketone positions on selected substrates. This was exemplified by the use of a steroid with diketone functionality, Figure 2.1 **I**, where it was possible to completely direct reduction to the 17-position using an imprinted polymer. Conversely, when in solution without the polymer, 99% of the product was reduced at the 3-position.

Semi-covalent methods were later employed for cholesterol by Whitcombe *et al.* [12] (discussed in detail in Section 1.3.3.1) and the first steroid to be imprinted by non-covalent means was hydrocortisone in the work of Ramström *et al.* [13] (discussed in detail in Section 1.3.2). Since this early work, a number of steroids have been studied and molecular imprinting technologies have been investigated for a number of purposes. Figure 2.2 presents a number of the steroids that have been investigated to date while examples of the steroid imprinted polymers that have been prepared are presented in Table 2.1.

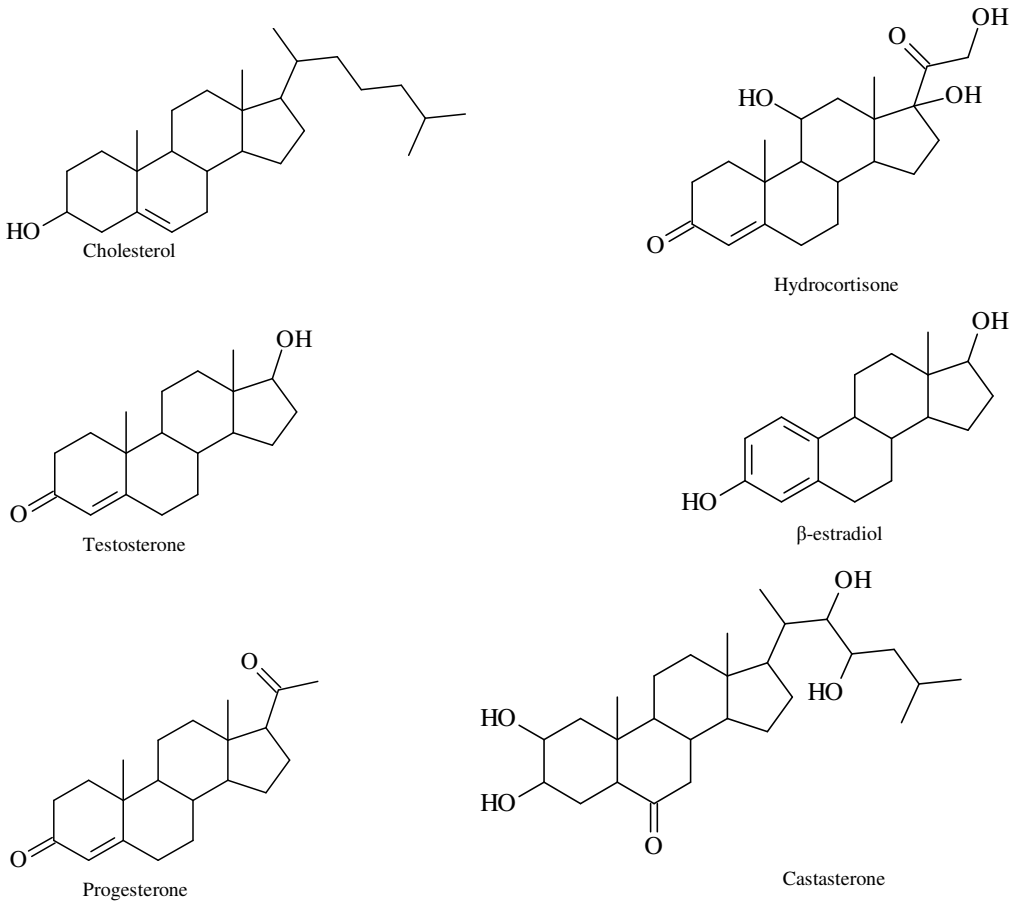



**Figure 2.2: Structures of some commonly imprinted steroids.**

**Table 2.1: Examples of some steroid selective molecularly imprinted polymers that have been reported in the literature.**

| Steroid | Template monomer interaction | Monomers | | Polymerisation strategy | References |
|---|---|---|---|---|---|
| | | Functional monomer | Crosslinking monomer | | |
| Cholesterol | Non-covalent | MAA | TRIM | Precipitation | [14] |
| | Non-covalent | MAA | EGDMA | Seeded suspension | [15] |
| | Semi-covalent | Synthesised from chitin | Toluene 2,4-diisocyanate | Bulk monolith | [16] |
| | Non-covalent | MAA | EDGMA/PEGDMA | Hydrogel film | [17] |
| β-estradiol | Non-covalent | TFMAA | TRIM | Precipitation | [7] |
| | Non-covalent | MAA | DVB/TRIM | Precipitation | [18] |
| | Covalent | Synthesised | DVB/EDGMA | Precipitation | [19] |
| | Non-covalent | MAA | TRIM | Precipitation | [20] |
| | Non-covalent | MAA | DVB/EGDMA | Precipitation | [21] |
| Testosterone | Non-covalent | MAA | EGDMA | Bulk monolith | [22] |
| | Non-covalent | MAA | EGDMA | Bulk monolith | [23] |
| | Non-covalent | MAA | EGDMA | Bulk monolith | [24] |
| Hydrocortisone | Non-covalent | MAA | EGDMA | Bulk monolith | [13], [25] |
| | Non-covalent | HEMA | EGDMA | Bulk monolith | [26] |
| | Non-covalent | HEMA | EGDMA | Bulk monolith | [27] |

Figure 2.2 and Table 2.1 serve to demonstrate that a number of steroids have been used as templates for the creation of MIPs prepared in a variety of formats. Much of the molecular imprinting of steroids has been carried out with a view to the chromatographic separation of steroids [25, 28] where the absolute binding capacity for the imprinted polymers is not of any particular relevance and as such, is often not calculated. Such applications aside, the absolute binding capacity is still rarely quoted and in instances where it is, the values are predominantly low, for example 2.9 mg/g for hydrocortisone [27] and 5 mg/g for testosterone [22]. In the majority of cases, relative values, i.e. values for the MIP versus the NIP, rather than absolute values are quoted. In these cases the conditions used for rebinding experiments may be a clue to the binding capacity. This is exemplified by the work of Ye *et al.* [20] and Wei *et al.* [21] where 30 mg of polymer was added to 1 mL of solvent containing 417 fmol of template. These relatively low capacities suggest that the creation of steroid selective binding sites within polymers is no trivial matter. The relevance of the binding capacity would be dependant on the end application, for example, MIPs for trace environmental analysis or sensing applications would not require a high capacity, however, long-term controlled release may require higher polymer loading capacities.

### 2.1.3 Strategies for the synthesis of steroid imprinted polymers

As demonstrated in Table 2.1 a number of different polymer formats have been used for the synthesis of steroid specific MIPs and while the majority of examples presented in this table are comprised of bulk monolith or precipitation, other formats have also been employed. Cholesterol has been imprinted by Lee *et al*. using polystyrene microbeads for seeded suspension polymerisation [15]. This template has also been imprinted by Spizzirri and Peppas [17] using hydrogels prepared from MAA as the functional monomer and either EGDMA or PEGDMA as crosslinking agents. These examples notwithstanding, the majority of steroid imprinting has been carried out using either bulk monolith or precipitation methods [7, 8, 20, 21, 29-31].

A number of studies have compared the performance of steroid imprinted MIPs that have been prepared using different strategies. One such study was that carried out by Ye *et al.* where imprinted polymers were prepared for 17$\beta$-estradiol by bulk monolithic and precipitated microspheres [32]. MAA was used as the functional monomer in all cases

while EGDMA was used as the crosslinking monomer in both methods. Additional microspheres were prepared using TRIM as the crosslinking monomer. Acetonitrile was the solvent used for all polymer synthesis. In the case of the monolithic polymer the MIP bound 30% of the added template while the NIP bound <13%, i.e. the MIP bound more than twice as much as the NIP. In the case of the microspheres using EGDMA as the crosslinking monomer, the MIP bound only 14% of the added template but the NIP in this case bound almost five times less template, at 3%.

While the bulk monolithic polymers bound more template than the microspheres, the binding was far more selective in the case of the microspheres. In an effort to improve the binding capacity of the microspheres, TRIM was used as crosslinking monomer to reduce the content of non-binding monomer in the composition of the polymer. The use of the tri-functional monomer TRIM allowed for a reduction in the percentage of crosslinker used, thereby increasing the percentage of functional monomer in the polymer composition. In this case, the polymers bound approximately 50% of the added template while the control microspheres bound three times less than this. Thus, with careful tailoring of the properties of the microspheres it was possible to achieve both a higher selectivity and a higher binding capacity than with the polymers prepared as bulk monoliths.

In a similar study, Wei and Mizaikoff compared the performance of 17$\beta$-estradiol imprinted polymers that were prepared in three formats: bulk monolith, microspheres and sub-microspheres [33]. They investigated the performance of the polymers in three areas: release studies, equilibrium binding and non-equilibrium binding. During equilibrium binding studies it was observed that in all cases the control NIPs bound less of the template than the MIPs but the control microspherical polymers rebound less template than the other two control polymers. This corroborates the findings of Ye *et al.* in that less non-specific binding occurred in the case of the microspheres (*vide supra*). Using binding isotherms based on the Freundlich model the microspheres showed a higher median binding affinity constant ($1.3 \times 10^{-2} \pm 4.8 \times 10^{-4}$ $mM^{-1}$) than the values for the bulk particulate ($9.0 \times 10^{-3} \pm 5.3 \times 10^{-4}$ $mM^{-1}$). During release studies the polymer (20 mg) was placed in 1 mL of acetonitrile and the amount of template released was determined by HPLC. The kinetics of template release was deemed to be comparable for all three formats but it was observed that overall, more template was

released from the microspheres. A possible reason for this was the increased mobility of the microspheres, thereby improving extraction conditions. Given the suggested superior performance of microspheres prepared by precipitation methods, and its relevance to this study, it is considered worthwhile to expand on the introduction to this method presented in Section 1.5.2.

### 2.1.4 Precipitation polymerisation

Early work on the preparation of polymeric microspheres without the use of stabilisers and surfactants was carried out by Naka *et al.* [34, 35]. This work involved the use of diethylene glycol dimethacrylate as the monomer and initiation was carried out by exposure of the monomers to ionising radiation. This yielded microspherical particles in good yields with a high degree of monodispersity. Findings in this early work pointed to the importance of the total monomer concentration with respect to solvent and above a certain 'critical' concentration, 'gelation' occurred and a polymeric gel was formed rather than microspherical particles. This was attributed to the ability of the polymer network to grow at a rapid rate under the conditions used. The evidence also suggested that 'good' solvents for the monomer, i.e. solvents with solubility parameters close to that of the monomer, were good solvents for the preparation of microspheres. The size of the microspheres ranged between 0.4 and 4.8 μm, depending on the solvents used.

A series of studies carried out by Stover *et al.* investigated the precipitation polymerisation of DVB to form microspheres between 2 and 7 μm [36-38]. In contrast to the work of Naka *et al.*, an initiator was used to carry out the polymerisation. In the first of these studies [36] Li and Stover stated that the precipitation of the microspherical particles was due to the decreased level of solubility of the growing polymer because of the rising degree of crosslinking. This was later termed 'entropic' precipitation [38].

In the initial study, the effect of monomer concentration, solvent type, initiator type and concentration were all investigated. It was found that here, as in the work of Naka *et al.*, that above a certain monomer concentration (in this case 5 vol%) coagulation of the particles occurred, to form either space-filling gels or coagulum and it was again

suggested that this was due to increased solvating power of the continuous phase leading to increased likelihood of coagulation.

Increasing the concentration of initiator increased the conversion of monomer but was incomplete even at high initiator concentrations while the type of initiator also had a profound effect on the conversion and size distribution of the particles. Later work [37] showed that the use of toluene as a co-solvent produced porous microspheres with surface areas of 800 $m^2/g$, in sharp contrast to values of approximately 9 $m^2/g$ for polymers prepared in neat acetonitrile. This was attributed to the fact that in a poor solvent for the growing polymer, such as acetonitrile, phase separation occurs early and leads to non-porous polymeric microspheres whereas the addition of toluene delays the onset of phase separation causing the entrapment of some solvent in the growing polymer ultimately leading to pore formation.

Ye *et al.* [20] first introduced the use of precipitation in the formation of molecularly imprinted polymers. Both theophylline and 17-$\beta$-estradiol were imprinted in polymers prepared using MAA and TRIM as the functional and crosslinking monomers respectively. Microspheres in the range of 0.2-0.3 μm were prepared in this fashion. Since this initial imprinting work with precipitated microspheres, a wide range of templates have been imprinted using this polymer format including the steroid dexamethasone [31], phenols [39], the alkaloid cinchonidine [40] and the antineoplastic agent, fluorouracil [41].

In addition to the work outlined above, comparing MIPs prepared by precipitation methods to those prepared by bulk monolith methods, Mayes and Pérez-Moral carried out a comprehensive investigation of the various polymeric formats used in molecular imprinting [42]. In this study five polymeric formats were investigated: bulk; suspension in fluorocarbon solvent; two-step swelling, emulsion core-shell and precipitation. MAA was the functional monomer used while EGDMA was used as the crosslinker and all of the polymers were imprinted with the β-blocker, propranolol.

In the radioligand binding assays that were used, the imprinted precipitation polymer re-bound 53% of the template in the toluene solution in comparison to the control polymer which bound only 3%. This suggests that the binding in the imprinted polymer was

almost 100% specific. The bulk imprinted polymer bound only 39% of the template in toluene. In aqueous solution, the imprinted precipitation polymer bound 43% of the template in aqueous buffer (the second highest percentage rebound) but none of the binding was specific, i.e. the same amount of template was bound to both the MIP and the NIP.

In 2011 the work of Mohajeri *et al*., performing a comparison of bulk monolith particles and precipitated beads for clozapine, showed improved specificity for the precipitated polymers with an imprinting factor of 3.26 compared to that of 1.95 for the bulk particles [43]. Scatchard analysis showed that the binding sites of the precipitated polymers were more homogenous than those of the bulk particles, with only one dissociation constant observed for the beads while two were observed for the bulk particles. The binding affinity was also stronger towards the beads, with the dissociation constant of only 0.45 μM for the beads but a value 32 times higher at 14.5 μM for the bulk particles.

#### *2.1.4.1 Advantages and disadvantages of precipitation polymers*

There are a number of advantages and disadvantages associated with the use of precipitation strategies and these are summarised in Table 2.2.

**Table 2.2: Advantages and disadvantages of the use of precipitation polymerisation**

| Advantages | Disadvantages |
|---|---|
| Controllable monodisperse particles in the micron and sub-micron range are easily synthesised. | Possible pressure build up on chromatographic columns due to small particle size. |
| No grinding or sieving necessary | Optimisation necessary for each polymer composition. |
| No additional surfactant needed. | |
| Rapid synthesis allowing for readily optimised conditions. | |
| High yield of usable polymer. | |

It is also worth noting that spherical particles are well suited for use in polymer based drug delivery systems [44]. Typically having a large surface area to volume ratio and an interior network capable of incorporating drugs and biomolecules [45], micro- and nanospheres can have a high degree of uptake into cells as well as causing minimal irritation if administered subcutaneously [4, 46].

## *2.2 Research aims and objectives*

The aims and objectives of this chapter were as follows:

- To prepare molecularly imprinted polymers for budesonide, a corticosteroid used for respiratory, bowel and eye conditions and which has been studied for lung cancer treatment.
- To determine the most suitable monomer composition based on previously reported MIPs for structural analogues of budesonide.
- To adapt the selected monomer composition for the preparation of polymers using precipitation methodologies outlined in the literature.
- To determine the factors affecting the polymerisation and thus optimise the conditions for the preparation of microspherical polymers.
- To develop a microspherical molecularly imprinted polymer capable of the selective uptake and release of budesonide.

## *2.3 Experimental*

### 2.3.1 Materials

**Table 2.3: List of materials employed during the course of this study.**

| Reagent | Assay | Supplier |
|---|---|---|
| 2-Hydroxyethyl methacrylate | 98% | Sigma-Aldrich |
| Ethylene glycol dimethacrylate | 98% | Sigma-Aldrich |
| Methacrylic acid | 99% | Sigma-Aldrich |
| Divinyl benzene | 80% | Sigma-Aldrich |
| 2,2'-Azobis(2-methylpropionitrile) | 98% | Acros |
| 1,1-Azo bis (cyclohexanecarbonitrile) | 98% | Sigma |
| 4',4-azobis(4-cyanovaleric acid) | 98% | Sigma |
| Acetonitrile | >99.9% | Romil |
| Acetonitrile-$d_3$ | 99.8 atom% D | Sigma-Aldrich |
| Toluene | >99.9% | Romil |
| Dichloromethane | >99.9% | Romil |
| Chloroform | >99.8% | Sigma-Aldrich |
| Chloroform-$d_3$ | 99.8 atom% D | |
| Methanol | >99.9% | Sigma-Aldrich |
| Acetic acid | >99.8% | Fluka |
| Acetic acid-$d_4$ | 99.5 atom% D | Sigma-Aldrich |
| Tetrahydrofuran | >99.8% | Romil |
| Dimethyl sulfoxide | ≥99.5% | Riedel-de-Haen |
| Budesonide | 98% | Eirgen Pharma |
| Prednisolone | 99% | Sigma |

All reagents from Sigma-Aldrich were obtained from Sigma-Aldrich (Dublin, Ireland) while all of the solvents from Romil were obtained from Lennox Laboratory Supplies (Dublin, Ireland). All solvents used were of HPLC grade. AIBN was obtained from Acros Chimica (Geel, Belgium) and was recrystallised from acetone prior to use.

Inhibitor removal was carried out for MAA by vacuum distillation at 80 °C; EGDMA was purified by sequentially washing with 10% NaOH, saturated NaCl, dried over magnesium sulphate and vacuum distilled at 120 °C; DVB was purified using a basic alumina column. HEMA was used as received and all monomers were stored at -20 °C prior to use.

### 2.3.2 Instrumentation

**Table 2.4: Instrumentation used for this study**

| Instrument | Model | Supplier |
|---|---|---|
| Photochemical mini-reactor (8 W lamps) | RMR-600 | Branford, CT, USA |
| UV-Vis spectrophotometer | UV-2401 PC | Shimadzu, Japan |
| Optical microscope | Olympus CH20 | Optical Co. Ltd., Japan |
| Microscope camera | MiniVid | LW Scientific, USA |
| Scanning electron microscope | Hitachi S-2460N | Oxford instruments, England |
| Shaking incubator | C24 | New Brunswick Scientific, USA |
| HPLC | 1200 | Agilent Technologies, Ireland |
| Particle size analyser | Mastersizer 2000S | Malvern, Ireland |
| NMR spectrometer | EX 400 | Jeol, England |

### 2.3.3 Preparation of polymers

The polymers were prepared in accordance with Table 2.5.

**Table 2.5: Polymer compositions and polymerisation conditions used for this study.**

| Polymer | Template | Functional monomer | Crosslinking monomer | Molar ratio [a] | Solvent | Polymerisation conditions |
|---|---|---|---|---|---|---|
| M1 | BDN | MAA | EGDMA | 1:10:50 | MeCN | UV; 350 nm 4 °C |
| | | | | | | Thermal 60/80 °C |
| M2 | BDN | MAA | EGDMA | 1:10:30 | MeCN | Thermal 60/80 °C |
| M3 | BDN | HEMA | EGDMA | 1:10:50 | MeCN | Thermal 60 °C |
| M4 | BDN | MAA | EGDMA | S[b]:10:50 | MeCN | Thermal 60 °C |
| M5 | PRD | MAA | EGDMA | 1:10:50 | MeCN | Thermal 60 °C |
| M6 | BDN | MAA | EGDMA | 1:10:50 | MeCN/TOL | Thermal 60 °C |
| | | | | | 3:1 and 4:1 (v/v) | |
| M7 | BDN | MAA | DVB | S[b]:10:50 | MeCN/TOL | Thermal 60 °C |
| | | | | | 3:1 and 4:1 (v/v) | |
| M8 | ____ | ____ | DVB | N/A | MeCN/TOL | Thermal 60 °C |
| | | | | | 3:1 (v/v) | |
| M9 | PRD | MAA | DVB | 1:10:30 | MeCN/TOL | Thermal 60 °C |
| | | | | | 3:1 (v/v) | |
| M10 | PRD | MAA | DVB | 1:10:50 | MeCN/TOL | Thermal 60 °C |
| | | | | | 3:1 (v/v) | |

[a] molar ratio of template:functional monomer:crosslinking monomer; [b] saturated solution of template in porogen used to drive complex formation

Polymers were prepared in accordance with Table 2.5. For ease of observation, the non-imprinted polymers are not included in the table; in all cases they were prepared in the same manner but without the presence of the template. Typically, the template and functional monomer were added to the solvent, sonicated then placed in ice and allowed to equilibrate for 20 minutes. The crosslinking monomer and initiator were then added. Further sonication was carried out to ensure a homogenous solution and the pre-polymerisation mixture was purged with $N_2$ gas for 5 minutes while placed in an ice bath. Total monomer concentration was adjusted from 0.5-6% (w/v) by varying the volume of the solvent or the total mass of the monomers.

Polymerisation was carried out either by thermal initiation or UV initiation. Thermal initiation was carried out in a shaking water bath with gentle agitation (20 RPM) where the temperature was raised from room, to the required temperature (Table 2.5) over a 2 hour period. UV initiation was carried out using Rayonet photochemical mini-reactor at 4 °C and the polymerisation vial was rotated about its long axis at 45 RPM.

Upon completion of polymerisation the polymers were filtered using 0.45 μm PTFE Cronus membrane filters from Antech (Waterford, Ireland). They were then rinsed with 50 mL of each porogen used. The polymers were then suspended in 4:1 MeOH:AcOH at a concentration of 1 g of polymer/50 mL of solvent. They were stirred with gentle heating for 2 hours, filtered and rinsed with three 30 mL aliquots of hot MeOH. This was repeated twice and the polymers were then suspended in hot MeOH at the same concentration and stirred for 2 hours, filtered and rinsed with 100 mL of hot MeOH. The removal of template and polymeric bleed was monitored by UV-Vis spectroscopy using the UV-2401 PC UV-Vis spectrophotometer from Shimadzu (Japan). The polymer particles were finally rinsed with HPLC grade acetone and allowed to dry for 48 hours at 35 °C.

*(Note on polymer notation: Throughout the text, M denotes precipitation polymer, MB bulk polymer, while N denotes precipitated non-imprinted polymer and NB, non-imprinted bulk polymer. The number, e.g. 1, 3, etc. serves to denote the formulation used. For example, N3 would be the non-imprinted polymer prepared using BDN, MAA and HEMA in a 1:10:50 ratio. Where only the formulation is discussed and not a comparison of MIP vs. NIP, M or MB will be used).*

### 2.3.4 Scanning electron and optical microscopy

Optical microscopy was carried by depositing the beads on a microscopic slide with gridlines 50 μm in length. Optical micrographs were obtained using the MiniVid microscope camera and images recorded using the ScopePhoto software program. These were used for preliminary basic particle size measurements. The size of the particles was determined as follows: using an optical micrograph obtained as described, the image of the 50 μm line on the slide was physically measured and the size of polymeric particles were calculated based on this physical measurement. Where a single value is quoted values were calculated by getting an average value for ten polymeric particles per image and an average of three images per polymer formulation

Prior to scanning electron microscopy (SEM) the polymers were sputter-coated with gold.

### 2.3.5 Equlibrium binding studies

For polymers M1-M7, dry polymer (50 mg) was placed in 1, 5 or 10 mL of a solution of the template used in polymer synthesis, ranging in template concentration from 0.0175 mM to 1 mM. The suspended polymer solution was then placed in a C24 incubator shaker from New Brunswick Scientific (Edison, NJ, USA) at 20 °C at 300 RPM. After 24 hours the suspended polymer solution was centrifuged at 45000 RPM for 1 hour and the supernatant was filtered through 0.45 and 0.2 μm syringe filters. The rebinding analyses were carried out by measuring the absorbance of the rebinding solution at the $\lambda_{max}$ of the template and comparing it to the original template solution to determine the percentage of template bound. The supernatant was also compared to a solution of the polymer and the rebinding solvent only, in order to determine the level of polymeric bleed that was present. Rebinding analyses were carried out using UV-Vis spectroscopy.

Polymers M8-M10 were tested using 30 mg dry polymer in a 0.9 mL solution of the target analyte in 1.5 mL HPLC vials, in concentrations ranging from 2-140 μg/mL. Equilibrium binding conditions were as above but the supernatant was filtered into fresh HPLC vials and the concentration of unbound steroid was quantified using reverse phase chromatography.

HPLC was carried out using a method previously reported for budesonide analysis [47]. Here, an Agilent 1200 series system from Agilent Technologies was employed, using an Agilent 150 × 4.6 mm 5 µm Eclipse XDB-$C_{18}$ column and a mobile phase composed of 69:31 methanol-aqueous buffer (v/v). The aqueous buffer was prepared using 0.1% acetic acid at pH 3 with a flow rate of 1 $mL.min^{-1}$. The run time was 8 minutes. A standard curve was constructed for the steroids analysed by plotting the peak areas against the concentration. The method was linear from 0.25-20 $\mu g.mL^{-1}$. The amount of steroid bound to the polymer, was again calculated by subtracting the concentration of the free steroid from the initial known concentration. All injections were carried out in triplicate. Imprinting factors, IF, in all cases were calculated using the following formula:

$$IF = \frac{\%Bound\ MIP}{\%Bound\ NIP} \qquad \textbf{Equation 2.1}$$

Where, *IF* is the imprinting factor and *%Bound MIP* and *%Bound NIP* are the percentages of the steroid bound to the imprinted and non-imprinted polymers, respectively.

### 2.3.6 Particle size analysis

Particle size analysis was carried out using a Malvern Mastersizer 2000S laser diffraction liquid particle size analyser. MeOH was used as the dispersant and a stirring speed of 1750 RPM was applied. 20 mg of dry polymer was suspended in 1 mL of MeOH and sonicated for 5 minutes. The polymer solution was then added to the dispersant until the laser obscuration was between 7 and 9%. Where particle aggregation was observed, sonication was applied until no evidence of aggregation was present. The optical properties were empirically determined post-analysis to obtain the lowest residual value, giving the best fit between the predicted and observed values. The d(0.1), d(0.5), d(0.8) and d(0.9) values were determined and particle size values are given as a range between d(0.1) and d(0.9), thus giving a size range that contains 80% of the particles analysed. Where aggregation persisted the size range given was determined based on the values between the d(0.1) and d(0.8) values, which contained 70% of the sample analysed. In a small number of cases, where particle aggregation

could not be prevented the size range quoted is between d(0.1) and d(0.5). All values given are an average of three analyses.

### 2.3.7 NMR analysis

Preliminary $^{1}H$ NMR spectra were recorded on a Bruker spectrometer operating at 500 MHz and a temperature of 25 °C, while a Jeol EX 400 spectrometer operating at 400 MHz at 18.5-18.9 °C was used for the bulk of the work. Chemical shifts were referenced to the TMS peak. Where relevant deuterated acetic acid, AcOD, was used as a functional analogue of MAA.

#### *2.3.7.1 Job plot analysis*

Equimolar solutions of the template and the functional monomer (or functional analogue) were prepared at the following concentrations: AcOD and BDN in $CDCl_3$: 0.04 M; AcOD and BDN in $CD_3CN$: 0.02 M; HEMA and BDN in $CDCl_3$: 0.03 M; HEMA and BDN in $CD_3CN$: 0.02 M. The ratios of the template:monomer were systematically varied between 1:9 and 10:0. The total concentration, of the monomers and template, was kept constant while the volume was held at 0.75 mL. The shim map was determined by carrying out a 'manual gradient shim' on the 8:2 template:monomer solution followed by fine tuning in manual mode. This was carried out for each separate Job plot analysis, which utilised 64 scans and 32768 data points per spectrum. The spectra were analysed and the data used to construct a plot of: $\Delta\delta \times \chi_{BDN}$ vs. $\chi_{BDN}$. Where $\Delta\delta = \delta_{free\ template} - \delta_{obs}$ and $\chi_{BDN}$ is the mole fraction of budesonide in the solution.

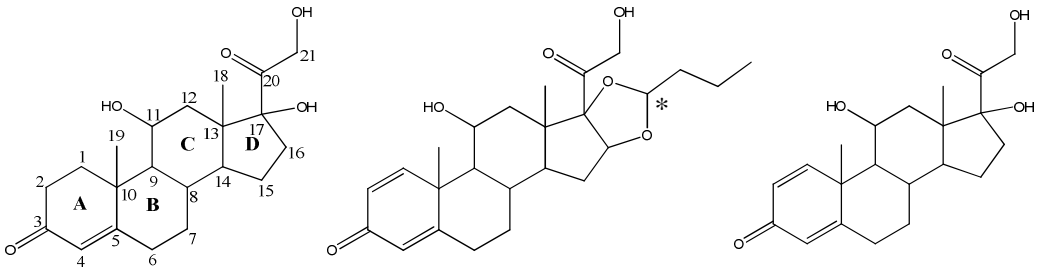
#### *2.3.7.2 NMR titration*

To a 500 μL sample of BDN (0.03 M) in $CDCl_3$, a mixture of BDN (0.03 M) and either AcOD (8.73 M) or HEMA (4.12 M) was added incrementally until there were 110 equivalents of AcOD or 90 equivalents of HEMA present. The shim map was determined by carrying out a manual gradient shim on the free template solution and tuned manually when necessary thereafter. 16 scans and 16384 data points were utilised to obtain the NMR spectra. The changes of the chemical shift values of the BDN protons were monitored and association constants were obtained using Prism, GraphPad software 4.0.

## *2.4 Results and discussion*

### 2.4.1 Initial polymer formulation

Given the advantages of the use of polymeric beads prepared by precipitation polymerisation outlined in Section 2.1.4 and the successful use of this method for imprinting steroids as outlined in Section 2.1.3, it was decided to use this polymer format for the synthesis of budesonide imprinted polymers. Budesonide was chosen as a target due to its use in asthma [3], Crohn's disease [4] and age-related macular degeneration [5]. This potent corticosteroid had, hitherto, not been used in molecular imprinting protocols, thus it was necessary to find a close structural analogue that had been successfully imprinted and adapt the methodology. This is often used as an initial guideline for the development of imprinting protocols [21]. The closest structural analogue observed in the literature was hydrocortisone which, as outlined in Section 2.1.2, has been successfully imprinted by the groups of Ramstrom *et al*., Baggiani *et al.* and Sreenivasan [13, 25, 26]. The structures of hydrocortisone and the two steroids used in this study, budesonide and prednisolone, are given in Figure 2.3.




Hydrocortisone Budesonide Prednisolone

**Figure 2.3: Structures of hydrocortisone, budesonide and prednisolone, demonstrating the structural similarity of the three compounds. C* is the epimeric carbon in budesonide.**

The structures of these steroids are quite similar, with OH groups on the C11 and C21 positions in all molecules as well as carbonyl groups at C3 and C20. Further, all three molecules contain conjugation in the A-ring but while the A-ring of hydrocortisone is a cyclohexenone, having only one internal double bond, both budesonide and prednisolone have two internal double bonds and as such the A-ring is a cyclohexadienone. Hydrocortisone and prednisolone both possess a further OH at the C17 position while budesonide has an acetal grouping at the C16 and C17 positions. It

must also be noted that budesonide is prepared and used as a 1:1 mixture of epimers with the epimeric carbon marked '*' in Figure 2.3 above.

The monomers chosen for initial investigation were MAA and EGDMA, as these monomers are commonly used in imprinting and had been successfully employed by Ramstrom *et al.* and Baggiani *et al.* [13, 25] at template:functional monomer:crosslinking monomer ratios of 1:10:50 and 1:10:90, respectively. A similarly high ratio of functional monomer to template has also been utilised for the imprinting of estradiol [7], cholesterol [48] and testosterone [22]. This is necessary to drive the equilibrium towards interaction, in particular when there can be a relatively low degree of functionality in comparison to the size and rigidity of the molecule.

As discussed in Section 2.1.4, it is the crosslinking process that induces entropic precipitation of the polymeric beads [38]. To exploit this characteristic Cormack *et al.* have used a ratio of 1:5 for functional to crosslinking monomer for precipitation polymerisations using MAA and EGDMA as the monomers for 17β-estradiol [20]. With this in mind, this ratio of crosslinking to functional monomer was chosen as a starting point for this study.

As well as the importance of the total monomer concentration relative to the reaction solvent, typically below 5% for imprinted polymers [32, 37], the concentration of the initiator used also plays a vital role and is typically used at concentrations of 1-3% of the total number of moles of polymerisable double bonds [36, 49]. Given these parameters, a total monomer concentration of 4% (w/v) was initially used, as this allowed for small scale syntheses using 30 mL sample vials for polymerisation reactions having theoretical yields of approximately 1 g of polymer. An initiator concentration of 3 mol% was decided upon in an effort to promote the growth of microspheres and ACCN was used as the initiator during initial trials, as this had previously been used to carry out successful polymerisation reactions within the research group.

The solvents that were initially tested were selected due to their use in the imprinting of steroids [50] and hydrocortisone in particular [13]; their use in precipitation polymerisation [49] and their polarities. These solvents, in order of increasing polarity,

were: toluene, dichloromethane, THF, chloroform, acetonitrile and DMSO. Thermal initiation was carried out as described in Section 2.2.2 and of the seven solvents tested with this monomeric concentration, beads were formed in acetonitrile only.

In all other solvents the polymers formed had the appearance of bulk monolith polymers but appeared to be less densely crosslinked as they did not form rigid materials, being readily broken apart with a spatula. The observed results appeared to be analogous to what was described as 'gelation' by Naka *et al.* [34] where the cause was ascribed to a high percentage of monomers. Sherrington refers to this as macrogelation where the "mass of growing polymer molecules dissolved in solution becomes crosslinked into one infinite network", the result of which is a swollen monolithic soft gel filling the containing vessel [51].

In the present study, this will be termed a space-filling gel. The fact that space-filling gels were formed in the solvents other than acetonitrile, can be attributed to the fact that the monomer loading may have been too high or the growing polymer was too soluble in the solvent system used. This may have led to rapid formation of the growing polymer network, thereby allowing the polymer chain to grow rapidly in a short space of time [35, 36]. The swelling effect of chlorinated solvents on polymers and the resultant difficulties with preparing microspherical beads has also been documented [52]. Some preliminary investigations were carried out on the original space-filling gels formed in these solvents. In these investigations it was observed that upon drying, the polymers (contained in a 30 mL vessel) lost up to 18 g of solvent. Upon addition of 30 mL of solvent, the polymers again swelled to their original size and absorbed the same amount of solvent that had been lost on drying. This suggested that the polymers were indeed loosely crosslinked.

To investigate whether these space-filling gels were caused by the solubility of the growing polymer or the monomer concentration, the total monomer concentration in relation to the solvent was varied from 0.5-4% for all of the solvents. As the concentration was reduced the polymer morphology changed from an opaque space filling gel to a translucent gel-like polymer that was suspended in the solvent and could only be investigated upon evaporation of the solvent. This suggested that the growing polymer was too soluble in the solvent for the preparation of polymeric beads. For this

reason, solvents other than acetonitrile were no longer investigated for the M1 composition.

Acetonitrile was considered to be an acceptable solvent for use in further investigations because of its initial success in bead formation and due to its previous use for imprinting steroids in general [53] and steroids in precipitation polymerisation [7, 20, 33]. Seminal work on the effect of the porogen on the morphology of polymers used for molecular imprinting by Sellergren and Shea quote MeCN, while being a polar solvent, as having poor hydrogen bonding capacity [54]. This would suggest that the interference with hydrogen bonding between the template and the functional monomer could be minimised. The M1 formulation was synthesised as outlined in Table 2.5, using thermal and photochemical initiation at 60 and 4 °C, respectively. SEM images of the imprinted and non-imprinted beads obtained from thermally and photochemically initiated polymerisations are shown in Figure 2.4.

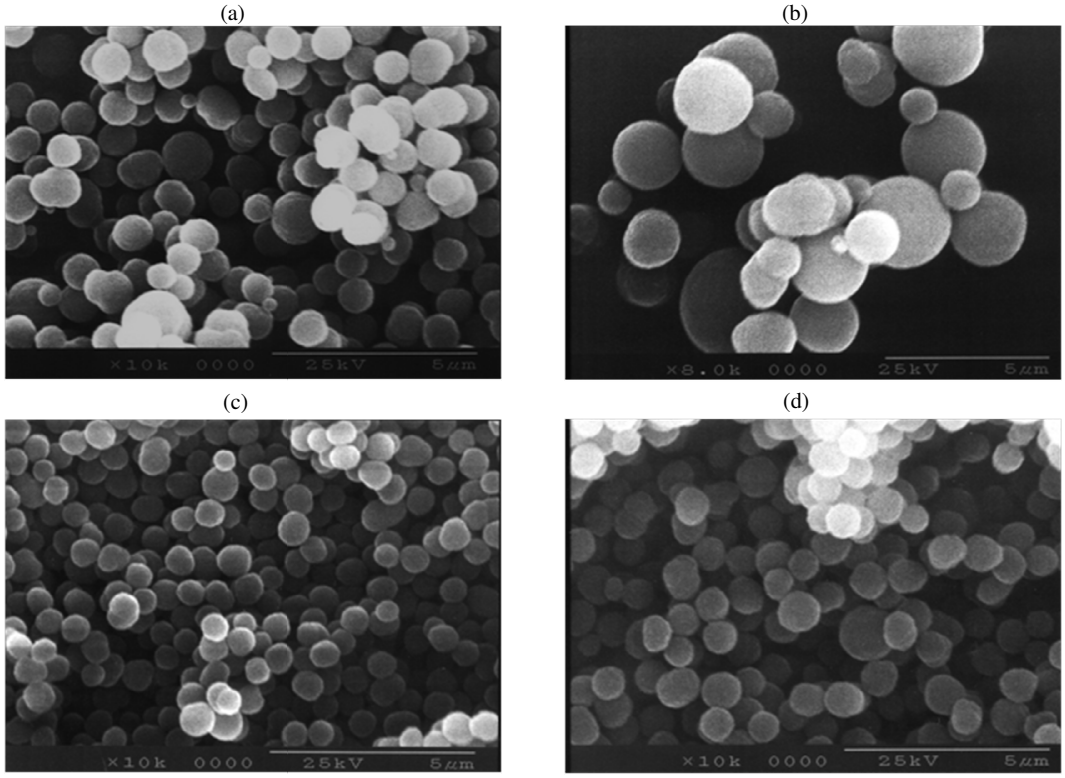



**Figure 2.4: Microspherical and sub-microspherical beads prepared with the M1 formulation at 4% (w/v) monomer concentration and 3 mol% ACCN as initiator. (a) M1 by thermal polymerisation; (b) N1 by thermal polymerisation; (c) M1 by UV polymerisation; (d) N1 by UV polymerisation.**

Figure 2.4 shows that for imprinted and non-imprinted polymers, spherical beads in the micron to sub-micron size range were formed in acetonitrile using thermal and photochemical polymerisation. Figure 2.4 also demonstrates that in both cases the imprinted polymer differs in size to the non-imprinted polymer. Particle size analysis showed that the thermally initiated MIP had a size ranging from 0.6-2.1 µm while the NIP had a range of 1.6-5.2 µm. For the UV initiated polymers the ranges were 0.6-1.9 µm for the MIP and 0.8-2.8 µm for the NIP.

Similar differences in particles size have been reported for a number of templates, e.g. sulfasalazine, where the MIP was larger than the NIP [44], theophylline, where the MIP was smaller than the NIP [55] and cinchonidine, where the non-imprinted polymers were monodisperse but those prepared in the presence of the template had a high degree of polydispersity [40]. While Wang *et al.* demonstrated that the morphology of the polymer was not affected to a large extent by the presence of the template [55], Yoshimatsu *et al*. discussed the effect of particle growth when the template was present in the pre-polymerisation solution [56]. They attributed this in part to the different forms of MAA in the pre-polymerisation solution for the MIP and the NIP. In the absence of the template, MAA can exist as the free monomer or as a dimer, whereas when the template is present interaction between the template and the monomer also plays a role in particle growth. Lai *et al*. also noticed a difference in the bead size of the MIP and NIP when imprinting di(2-ethylhexyl)phthalate [57]. In this instance, the difference in bead size was attributed to a difference in polarity of the polymerisation solution brought about by the presence of the template.

Another notable point observed in Figure 2.4 is that the beads formed during UV polymerisation were more monodisperse than those prepared by thermal methods but were much smaller also. This was confirmed by particle size analysis for the non-imprinted polymers in both cases (unless otherwise stated, discussion of particle size will state the size of the NIP as this negates the involvement of the template and demonstrates the effect of the polymerisation conditions on the polymeric system in question). The polymer prepared by thermal initiation had a particle size range of 1.6-5.2 µm while the UV initiated polymers had a particle size range of 0.8-2.8 µm. It is necessary to note that differences in particle size from the dry (optical or scanning

electron microscopy) or solvated state (particle size analysis) are due to different swelling behaviours of the polymers. The particle size distributions of the polymers are shown in Figure 2.5.

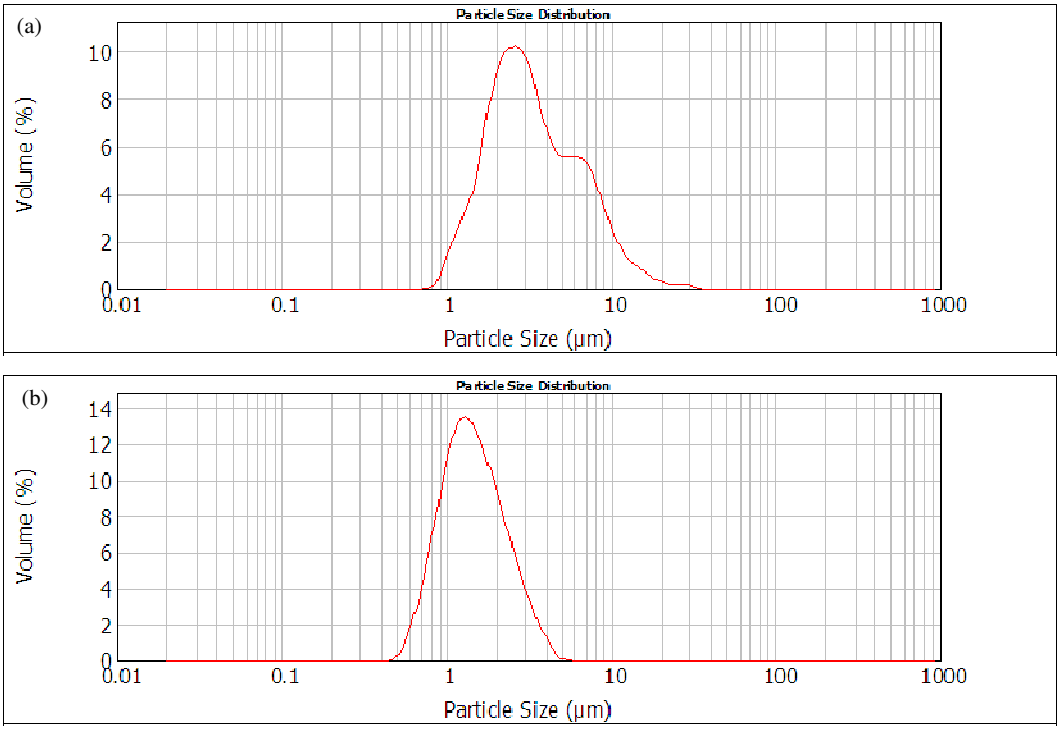



**Figure 2.5: Particle size distribution of (a) the thermally initiated N1 polymer and (b) the photochemically initiated N1 polymer.**

It has been suggested that particle growth is dependent on exhaustion of the initiator, which in this case could indicate that there is a difference between the rate or level of initiation between UV and thermal methods [36]. It is possible that dissociation of the initiator occurs almost instantaneously for photochemical initiation causing the particles to start forming at the same time, whereas in thermal initiation, there may be some heterogeneity in terms of heat transfer. This would suggest a difference in the efficiencies of the initiator based on the method of initiation. This possible difference could determine the number and respective sizes of the beads, depending on the number of particles that start growing and the time at which they begin to grow. While temperature will affect the solubility of the growing polymer and may delay precipitation of the particles, Downey *et al*. suggest that the growth mechanism involves reaction of the vinyl groups on the surface of the particle with oligomers still dissolved in solution [38].

It was decided to employ simple UV-Vis spectroscopy to carry out equilibrium binding studies. To ascertain if this was a suitable method of analysis, the polymer was suspended in the rebinding solvent at the concentration at which the studies were to be carried out and to then investigate if there was any material present that would interfere with the absorbance spectrum of the template. During the course of this investigation it was observed that there was a considerable amount of material leaching out of the polymer after template extraction and washing procedures had been carried out. This was observed by leaving 50 mg of the treated polymer in the rebinding solvent for 24 hours. The result of this is presented in Figure 2.6.

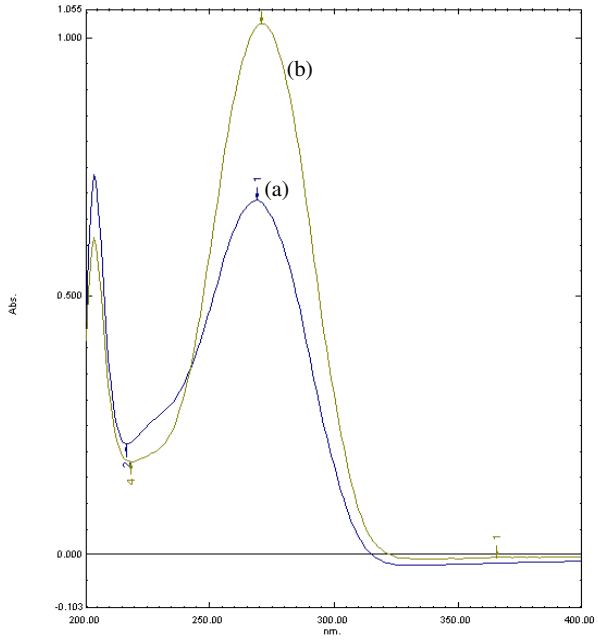



**Figure 2.6: UV-Vis spectra of the (a) M1 and (b) N1 thermally initiated polymers after initial washing was carried out. The peaks are at 210 nm and 270 nm.**

Figure 2.6 is representative of the absorbance spectra obtained for the filtrate obtained from a suspension of polymer left overnight in solvent. The peaks were at 210 nm and 270 nm, which are the $\lambda_{max}$ values for EGDMA and ACCN respectively. The polymers were analysed further using GC-MS based thermal desorption studies [58]. This allowed for an investigation of the material that was leaching from the polymer. A representative spectrum is shown in Figure 2.7.

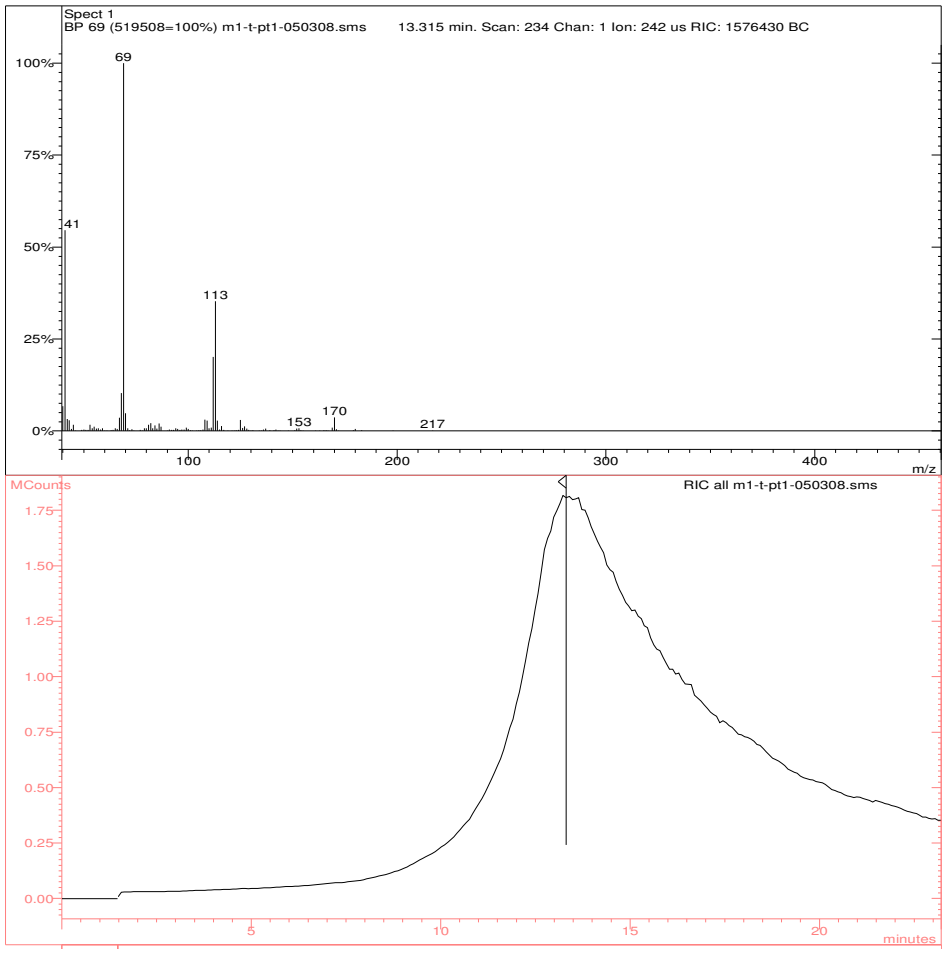



**Figure 2.7: Representative GC-MS thermal desorption profile for the M1 polymers with the fragmentation pattern showing evidence of the presence of EGDMA, having peaks with m/z values of 69, 113 and 170.**

GC-MS thermal desorption profiles similar to that depicted in Figure 2.7 suggested that the main component of the polymeric bleed was the crosslinking monomer, EGDMA. This was not unexpected, as this was the main component of the polymers. It was postulated that this unreacted crosslinker was due to poor dissociation of the initiator and the presence of absorbance peaks at both $\lambda_{max}$ values was due to oligomers and growing polymer chains with initiator molecules at the chain end. The possible poor dissociation of the initiator was proposed to be related to the temperature at which polymerisation was carried out. Since the decomposition temperature of ACCN was 88 °C, the synthesis was repeated at 80 °C (the boiling point of MeCN is 79 °C).

The microspherical polymeric particles prepared at 80 °C resulted in beads that were readily prepared, tested and cleaned of any partially polymerised material. The

increased temperature produced smaller beads, with particle size ranges of 0.46-1.15 µm and 0.46-1.76 µm for the MIP and NIP, respectively. This may have been due to the fact that greater rate of initiator dissociation had taken place, producing more radical centres thereby creating a greater number of beads but with a smaller average size. Tunc *et al*. have recently seen a similar effect when raising the polymerisation temperature from 60 to 80 °C for polymers using EGDMA or TRIM as the crosslinking monomers [59]. They also attributed the decrease in bead size to an increase in the number of radicals and, thus a smaller average bead size. This was similar to using UV as the method of initiation, which was further evidence that the original difference between thermal and UV initiation was due to the initiation efficiency. SEM images in Figure 2.8 demonstrate the difference in polymer morphology in the dry state.

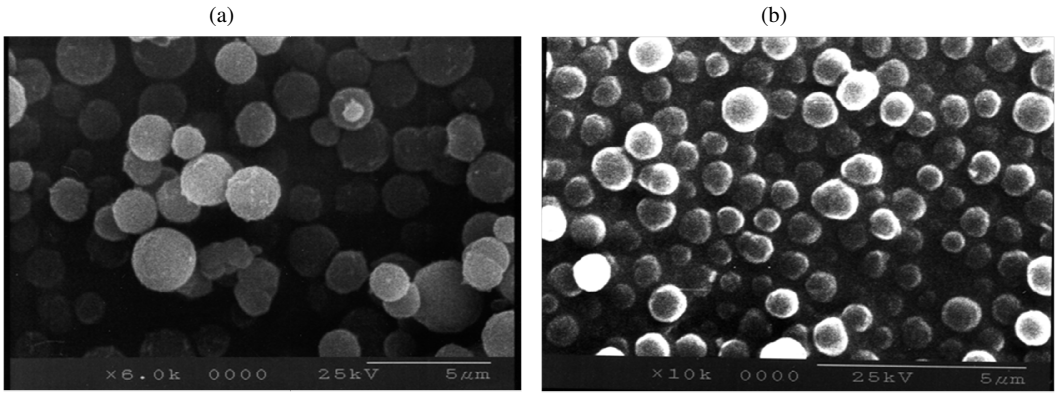



**Figure 2.8: SEM images of (a) N1 using ACCN as initiator and prepared at 60 °C and (b) N1 using ACCN as initiator and prepared at 80 °C.**

*2.4.1.1 Equilibrium binding studies on the M1 formulation*

In order to test the polymers for selectivity and binding capacity the equilibrium binding studies in the porogen in which the polymers were prepared were carried out. Conventional wisdom in molecular imprinting states that rebinding is considered to be most successful in the porogen in which the polymer is made. Shea and Spivak postulate that the swelling of the polymer in the porogen gives the desired functional group proximity and location and therefore enhances binding site fidelity [60].

The studies were carried out as described in Section 2.2.4. Briefly, 50 mg of dry polymer were placed in 5 mL of template solution, agitated for 24 hours, centrifuged and the supernatant was filtered and analysed to test the concentration of template. Template solutions of 0.07 mM and 0.035 mM were prepared in MeCN. Using a

template solution of 0.035 mM equates to carrying out the binding study at a concentration of 1.5 mg of budesonide per gram of polymer. This would allow for a very low binding capacity for the imprinted polymers. A sensitivity study was carried out for the UV-Vis spectrophotometer and it was shown that a drop in concentration of only 5% (equating to a binding capacity of only 0.075 mg/g) could be observed. No affinity, either selective or non-selective was observed in the rebinding studies.

To ascertain the cause of this apparent lack of affinity a number of different rebinding strategies were applied. The first of these was to increase the concentration of the template in solution in a bid to drive the equilibrium towards binding. Concentrations of 0.5 mM and 1.0 mM were then tested. The binding studies were carried out exactly as before but in order to read the absorbance of the rebinding solvent, it had to be diluted prior to analysis. Again, no affinity was observed.

It was then postulated that the affinity of the template for the solvent was greater for the polymer. In order to test this, rebinding was carried out in a mixture of MeCN and $H_2O$ in a 4:1 ratio, as BDN is practically insoluble in $H_2O$. This again showed no affinity. MeOH was also tested to determine whether or not a different polar solvent would drive the mainly hydrophobic template towards the relatively hydrophobic polymer. This again proved unsuccessful. At this stage it was decided to try a range of different polymer formulations and template analogues in an attempt to effect affinity for budesonide in a polymer.

### 2.4.2 Systematic synthesis and testing of polymers M1-M7

Once the initial polymer formulation had proved unsuccessful over a range of template concentrations and rebinding solvents, it was decided to systematically test a range of polymers by varying one factor at a time, to ascertain what properties were responsible for the lack of affinity and to use this information to prepare a budesonide selective polymer. A detailed list of the compositions of these polymers is presented in Table 2.4 while a summary of the results is presented in Table 2.6.

**Table 2.6: Systematic investigation of polymer properties and their effect on template affinity (particle size ranges include smallest to largest for both MIP and NIP combined).**

| Polymer formulation | Property investigated | Change to polymer composition | Affinity studies | Particle size (µm) |
|---|---|---|---|---|
| M2 | Effect of the degree of crosslinking | Ratio of crosslinker to functional monomer changed from 5:1 to 3:1 | No affinity observed | 0.3-1.2 |
| M3 | Affinity of functional monomer for template | Functional monomer changed from MAA to HEMA | No affinity observed | 1.7-4.7 |
| M4 | Concentration of template in pre-polymerisation solution | Polymerisation carried out in a saturated template solution | No affinity observed | 0.5-3.6 |
| M5 | Effect of template on affinity for polymer | Template changed from budesonide to prednisolone | No affinity observed | 1.5-3.7 |
| M6 | Polarity of solvent/ morphology of polymer due to solvent | MeCN/Toluene mixtures used instead of MeCN alone | No affinity observed | 0.2-0.9 |
| M7 | Effect of crosslinking monomer | DVB used as crosslinking monomer instead of EGDMA | No affinity observed | 0.5-6.6 |

The polymers outlined in Table 2.6 were prepared in an effort to systematically investigate the properties of the polymer and method of polymerisation that may be responsible for the apparent lack of affinity for the template.

As discussed in detail in Section 2.4.1 the high degree of crosslinking monomer is typical for imprinting steroids. It was considered that this high degree of crosslinking monomer in the polymer composition may have caused inaccessibility to the binding sites. For this reason, the M2 polymers were prepared where the monomers remained the same but the ratio was changed from 1:10:50 to 1:10:30. No affinity was observed; ruling out problems with the degree of crosslinking monomer and the 1:5 ratio of functional to crosslinking monomer was continued for future investigations at this stage. It was deemed possible that the monomer may not have sufficient affinity for the template and might have caused the lack of affinity for budesonide. Since HEMA had been used successfully as a functional monomer for the structural analogue of the target, hydrocortisone [26], it was decided to test this as the functional monomer, for the M3 composition but using the same template to monomer ratio of 1:10:50. Again, MeCN was used as the porogen. While microspherical beads of a suitable size, 1.7-4.7 μm, were obtained, no affinity was observed.

In early work on imprinting steroids by precipitation Mosbach *et al*. reported the use of a saturated template solution to enhance complex formation in such dilute conditions [20]. M4 polymers were prepared using the same ratio of functional to crosslinking monomer but with a saturated template solution. While this appeared to have the effect of forming more monodisperse polymeric beads, in the range of 0.5-3.6 μm, it did not produce affinity in the resultant polymer, with no binding of the template observed to either MIP or NIP.

In order to determine whether it was the template itself causing the lack of affinity, i.e. due to lack of functionality or steric issues, the M5 formulation was tested. This formulation incorporated prednisolone (Figure 2.3), a close structural analogue of budesonide, which instead of the acetal grouping on the C16 and C17 positions, has an extra OH at C17. The composition of the polymer was the same in all other respects but again, no affinity was observed for either the imprinted or the non-imprinted polymer.

It has been reported that the use of MeCN alone can lead to non-porous polymeric particles when preparing precipitated beads using DVB as the crosslinking monomer [37]. In that study toluene was used as a co-solvent to impart porosity, as it was for EGDMA based polymers by Puoci *et al.* [44]. M6 polymers were prepared in 3:1 and 4:1 mixtures of MeCN/Toluene. It was also hoped that the presence of a non-polar co-solvent would enhance hydrogen bonding between the monomers and the template. Neither of these factors appeared to improve affinity and again, none was observed during rebinding studies.

M7 polymers were prepared using divinyl benzene as the crosslinking monomer to investigate the role of the crosslinking monomer. It was suggested that perhaps repulsion of the hydrophobic template from a relatively polar polymer molecule was taking place. This was also tested due to the fact that Wallimann *et al.* state that steroids are best bound to biological molecules comprised mainly of hydrophobic aromatic amino acid side chains incorporating a number of hydrogen bonding sites for interaction [1]. The ratio of functional to crosslinking monomer used for M7 polymers was 1:5 and budesonide saturated MeCN/TOL mixtures were used in 3:1 and 4:1 ratios. No affinity was observed for these polymers.

After the systematic study on a range of polymer compositions based on factors reported in the literature to affect polymerisation and affinity, a polymer with affinity for budesonide had not been successfully synthesised. Although a budesonide specific polymer was not prepared, during the course of synthesising polymers M1-M7 a good deal of information regarding the factors that affected the polymerisation process was obtained. This information would prove beneficial for the rapid optimisation of polymers once baseline affinity was achieved. The following sections present a summary of this information.

### 2.4.3 Factors affecting polymerisation

Starting from the initial M1 formulation a number of factors were varied to determine their impact on the resultant polymers. These factors included the type and concentration of components, such as the initiator and the monomer, as well as the solvent composition. Since no affinity was observed for any of the polymers formed,

this could not be used as a guide to the success or otherwise of a polymer formulation. For this reason it was necessary to outline a set of parameters by which the success of the polymer formulations and conditions could be judged. To this end, for a polymer to be considered successful it was decided that the synthesised polymers must have the following properties:

- Bead size in the range of 0.5-10 μm (beads <0.5 μm could not be readily cleaned and analysed due to pore size of filters, etc.)
- The template must be readily extracted.
- The level of polymeric bleed after washing should be negligible (polymer bleed would be an undesirable characteristic for applications such as drug delivery)

Based on these guidelines the following factors were investigated for their affect on polymerisation conditions in this systematic study.

*2.4.3.1 Concentration and type of initiator*

As stated in Section 2.1 above, the role of the initiator is an important one in the preparation of polymers by precipitation methods [36, 38] and during the course of these investigations, this was seen to be the case, as the type and concentration of the initiator had a profound effect on the size and monodispersity of the polymeric beads. Figure 2.9 shows the structures of the three initiators used in this study.

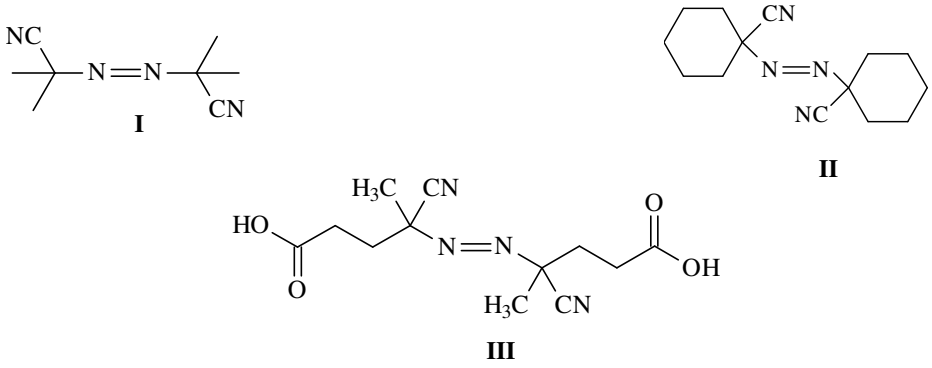



**Figure 2.9: Structures of the initiators used in this study: I azobisisobutyronitrile (AIBN); II 1,1'-azobis(cyclohexanecarbonitrile) (ACCN); III 4,4'-azobis(4-cyanovaleric acid) (CVA).**

The 10 hour half-life decomposition temperatures of the initiators used in this study are 88 °C, 65 °C and 70 °C for ACCN, AIBN and CVA, respectively. While these temperatures are given for specific solvents, they serve as a guide as to the relative decomposition temperatures. A summary of the main observations on varying the initiator type are presented in Table 2.7.

**Table 2.7: Summary of the observations made upon investigation of type of initiator**

| Polymer formulation | Solvent | Initiator[a] | Bead size[b] (µm) | Observation |
|---|---|---|---|---|
| M1 | MeCN | ACCN (80 °C) | 0.5-1.8 | CVA produced the most monodisperse beads of the largest size at the lowest temp |
| | | CVA | 1.6-5.4 | |
| | | AIBN | 0.5-1.3 | |
| M3 | MeCN | ACCN (80 °C) | 1.7-4.3 | CVA produced the largest beads at the lowest temp and were most readily cleaned |
| | | CVA | 1.7-4.7 | |
| M7 | MeCN | AIBN | 2.2-4.1 | AIBN produced the biggest particles for both solvent compositions |
| | | CVA | 1.7-3.1 | |
| | MeCN:TOL (3:1) | AIBN | 3.2-6.6 | |
| | | CVA | 1.3-3.5 | |

[a] Initiator concentration was maintained at 3 mol% and temperature was 60 °C in all cases except where otherwise stated.
[b] Size range quoted for non-imprinted polymers only.

The variation in the bead size of the various formulations shown in Table 2.7 demonstrates that the initiator plays an important role in the resultant bead size when other parameters are kept constant. The first point to note is that due to the higher decomposition temperature of ACCN compared to CVA and AIBN, it was necessary to carry the polymerisation out at a higher temperature, to ensure complete polymerisation and to produce particles with a low level of polymeric bleed, as discussed in Section 2.4.1. Since this proved to be the case for both M1 and M3, the use of ACCN was

discontinued as high polymerisation temperatures could interfere with the interaction of the functional monomer and the template during polymerisation.

The use of CVA produced larger beads in the EGDMA based M1 formulation, 1.6-5.4 µm, than the use of AIBN, 0.5-1.3 µm. In the DVB based M7 formulation the reverse was observed with beads of 2.2-4.1 µm and 1.7-3.1 µm for AIBN and CVA, respectively, in MeCN and 3.2-6.6 µm and 1.3-3.5 µm for AIBN and CVA, respectively, in the MeCN/TOL mixture.

An explanation of the results observed is not straightforward, due to the complex factors involved in polymer particle growth, but a discussion of the fundamental aspects of polymerisation processes and precipitation methods in particular may be beneficial. During polymerisation, particle size is related to the rate of initiator decomposition and if the rate of decomposition is high, a large number of growing reactive centres or nuclei will be generated, which may lead to a higher rate of termination and low molecular weight polymers [59, 61]. Lower molecular weight polymer molecules will be more soluble in the polymerisation solvent and this will affect critical polymer chain length, thereby influencing the size of the polymer particles that precipitate [62].

In looking at the effects of the initiators in the two examples above, a direct comparison between the rates of decomposition cannot be made as the opposite effects took place with each formulation. This would indicate that the differences in bead size are related to the monomers used rather than the initiator. It may suggest, however, that the decomposition temperature or rate of initiation is altered by a change in the properties of the solvent, due to the presence of a particular monomer or set of monomers. While different rates of decomposition would lead to different degrees of aggregation early in the polymerisation, and therefore particle size, these effects may be offset by different rates of crosslinking, which is responsible for the stabilisation of particles against aggregation [36, 57]. This complex set of factors would suggest that for each polymer formulation, a number of small scale syntheses should be carried out to determine which initiator is most suitable for the particular formulation.

One notable difference, between precipitated beads and monolithic preparation strategies for imprinted polymers, is the amount of initiator used. The amount of

initiator is considerably higher in the former, with values of 1-3 mol% of the polymerisable double bonds commonly used [38, 49, 56]. The effect of the initiator concentration was investigated for the M1 formulation only and it was observed that upon increasing the concentration of the initiator from 1 to 3 mol% the bead size range increased from 0.8-1.5 µm to 1.6-5.4 µm. This difference is demonstrated in the images presented in Figure 2.10.

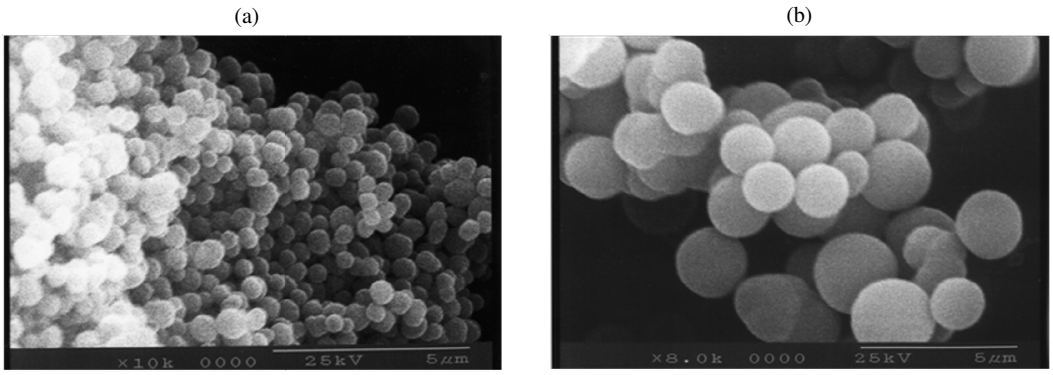



**Figure 2.10: Investigation into the effect of initiator concentration (a) M1 polymer prepared with 1 mol% CVA; (b) M1 polymer prepared with 3 mol% CVA.**

It is readily observed in Figure 2.10 (a) and (b) that the change in the concentration of the initiator produces quite a notable difference in the bead size of two polymers prepared under identical conditions. This effect has also been reported by Li *et al.* and Tunc *et al.* and has been attributed to a number of reasons [59, 62]. Firstly, an increase in initiator makes the reaction more exothermic during the initial phase of the polymerisation, possibly leading to the formation of larger particles. A second possibility is that with an increase in the concentration of the initiator, the rate of radical creation and chain termination could be increased thus decreasing the average molecular weight of the polymer. A decrease in the molecular weight and perhaps, the degree of crosslinking, would make the growing polymer chain more soluble in the continuous phase. If the growing polymer chain is more soluble in the continuous phase, entropic precipitation of the bead may be delayed, thereby allowing the polymer particle to increase in size before precipitation. This would suggest that where larger particles are required, a higher concentration of initiator should be employed.

*2.4.3.2 Monomer concentration*

During the course of this work the growing polymers resulting from the formulations used, proved to be too soluble in a number of the solvents tested and failed to form precipitated microbeads. These solvents were dichloromethane, chloroform, DMSO and THF and despite testing the total monomer concentration over a range of 0.25-4% no beads were formed. The following discussion outlines the cases where beads were formed and the effects observed upon varying the monomer concentration in these solvents.

The concentration of the monomers in relation to the total volume of the solvent plays an important role in precipitation methodologies and it is generally considered that the concentration should be from 2-5% (w/v) [49]. The varying effects of monomer concentrations in different polymeric systems prepared by precipitation methods have been reported [35, 36, 59, 62, 63]. These varying effects are attributed to different solvation properties of the solvents for the monomers and growing polymers, as well as the ability of the growing polymer chains to aggregate and form large networks before entropic precipitation takes place.

The effect of varying monomer concentration was studied and an example of where a change in the monomer concentration influenced the resultant bead size is illustrated in Figure 2.11 while the observations are summarised in Table 2.8.

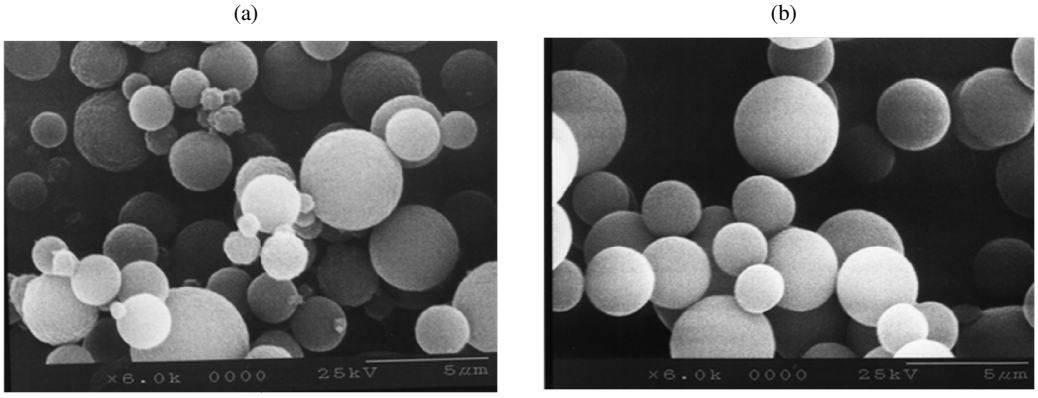



**Figure 2.11: Change in bead size observed upon increasing the total monomer concentration of M7 from (a) 2% (w/v) to (b) 4% (w/v) in MeCN/TOL 3:1 (v/v).**

**Table 2.8: Results obtained during investigation into the effects of monomer concentration[a]**

| Polymer | Solvent | Monomer Conc. (% w/v) | Polymer morphology[c] | Observation |
|---|---|---|---|---|
| M1 | MeCN | 6 | 0.8-2.3 | 4% (w/v) was the optimum monomer concentration |
| | | 5 | 0.6-1.5 | |
| | | 4 | 1.6-5.4 | |
| | | 3 | Soluble polymer | |
| | MeCN/ TOL[b] | 4 | SFG | This solvent composition produced no suitable particles |
| | | 3 | Mixture | |
| | | 2 | 0.2-0.8 | |
| | | 1 | 0.2-0.9 | |
| M3 | MeCN | 4 | SFG | 1% (w/v) was the optimum monomer concentration |
| | | 2 | Mixture | |
| | | 1 | 1.7-4.7 | |
| | | 0.5 | 1.3-2.3 | |
| M7 | MeCN | 4 | 1.8-3.42 | 4% (w/v) was the optimum monomer concentration for both solvent compositions |
| | | 2 | 0.2-2.1 | |
| | MeCN/ TOL (3:1) | 4 | 3.2-6.6 | |
| | | 2 | 1.5-6.1 | |

[a] Reaction conditions: CVA was initiator for M1 and M3, AIBN was the initiator for M7; Initiator concentration was 3 mol% of polymerisable double bonds and initiation temperature was 60 °C;
[b] Tested for ratio of 4:1, 3:1 and 2:1 and similar results observed .
[c] Bead size given in μm; SFG: space-filling gel.; Mixture: combination of coagulum, fused particles and spherical beads

As is demonstrated in Table 2.8 and illustrated in Figure 2.11, the monomer concentration had a considerable effect on the resultant polymer morphology, with many smaller particles observed for M7 using 2% (w/v) compared to 4% (w/v). The 2% (w/v) concentration also contained more fused particles and particles with a less defined shape than the 4% (w/v). This may have been due, in part, to the solubility of the growing polymer in the continuous phase. Li and Stover report that coagulation of DVB

microspheres is prevented by their crosslinked surface and that the particles precipitate out of solution, due to insolubility, before coagulation can occur [36].

In formulations such as M1 in MeCN/TOL mixtures and M3 in MeCN, it was observed that at high monomer concentrations a coagulum (or a space-filling gel) was formed, while at lower concentrations discrete particles were prepared. This may be a result of an increased solvating power (due to changes in the monomer concentration) of the continuous phase leading to swelling of the particle surface and an increased likelihood of coagulation [36].

In some cases a shift from discrete particles to coagulum with an increase of just 0.1% was observed when using TRIM as a crosslinker [59]. The increased monomer concentration would also mean an increase in the concentration of the initiator in relation to solvent volume, thereby causing a more exothermic environment which may also affect the solvating ability of the continuous phase, and so, produce the same effect. Naka *et al.* also relate the formation of coagulated material upon increased monomer concentration, to solubility in the solvent and the fact that the polymer network grows at a faster rate than that which would allow discrete spherical particles to precipitate [34, 35].

In the case of M3 it can be seen that at 2% (w/v) a mix of coagulated material, agglomerates and spherical particles were obtained but on further reduction to 1% (w/v) particles of 1.7-4.7 µm were observed and reduction again to 0.5% (w/v) gave particles of 1.3-2.3 µm. A similar effect was observed for the M7 formulation where a decrease in monomer concentration resulted in a decrease in particle size, i.e. from 3.2-6.6 µm at 4% (w/v) to 1.5-6.1 µm at 2% (w/v), an effect also observed by Yang *et al.* [63]. This could suggest that as the concentration is reduced the growing particles are too dilute in solution to allow them to capture nascent oligomers, thereby preventing particle growth. The opposite effect was observed for M1 polymers however, in that as the concentration was increased from 4 to 6% (w/v), the bead size dropped from 1.6-5.4 µm to 0.8-2.3 µm. As with initiator type, the most suitable monomer concentration must be determined using small scale syntheses to test the effects of varying monomer concentration.

An understanding into the control of the factors affecting polymerisation is necessary for optimising bead formation for the required application; at this stage, however, there was still no affinity, specific or otherwise, for the template. To gain a better understanding of why this was the case, nuclear magnetic resonance (NMR) spectroscopy, was employed to investigate the interaction between the components in the pre-polymerisation solution.

### 2.4.4 NMR Spectroscopy

NMR is one of the most widely used techniques for the investigation of molecular interaction in organic chemistry and may be used to obtain information regarding complex stoichiometry in solution and the extent of that binding based on association constants [64-66]. In order to determine the different factors involved in molecular recognition and interaction a number of different experiments may be conducted by NMR. The Job plot, or method of continuous variation, is commonly used for determination of the complex stoichiometry [64, 65, 67]. The construction of a Job plot involves the preparation of a series of solutions where the mole fraction of the substrate and ligand vary but the total substrate and ligand concentration, together with the total volume, remains constant. A measurable property, e.g. the chemical shift of a proton, which changes in relation to the complex formed, is monitored and plotted against the mole fraction of either component. The maxima of the resultant plots are capable of yielding the ratio of substrate to ligand.

An NMR titration is commonly carried out to determine the strength of interaction based on the binding constants [64-66]. Here, the concentration of the substrate remains constant while the change in the chemical shift of a chosen proton or carbon nucleus is monitored upon each addition of a ligand/template solution. The application of linear or non-linear mathematical models to the resulting data allows for the determination of the binding constants and hence, an indication of the strength of interaction.

Since the performance of non-covalent molecularly imprinted polymers is generally considered to be influenced by the number, nature and extent of the molecular interactions between the functional monomer and the template in the pre-polymerisation reaction solution [68-70], the use of NMR studies to determine the nature and strength

of these molecular level interactions is therefore a valuable tool in both understanding the processes involved in molecular imprinting as well as the rational design of MIPs in general.

With this in mind, NMR spectroscopy was used to study the interaction of the template with the functional monomers (or analogues thereof) used in the polymerisation strategies previously outlined, in a bid to understand the reasons for the lack of affinity observed to this point. The construction of Job plots was carried out to determine the stoichiometry of the complexes that could possibly be formed in the pre-polymerisation solution, while NMR titrations were carried out to determine the strength of these interactions. While HEMA was used in these experiments deuterated acetic acid (AcOD) was used as a functional analogue of methacrylic acid (MAA) as this has been previously used to simplify the resulting NMR spectrum [71]. This is particularly useful given the complex spectrum for budesonide and the potential for overlapping of the vinyl protons of MAA with similar protons in budesonide. To aid discussion of the results, the NMR spectrum of budesonide, its chemical structure with the relevant positions numbered and potential sites of hydrogen bonding interaction outlined, are presented in Figure 2.12. (Assignment of the relevant protons was carried out by $^{1}H$ and COSY NMR experiments and was aided by analysis of literature reports for analogous compounds [72-75]).

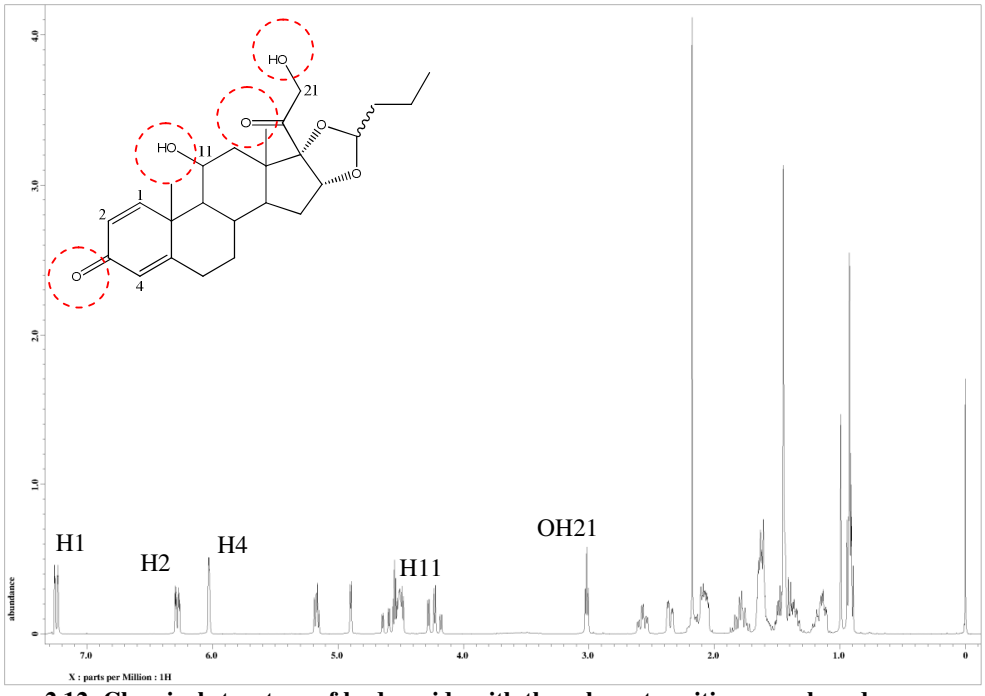



**Figure 2.12: Chemical structure of budesonide with the relevant positions numbered.**

*2.4.4.1 Job plot analyses*

For Job plot analysis it is recommended to use the highest concentration possible for the method of analysis employed [67]. During the course of this study, however, it was observed that both the solvent and the functional monomer, or functional analogue used, played a role in both the solubility of the mixture and maintaining an appropriate shim lock on the solvent. For this reason the concentration values had to be adjusted, not only on changing the solvent but also on changing the functional monomer. Despite careful adjustment of these values, there appeared to be some problems with maintaining a shim lock on the solvent. This seemed to have been caused in part by abrupt changes in the composition of the mixture upon varying the ratio of template to functional monomer and in part by the occasional formation of precipitate in the solvent.

The precipitate was also a problem observed by Ansell *et al.* upon titration of itaconic acid and ephedrine where, as here, it did not hinder the collection of NMR data [76]. To counter these problems a manual gradient shim was carried out on a mixture containing both the functional monomer and the template. The obtained shim map was then carefully fine-tuned by manual shimming to obtain the maximum lock on the mixture and in turn produce the most accurate spectrum. This was quite effective and allowed for the collection of reliable NMR data.

The Job plot analyses carried out looked at the interactions of the functional monomers in both acetonitrile and chloroform. In acetonitrile, the Job plots suggested no straightforward interaction between either HEMA or AcOD with no single maximum obtained for either monomer. This suggested that the polar solvent was preventing any meaningful or predictable interactions between budesonide and the monomers. Despite this setback, it was considered worthwhile to investigate any possible interactions that could take place in the less polar solvent, chloroform. For this reason, the remainder of the discussion is focussed on the interactions that were observed in deuterated chloroform. In the less polar solvent the situation was somewhat different, with points of interactions observed for both AcOD and HEMA and the template. Job plots of the type shown in Figure 2.13, for the interaction of budesonide and HEMA in $CDCl_3$, were observed in both cases and the results obtained for budesonide with AcOD and HEMA in $CDCl_3$ are summarised in Table 2.9.

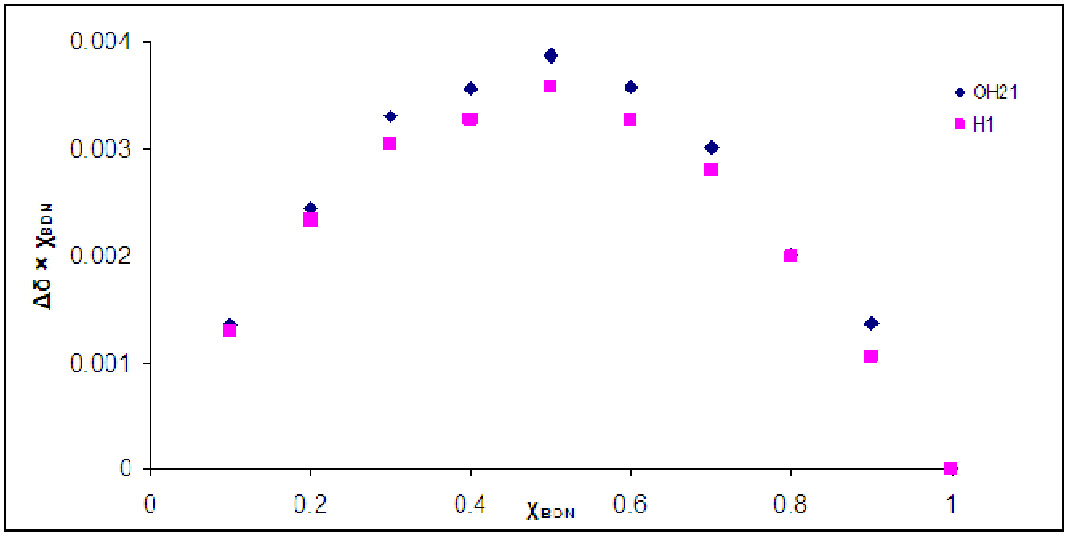



**Figure 2.13: Representative Job plot for the interaction between 0.03M BDN and 0.03 M HEMA in $CDCl_3$ with maxima observed at $\chi_{BDN}$= 0.5 for both plots.**

**Table 2.9: Summary of Job plot analyses for budesonide with AcOD and HEMA in $CDCl_3$.**

| Proton | AcOD | | HEMA | |
|---|---|---|---|---|
| | $\chi_{BDN}$ | Inference | $\chi_{BDN}$ | Inference |
| H1 | 0.3 | 2:1 complex due to interaction at C3 carbonyl and OH11 | 0.5 | 1:1 complex with interaction at OH11 |
| H2 | 0.5 | 1:1 complex with interaction at C3 carbonyl | n.d | |
| H4 | 0.5 | 1:1 complex with interaction at C3 carbonyl | n.d | |
| H11 | 0.5 | 1:1 complex with interaction at OH11 | ** | Possible interaction at OH11 |
| OH21 | * | Possible interaction at either C20 carbonyl or OH21 | 0.5 | 1:1 complex with interaction at OH21 |

* no plot constructed but signal shifted and changed from a triplet to a broad singlet
** complex multiplet that shifted but was unreliable for tracking and construction of plot
n.d.: no data

The results presented in Table 2.9 serve to demonstrate that interaction did take place between the monomers/analogues tested and the template. The results also suggest that the interactions between the template and the monomers were different from each other. For AcOD maxima were observed for H2 and H4 at 0.5 for the mole fraction of budesonide. This suggests a 1:1 complex between the template and the AcOD. This would be expected as the carbonyl on the A-ring of the steroid lies between these two protons and it would be expected that the acetic acid would interact at this point. The maximum for the H1 proton was at 0.3, suggesting a 2:1 interaction of monomer:template.

There was some evidence of interaction at the OH11 position with a 1:1 complex predicted by the construction of a Job plot for H11 and a maximum observed at 0.5. The possibility of interaction at the C3 carbonyl and the OH11 position could be a reason for the 2:1 prediction for the H1 proton. An example of this in the literature can be seen in the binding of 3-aminopyrazole with different amide protons of *N*-acetyl-*L*-valyl-*L*-valine methyl ester, as studied by Kirsten and Schrader and discussed by Wulff and Knorr [77]. Here the overlaid Job plots of the two amide protons showed a 1:1 complex forming for one of the protons and a 2:1 complex forming with a much lower overall change in chemical shift. This would be analogous to the observed interactions for BDN and AcOD in $CDCl_3$.

As well as the evidence suggesting two sites of interaction, analysis of the NMR spectra of the Job plots suggested a possible third site of interaction. For the NMR spectrum of budesonide alone, a triplet was observed at 3.03 ppm. Analysis of separate NMR spectra determined that this was the signal of the OH21 proton, which it is predicted, would be involved in intramolecular hydrogen bonding to the oxygen of the C20 carbonyl. This intramolecular bond enables the splitting of the signal by the two H21 protons. This was confirmed by 1-D COSY experiments. The signal was seen as a triplet rather than a quartet due to the overlapping of the two centre peaks of the quartet. Upon increased presence of the AcOD, this peak shifted downfield and broadened to the typical peak seen for an OH proton. This suggests the breaking of the intramolecular hydrogen bond caused by preferential interaction at either the C20 carbonyl or the OH21 proton.

When HEMA was used as the functional monomer, the interaction observed at the OH21 site was more pronounced, with definite shifts observed in the signal for the OH21 proton, allowing for the construction of a Job plot giving a maximum at a budesonide mole fraction of 0.5. While no observable shifts were present for the H2 or H4, suggesting no interaction at the C3 carbonyl, there was a shift, again more pronounced, for the H1 proton, this time suggesting a 1:1 interaction at the OH11 site. Due to the fact that these sites were at the antipodes of the molecule, the combined 1:1 interactions could in fact suggest a 2:1 monomer:template interaction, as opposed to the possible 3:1 monomer:template interaction observed for AcOD. To further investigate these interactions and to try and determine their magnitude, NMR titrations were then carried out in chloroform.

*2.4.4.2 NMR titration analysis for budesonide and $d_4$-acetic acid*

Due to the fact that AcOD produced no signal in the resultant NMR spectra it was possible to follow a number of template peaks of interest and it is worth noting that while many of the overall chemical shifts observed are relatively small (*vide infra*) the plots presented in Figure 2.14 suggest that the results are meaningful.

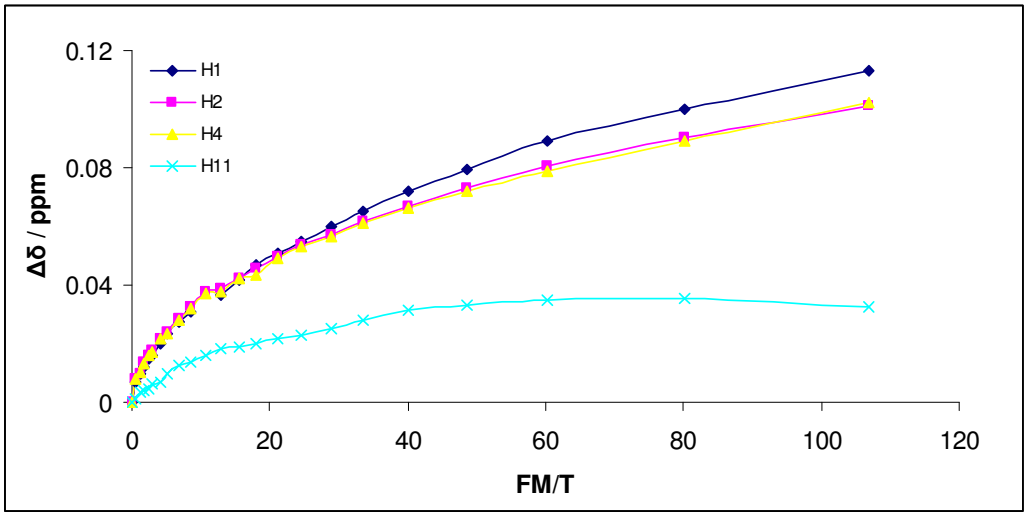



**Figure 2.14: Overlaid plots of the change in chemical shift of the protons: H1, H2, H4 and H11 observed in the titration of BDN with AcOD in $CDCl_3$, where FM/T is the ratio of functional monomer to template.**

The plots of the H2 and H4 vinyl protons of the A-ring in BDN changed in quite similar fashions during the titration with AcOD. H1 also acted in quite a similar manner at the

start of the titration but changed slightly after an FM/T ratio of approximately 25. Even at functional monomer to template ratios of 110, saturation had still not been reached for any of the vinyl protons and the overall change in the chemical shift of the protons are: 0.113, 0.101 and 0.102 ppm for H1, H2 and H4 respectively. These were all downfield shifts suggesting that the nuclei deshielded to a greater extent possibly due to the presence of the electronegative carboxyl group of AcOD in close proximity while binding with the carbonyl at C3.

These changes are relatively low when compared with changes in shifts of 1.2 ppm observed for the $NH_2$ protons of 2-aminopyridine upon interaction with AcOD observed by Osmani *et al.* [71], 2 ppm for $NH_2$ protons of trimethoprim interacting with MAA [78] and 3.23 and 4.55 ppm for the OH3 proton of estradiol with AcOD and deuterated pyridine respectively, in toluene [79]. Svenson *et al.* observed shifts of up to 1.2 ppm for protons in the interaction of nicotine and AcOD at functional monomer to template ratios as low as 35 [80]. It is necessary to note, however, that strong H-bonding would be expected between carboxyl and amino groups.

For H11, which is an indicator of the interaction at the OH11 proton, the overall change in shift was smaller again, with a value of 0.033 ppm. This change in chemical shift is in an upfield direction which indicates that the H11 nucleus is in a less deshielded environment. This may be due to the fact that the O atom of the OH21 is involved in hydrogen bonding with AcOD, thus diminishing some of its deshielding effects. The interaction does, however, reach saturation at an FM/T ratio of about 40. It is worth noting that the differences in magnitude between the overall change in chemical shifts values correspond with the results seen for the Job plots, where the shift change was again, considerably smaller for H11 than for the vinyl protons. This indicates that interaction at the C3 carbonyl is favoured over interaction at the OH11 proton.

Another point worth noting is that the slight difference in the plot for H1 plus the slightly larger change in shift (~10%) in comparison to the other two vinyl protons may also be an indication of the effect on H1 by the interaction at OH11. Further evidence of the presence of higher order complexes, i.e. 1:2 as well as 1:1, is provided by comparison of the relative changes in complexation induced shifts for the different protons. As stated by Connors [64] and utilised by Quaglia *et al.* [78], the ratio of the

change in shifts due to complexation, should be the same for two different protons when only 1:1 complexes occur. This is not the case when 1:2 complexes occur and the ratio is expected to vary with the change in the concentration of the ligand. This was shown to be the case for the interaction between BDN and AcOD when the ratio: $\Delta\delta/\Delta\delta_{max}$, for each proton was plotted against the total concentration of AcOD in a manner analogous to that employed by Quaglia *et al.* [78], as illustrated in Figure 2.15.

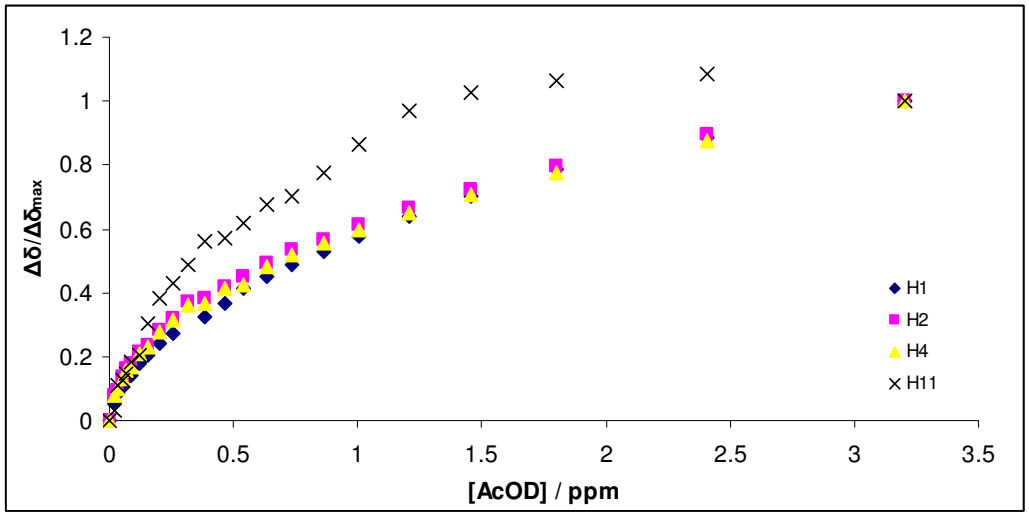



**Figure 2.15: Comparison of plots of the relative changes in shifts of the protons of BDN against the total concentration of AcOD in $CDCl_3$.**

The occurrence of non-parallel plots for the relative changes in shifts of the protons in Figure 2.15, suggests the presence of higher order complexes in the interaction between AcOD and BDN [78]. This combination of evidence suggests that there are at least two points of interaction between AcOD and BDN and a possible third based on the effect on the OH21 resonance signal as discussed in Section 2.4.2.1.

In order to determine the association/dissociation constants, the data was manipulated using non-linear regression in Prism, GraphPad software 4 [80, 81]. The likelihood of weak complexation, due to small shift changes, presents its own problem in that it is necessary to use high monomer concentrations to drive the equilibrium [64]. This can lead to problems with medium effects and possible self-association of the ligand. Where a system may contain 1:1 and 1:2 complexes, the binding constants should be considered as approximate values rather than exact numbers due to the fact that an increase in the number of parameters (because of the increased number of complexes)

can affect the reliability of the data [64, 82]. This being said, the values of $K_{diss}$ for the protons affected by interaction during the titration are given in Table 2.10.

**Table 2.10: $K_{diss}$ values for the protons in which a complex-induced change in chemical shift occurred during titration with AcOD in $CDCl_3$.**

| Proton | $K_{diss}$ / M |
|---|---|
| H1 | 1.094 ± 0.11 |
| H2 | 0.732 ± 0.08 |
| H4 | 0.775 ± 0.09 |
| H11 | 0.534 ± 0.04 |

Table 2.10 shows that the value for H1 indicates weaker association than that observed for H2 and H4, while the latter two protons have very similar $K_{diss}$ values. This would be expected as H1 is further away from the expected site of interaction. The low association predicted by these values are in agreement with the small values for the change in chemical shift. The value for H11 points to slightly higher association which would be in agreement with the achievement of saturation for this proton over the concentration range used.

*2.4.4.3 NMR titration analysis for budesonide and HEMA*

There were a number of differences observed in the titration of BDN and HEMA in $CDCl_3$ in comparison to the BDN and AcOD titration in $CDCl_3$; one such difference involved the signals for H2 and H4. In this case, there was no observable pattern in the change in shift, suggesting that no interaction took place at the C-3 carbonyl. This was in agreement with the findings from the Job plots. For this reason only the H1, H11 and OH21 peaks are presented.

Another difference lay in the fact that it was not possible to carry out the titration of BDN and HEMA to the same extent as BDN and AcOD. This was because once the ratio of 1:90 had been reached it was not possible to get an appropriate shim-lock and the spectra could not be considered to be reliable. It was possible to track the change in chemical shift of the H1 and H11 signals up to the ratio of 1:90 but this was not the case with the OH21 proton, which could only be tracked to the 1:50 ratio due to overlap of

peaks from the HEMA. The HEMA peak was at a much greater intensity than the OH21 peak due to the far greater concentration in the mixture. An overlay of the plots is given in Figure 2.16.

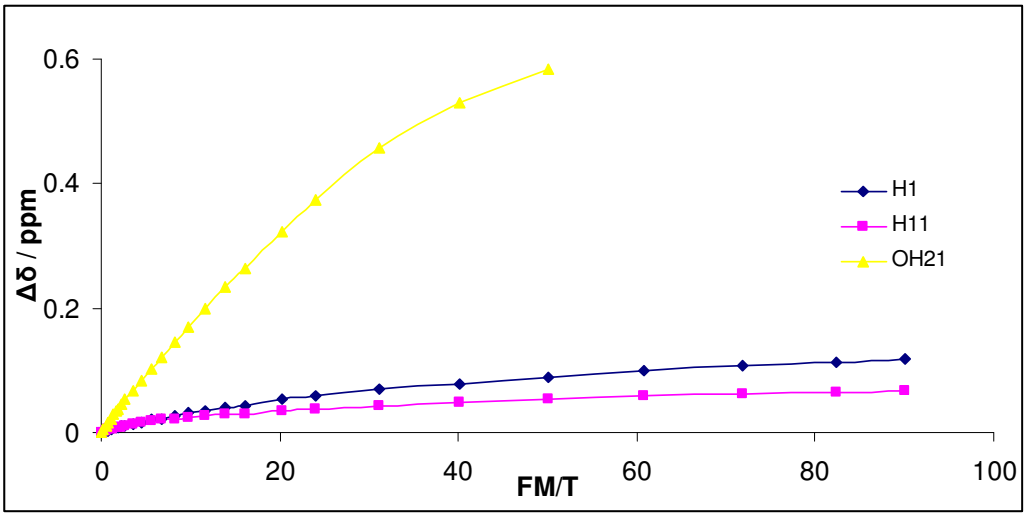



**Figure 2.16: Overlaid plots of the changes in chemical shift in the H1, H11 and OH21 protons of BDN, during titration of BDN and HEMA in $CDCl_3$.**

There was a considerable difference between the overall change in shift of the OH21 proton and the H1 and H11 protons. The overall value for the OH21 proton was a shift of 0.584 in a downfield direction. This suggested that the proton was becoming more deshielded, i.e. was in a more electronegative environment. This indicated that that the proton was taking part in a hydrogen bond with an O atom on the HEMA molecule. While it is not possible to track this peak to the full extent of the titration, the obtained data suggests that this interaction is the strongest of the interactions seen thus far.

The overall changes in chemical shifts of the H1 and H11 protons had values of 0.119 ppm downfield and 0.067 ppm upfield respectively. The direction of chemical shift change is analogous to the changes observed for the same nuclei in the titration with AcOD. This would suggest that again, the OH11 atom was involved in hydrogen-bonding to a H atom of an OH group on the HEMA. This was thus brought in close proximity to H1, thereby bringing it into a more electronegative environment. At the same time, this would reduce some of the deshielding power of the OH11 oxygen causing the upfield shift in H11. Overall chemical shift values were larger in the HEMA titration, albeit only by 5% in the case of the H1 shift change but this value increased to

50% in the case of the H11 shift change. What may be more noteworthy is the fact that this occurred at a 1:90 ratio, for HEMA and a 1:106 for AcOD. The overall size of these shift changes were still quite small in relation to the majority of the values quoted in the literature for NMR titrations, as the examples given in Section 2.4.4.2 demonstrate.

While it may appear from Figure 2.16 that the plots of the chemical shift change for H1 and H11 were similar, when these plots are put on a similar scale it can be seen that this was not the case, as shown in Figure 2.17.

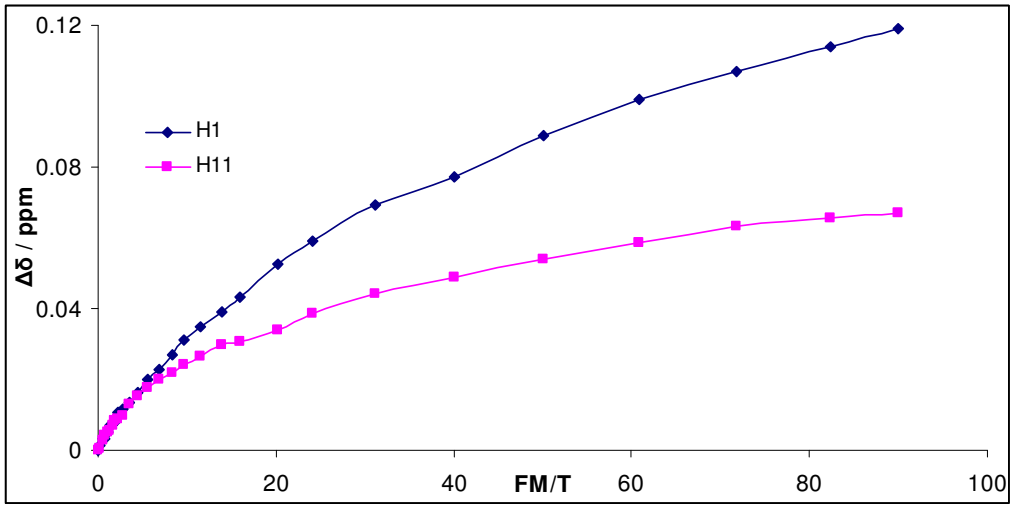



**Figure 2.17: Plot of H1 and H11 protons of BDN obtained during titration of BDN and HEMA in $CDCl_3$.**

Figure 2.17 suggested that, as with AcOD, the interaction for H11 may have neared saturation, while this was not the case for H1 although the overall change in shift was greater for the latter. As discussed in Section 2.4.4.2 a comparison of the ratio of the change in chemical shift values yields information as to the number of complexes present [64, 78]. This has also been applied to the interaction of BDN with HEMA, the plots for which are presented in Figure 2.18.

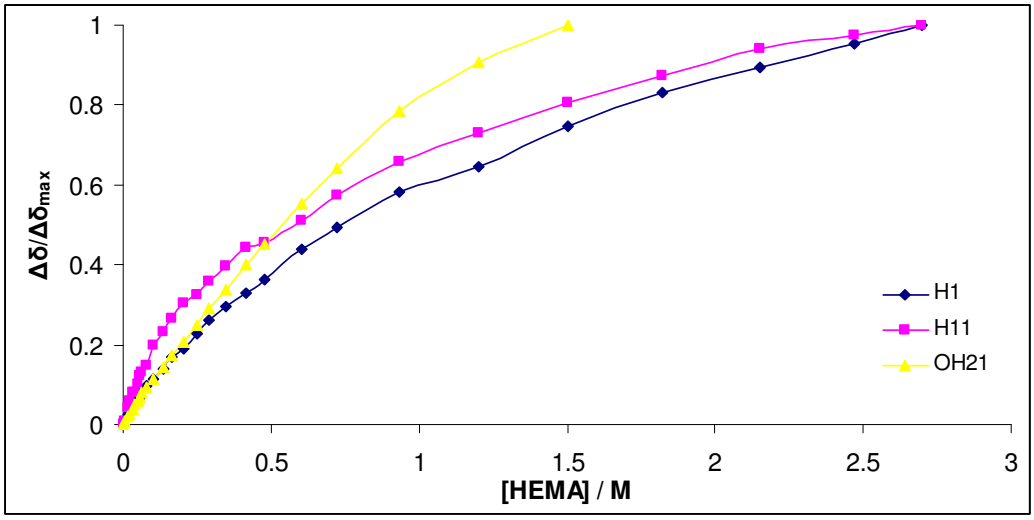



**Figure 2.18: Relative changes for the complex-induced shifts for each of the protons plotted against the total concentration of the functional monomer, HEMA, in $CDCl_3$.**

As before, a difference in the relative complexation induced shifts for the different protons suggests the presence of higher order complexes, e.g. 1:2. This was also seen to be the case in Figure 2.18 suggesting the presence of more than a 1:1 complex. Since there was evidence of interaction at two sites, i.e. OH11 and OH21, in the Job plots shown in Figure 2.13, where shift changes occurred at the H1 and OH21 protons, this would be in agreement with the data presented in Figure 2.18. The $K_{diss}$ values calculated for the three protons are presented in Table 2.11.

**Table 2.11: $K_{diss}$ values determined for H1, H11 and OH21 using Prism, GraphPad software.**

| Proton | $K_{diss}$ / M |
|---|---|
| H1 | 1.465 ± 0.06 |
| H11 | 0.726 ± 0.08 |
| OH21 | 2.130 ± 0.11 |

The results for the apparent $K_{diss}$ seen in Table 2.11 show that the values obtained for H1 and H11 are quite similar to those obtained for the titration of BDN and AcOD, given in Table 2.10. It must be noted that while the value for OH21 would appear to give very weak association, it must be taken into consideration that the full set of values

were not obtained because of overlapping peaks; the size of the complex induced shift, however, suggested the possibility that the interaction may have been stronger.

### *2.4.4.4 Summary of NMR titration investigations*

A comparison of the effects seen during titration of HEMA, AcOD and BDN in $CDCl_3$ is presented in Table 2.12.

**Table 2.12: Comparison of data obtained during titration of BDN with HEMA and AcOD in $CDCl_3$.**

| Proton | AcOD | | | HEMA | | |
|---|---|---|---|---|---|---|
| | $\Delta\delta_{max}$ | $K_{diss}$ /M | Inference | $\Delta\delta_{max}$ | $K_{diss}$ /M | Inference |
| H1 | 0.113 | 1.094 ± 0.11 | Interaction at C3 CO and OH11 | 0.119 | 1.465 ± 0.06 | Interaction at OH11 |
| H2 | 0.102 | 0.732 ± 0.08 | Interaction at C3 CO | n.d. | n.d. | n.d. |
| H4 | 0.101 | 0.775 ± 0.09 | Interaction at C3 CO | n.d. | n.d. | n.d. |
| H11 | 0.033 | 0.534 ± 0.04 | Interaction at OH11 | 0.067 | 0.726 ± 0.08 | Interaction at OH11 |
| OH21 | n.c. | n.d. | n.d. | 0.584 | 2.130 ± 0.11 | Interaction at OH21 |

n.d.: no data; n.c.: no curve, i.e. interaction detected but not possible to track change.

The proposed interactions summarised in Table 2.12 are depicted in Figure 2.19.

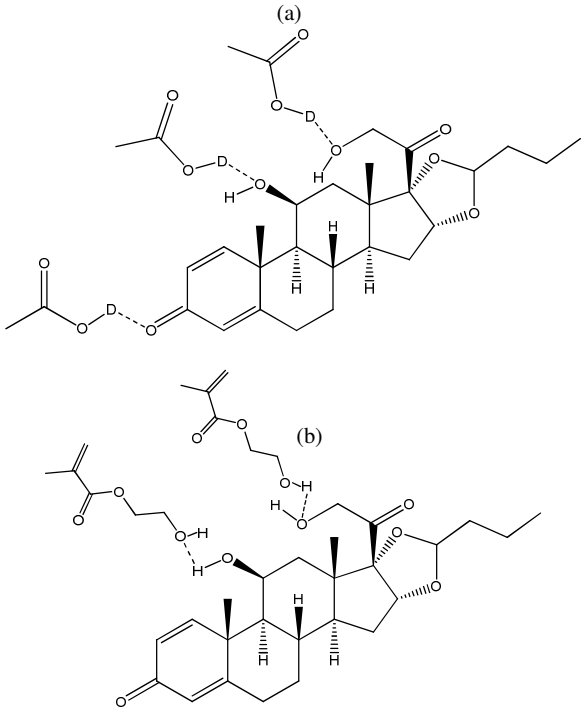



**Figure 2.19: Proposed interactions for (a) AcOD and BDN and (b) HEMA and BDN**

Table 2.12 outlines the results obtained during the NMR investigations of AcOD and HEMA with BDN in $CDCl_3$. In all cases the values for the dissociation constants are high when compared to values obtained by other groups. As no titration data for these monomers and corticosteroids have been reported, it is necessary to draw comparisons with studies carried out for different templates used in molecular imprinting. For example Urraca *et al*. obtained values for $K_{diss}$ as low as $2.12 \times 10^{-5}$ M for interaction between 1-allylpiperazine and the template cyclododecyl 2,4-dihydroxybenzoate [82] while Ansell and Kuah obtained values of $1.4 \times 10^{-4}$ M for interaction between ephedrine and MAA [83]. Interestingly, O'Mahony *et al*. obtained values as low as 0.045 M for π- π interactions (a relatively weak interaction compared to hydrogen bonding, etc.) between 4-vinyl pyridine and 2,4-dichlorophenoxy acetic acid [81]. This suggests that with dissociation constants as high as were observed in Table 2.12, any interaction between template and functional monomer could be considered very weak. This indication of very weak interaction between the template and monomers, even in

non-polar solvent such as chloroform, may explain the lack of any apparent affinity in the polymers, M1-M7.

The work of Long *et al*., discussed in Section 1.5.2.2, where the degree of interaction between a template, testosterone, and the functional monomer, MAA, was seen to be relatively weak, only 20% of the template was shown to be bound to the polymer after 24 hours of reaction in a precipitation polymerisation process [84]. In this and any analogous situation, such as the one presented here, the number of binding sites on the polymer itself is probably relatively low. With this in mind, a number of new precipitated polymers were prepared and subsequent binding studies were carried out at a much lower concentration. For quantitative analysis at such low concentrations and with the possibility of interfering compounds (such as polymeric bleed) it was necessary to employ HPLC for analysis of the rebinding solutions.

### 2.4.5 Initial studies towards the use of a template analogue approach

UV-Vis analysis had shown that a drop in concentration of 75 µg in the binding solution could be detected but, as stated above, it was now presumed that this could possibly be far higher than the actual capacity of the polymers. The more sensitive method of HPLC, was therefore employed for the quantitative analysis of the steroid in the binding solution. The construction of standard curves using HPLC showed that analysis of budesonide, based on a method by Faouzi *et al*. [47], was linear down to at least 0.25 µg/mL. Robust and reliable detection at this level allowed for the quantitation of any material bound to the polymers, even at very low levels.

Given the fact that NMR studies had shown that interaction between MAA and HEMA would be very weak in polar solvents, it was decided to first test non-imprinted polymers using the crosslinking monomer alone, in aqueous solutions of an organic solvent. This was an effort to promote the hydrophobic interactions, which would most likely predominate with a molecule such as a steroid, with its largely hydrocarbon skeleton. A polymer, M8 (see Table 2.4), composed of DVB only was prepared in acetonitrile-toluene at a ratio of 3/1 (v/v). In aqueous solutions of methanol, this DVB NIP bound 100% of budesonide at concentrations up to 20 µg/mL. When polymers prepared with EGDMA only were tested at these concentrations, only 30% of the

material was bound to the polymer. For this reason a number of polymers were prepared using DVB as a crosslinking monomer and MAA as the functional monomer.

When HPLC analysis was carried out on a number of the previously prepared polymers (M1 and M7), it was observed that this more sensitive technique revealed a small but significant quantity of template bleeding. This fact, coupled with the by now prohibitive cost of budesonide (€1000 per gram), suggested long-term use of a template analogue strategy. The template analogue approach has been utilised in molecular imprinting applications for a range of templates [25, 85, 86] and can be necessary for imprinting when template bleeding poses a significant problem or when using the actual target is unsuitable due to factors such as cost or toxicity. Prednisolone, as shown in Figure 2.3, has a structure very similar to that of budesonide with only the acetal grouping and alkyl chain attached to the D ring of the steroid in the difference. While it was considered that this steric factor could play a role, the difference in the retention times of these two analytes (~5 minutes), as opposed to more sterically similar steroids made prednisolone an attractive template analogue. Thus, polymers M9 and M10, i.e. polymers with ratio of prednisolone:MAA:DVB of 1:10:30 and 1:10:50, respectively, were prepared, using acetonitrile-toluene 3:1 (v/v) as the porogen.

As this was a target analogue approach, budesonide was again used as the target for equilibrium binding studies. These binding studies were carried out as before, with the exception that acetonitrile or acetonitrile-water 90:10 (v/v) was used and 30 mg of polymer were added to 0.9 mL of binding solution over a range of template solutions (2-100 µg/mL). While no repeatable imprinting effect was observed for the M10 polymer, some degree of imprinting seemed to be present for the M9 polymer. In acetonitrile, at a budesonide concentration of 100 µg/mL an average imprinting factor of 1.15 was obtained. Similarly, in the 10% water solution at a concentration of 10 µg/mL, an imprinting factor of 1.46 was observed. While these results seemed somewhat promising, the actual difference between the percentage rebound for the MIP and NIP was very low, for example, in 10% water, the MIP bound 6.3% while the NIP bound 5.2%. Low as this was, however, it was repeatable, and suggested that it was possible to use the combination of a template analogue and a method of enhancing template-monomer interaction to effect some degree of molecular imprinting and subsequent rebinding of a target steroid.

## *2.5 Conclusions*

The above investigations were carried out in a bid to prepare a polymer selective for budesonide. The initial polymer formulation was selected on the basis of published methods for the imprinting of structural analogues of budesonide. When no affinity was observed, the components and parameters were varied in a systematic fashion in a bid to discern the reason for this apparent lack of affinity. Despite this systematic study of polymer composition and reaction conditions, no affinity was observed for initial polymer formulations.

During the systematic study, however, considerable information was obtained as to the factors which play a role in the preparation of successful polymers, in relation to bead size, ease of preparation and lack of polymeric bleed, etc. Due to the complex set of factors involved in this method of polymerisation, no clear trends were observed. It could be said, however, that for each combination of functional and crosslinking monomer, the solvent composition, monomer concentration, initiator type and concentration must be carefully chosen to achieve the most desired result. The ease of this method and the lack of labour intensive processes such as grinding and sieving should allow for rapid and efficient optimisation of the polymer compositions and conditions for future work and the knowledge gained from these preliminary investigations should prove applicable and beneficial to future polymer synthesis once affinity has been achieved.

NMR studies were applied to the pre-polymerisation solutions to determine possible causes for this apparent lack of affinity. These studies indicated the presence of weak interactions between the template and the functional monomers used, which could lead to a low number of binding sites within the polymeric matrix. This rational understanding of the limitations of corticosteroid imprinted polymers by precipitated means led to studies using much lower concentrations than had previously been employed. These studies revealed that the polymers had a degree of inherent affinity for the target which, while low, suggested the potential to improve this affinity by molecular imprinting means.

Problems with template bleed and cost suggested the use of a target analogue approach, and subsequent experiments using prednisolone as the template indicated some initial imprinting effects. Low though these effects were, they suggested that specific rebinding of budesonide using a target analogue approach was possible. These results, coupled with the information gleaned from NMR studies suggested that an approach that would promote the relatively weak interaction between the template and functional monomers in the pre-polymerisation studies was required to enhance the imprinting effect observed.

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# Chapter 3

# The synthesis of a molecularly imprinted polymer using the target analogue approach

# 3. The synthesis of a molecularly imprinted polymer using the target analogue approach

## *3.1 Introduction*

### 3.1.1 Target analogue imprinting

Since Andersson *et al*. first utilised a target analogue as the template for the synthesis of a sameridine selective polymer [1], the use of the so-called 'dummy template' or 'template analogue' approach has become widespread in molecular imprinting [2-4]. (As the preceding sentence suggests, this approach can be described using a number of names. In this chapter, these names will be interchanged but as the template used for all polymers is an analogue of the primary target, 'target analogue' is primarily used). As with the first occurrence of this method, the vast majority of work employing this strategy involves solid phase extraction, where the presence of even trace quantities of template 'bleed' will negatively effect the ultimate quantitation of the analytes of interest, often only present themselves at part per billion or part per trillion levels [5-7]. This technique is used, however, for other applications, such as the synthesis of a molecularly imprinted membrane by Wang *et al*. for lovastatin acid using lovastatin as a target analogue [8] or the preparation of explosive sensing technologies, using carboxylic acid analogues of the targets by Riskin *et al*. [9].

As stated above, where trace quantities of analytes are to be detected, any template that remains in the polymer matrix, commonly quoted as greater than 1% [7, 10], has the potential to leach from the matrix and interfere with quantitation, thus necessitating the use of a target analogue as the template species. Despite this being the predominant reason for their use, target analogues may also be employed for a variety of other reasons; Khorrami *et al*., for example, used oxindole as a cost effective target analogue for the toxin patulin [11], while Kubo *et al*. cite toxicity or rarity of targets as suitable reasons for utilising a target analogue [12]. Whatever the reason for its use, the process of molecular imprinting dictates that there are certain criteria that the target analogue must fulfil to make it suitable for use, including functionality, as well as molecular size and shape. An example of this can be seen in the aforementioned work by Andersson *et*

*al*., where the template analogue differed from the target by only the substitution of a methyl group for an ethyl group [1].

While many template analogues are commercially available, special requirements may necessitate the synthesis of a particular compound, such as the work of Kubo *et al*. where the intended target was a cyanobacterium toxin [12]. The toxin contained opposing ionic groups at a particular interval and the target analogue was synthesised to have the same interval between ionic groups, thereby organising the binding sites in such a way that the target could be recognised based on this "interval immobilisation". As discussed in Section 1.6.1, Baggiani *et al*. have also synthesised a "template mimic", a chlorinated azo-dye, for targeting Sudan dyes in food samples [13]. Interestingly, this imparted selectivity to the polymer not just for the main target, Sudan I, but also group selectivity for several of its analogues. Group selectivity was also imparted upon polymers prepared by Baggiani *et al*. for the recognition of corticosteroids, where hydrocortisone, or cortisol, was used as a template and a vast range of polymers were studied for their selectivity towards fourteen related corticosteroids [14].

### 3.1.2 MIP Chromatography

Chromatography, as a method for evaluating the success of the templating process for MIPs, has been in use for almost as long as the modern era of molecular imprinting itself. Since Wulff *et al*. first applied affinity chromatography to a ground, sieved and packed imprinted polymer to evaluate the racemic separation of D- and L-4-nitrophenyl mannopyranoside [15, 16], this technique has been consistently employed for investigations into the factors contributing to the recognition capabilities of imprinted polymers [17-22].

Despite the usefulness of this technique in comparing MIP to NIP and investigating the fidelity of binding recognition, MIP chromatography as an analytical method in its own right has a number of problems. The previously mentioned work by Wulff *et al*. described the presence of "broad peaks with strong tailing" and attributed this to the mechanisms by which binding takes place in these covalent polymers and the resulting exchange equilibria [15]. While early work by Sellergren *et al*. suggested that these problems could be overcome by using non-covalent interactions [17], these problems

have continued to plague the use of molecularly imprinted polymers as successful stationary phases and, over a decade and a half later, the same author cited ten problems preventing their use for commercial applications, including binding site heterogeneity, slow mass transfer and swelling/shrinkage problems [23].

In a bid to overcome some of these problems, a range of techniques have been employed with considerable focus on particle morphology and size distribution, factors which the use of ground and sieved particles exacerbate. To this end, polymerisation techniques including those discussed in detail in Section 1.5.2, i.e. suspension, precipitation and core-shell methods, have been investigated for use as polymeric stationary phases. Some notable examples include the separation of racemic ketoprofen on surface imprinted core-shell beads by Jiang *et al*. [24] and the separation of cholesterol from a number of other steroids, on beads prepared by seeded suspension by Lee *et al*. [25]. The separations achieved by these groups are exemplified by Figure 3.1.

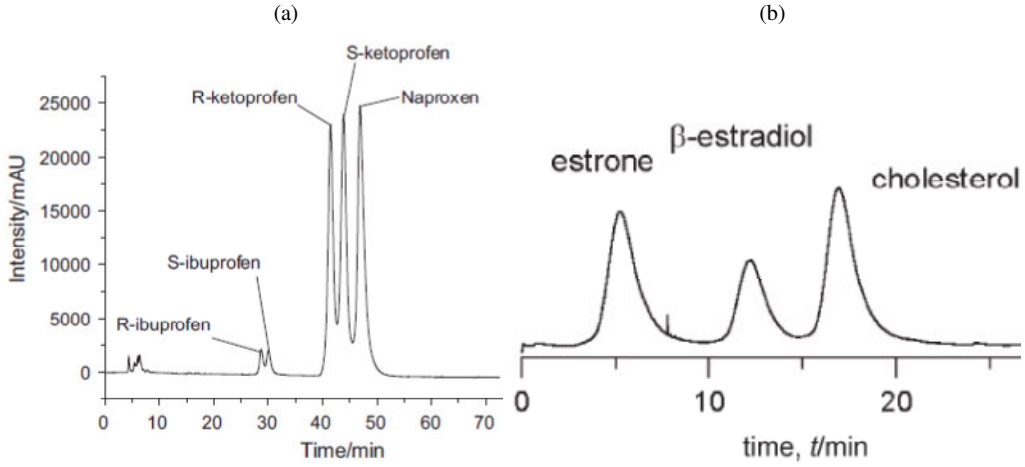



**Figure 3.1: Separations achieved on molecularly imprinted stationary phases for (a) racemic non-steroidal anti-inflammatory drugs [24] and (b) for a range of steroids [25].**

For applications where a molecularly imprinted stationary phase is not the ultimate aim, MIP chromatography has been ubiquitously used throughout the field as a reliable method of investigating polymeric properties [22, 26, 27]. Sellergren *et al*. have used chromatographic evaluation for imprinted polymers prepared for phosphorous esters [28] and riboflavin [29], while Baggiani *et al*. have employed MIP chromatography for evaluation of polymers prepared for a number of templates, including fungicides [30],

polycyclic aromatic hydrocarbons [31] and the steroid, hydrocortisone [32]. Indeed, the effectiveness of the imprinting process for a range of steroids has been studied using MIP chromatography, including cholesterol [33], estradiol [27] and testosterone and its derivatives [34]. The latter example succeeded in separating testosterone and epitestosterone on a MIP based column.

The retention behaviour of an analyte on different polymer columns can be used to draw conclusions about the molecular recognition properties of the materials. It can often be necessary, however, to carry out physical characterisation of the materials to ensure that any differences observed between the polymers are due to differences in the manner and extent of chemical interactions between the polymers, rather than simply physical differences [35]. A number of physical characterisation strategies are outlined in Section 1.6, including scanning electron microscopy (SEM), binding site characterisation and nitrogen sorption porosimetry. To supplement the brief introduction to the latter technique, given in Section 1.6, a more detailed discussion is presented in Section 3.1.3.

### 3.1.3 Nitrogen sorption porosimetry

The physisorption or adsorption of gases, typically nitrogen, is a general method for the physical characterisation of materials, capable of providing information on the surface area and porosity of the materials analysed [36]. As such, this method has been widely applied to molecularly imprinted polymers [37-39]. This technique has been used to analyse polymer morphologies and in so doing to gain a more detailed understanding of the physical factors that can affect molecular recognition. This technique involves the construction of adsorption-desorption isotherms by exposing a fixed mass of dry material to a gas at a constant temperature and monitoring the equilibrium pressures as a series of known quantities of gas are added to the sealed chamber [36, 40, 41]. The isotherms can provide information on specific surface area, pore diameter and volume, as well as pore size distribution. Regarding porous materials the following classifications have been made by the IUPAC relating to pore sizes [36]:

- Micropores: pores with widths not exceeding about 2 nm
- Mesopores: pores with widths between 2 nm and 50 nm
- Macropores: pores with widths exceeding about 50 nm

The isotherms obtained from gas sorption studies follow six basic types, as shown in Figure 3.2 (a).

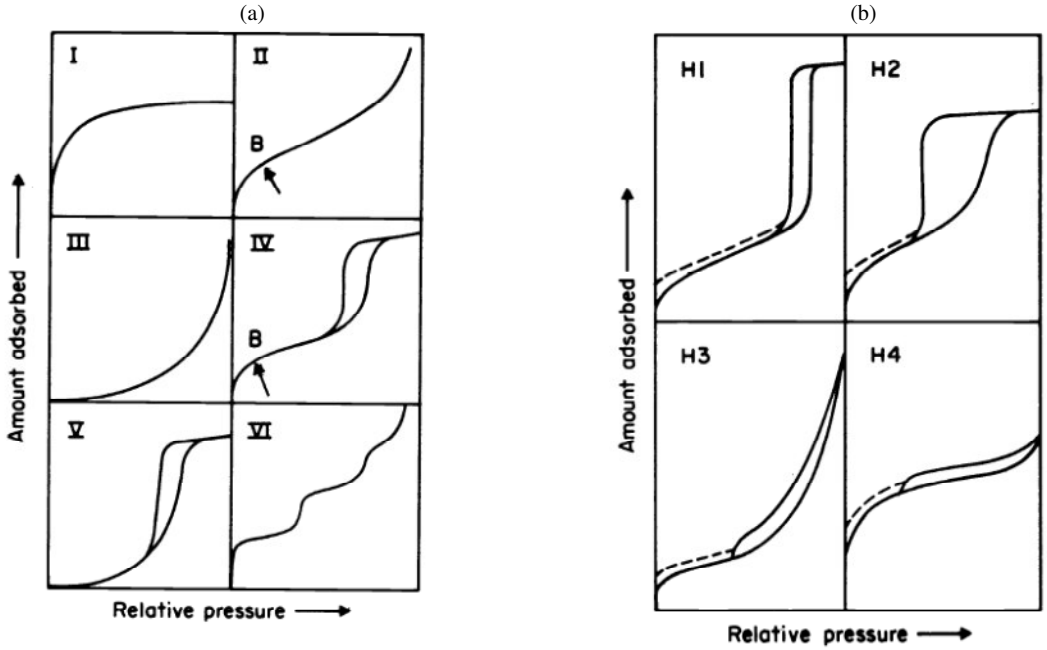



**Figure 3.2: (a) Six basic types of physisorption isotherms and (b) four possible types of hysteresis loops obtained [36].**

The reversible isotherms are defined by the IUPAC as follows [36]:

- Type I isotherms are observed for microporous solids that have relatively small external surfaces.
- Type II isotherms are the typical form of the isotherm obtained for a non-porous or macroporous adsorbent. This isotherm represents unrestricted monolayer-multilayer adsorption.
- Type III isotherms are uncommon and represent materials where adsorbate-adsorbate interactions are important.
- Type IV isotherms are characterised by their hysteresis loop, a characteristic related to capillary condensation taking place in mesopores, and as such, this type of isotherm indicates a mesoporous material.
- Type V isotherms are related to Type III and are again uncommon.
- Type VI isotherms represent stepwise multilayer adsorption on non-porous surfaces.

The different shapes of the hysteresis loops obtained, as shown in Figure 3.2 (a), while not fully understood, can be used to identify specific pore structures [36], for example H1 type hysteresis can be associated with porous materials consisting of agglomerates while porous adsorbents with poorly defined size and shape distribution can give H2 type loops.

In order to determine the surface area of the materials analysed by such methods, the Brunauer-Emmett-Teller (BET) method [42] has been widely used and has been applied to molecularly imprinted materials [36, 37, 43, 44]. The BET method employs the following equation in the linear form:

$$\frac{p}{n^a(p^0-p)} = \frac{1}{n^a.C} + \frac{(C-1)\,p}{n_m^a.C\,p^0} \qquad \textbf{Equation 3.1}$$

Where:

$P$ is the equilibrium pressure;

$P_o$ is the nitrogen saturation pressure at 77 K;

$n^a$ is the amount adsorbed at the relative pressure $P/P_o$;

$n_m^a$ is the monolayer capacity;

C is a constant dependent on the shape of the isotherm.

To calculate the surface area, the BET plot is constructed using the linear relationship between $p/n^a(p^0-p)$ and $p/p^0$ with the range of linearity restricted to the linear part of the isotherm, typically between 0.05 an 0.30 [36].

The surface area is then determined using the following equation:

$$a_s = n_m^a L a_m \qquad \textbf{Equation 3.2}$$

Where:

$a_s$ is the surface area;

$L$ is Avogadro constant;

$a_m$ is the molecular cross-sectional area of the adsorptive.

It is necessary to note that Sing states that the BET method, while generally suitable for Type II and Type IV isotherms as long as the C value is neither too high nor too low, it is not considered likely that it will yield reliable values for the actual surface area of Type I or Type III isotherms [36]. It is also worth mentioning the limitations of nitrogen sorption data relating to MIPs, in that the information gleaned from such experiments relates to polymers in the dry state. While this information is important and it allows us to draw comparisons between the MIP, the NIP and other polymers, it must be remembered that the polymers are typically used in conjunction with a solvent. As such, conclusions are drawn on information acquired from physisorption experiments in the absence of solvent and these conclusions are used to rationalise polymer behaviour in the presence of solvent.

## *3.2 Research aims and objectives*

The aims and objectives of this chapter were as follows:

- To prepare molecularly imprinted polymers for corticosteroids with a particular focus on budesonide by use of the target analogue imprinting approach.
- To test the prepared polymers using MIP chromatography and so ascertain the binding characteristics of the polymers and the factors driving recognition.
- To carry out a physical characterisation on the polymers prepared in order to gain a full understanding of the morphological effects of different polymerisation conditions and to investigate the effect of such differences on molecular recognition.

## *3.3 Experimental*

### 3.3.1 Materials

Hydrocortisone-17-butyrate (HCB), hydrocortisone (HYD), hydrocortisone-21-acetate (HAC) and prednisolone-21-acetate (PAC) (all ≥97% purity or better) and trimethylolpropane trimethacrylate (TRIM) and pentaerythritol triacrylate (PETRA) (both technical grade) were all purchased from Sigma-Aldrich, Wicklow, Ireland. All other materials and reagents used were as per Table 2.3 and treated as per Section 2.3.1.

### 3.3.2 Instrumentation

**Table 3.1: Instrumentation used for this study.**

| Instrument | Model | Supplier |
|---|---|---|
| Photochemical mini-reactor (8 W lamps) | RMR-600 | Branford, CT, USA |
| Column packer | Preparatory pump | JVA analytical, Ireland |
| Nitrogen sorption porosimeter | Micromeritics Gemini VI | Particular sciences, Ireland |
| Scanning electron microscope | Hitachi S-2460N | Oxford instruments, England |
| Shaking incubator | C24 | New Brunswick Scientific, USA |
| HPLC | 1200 | Agilent Technologies, Ireland |
| Particle size analyser | Mastersizer 2000S | Malvern, Ireland |

### 3.3.3 Preparation of polymers

Polymers were prepared as per Table 3.2

**Table 3.2: Polymer compositions and conditions used for this study**

| Polymer | Template | Functional monomer | Crosslinking monomer | Molar ratio [a] | Solvent | Polymer format [b] |
|---|---|---|---|---|---|---|
| MB3 | HCB | MAA | EGDMA | 1:10:30 | MeCN | Bulk |
| MB4 | HCB | MAA | DVB | 1:10:30 | MeCN/ TOL 3:1 (v/v) | Bulk |
| MB5 | HCB | MAA | TRIM | 1:10:15 | MeCN | Bulk |
| MB6 | HCB | MAA | PETRA | 1:10:15 | MeCN | Bulk |
| MB8 | HCB | ---- | EGDMA | 1:0:30 | MeCN | Bulk |
| MB10 | HCB | HEMA | EGDMA | 1:10:30 | MeCN | Bulk |
| MB12 | HCB | MAA | EGDMA | 1:10:30 | $CHCl_3$ | Bulk |
| MB13 | HCB | MAA | TRIM | 1:10:15 | $CHCl_3$ | Bulk |
| M13 | HCB | MAA | EGDMA | 1:10:30 | MeCN | PPT |
| M14 | HCB | MAA | DVB | 1:10:30 | MeCN/ TOL 3:1 (v/v) | PPT |
| M23 | HCB | MAA | TRIM | 2:4:2 | DCM | PPT |

[a] Ratio of template:functional monomer:crosslinking monomer; [b] PPT: precipitation

Polymers MB3-MB13 were prepared as bulk monolith polymers at 60 °C for 24 hours at a monomer concentration ranging between 75 and 85% (w/v). These polymers were crushed and roughly sieved before Soxhlet extraction in MeOH for 24 hours before final grinding and sieving to size ranges of either 25-75 µm or 38-53 µm. Fine particles were sedimented in MeOH-$H_2O$ 80/20 (v/v). Polymers M13 and M14 were prepared as per Section 2.3.2 by thermal initiation and at a monomer concentration of 4% (w/v). Precipitated beads, M23, were prepared using a method based on that employed by Ye *et al.* [45]. Dichloromethane was used as the porogen and the polymerisation was carried out at 4 °C and photochemically initiated at 350 nm. Polymerisation was carried out in a round-bottom flask in the UV reactor while being rotated around its long axis at

a speed of 20 RPM. The monomer was 2% (w/v) and precipitated beads were cleaned on-line by washing the column with acidified MeOH with 5% AcOH at 0.2 mL/min until a stable baseline was achieved.

### 3.3.4 Chromatographic polymer evaluation

*3.3.4.1 Column packing*

50 × 4.6 mm i.d. columns were used in all cases for polymer packing. Polymers were first suspended and sonicated in the packing solvent, which was composed of either aqueous solutions of MeOH and MeCN or MeCN alone. This slurry was then added to the packing chamber and the solvent was pumped through the column, at flow rates which varied between 0.2 and 0.5 mL/min for precipitated beads and 18 mL/min for ground bulk particles, until three chamber volumes had been pumped through. The polymers were tested for packing uniformity by equilibrating the column with acetonitrile at the required analytical flow rate and injecting 5 µL of a 20 µL/mL solution of acetone in the mobile phase and monitoring the retention time and shape of the resulting peak.

*3.3.4.2 Zonal chromatography*

Suitably packed columns were equilibrated with the mobile phase, either MeCN or MeCN/$H_2O$, at a flow rate of 0.5 mL/min for bulk particles or 0.2-0.5 mL/min for precipitated beads, until a stable baseline was achieved. Stock steroid solutions were prepared at a concentration of 2.5 mM (ca. 1 mg/mL for each steroid) in acetonitrile and stored at -20 °C. Steroid solutions for injection onto the imprinted and blank columns were prepared by carrying out a 1 in 50 dilution on the stock solution, giving a final concentration of $5 \times 10^{-5}$ M for each analyte. 5 µL of the steroid solutions were injected on to the column and eluted at the flow rates listed above, while monitoring the absorbance at 240 nm. Injections were carried out in duplicate, or triplicate where necessary, to ensure repeatability. The capacity factor, $k'$, was calculated using Equation 3.3.

$$k' = \frac{(t - t_0)}{t_0} \qquad \textbf{Equation 3.3}$$

Where $k'$ is the capacity factor or retention factor, $t$, is the retention time for the analyte and $t_0$ is the retention time for the column void marker, obtained by injecting 5 µL of a 20 µL/mL acetone solution. Equation 3.4 was used to calculate the imprinting factor (IF) for each steroid.

$$IF = \frac{k'_{MIP}}{k'_{NIP}} \qquad \textbf{Equation 3.4}$$

where $k'_{\mathrm{MIP}}$ and $k'_{\mathrm{NIP}}$ are the capacity factors obtained on the MIP and NIP column, respectively.

*3.3.4.3 Frontal chromatography*

Staircase frontal chromatography was carried out on all polymers as described by Hall *et al*. [46]. This technique acquires analyte adsorption data over a wide range of concentrations, typically covering at least three orders of magnitude. Here, three stock solutions of HCB were prepared in acetonitrile at concentrations of 0.001, 0.01 and 0.1 mM. These solutions were used as the mobile phase in a step gradient using acetonitrile as the co-solvent. Beginning with 100% acetonitrile and the lowest concentration template solution, the composition of the mobile phase was changed in a stepwise fashion by increasing the percentage of the template solution until 100% template solution was flowing through the column. Without washing the column, this was continued with the higher concentration template solutions, resulting in three separate staircase-type chromatograms, each comprised of ten steps.

The results obtained by frontal chromatography are plotted in binding isotherms that were subsequently fitted to the Freundlich adsorption model using Prism, GraphPad software 4.0 and thermodynamic constants were derived using affinity distribution analysis (FIAD), as described by Rampey *et al*. [47]. The Freundlich isotherm is shown in Equation 3.5.

$$q = aC^m \qquad \textbf{Equation 3.5}$$

where $q$ is the amount of analyte bound on the stationary phase and $C$ is the concentration of free steroid, while $a$ and $m$ are fitting constants. The concentration of

analyte bound to the stationary phase for each step, $q^*$, is calculated using the following equation:

$$q^* = \Delta C_m (V_{1/2} - V_0) \qquad \textbf{Equation 3.6}$$

where $\Delta C_m$ is the change in concentration of analyte in the mobile phase at each step, $V_{1/2}$ is the retention volume at half-height of the corresponding step and $V_0$ is the void volume of the system including the column. $V_0$ was measured by either using the half-height of an un-retained analyte, i.e. acetone, or by measuring the volume of the system and the column separately, using acetone as a void marker.

### 3.3.5 Nitrogen sorption porosimetry

All polymers were dried under vacuum at 50 °C and degassed overnight at 50 °C under a constant flow of nitrogen. A known amount of polymer, between 50-200 mg, was placed in the analysis tube and nitrogen physisorption isotherms were measured at 77 K. The BET method, as described in Section 3.1.3, was used to calculate the specific surface area while the Barret-Joyner-Halenda (BJH) method was applied to physisorption isotherms to determine pore size distribution as carried out by Urraca *et al*. [38].

### 3.3.6 Particle size and scanning electron microscopy analysis

All particle size and SEM analyses were carried out as per sections 2.3.3 and 2.3.5.

## *3.4 Results and discussion*

### 3.4.1 Target analogue selection

Initial efforts towards the use of a target analogue to prepare molecularly imprinted polymers selective for corticosteroids, with a particular focus on budesonide, employed prednisolone as the template for use as a target analogue. While the imprinting factors obtained were relatively low, i.e. 1.15-1.46, they suggested that the use of a cost effective target analogue, coupled with a means of enhancing the weak template-monomer interactions in the pre-polymerisation solution, would be a viable approach for effecting corticosteroid selectivity within imprinted polymers. In order to choose the most suitable target analogue, a number of suitably priced and structurally analogous steroids were investigated. Five corticosteroids, bearing similar functionalities and ranging in price from ten to forty times less than budesonide, were selected as possible target analogues. These compounds were also used for an investigation of the factors driving recognition and selectivity. The steroids used in this study are presented in Figure 3.3.

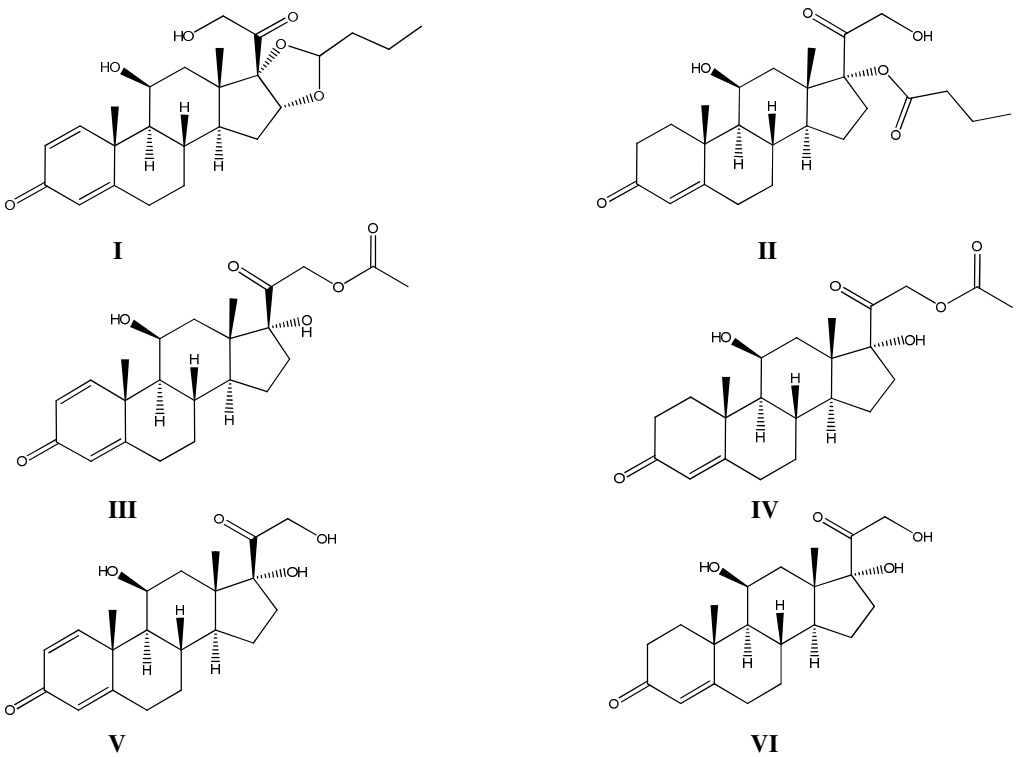



**Figure 3.3: Corticosteroids used in this study: I Budesonide (BDN); II Hydorcortisone-17-butyrate (HCB); III prednisolone-21-acetate (PAC); IV hydrocortisone-21-acetate (HAC); V prednisolone (PRD); VI hydrocortisone (HYD)**

As discussed in Section 3.1, the target analogue must fulfil certain criteria for it to be suitable for use. As such, the target analogue must have similar size, shape and functionality to the target. As can be seen from Figure 3.3, all of the steroids have similar structure and functionality on rings A-C. In the case of **I** budesonide (BDN), **III** prednisolone-21-acetate (PAC) and **V** prednisolone (PRD), there are two double bonds in the A-ring of the steroid. This is opposed to only one for steroids **II** hydrocortisone (HYD), **IV** hydrocortisone-21-acetate (HAC) and **VI** hydrocortisone-17-butyrate (HCB). The second double bond being positioned between C-1 and C-2 on the former group of steroids. The effect of this structural difference has been discussed by Baggiani *et al.* where the planar configuration of the A ring, resulting from the second double bond, was seen to have a considerable affect on polymer recognition and determined to be "one of the main discriminating structural differences of a steric nature" for MIPs with group selectivity for corticosteroids [14].

Figure 3.3 shows that it is on the D-ring of the steroid that a number of differences exist between the principal target, budesonide, and the structural analogues. BDN possesses a hydroxyl group on the C-21 position, as do steroids, **II**, **V** and **VI**, while **III** and **IV** have bulky acetate groupings at this position. It is worth noting that evidence of interaction at OH-21 was observed using NMR investigations, as reported in Section 2.4.4 and disruption of this interaction with an acetate group, would be expected to have a deleterious effect on recognition for the target. While steroids **III**-**VI** have another hydroxyl group on the 17α position, BDN possesses an acetal grouping on positions 16 and 17, forming an extra ring structure, from which a propyl chain extends. The presence of this bulky substituent in **I** and the lack of any such substituents on **III-VI** would be expected to play a major role in the steric recognition for the steroid and it was postulated that the creation of an imprinted site without the proper steric requirements might inhibit recognition. With this in mind, the presence of a bulky hydrophobic group in this position, as with **II** above, would be considered to be advantageous.

With a view to predominantly aqueous biological applications for the corticosteroid selective polymers and preliminary results showing that hydrophobic interactions would be important, it was deemed pertinent to also take the hydrophobicity of the steroids into consideration. The predicted log P values were calculated using ChemBioDraw

Ultra 11.0, giving a value of 2.73 for BDN and 2.86 for **II**, while steroids **III-VI** all had much lower predicted log P values than BDN, with values ranging from 1.3-1.7. This was considered to be an appropriate method for log P predictions as the predicted value for prednisolone was 1.71, which was in close agreement to the literature value of 1.62 [48]. The combination of these factors suggested hydrocortisone-17-butyrate (HCB) as a likely target analogue wit NMR studies carried out to further investigate its suitability.

### *3.4.1.1 NMR studies using HCB*

Job plot experiments were carried out with HCB and deuterated acetic acid in deuterated chloroform in a manner analogous to that described in Section 2.4. While many of the shift changes that occurred could not be accurately mapped due to overlapping signals, the shift change for the H4 proton was readily tracked and the Job plot obtained suggested a possible 1:1 interaction between template and monomer, as shown in Figure 3.4.

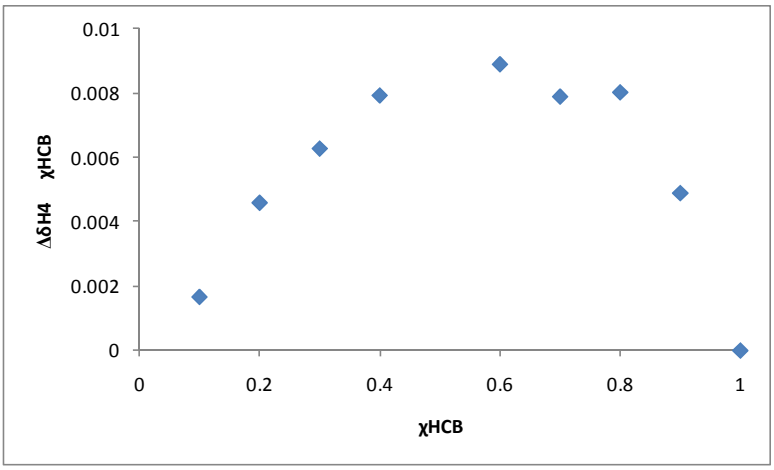
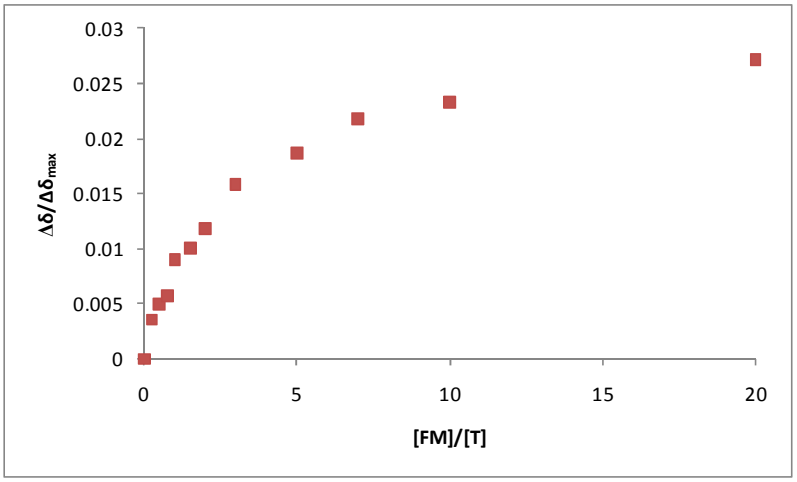



**Figure 3.4: Job plot obtained for interaction between HCB and AcOD in $CDCl_3$ with a maximum at χHCB 0.5 suggesting a possible 1:1 interaction taking place at the C-3 carbonyl.**

While the Job plot obtained suggested the likelihood of a 1:1 interaction between HCB and AcOD, when a titration was carried out the points of interaction between HCB and deuterated acetic acid were similar to those observed for BDN and acetic acid under similar conditions (as outlined in Section 2.4.4). It must be noted that there was potential evidence of 1:2 interaction, with the possible presence of another maximum at 0.7, however, further investigation would be necessary to determine this.

As with BDN, evidence was observed that suggested interaction at the C-3 carbonyl, OH11 and OH21. As H11 was a complex multiplet it was not possible to map the change in shift but the changes in chemical shifts for H4 and H21$_{\alpha,\beta}$ were readily observed for titration experiments and the plots presented in Figure 3.5 were obtained.

(a)




(b)

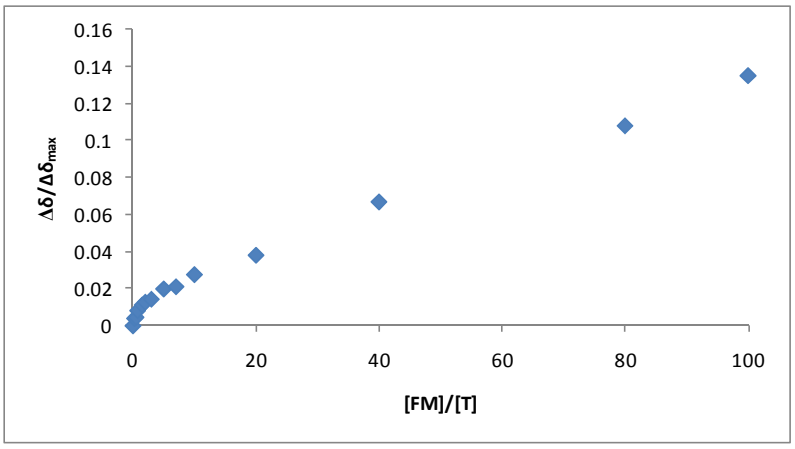



**Figure 3.5: Plots obtained for chemical shift changes observed during NMR titrations of HCB and AcOD in $CDCl_3$ for (a) H4 and (b) H21.**

The interaction observed at the C-3 carbonyl, Figure 3.5 (a), appeared to become saturated at a functional monomer:template ratio of 1:20 and the dissociation constant, $k_{diss}$, obtained was considerably lower than any for BDN, at 0.058 M. This suggested that the degree of interaction was stronger at this point than that of BDN and AcOD ($k_{diss}$ 0.774 M) and this may have been due to the shape of the A ring of the steroid. The interaction observed at the OH21, however, appeared to be more analogous to that of BDN, as even at FM/T ratios of 100:1, saturation was not achieved and the $k_{diss}$ value

was 0.767 M. Interactions observed at similar points on HCB as BDN indicated that HCB would be a suitable target analogue based on NMR investigations.

The combination of the bulky hydrophobic substituent on the 17α position, the hydroxyl group in the 21 position, the similar hydrophobicity of **II**, hydrocortisone-17-butyrate, to BDN and the analogous interactions observed by NMR studies, suggested its use as a target analogue. Further, it was postulated that these factors could possibly overcome any problems posed by the difference in structure of ring-A of the two steroids. As such, HCB was chosen as the template for use as a target analogue for the synthesis of the polymers discussed in the following sections.

### 3.4.2 Preparation and analysis of HCB imprinted polymers

In order to investigate the variety of factors contributing to selective imprinting, the polymers outlined in Table 3.2 were prepared. These polymers were prepared to study the effect of a range of parameters including the type of crosslinking and functional monomers, the porogen and the polymer morphology.

Initial results are summarised in Table 3.3. This table also summarises the imprinting factors (*IF*) obtained for the polymers prepared during this study in both 100% acetonitrile and acetonitrile-water 30/70 (v/v). These solvents were chosen as the mobile phase for chromatographic investigation into the imprinting effect for a number of reasons; 100% acetonitrile was chosen because this solvent was used as either the porogen or co-porogen for the majority of cases and is widely used in analysis. The aqueous solution of acetonitrile allowed for a study of the behaviour of the polymers in predominantly aqueous systems but had a sufficient quantity of acetonitrile present to prevent the hydrophobic analytes from being retained to an impractical extent on the majority of the polymeric columns.

**Table 3.3: Result summary for HCB imprinted polymers**

| Polymer | Functional monomer | Crosslinking monomer | Solvent | Polymer[1] morphology | IF[2] MeCN 100% | IF[2] MeCN/ $H_2O$ (30/70) |
|---|---|---|---|---|---|---|
| MB3 | MAA | EGDMA | MeCN | GP | 7.5 | 2.0 |
| MB4 | MAA | DVB | MeCN/ TOL 3:1 (v/v) | GP | 3.0 | 2.2 |
| MB5 | MAA | TRIM | MeCN | GP | 2.9 | 1.7 |
| MB6 | MAA | PETRA | MeCN | GP | N.E. | NE |
| MB8 | ---- | EGDMA | MeCN | GP | 1.7 | 1.3 |
| MB10 | HEMA | EGDMA | MeCN | GP | N.E. | N.E. |
| MB12 | MAA | EGDMA | $CHCl_3$ | GP | 1.9 | 1.5* |
| MB13 | MAA | TRIM | $CHCl_3$ | GP | 2.8 | 2.0 |
| M13 | MAA | EGDMA | MeCN | MS | N.E. | 1.5 |
| M14 | MAA | DVB | MeCN/ TOL 3:1 (v/v) | MS | 1.5 | N.E.** |
| M23 | MAA | TRIM | DCM | MP | 1.4/1.7 2 | N.E.** |

[1] GP: ground bulk particles; MS: microspheres; MP: microparticles; [2] IF: imprinting factor as calculated as per Equation 3.4
N.E.: no imprinting effect observed
* result obtained from single sample run; ** analytes retained for more than three hours on both MIP and NIP columns
Flow rates: 0.5 mL/min for ground particles; 0.2 mL/min for microspherical beads/particles except for M23 where first value was at 0.5 mL/min and second value at 0.2 mL/min.

Table 3.3 shows a wide range of behaviour in the two solvents for all polymers and to obtain a clearer understanding of the factors driving these behaviours, the affect of the different variables were examined. Polymer composition, solvent and polymerisation strategy all play important roles in determining the success of molecular imprinting and while detailed results are presented in Table 3.4, the results will be discussed in such a manner as to isolate the discussion of each variable as much as possible.

**Table 3.4: Comparative result data for ground monolith particles prepared using EGDMA (MB3 and MB12), DVB (MB4) and TRIM (MB5 and MB13) as crosslinking monomers from nitrogen physisorption, chromatographic binding site characterisation and chromatographic evaluation of imprinting factors.**

| Polymer | Surface area ($m^2.g^{-1}$) | Pore [1] volume ($cm^3.g^{-1}$) | Pore [1] diameter (nm) | $m^2$ | $K^3$ ($L.mol^{-1}$) | $N^4$ ($\mu mol.g^{-1}$) | k'[5] MeCN | IF MeCN | k' MeCN-$H_2O$ | IF MeCN-$H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|
| MB3 | 265 | 0.529 | 11 | 0.8769 | $9.37 \times 10^4$ | 34.8 | 1.42 | 7.5 | 7.22 | 2.0 |
| NB3 | 251 | 0.457 | 10 | 0.9684 | $7.49 \times 10^4$ | 6.31 | 0.19 | | 3.65 | |
| MB4 | 466 | 0.425 | 6.2 | 0.7618 | $1.34 \times 10^5$ | 5.06 | 0.75 | 3.0 | 14.87 | 2.2 |
| NB4 | 468 | 0.418 | 6.3 | 0.7326 | $1.47 \times 10^5$ | 1.79 | 0.25 | | 6.68 | |
| MB5 | 331 | 0.604 | 9.8 | 0.5891 | $2.30 \times 10^5$ | $1.1 \times 10^{-2}$ | 2.11 | 2.9 | 9.34 | 1.7 |
| NB5 | 320 | 0.606 | 8.6 | 0.8529 | $8.70 \times 10^4$ | $1.2 \times 10^{-2}$ | 0.73 | | 5.43 | |
| MB12 | 277 | 0.480 | 9.6 | n.d. | n.d. | n.d. | 0.70 | 1.9 | n.d. | n.d. |
| NB12 | 240 | 0.336 | 7.5 | n.d. | n.d. | n.d. | 0.36 | | n.d. | |
| MB13 | 246 | 0.242 | 5.1 | 0.8822 | $9.43 \times 10^4$ | $1.8 \times 10^{-2}$ | 2.47 | 2.8 | 25.07 | 2.0 |
| NB13 | 211 | 0.280 | 6.8 | 0.6842 | $1.72 \times 10^5$ | $4.8 \times 10^{-3}$ | 0.87 | | 12.67 | |

[1] values obtained from the adsorption curve; [2] heterogeneity index obtained from Freundlich isotherm (FI); [3] apparent weighted average affinity constant of binding sites from FI; [4]apparent number of sites from FI; [5]capacity factor of the analytes from Equation 3.3.
n.d.: no repeatable data
Frontal chromatography carried out in MeCN and all chromatography carried out at flow rate of 0.5 $mL.min^{-1}$

### 3.4.3 Polymers prepared by bulk methods

*3.4.3.1 The effect of the crosslinking monomer on ground bulk particles*

Four crosslinking monomers, divinyl benzene (DVB), ethylene glycol dimethacrylate (EGDMA), trimethylolpropane trimethacrylate (TRIM) and pentaerythritol triacrylate (PETRA), were chosen as the crosslinking monomers for use in this study, the structures of which are given in Figure 3.6.

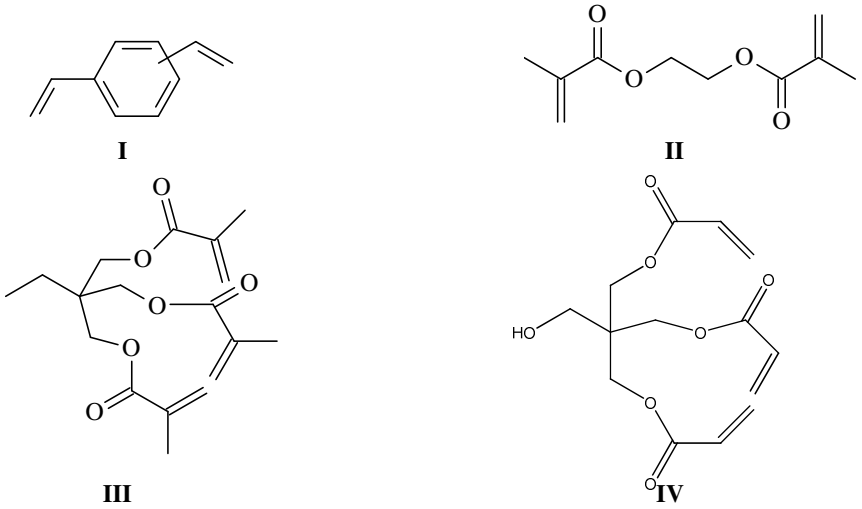



**Figure 3.6: Crosslinking monomers used in this study: I DVB; II EGDMA; III TRIM; IV PETRA.**

With the exception of PETRA, MIPs prepared using the above crosslinking monomers exhibited promising imprinting effects. It was postulated that the use of the hydrophilic crosslinker PETRA [29] would have given the polymer a degree of hydrophilicity that repelled the predominantly hydrophobic steroidal compounds and as such PETRA polymers were not investigated further. Using the remaining three crosslinking monomers, polymers were prepared using both precipitation and bulk methods.

*3.4.3.2 Zonal Chromatographic evaluation of polymer binding properties*

Zonal chromatography was employed to investigate the difference in retention behaviour of the analytes on the imprinted and non-imprinted polymers. Chromatograms typical of the type observed can be seen in Figure 3.7.

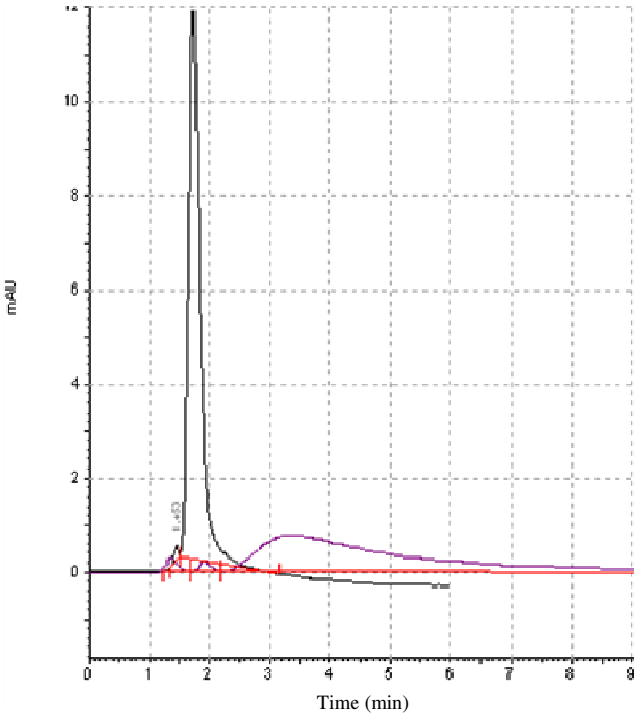



**Figure 3.7: Representative chromatograms of the template, HCB, when injected on the MIP column (purple trace) and the NIP column (black trace). Example shown: MB3 in MeCN.**

The chromatograms presented in Figure 3.7, exhibited the broad peaks and tailing that are typical of those observed in MIP chromatography [25, 49]. This broadness and tailing can be due to the factors mentioned in Section 3.1.2, including binding equilibria and binding site heterogeneity [15, 23]. Since polymers MB3 and MB5 were prepared in acetonitrile and MB4 in acetonitrile-toluene, the differences due to crosslinking monomer alone are most readily discussed by an initial comparison of these three polymers and the retention behaviour in the two solvents are presented in Figure 3.8 (a) and (b).

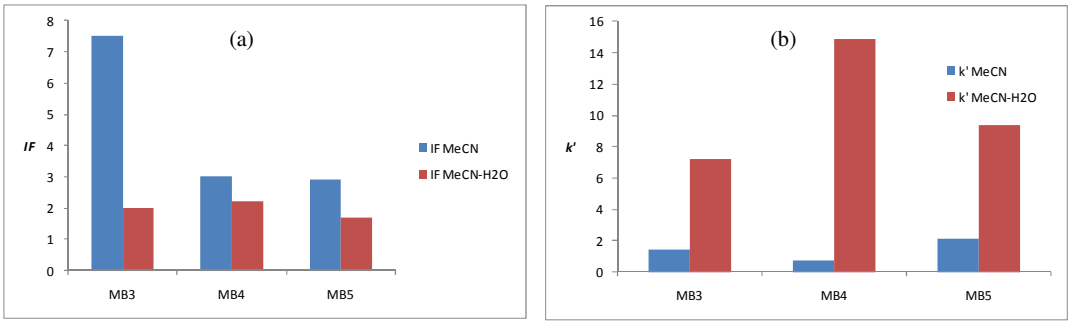



**Figure 3.8: Effect of crosslinker on: (a) imprinting factors and (b) capacity factors for the three polymers, MB3 (EGDMA), MB4 (DVB) and MB5 (TRIM), prepared with acetonitrile/acetonitrile mixtures as porogen in two mobile phase compositions.**

As can be seen from Figure 3.8 (a), in the case of these polymers the imprinting effect was most pronounced when MeCN was used as a the mobile phase. This was not unexpected, as enhanced specific recognition in the porogen due to the so-called 'solvent memory effect' has been well documented, and Spivak *et al*. have suggested that this is due to the solvent swelling the polymer to a state where the arrangement and proximity of the functional groups of the binding sites achieve the dimensions created during the imprinting process [19, 20, 50]. The much larger $k'$ values obtained in the aqueous based mobile phase for all polymers (Figure 3.5 (b)) suggest that the hydrophobic nature of the steroids facilitated enhanced hydrophobic interactions between the polymer and the analyte in all cases. Given the monomers used, this would be expected to be largely non-specific and indeed, the larger $k'$ values for the MIP were accompanied by larger $k'$ values for the NIP in all cases, resulting in a corresponding decrease in *IF* values. Despite the decrease in *IF* values specific recognition, which was presumably due mainly to hydrophobic interaction, was observed in aqueous based systems for the three polymers.

The difference between the two solvent compositions was most noticeable for MB3, which had the highest *IF* in acetonitrile but had an *IF* almost four times lower in the aqueous mobile phase. As expected, for MB4, the polymer based on DVB, the difference between *IF* for the different mobile phases was less pronounced, with a decrease of only 26% observed in the aqueous system. The increase in the $k'$ value was also the largest in this case and was believed to be due to the hydrophobic nature of the vinyl benzene crosslinking monomer. MB5, the TRIM based polymer appeared to give intermediate results in all cases. While the $k'$ values in acetonitrile were the largest for both MIP and NIP, the corresponding *IF* value was the lowest by a small margin. It is here postulated that this was due to an inherent affinity of the polymer for the template HCB, resulting in a non-imprinted polymer with a good deal of attraction for the template and a correspondingly low imprinting factor.

Spivak discusses the need to compare binding results with results determined using the same technique, as chromatographic and batch methods do not "correspond quantitatively" with one another [51]. There is, however, a limited number of publications dealing with corticosteroid imprinting and an even more limited number using chromatographic determination of *IF* values. In the aforementioned work by

Baggiani *et al*. where hydrocortisone was imprinted [32], chromatographic evaluation demonstrated an imprinting factor of 9.71 for the template. The values obtained in this study compared favourably with those results, with the highest value for the template reaching 7.5.

*3.4.3.3 Evaluation of binding site characteristics by frontal chromatographic evaluation*

To elucidate fully the behaviour of polymers MB3, MB4 and MB5 in zonal chromatographic experiments, frontal chromatography was carried out as outlined in Section 3.4. As discussed in Section 1.6.3, quantitation of the number (*N*) and affinity (*K*) of binding sites is typically achieved by acquiring analyte adsorption data for a polymer over a specified concentration range, where concentrations of bound (*B*) and free (*F*) analyte are measured. This data is then treated using a suitable mathematical model, typically Scatchard analysis, Langmuir-Freundlich (LFI) or Freundlich (FI) isotherms. Rampey *et al*. state that while the LFI is more universally applicable, it is not usually necessary and the FI, which is more easily applied, can be used [52]. This is because sub-saturation concentrations are typically used and the FI is used for sub-saturation conditions. The FI was chosen for this study and Equation 3.6 was employed to fit the adsorption data obtained from multistep staircase analyses to the FI as it has been shown to be suitable for use for MIPs due to the heterogeneous nature of the binding sites within such polymers [53].

The experimental data were fit to the power function of the FI using GraphPad Prism software and curves with a degree of correlation ($R^2$) >0.99 were obtained for all three MIPs and NIPs, demonstrating the suitability of this model for the experimental data acquired. A representative example of the experimental and 'fit' adsorption isotherms is presented in Figure 3.9.

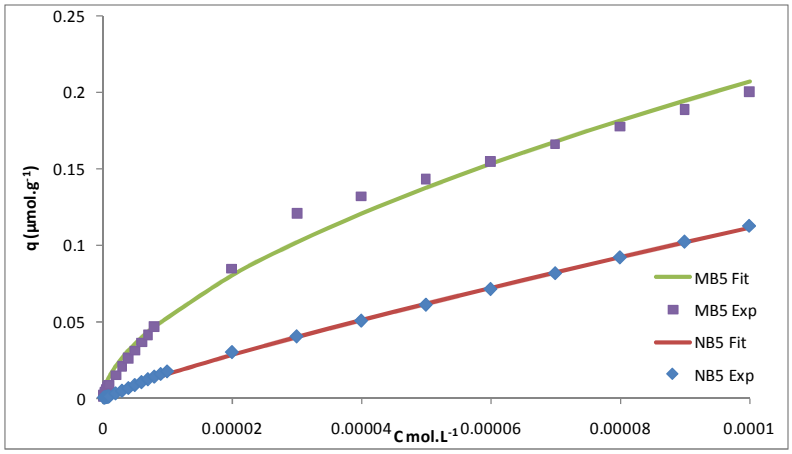



**Figure 3.9: Representative adsorption curves obtained for fitting the experimental data (dots) with FI function fit data (solid lines) for MIP and NIP. The example shown is for MB5 and NB5 (TRIM polymers).**

Fitting of this data in the manner demonstrated in Figure 3.9 provides values for *a* and *m* (Equation 3.5), with the *m* value being of particular interest, as this is the heterogeneity index and indicates the degree of heterogeneity present within the binding sites of the polymer, while *a* is related to the median binding affinity [53]. It can be seen in Table 3.3, that for MB3 and MB5, the *m* values for the corresponding non-imprinted polymers, NB3 and NB5, respectively, are larger. This suggests a greater degree of heterogeneity for the imprinted polymers. The opposite appeared to be the case for the MB4 polymers with the non-imprinted polymer having a lower *m* value. Of the polymers prepared from the three crosslinking monomers, the difference in *m* value for MIP and NIP was greatest for polymer MB5, suggesting that the presence of the template in the pre-polymerisation complex affected the heterogeneity of the sites to the greatest extent. MB3 had the highest heterogeneity index for all three imprinted polymers, suggesting that the template had a positive directing step for the binding sites in this polymer, as the absence of the template would lead to a more homogenous polymer.

When the experimental data and fit parameters are used in conjunction with the Freundlich isotherm affinity distribution (FIAD), information can also be provided on the number and strength of the binding sites within the polymer matrix [46, 47, 54]. The FIAD results in the calculation of the apparent number of sites, *N*, and the apparent

weighted average affinity constant, *K* and the affinity distribution can be represented in a linear log *N*-log *K* plot. Figure 3.10 illustrates the linear nature of these plots for polymers MB3 (EGDMA), MB4 (DVB) and MB5 (TRIM), which demonstrates the suitability of the use of the FI for these polymeric systems [47].

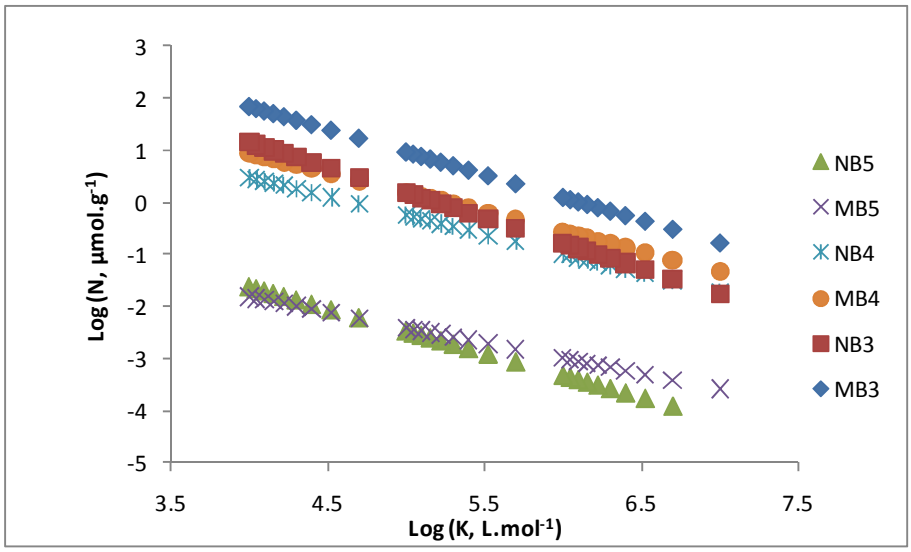



**Figure 3.10: Affinity distributions for MB3-MB5 using log *N* vs. log *K* format**

Of the three polymers, MB3 had the highest *N* value at 34.8 µmol.g$^{-1}$, while its corresponding NIP had only 6.31 µmol.g$^{-1}$. Since there was only a marginal difference observed for the affinity constants at 9.37 × 10$^{4}$ and 7.49 × 10$^{4}$ L.mol$^{-1}$ for MIP and NIP, respectively, it would appear that it was the difference in the number of the binding sites that resulted in the high imprinting factor obtained for MB3. MB4 and NB4 had average affinity constants an order of magnitude higher than MB3 at 1.34 × 10$^{5}$ and 1.47 × 10$^{5}$ L.mol$^{-1}$, respectively, but the number of binding sites were much lower for both MIP and NIP in these cases. It must also be taken into consideration that the pore diameter for DVB polymers was also much smaller than that of the EGDMA polymers, with values of 6 and 11 nm, respectively and this may have had an effect on access to sites. What was interesting here was that *K* was slightly higher for NB4 than MB4 but due to the lower number of binding sites, 5.06 µmol.g$^{-1}$ for the MIP and 1.79 µmol.g$^{-1}$ for the NIP, the template was still retained more strongly on MB4. MB5 had a marginally lower number of binding sites, 1.1 × 10$^{-2}$ µmol.g$^{-1}$, compared to NB5, 1.2 × 10$^{-2}$ µmol.g$^{-1}$, but due its far higher *K* value, 2.3 × 10$^{5}$ L.mol$^{-1}$ as opposed to 8.7 × 10$^{4}$

L.mol$^{-1}$ for NB5, the template was again retained more specifically on the MIP. These results, while varying greatly from each other, nevertheless provide physical data explaining the different retention behaviour for MIP vs. NIP in each. Interestingly, the polymer which had the greatest difference in the number of binding sites between MIP and NIP, MB3, rather than that which had the largest difference between the average affinity constants, MB5, resulted in the polymer with the highest imprinting factor.

### *3.4.3.4 Nitrogen sorption porosimetry*

To probe whether the retention behaviour of the imprinted polymers compared to that of the non-imprinted polymers was due to molecular interaction rather than mere physical and morphological factors, nitrogen sorption porosimetry was carried out, the results of which are presented in Table 3.3. The first point to note was that there were no relevant differences for the surface area of any of these MIP and NIP pairs, i.e. 265 and 251 $m^2.g^{-1}$ for MB3 and NB3, respectively, 331 and 320 $m^2.g^{-1}$ for MB5 and NB5, and 466 and 468 $m^2.g^{-1}$ for MB4 and NB4, respectively. While MB3 polymers had the lowest surface area, they had the largest pore diameter and conversely, MB4 polymers had the highest surface area but the lowest pore diameter. As with the binding characteristics, MB5 had intermediate surface area and pore diameters compared to the other two. In all cases, the nitrogen physisorption isotherms obtained were Type IV isotherms and had hysteresis resembling H4 type loops. This isotherm is typically related to meso-macroporous materials [38]. The isotherms for MB3, MB4 and MB5 are shown Figure 3.11 and the corresponding non-imprinted polymers displayed analogous isotherms.

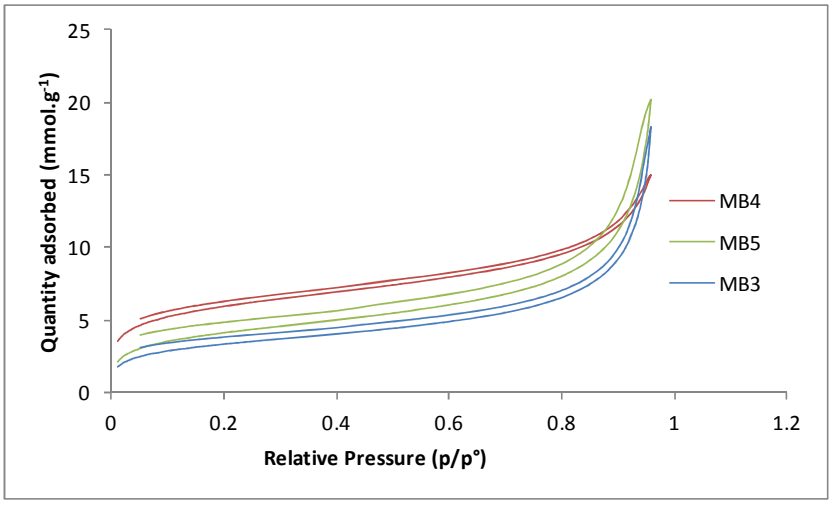



**Figure 3.11: Representative physisorption isotherms displaying isotherms for MB3-MB5.**

Hysteresis, which Sing states is associated with capillary condensation within mesopores [36] and is due to differences between the adsorption and desorption behaviours, is present even at low pressure ranges. This behaviour was also observed by Urraca *et al.* who suggested that this was related to the presence of narrow pores that were more accessible at higher gas pressures but from which the adsorbate was not completely removed [38] and was also reported by Sellergren and Shea to be related to entrapment of nitrogen within the polymer matrix [18]. The pore distributions obtained from adsorption and desorption segments of the physisorption isotherms using the BJH method are presented in Figure 3.12 (a) and (b).

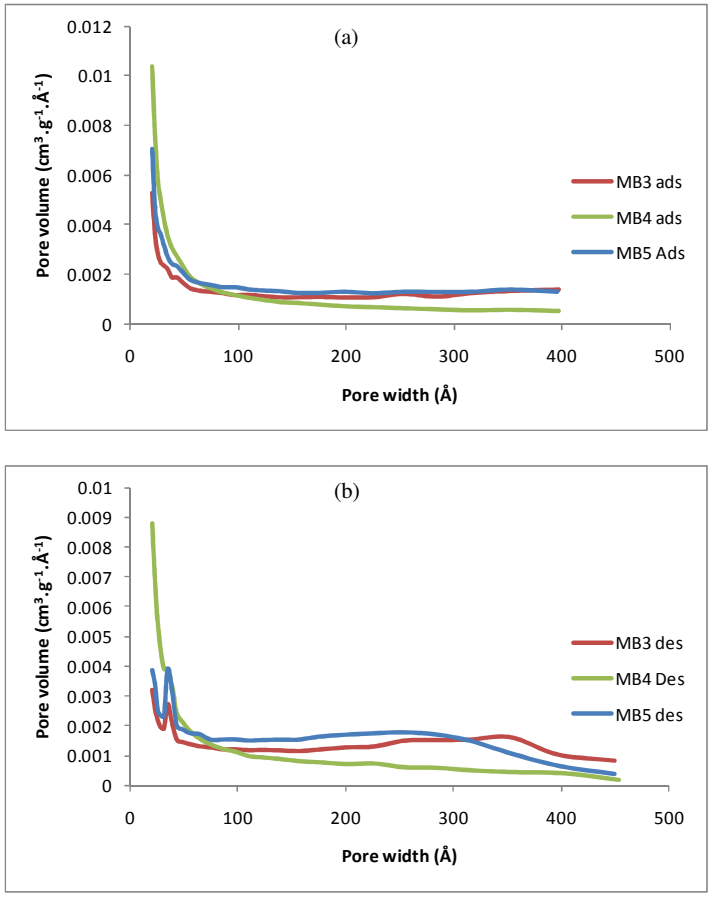



**Figure 3.12: Pore size distributions attained using the BJH method for (a) adsorption and (b) desorption.**

Figure 3.12 (a) demonstrates that the pore size distribution was largely heterogeneous and in the mesoporous 2-50 nm range. The desorption profile in Figure 3.12 (b) suggests that there is evidence of a sharp peak in the low nm range in the desorption

curve for MB3 and MB5 with a broad peak in higher ranges but Sing states that the desorption profile can be unreliable if pore blocking effects occur [36]. The significance of these results in this case is predominantly that the crosslinking monomer does indeed affect the morphological characteristics of the resulting polymers but for individual MIP/NIP pairs, the presence of the template appeared to have very little effect on the morphology of these bulk particles. This would suggest that the differences in retention behaviour were indeed due to the imprinting process and both frontal and zonal chromatographic experiments corroborate this.

*3.4.3.5 The effect of the functional monomer*

To better probe the role of the functional monomer in the recognition process it was deemed pertinent to prepare a polymer using crosslinking monomer alone. As Table 3.3 demonstrates, the highest imprinting effect was observed for MB3, where EGDMA was used as crosslinker and this polymer was used as the basis for functional monomer studies.

Table 3.1 shows that polymer MB8 was synthesised using only the template, HCB, and the crosslinker, EGDMA, in a 1:30 ratio in acetonitrile and Table 3.3 shows that *IF* values of 1.7 and 1.3 were obtained in MeCN and MeCN-$H_2O$ (30/70), respectively. This suggested that there was some degree of selectivity imparted upon the polymer but it must be noted that the $k'$ values for MeCN were 0.19 and 0.11 for MIP and NIP, respectively, so retention on the polymers in MeCN was limited. When MAA was used as the functional monomer (MB3 polymers), the $k'$ value increased to 1.14 for the MIP but only 0.19 for the NIP. This large increase in the MIP relative to that of the NIP suggested that it was not just an inherent increased affinity for the template due to the presence of MAA but rather the creation of imprinted sites, which caused an increase in the degree of interaction between the template and the polymer.

MB10 was prepared as a polymeric analogue to MB3, replacing MAA with HEMA as the functional monomer as NMR studies had suggested a degree of interaction of HEMA with steroidal templates (Section 2.4.). Despite the possible interaction demonstrated by NMR studies, this polymer displayed no imprinting effect and it was postulated that, as with PETRA, the slightly hydrophilic character of the monomer

prevented any selective interaction between the hydrophobic steroid analytes and the polymer. Another factor could be interaction between the solvent and the sites, effectively forming a barrier between the steroid and the sites. For this reason, MAA was chosen as the functional monomer for the preparation of all further polymers.

*3.4.3.6 The effect of the porogen on ground bulk polymers*

The results shown in Table 3.3 and discussed in the preceding sections suggested that the most promising bulk polymers were those prepared with EGDMA and TRIM as the crosslinking monomers. Since MB3 and MB5 were prepared in acetonitrile, it was decided to prepare similar polymers but using chloroform as the porogen as a route to understanding the role of the porogen. The polymers so prepared were MB12 and MB13 for EGDMA and TRIM, respectively. Table 3.3 demonstrates that while an imprinting factor of 1.9 was achieved for MB12 in acetonitrile, this was over three times lower than the corresponding *IF* value obtained for MB3 in acetonitrile. It was also not possible to obtain a repeatable retention time for any of the injections carried out in the MeCN-$H_2O$ mobile phase and for this reason no further chromatographic studies were carried out. It was interesting to note, however, that nitrogen sorption analysis suggested that there was very little difference between the surface areas, pore volumes or pore diameters of the polymers prepared in the different porogens and isotherms obtained were of the same type, despite the fact that polymers prepared in chloroform are typically non-porous [50].

This similarity in morphology was not repeated for the TRIM polymers prepared in chloroform. In this case, where MB5 (prepared in MeCN) polymers produced Type IV isotherms with H4 hysteresis loops, MB13 (prepared in $CHCl_3$) produced again Type IV isotherms but appeared to produce a H2 hysteresis loop for the MIP and a H4 hysterisis loop for the NIP, as demonstrated in Figure 3.13 (a).

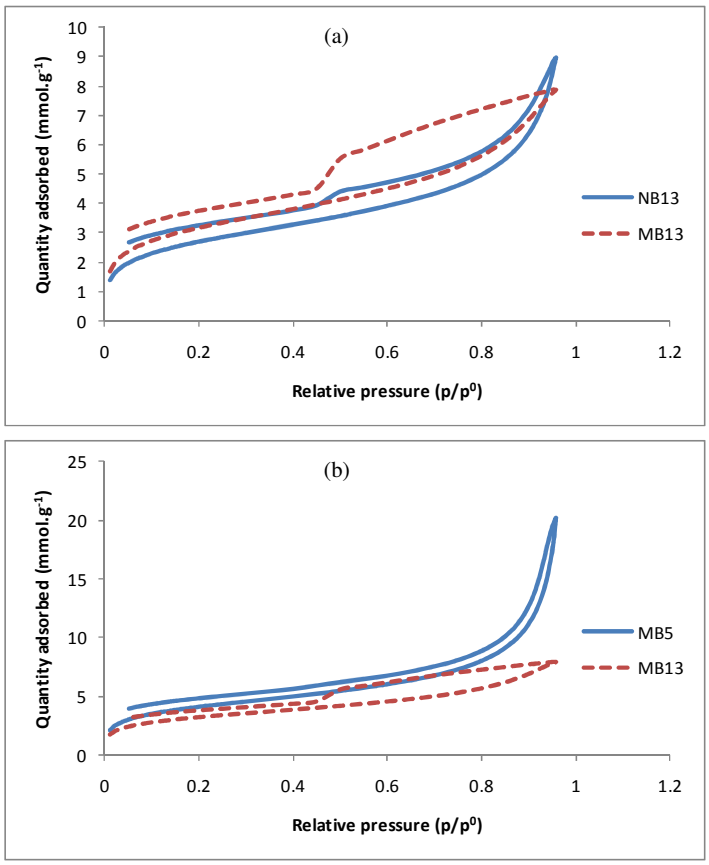



**Figure 3.13: Comparison of physisorption isotherms obtained for (a) MB13 and NB13 and (b) MB5 and MB13.**

While H2 hysteresis loops are considered difficult to accurately interpret [36], the differences exhibited for the templated polymer prepared in $CHCl_3$ suggest that the template plays a major role in the type of polymer morphology obtained when TRIM and MAA are used as the monomers with chloroform as a solvent. The increased morphological affect of the template was postulated because H4 hysteresis loops were obtained in all other cases for bulk polymers, for both imprinted and non-imprinted polymers. Table 3.3 shows that the pore volumes and pore diameters were also reduced in the polymers prepared in chloroform using TRIM as the crosslinking monomer.

Frontal analysis on these MB13 polymers also uncovered different binding properties than the acetonitrile prepared polymers. The average affinity constant for NB13 was actually higher, at $1.72 \times 10^5$ L.mol$^{-1}$, than the value of $9.43 \times 10^4$ L.mol$^{-1}$ for MB13, while the opposite was observed for MB5. The number of binding sites, *N*, for MB13 was higher by an order of magnitude than NB13, with values of $1.8 \times 10^{-2}$ and $4.8 \times 10^{-3}$

$\mu mol.g^{-1}$ for MIP and NIP, respectively. The latter value was the lowest number of binding sites for all of the TRIM based polymers. The heterogeneity index was also higher for MB13 than NB13 suggesting that the presence of the template in the pre-polymerisation mixture had an effect on the creation of the binding sites when chloroform was used as the porogen.

Despite the differences in polymer morphology and binding characteristics observed for frontal analysis, the results obtained for zonal chromatography were remarkably similar with an imprinting factor of 2.8 obtained in acetonitrile for MB13, compared to the value of 2.9 for MB5. Using MeCN-$H_2O$ (30/70) as the mobile phase, the *IF* value was actually higher for MB13 than MB5, with values of 2 and 1.7, respectively. These results suggested that while the porogen had pronounced effects on the morphology and individual binding characteristics of the imprinted polymer, the overall effect on the selective binding of the analyte was negligible and the solvent memory effect was negligible. This was contrasted by the EGDMA based polymer, MB12, where the solvent memory effect deleteriously affected binding selectivity. These results suggest that the use of the tri-functional crosslinker TRIM, as opposed to the di-functional EGDMA, while reported to increase load capacity and improve resolution of imprinted polymers [55], may also contribute to a more rigidly crosslinked polymeric matrix in which the binding sites created are less susceptible to swelling effects by the various solvents used. This factor could prove to be advantageous in that polymers could be prepared in non-polar solvents, thereby promoting hydrogen bonding interactions but could be utilised in aqueous based or polar solvents where the rigid binding sites would remain intact for rebinding.

*3.4.3.7 Selectivity studies*

To study the selectivity of the polymers and explore the factors driving recognition in the polymers, zonal chromatography was carried out on the most successful bulk polymers using all six steroidal analogues (Figure 3.2). The results for MeCN and MeCN-$H_2O$ (30/70) are given in Table 3.5 (a) and (b) and all data is the average of at least two repetitions.

**Table 3.5: Summary for the retention behaviour of ground bulk particles, MB3 (EGDMA), MB4 (DVB), MB5 (TRIM with MeCN as porogen) and MB13 (TRIM with $CHCl_3$ as porogen), using all six steroidal analogues in two mobile phases: (a) acetonitrile and (b) acetonitrile-water (30/70). (*k'* values quoted are MIP only).**

(a)

| Analyte | MB3 | | MB4 | | MB5 | | MB13 | |
|---|---|---|---|---|---|---|---|---|
| | ***k'*** | ***IF*** | ***k'*** | ***IF*** | ***k'*** | ***IF*** | ***k'*** | ***IF*** |
| HCB | 1.42 | 7.5 | 0.75 | 3.0 | 2.11 | 2.9 | 2.47 | 2.8 |
| BDN | 1.57 | 4.9 | 0.88 | 2.2 | 2.13 | 1.8 | 2.92 | 1.9 |
| HYD | 2.73 | 3.9 | 0.99 | 1.7 | 4.90 | 1.6 | 4.95 | 1.8 |
| PRD | 3.15 | 9.3 | 1.10 | 1.5 | 6.16 | 1.7 | 6.00 | 1.7 |
| HAC | 0.68 | 3.1 | 0.28 | 2.9 | 0.56 | 1.6 | 1.38 | 1.3 |
| PAC | 0.91 | 2.7 | 0.38 | 2.5 | 0.77 | 1.4 | 1.03 | 1.3 |

(b)

| Analyte | MB3 | | MB4 | | MB5 | | MB13 | |
|---|---|---|---|---|---|---|---|---|
| | ***k'*** | ***IF*** | ***k'*** | ***IF*** | ***k'*** | ***IF*** | ***k'*** | ***IF*** |
| HCB | 7.22 | 2.0 | 14.87 | 2.2 | 9.34 | 1.7 | 25.07 | 2.0 |
| BDN | 6.66 | 1.7 | 14.37 | 2.0 | 9.29 | 1.3 | 28.86 | 1.7 |
| HYD | 1.58 | 1.7 | 1.16 | 2.0 | 1.59 | 1.3 | 5.19 | 1.8 |
| PRD | 3.84 | 1.7 | 1.06 | 2.0 | 1.48 | 1.2 | 5.03 | 1.8 |
| HAC | 6.33 | 1.6 | 8.08 | 1.9 | 6.69 | 1.2 | 22.75 | 1.8 |
| PAC | 6.09 | 1.6 | 7.13 | 1.8 | 7.00 | 1.2 | 23.51 | 1.8 |

These results for the imprinting factors in the two solvent compositions are also presented graphically in Figure 3.14.

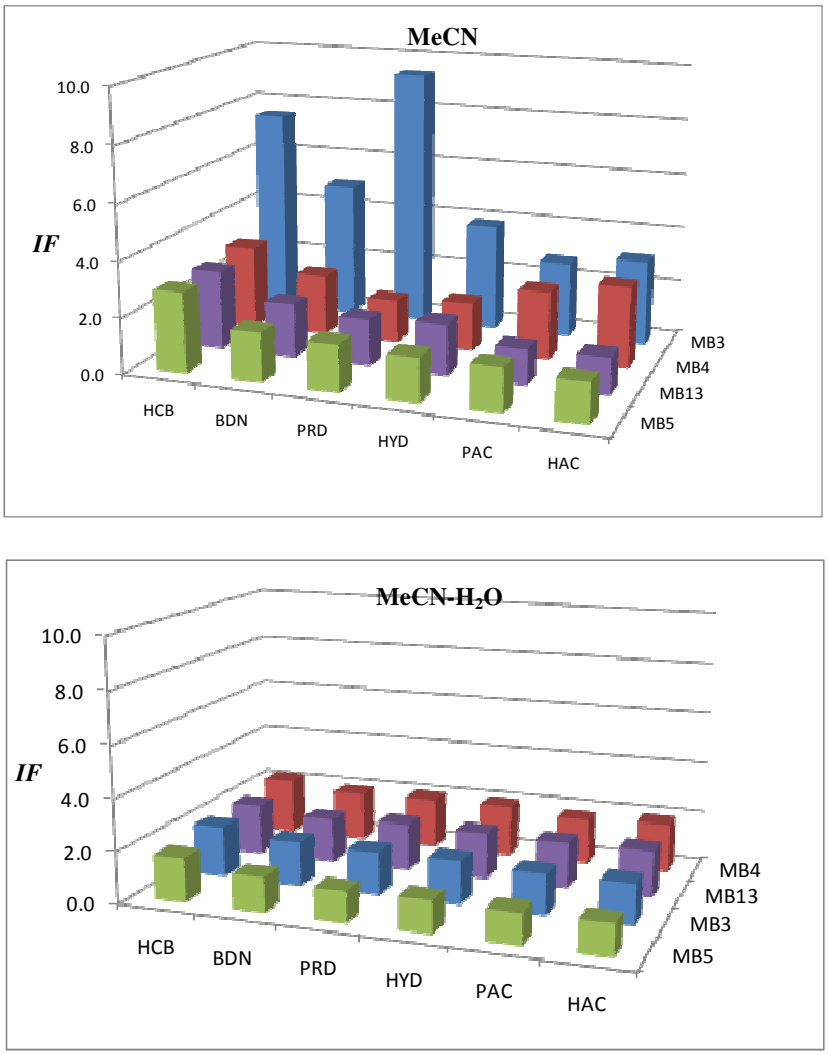



**Figure 3.14: Imprinting factors obtained for ground bulk particles, MB3, MB4, MB5 and MB13, in MeCN and MeCN-$H_2O$ (30/70).**

While analysis of the data presented in Table 3.5 and Figure 3.14 demonstrate that there are a number of differences between the imprinting effects observed in MeCN and in MeCN-$H_2O$, it can also be seen that there are a number of discernible patterns. Firstly, with the exception of one anomalous result, the template, HCB, had the highest *IF* value for all polymers in both solvents. This would be expected to be the case as the binding sites created within the imprinted polymers should most closely match the shape, size and functionality of the template. The exception to this was in the case of prednisolone in MB3 (EGDMA) using acetonitrile as the solvent.

In 100% MeCN, MB3 appears to display shape and size selectivity as, apart from the result for PRD, the highest *IF*, 7.5, was obtained for the template, HCB, followed by BDN at 4.9. The structure of BDN was used to choose the template and as such, the cavity formed by the template should match the shape and size of BDN to a greater extent than the other analytes. PRD and HYD possess no acetate, acetal or butyrate groupings on the C-17 or C-21 positions and, in theory, should 'fit' into the templated cavity with relative ease. The high *IF* obtained for PRD shows this to be the case while HYD had an *IF* of 3.9, next to BDN. HAC and PAC, both possessing acetate groupings on the C-21 position had the lowest *IF* values at 3.1 and 2.7, respectively. This suggested that the shape of these molecules obstructed rebinding to the same extent as the more sterically suitable steroids. A somewhat similar pattern was observed for MB13, although the *IF* values were lower than those of MB3 in all cases. That being said, shape and size selectivity still appeared to play an important role in analyte recognition.

The TRIM polymer prepared in acetonitrile, MB5, had an *IF* of 2.9 for the template and again, BDN had the closest imprinting factor but in this case the distinction between the remaining analytes was less clear. Here, the difference between the *IF* values for HCB and BDN was 1.1 but the difference between BDN and the lowest, PAC, was only 0.4. This suggested that MB5 had a high degree of fidelity for the template but rebound the remaining analytes to a more or less similar extent. It would be expected that TRIM, due to its trifunctionality, should give a more rigid structure with better shape and functional group selectivity for the template.

MB4, the DVB polymer, while having relatively low $k'$ values for the majority of analytes still managed to achieve the highest imprinting factor for HCB. For this polymer, hydrophobicity of the analytes appeared to play a more significant role as HYD and PRD, the analytes with the lowest predicted log P values, had the lowest *IF* values. This suggested that while these analytes could fit in the binding sites quite readily, they were repelled by their extra hydroxyl groups from the relatively hydrophobic polymer backbone, comprised mainly of DVB.

For analyses carried out using the predominantly aqueous mobile phase, i.e. MeCN-$H_2O$ (30/70), Table 3.5 (b) and Figure 3.14 (b) show that imprinting factors in all cases were

reduced and in the case of MB3, reduced significantly. The results also show that this reduction in imprinting factors coincided with an increase in the $k'$ values in the majority of cases. PRD and HYD were exceptional in this trend and this may have been due to their extra hydroxyl group at the C-17 position, imparting greater affinity towards the aqueous mobile phase.

Despite the reduction in *IF* values it was seen that a number of patterns still emerged, such as the highest imprinting factor in all cases for the template. MB5 still displayed a degree of fidelity for the template, with an *IF* of 1.7, while showing little or no discrimination between the other analytes with *IF* values ranging from 1.2-1.3. MB13, MB4 and MB3 did display marginally higher degrees of specificity for the template with *IF* values of 2.2, 2 and 2, respectively, but the difference between template values and other analyte values was no greater than 0.4 in any case. These results suggest that the hydrophobic interactions taking place in the predominantly aqueous solutions cause the analytes to be retained to a greater extent on both MIP and NIP in all cases. This still allowed for the analytes to interact with the specific binding sites on the MIP to a greater extent, thus demonstrating an imprinting effect in all cases.

Overall, these results show a high degree of recognition in these bulk polymers prepared with different crosslinking monomers. The highest degree of shape and size selectivity was demonstrated by MB3, the polymer prepared with EGDMA, in acetonitrile. This was not unexpected as the combination of the "reactive and prochiral methacrylate ester with a short spacer" allows for a large degree of conformational possibilities to facilitate imprinting and a highly rigid polymer matrix [56]. While the affinity of the polymers for the analytes was increased by the use of the tri-functional TRIM, where both MIP and NIP had a high $k'$ values, the shape and size differentiation was somewhat diminished in the case of MB5, although fidelity for the template was relatively high. What the combination of these results suggest is that the 'best' polymer would not be chosen based on a comparison of the individual characteristics but rather on the ultimate application for the material. For example sensing applications, where sensing of closely related structural analogues was necessary, MB3 with the obvious shape and size selectivity may best be utilised. Whereas for an application that required the slow release of an analyte, such as controlled release applications, the material with the highest affinity for the drug, such as MB5 or MB13 may best be employed.

### 3.4.4 Polymers prepared by precipitation

The preparation of bulk polymers prepared with HCB as the template suggested the suitability of this template as a target analogue for corticosteroid recognition and as such, was used as the template for the synthesis of imprinted polymers prepared by precipitation methods. The information gleaned from the work discussed in Chapter 2 allowed for the rapid synthesis of polymeric microspheres and microparticles with a high degree of control over particle size. The polymers that were synthesised in this way (Table 3.1) all used MAA as the functional monomer and employed EGDMA (M13), DVB (M14) and TRIM (M23) as crosslinking monomers.

M13 and M14 were prepared in acetonitrile and acetonitrile-toluene 3:1 (v/v), respectively, using thermal initiation while M23 was prepared in dichloromethane using UV irradiation to initiate polymerisation. While M13 and M14 were prepared based on information acquired in Chapter 2 it was deemed suitable to use a non-polar solvent to synthesise M23 based on the results given in Section 3.4.3. These results demonstrated the similar behaviour of TRIM polymers prepared in polar and non-polar solvents, with slightly improved imprinting effects observed for those prepared in non-polar porogens. The use of dichloromethane, however, necessitated the use of a low temperature polymerisation strategy and a method was developed based on that employed by Ye *et al*. for the preparation of TRIM based microspheres with estradiol selectivity. SEM micrographs of the precipitated polymers investigated in this study can be seen in Figure 3.15

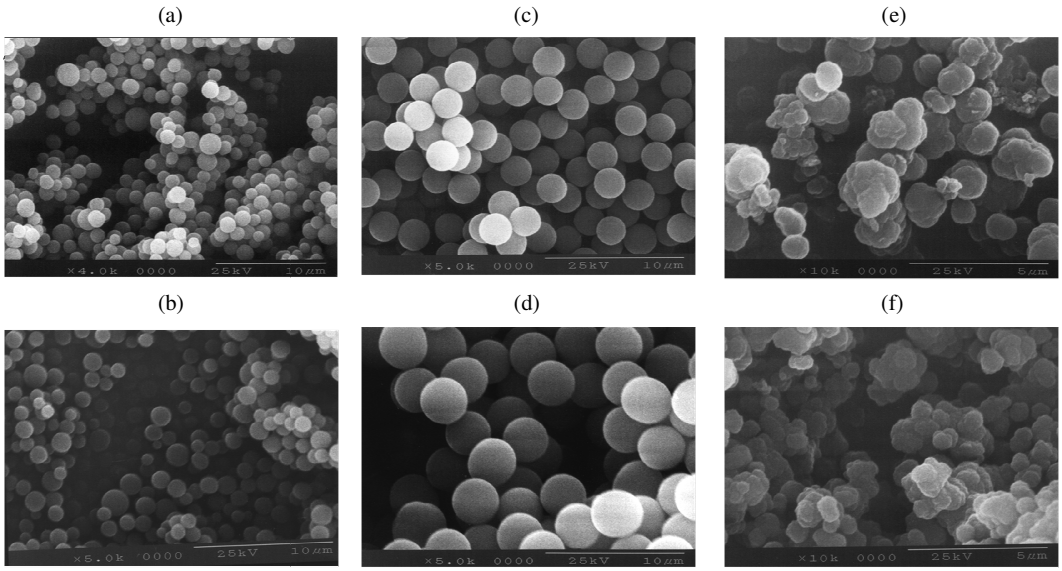



**Figure 3.15: Scanning electron micrographs of: (a) M13; (b) N13; (c) M14; (d) N14; (e) M23; (f) N23.**

The SEM images in Figure 3.15 (a)-(f) show that polymers with a microspherical morphology were obtained for M13 and M14 formulations but the M23 formulation, while producing precipitated microparticles, did not possess the smooth spherical shape of the other formulations. Instead, a mixture comprising of a small percentage of microspheres but predominantly rough agglomerated particles in the low micron range were obtained. The particle sizes for the three polymer formulations are presented in Table 3.6.

**Table 3.6: Particle size data for polymer formulations M13, M14 and M23**

| Polymer formulation | M13 | | M14 | | M23 | |
|---|---|---|---|---|---|---|
| | MIP | NIP | MIP | NIP | MIP | NIP |
| Particle size (µm) | 1.1-3.2 | 1.0-2.8 | 1.5-3.7 | 1.7-7.3 | 2.3-8.1 | 2.4-12.4 |

Figure 3.15 and Table 3.6 demonstrate that a high degree of control over particle size was attained. For M13, the EGDMA based polymer, the template appeared to have little effect on the particle size. The imprinted beads were slightly larger than the non-imprinted beads, which was the reverse effect of the use of budesonide as the template. The bead size was similar to those prepared using the same formulation, in Chapter 2. M14, the DVB polymer had a similar size distribution as the budesonide imprinted

polymer and the same effect of the template, i.e. smaller particles for the MIP was observed. Overall, the effect of changing templates for EGDMA and DVB based beads was relatively minor.

M23, the TRIM based polymer, had not been prepared for budesonide. Interestingly, as stated above, the smooth spherical shape was absent for these polymers. This has been observed in the work of Yoshimatsu *et al*. where varying amounts of TRIM were added to DVB formulations and in some cases the particles lost their spherical shape [57]. Precipitated particles prepared with TRIM by Yong Jiang *et al*. also lacked this spherical shape [58]. While spherical TRIM particles have also been prepared [59], it appears to be the case that for certain formulations, this crosslinker forms irregular shaped particles rather than spheres. This may be related to the trifunctionality of the monomer and the rapid nature of the growing network, which is ultimately prevented from forming a space filling gel due to dilution.

The primary focus of this study, however, was to investigate the applicability of the target analogue approach for imprinting polymeric microspheres and to determine if MIP chromatography might be a suitable probe for any selective recognition created within the polymers during the molecular imprinting process. To this end, the results obtained for these polymers are presented in Table 3.7.

**Table 3.7: Comparative result data for polymers prepared by a precipitation polymerisation approach using three different crosslinking monomers: EGDMA (M13), DVB (M14) and TRIM (M23) from nitrogen physisorption, chromatographic binding site characterisation and chromatographic evaluation of imprinting factors.**

| Polymer | Surface area ($m^2.g^{-1}$) | Pore [1] volume ($cm^3.g^{-1}$) | Pore [1] diameter (nm) | $m^2$ | $K^2$ ($L.mol^{-1}$) | $N^3$ ($\mu mol.g^{-1}$) | k' MeCN | IF MeCN | k' MeCN-$H_2O$ | IF MeCN-$H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|
| M13 | 5.54 | 0.012 | 14 | n.d. | n.d. | n.d. | n.r. | n.d. | 5.84 | 1.5 |
| N13 | 4.28 | 0.015 | 17 | n.d. | n.d. | n.d. | n.r. | | 3.94 | |
| M14 | 596 | 0.142 | 2.9 | 0.8556 | $1.02 \times 10^5$ | 18.1 | 1.85 | 1.5 | n.d. | n.d. |
| N14 | 631 | 0.154 | 2.8 | 0.8267 | $1.11 \times 10^5$ | 16.4 | 1.26 | | n.d. | |
| M23 | 5.47 | 0.011 | 8.9 | 0.9554 | $7.74 \times 10^4$ | $7.3 \times 10^{-2}$ | 4.13 | 1.7 | n.d. | n.d. |
| N23 | 21.24 | 0.025 | 7.7 | 0.9627 | $7.60 \times 10^4$ | $3.1 \times 10^{-2}$ | 2.4 | | n.d. | |

[1] values obtained from the adsorption curve; [2] heterogeneity index obtained from Freundlich isotherm (FI); [3] apparent weighted average affinity constant of binding sites from FI; [4]apparent number of sites from FI;
n.d.: no repeatable data; n.r.: not retained
*k'* and IF values obtained at 0.2 $mL.min^{-1}$ and frontal chromatography carried out at 0.5 $mL.min^{-1}$ in MeCN

The results presented in Table 3.7 demonstrate that the effect of imprinting on the precipitated particles was not as clear-cut as in the case of the ground bulk particles. This was to be expected given the results obtained for precipitated polymers in Chapter 2 and the NMR studies showing weak interaction between the template in those studies. This fact notwithstanding, there were still a number of positive results obtained for the precipitated particles. While no polymer yielded imprinting factors in both solvent compositions, each polymer displayed imprinting effects in one solvent system.

For analysis where acetonitrile was used as the mobile phase, HCB was not retained past the void marker on M13 or N13. Some clue to the reasons for this can be seen in the low surface areas observed for these polymers, 5.5 $m^2.g^{-1}$ and 4.28 $m^2.g^{-1}$, for MIP and NIP, respectively. The isotherms (Figure 3.16) demonstrate Type II behaviour.

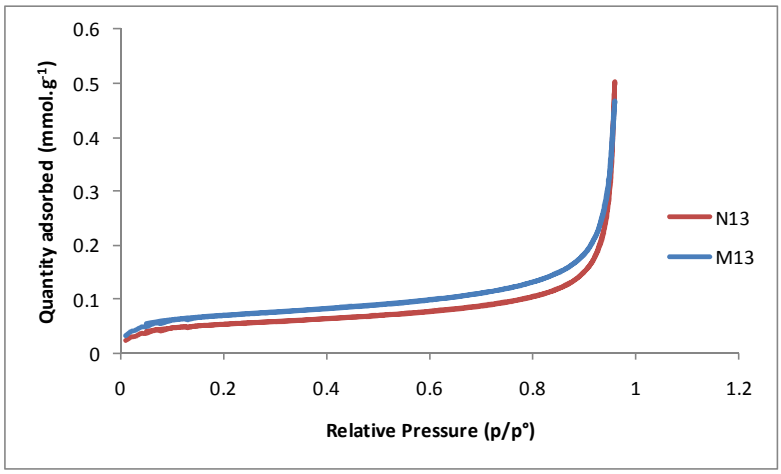



**Figure 3.16: Physisorption data obtained for M13 and N13 displaying Type II isotherms.**

The Type II physisorption isotherms obtained for M13 and N13, coupled with the low BET surface area suggest that these polymers were non-porous adsorbents. While relatively large average pore diameters were calculated, these appear to be somewhat misleading as the pore volumes were low, with values of 0.012 $cm^3.g^{-1}$ for M13 and 0.015 $cm^3.g^{-1}$ for N13. The low surface area and poor porosity of these polymers would suggest that acetonitrile was a relatively 'bad' thermodynamic solvent for the growing polymer, causing it to phase separate at an early stage resulting in non-porous microspheres with low surface areas [60]. The poor porosity and low surface area of the polymers coupled with a greater affinity of the analyte for the solvent may have

prevented the analytes from being retained on the polymeric column, even in imprinted sites.

The non-retention of the analyte prevented further investigation of the binding site characteristics by frontal chromatography. It did not prevent, however, the analysis of these polymers in the acetonitrile-water mobile phase and it can be seen from Table 3.7 that an imprinting factor of 1.5 was achieved for this analysis. Again, the *k′* values were increased considerably due to presumably hydrophobic interactions and this facilitated interaction with specific binding sites present on the surface of these polymers.

Precipitated polymers prepared using DVB as the crosslinking monomer, M14 and N14, displayed quite different behaviour in a number of cases. Here acetonitrile-toluene in a 3:1 (v/v) ratio was used, as this had been shown in Chapter 2 to prepare the largest and most spherical particles for polymers based on this crosslinker. Coupled with this, toluene is a thermodynamically 'good' solvent for polymers prepared with DVB, as its addition delays phase separation of the growing polymer allowing for the creation of pores [60, 61]. The BET surface areas for these polymers would therefore be expected to be high and this was shown to be the case, with values of 596 $m^2.g^{-1}$ and 631 $m^2.g^{-1}$ for MIP and NIP, respectively. The pore volumes, 0.142 $cm^3.g^{-1}$ for the MIP and 0.154 $cm^3.g^{-1}$ for the NIP were also considerably larger than for M13 or M23. The physisorption isotherms for M14 and N14 are shown in Figure 3.17 and the presence of hysteresis appears to suggest Type IV behaviour similar to that seen with the ground bulk particles and typical for meso-macroporous materials. Again, the gas was not fully desorbed at low $P/P^0$ values and as with the ground bulk particles, this was probably due to the presence of narrow pores from which it was difficult for the gas to be removed [38].

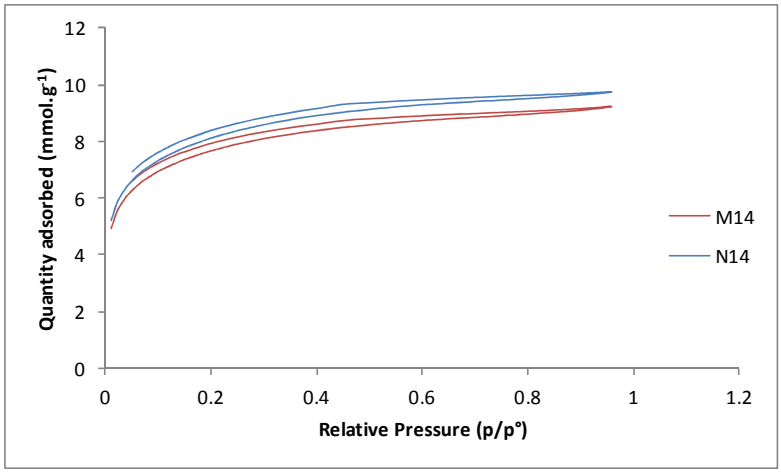



**Figure 3.17: Physisorption isotherms obtained for M14 and N14.**

As well has having different morphological characteristics, the M14 polymers also had different retention behaviours for the template. While the large surface areas and strong hydrophobic nature of the polymer retained the analytes on the columns for over three hours in the case of both MIP and NIP in highly aqueous mobile phases, an imprinting factor of 1.5 was achieved in MeCN.

Frontal chromatography showed that there was very little difference between the heterogeneity indexes for the MIP and NIP. While they both displayed a strong degree of affinity for the template, with values of $1.02 \times 10^5$ L.mol$^{-1}$ and $1.11 \times 10^5$ L.mol$^{-1}$ for MIP and NIP, respectively, the MIP had a marginally higher *N* value at 18.1 µmol.g$^{-1}$ compared to 16.4 µmol.g$^{-1}$ for the NIP.

These results suggested that while imprinting did indeed take place, the number of specific binding sites created within the polymer matrix was minimal. The log *N* vs. log *K* plot shown in Figure 3.18 demonstrated the almost negligible differences for the two polymers.

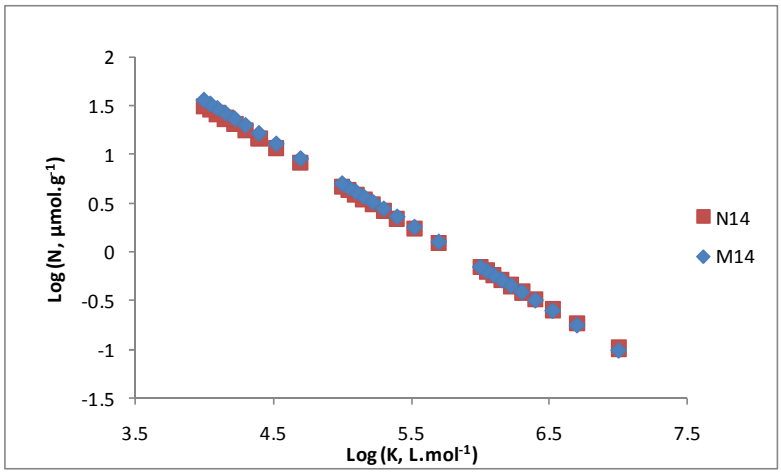



**Figure 3.18: Log *N* vs. log *K* plot obtained for M14 and N14 showing the marginal differences between the MIP and NIP.**

M23 exhibited similar behaviour to M14 in that an *IF* value of 1.7 was achieved for HCB in MeCN. This was the highest *IF* observed for the HCB for the precipitated polymers. This was despite that fact that the MIP had morphological characteristics similar to those of M13, with a surface area of only 5.47 $m^2.g^{-1}$ and displaying a Type II isotherm. Interestingly, N23 had a considerably higher surface area (though by no means a high surface area) with a value of 21.24 $m^2.g^{-1}$. It was noteworthy that N23 had a Type IV isotherm with H4 hysteresis, similar to all of the ground bulk particles and M14. The differences between the two isotherms is depicted in Figure 3.19.

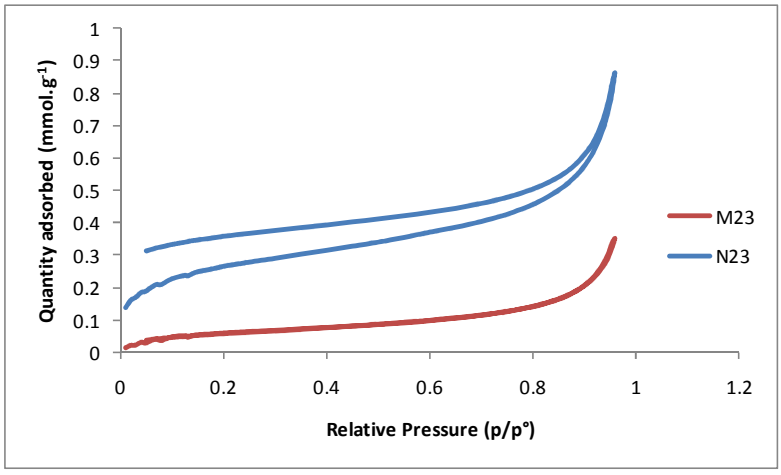



**Figure 3.19: Physisorption isotherms obtained for M23 (Type II) and N23 (Type IV).**

With the data for M23 suggesting a non-porous material and that for N23 suggesting a meso-macroporous material, it would appear that the presence of the template in the pre-polymerisation complex had a profound effect on the morphological characteristics

of the resultant polymer. While the SEM images in Figure 3.15 (e) and (f) show that microparticles rather than microspheres were formed in both cases, there did appear to be some differences in the morphologies of the polymers in that the NIP appeared to have slightly larger agglomerates than did the NIP. This would suggest, as would the gas sorption data, that the addition of template to the complex mixture reduced its thermodynamic favourability for the polymer and caused the precipitation of the MIP at an earlier stage and resulting in non-porous microparticles.

While gas sorption data showed differences between the morphologies of the polymers, frontal chromatographic analysis demonstrated that differences also existed for the binding characteristics of the two polymers. The heterogeneity index, *m*, was slightly lower for M23 at 0.9554 compared to 0.9627 for N23, suggesting the presence of a marginally greater degree of heterogeneity. *K* values were $7.74 \times 10^4$ L.mol$^{-1}$ and $7.60 \times 10^4$ L.mol$^{-1}$ for MIP and NIP, respectively while the *N* value was twice as high at $7.3 \times 10^{-2}$ µmol.g$^{-1}$ for M23 and $3.1 \times 10^{-2}$ µmol.g$^{-1}$ for N23. These results are represented graphically in Figure 3.20.

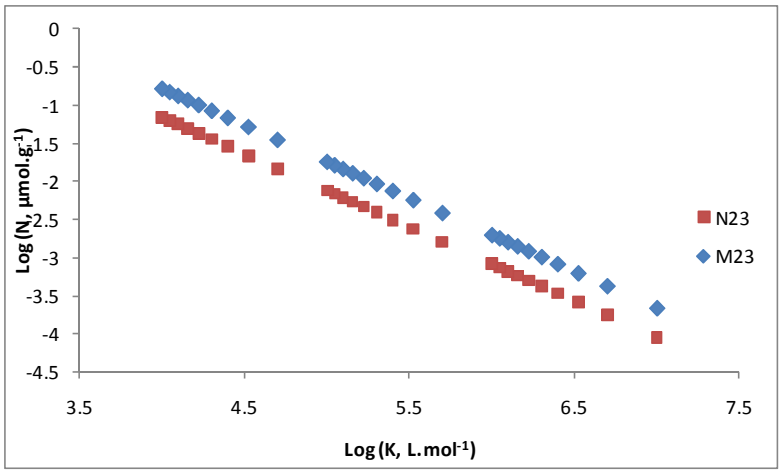



**Figure 3.20: Log *N* vs. log *K* plot for M23 showing differences in binding strength and the number of binding sites.**

While these differences were relatively marginal, they were sufficient to impart a degree of specific binding to the MIP. As was the case for M14 the steroid was retained for over three hours on both MIP and NIP when the aqueous mobile phase was used, presumably due to strong hydrophobic interactions and no repeatable or reliable data could be obtained for acetonitrile-water mixtures.

*3.4.4.1 Selectivity studies for precipitation polymers*

The six steroidal analogues were again injected onto the imprinted and control polymers to attempt to probe the factors driving recognition within the precipitated polymers. These studies were only carried out for each polymer in the solvent in which imprinting effects could be measured, therefore all three polymers could not be directly compared. For this reason the results in Table 3.8 and Figure 3.21 are presented for analysis of M13 in acetonitrile-water and for M14 and M23 in acetonitrile. All results given are an average of at least two repetitions.

**Table 3.8: Result data for selectivity studies on the six structural analogues for precipitated polymers M13, M14 and M23.**

| Analyte | M13 (MeCN-$H_2O$) | | M14 (MeCN) | | M23 (MeCN) | |
|---|---|---|---|---|---|---|
| | ***k'*** | ***IF*** | ***k'*** | ***IF*** | ***k'*** | ***IF*** |
| HCB | 5.84 | 1.5 | 1.85 | 1.5 | 4.13 | 1.7 |
| BDN | 8.29 | 1.4 | 2.41 | 1.1 | 7.70 | 1.7 |
| HYD | 1.89 | 1.5 | 3.10 | 1.7 | 10.73 | 1.7 |
| PRD | 1.84 | 1.5 | 3.61 | 1.6 | 14.47 | 1.7 |
| HAC | 7.92 | 1.5 | 1.41 | 1.3 | 4.07 | 1.7 |
| PAC | 7.32 | 1.4 | 1.75 | 1.1 | 5.96 | 1.6 |

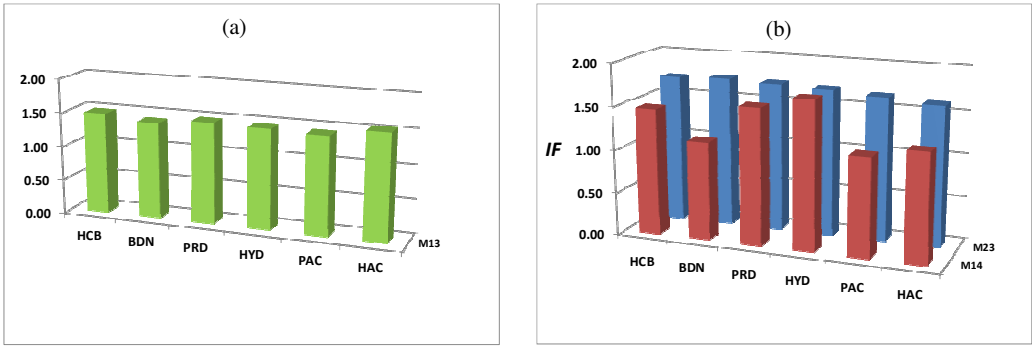



**Figure 3.21: Imprinting factors observed for the six structural analogues for (a) M13 in MeCN-$H_2O$ (30/70) and (b) M14 and M23 in MeCN.**

As Table 3.8 and Figure 3.21 demonstrate, there was almost no difference present for the different analytes on polymers M13 or M23, with differences no greater than 0.1 for the imprinting factors of any of the analytes. M14 displayed some small differences in

*IF* values, with low values for BDN, HAC and PAC, ranging from 1.1 to 1.3. The imprinting effect was more pronounced for the template, with a value of 1.5 but the highest values were for PRD and HYD at 1.6 and 1.7, respectively. While the higher *IF* for the template would have been expected, the higher values for HYD and PRD were accompanied by increased retention and while this may have been due to slight incompatibilities with the solvent, it allowed for a greater degree of interaction between these analytes and the specific binding sites on the imprinted polymers.

While the results obtained for all of the precipitated beads were, as previously stated, less clear-cut and without the discernible patterns of the ground bulk particles, they nonetheless demonstrated the first instance of corticosteroid specific molecularly imprinted polymers prepared *via* precipitation methods, using a target analogue approach. While many of the differences were small, it must be noted that these results were highly repeatable and averages of at least two values are quoted. The lower imprinting effects observed in the precipitated beads than the bulk particles corroborated the findings of Chapter 2, which suggested that a means of promoting the interaction between the template and monomers in the pre-polymerisation solution for dilute precipitated methodologies would be necessary. It was shown here that even the use of a non-polar solvent, DCM, and UV polymerisation at 4 °C could not promote the interaction enough to significantly increase the number of binding sites within the polymer matrix.

## *3.5 Conclusion*

Molecularly imprinted polymers with group selectivity for corticosteroids have been prepared using a ground, bulk particle format by the target analogue approach. Hydrocortisone-17-butyrate was chosen as the template that most closely matched one of the primary targets, budesonide and the success of this strategy was demonstrated by the high degree of affinity observed for budesonide over analytes other than the template in the majority of cases. This suggested the suitability of this approach for the preparation of polymers for use in solid-phase extraction applications where HCB imprinted polymers could specifically retain BDN. This is discussed in Chapter 4.

Novel corticosteroid imprinted microspheres and microparticles were prepared using three crosslinking monomers. A high degree of control over bead size and morphology was demonstrated for these beads. Binding site characterisation and chromatographic evaluation suggested that the molecular imprinting effect was less pronounced than in the case of the bulk particle strategy. This corroborated findings in Chapter 2, which suggested that this was related to weak interaction between the template and monomer in the pre-polymerisation mixture for such dilute solutions and efforts to enhance these interactions will be discussed in Chapter 5.

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# Chapter 4

# Applications of corticosteroid imprinted polymers

# 4. Applications of corticosteroid imprinted polymers

## *4.1 Introduction*

A number of applications, for which molecularly imprinted polymers have been developed, have been discussed in detail in Section 1.6 and Section 3.1. These examples included solid phase extraction (SPE) [1, 2], catalysis [3, 4], drug delivery [5, 6] and chromatography [7, 8]. Many other applications, however, exist, including the use of MIPs in sensor platforms, such as that prepared by Zhou *et al*. [9]. This involved the imprinting of an analogue of domoic acid on a polydopamine film, which was coated onto a quartz crystal microbalance (QCM) sensor. The use of the target selective imprinted polymer as the sensing element for the highly sensitive gravimetric sensor enabled detection of domoic acid down to 5 ppb and could discriminate between the target and two close structural analogues.

QCM sensors incorporating imprinted polymers have also been prepared by, amongst others, Yang *et al*. for billirubin [10] and Lee *et al*. for salivary proteins [11]. Liang *et al*. [12] and Guerreiro *et al*. [13] have employed MIPs as the sensing element in ion selective electrodes for the pesticide chlropyrifos and the antimicrobial chlortetracycline, respectively, while Riskin *et al*. have used imprinted films for the detection of explosives using surface plasmon resonance [14].

Depending on the application, and indeed, the specific end use for each application, different characteristic behaviours of MIPs can be exploited. The use of imprinted polymers in SPE, for example, can employ MIPs capable of targeting a group of analogous molecules in a system, as was the case for the imprinted polymers prepared for the extraction of banned Sudan dyes in food by Baggiani *et al*. [1] and organic acids from a herb sample by Zhu *et al*. [15]. The ability of MIPs to selectively recognise the template molecule when in the presence of close structural analogues was a characteristic of this technology exploited by Jiang *et al*. [7] and Lee *et al*. [8] for the chromatographic separation of structurally similar non-steroidal anti-inflammatory drugs and closely related steroids, respectively.

The potential use of corticosteroid imprinted polymers for various applications has been investigated by a number of research groups, for example, Ramstrom *et al*. have

discussed their potential use in biotechnological industrial processes that utilise catalysts for steroid transformation [16]. The same group has studied the use of molecularly imprinted polymers for corticosterone in the screening of chemical combinatorial libraries [17] while Sreenivasan has prepared a hydrocortisone imprinted polymer capable of the switchable release of testosterone [18].

Baggiani *et al*. have synthesised hydrocortisone imprinted polymers for application as steroid selective adsorbents for solid phase extraction [19, 20]. In the first of these studies, the binding properties of the imprinted polymer were studied using liquid chromatography and while the imprinting factor was highest for the template, at 9.71, significant binding was observed for a number of the structural analogues. The need for adsorbents with group selectivity for corticosteroids was highlighted in the second of these studies, due to the need for regulation and monitoring of steroids in food products and the inherent complexity of such samples.

A 2011 review by Gorog, dealt with 213 articles and evaluated the analysis of steroidal drugs in pharmaceutical and environmental samples [21]. That review highlighted the relevance of developing analytical strategies for steroidal compounds as it discussed the reliance on non-selective methods of steroid analysis of bulk drug materials, quoting figures of 75% of the assays of the Unites States Pharmacopoeia relying on selective chromatography, while the European and Japanese Pharmacopoeias use HPLC for 19% and 54%, respectively. The author goes on to discuss the inherent lack of suitability of many of the non-selective methods for the accurate quantitation of many impurities and degradation products.

The preceding examples demonstrate the need for materials that are capable of group selectivity for corticosteroid compounds, as well as the ability to prepare materials that can separate and quantify corticosteroid compounds with almost identical structures. To this end, corticosteroid imprinted polymers discussed in the preceding chapters have been investigated for use as adsorbents for solid phase extraction using a target analogue approach and for highly selective chromatographic stationary phases capable of differentiating between nearly identical steroidal compounds.

## *4.2 Research aims and objectives*

The aims and objectives of this study were to:

- Investigate the use of the corticosteroid imprinted polymers outlined in the preceding chapters for solid phase extraction using a target analogue approach
- Investigate the use of novel corticosteroid imprinted polymers prepared by precipitation polymerisation for the chromatographic separation of structurally similar corticosteroid analytes

## *4.3 Experimental*

### 4.3.1 Materials

Solid phase extraction (SPE) cartridges and frits were obtained from Sigma-Aldrich, Ireland. All other materials were as described in Sections 2.3 and 3.3.

### 4.3.2 Molecularly imprinted solid phase extraction

Dry polymer (MB3 or MB5) (200 mg), prepared as per Section 3.3.2, was packed into 3 mL polypropylene SPE cartridges containing frits with a 20 µm porosity. The columns were connected to a vacuum manifold and washed with 50 mL acetonitrile to ensure the polymer was evenly packed. Prior to use, packed SPE cartridges were conditioned with 2 × 1 mL acetonitrile followed by 2 × 1 mL $H_2O$ and vacuum dried to remove all traces of solvent. The columns were reconditioned using 2 × 2 mL 10% acetic acid in acetonitrile followed by 2 × 2 mL acetonitrile.

A number of molecularly imprinted solid phase extraction (MISPE) protocols were used (protocol 1-5). Unless otherwise stated the loading step involved the loading of budesonide (BDN) onto conditioned columns in 1 mL $H_2O$ at a concentration 10 $\mu g.mL^{-1}$:

Protocol 1 (P-1):

- Molecular recognition/wash: 1 mL aliquots of water-acetonitrile solutions, composed of sequentially increasing acetonitrile content from 10% to 50%.
- Elution: 1 × 1 mL acetonitrile followed by 3 × 1 mL 10% acetic acid in acetonitrile.

Protocol 2 (P-2):

- Molecular recognition/wash: 1 × 1 mL water-acetonitrile (MB3 – 60% $H_2O$; MB5 – 50% $H_2O$)
- Elution: 1 × 1 mL acetonitrile followed by 3 × 1 mL 10% acetic acid in acetonitrile

Protocol 3 (P-3):

- Molecular recognition/wash: 1 × 2 mL water-acetonitrile (60% $H_2O$)
- Elution: 1 × 2 mL acetonitrile followed by 1 × 1 mL acetonitrile

Protocol 4 (P-4):

- Load: 1 mL 5 $\mu g.mL^{-1}$ budesonide solution in $H_2O$
- Molecular recognition/wash: 1 × 1 mL water-acetonitrile (60% $H_2O$)
- Elution: 2 × 2 mL acetonitrile

Protocol 5 (P-5):

- Conditioning: 1 × 2 mL MeOH followed by 1 × 1 mL 0.1 M KOH in MeOH followed by 2 × 5 mL $H_2O$
- Molecular recognition/wash: 1 mL aliquots of water-acetonitrile solutions, composed of sequentially increasing acetonitrile content from 10% to 40%.
- Elution: 1 × 2 mL acetonitrile.

P-1 was repeated three times for MB3 and MB5 with stated results calculated as the average of the three individual results. P-2 was carried out in triplicate for MB3 (with results calculated as per P-1) and carried out once for MB5. P-3, P-4 and P-5 were performed once for MB3.

### 4.3.3 Preparation and evaluation of prednisolone imprinted polymers (M25-M27)

Polymers M25-M27 were prepared using a formulation based on M23 (as outlined in Section 3.3.2) and polymers prepared by Ye *et al*. [22]. Prednisolone was used as the template and a saturated template solution was prepared in dichloromethane (0.27-0.8 g depending on the volume of solvent). Methacrylic acid was then added, followed by trimethylolpropane trimethacrylate (TRIM). The ratio of functional to crosslinking

monomer was held constant at 8:4 while the monomer concentration was set at 2% (w/v) for M25 and M26, while a concentration of 1% (w/v) was used for M27. Azobisisobutyronitrile was used as the initiator at a concentration of 3 mol%. Polymerisation was carried out at 4 °C using UV initiation at 350 nm. The control polymer was prepared as per N23 (Section 3.3.2).

Polymers were washed on-line using MeOH acidified with 5% acetic acid until a stable baseline was achieved. Chromatographic evaluation, nitrogen sorption porosimetry and particle size analysis were carried out as per sections 3.3.3, 3.3.4 and 3.3.5, respectively.

## *4.4 Results and discussion*

### 4.4.1 Molecularly imprinted solid phase extraction of budesonide

#### *4.4.1.1 MISPE protocol P-1*

Budesonide (BDN), one of the primary steroidal targets for this study, is a corticosteroid used for a range of medical conditions, including Crohn's disease [23], asthma [24] and has been investigated for use in combating lung cancer [25]. Thus, the ability to extract and quantify budesonide from complex samples could be beneficial for a range of analytical processes. Chapter 3 discusses the use of the target analogue approach to molecular imprinting where a close structural and functional analogue of budesonide, hydrocortisone-17-butyrate (HCB) was used to prepare imprinted polymers with group selectivity for a number of corticosteroids, with a particular emphasis on affinity for budesonide.

In Chapter 3, HCB imprinted polymers MB3 and MB5, prepared using ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM), respectively, as crosslinking monomers and methacrylic acid (MAA) as the functional monomer, had the most suitable affinity for budesonide. Table 3.4 shows that in all but two cases the imprinting factor for budesonide was closest to that of the template, HCB, for these polymers. While the TRIM based polymer prepared in chloroform also gave a high imprinting factor in acetonitrile-water mixtures, the *k*' value was extremely high, which could pose problems during analyte elution. MB3 and MB5 were thus chosen for preliminary MISPE studies.

MISPE protocols P-1 to P-5 were investigated for solid phase extraction experiments, in an effort to observe the greatest degree of molecular recognition in the imprinted polymers while using the lowest number of sample treatment steps. To this end, P-1 employed a molecular recognition step that utilised water-acetonitrile washes increasing in acetonitrile concentration up to 50% (v/v). The elution profiles for P-1 are presented in Table 4.1 and Figure 4.1.

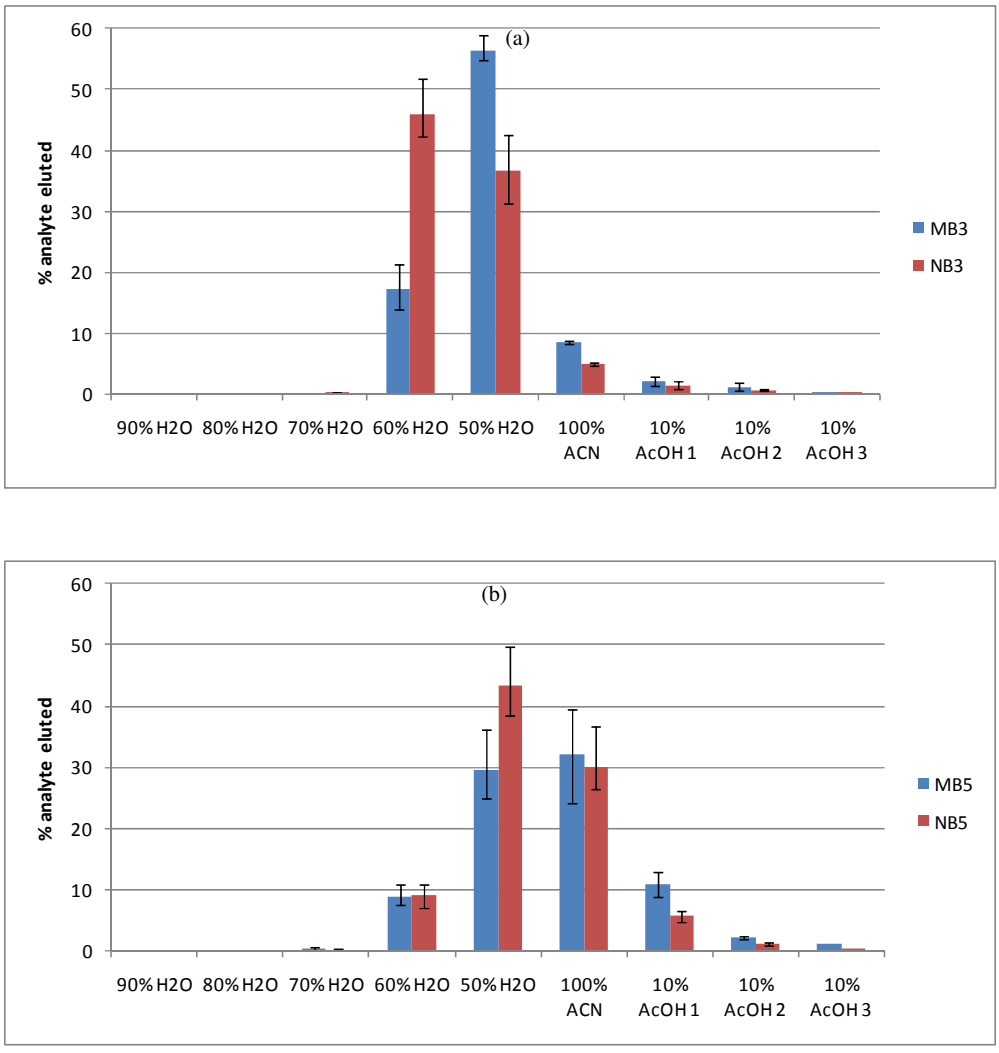


**Figure 4.1: BDN elution profiles using MISPE P-1 for HCB imprinted polymers: (a) MB3 (EGDMA) and (b) MB5 (TRIM).**

**Table 4.1: BDN elution profiles for HCB imprinted polymers: MB3 (EGDMA) and MB5 (TRIM) using MISPE protocol P-1.**

| Solvent composition | % Budesonide eluted | | | |
|---|---|---|---|---|
| | MB3 | NB3 | MB5 | NB5 |
| 90% $H_2O$ | 0 | 0 | 0 | 0 |
| 80% $H_2O$ | 0 | 0 | 0 | 0 |
| 70% $H_2O$ | 0 | 0.13 (± 0.1) | 0.4 (± 0.3) | 0.2 (± 0.2) |
| 60% $H_2O$ | 17.2 (± 4.1) | 45.9 (± 5.8) | 9.0 (± 2.1) | 9.1 (± 2.1) |
| 50% $H_2O$ | 56.4 (± 2.5) | 36.7 (± 5.8) | 29.5 (± 6.6) | 43.4 (± 6.2) |
| 100% MeCN | 8.5 (± 0.3) | 4.9 (± 0.4) | 32.1 (± 8.1) | 30.1 (± 6.6) |
| 10% acetic acid -1 | 2.3 (± 1.0) | 1.4 (± 0.7) | 10.8 (± 2.2) | 5.7 (± 0.9) |
| 10% acetic acid -2 | 1.2 (± 0.7) | 0.7 (± 0.2) | 2.3 (± 0.3) | 1.1 (± 0.3) |
| 10% acetic acid -3 | 0.4 (± 0.1) | 0.5 (± 0.1) | 1.3 (± 0.1) | 0.5 (± 0.2) |
| Total recovered | 86.1 (± 8.0) | 90.1 (± 13.2) | 85.3 (± 18.8) | 90.2 (± 15.8) |

Protocol P-1 used a gradient molecular recognition step to enhance the interactions between budesonide and the specific sites in the imprinted polymer with concomitant removal of the budesonide molecules non-specifically bound to both imprinted and control polymers. Figure 4.1 and Table 4.1 demonstrate that for this molecular recognition profile, no budesonide was detected during the 90% and 80% water steps for any of the polymers. This was expected due to the hydrophobic nature of the interaction between BDN and the polymers.

Figure 4.1 (a) shows that when the water content was reduced to 70% 0.13% of the budesonide was eluted from the NB3 column while no budesonide was detected for the MIP. As the MeCN content was further increased and the water reduced to 60%, 45.9% of the budesonide was eluted from NB3 while over 2.5 times less was eluted from MB3. This suggested that the gradient steps used did indeed drive the interaction between the analyte and the specific sites on the polymer. The material so adsorbed, however, was then removed during the next steps where 50% and 100% acetonitrile were used, due to increasing affinity of the solvent for the analyte.

These results suggested that specific sites created for HCB were capable of specifically retaining BDN. This was in agreement with the zonal chromatography on this polymer,

discussed in Section 3.4.2.4, which showed that imprinting factors of 4.9 and 1.7 were obtained for budesonide in MeCN and MeCN-$H_2O$ (30/70), respectively. This was also in agreement with the results for frontal chromatography, which determined that while the difference between the affinity constants was marginal, the MIP had over five times more sites than the NIP (34.8 µmol.$g^{-1}$ compared to 6.31 µmol.$g^{-1}$).

Figure 4.1 (b) shows that again, the affinity of the solvent for the analyte played a significant role in elution. Similar behaviour was observed for the TRIM based polymers in that it was at 70% water where some budesonide began to elute from both columns. An increase in the acetonitrile content to 40% did not result in any difference between the MIP and the NIP, however, further increase to 50% acetonitrile resulted in 14% more budesonide being retained on MB5 than NB5. As with MB3, further increase to 100% acetonitrile and the use of acidified acetonitrile resulted in the elution of the specifically bound analyte. These results were again in agreement with the chromatographic evaluation of MB5 in Chapter 3, where imprinting factors of 1.8 and 1.3 were obtained for BDN in MeCN and MeCN-$H_2O$ (30/70), respectively. The results from frontal chromatography on this polymer suggested that the number of binding sites was similar for MIP and NIP but that the affinity constant was an order of magnitude higher for the MIP at $2.3 \times 10^5$ L.$mol^{-1}$ compared to the NIP at $8.7 \times 10^4$ L.$mol^{-1}$.

A comparison of the two polymer suggested correlation between the results from the MISPE experiment using P-1 and the imprinting factors observed by chromatographic evaluation. The greatest imprinting factors obtained, i.e. for MB3, resulted in the greatest difference between material specifically retained on the SPE column for a single molecular recognition step. This was during the 60% $H_2O$ step where 17.2% (± 4.1) was eluted from the MIP while 45.9% (± 5.8) was eluted from the NIP.

An interesting point to note is that for both the EGDMA and TRIM based polymers, the recovery from the NIP was greater by approximately 5%. This suggested that some material was more strongly bound to the MIPs in both cases, a factor that could be related to a greater number of binding sites and to stronger interaction between the analyte and these binding sites, as predicted by frontal chromatography.

Protocols P-2 to P-5 were investigated to determine if the results obtained in P-1 could be improved upon, in terms of molecular recognition or a reduction in the number of steps used. Only the most successful protocols were carried out in duplicate.

*4.4.1.2 MISPE protocols P-2 – P-5*

Protocol P-2 was an exploratory study carried out to ascertain whether the number of steps utilised in P-1 could be reduced, and if indeed, this would improve the retention of the analyte on the imprinted polymer.

P-2 investigated the possibility of not using the gradient mobile phase increments and instead using the wash step that demonstrated the highest elution of non-specifically bound budesonide in P-1. For MB3 this involved a wash step using 60% $H_2O$ and continuing with the subsequent elution steps as P-1, while the molecular recognition step for MB5 necessitated 50% $H_2O$. P-2 for MB5 resulted in a difference of only 2% in the quantity of budesonide specifically retained on the imprinted polymer column and, since this polymer also displayed decreased performance in P-2, no further investigations were carried out for MB5. The averaged results for the P-2 protocol carried out in triplicate for MB3 are presented in Table 4.2 and represented graphically in Figure 4.2.

**Table 4.2: BDN Elution profile for HCB imprinted polymer MB3 (EGDMA) using MISPE protocol P-2.**

| Solvent composition | % Budesonide eluted | |
|---|---|---|
| | MB3 | NB3 |
| 60% $H_2O$ | 2.3 (± 0.9) | 16.3 (± 9.7) |
| 100% MeCN | 82.5 (± 10) | 71.3 (± 16.7) |
| 10% acetic acid -1 | 8.7 (± 3.1) | 6.7 (± 1.7) |
| 10% acetic acid -2 | 2.6 (± 1.3) | 1.8 (± 0.7) |
| 10% acetic acid -3 | 0.6 (± 0.3 ) | 0.43 (± 0.43) |
| Total recovered | 96.7 (± 16.2) | 96.5 (± 19.1) |

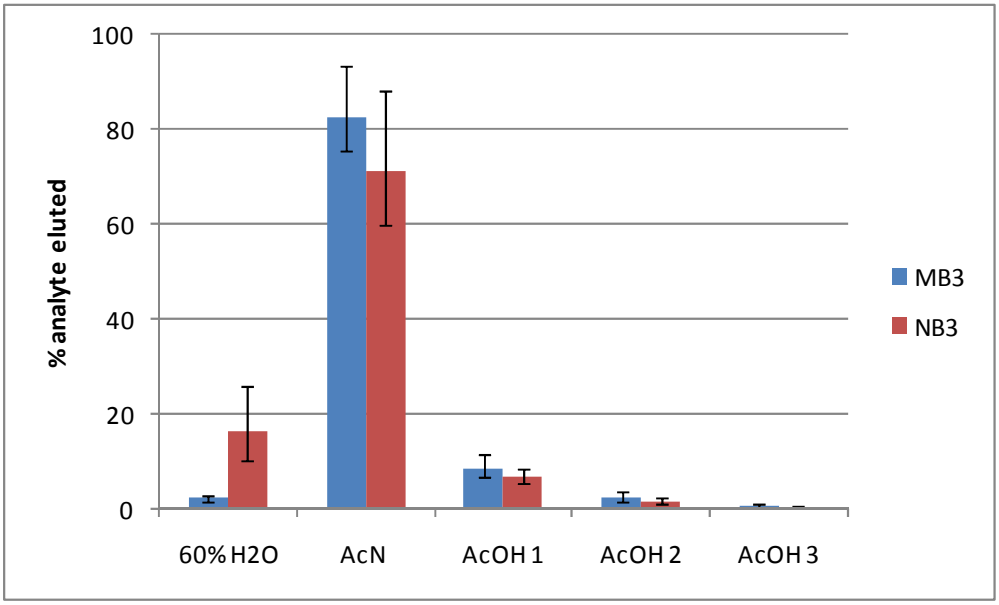


**Figure 4.2: Elution profile for MB3 (EGDMA) using the P-2 protocol.**

The results presented in Table 4.2 and Figure 4.2 show that the molecular recognition wash step induced specific retention of budesonide on the imprinted polymer. In this case, seven times more material was retained on the imprinted polymer than the non-imprinted polymer with 2.3% (± 0.9) eluted from the MIP and 16.3% (± 9.7) eluted from the NIP. As with P-1, the difference in the amount of material was balanced in the following elution steps. It is worth noting that a greater amount of analyte was recovered for this protocol than for P-1, with ca. 96% recovery for P-2. This may suggest that budesonide was indeed eluting from the columns during the initial molecular recognition steps for P-1 but that the concentration of BDN in these steps was such that it was not distinguishable from baseline noise.

P-3 doubled the volume of the 60% $H_2O$ wash step to determine the effects on the specific retention of budesonide on the imprinted polymers. This resulted in the elution of 71.2% and 80.7% of the loaded analyte for the MIP and NIP, respectively. This suggested that the increased volume of acetonitrile reduced the ability of the polymer to specifically retain budesonide and to discriminate between specific and non-specific binding, as shown in Figure 4.3.

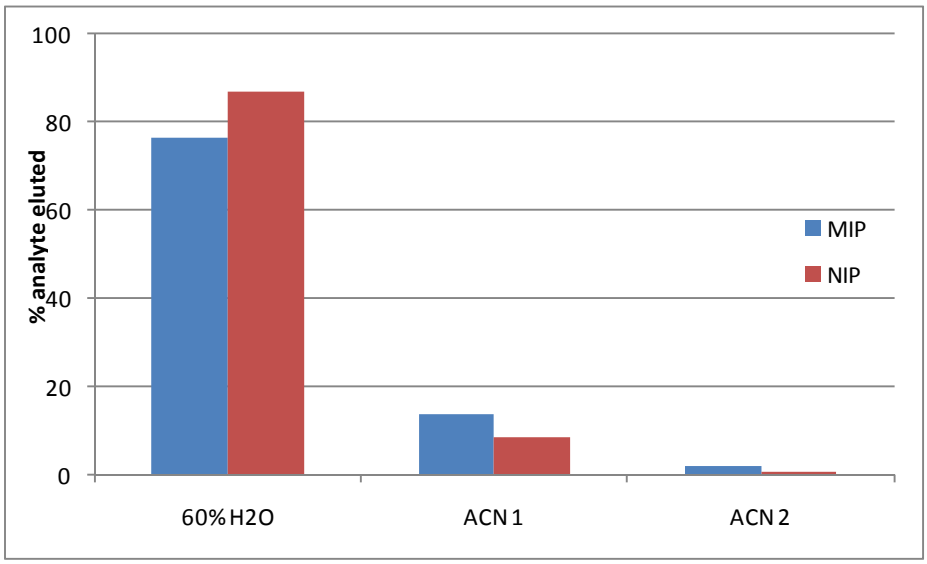


**Figure 4.3: Elution profile for the P-3 protocol.**

A decreased concentration of budesonide (5 µg.mL$^{-1}$) was loaded onto the polymers to determine if this would allow for enhanced molecular recognition, in the event that the original concentration was saturating the specific binding sites. This approach yielded quite similar results to P-2, in that 4.4% and 18.8% budesonide was eluted from the MIP and the NIP, respectively. This is represented graphically in Figure 4.4.

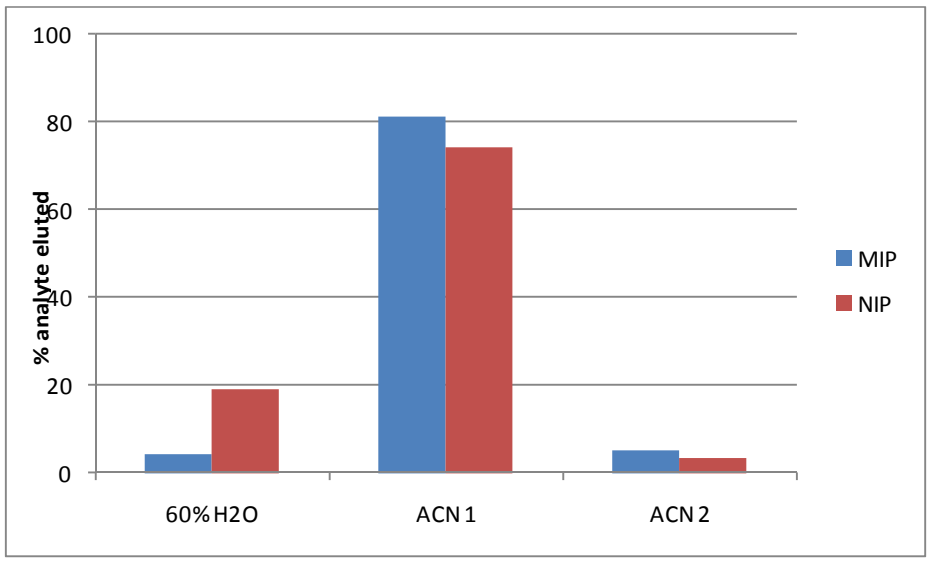


**Figure 4.4: Elution profile for MB3 using P-4.**

Preliminary work on semi-covalent polymers suggested that treatment of polymers using a basic solution increased the retention of corticosteroid analytes. The P-5 protocol sought to exploit this characteristic of the polymers by pre-conditioning the column with 0.1 M KOH in MeOH. Only minimal differences, however, were observed between the MIP and the NIP during this investigation.

#### *4.4.1.3 Summary of MISPE investigations*

The preliminary solid phase extraction studies carried out in this work suggested that with appropriate optimisation of the protocols, the steroid specific binding sites on the hydrocortisone-17-butyrate imprinted polymers could be exploited to retain budesonide to a greater extent than on the non-imprinted polymers. For polymers where trace analysis using SPE is the final application, it is generally necessary to use such a target analogue approach, as template bleed could give false quantitation results.

### 4.4.2 MIP chromatography for the separation of closely related steroidal compounds

The MISPE experiments outlined in Section 4.4.1 exploited the potential for target analogue imprinting of the HCB imprinted polymers prepared in Chapter 3, allowing for the specific retention of BDN. Another important requirement for molecularly imprinted polymers is the ability to discriminate between closely related compounds. An important application of this would be the separation of structural analogues, for example impurities or related compounds in a system. This would have potential for use in process purification as well as chromatographic applications.

In Chapter 3 of this study, zonal chromatographic evaluation of the polymers prepared suggested that in the majority of cases, steroids with similar log P values and functional moieties appeared to be retained to similar extents on both imprinted and non-imprinted polymers. This resulted in similar retention profiles for the steroids in groups of two, for example, BDN and HCB often behaved similarly, as did hydrocortisone (HYD) and prednisolone (PRD). This was not unexpected, and in fact it was this very property of molecularly imprinted polymers that allowed for use of the target analogue approach for the preparation of the imprinted polymers prepared in Chapter 3.

Somewhat anomalous behaviour, however, was observed for hydrocortisone and prednisolone for the M23 polymers. These polymers were prepared as MAA-co-TRIM polymers, using a precipitation strategy with dichloromethane as the porogen. As stated, in the majority of cases the $k'$ values for HYD and PRD differed by no more than 1.5. For M23, however, the difference was almost 4. The difference between $k'$ values of these analytes on the NIP, N23, was higher than all of the other MIPs, with a value of 2. This suggested that MAA-co-TRIM polymers prepared in this fashion had an inherent affinity for prednisolone. A molecular imprinting strategy using PRD as the template was applied to these polymers, to enhance this inherent affinity and determine if the polymers so prepared could separate these closely related structural analogues.

NMR studies carried out in Chapter 2 and Chapter 3, coupled with the results obtained for precipitation polymers in Chapter 3, indicated that to maximise interaction in the pre-polymerisation complex the concentration of the template should be as high as possible. The solubility of PRD in dichloromethane was relatively low at less than 1 mg/mL. Three polymer formulations were tested for the preparation of PRD imprinted polymers: M25, M26 and M27.

These polymers differed only in the concentration of the template and the monomer concentration. M25 was prepared by saturating the porogen with the template followed by addition of the remaining components to a monomer concentration of 2% (w/v). M26 and M27 used the combined solvating powers of the monomers and the solvent to allow for increased template concentration by first adding the monomers to the porogen, followed by addition of the template. M26 used a 2% (w/v) monomer concentration and achieved an increase of 50% in the concentration of the template. M27 reduced the monomer concentration to 1% (w/v) and in doing so the concentration of the template in the solution was double that of M26 and triple that of M25.

The different polymerisation conditions resulted in particle size ranges of 0.8-7.4 µm for M25, 0.7-4.8 µm for M26 and 0.5-4.2 µm for M27. These results suggest that the particle size range became smaller with increased template or with decreased monomer concentration. The effect on particle morphology can be seen in the SEM images presented in Figure 4.5, showing that predominantly discrete and roughly spherical microparticles were prepared for M25-M27.

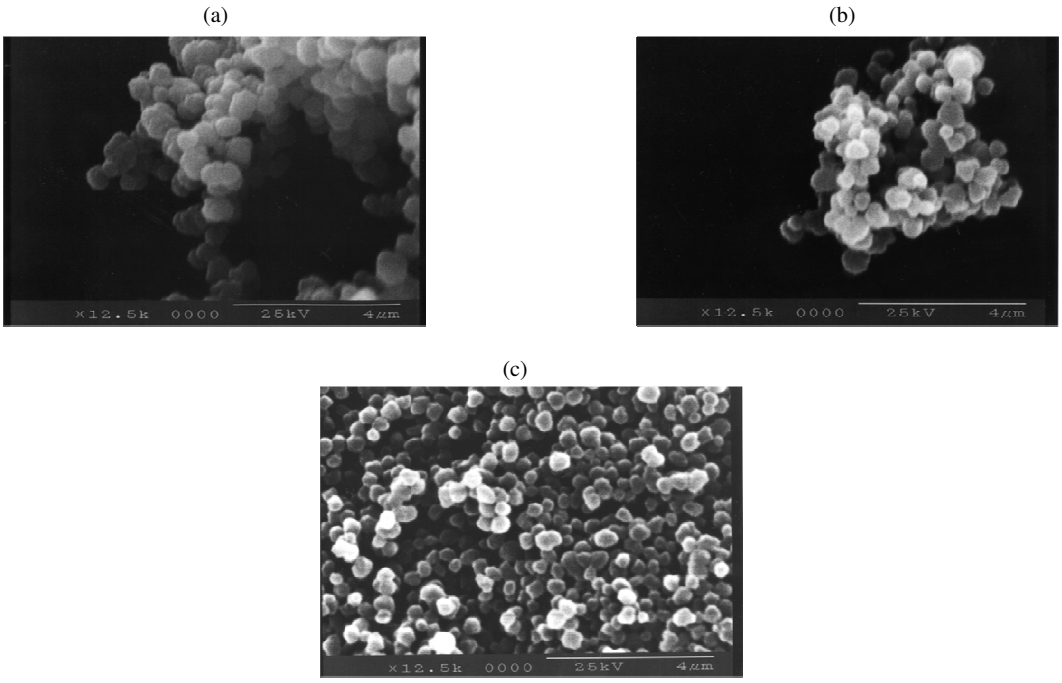


**Figure 4.5: SEM images for (a) M25; (b) M26; (c) M27.**

The primary objective of this study was to achieve the separation of prednisolone and hydrocortisone using, prednisolone imprinted polymers. This was achieved to varying degrees for all three polymers prepared utilising a precipitation polymerisation strategy and 5 cm columns. The separation achieved for M26 at 20 °C at a flow rate of 0.3 mL.min$^{-1}$ is shown in Figure 4.6.

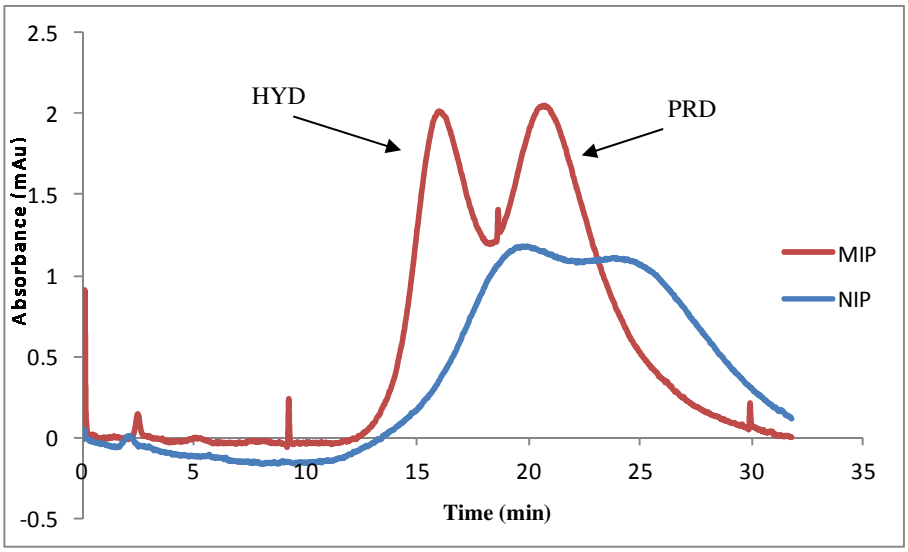


**Figure 4.6: Chromatographic separation of HYD and PRD on PRD imprinted polymers in acetonitrile at 0.3 mL.min$^{-1}$.**

Figure 4.6 shows that the non-imprinted polymers, while displaying different analyte retention times when injected separately, the polymer did not have the capability to resolve the peaks of the two analytes to any significant extent when injected together. As discussed, this suggested that the polymer had an inherent affinity for PRD. Molecular imprinting, using PRD as the template, enhanced this affinity and resulted in the separation of HYD and PRD shown in Figure 4.6. This separation for compounds that differ only in the presence of an extra double bond in the A-ring of PRD, demonstrates the ability of the imprinting process to enhance the inherent affinity of a polymer for a compound and so achieve a high degree of molecular recognition. The selectivity factor, α, for this separation was relatively high, at a value of 1.37, however, the resolution was not optimal because of the typically broad peaks observed with molecularly imprinted polymers. The separation temperature was varied to study the effects on the chromatographic separation and the results are presented in Table 4.3.

**Table 4.3: The effects of temperature on the chromatographic separation of HYD and PRD.**

| Temperature (°C) | $k'_{HYD}$ | $k'_{PRD}$ | α |
|---|---|---|---|
| 2 | 6.41 | 8.99 | 1.40 |
| 10 | 5.57 | 7.74 | 1.39 |
| 20 | 4.85 | 6.63 | 1.37 |
| 30 | 4.71 | 6.17 | 1.31 |
| 50 | 3.40 | 4.49 | 1.32 |
| 60 | 2.96 | 3.90 | 1.32 |

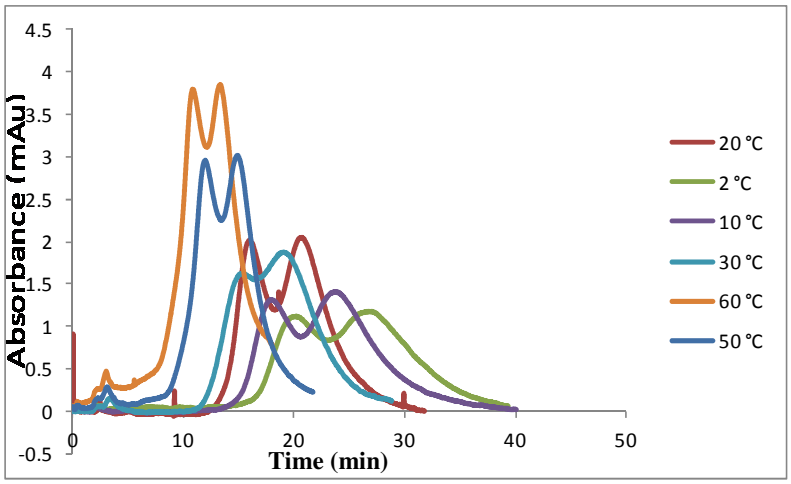


**Figure 4.7: Temperature study for the chromatographic separation of HYD and PRD on the PRD imprinted M26 polymer. Acetonitrile was the mobile phase and the flow rate was 0.3 mL.min$^{-1}$.**

Table 4.3 and Figure 4.7 show that while the α value increased as the temperature was decreased, this was accompanied by a concomitant increase in retention time and loss of resolution. While an increase in temperature brought a decrease in retention times, the resolution was again negatively affected. The optimum temperature was seen to be 20 °C as this had an average retention time with the most resolved peaks.

In an effort to improve the resolution, a higher concentration template solution was used in conjunction with a more dilute monomer loading. This resulted in a higher ratio of template to monomer. Investigations in Chapter 2 into the factors affecting particle formation suggested that at lower monomer loading, smaller, more discrete particles should be formed. To this end, M27 was formed. Figure 4.5 (a)-(c) shows that this polymer had a more discrete, microspherical morphology than M25 or M26, with a smaller particle size range of 0.5-4.2 µm. The separation achieved on this polymer is shown in Figure 4.8.

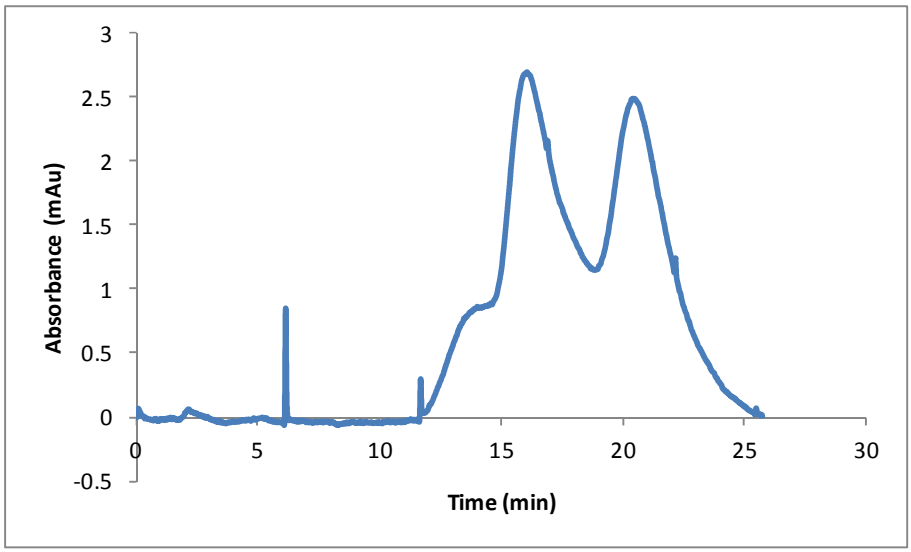


**Figure 4.8: Chromatographic separation of HYD and PRD on M27 in MeCN at 0.3 mL.min$^{-1}$.**

The α value for this separation was similar, though slightly higher, to that for M26, at 1.33. The peaks in this case, however, were resolved to below half the peak height. This allowed for calculation of the resolution ($R_s$), which gave a value of 0.75. This increase in resolution may have been due to a number of factors, such as the increase in the concentration of the template in the polymerisation solution leading to a possible increase in the number of binding sites or the decrease in particle size leading to increased mass transfer properties, or indeed, a combination of the two. The decrease in

particle size, however, was accompanied by an increase in back pressure, as can be seen from the spikes observed in Figure 4.8, as well as the shoulder on the hydrocortisone peak. This is most likely due to the fact that the 10% of the particles in the solution were below 0.5 µm and as such, blockages of the 0.5 µm frits used could occur.

## *4.5 Conclusions*

The investigation into the use of molecularly imprinted polymers for SPE and chromatography demonstrate that different characteristics of MIP behaviour may be exploited to achieve the specific requirements for the chosen application. The ground bulk particles used for SPE demonstrated the success of the target analogue approach for use in applications where template bleed would interfere with accurate quantitation in trace analysis. Correlations were shown between chromatographic evaluation of the EGDMA and TRIM based polymers used and their selective retention behaviours in SPE. This demonstrated the power of chromatographic evaluation of imprinted polymers during development of polymers for a chosen application.

Novel microspherical prednisolone imprinted polymers were prepared by precipitation strategies and these polymers achieved the successful chromatographic separation of the structural analogues, HYD and PRD, on 5 cm columns. This demonstrated the ability to identify a polymer with inherent affinity for an analyte and to greatly enhance this affinity by molecular imprinting strategies, which increased the number of selective binding sites within the polymer matrix. Knowledge gained from the studies in Chapter 2, on the development of precipitated polymers allowed for an improvement of chromatographic separation and resolution of the target analytes based on altering the morphology of the polymers used. These polymers demonstrated the potential for use in applications such as process purification as well as their suitability for use in conjunction with chromatographic instrumentation capable of dealing with small particle size and high back pressures, such as ultra high performance liquid chromatography (UHPLC).

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# Chapter 5

# The synthesis of tailor-made functional monomers for corticosteroid molecular imprinting

# 5. The synthesis of tailor-made functional monomers for corticosteroid molecular imprinting

## *5.1 Introduction*

The strategies employed to create binding sites within molecularly imprinted polymers, as discussed in Section 1.3, include covalent, non-covalent, semi-covalent and stoichiometric non-covalent methodologies. Of these routes to binding site creation, the non-covalent approach has been utilised to the greatest extent [1, 2]. Due to wide ranging applicability, the use of hydrogen bonding and ionic interactions appear to have predominated in this strategy, with monomers such as methacrylic acid [3] and 4-vinyl pyridine [4] typically employed for the former, while 2-(diethylamino) ethyl methacrylate [5] and 2-acryloylamido-2,2'-dimethylpropane sulfonic acid [6] have been utilised as cationic and anionic functional monomers, respectively.

However, for true biomimetic imprinting, templating techniques must exploit as many of the possible interactions, weak or otherwise, between the template and the interacting species. To this end, non-covalent molecular imprinting has also taken advantage of hydrophobic interactions, and, in a manner befitting biomimetic polymers, has been exemplified by the imprinting of the peptide, melittin, using the hydrophobic monomer N-*t*-butylmethacrylamide, by Hoshino *et al*. enabling *in vivo* recognition of the template by so-called 'plastic antibodies' [7, 8].

While some hydrophobic interactions could be considered relatively weak, there are compounds that can achieve high association constants based on predominantly hydrophobic interactions. One such set of compounds are the cyclodextrins, cyclic oligosaccharides composed of D-glucose units joined by α-1,4-glucosidic linkages [9]. Due to steric factors, cyclodextrins (CD) composed of less than six glucose units do not exist and the most common types of CD contain six, seven or eight glucose units and are named α-CD, β-CD and γ-CD, respectively [10], the chemical structures of which are presented in Figure 5.1.

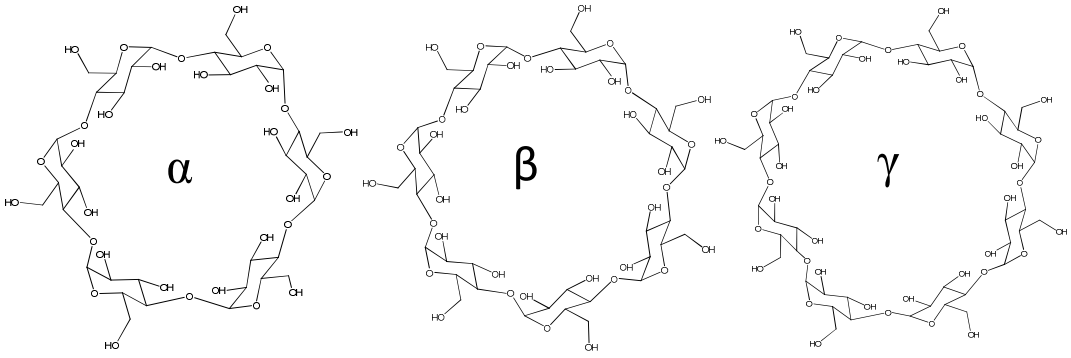
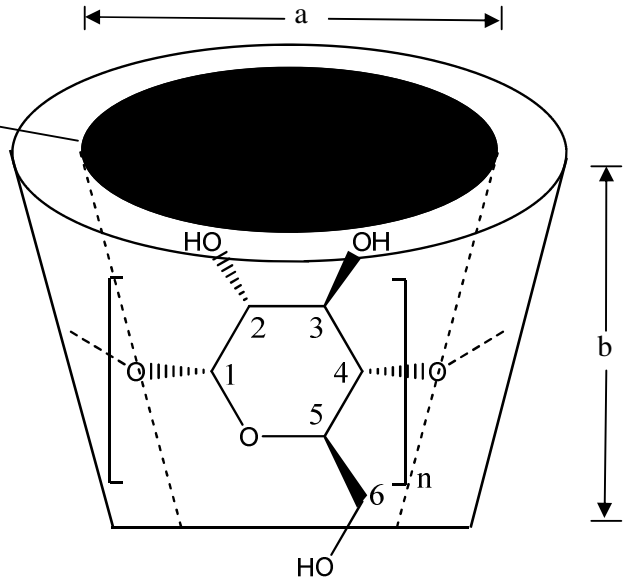



**Figure 5.1: Chemical structures of α-CD, β-CD and γ-CD.**

Cyclodextrins have a three-dimensional structure that is generally considered to be a truncated cone, bearing the hydroxyl groups on the outer surface of the cone [10]. Primary and secondary hydroxyl groups are located on the narrow and wider side of this cone, respectively (Figure 5.2).




| CD | Dimensions (nm) | |
|---|---|---|
| | a | b |
| αCD | 0.57 | 0.78 |
| βCD | 0.78 | 0.78 |
| γCD | 0.95 | 0.78 |

**Figure 5.2: Three-dimensional structure of the cyclodextrin molecule represented as a truncated cone with n= 6, 7 and 8 for α-CD, β-CD and γ-CD, respectively [10].**

The hydroxyl groups, on what is essentially the exterior rim of the CD molecule, makes them water-soluble while the interior is composed of C-C bonds and ether linkages, making it relatively hydrophobic. It is this hydrophobic cavity that allows for the formation of inclusion complexes with lipophilic compounds of appropriate size that display high association constants, often in the order of $10^3$ or $10^4$ $M^{-1}$ [10-13].

Inclusion complexes (driven primarily by hydrophobic and van der Waal's interactions [10]) may be represented schematically, as in Figure 5.3.

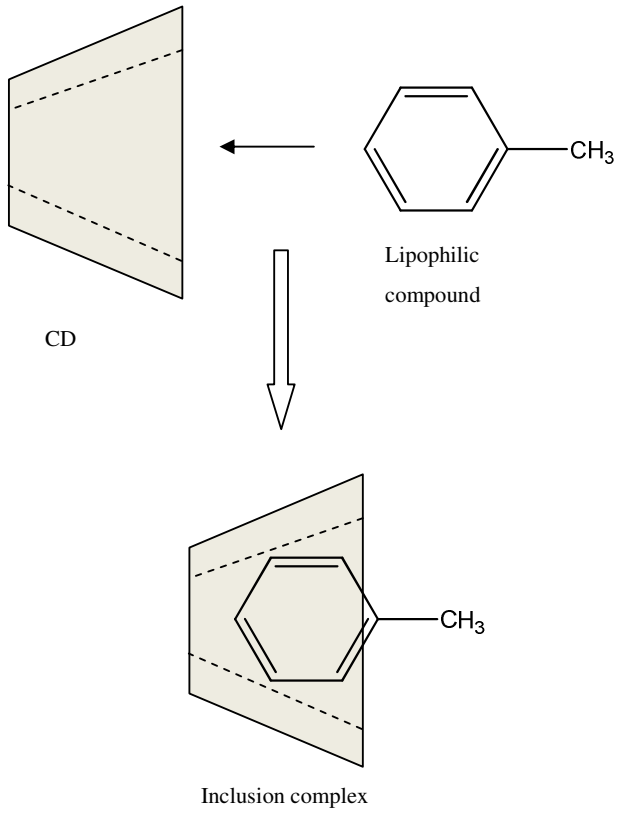



**Figure 5.3: Schematic representation of the formation of an inclusion complex formed by a cyclodextrin and a lipophilic compound.**

In light of the ability of cyclodextrins to form such inclusion complexes, it is little wonder that their use as functional monomers in molecular imprinting technologies has been investigated for a range of compounds, including pharmaceutical actives such as dextromethorphan [14], herbicides such as aciflourfen [15] and aromatic amino acids [16].

The relatively hydrophobic nature of the steroidal skeleton suggests these compounds as likely candidates for strong interaction with cyclodextrins and this has been confirmed by the wealth of literature investigating these interactions [17-20]. As such, a number of groups have employed these cyclic oligosaccharides as the complex-forming monomers for steroids, most notably that of Komiyama, where different steroids have been imprinted using cyclodextrins as functional monomers.

Komiyama *et al*. have used diisocyanate compounds to crosslink the cyclodextrin 'monomers' in a condensation reaction that forms a monolithic polymer and have achieved imprinting effects that resulted in fifteen times more material adsorbed to the cholesterol-imprinted polymer than the non-imprinted polymer [21]. Using cholesterol specific imprinted polymers, also prepared with toluene-diisocyanate and β-cyclodextrin, they achieved cholesterol retention times up to twenty four times longer on imprinted columns than non-imprinted columns [22].

Komiyama has also studied the use of 'tailor-made' vinylated cyclodextrin monomers which allowed the use of conventional crosslinking monomers, such as *N,N'*-methylenebisacrylamide [23], for imprinting on vinylated silica particles [24]. In these studies, the vinyl monomers of 6-*O*-α-D-glucosyl-β-cyclodextrin and α-CD were synthesised by way of an ester exchange reaction with *m*-nitrophenyl acrylate. $^{1}$H NMR and MALDI-TOFMS were used to characterise the vinylated cyclodextrin. It was shown that despite testing various reaction conditions, between one and three vinyl groups were invariably added to the CDs, with column chromatography unable to isolate individual compounds from these mixtures. $^{1}$H NMR provided average numbers of 1.3 and 1.2 vinyl groups per CD for 6-*O*-α-D-glucosyl-β-cyclodextrin and α-CD, respectively.

Custom-made functional monomers based on modified cyclodextrins have also been employed by Piletsky *et al*., in conjunction with an ionic monomer, for the imprinting of phenylalanine [6, 25]. As was the case for Komiyama *et al*., the vinylated cyclodextrin was again synthesised as a mixture of differently substituted CDs, which $^{1}$H NMR showed had an average of 1.9 vinyl groups per CD molecule.

Liu *et al*. employed a different approach in which spherical poly(glycidyl methacrylate) with β-cyclodextrin anchored to the surface, was used to prepare molecularly imprinted microspheres for use in the solid phase extraction of ursolic acid [26]. Ursolic acid was bound within the hydrophobic cavity of the cyclodextrin and was subsequently entrapped by crosslinking of surface bound 2-hydroxyethyl methacrylate (HEMA) and the crosslinker, ethylene glycol dimethacrylate. This resulted in molecularly imprinted microspheres where the capacity of the MIP for ursolic acid was 42.5 $\mu mol.g^{-1}$, while the capacity of the NIP was almost ten times less, at 4.9 $\mu mol.g^{-1}$.

Sreenivasan, however, in a study using cyclodextrin modified HEMA, adds a cautionary note to the use of cyclodextrin monomers for the imprinting of hydrophobic molecules as their ability to form inclusion complexes with any suitably sized lipophilic molecule, may significantly reduce the selectivity of the resultant polymer [27].

Another approach, which involves the use of custom-made monomers, is the semi-covalent strategy. This method is discussed in detail in Section 1.3.3 and for this approach the monomers are not prepared to interact in a particular fashion with the template. Rather, a polymerisable analogue of the template is synthesised in an effort to construct binding sites with the fidelity achieved by covalently formed sites, while simultaneously allowing for the rapid rebinding kinetics of non-covalent interactions [28, 29].

As discussed in Section 1.3.3, this approach has been used for a range of templates including glucopyranoside [29], propazine [28] and bisphenol-A [30]. Where binding sites are created by relatively weak interactions, such as in the case of templates with limited functionality, it would be expected that a more directed structuring of the interacting functional moieties within the binding cavities could lead to a higher degree of specificity and selectivity. Given the weak interaction observed between corticosteroids and functional monomers throughout this study, the semi-covalent approach was considered a potentially useful strategy for the creation of corticosteroid molecularly imprinted polymers.

## *5.2 Research aims and objectives*

The aims and objectives of this study were:

- To synthesise tailor-made functional monomers for use in the preparation of corticosteroid molecular imprinting.
- To study the interaction between cyclodextrins and budesonide and to assess the feasibility of cyclodextrin based functional monomers for corticosteroid imprinting.
- To carry out an investigation of a number of synthetic strategies for the preparation of polymerisable cyclodextrin monomers.

- To synthesise a polymerisable template-monomer based on a suitable corticosteroid compound for use in a semi-covalent imprinting strategy.
- To carry out preliminary investigations into the use of the prepared custom monomers for the synthesis of corticosteroid molecularly imprinted polymers.

## *5.3 Experimental*

### 5.3.1 Materials

β-cyclodextrin (β-CD) (≥ 97%), γ-cyclodextrin (γ-CD) (≥ 99%), toluene-2,4-diisocyanate (TDI) (≥ 98%), acryloyl chloride (AC) (≥ 97%), methacryloyl chloride (MAC) (≥ 97%), N-(hydroxymethyl) acrylamide (NHA) (48% (w/v) in $H_2O$), dimethyl sulfoxide-$d_6$ (99.9% atom D), deuterium oxide (99.99% atom D), anhydrous pyridine (≥ 99.8%), anhydrous dimethyl sulfoxide (≥ 99.9%), *N*, *N'*-methylene bisacrylamide (MBA) (≥ 98%), 4-(dimethylamino)pyridine (DMAP) (≥ 99%), triethylamine (TEA) (≥ 99%) and hydrochloric acid (37%) were all obtained from Sigma-Aldrich, Wicklow, Ireland. All other solvents and reagents used were as per Table 2.3 and Section 3.3.1.

### 5.3.2 Instrumentation

LC-MS analysis was carried out on using an Agilent 1200 series LC coupled to an XCT Ultra ion trap. All other instrumentation was as per Table 2.4 and Table 3.1.

### 5.3.3 Monomer synthesis

#### *5.3.3.1 Cyclodextrin methacrylate*

Cyclodextrin methacrylate (CDM) was prepared using a method adapted from that used by Xu *et al*. [15, 31]. β-cyclodextrin (1 mmol) was dissolved in 50 mL freshly distilled anhydrous pyridine and the temperature was reduced to 0° C. Methacryloyl chloride (2.2-5 equivalents) was dissolved in 10 mL sodium-dried ether at 0 °C. The methacryloyl chloride solution was added in a drop-wise manner to the cyclodextrin solution and the reaction was allowed to proceed for 24 hours. The pyridine was removed by evaporation *in vacuo* at 50 °C and the resultant solid was re-dissolved in ethanol. The ethanol was then removed by evaporation *in vacuo* at 50 °C and the resultant solid was stored at 4 °C until further use.

*5.3.3.2 Acrylamidomethyl cyclodextrin*

Acrylamidomethyl cyclodextrins (β-CDA and γ-CDA) were prepared in a fashion based on the method used by Lee *et al*. for the synthesis of polymerisable cyclodextrin monomers [32]. In the present study, β- or γ-cyclodextrin and N-(hydroxymethyl) acrylamide (NMA) were dissolved in water, using ratios ranging from 1:10 to 1:90, and the temperature was raised to 80 °C. Sufficient hydrochloric acid was added to form a 1% (v/v) solution and the reaction was allowed to proceed for between 5 and 45 minutes. The reaction mixture was then added to acetone (50 mL reaction mixture to 200 mL acetone) to stop the reaction and precipitate the product. The reaction so quenched was then stored overnight at 4 °C. The resultant precipitate was removed by filtration, washed repeatedly with acetone, and stored at 4 °C until further use.

*5.3.3.3 Hydrocortisone methacrylate*

Hydrocortisone (2.5 mmol), triethylamine (3 mmol) and a catalytic amount of 4-(dimethylamino)pyridine (11-12 mg) were dissolved in 50 mL THF and the solution was cooled to 0 °C. Methacryloyl chloride (3 mmol) was dissolved in ice-cooled THF and added to the magnetically stirred reaction mixture in a drop-wise fashion. The reaction was allowed to proceed for 24 hours and then filtered. Dichloromethane was added the solution was washed with water three times. After separation of the two layers the remaining dichloromethane was dried over magnesium sulphate and the solvent was evaporated *in vacuo* to give a solid, which was stored at 4 °C until further use.

### 5.3.4 Polymer synthesis

*5.3.4.1 Cyclodextrin molecularly imprinted polymer 1 (CDMP1)*

Cyclodextrin polymer 1 (CDMP1) was prepared using a method employed by Hishiya *et al*. [33], which used toluene-2,4-diisocyanate (TDI) as the crosslinking component. In the present study, the template (budesonide) (0.3 mmol) and β-cyclodextrin (0.88 mmol) were dissolved in anhydrous dimethyl sulfoxide (10 mL) and allowed to equilibrate for 24 hours at room temperature. TDI (5.6 mmol) was then added and the temperature was raised to 65 °C for two hours with magnetic stirring. The resultant polymer was then precipitated by adding the reaction mixture to acetone (200 mL). The irregular shaped microparticles obtained were washed repeatedly with hot water,

ethanol and tetrahydrofuran. Cyclodextrin non-imprinted polymer 1 (CDNP1) was prepared in a similar manner but in the absence of the template.

*5.3.4.2 Cyclodextrin molecularly imprinted polymer 2 (CDMP2)*

Cyclodextrin polymer 2 (CDMP2) was prepared using hydrocortisone-17-butyrate (HCB) as the template and an acrylamidomethyl cyclodextrin monomer (CDMA) as the functional monomer. CDMA and HCB were dissolved in dimethyl formamide (DMF) and allowed to equilibrate for 24 hours at room temperature. *N, N'*-methylenebisacrylamide was utilised as the crosslinking monomer and was added after 24 hours. The template:functional monomer: crosslinking monomer ratio was 1:3:20 and the monomer loading was 28% (w/v). Azobisisobutyronitrile was used as the initiator, at a concentration of 1 mol% and polymerisation was carried out at 60 °C for 24 hours. Soxhlet extraction was carried out in methanol and the polymer was ground and sieved as per Section 3.3.2. Cyclodextrin non-imprinted polymer 2 (CDNP2) was prepared in the same manner but without the template.

*5.3.4.3 Cyclodextrin molecularly imprinted polymer 3 (CDMP3)*

Cyclodextrin polymer 3 (CDMP3) was prepared by precipitation methods. HCB was used as the template, acrylamidomethyl γCD (γ-CDA) and methacrylic acid (MAA) as co-functional monomers and ethylene glycol methacrylate (EGDMA) as the crosslinking monomer in a 1:2:4:30 ratio. Acetonitrile-water 60/40 (v/v) was used as the porogen and the monomer concentration was 1% (w/v).

*5.3.4.4 Hydrocortisone methacrylate imprinted polymer 1 (HMAMP1)*

Hydrocortisone methacrylate imprinted polymer 1 (HMAMP1) was prepared in bulk format using hydrocortisone methacrylate, methacrylic acid and divinylbenzene in a 1:2:20 ratio in acetonitrile-toluene 3:1 (v/v) at a monomer concentration of 70% (w/v). AIBN was used as the initiator at 1 mol% and polymerisation was carried out at 60 °C for 24 hours. The polymer was ground and sieved to 25-53 µm size range. Basic hydrolysis was carried out by reflux in 1 M methanolic KOH for 6 hours followed by washing with dilute HCl using a method based on that employed by Cacho *et al*. [28]. The control polymer (HMANP1) was prepared without the template-monomer and to ensure the presence of a similar proportion of functional groups a MAA:DVB ratio of 4:20 was used.

*5.3.4.5 Hydrocortisone methacrylate imprinted polymer 2 (HMAMP2)*

Hydrocortisone methacrylate imprinted polymer 2 (HMAMP2) was prepared *via* a precipitation strategy based on that used for DVB in chapters 2 and 3. The same ratio of HMA:MAA:DVB was employed as for HMAMP1 but the monomer concentration was reduced to 4% (w/v). AIBN was used as the initiator at a concentration of 3 mol% and polymerisation was carried out at 60 °C for 24 hours with agitation at 20 RPM. The non-imprinted polymer, HMANP2, was prepared in the same manner with the component ratio as per HMANP1, again, in the absence of the template.

### 5.3.5 NMR

NMR analyses were carried out at 18.7 ± 0.2 °C. For routine identification, chemical shifts were referenced to the TMS peak. NMR binding studies were carried out by preparing a series of solutions where the concentration of budesonide was held constant (typically between 2-5 mM) and the concentration of the β-cyclodextrin was varied, giving molar ratios ranging from 0 to 20. The solutions were allowed to equilibrate for 24 hours before analysis and methanol was used as the internal reference for chemical shift determination.

*5.3.5.1 NMR characterisation*

The $^{1}$H NMR peaks of β-CD in DMSO-$d_6$ were assigned as follows: H-1 = 4.68; H-2 = 3.29; H-3 = 3.64; H4 =3.34; H-5 = 3.59; H-6 = 3.64; OH-2 = 5.52; OH-3 = 5.48; OH-6 = 4.26 [9]. Vinyl protons for β-CD methacrylate (CDM) were assigned to the multiplets from 5.5-6 ppm. Vinyl protons for acrylamidomethyl β-CD (β-CDA) were assigned to the multiplets between 5.5-6.3 ppm. The $^{1}$H NMR peaks of γ-CD in $D_2O$ were assigned as follows: H-1 = 5.11 ppm; H-2 = 3.65 ppm; H-3 = 3.93 ppm; H-4 = 3.59 ppm; H-5 = 3.86 ppm; H-6 = 3.87 ppm [34]. Vinyl protons were assigned to multiplets at 5.5-6.5 ppm. Vinylic carbons for all derivatised CD compounds were assigned to peaks at 120-130 ppm and carbonyl carbons were assigned to peaks at 170 ppm.

Vinyl protons for hydrocortisone methacrylate were assigned to multiplets at 5.3-6.0 ppm. Vinylic carbons for all derivatised compounds were assigned to peaks at 120-130 ppm and carbonyl carbons were assigned to peaks at 170 ppm.

### 5.3.6 LC-MS

LC-MS analyses employing acetonitrile-water mixtures were used as the mobile phase at a ratio of either 95:5 or 50:50 (v/v) and the flow rate was set at 0.5 $mL.min^{-1}$. An Agilent 150 × 4.6 mm 5 µm Eclipse XDB-$C_{18}$ column was used for all analyses.

#### *5.3.6.1 LC-MS characterisation*

MS assignment for β-CD: $MNa^+$ = m/z 1157.5; CDM: monosubstituted: $MNa^+$ = m/z 1225.5; disubstituted: $MH^+$ = 1271.5; trisubstituted: $MH^+$ = m/z 1339.5; β-CDA: monosubstituted: $MH^+$ = m/z 1218.5; disubstituted: $MH^+$ = m/z 1323.5; γ-CD: $MH^+$ = m/z 1298.5; $MNa^+$ = m/z 1319.5; γ-CDA: monosubstituted: $MH^+$ = 1380.5; disubstituted: $MNa^+$ = m/z 1485.5; trisubstituted: $MH^+$ = m/z 1568.6; Hydrocortisone: dimer = m/z 725.6; hydrocortisone methacrylate: monosubstituted: $MH^+$ = 431.6; dimer = m/z 861.5.

### 5.3.7 SEM and nitrogen sorption porosimetry

SEM and nitrogen sorption porosimetry were carried out as per Sections 2.3.5 and 3.3.4, respectively.

### 5.3.8 Equilibrium binding studies

Equilibrium binding studies were carried out as per Section 2.3.4. Briefly, dry polymer (50 mg) was placed in MeOH-$H_2O$ (70/30) solution at budesonide concentrations of 2-6 µg/mL. The suspended polymer solution was then placed in a C24 incubator shaker from New Brunswick Scientific (Edison, NJ, USA) at 20 °C at 300 RPM. After 24 hours the suspended polymer solution was centrifuged at 45000 RPM for 1 hour and the supernatant was filtered through 0.45 and 0.2 µm syringe filters into clean HPLC vials. This was then analysed as outlined in Section 2.3.4.

### 5.3.9 Column packing and chromatographic evaluation

Column packing and chromatographic evaluation were carried out as per Section 3.3.3.

## *5.4 Results and discussion*

Chapters 2 and 3 of this study outlined the preparation and characterisation of molecularly imprinted polymers capable of the selective recognition of corticosteroids. NMR studies were carried out with a view to achieving a rational understanding of the performance of these polymers (sections 2.4.4 and 3.4.1). These studies, coupled with the results observed for novel corticosteroid-imprinted precipitated microspheres and microparticles (Section 3.4.4), suggested that the weak interactions observed between template and functional monomer in the pre-polymerisation mixture resulted in the creation of a relatively low number of binding sites in the resultant polymers, particularly for polymers prepared in dilute solutions such as precipitation polymers.

This chapter focuses on the synthesis of tailor-made functional monomers capable of enhancing the number of corticosteroid specific binding sites within the polymeric matrix, using two separate strategies. The first strategy involved promotion of template-monomer complex formation in the pre-polymerisation complex and the second involved the use of the synthesis of a polymerisable template-monomer for use in a semi-covalent imprinting strategy.

### 5.4.1 Cyclodextrin compounds as complex-forming agents for molecular imprinting

*5.4.1.1 NMR studies on cyclodextrin-steroid interaction*

The strong interaction between steroids and cyclodextrins discussed in the introduction, points to the applicability of cyclodextrins for use as complex-forming agents in molecular imprinting. To investigate their potential for interaction with the corticosteroids used in this study, BDN and HCB, binding studies were carried out to elucidate the strength and nature of the interactions taking place. α-cyclodextrin was not investigated, as a number of studies have suggested that prednisolone and hydrocortisone, i.e. structural analogues of BDN and HCB, can not enter the α-CD cavity [17, 18]. Due to the cost of γ-CD, preliminary studies were carried out using β-CD. The binding interactions taking place between BDN and β-CD were studied in DMSO using NMR titration investigation. The protons which were most responsive to complexation and produced the most notable complex induced shifts (CIS) were monitored [35]. The resonance signals for the H-1 proton on the A-ring of BDN and the

H-3 proton for β-CD were observed to be the most responsive and plots (presented in Figure 5.4) were constructed.

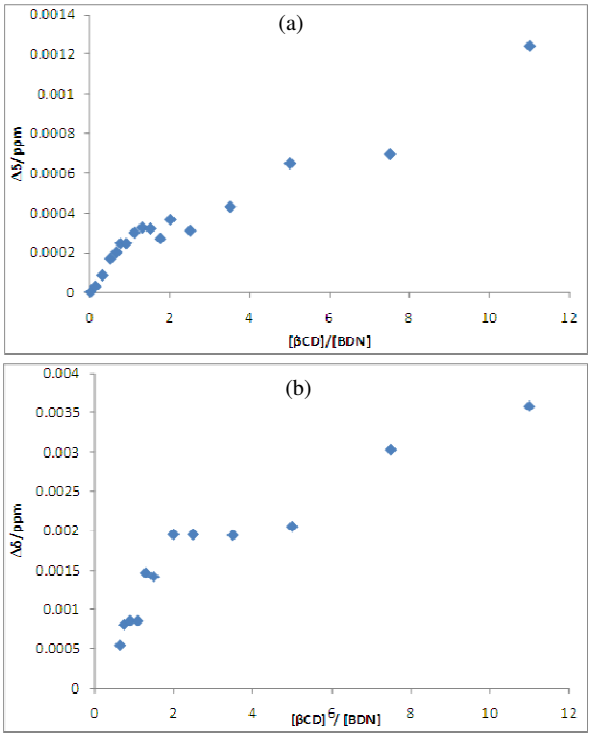



**Figure 5.4: Plots constructed for Δδ vs. [β-CD]/[BDN] for (a) BDN H-1 proton and (b) β-CD H-3 proton obtained from NMR binding studies carried out in DMSO.**

The CIS of the steroidal H-1 suggested that the interaction between the two compounds involved the A-ring of the steroid. The stereochemistry of cyclodextrins is such that the H-3 and H-5 protons point inwards to the cavity of the three-dimensional truncated cone represented in Figure 5.2, and as such, CIS observed for these protons are indicative of inclusion into the hydrophobic cavity of the CD [12, 17]. The shift observed for the CD H-3 proton is therefore evidence of the inclusion of BDN into the hydrophobic cavity [12].

The combination of the CIS observed for both locations, coupled with the absence of any significant CIS for the H-5 proton suggested the interaction represented schematically in Figure 5.5 where the A-ring of the steroid interacts with the hydrophobic cavity of β-CD

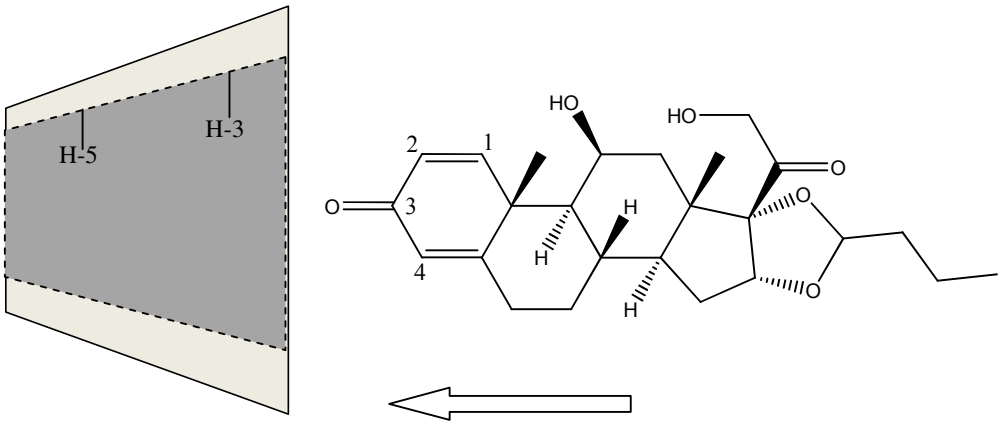



**Figure 5.5: Proposed interaction between budesonide and β-CD.**

Another factor, suggesting the interaction represented in Figure 5.5, was the almost identical dissociation constants observed for the two protons, having values of $2.2 \times 10^{-3}$ M and $2.5 \times 10^{-3}$ M, for the steroidal H-1 and the cyclodextrin H-3, respectively. It is worth noting that these dissociation constants suggested far greater interaction than had been observed for BDN and acetic acid or 2-(hydroxyethyl) methacrylate in Chapter 2 ($k_{diss}$ 0.058-1.46 M).

This evidence suggesting strong hydrophobic interaction between BDN and β-CD and the formation of a complex in polar solutions, demonstrated the potential for using CD based functional monomers to enhance the formation of template-monomer complexes in the pre-polymerisation solution. Based on this, a number of strategies were investigated for their capability to exploit this enhancement of complex formation.

### *5.4.1.2 Synthesis and evaluation of cyclodextrin molecularly imprinted polymer 1 (CDMP1)*

CDMP1 was prepared using a method analogous to that used by Komiyama *et al*. [21, 33, 36]. This method utilises toluene-2,4-diisocyanate (TDI) to crosslink native cyclodextrins *via* a condensation reaction of the OH groups of the cyclodextrin, as depicted in Figure 5.6.

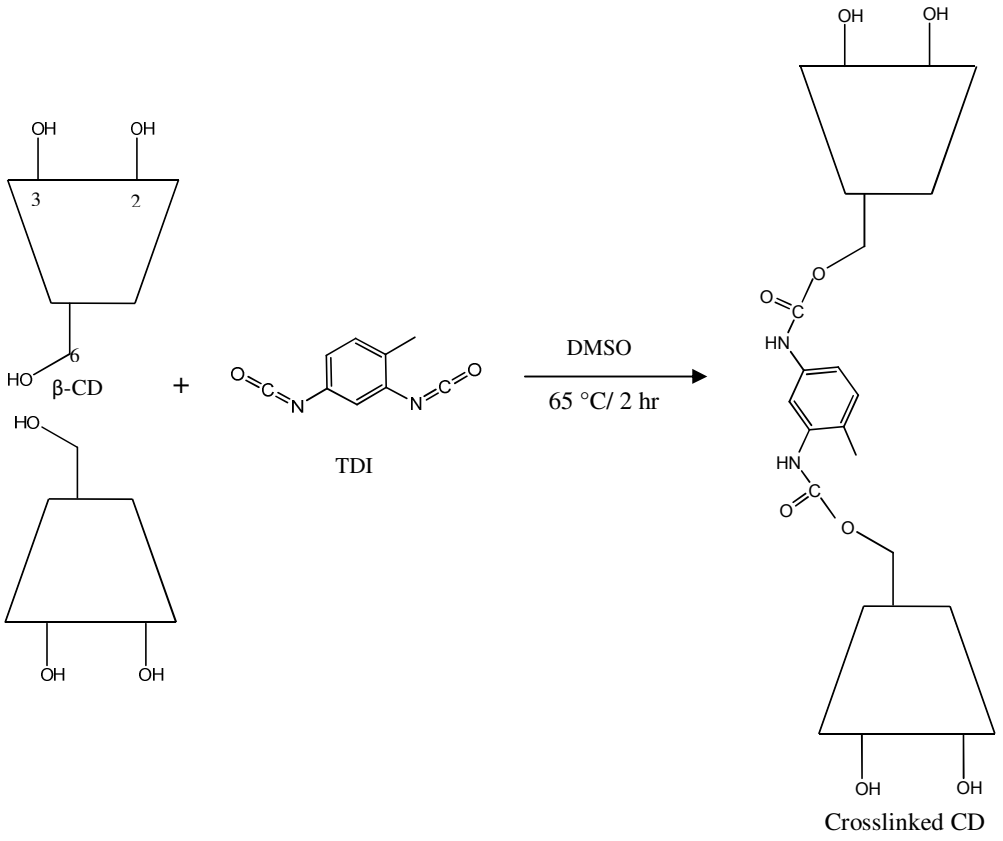



**Figure 5.6: Crosslinking of cyclodextrins with toluene-2,4-diisocyanate showing interaction at C-6 OH [21, 33, 36].**

In the present study, BDN and β-CD were added to DMSO and allowed to equilibrate for 24 hours. TDI was then added and the temperature was raised to 65 °C for 2 hours. The resultant polymer did not need to be ground and sieved as irregular shaped particles in the micron size range were formed. It was decided to first test the capacity of these polymers using only the non-imprinted polymer in equilibrium binding studies described in Section 5.3.7. The concentration range tested was between 2 and 6 µg/mL and the results for non-specific binding on the non-imprinted polymer are shown in Figure 5.7.

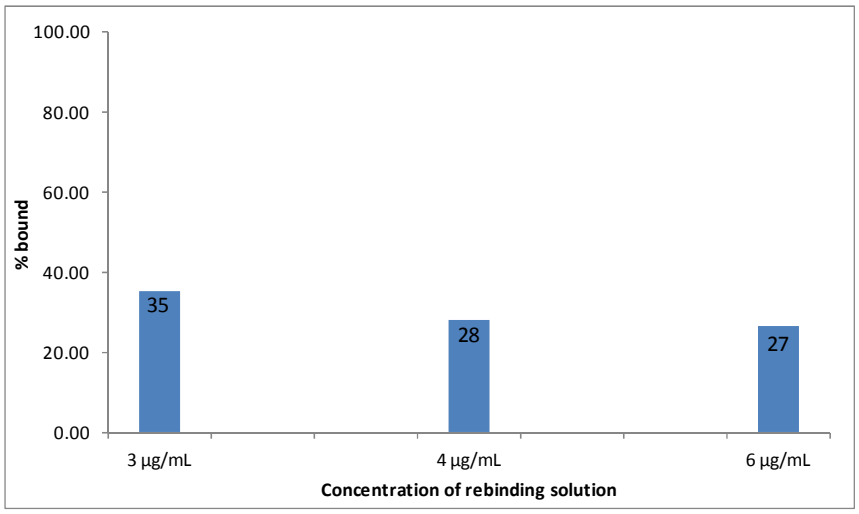



**Figure 5.7: Percentage of the template bound by the non-imprinted polymer CDNP1 over a range of template concentrations.**

The results presented in Figure 5.7 show that the percentage of BDN bound to the non-imprinted polymer decreased as the concentration of the template increased. This suggested that the lowest concentration solution, 3 µg/mL, should be used for binding studies using the MIP. This template solution concentration was applied during equilibrium binding studies on CDMP1 and CDNP1. During these studies it was observed that the amount of template bleed from the imprinted polymer was ten times the concentration of the rebinding solution. This prevented further use of these polymers as it appeared that not only was binding capacity relatively low in the first place, the amount of entrapped template that leeched from the polymer during binding studies was too high for any meaningful investigation.

The high degree of entrapped template may have been due to the nature of the crosslinking component, TDI, which would form a relatively short spacer between two cyclodextrin monomers (Figure 5.7). Hishiya *et al.* have carried out spectroscopic analysis of the complexes formed with TDI and have observed intra molecule linkages as well intermolecular crosslinkers involving more than one TDI molecule [37]. In the present study, the formation of such complexes may have played a role in the high degree of template bleed. There was also some evidence of solubilisation of the polymer in particular solvents, such as DMSO and solutions with high water contents. The combination of these factors suggested the necessity to prepare polymers using more conventional crosslinking monomers. This in turn, required the synthesis of tailor-made cyclodextrin monomers containing polymerisable vinyl groups.

*5.4.1.3 Synthesis of polymerisable cyclodextrin monomers*

The objective of this study was to prepare CD-based compounds, derivatised with polymerisable vinyl groups, for use in the preparation of corticosteroid molecularly imprinted polymers. A number of strategies were investigated for suitability. The aim was to successfully derivatise β-CD and γ-CD and to characterise them as fully as possible by NMR and LC-MS, given the limitations of both the synthetic and analytical methods described in Section 5.1, relating to previously published studies, i.e. the ability to provide an average number of substituted groups per CD molecule [6, 23, 25].

Cyclodextrin methacrylate (CDM) was prepared according to a method employed by Xu *et al.* (CDM) [15, 31]. This was a relatively straightforward synthetic strategy (Figure 5.8), where 2.2 equivalents of methacryloyl chloride (MAC), dissolved in ether, were added in a drop-wise fashion to a solution of β-CD in freshly distilled pyridine at 0 °C and reacted for twenty-four hours.

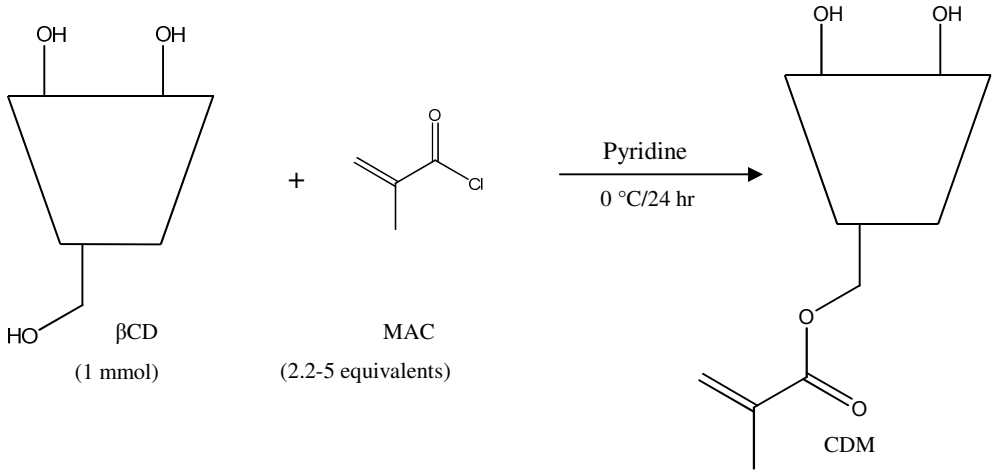
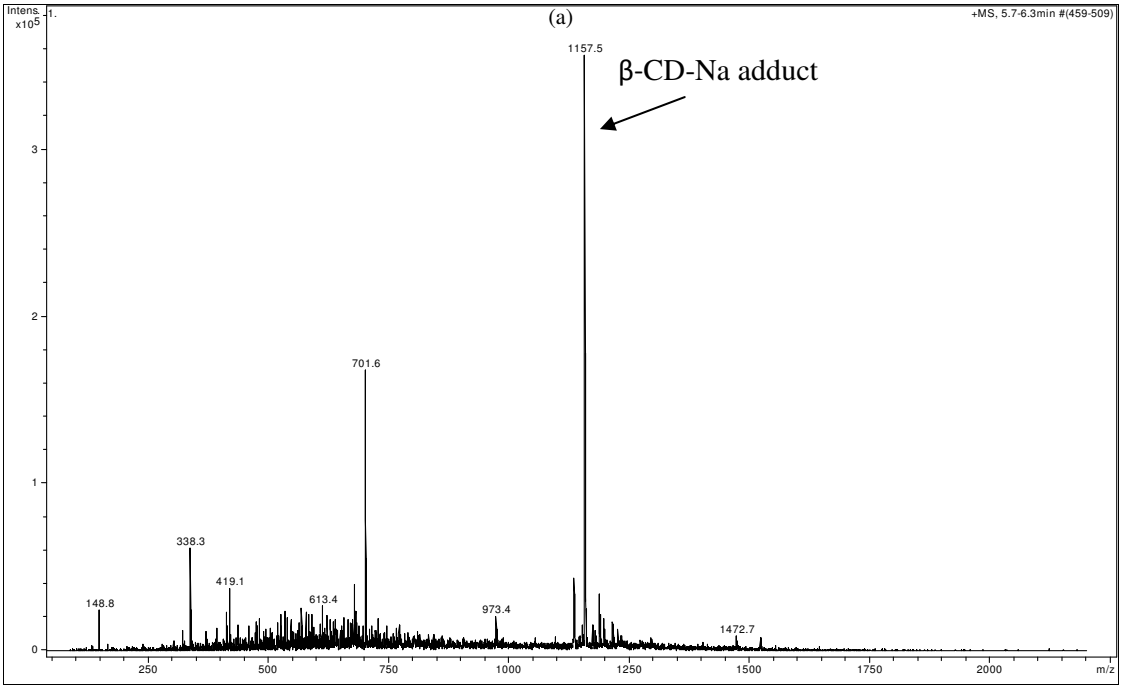
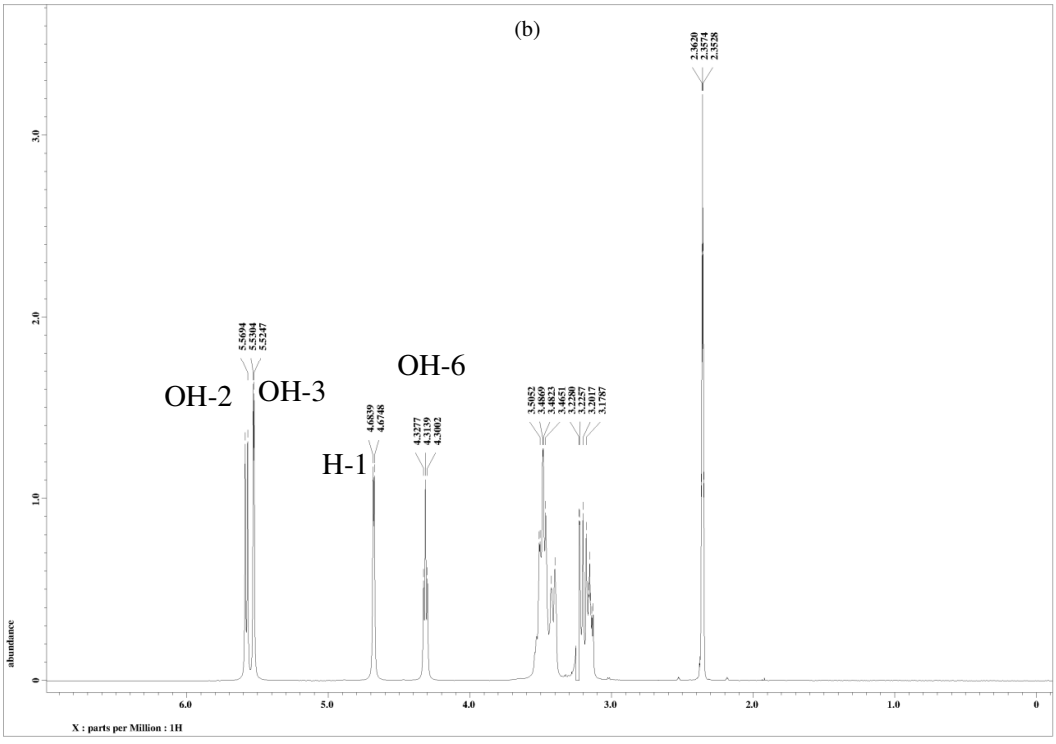



**Figure 5.8: Synthesis of CDM showing reaction at C-6 OH.**

The synthetic strategy for the derivatisation of β-CD using MAC in Figure 5.8 depicts derivatisation of the primary C-6 OH position of the CD. While this would be expected to react preferentially with the acid chloride, the secondary hydroxyl groups, C-2 OH and C-5 OH, could also react and the extent to which each group becomes derivatised would be difficult to accurately quantify using the methods outlined in this study. As stated in the introduction these monomers are typically prepared as a mixture of differently substituted CDs and an average figure for derivatisation is usually assessed.

The success of this synthetic strategy was monitored using both NMR and LC-MS to identify the obtained derivatised monomers and to quantify the average number of vinyl groups per cyclodextrin monomer. The MS, $^{1}H$ NMR, and $^{13}C$ NMR spectra for the starting material, β-CD, are presented in Figure 5.9 (a)-(c). (Peak assignment of the $^{1}H$ NMR spectrum from reference [9]).



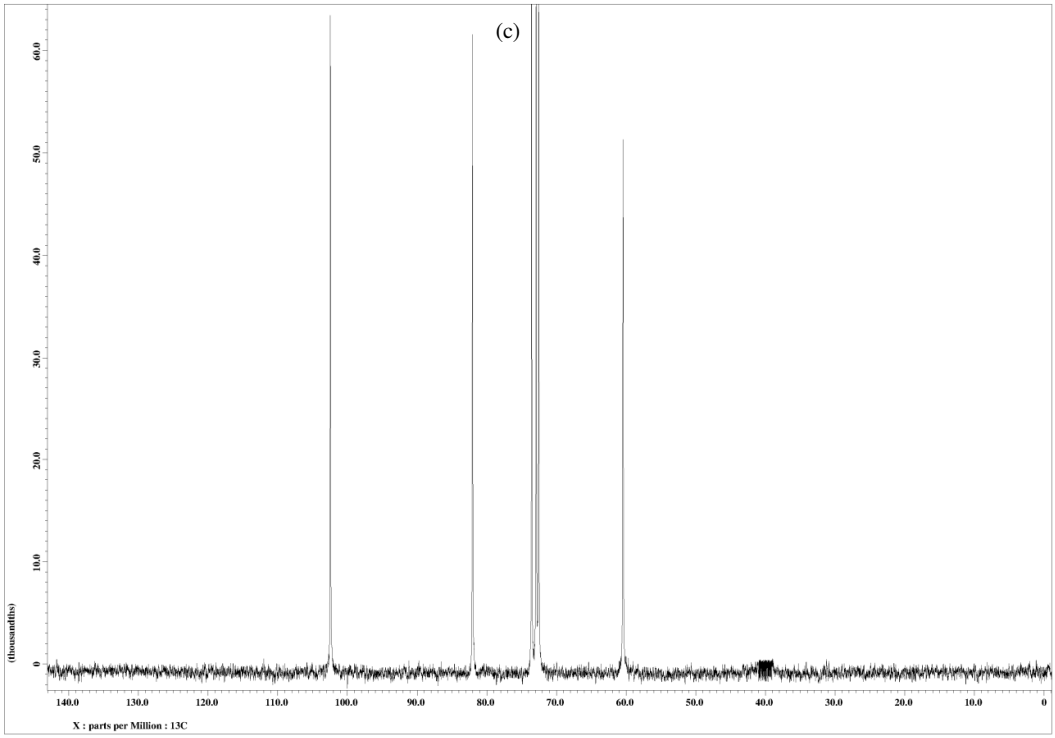
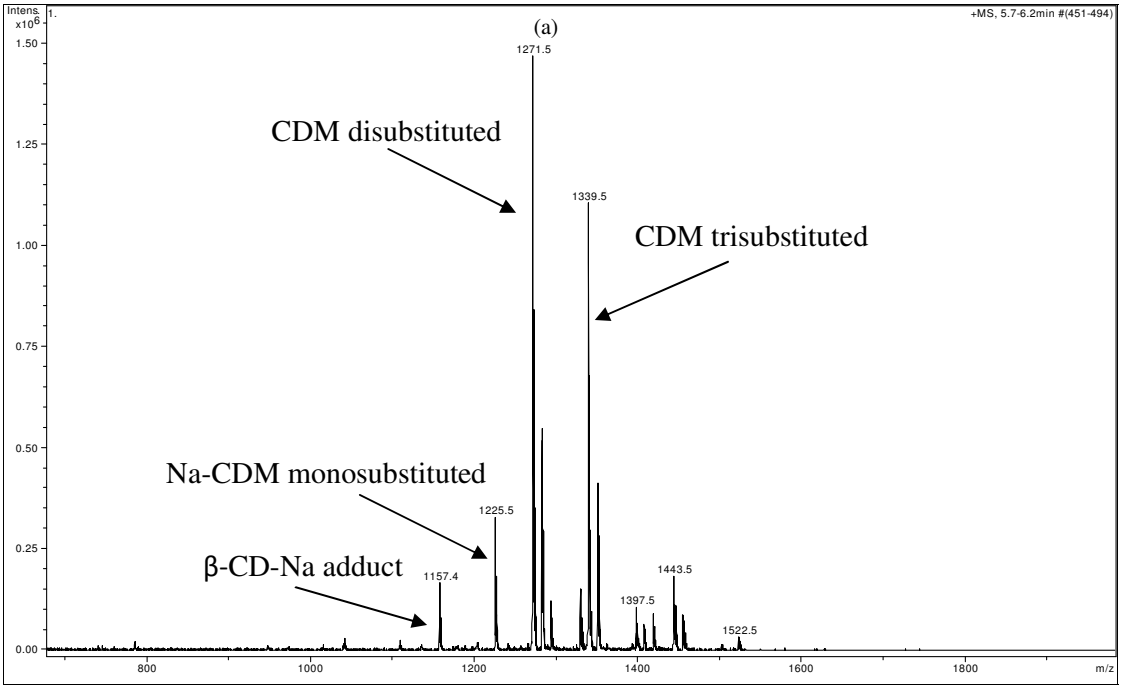
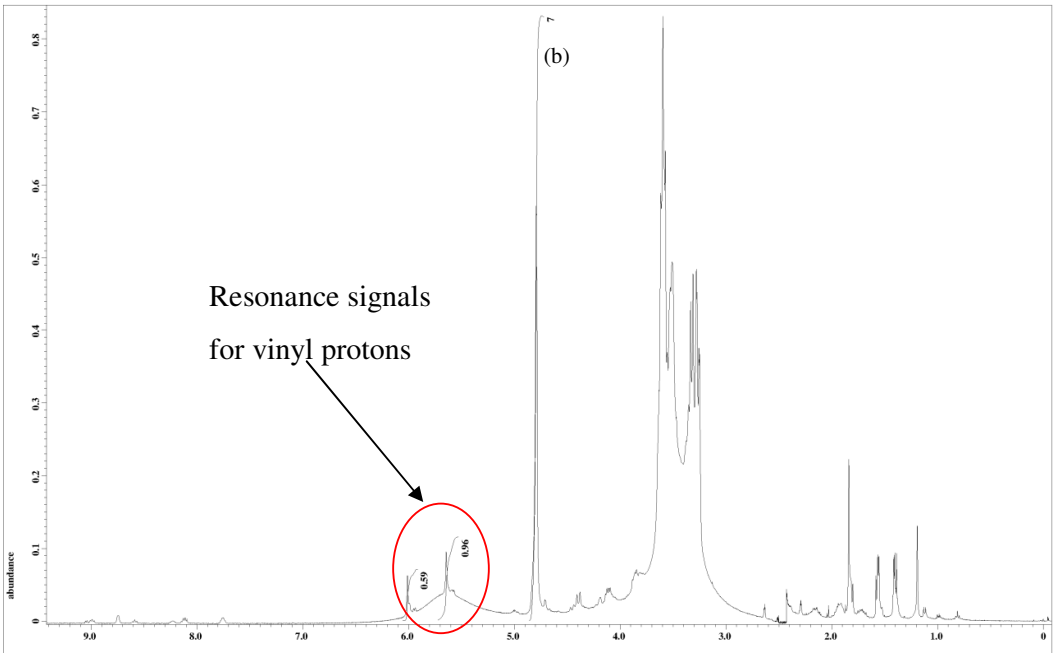



**Figure 5.9: Spectra obtained for β-CD using (a) LC-MS, (b) $^{1}$H NMR, (c) $^{13}$C NMR (NMR carried out in DMSO-$d_6$).**

It is important to note the presence of sharply defined OH peaks in Figure 5.9 (a), as a reduction or broadening of these peaks, with concomitant appearance of vinyl proton signals would indicate the addition of the vinyl groups at these sites. The absence of any vinylic or carbonyl carbon resonance signals in Figure 5.9 (c) is also important, as the introduction of these groups would suggest success of the reaction.

A number of different ratios of β-CD to MAC were tested ranging from 1:2.2-1:5 and it was observed that the most successful ratio was 1:2.2. The spectra obtained for the CDM product are presented in Figure 5.10 (a)-(c).


(a)
CDM disubstituted
CDM trisubstituted
Na-CDM monosubstituted
β-CD-Na adduct
1271.5
1339.5
1225.5
1157.4
1443.5
1397.5
1522.5
m/z


(b)
Resonance signals
for vinyl protons
0.59
0.96
abundance

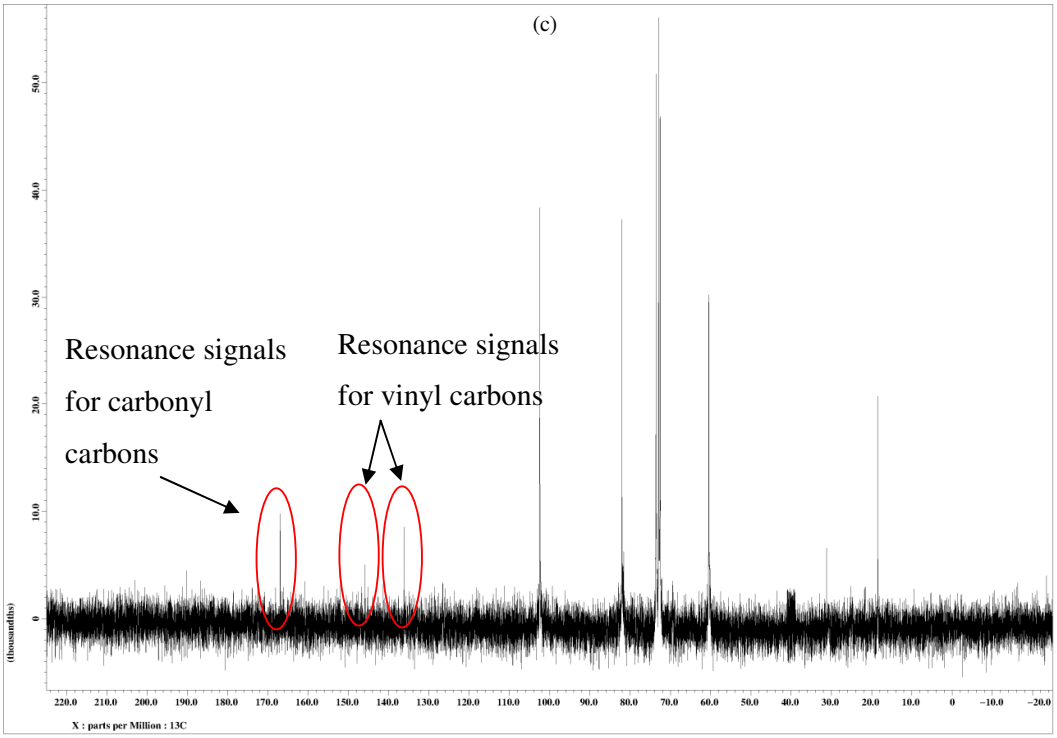



**Figure 5.10: Spectra obtained for the CDM product: (a) MS, (b) $^{1}$H NMR and (c) $^{13}$C NMR (NMR carried out in DMSO-$d_6$).**

Figure 5.10 (a)-(c) suggested that β-CD was successfully derivatised using methacryloyl chloride, with a number of different substituted vinyl derivatised CD monomers. Figure 5.10 (a) shows the mass spectrum of the product. The peak at m/z 1157.5, the sodium adduct of β-CD, suggests that some starting material remained. There was strong evidence for the presence of β-CD derivatised to various extents, with peaks at m/z 1225.5 signifying the sodium adduct of the monosubstituted product), m/z 1271.5 for the disubstituted product and m/z 1339.5 for the trisubstituted monomer. There was evidence of further substitution but these peaks were at a very low intensity.

$^{1}$H NMR was used to provide an average number of methacryloyl groups per cyclodextrin unit [6, 25]. Figure 5.10 (b) shows that the OH-6 peak at 4.3 ppm had all but disappeared, suggesting that this was the primary site of reaction. The OH-3 and OH-2 peaks changed from sharp peaks at 5.5 ppm to a broad signal, overlapping with the vinyl protons at 5.5-6.0 ppm. The integration for the CDM H-1 proton was normalised for a value of 7 and comparison of the integrals for the suspected vinyl protons gave a combined value of 1.6, indicating an average of 0.5 groups substituted per cyclodextrin molecule. Given the results obtained from LC-MS, this appeared to be

very low, which suggested that the presence of the broad signal overlapping the vinyl protons prevented accurate determination of the average number of groups per molecule.

There was evidence of the presence of vinylic carbons in the $^{13}C$ spectrum (Figure 5.6 (c)), at 130 and 150 ppm, suggesting the presence of vinyl groups of different types. A peak at 170 ppm indicated the presence of a carbonyl carbon.

The combination of evidence from MS and NMR suggested the successful derivatisation of β-CD, however, downfield peaks unrelated to the cyclodextrin monomer were observed in the $^{1}H$ NMR spectrum for CDM at 8-9 ppm. Analysis of the $^{1}H$ spectrum of pyridine suggested that these peaks were due to the presence of pyridine entrapped within the hydrophobic cavity. While the wash step with ethanol was carried out in a bid to reduce this problem, neither this step nor various vacuum drying steps could remove the pyridine. This was considered to be problematic as the pyridine molecules were likely to reside in the hydrophobic cavity intended for interaction with the steroidal template in the pre-polymerisation complex. To combat this, a synthetic strategy using a non-competitive solvent was investigated.

The approach developed by Lee *et al*. [32] and employed by Siemoneit *et al*. [34] for the synthesis of polymerisable β- and γ-cyclodextrin monomers, was studied. This method involved the synthesis of acrylamidomethyl cyclodextrin (β- or γ-CDMA) by preparation of an aqueous cyclodextrin solution followed by the addition of *N*-(hydroxymethyl)acrylamide (NMA). The reaction mixture was then heated to 80 °C and sufficient HCl was added to prepare a 1% (v/v) solution. The synthetic strategy employed is schematically depicted in Figure 5.11.

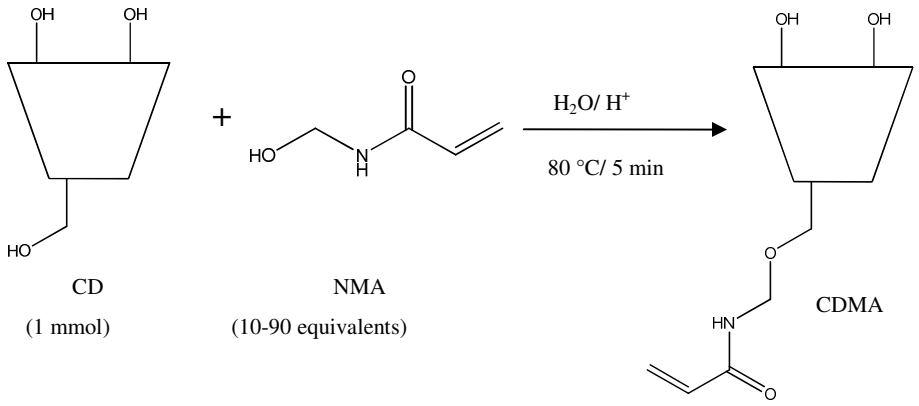
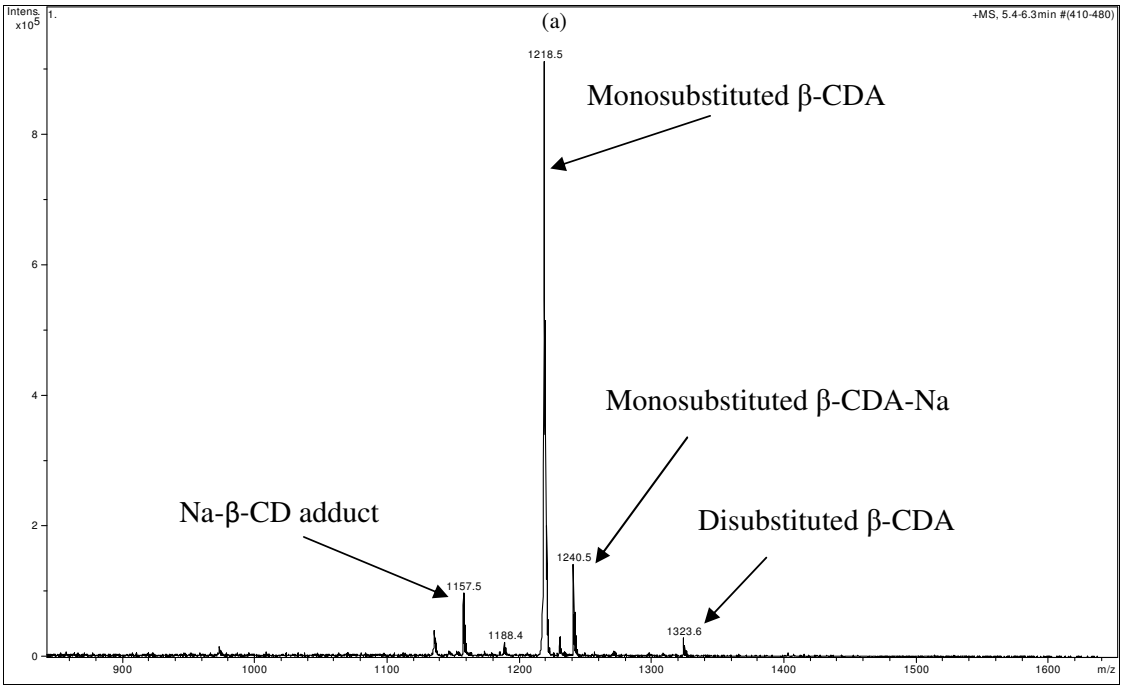
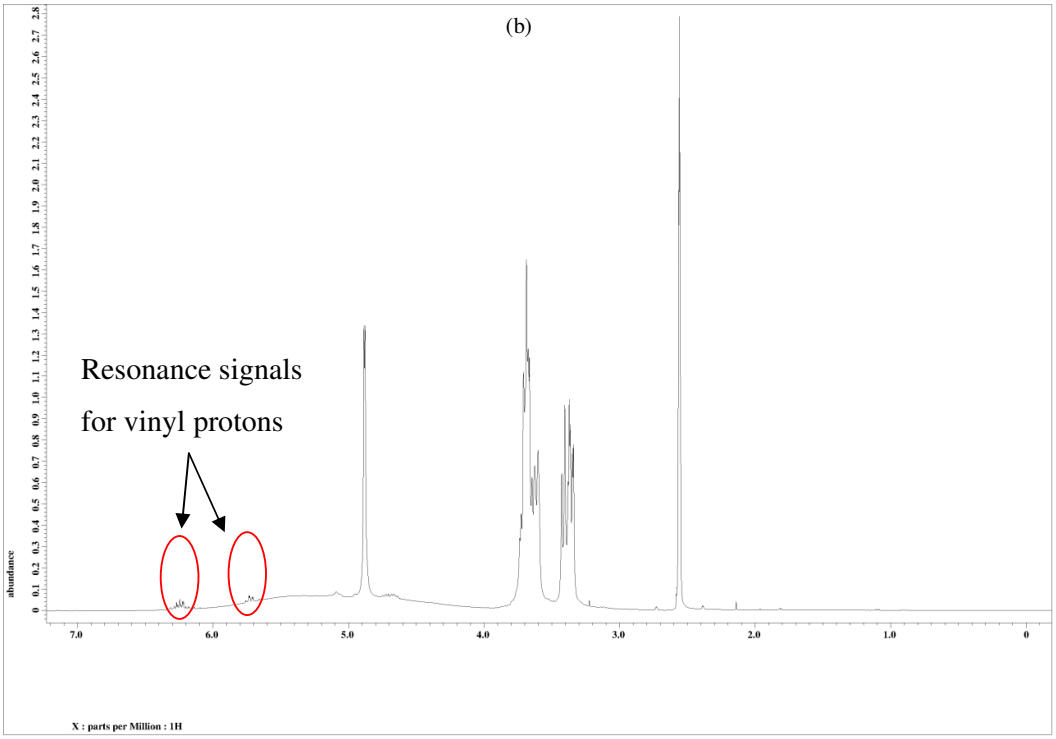



**Figure 5.11: Synthesis of acrylamidomethyl cyclodextrins (CDMA) showing reaction at C-6 OH.**

The derivatisation of CDs by this method had two distinct advantages over the preparation of CDM, namely, the short reaction time of five minutes and the use of a solvent that would be unlikely to compete with the steroid templates for interaction with the hydrophobic cavity. Preliminary investigations for β-CD used the 1:10 ratio of cyclodextrin to NMA reported in the literature by Siemoneit *et al*. for the derivatisation of γ-CD [34]. However, LC-MS analysis suggested that very little product was formed. This was possibly due to the difference in solubility between β-CD and γ-CD (the latter being over ten times more soluble in water). To promote interaction the concentration of NMA was increased three-fold and the following mass and NMR spectra (Figure 5.12) demonstrate the success of this reaction.





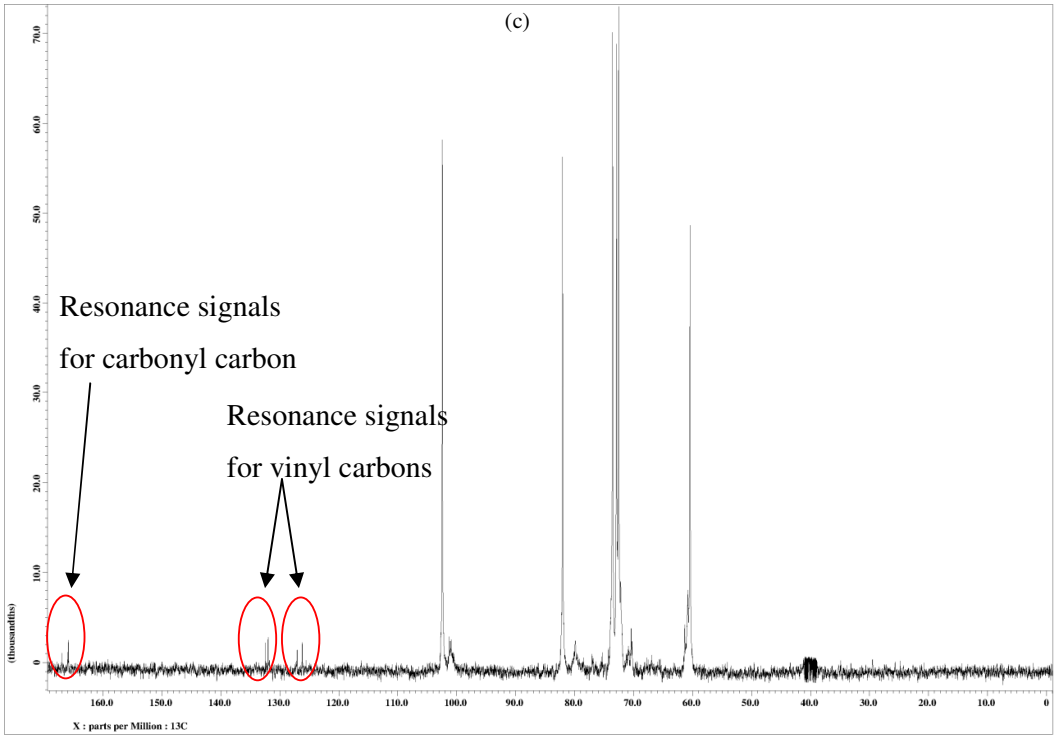
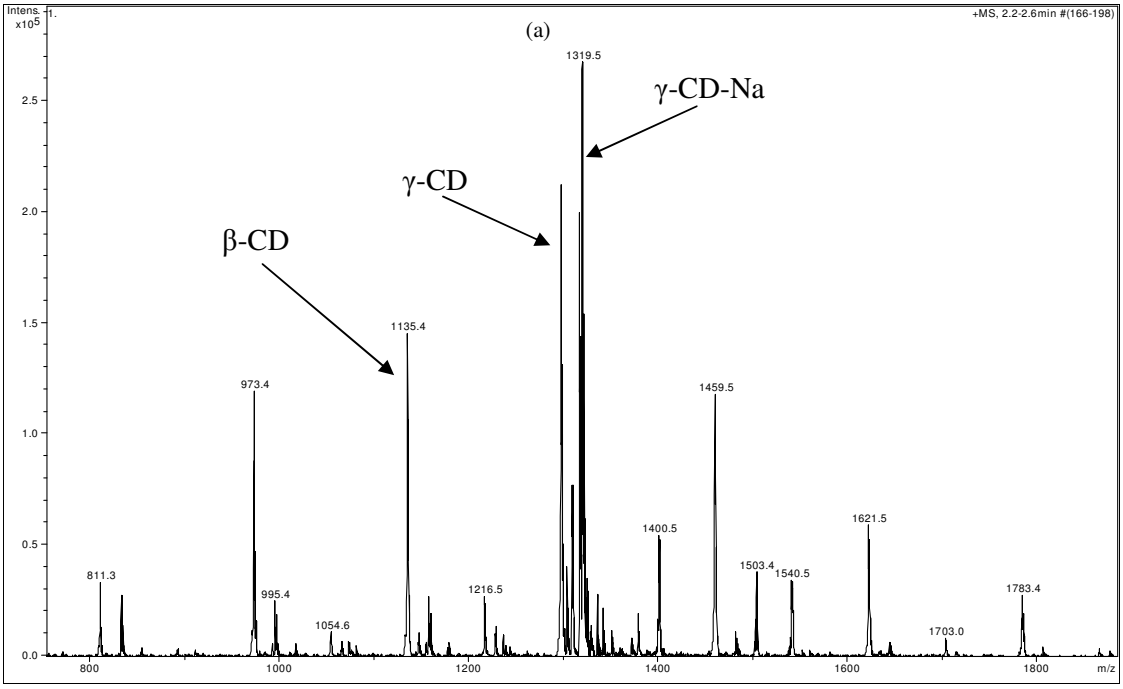




**Figure 5.12: Spectra obtained for β-CDA: (a) MS, (b) $^{1}$H NMR, (c) $^{13}$C NMR (NMR carried out in DMSO-$d_6$).**

The major peak at m/z 1218.5 in the mass spectrum shown in Figure 5.12 (a) suggests that the monosubstituted compound was the major product as this was the sodium adduct. Evidence for the disubstituted product was also present with a peak at m/z 1323.6. The $^{1}$H NMR spectrum shows evidence of vinyl protons at 5.5-6.3 ppm. Quantitation was again complicated by the presence of the broad overlapping peak in the region of the signals observed for the vinyl protons and a value could not be accurately obtained. The $^{13}$C NMR spectrum in Figure 5.12 (a) showed evidence of vinylic carbons at 120-130 ppm and a carbonyl group is indicated by the resonance signal observed at 170 ppm. These results again suggested the presence of a mixture of derivatised β-CD compounds and the low intensity of all signals may suggest that the monosubstituted product was the major component.

Due to the modest success of this reaction, this approach was applied to γ-CD for the preparation of γ-CDA. The mass and NMR spectra for the starting material, γ-CD, are presented in Figure 5.13 ($^{1}$H NMR peak assignment from reference [34]).



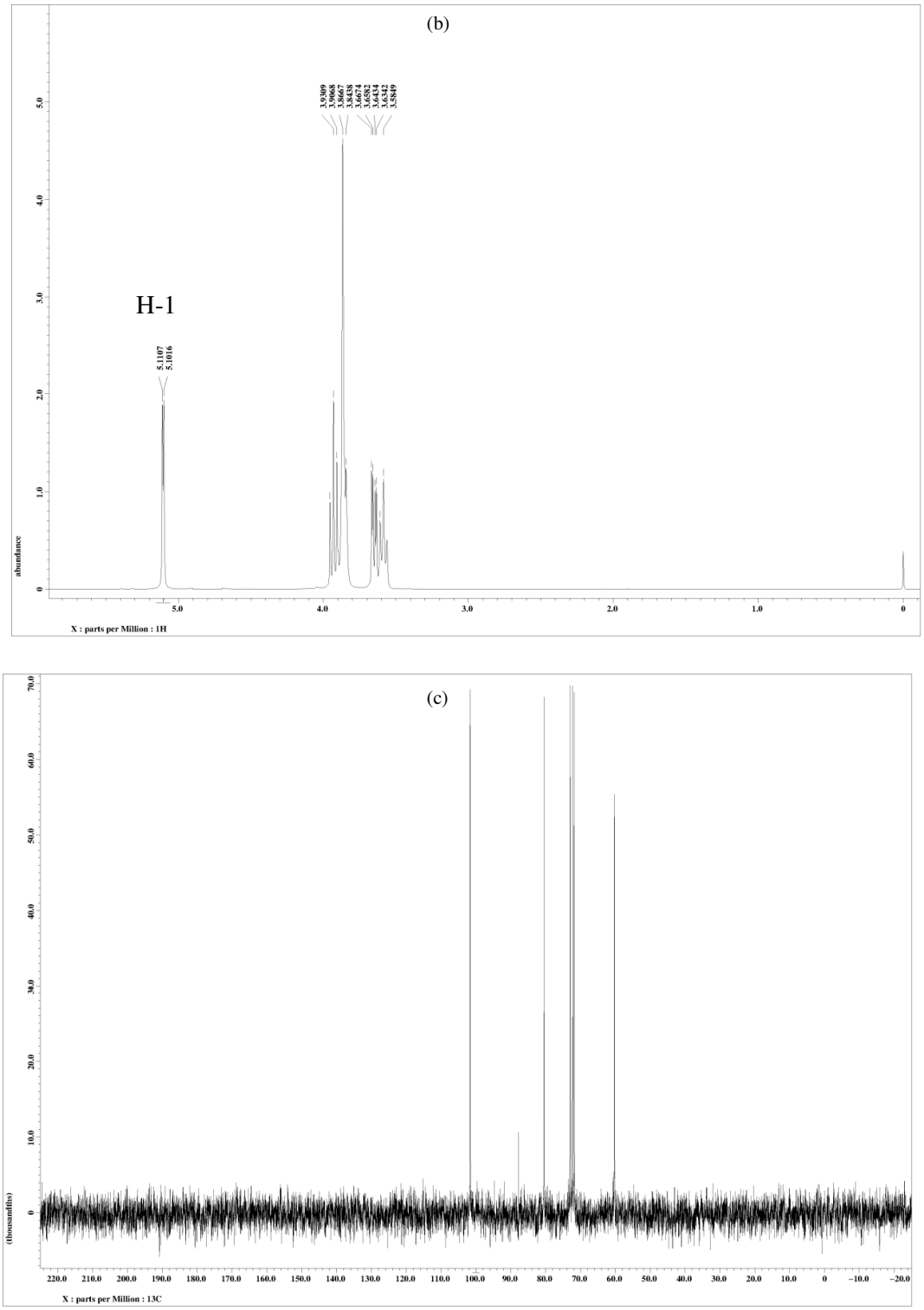
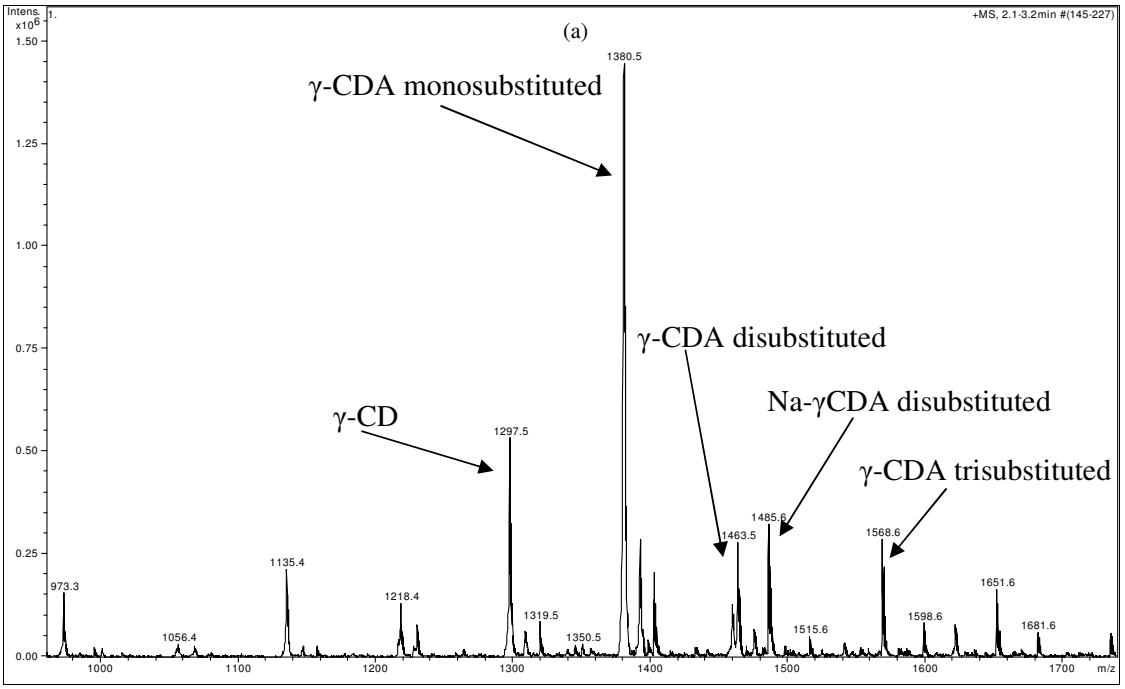
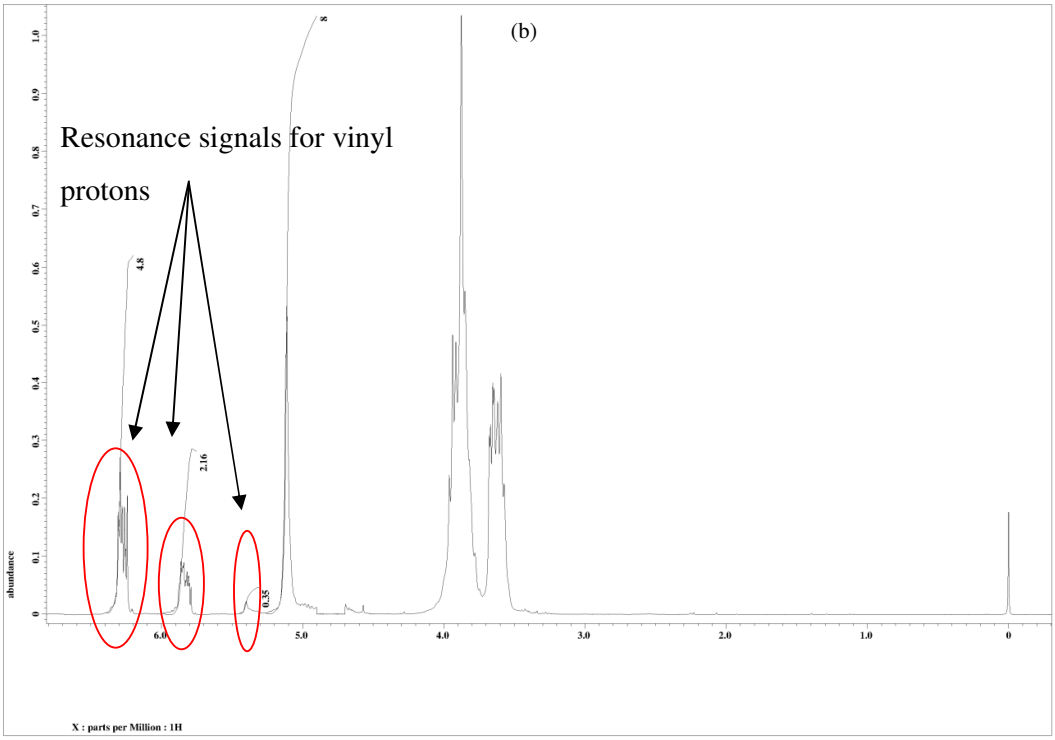



**Figure 5.13: Spectra for γ-CD starting material: (a) MS, (b) $^{1}H$ NMR and (c) $^{13}C$ NMR (NMR carried out in $D_2O$).**

The preparation of the γ-CD based monomer initially attempted using the 1:10 CD:NMA ratio but as with β-CD, very little product was formed. In this case, the most successful ratio for CD:NMA was found to be 1:50 and the relevant spectra are presented in Figure 5.14.



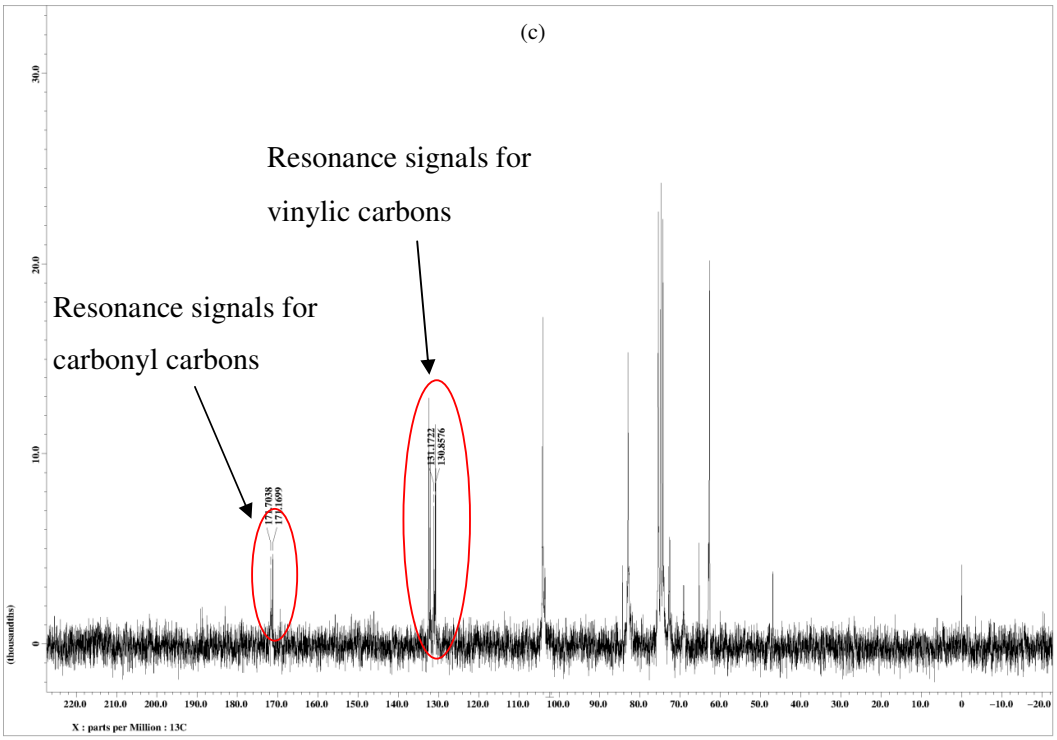



**Figure 5.14: Spectra for γ-CDA: (a) MS, (b) $^{1}$H NMR and (c) $^{13}$C NMR (NMR carried out in $D_2O$).**

The mass spectrum in Figure 5.14 (a) shows peaks for the monosubstituted γ-CDA (m/z 1380.5), disubstituted γ-CDA (m/z 1463.5) and trisubstituted γ-CDA (m/z 1568), as well as low intensity peaks for further substituted products. Using the H-1 CD proton in Figure 5.14 (b), the $^{1}$H NMR spectrum, to normalise the integration of the three vinyl proton groups observed, the average number of acrylamide groups per CD was calculated to be 2.5. The $^{13}$C NMR spectrum (Figure 5.14 (c)) contains peaks at 170 ppm and 130 ppm, which are indicative of the presence of carbonyl carbons and vinylic carbons, respectively. These peaks were not present in the starting material, γ-CD, as is shown in Figure 5.13 (c). These results suggested the successful synthesis of derivatised γ-CD composed of a mixture of differently substituted compounds.

Although the purity and yield of the successfully derivatised CD compounds was not fully optimised at this stage, preliminary studies were carried out on the use of these monomers for the preparation of corticosteroid-imprinted polymers.

*5.4.1.4 Preliminary studies on β-CDA and γ-CDA based molecularly imprinted polymers*

Due to the high cost of γ-CD, the majority of method development was carried out using β-CD based monomers, i.e. β-CDA. This monomer was used in the preparation of cyclodextrin molecularly imprinted polymer 2 (CDMP2). The solubility characteristics of the polymeric components, necessitated the use of dimethyl formamide (DMF) as the solvent as, of a range of solvents tested, this was the only solvent in which the template (hydrocortisone-17-butyrate), the crosslinker (*N,N*-methylene bisacrylamide) and β-CDA were soluble. Ethylene glycol dimethacrylate (EGDMA) was tested for use as crosslinker and while this monomer was soluble in a range of solvents, it was not possible to simultaneously dissolve EGDMA and β-CDA in any of the solvents studied. For this reason *N, N*-methylene bisacrylamide was investigated for use and while solubility was still an issue to some extent, it was possible to dissolve all of the components simultaneously in DMF. The maximum monomer concentration, however, was still quite low with a limit of 28% (w/v).

CDMP2 was prepared as a bulk monolith and ground and sieved to a range of 23-58 μm. This polymer fraction was then extracted using Soxhlet apparatus and was packed into HPLC columns as per Section 5.3.8. The polymer was tested and compared to the non-imprinted control polymer (CDNP2) using chromatographic evaluation to investigate the imprinting effect. Acetonitrile and acetonitrile-water compositions were used as the mobile phase in mixtures ranging from 10/90 to 90/10 (v/v). No difference was observed in the retention behaviour on the MIP and NIP column for either the template or any of the six steroidal analogues investigated in this study. A possible reason for this may have been the extremely low surface area of the polymer, which was less than 1 $m^2.g^{-1}$. Solvent competition for the CD cavity in the pre-polymerisation complex may also have had a negative effect on imprinting attempts. The lack of any observable imprinting effect, despite analysis in highly aqueous solutions where hydrophobic interactions should predominate, suggested that this polymer was not suitable for further investigations. Rather, other avenues for the exploitation of CD and steroid interaction in the pre-polymerisation solution were pursued.

Since a high monomer percentage is required for bulk monolith preparation, the aforementioned high cost of γ-CD prevented the cost effective synthesis of 'bulk' polymers using γ-CDA. In contrast, the low monomer percentage necessary for the

preparation of precipitated polymers facilitated preliminary investigations using γ-CDA as a co-monomer. Since NMR studies outlined in Section 5.4.1.1 showed strong interaction between β-CD and BDN, it was postulated that the interaction between γ-CD and steroidal templates (in this case HCB) would be of the same magnitude. In fact, literature reports have shown the interaction between γ-CD and steroidal analytes to be stronger than that of β-CD [12]. This enhanced interaction should allow for an improved imprinting effect for precipitated polymers over those outlined in Chapters 2 and 3.

To further drive reaction, the use of aqueous solutions for the preparation of such polymers would be expected to be advantageous, due to the hydrophobic nature of the interaction between steroids and CDs. While the inherently high monomer loading of bulk strategies prevented the use of water for this approach, the necessarily high solvent to monomer ratios required for precipitation strategies facilitated investigation of the use of water as the porogen for steroidal polymers. To this end, precipitated polymers using γ-CDA as the functional monomer and aqueous mixtures of acetonitrile as porogen were investigated.

It was decided to prepare precipitated polymers based on M13, as outlined in Chapter 3, which employed methacrylic acid (MAA) as the functional monomer, EGDMA as the crosslinking monomer and acetonitrile as the porogen. The amount of MAA could be reduced as the template could interact with the cyclodextrin monomer as well. The chosen ratio of template (HCB):γ-CDA:MAA:EGDMA was therefore selected as 1:2:4:30. Different ratios of acetonitrile to water were tested empirically to determine the composition in which all components could be dissolved and the highest monomer concentration that could be used. This resulted in the use of an acetonitrile-water 60/40 (v/v) mixture and a monomer concentration of 1% (w/v). This resulted in precipitated microparticles produced in the size range of 0.27 – 7.23 μm, of which an SEM image can be seen in Figure 5.15.

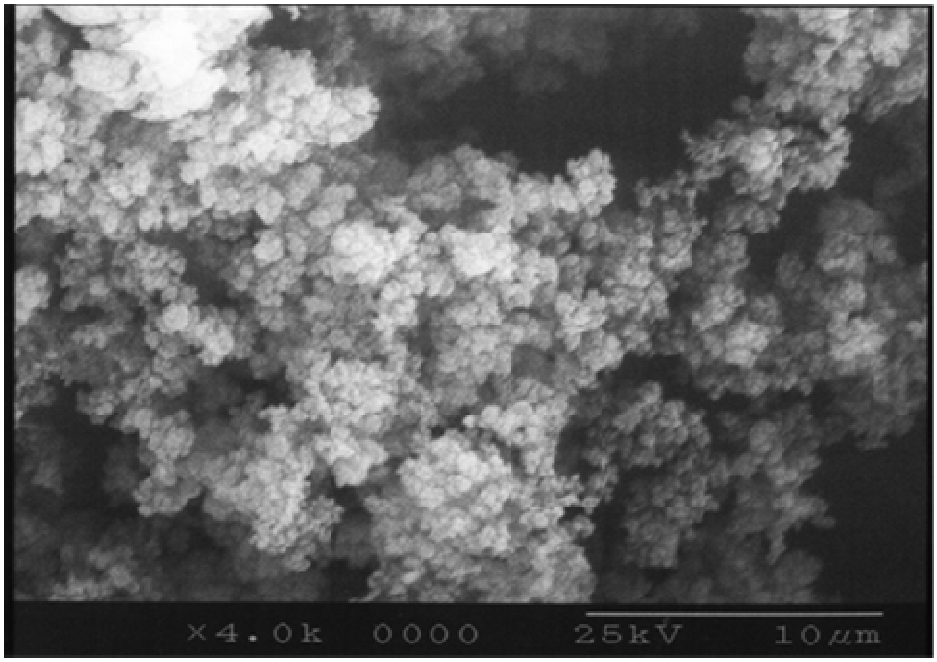



**Figure 5.15: SEM image of microparticles formed by precipitation polymerisation using aqueous acetonitrile as the porogen and γ-CDA as the co-monomer.**

Time constraints prevented any binding analysis or physical characterisation of this polymer, however, the ability to use CD based functional monomers in a highly aqueous solvent to prepare precipitated microparticles suggests the possibility of preparing molecularly imprinted polymers with an increased capability for selective corticosteroid rebinding.

### 5.4.2 The synthesis of a polymerisable template-monomer for use in semi-covalent corticosteroid MIPs

As discussed in detail in Section 1.3.3, the semi-covalent method of molecular imprinting attempts to utilise covalent linkages for the creation of binding sites with the homogeneity and fidelity synonymous with covalent imprinting, while achieving the fast rebinding kinetics inherent to non-covalent binding events. In an effort to achieve this for the corticosteroid templates focussed on in this study it was decided to use hydrocortisone as the starting material for derivatisation reactions. Hydrocortisone possesses three hydroxyl groups, at the C-11, C17 and C-21 positions, with the potential for derivatisation and subsequent addition of between one and three polymerisable vinyl groups. The method outline in Section 5.3.3.2 was used to synthesise hydrocortisone methacrylate (HMA) and the reaction scheme is shown in Figure 5.16.

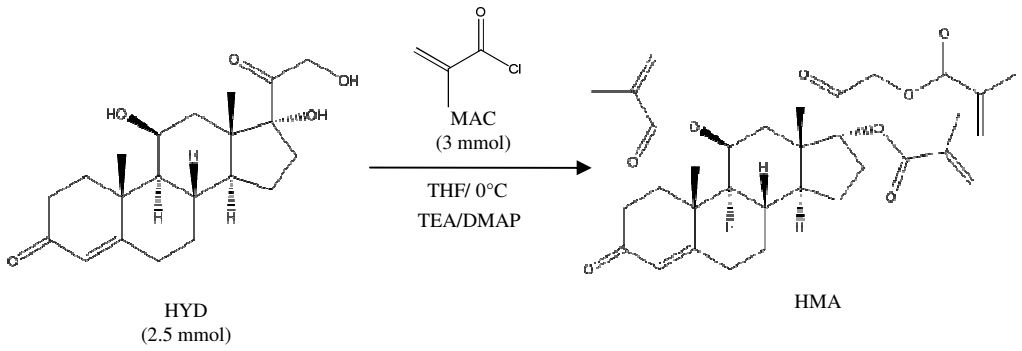
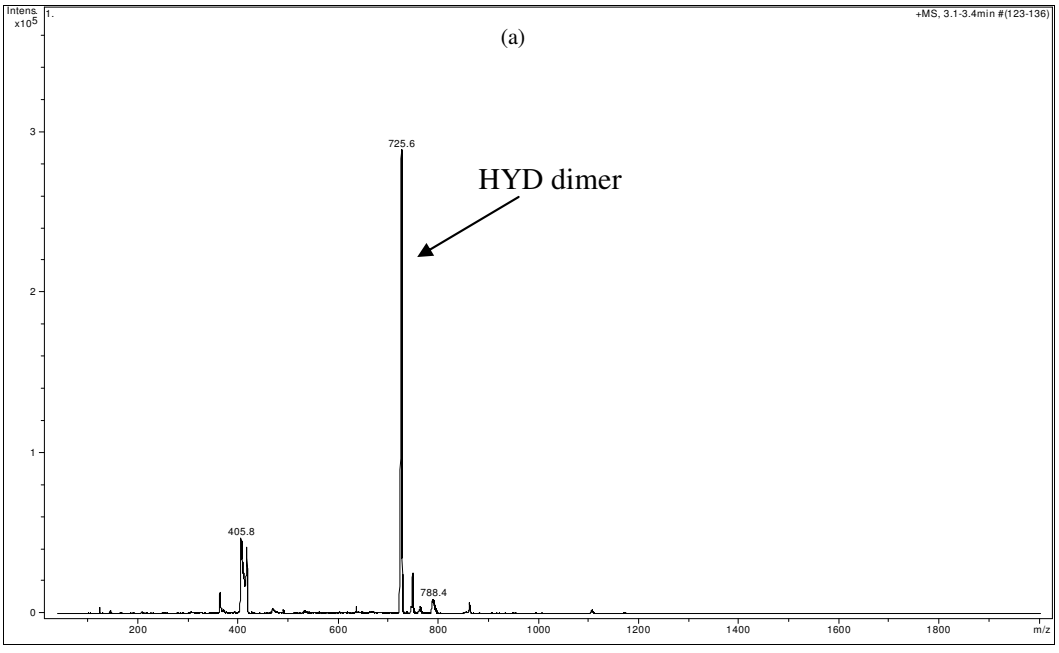



**Figure 5.16: Reaction scheme for the synthesis of HMA with all three potential vinyl groups shown.**

Time constraints again dictated that the synthesis of this polymerisable template-monomer was a preliminary feasibility study into the ease of preparation of the monomer and the possibility of its use for the preparation of corticosteroid imprinted polymers by semi-covalent means. As can be seen from the following discussion, LC-MS and NMR analysis were used to obtain evidence for the derivatisation of hydrocortisone and rather than carrying out a full characterisation of the prepared template-monomer, the discussion will centre on comparisons of the NMR spectra of hydrocortisone (HMA) and hydrocortisone methacrylate (HMA). To facilitate this, the mass spectra of both compounds can be seen in Figure 5.17 (a) and (b).



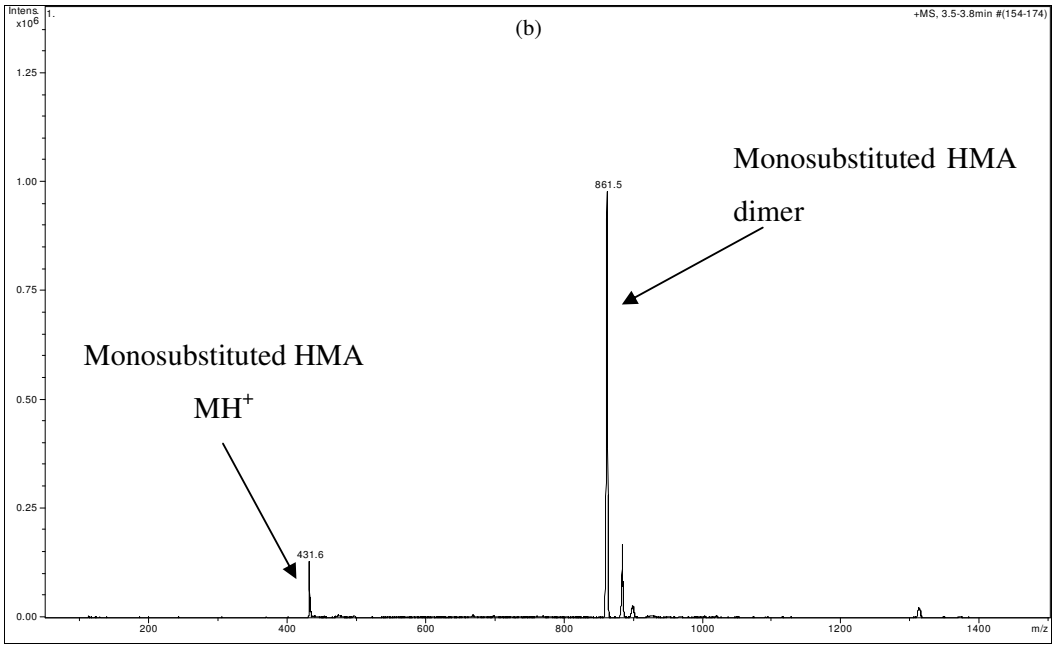
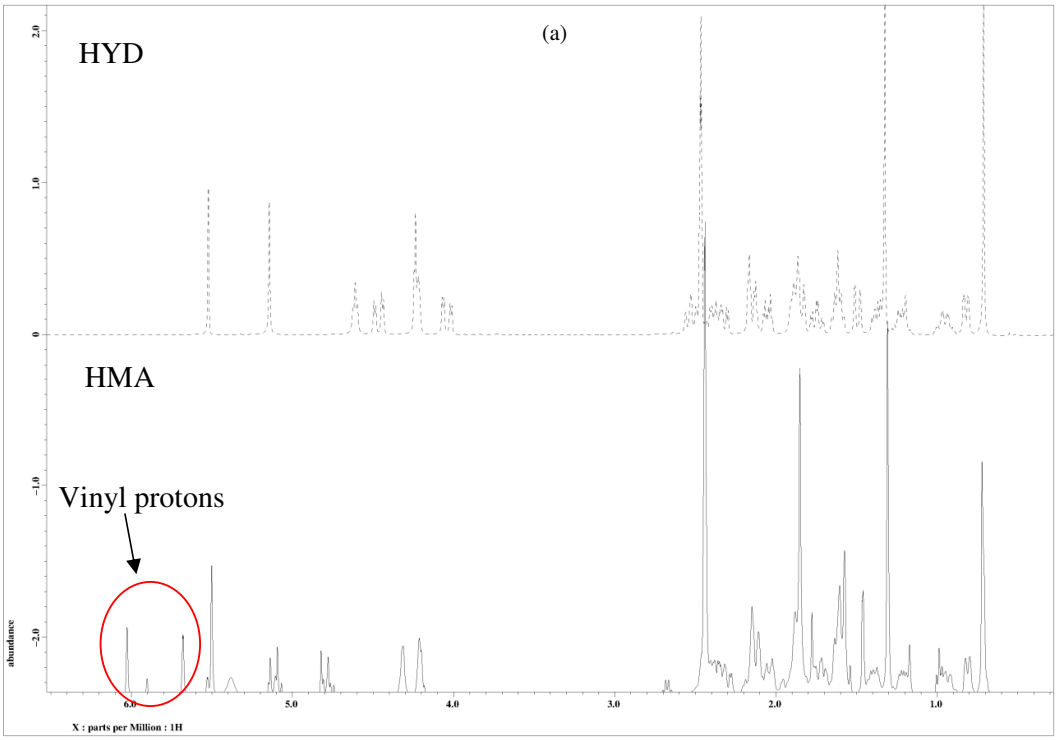



**Figure 5.17: Mass spectra for (a) HYD and (b) HMA**

The mass spectra shown in Figure 5.17 (a) and (b) suggest the synthesis of monosubstituted HMA based on peaks at m/z 431.6 and 861.5 for the $MH^+$ and dimer ions, respectively. The NMR spectra for HYD and HMA is presented in Figure 5.18.



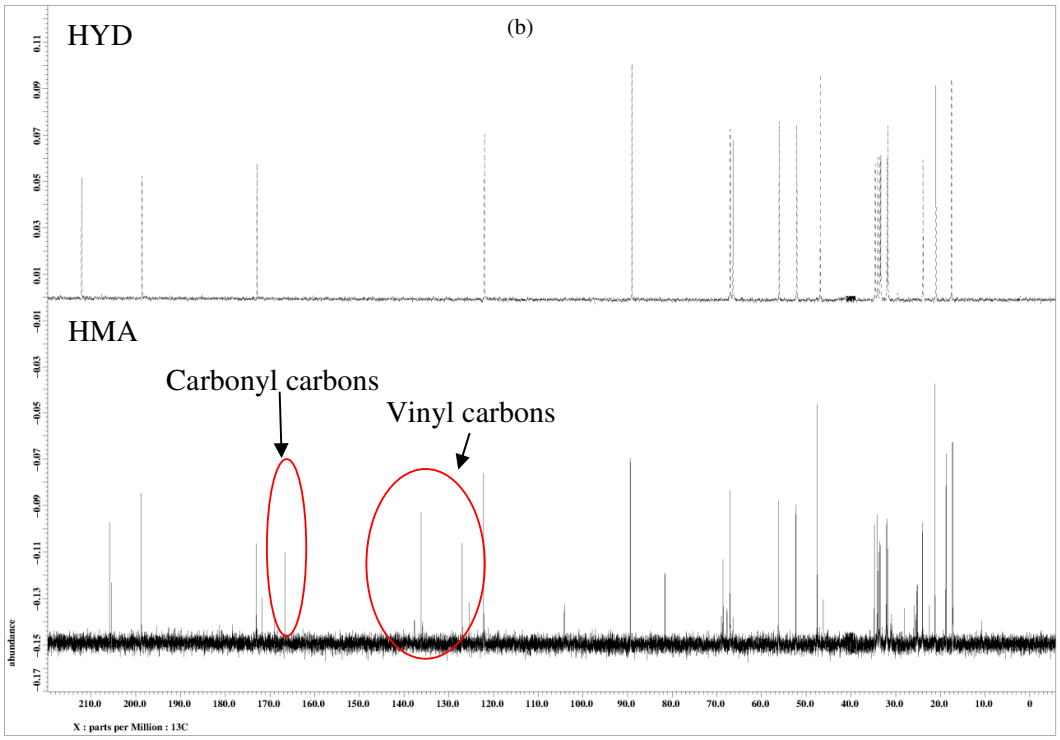



**Figure 5.18: Comparative spectra highlighting the differences between HYD and HMA in (a) $^{1}$H NMR and (b) $^{13}$C NMR.**

Figure 5.18 (a) demonstrates that while the main steroidal skeleton (chemical shifts between 0-4 ppm) remained unchanged, there was evidence to suggest the presence of a number of different vinyl protons at 5.6-6 ppm. Further evidence of the presence of different vinyl groups was observed on the $^{13}$C spectra; where extra peaks for HMA at 125-130 ppm suggested the presence of different types of vinyl protons while extra peaks at 167 ppm suggested the presence of two types of carbonyl groups. The evidence observed from MS and NMR analysis suggested that HYD had been successfully derivatised and while MS pointed to the monosubstituted compound, NMR analysis, where evidence of different vinyl protons was present, suggested the possibility of more than one type of monosubstituted compound. No evidence of starting material was observed in the MS spectrum, suggesting that the reaction went to completion. The monomer so prepared was utilised in preliminary investigations into corticosteroid imprinted polymers prepared by a semi-covalent strategy.

### *5.4.2.1 Towards the semi-covalent imprinting of corticosteroids*

The semi-covalent approach to the molecular imprinting of corticosteroids was investigated using the polymerisable template-monomer, HMA, discussed in the

preceding section. The bulk imprinted polymer, hydrocortisone methacrylate molecularly imprinted polymer, HMAMP1, and non-imprinted polymer, HMANP1, were prepared (Section 5.3.4.2).

Prior to removal of the template-monomer by basic hydrolysis, the polymers were packed into stainless steel columns and the retention behaviours of the imprinted and control polymers for hydrocortisone were chromatographically evaluated. This involved using 100% acetonitrile as the mobile phase. Almost identical retention times of 2.3 minutes were obtained for both polymers.

The template-monomer was then removed by refluxing the polymer with 1 M KOH prepared in MeOH in a manner analogous to that used by Cacho *et al*. [28]. After washing with dilute HCl to neutralise the functional groups on the polymer, the ground and sieved particles were packed into stainless steel columns. The retention behaviours for polymers were drastically different at this stage, with retention times of 4.5 minutes obtained for both polymers. While no imprinting effect was observed, i.e. no difference between HMAMP1 and HMANP1, the basic treatment of the polymer appeared to have dramatically enhanced the affinity of the polymer for the steroidal template.

Physical characterisation by nitrogen sorption porosimetry showed that there was no significant differences between surface area (570 $m^2.g^{-1}$ ±10), pore volume (0.80 $cm^3.g^{-1}$ ± 0.1) or pore diameter (8.5 nm ± 1.0) for the imprinted polymers before and after hydrolysis. No morphological differences were observed upon hydrolysis for the control polymer, though they had a slightly larger surface area at 620 $m^2.g^{-1}$. Given that differences in retention were not due to physical characteristics, it was postulated that it was due to chemical changes in the polymer upon hydrolysis.

This behaviour was also observed by Cheong *et al*. [38], in a study carried out for testosterone imprinted polymers. In that investigation an imprinting strategy was referred to, where testosterone methacrylate was prepared and used in the preparation of EGDMA based polymers. Removal of this template-monomer was carried out by reflux in methanolic NaOH. Cheong *et al*. observed no imprinting effect but did see drastically increased retention behaviour on both imprinted and control polymers. They attributed this increased retention to the presence of sodium carboxylate functionalities that interacted more strongly with the steroidal analytes. This may have been the case in the

present study, with potassium carboxylate functionalities being responsible for the increased affinity.

It is worth noting that Cheong *et al*. point to the work of Bystrom *et al*. [39], where it was observed that NaOH/MeOH and NaOMe/MeOH treatment of the polymer had no affect on the ester groups of the steroid-methacrylate template-monomers. Instead, Bystrom *et al*. required the use of $LiAlH_4$/THF to successfully remove the template-monomer. In the present study, no effort was made to quantify the amount of hydrocortisone removed from the imprinted polymer and these literature examples would suggest that the use of a stronger hydrolysis agent would be required for removal of the template.

The potential to use the prepared template-monomer, HMA, for precipitated microspheres was also demonstrated in the present study. The incorporation of the template-monomer complex into polymers prepared in dilute solution could increase the number of binding sites in the resultant polymer, despite being prepared in dilute solutions. This polymer, HMAMP2, was prepared as outlined in Section 5.3.4.2 and resulted in the synthesis of discrete microspherical particles in the 1.2-5.1 μm size range. Again, time constraints prevented any analysis of these polymeric microspheres.

## *5.5 Conclusion*

The primary focus of this study was to synthesise tailor-made functional monomers capable of increasing the number of corticosteroid selective binding sites within polymeric matrices by enhancing complex formation in the pre-polymerisation solution or incorporating polymerisable template-monomers into the growing polymer network. Regarding the former, enhanced interactions between β-CD and BDN were observed during NMR binding studies.

This chapter demonstrated that cyclodextrin compounds were successfully derivatised using two cyclodextrin compounds, β-CD and γ-CD. Two methods were investigated and it was demonstrated that the most successful strategy resulted in the addition of an average of 2.5 polymerisable acrylamide units per γ-CD molecule. The monomers prepared showed the capability of being incorporated into corticosteroid imprinted

polymers. While time constraints prevented a full investigation into the ability of these polymers to specifically bind corticosteroid targets, precipitated microparticles incorporating γ-CD based monomers were prepared in highly aqueous porogens, using HCB as a template. Bulk polymers were also prepared using β-CD based monomers, showing the versatility of these compounds for use in a range of polymer formats and solvents.

This study also demonstrated the successful synthesis of a polymerisable template-monomer based on hydrocortisone. This template-monomer, hydrocortisone methacrylate, was prepared in what evidence suggested was a mixture of monosubstituted compounds with different positions of derivatisation. This polymerisable template was used in the preparation of bulk polymers and perhaps more interestingly, novel precipitated microspheres. This again showed the potential for the use of successfully derivatised compounds for a range of polymer formats.

Although it was not possible to fully investigate the polymers prepared from the synthesised functional monomers in either case, and so fully exploit the potential of these monomers, it was shown that polymerisable functional monomers with the potential for dramatically increasing the number of bindings sites within polymers for poorly functionalised templates were prepared.

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# Chapter 6

# Conclusions and future work

# 6. Conclusions and Future work

Molecularly imprinted polymers with corticosteroid recognition capabilities have been prepared in this study. These polymers have been synthesised in a range of polymer formats and while some of the polymers have demonstrated group selectivity towards six steroidal analytes, others have demonstrated the ability to differentiate between closely related structural analogues.

Novel corticosteroid-imprinted polymers have been prepared by precipitation methods, with both microspherical and microparticular morphologies. The development of these precipitated polymers resulted in the compilation of detailed knowledge for the preparation of such polymers and the factors affecting polymerisation. It was shown that monomer concentration, monomer ratios, initiator type and concentration as well as temperature and initiation methods all played a role in the ability to control the resultant polymers. Polymers were prepared with a high degree of control over particle size and morphology, using three crosslinking monomers, two templates and a range of solvent compositions. The control of particle size and morphology is demonstrated by the microspherical EGDMA and DVB polymers shown in Figure 6.1.

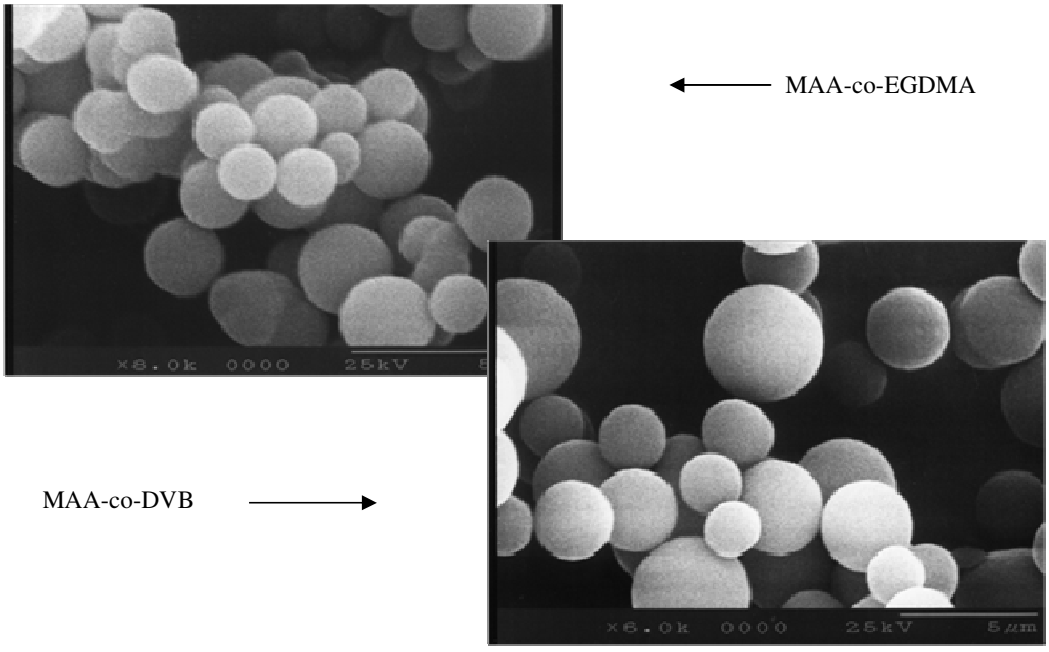


**Figure 6.1: Examples of the novel microspherical corticosteroid-imprinted polymers prepared in this study.**

The novel microspheres and microparticles, while exhibiting both group and specific target selectivity, demonstrated that these polymers, prepared by precipitation strategies, had a relatively low number of specific binding sites. A range of NMR studies, including titration based binding studies and Job plot analyses, were successfully carried out. These studies revealed weak interactions between two of the templates, budesonide (BDN) and hydrocortisone-17-butyrate (HCB), and commercially available functional monomers or monomer analogues. This led to a rational understanding of the effects of the combination of these weak interactions and the high dilution conditions required for precipitation strategies.

This knowledge was applied to the preparation of bulk polymers in an effort to enhance the interaction between templates and functional monomers in the pre-polymerisation complex by the inherent increase in monomer loading for such strategies. The target analogue approach was employed, using HCB as the template for polymers synthesised to have a high degree of affinity for BDN. A range of bulk polymers were prepared using three crosslinking monomers, DVB, EGDMA and TRIM, and a number of different porogen compositions. Evaluation of these polymers displayed polymers with a number of different characteristics and showed that the crosslinking monomer had profound effects on the molecular recognition properties of the polymers, as well as impacting on the driving forces behind recognition.

These polymers were investigated for use in solid phase extraction applications and HCB imprinted polymers were shown to specifically recognise and retain the target, BDN, using a number of different protocols. Precipitated microparticles, using prednisolone (PRD) as the template, were shown to be capable of the chromatographic separation of PRD and the closely related, hydrocortisone, as shown in Figure 6.2.

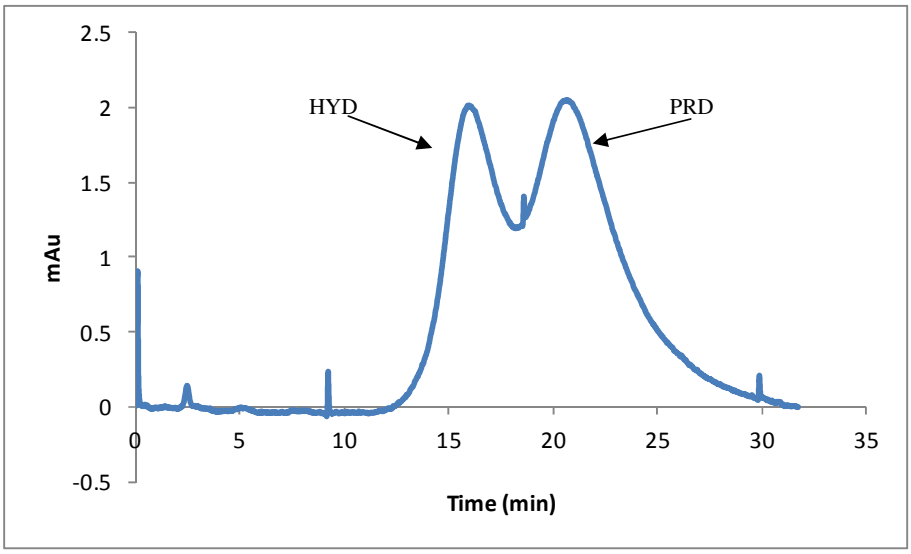


**Figure 6.2: Chromatographic separation using precipitated polymers to separate prednisolone and hydrocortisone**

Separations of these compounds on 5 cm columns achieved α values ranging from 1.3-1.4 at different temperatures and using optimised polymers. Information obtained on the factors affecting precipitation polymerisation allowed for tailoring of particle morphology to achieve analyte resolution of 0.75. Future work for this polymer will focus on increased control over particle morphology and the use of chromatographic equipment suited to smaller particles and higher pressures, such as ultra pressure liquid chromatography (UPLC). In such instrumentation the high pressures needed for small particles can be achieved. Future investigations will also include studies on the effect of an increased amount of template in the porogen, to increase the number of selective binding sites or the use of a functional monomer capable of increasing the number of sites within the polymer.

These polymers suggested the potential for cheap and efficient bulk preparation of selective materials, capable of differentiating between close structural analogues. While the focus for MIP application has often been on their use for analytical separations such as in chromatography, realisation of their commercialisation has been slow. Some of the reasons for this, such as peak tailing and band broadening have been highlighted in this study, and while approaches such as the use of polymers with more defined and controllable morphology can be applied, they too have their limitations. Consideration must be given to where the focus of commercialisation hopes for MIPs should lie. They have definite and proven strengths, such as the cheap and inexpensive production of highly selective materials, which can be prepared for myriad targets. Solid-phase

extraction is an application that exploits such strengths and has achieved commercial success. This success could be built upon by the use of MIPs in process applications where they are employed in bulk for the selective purification of a product or process intermediate. This can be achieved by retention of either the product or retention of impurities that need to be removed from the process. The use of MIPs in membranes for such applications is a growing field and the ability to create strong, specific interaction for targets of all types is essential for this area to realise its full potential.

The need for strong, specific interaction was also highlighted during the course of this study, where the weak interaction between widely used functional monomers and the steroidal templates was seen to have a profound effect on the binding characteristics of the resultant polymers. This further demonstrated the need for tailor-made functional monomers with the ability to increase the number of selective binding sites within the polymeric matrix. To this end a number of tailor-made functional monomers were successfully synthesised using a range of approaches. One strategy involved the preparation of derivatised cyclodextrin (CD) compounds. In this study, CDs were shown to interact to a strong degree with BDN in polar solvents. Native β- and γ-CD were then successfully derivatised to form polymerisable functional monomers containing, for the most successful reaction, an average of 2.5 polymerisable vinyl groups per γ-CD. The successfully vinylated CDs were shown to have the capability to be incorporated into polymers prepared in either bulk or precipitation strategies in both organic and aqueous-organic porogens.

While time constraints prevented the full development of these polymers, the capability to prepare polymers using precipitation strategies in aqueous-based porogens has potential for the preparation of imprinted polymers with enhanced selectivity for poorly functionalised corticosteroids. Future studies for this strategy involve optimisation of the polymer morphology and a detailed binding study, evaluating the imprinting power for such polymers and corticosteroids.

The semi-covalent approach was investigated in this study and a polymerisable steroidal template-monomer was successfully prepared by derivatisation of hydrocortisone. The use of derived template-monomers negates the need for strong interaction in the pre-polymerisation stage and incorporates the template into the growing polymer by way of direct polymerisation. Hydrocortisone methacrylate was successfully prepared in a

mixture of monosubstituted compounds and this monomer was shown to be capable of incorporation into a precipitated microsphere with highly controllable morphology. Time constraints prevented the full development of this strategy and future work would involve a detailed investigation into the removal of the template from the polymer. This would allow for a detailed binding study on the polymer, allowing for quantitation of the effect on the strength and quantity of the binding sites present within the polymeric matrix. The potential to increase the number of binding sites on a polymer, for which the morphological properties can be accurately and readily adjusted, suggests the potential for use of such polymeric strategies in a wide range of applications.

This study has highlighted the urgent need in the field of molecular imprinting to focus on the development of tailor-made and custom monomers for use with challenging templates like corticosteroids. The ability to design and synthesis monomers capable of exploiting all of the possible molecular interactions, as well as having the potential for use in a range of highly controllable polymer formats, will widen the applicability of molecular imprinting, not just in terms of target choice but also in the realisation of their full commercial potential.