Analyte rebinding of optimized molecularly imprinted polymers in water

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Analyte rebinding of optimized molecularly imprinted polymers in water

Abstract
A series of non-covalent Molecularly Imprinted Polymers (MIPs) were prepared using caffeine and guanosine separately as template molecules. A wide variety of MIPs were prepared by varying a number of formulation parameters, including the template, recognition monomer, crosslinking agent and porogen types and concentrations. The specificity and selectivity of the MIPs were determined by rebinding studies in an aqueous medium using HPLC detection and the results were supported by gas adsorption experiments. Rebinding conditions, such as the pH and MIP concentration were also varied to determine the best conditions for analyte recognition. By using variety of polymerization conditions the particle size of the MIPs ranged from 500 nm to 200 mum. Their rebinding characteristics were analyzed.

An optimized formulation with suitable rebinding conditions was determined. By balancing the binding strength and specific site density the highest recognition performance was identified.

Keywords
Engineering, Materials Science, Chemistry, Polymer

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ANALYTE REBINDING OF OPTIMIZED
MOLECULARLY IMPRINTED POLYMERS IN WATER

BY

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THESIS

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in

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December, 2006
This thesis has been examined and approved.

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December 20th, 2006
Date
DEDICATION

This thesis is dedicated to my family especially my mom. Without their love, support and encouragement, this work would not have been possible.
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ABSTRACT

ANALYTE REBINDING OF OPTIMIZED MOLECULARLY
IMPRINTED POLYMERS IN WATER

by

Sayantan Roy

University of New Hampshire, December 2006

A series of non-covalent Molecularly Imprinted Polymers (MIPs) were prepared using caffeine and guanosine separately as template molecules. A wide variety of MIPs were prepared by varying a number of formulation parameters, including the template, recognition monomer, crosslinking agent and porogen types and concentrations. The specificity and selectivity of the MIPs were determined by rebinding studies in an aqueous medium using HPLC detection and the results were supported by gas adsorption experiments. Rebinding conditions, such as the pH and MIP concentration were also varied to determine the best conditions for analyte recognition. By using variety of polymerization conditions the particle size of the MIPs ranged from 500 nm to 200 μm. Their rebinding characteristics were analyzed.

An optimized formulation with suitable rebinding conditions was determined. By balancing the binding strength and specific site density the highest recognition performance was identified.
INTRODUCTION

The Molecularly Imprinted Polymer (MIP) is generally described as a plastic cast or mold of the molecule of interest, where recognition is based on shape, much like a lock and key. MIPs are made by adding the molecule of interest to a solution of binding molecules that can be chemically incorporated into a polymer. These binders usually have an affinity for the target and form a complex.

This technology was applied for imprinting caffeine and the effectiveness of the polymer was examined through analytical technique for both caffeine and its structural analogue theophylline. Caffeine was selected due to its simple structure and because of the extensive literature existing on the detection of caffeine by a MIP. The binding constant and site density were obtained from the characterization of MIPs and the findings were supported using a gas adsorption technique to conduct a surface area analysis.

It was the goal of this research to better understand some of the fundamentals of the science in MIPs in order to be able to predict what type of formulation would provide for better recognition of the target molecules. The research we performed would be useful to provide the basic understanding of the binding to the MIPs and to investigate the impact of the constituents on a MIP’s performance with regard to the specificity of molecular recognition.
For our study, non-covalent imprinting, the most widely used form of imprinting in polymers was used. Keeping the template molecule constant, all other constituents were varied to determine optimum formulations. The conventional bulk polymerization method was employed for the research and nanoparticles formation was targeted since it has the advantage of higher surface area, which was expected to be a speculated advantage during the binding of a guest template. Starting with an organic porogen, we examined the aqueous medium to formulate the MIPs and the rebinding characteristics were tested in each variations employed. Caffeine is very water soluble and we were interested to evaluate if the aqueous medium would provide a stable interaction between the recognition monomer and the template caffeine. Aqueous medium was employed as a rebinding medium for the template due to the prospective application of the MIP in a field sensor.

The thesis is constructed as follows: The background chapter will include basic information about molecular imprinting technology, its characterization techniques and its application in sensors. The results obtained from different experimental techniques will be summarized with plausible explanations in the third chapter that is followed after an experimental section.

One of the initial driving forces of this research project was the sensor detection of a marine bio-toxin named saxitoxin. Saxitoxin causes paralytic shellfish poisoning and has a long history in New England region. The background information of the saxitoxin, its present detection methods and the closeness of our research work towards it, are sited in the Appendix A. Due to the structural similarity with saxitoxin; guanosine was tested as a
template and effectiveness of the MIPs were examined. Lack of success in the field did not allow us the manipulation with saxitoxin.

The focus of this thesis is based on the testing the specificity and selectivity of the MIPs prepared by conventional and nanodispersion technique. This project was a multi-investigator project in co-operation with Marine J Barasc and implementation of the latex technology and its possible application in sensor were examined by her. Hence that part is not included in this thesis. This project had a long term objective of facilitating coastal ocean characterization.
CHAPTER I

BACKGROUND AND LITERATURE REVIEW

1 MOLECULARLY IMPRINTED POLYMER IN RECOGNITION

1.1 Historical perspective

Molecularly imprinting has gained wide interest in recent decades. Among 1670 papers in the last 23 years, 250 original research papers published in 2005 (Figure 1).

Figure 1. Graphical Representations illustrating the number of original papers published in the field of molecular imprinting between 1970 and 2005\textsuperscript{1,2}

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In the 70's, molecular imprinting was first reported in synthetic polymers. Primarily it was targeted to obtain a specific binding cavity by arranging functional groups three-dimensionally with a pre-determined size and shape. Eventually it was demonstrated that those functional cavities could be customized to closely imitate the active sites of enzymes.²

1.2 Principles of Molecularly Imprinted Polymer (MIP)

In general, the monomer-template combination is copolymerized to form a rigid macroporous structure (Figure 2) in the presence of an excess of a cross-linking agent and a porogenic solvent. During the crosslinking polymerization the reduction in entropy of the imprinted polymer results in the phase separation with the pores occupied by the solvent. Subsequent removal of templates leaves three dimensional complementary functionalized voids in the polymer, where the size and shape imitates the template molecule.² These functional cavities are tailor made to recognize the template molecule specifically and selectively.

Figure 2. Scanning electron micrograph of a macroporous resin fracture section (magnification 5000X)³ Scale bar 100 μm
1.2.1 Types of molecular imprinting

Development of a MIP was initially being achieved by optimization of the structure through covalent interaction between the functional monomer and the template molecules. Since most of the biological interactions in nature are conveyed through non-covalent interactions, research work started progressing to imitate the natural phenomenon. Although covalent interaction is superior in terms of strictness of imprinting, in general non-covalent imprinting is easier to achieve and applicable to a wider variety of template. Depending on the need and situation researchers have chosen either of these methods. The advantages and disadvantages of two types of imprinting are summarized in Table 1.

Table 1. Issues in covalent and non-covalent imprinting

<table>
<thead>
<tr>
<th>Aspects</th>
<th>Covalent interaction</th>
<th>Non-covalent interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of monomer-template assembly</td>
<td>Necessary</td>
<td>Unnecessary</td>
</tr>
<tr>
<td>Polymerization condition</td>
<td>Wide range</td>
<td>Restricted</td>
</tr>
<tr>
<td>Template removal</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
<tr>
<td>Analyte binding and release</td>
<td>Slow</td>
<td>Fast</td>
</tr>
<tr>
<td>Structure of guest-binding site</td>
<td>clearer</td>
<td>Less clear</td>
</tr>
</tbody>
</table>
1.2.1.1 Covalent Imprinting

In covalent imprinting the functional monomer and the templates are bound to each other by covalent linkage, forming a covalent conjugate, which is polymerized in a condition where it remains intact. Template removal of the imprinted polymer is achieved by chemically cleaving the covalent linkage and guest binding is then operated by reforming it (Figure 3a). Formation of MIP by covalent bonding is relatively clear-cut as the monomer-template conjugates are stable and stoichiometric. Due to the stability of covalent linkage a wide variety of polymerization conditions can be used. Wide ranges of temperature, pH and polarity of the medium have no effect. Despite the advantages, the synthesis of a polymerizable monomer-template conjugate necessary for covalent imprinting is often troublesome and overall less economical. Availability of reversible covalent linkage is also limited, and due to the strong interaction, binding and release of the template is slow.5
1.2.1.2 Non-Covalent Imprinting

Hydrogen bonding, electrostatic interaction or co-ordination bond formation is employed in non-covalent interaction to connect the functional monomer and template. Thus the assembly of monomer and template is formed in-situ simply in the reaction mixture (Figure 3b). An appropriate solvent is used to remove the template and guest binding is achieved by the corresponding non-covalent interaction. This technique discards the synthesis of the conjugate between monomer and template and post-polymerization operations can be performed under milder conditions than required for covalent interactions. On the other hand the imprinting process is less clear cut as the assembly is not strictly stoichiometric. The assembly is labile; hence the polymerization
and the rebinding condition should be carefully chosen to maximize the interaction between the functional monomer and the template. In order to displace the equilibrium for adduct formation the functional monomer needs to be present in excess. This often produces the non-specific binding sites which in turn diminish the binding selectivity.

Many of the practically important molecules possess polar groups such as hydroxyl, carboxyl, amino and amide groups which are suitable for non-covalent interaction. Although any kind of non-covalent interaction is effective and employed for the imprinting, hydrogen bonding interaction is often chosen over electrostatic interaction.

Hydrogen bond strength depends almost linearly with the length (shorter length gives stronger hydrogen bonding) and the electrostatic interaction is inversely proportional to the square of the separation distance between the two objects. Thus hydrogen bonding is highly dependent on the distance and also with the direction between the monomer and templates, hence helpful for precise molecular recognition. On the other hand electrostatic interaction is less dependent on distance and direction; hence it is unfavorable for precise recognition.

In reality non-covalent interaction is the contribution of both hydrogen bonding and electrostatic interaction. The extent to which it is present depends upon the pKa values (for definition see Appendix B) of both functional monomer and template. If both the acid and base are very strong, the proton is completely transferred from the acid to the base and the adduct formation is governed by electrostatic interaction only. With a combination of acid and base of intermediate strength the proton exists between the acid and base and yields dominant hydrogen bonding. Efficient imprinting is
expected under this condition. For recognizing caffeine, methacrylic acid (MAA) works well, as along with favorable hydrogen bonds, there also exists a secondary acid/base interaction between caffeine and MAA. MAA has a pKa value of 4.66 and caffeine has a pKa value of around 8. If both acid and base are weak, the resulting weak interaction proves inappropriate for imprinting.5

Non-covalent interaction has the advantage of comparatively easy achievement of mimic structure. Moreover for achievement of fast and reversible binding non-covalent interaction is favored as the activation energy for non-covalent interaction is lower than that of covalent bonding. Considering that non-covalent bonding offers experimental easiness, versatility and allows performing the operation in mild conditions,9 we are interested in making MIP using that approach.

Scientists in different parts of the world gradually adapted and evolved the production methods of MIP and exploited its molecular recognition property in various forms and applications to fit their specific requirement.10 This technique provides a handy way of molecular recognition used in sensors6,11,12,13, membranes9,14, stationary phases in chromatography4,15,16,17,18,19, solid phase extraction sorbent20,21,22,23,24, enantiomeric separations25,26 and so forth.

1.3 Design and synthesis of MIP

1.3.1 Free radical polymerization

Free radical polymerization is the most common method adopted nowadays as numerous vinyl monomers can be polymerized effectively by this method. To initiate the polymerization, a radical is generated from the initiator thermally or by homolytic dissociation. Although the choice of appropriate thermal initiator is limited, peroxides are

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often used as radical sources. Apart from peroxides, the free radical initiator AIBN (azobisisobutyronitrile) is capable to initiate the growth of vinyl monomers. Additionally it is decomposed easily by thermolysis or photolysis, so perfect for rapid initiation. The formation of stable nitrogen compound is the driving force for the fast homolysis of AIBN (Figure 4).

\[
\begin{align*}
H_3C\text{-}C\overset{\text{CN}}{\text{C}}\overset{\text{CN}}{\text{N}}\overset{\text{C}}{\text{-}}\overset{\text{CH}_3}{\text{CH}_3} & \rightarrow H_3C\text{-}C\overset{\text{CN}}{\text{C}}\overset{\text{N}}{\text{N}}\overset{\text{C}}{\text{-}}\overset{\text{CH}_3}{\text{CH}_3} + \overset{\text{CH}_3}{\text{CH}_3} \\
\overset{\text{CH}_3}{\text{C}=\text{O}}\text{-}\overset{\text{OMe}}{\text{OMe}} & \overset{\text{AIBN}}{\text{UV or heat}} \rightarrow \overset{\text{CH}_3}{\text{C}}\text{-}\overset{\text{OMe}}{\text{OMe}} \overset{\text{CH}_3}{\text{C}}\overset{\text{n}}{\text{n}}
\end{align*}
\]

Figure 4. AIBN decomposition and radical polymerization of methylmethacrylate (MMA) to poly (MMA)

The decomposed radical reacts with the monomer and produces the propagating radical by opening the π-bond of the monomer. The reactive monomer radical reacts with more monomer molecules to form a continuously growing chain (Figure 5). Depending on the reaction condition, at some point the polymer growth is terminated by the destruction of the reactive center. Molecular oxygen needs to be removed from the polymerization mixture since it can retard this polymerization growth. In order to remove the oxygen, degassing with nitrogen or freeze-and-thaw cycles is often employed. Overall, this polymerization procedure is very simple and economical.
Figure 5. Formation of poly (MAA) by free radical polymerization (R' is radical initiator)

Some non-covalent adducts between functional monomer and template are unstable at high temperature\textsuperscript{33}, thus demands an initiator that can be activated in low temperature. Photo initiation by UV-light irradiation is often employed\textsuperscript{18,19,33} to overcome this problem. A redox initiation system at ambient temperature was reported\textsuperscript{28} to produce a MIP with higher affinity over photopolymerization.

Also non-selective binding sites are less prone to be created at lower temperature; thus imprinting efficiency becomes high.\textsuperscript{5} The study performed in Cranfield University\textsuperscript{29} revealed that the MIP specificity strongly depends on the polymerization temperature. Both the quantity and quality of the monomer-template interaction is
affected by the temperature used to polymerize a MIP. O'Shannessy et al.\textsuperscript{30} compared
sets of MIPs prepared at temperatures ranging from 0 to 60°C and reported that the
polymers prepared at low temperature had better specificity compared to those prepared
at higher temperature. Piletsky et al.\textsuperscript{31} performed their analysis by preparing sets of MIPs
at a range of temperature from -30°C to 85°C. Their result revealed that although binding
sites having high affinities to the template do not vary in concentration for MIPs prepared
at different temperature; it does significantly for non-specific binding sites. The binding
affinity towards both types of sites was found higher for MIPs prepared at lower
temperature.

1.3.2 Variables those influence the performance of MIP

Based on this initial idea several researchers started taking the challenge of
designing and synthesizing MIPs. Other experimental variables involved are template
type, nature of functional monomer and crosslinking agent, porogen and depending
upon that, sometimes initiator.

1.3.2.1 Template

Template is of the foremost importance in preparing MIPs as it
directs the organization of the functional groups from the recognition monomer. But the
template does not contribute in the polymerization. In general a template is suitable to use
in a free radically polymerized MIP if it is chemically inert and stable during the
polymerization. It also should not inhibit the free radical polymerization.\textsuperscript{10}

Varieties of template molecules have been explored throughout the years. Caffeine was found to be an interesting template\textsuperscript{12,13,19,20,21,22,23,24,32} due to its

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simple highly polar rigid planar structure (Figure 6). During non-covalent imprinting it has the convenient capability of having sufficient interaction with the vinyl recognition monomer. Molecular modeling (with partial charges evaluation) carried out by Farrington et al. reveals that there are several sites on the molecule which are capable of undergoing electrostatic or hydrogen bonding interactions. The two nitrogen atoms of the five-membered ring and both of the oxygen atoms are the hydrogen bond acceptors for the proton present in a functional monomer (Figure 7).

![Caffeine 2D structure](image)

**Figure 6. Caffeine 2D structure**

### 1.3.2.2 Functional monomers

Functional recognition monomers are responsible in the formation of imprinted binding sites. Suitable functional groups from vinyl monomers serve the purpose efficiently for non-covalent imprinting. To facilitate the imprinting, formation of an effective complex is necessary. In order to do that it is important to match the functionality of the monomer to the functionality of the template. The matching should be complementary, that means if the template molecule is a hydrogen acceptor, the monomer chosen should be a hydrogen donor.

Mosbach et al. introduced and further developed the non-covalent approach of molecular imprinting using the functional monomer MAA. The carboxylic acid functionality of MAA helps to form the ionic interaction with the amino group of the print molecule and the hydroxyl functionality forms hydrogen bonds with the polar
functions of the template molecule (Figure 7). Dipole-dipole and hydrophobic interaction also participates in non-covalent bonding between the recognition monomer and the template molecule. Even though only limited numbers of functional monomers have been used so far for non-covalent imprinting, some of the existing monomers like MAA have been successfully used to achieve highly stereoselective and enantioselective separations.

Figure 7. Complex formed between the caffeine molecule and two MAA molecules. (a) N atoms in caffeine are forming hydrogen bonds with the –OH functional groups of the recognition monomer (MAA). (b) Alternate possible configuration: Carbonyl group of caffeine is forming hydrogen bond with the –OH functional group of MAA along with the common configuration. Molecular modeling was done by Farrington et al. Nitrogen = blue, carbon = turquoise, oxygen = red, hydrogen = white.
Researchers explored the fact that MAA has all the possible non-covalent interactions for its template caffeine. Screening of a library of monomers in the molecular modeling software also proves the fact.\textsuperscript{21} As a result in case of template caffeine, MAA is often used as a functional monomer\textsuperscript{12,13,19,20,21,22,23,49,83}

A MIP formed by a non-covalent approach needs excess moles of functional monomer relative to the template in order to favor the formation of monomer-template assembly.\textsuperscript{10} The mole ratio varies depending upon the constituents. The traditional approach to achieve the best combination is by trial and error. For the caffeine-MAA combination, Farrington et al.\textsuperscript{21} recently established an optimal ratio of 1:2 (template: monomer) by using a computational approach (Figure 7). In their molecular modeling software although binding energy increased by the addition of another molecule of MAA in the 1:1 complex of MAA-caffeine; the binding energy was stable after further additions. This shows the possibility of finding an optimum ratio of the monomer to template in order to get it fully complexed. UV–vis spectroscopic studies also support this 2:1 molar ratio of MAA and caffeine when acetonitrile is used as a solvent for polymerization.\textsuperscript{21}

1.3.2.2.1 Selection of a recognition monomer

The success of imprinting theoretically depends on the formation of a monomer-template complex and preservation of that structure during the polymerization. Thus complex formation mostly depends on the selection of the recognition monomer for a particular template. For caffeine imprinting we did not vary the recognition monomer type as it was pre-determined. But for an unexplored template, the suitable selection of recognition monomer is a challenge.

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Simulations were often employed\textsuperscript{21,34,35,36} to better understand the underlying network structure that gives rise to the formation of an effective monomer-template complex. A computational approach for screening appropriate monomer can be achieved by using the binding energy (\(\Delta E\)) between the functional monomer and the template. The binding energy indicates the measurement of the interaction between those. For a particular template, a monomer having higher associated \(\Delta E\) is found suitable for preparing the MIP. By simulation, several possible conformations of the template, monomer and the monomer-template complex, can be optimized and the interaction energies can be calculated from the minimal conformations and their related energies. The interaction energy (\(\Delta E\)) can be calculated in the following manner:\textsuperscript{35,36}

**Equation 1. Calculation of interaction energy in simulation approach**

\[
\Delta E = E(\text{template-monomer complex}) - \Sigma E(\text{template}) - \Sigma E(\text{monomer(s)})
\]

Subsequent study and comparison of the strength of monomer-template interaction could help researchers to select the suitable recognition unit which possesses a higher complexation affinity towards a specific template.

Dong et al.\textsuperscript{34} explored this approach and for imprinting theophylline they reported trifluoromethacrylic acid (TFMAA) as the best recognition unit over acrylamide (AA) and MAA. Their computational approach was supported by their experimental result obtained from NMR, which revealed that TFMAA can form the strongest H-bonding interaction with theophylline and with AA the interaction is the weakest. Farrington et al.\textsuperscript{21} found MAA as the best recognition unit for caffeine after
screening the library for functional monomers. Among 2-vinylpyridine, 4-vinylpyridine and MAA, the highest binding energy for caffeine was found for MAA. The simulation was supported by their experimental results. Greater rebinding and selectivity was found for the caffeine-MAA MIP over other monomers evaluated.

Thus a computational approach facilitates the selection of the best monomer suited for MIP formation that produces high affinity to the template. This simulation also allows imitating the specific polymerization or binding conditions by changing the dielectric constant and atomic charges of the monomer/template models. A relative polymer specificity and affinity can also be extracted from the simulation technique (Figure 8).37

UV-vis spectroscopic studies of the pre-polymerization mixture are reported to be useful to investigate the stoichiometry of the complex formation.21,70 An optimum ratio between the recognition monomer and template can be achieved by employing a varying molar ratio of both the components.

This approach could be useful to better understand the underlying characteristics in formation of a monomer-template complex, which allows the formation of an efficient MIP.
Figure 8. Pavel et al. sited a typical simulated equilibrated conformation of the support with one molecule of theophylline-8-butanoic acid (ligand) chemically attached and three polymeric chains of polymethacrylic acid. The conformation suggests that the polymer chains do not prefer to interact with the ligand and hence a poor rebinding.
1.3.2.3 Crosslinking Agent

The primary responsibility of the crosslinking agent is to stabilize the binding imprinted site by fixing the structural integrity of the monomer-template assembled structure through copolymerization (Figure 9). To achieve a permanent porous structure in the polymer matrix, high cross-linking density is preferred. The secondary purpose is to control the morphology of the resulting polymer and to impart the mechanical stability.

Figure 9. Schematic diagram of the molecular imprinting for caffeine using MAA as a recognition monomer and EGDMA as a cross-linker.
To achieve an effective imprinting, homogeneous crosslinking is desired throughout the polymer network so that the functional residues of the recognition monomer get uniformly distributed. The choice of a crosslinking agent whose reactivity is similar to the functional monomer serves this purpose. If the reactivity deviates one or both of the monomer polymerize to form homopolymer of their own.

Since it has a similar chemical structure, ethylene glycol dimethacrylate (EGDMA) (Figure 10) is often selected for a system having MAA as the functional monomer. Both copolymerize almost randomly as desired and yield the uniform distribution of the carboxylic acid groups in the polymer network (Figure 11). MAA-EGDMA produces a MIP with greater selectivity over other common crosslinking agent like divinyl benzene or butanediol dimethacrylate. In most studies EGDMA was the crosslinker used which also helps to obtain a MIP with good thermal and mechanical properties. As EGDMA is compatible in nearly all organic solvents it is also easier to employ in the formulation.

![Chemical structure of EGDMA](image)

**Figure 10. Ethylene glycol dimethacrylate**
Figure 11. Schematic diagram of MAA-EGDMA crosslinked structure

Appropriate level of crosslinking is necessary for efficient imprinting. At low levels of cross-linking, functional groups are not fixed sufficiently, which yields poor efficiency. At other extreme i.e. at very high level, diffusion of substrate during rebinding becomes difficult. The polymer matrix should be rigid enough to sufficiently preserve the imprinted molecular memory and also flexible enough to facilitate the release and rebinding of the template into the molecular cavity. In general, the bifunctional crosslinker EGDMA is commonly used at a molar ratio of 1:4 to the monomer. For trifunctional crosslinkers the amount could be reduced to achieve the same crosslinking density.

The polymer matrix not only has the role to contain the binding sites in stable arrangement, but also needs to provide porosity to allow for easy access of
guest template to all sites. The formation of porosity is achieved by selecting a suitable solvent.

1.3.2.4 Solvents (Porogen)

Solvent should be inert. The primary purpose of the solvent is to dissolve all the components, i.e. to bring template, recognition monomer, crosslinking agent and initiator into one single phase before polymerization. Through this complete dissolution process, a solvent helps to form uniform distribution of crosslinking in the polymer matrix. The solvent should not swell the crosslinked polymer. Therefore, the choice of solvent can be difficult, as it should be able to dissolve the monomer and unable to dissolve the linear polymer.

A good solvent ensures the solvent-swollen crosslinked network that helps the extension and flexibility of the structure formation. The uniform distribution of crosslinking facilitates the diffusion of template into the polymer structure during rebinding and a deeper access into the structure. A poor solvent for the polymerization mixture often yields heterogeneous distribution of crosslinking.

At some point of the polymerization the growing cross-linked form of the polymer becomes insoluble in the solvent and precipitates out. The solvent used during the polymerization remains inside the cross-linked product until it is later removed by post treatment of template removal. The space originally occupied by the solvents is left as pores inside the polymer network (Figure 12). Thus polymers prepared without solvents form a firm and dense structure, which does not allow easy rebinding of guest template.
In the macroporous polymer the purpose of the solvent is to create pores (Figure 12) and for that reason in MIP technology a solvent is referred to as a 'porogen'. When a porogen is present in the comonomer mixture this causes the phase separation of the polymer matrix and a macroporous resin is thus formed. The phase separation depends upon the nature of the porogen and its level of usage.

At a full conversion each polymer bead consists of a polymer phase which is crosslinked and a discrete porogen phase. The porogen phase acts as a template to form the permanent porous structure of the resin (Figure 12). The permanent pores which are formed during the polymerization upon drying collapse to different extent depending on the quality of the solvent. The rigid opaque and permanently porous beads are thus formed after the removal of porogen and drying of the polymer.³

The nature and the level of porogen control the morphology (Figure 13) and the pore volume. A thermodynamically good porogen provides well developed pore structure and high specific surface area; the development is just the opposite in case of a poor porogen.¹⁰ If the pores are sufficiently large, small molecules can diffuse freely into the pores. Higher surface area and pore volume indicates well formed and uniform pore structure development. In an otherwise identical polymer, pore
volume tends to increase with increasing volume of porogen. With increasing pore
diameter it is more likely to get a higher rebinding rate due to the reduced steric
hindrance of analyte inside the pore.\textsuperscript{21}

These fine details can be adjusted by the nature and proportion of
the porogen. During polymerization the point of time at which the phase separation
occurs, is the crucial point to determine the morphological characteristics.\textsuperscript{3}

Figure 13. Resin pseudo-phase diagram can anticipate the morphology for a given
type and level of porogen and crosslinker: I. Gel-type resin, II. Macroporous resin
(figure 11), III. Microgel powder\textsuperscript{3}

A solvent should create a favorable environment for non-covalent
imprinting. It needs to be chosen in such a way so that it could maximize the likelihood
of formation of monomer-template assembly. By stabilizing the assembly it is possible to
increase the number of imprinted sites. This minimizes the number of non-specific
binding sites as there is a reduction in the concentration of free non-associated functional
monomer.\textsuperscript{5}
As hydrogen bonding is the most common interaction for non-covalent imprinting, apolar and non-protic solvent is suitable. In general, the more polar the porogen is, the weaker the imprinting efficiency. The highly polar solvent weakens the interaction forces formed between the template and the functional monomer.

The use of water as a solvent is still under development since being a polar protic medium it considerably weakens the electrostatic and hydrogen bonding interaction. This decreases the specificity and selectivity of MIP for the guest analyte. Hydrogen bonds, which are preferentially used for pre-organization of template-monomer assembly during non-covalent imprinting, are easily affected in bulk water. So when it is present in excess, it prevents the formation of hydrogen bonded monomer-template assembly. Since water is both hydrogen donor and acceptor, it forms hydrogen bond with both hosts and guests.\(^5\) To promote a sufficient template-functional monomer interaction in water, hydrophobic\(^{24,32}\) and ionic bonding\(^{45}\) seems promising.

1.3.2.5 Miscellaneous effect of constituent concentration

As discussed in the previous sections, ratio of the constituent concentration needs to be optimized in order to produce a highly sensitive MIP. Keeping other factors constant, while increasing the recognition element and template concentration, proves beneficial.\(^9\) However, when precipitation polymerization is used to prepare microspheres of MIP, excess of template or recognition monomer often forms aggregation of particles. This can be explained by the fact that the high concentration of monomer-template mixture results in a high concentration of hydrogen bonded complex and eventually forms the coagulum. On the other hand, a low concentration yields amorphous particles.\(^{46}\) If only template concentration is increased, it produces a MIP with
higher capacity of rebinding. This is because the higher concentration of template generates higher concentration of binding sites (Figure 14).

![Diagram](https://via.placeholder.com/150)

**Figure 14. Effect of increasing template concentration**

As evident, monomer-crosslinker ratio is always important in preparation of efficient MIP. Selectivity of a MIP towards its template can be controlled by the ratio. A monomer crosslinker molar ratio of around 1:4 produces a good enantioselective molecular recognition.

1.3.2.6 Polymerization type and particle size effect

Conventionally MIP is prepared by the bulk (solution precipitation) polymerization method. Simply all the constituents are introduced in the reaction vessel and polymerization by radical mechanism is initiated by thermal or UV radiation in an inert atmosphere. All of the polymers discussed when prepared conventionally, are produced by the bulk.
polymerization method in the presence of a solvent. Thus the process is technically a solution precipitation polymerization, but for the ease of discussion the polymers prepared conventionally will be referred to as a bulk polymer.

1.3.2.6.1 Bulk and/or solution polymerization

The bulk polymerization method is used conventionally as it is simple, and only a few ingredients are required. This method is quick and conventionally polymers can be used after preparation. Autoacceleration could be a problem when prepared without a solvent. Similar to normal polymerization, a solvent in MIP polymerization also helps to disperse the heat generates during the process. Otherwise the locally elevated temperature of reaction could give rise to undesired side reactions. The absorption of the exotherm of polymerization by solvent can be also helpful to avoid autoacceleration. 47

Figure 15: Schematic diagram of typical Bulk method of Polymerization 44

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Bulk polymerization results a monolithic hard polymer which can be ground mechanically to obtain small particles to yield a large surface area. Particles formed this way have extremely broad size and shape distribution (Figure 16) which is often sieved to get a narrower range. Since print molecules are embedded in the polymer, unless the particles are small, it is difficult to extract the template and allow for rebinding.

Figure 16. SEM of conventional MIP particles obtained after grinding. Magnification 525X. Scale bar 100 μm.

To overcome the difficulty of controlling size through bulk polymerization colloidal preparation of MIP was eventually developed. Colloidal MIPs can be produced in a defined way with controlled size and shape distribution. The particles are produced at a submicron size level. Due to its smaller size it has the advantage of easy accessibility of sites for extraction and rebinding when compared to a bulk MIP. The complete removal of remnants of template can be confirmed experimentally by using a radiotracer technique. Defined structure of microspheres and

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nanospheres can be obtained by emulsion\textsuperscript{24,32,41} and mini-emulsion\textsuperscript{40} polymerization in aqueous phase and by precipitation polymerization\textsuperscript{19,39,46} in organic phase.

### 1.3.2.6.2 Emulsion Polymerization

The mechanism of emulsion polymerization (Figure 17) is not as simple as the bulk polymerization method. The mechanism is more complicated and needs a water insoluble monomer and a water soluble initiator. A surfactant is used to stabilize the particles formed in aqueous medium. During polymerization, the monomer droplets are surrounded by surfactant molecules. The water soluble initiator forms free radicals in the water and finds some monomers with which to react. In order for polymerization to occur, a few radicals need to diffuse into the monomer swollen micelles where there is a high concentration of monomer to start the chain reaction (Figure 18).\textsuperscript{47}

![Figure 17. Mechanism of emulsion polymerization\textsuperscript{47}](image)
In case of emulsion polymerization a hydrophobic interaction is desired and for caffeine imprinting oleyl phenyl hydrogen phosphate is used\textsuperscript{24,32} in coordination with EGDMA as a crosslinker. The nitrogen donor atom of caffeine can bond with the phosphate group. The conventional functional monomer MAA is soluble in water thus it can hinder the random copolymerization with the water insoluble crosslinker EGDMA.

The miniemulsion polymerization is often employed because it has some unique properties not characteristic of normal emulsion procedure. Miniemulsion gives uniform copolymer compositions and has greater shear stability. In miniemulsion, a hydrophobic molecule is used to suppress Ostwald-ripening. After shearing large surface area is generated due to the high surfactant concentration; minidroplets are formed and act as nucleation sites.\textsuperscript{50}

![Figure 18. Schematic diagram of emulsion polymerization](image)
Another way to overcome the drawback of tight entrapment of the template in bulk MIP is achieved by employing the core shell morphology\textsuperscript{56,60,65} (Figure 19) It is often employed because it is very surface active. Core-shell particles are imprinted at the surface and prepared by using a two step emulsion polymerization process. This process provides for the fast and easy removal of the template and in turn an easy rebinding. But since the core becomes inactive throughout the process and only surface imprinting is initiated, a lowering of site density could result in comparison to the polymer prepared conventionally (as depicted in Figure 14).

![Figure 19. Principle of surface imprinted nanoparticles](image)

The basic principle of the emulsion polymerization is oil-water incompatibility. But as caffeine is water soluble, imprinting may not be successful since the polymerization occurs in the oil phase. The small difference in surface area of a core shell MIP compared to a NIP measured by Tovar et al.\textsuperscript{40} might support this fact. Thus polymerization in entirely organic media is desirable and precipitation polymerization in this regard is suitable.
1.3.2.6.3 Precipitation Polymerization

This is a polymerization reaction in which the polymer formed is insoluble in its own monomer or in the monomer-solvent combination and thus precipitates out after it is formed. This method does not require the use of special dispersing phase or surfactants as in emulsion or miniemulsion process. This process is simple, easy to handle and produces high yield. Organic media is used in case of precipitation polymerization (Figure 20) where controlled size distribution can be obtained (Figure 21) similar to the emulsion polymerization. In this respect an organic solvent like dichloromethane or acetonitrile found to be capable of forming satisfactory microspheres with a good yield. Similar to the bulk method the polymerization through radical mechanism is initiated by thermal or UV radiation, but from a dilute monomer solution. Eventually the micro or nanospheres obtained are collected by centrifugation. The total monomer volume in the polymerization solution is generally restricted to 2% of the porogen volume. Apart from emulsion polymerization, crosslinked monodispersed microspheres can be formed by this technique without the need of any stabilizer.

![Figure 20. Schematic diagram of precipitation polymerization](image)

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The rebinding of the template depends upon aforementioned varieties of polymerization techniques. Micro-/nanospheres are mostly formed in a diameter range around or below 1 micron and monoliths can be produced in diameter over 10 microns. A broad range of micron sized particles often respond slowly to achieve the binding equilibrium. This occurs due to two simultaneously occurring process; the diffusion of analyte from solution to pores and the actual binding kinetics between the analyte and the cavities in the MIP. A reduction in response time can be achieved by lowering the particle size diameter and narrowing the size distribution.

Figure 21. SEM photograph of imprinted spheres prepared by precipitation polymerization (average diameter 300nm). Magnification 15000X48 Scale bar 100nm

Although both monolith MIP and microspheres show specificity towards their analyte, the binding site density is found higher in the later case. On the contrary, the excess amount of porogen present during the polymerization can cause an adverse effect and often the imprinting efficiency and analyte binding of colloidal MIPs are found lower than in the conventional monolith. This is due to the excess porogen

34
interfering with the molecular interaction formed between the functional monomer and template molecule. Marginal difference in adsorption between MIP microspheres and its corresponding NIP (Figure 22) as argued by Row et al.49, may suggest similar phenomenon.

![Graph showing adsorption of caffeine on imprinted and blank microspheres fitted with Langmuir isotherm model.](image)

**Figure 22. Adsorption of caffeine on imprinted and blank microspheres fitted with Langmuir isotherm model.**

1.3.3 Bulk monolith versus nanoparticles MIP

By analyzing the SEM study, Mosbach et al.19 reported that during the bulk polymerization technique the initially formed globular microgels densely fuse together to form the monolith (Figure 23). The polymer block thus obtained are composed of densely fused interlinked microgels that offers a high mechanical strength and able to withstand harsh conditions without losing the binding specificity. Starting with a dilute monomer solution, discrete microgels can be produced that gives rise to uniformly size
narrow-dispersed micro-/nanospheres (Figure 21). Through their investigation on MIP microspheres Mosbach argued that the cavities created by the template are still kept intact in the submicron ranged discrete polymer particles. SEM micrographs as sited by Wang et al.\textsuperscript{22} clearly show the imprinting on nanoparticles (Figure 24). Jiang et al.\textsuperscript{46} reported a better selectivity of imprinted nanoparticles over the conventional one.

Figure 23. SEM of ground monolith MIP, with 525X magnification and 30,000X magnification respectively.
1.4 Characterization of MIP

The most important aspect of characterization regarding molecular recognition of a MIP is commonly determined by a batch rebinding experiment for its template. A rebinding study can be performed using several procedures: particle suspension in a medium which is subjected to adsorb analyte$^{32,39,40,41,46}$, with the help of adsorption by
packing the particles in a HPLC column\textsuperscript{12,18,19} or in a solid-phase extraction (SPE) cartridge\textsuperscript{20,21,22,24}.

Morphological characterization is possible by implementing nitrogen sorption and mercury intrusion porosimetry. The porosimetry technique involves the measurement of gas or mercury adsorption into the pores. With the help of theoretical and mathematical models specific surface area, specific pore volume, average pore diameter and pore size distribution can be calculated\textsuperscript{52}. As porous surfaces could be seen by SEM (Figure 2, Figure 16, Figure 21), this is also often used to obtain the morphology by imaging the macropores\textsuperscript{10}.

We restricted our characterization mainly to a rebinding study and supported our result through BET gas adsorption measurement.

\textbf{1.4.1 Specificity and selectivity}

Specificity is the measure of how accurately a substance can be identified. This is the quality of being specific rather than general and pertaining to a specific case. Specificity can be judged by the ability to identify the substance of interest and selectivity is examined by other substances that could exhibit a degree of preference with the substance of interest. In case of MIP, the selectivity refers to the extent to which it can recognize its particular analyte in a mixture without having the interference with other analyte(s) present there\textsuperscript{53}.

Thus specificity of the MIP is obtained from its template adsorption data and selectivity can be judged by performing a competitive test of adsorption for the structural analogue of the template\textsuperscript{4,9,13,23,24,32,39,46}. For caffeine imprinted polymer, structurally similar theophylline is often employed\textsuperscript{12,13,22,32,54} to examine its selectivity.
Caffeine and theophylline differ only by a methyl group, thus the difference in the rate of adsorption between the two indicates a high selectivity of the MIP. Regardless of the polymerization technique, caffeine imprinted MIP is found to have a greater and more rapid binding capability towards caffeine over theophylline. Preparation of a non-imprinted polymer is a common practice for a comparison with MIP. For an efficient MIP the specificity or selectivity is higher than its non-imprinted counterpart.

![Graph showing caffeine and theophylline uptake over time](image)

**Figure 25. Time dependent competitive adsorption for caffeine and theophylline to caffeine imprinted MIP**

### 1.4.2 Rebinding study

A batch rebinding study is the most common method employed to examine the specificity and selectivity of MIP. This can be evaluated quantitatively by employing an analytical technique to measure the template concentration. For solid MIP particles, suspension of the same is generally exposed to a fixed or an incremental
amount of template concentration. After binding equilibration, the supernatant is analyzed by common spectroscopic techniques. The basic analysis of a rebinding study is similar when particles are packed in a HPLC column or in a SPE cartridge. In case of template caffeine, it can be detected by HPLC at the UV wavelength of 270 nm using a 5µm particle size C18 column$^{20,22}$. (Figure 26).

![Figure 26. HPLC separation of caffeine from coffee. Conditions: column, 15cmX 2mm i.d.; stationary phase, Octadecyl silica; mobile phase, 0.3 ml/min water-acetonitrile (8: 2); UV detector 272 nm.$^{37}$]
1.4.2.1 HPLC—a powerful separation method

A HPLC chromatogram directly provides (Figure 27) both quantitative and qualitative information of analytes. Both normal and reversed stationary phases are used for analysis which operates on the basis of hydrophilicity. A normal phase consists of a polar stationary bonded phase and needs a non-polar solvent as a mobile phase, while a reversed phase contains non-polar stationary phase and needs a polar solvent. We are particularly interested in the reversed stationary phase for the analytes with which we are concerned. The reversed stationary phase consists of n-alkyl chains bonded with silica based packing. Common reversed phase packings are listed in Table 2. The higher the hydrophobicity of the matrix, the higher the tendency the column has to retain hydrophobic moieties. In this configuration hydrophobic non-polar compounds elute later than hydrophilic polar compounds.
Figure 27. Schematic diagram of an HPLC unit. 1=solvent reservoir; 2=transfer line with frit; 3=pump (with manometer); 4=sample injection; 5=column (with thermostat); 6=detector; 7=waste; 8=data acquisition.

Table 2. Common ligands attached covalently to the silica support particle (Si-O-) for reversed phase packing

<table>
<thead>
<tr>
<th>Alkyl chain</th>
<th>Ligand name</th>
<th>Ligand structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td>Ethyl Silyl</td>
<td>-Si-CH$_2$-CH$_3$</td>
</tr>
<tr>
<td>C2</td>
<td>Dimethyl</td>
<td>-Si-(CH$_3$)$_2$</td>
</tr>
<tr>
<td>C8</td>
<td>Octyl Silyl</td>
<td>-Si-(CH$_2$)$_7$-CH$_3$</td>
</tr>
<tr>
<td>C18</td>
<td>Octadecyl Silyl</td>
<td>-Si-(CH$<em>2$)$</em>{17}$-CH$_3$</td>
</tr>
</tbody>
</table>
Common reversed phase polar solvents are methanol, acetonitrile or tetrahydrofuran. More polar solvents such as water elute the analyte more slowly than a less polar solvent acetonitrile. Isocratic elution operates in a constant mobile phase composition to elute the analyte. In this condition analytes begin the migration through the column at the onset but since analytes migrate at a different rate it results faster or slower elution time. Gradient elution works on the basis of increasing the strength of organic solvent to elute different compounds. Starting from a weaker mobile phase the strength is raised gradually by increasing the organic solvent fraction which in turn results in the elution of the retained compounds.

The UV detector is the most popular and useful detector available at this time. Multi-wavelength technology is used and the detector can function on the capacity of many compounds to absorb light in the wavelength range of 180-350 nm. Many substances those having a π-bond or unshared electrons absorb light in this wavelength range.

1.4.2.2 Binding isotherm

Adsorptive interaction requires information on equilibrium properties which is commonly referred to as the adsorption isotherm. Adsorption isotherm is an isothermal equilibrium relationship between the concentrations of a compound adsorbed in the solid phase to the concentration of the same compound in the bulk solution phase (or the partial pressure in the gas phase, see section 1.4.3.1), which is in contact with the solid phase. This is a boundary on a phase diagram that expresses the partitioning of a compound between the solid and liquid phase. The isotherm, derived from the thermodynamic theories, give rise to useful thermodynamic parameters such as
equilibrium constant and a maximum adsorption capacity. For the interaction of small molecules with the adsorbent surfaces adsorption isotherm provides information regarding binding energies and site distribution of the surface. However, the equation applied must satisfy the necessary thermodynamic boundary condition and must reduce to linear isotherm of Henry’s law (see appendix B) at a very low surface coverage. Assuming a uniform surface with a single layer of adsorbed material at a constant temperature the following equilibrium exists (Equation 2).

Equation 2. Equilibrium for homogeneous single site model

Analyte + binding sites $\leftrightarrow$ binding sites saturated with analyte

In case of MIP in liquid phase the adsorbate molecule interacts with the binding sites of the solid adsorbent. The binding isotherm is then obtained by simply plotting the equilibrium concentration of free analyte ($C$) versus the bound analyte ($q$). To explain the isotherm, several mathematical models have been used to fit with the experimentally obtained adsorption data. Langmuir type and Freundlich type of adsorption isotherm is often assumed (Figure 28) in this regard.
1.4.2.2.1 Mathematical models

For a single solute system the popular isotherm is proposed by Freundlich; expressed as in Equation 3. \( q(C) \) is bound template and \( C \) is free template concentration at equilibrium and 'a' and '1/n' are constants. For a highly heterogeneous surface this empirical equation is suitable and often works over a restricted range of concentration. Freundlich expression does not reduce to Henry's law at infinite dilute concentration.\(^6^1\)

Equation 3. Freundlich model

\[
q(C) = aC^{1/n}
\]

or \( \ln q(C) = \ln a + (1/n) \ln C \)
The simplest model is the Langmuir type adsorption, in which the isotherm is suited to describe the adsorption behavior of homogeneous surface. In this model the system is considered to be ideal and the adsorbent is assumed to have one type of site with uniform energy distribution and no adsorbate-adsorbate interaction. Monolayer coverage is assumed and the mathematical expression for Langmuir is written as in Equation 4 (the derivation from the equilibrium reaction is sited in Appendix B), where $a_l$ and $b_l$ are Langmuir parameters. The isotherm depends on saturation capacity $q_s$ and the binding constant $b_l$. Site density can be obtained from the saturation capacity and association binding constant (see Appendix B) $K_a$ is calculated by Equation 5; where $M_w$ is the molecular weight of the adsorbate. The monolayer saturation capacity describes a monomolecular layer of adsorbate on the surface of unit weight solid. The adsorbed molecules in the saturated monolayer are reasonably under the closest packing conditions and at a maximum orientation in the interface.$^{61}$

**Equation 4. Langmuir model**

$$q(C) = \frac{a_l C}{1 + b_l C} = q_s \frac{b_l C}{1 + b_l C}$$

where, $q_s = \frac{a_l}{b_l}$ (g/l)

**Equation 5. Association binding constant from Langmuir model**

$$K_a = b_l M_w \text{ (/mol)}$$

In reality most of the adsorbents are heterogeneous as they contain sites in a wide energy range. Sites could be polar or non-polar. Due to the exposure to the environment, adsorbed impurities such as moisture and organic
contaminants effect the outcome. Therefore in most systems, assumptions that follow the Langmuir model are far from reality. Nevertheless, due to its versatility, experimental data often fits well to the Langmuir equation and parameters governing the adsorption can be resolved. Langmuir equation also obeys the boundary condition of Henry’s law.

The presence of excess recognition monomer in a MIP produced by non-covalent interaction presumably creates heterogeneous distribution of sites. Some sites created are specific to template and some sites are not specific. The non-specific sites are the surface sites which have a non specific affinity. The number of non-specific sites grows directly with the surface area. Any experimental condition which generates the surface area generates the non specific sites. The former type of sites is fewer, but their binding energy is large and saturation capacity is low. On the other hand non-specific sites account for a larger fraction of total sites and have a larger saturation capacity but lower binding energy.

**Equation 6. Langmuir – Freundlich model**

\[
q(C) = q_s b_1 C^m / (1 + b_1 C^n)
\]

To explain the heterogeneity of sites in MIP a combination of two models could work well (Figure 29). A three parameter model was developed by Radke and Prausnitz which combine both the Langmuir and Freundlich (LF) (Equation 6) isotherm. \( m \) is the heterogeneous index and can be determined from the statistical fit of the experimental data. LF isotherm can extract the direct measurement of binding property of a MIP and could compare the behavior of different compounds towards the same MIP. At high concentration the LF model expresses the Freundlich
equation. The LF isotherm reduces to Langmuir isotherm at the limiting case of $m=1$, i.e. when the material is homogeneous. When $m$ is between 0 and 1 the material is heterogeneous. With the help of LF isotherm it is possible to characterize MIPs having both homogeneous and heterogeneous distributions of sites and at both high and low concentration of the analytes during rebinding.\cite{61}

![Log plot of binding isotherm of propazine to propazine imprinted polymer. The experimental data is fitted to Langmuir (dashed line), Freundlich (dotted line), and LF (solid line) isotherms.]

In the case of MIPs, the existences of two types of sites (specific and non-specific) indicate the presence of two equilibriums (Equation 7). What attracted our attention was the possibility of predicting the two site model from a single component adsorption.

**Equation 7. Equilibrium for two site model**

\[
\text{Analyte + specific binding sites} \rightleftharpoons \text{specific binding sites saturated with analyte}
\]

\[
\text{Analyte + non-specific binding sites} \rightleftharpoons \text{non-specific binding sites saturated with analyte}
\]

**Equation 8. Bi-Langmuir model**

\[
q(C) = q_{si} \frac{b_1 C}{(1+b_1 C)} + q_{ns} \frac{b_2 C}{(1+b_2 C)}
\]
Since MIPs contain heterogeneous distribution of sites with different affinities towards the template, multiple binding constants can be obtained.\textsuperscript{33} Due to the presence of mainly two different types of binding sites in MIP, application of the model considering two types of sites are common.\textsuperscript{62,65,67} The Langmuir binary site model or bi-Langmuir model (Equation 8) is the sum of two Langmuir isotherm. This model therefore assumes two different site classes in a heterogeneous adsorbent surface. In contrast, Freundlich isotherm model (Equation 3) assumes a continuous distribution of sites with different binding energies, but without any saturation capacity.\textsuperscript{2}

![Scatchard binding curve of caffeine to a caffeine imprinted MIP](image)

**Figure 30. Scatchard binding curve of caffeine to a caffeine imprinted MIP\textsuperscript{24}**

The bi-Langmuir model can be taken into account by the limiting slope analysis from the Scatchard plot (Figure 62). Scatchard plot is obtained from the bound analyte concentration plotted against bound and free ratio of the same. The Scatchard expression\textsuperscript{2} (Equation 9) is used to obtain an average association or dissociation constant\textsuperscript{18,33} along with the number of sites. The experimental plot is expected to exhibit two fairly distinct types of regions that can be fitted with linear
regression. The binding constant is obtained from the linear fit of the slope and number of sites from the intercept. For an efficient MIP this plot reveals mainly two linear regions representing two classes of binding sites.

**Equation 9. Expression for Scatchard format**

\[
\frac{q(C)}{C} = -bq + bq_s
\]

1.4.2.3 **Rebinding medium**

Organic solvent is often used as an incubation medium. In general a MIP shows better efficiency in the medium in which it is prepared. This is due to the fact that it achieves the same condition of self assembly when they are exposed to similar polymerization conditions. Acetonitrile was found to be a good porogen for self assembly of caffeine with the recognition monomer and it can be used as a rebinding media. On the other hand the porogen acetonitrile was reported inferior for rebinding due to the total solubility of caffeine in acetonitrile.

1.4.2.3.1 **Water as a rebinding medium**

It is a general observation for the imprinted polymers that the best rebinding occurs when it is exposed to its similar polymerization condition. Optimum binding condition was also reported in the same solvent in which a MIP is polymerized. The reason, as reported, is based on the origin of the mechanism of template recognition by a MIP.

The recognition arises from the shape of the imprint created by the spatial arrangement of the functional group in the polymer. The formed
complex is integrated into the polymer network and the orientation as well as distance between the groups can be affected by swelling with the solvent. Thus specificity can be lost if the MIP is placed in a wrong solvent during the rebinding.\textsuperscript{31}

Although in the aqueous medium, high rebinding affinity of the MIP was observed by Theodoridis et al.,\textsuperscript{23} they assigned it as non-specific rebinding since the control polymer, the NIP showed the similar adsorption trend. Anderson\textsuperscript{91} highlighted the problems with non-specific adsorptions in the aqueous medium. He argued that the presence of non-templated carboxylic acid residues dominate the polar type of non-specific interaction in an organic solvent. For the aqueous medium the hydrophobic interaction increases significantly and a strong binding is often observed.

1.4.2.3.2 Effect of pH in rebinding

The pH effect is pronounced in rebinding when the recognition monomer forms an assembly with acidic functionality (Figure 31). MAA is used conventionally and the carboxylic functional group acts as the recognition key to the template. Each site thus formed is an organic multi-acid and template rebinding to the same is dependent upon the pH of the medium\textsuperscript{12} (Figure 32). Adsorption of NIP with acidic functional group also shows pH dependence, but with a low adsorbability (Figure 33). Anderson\textsuperscript{91} found that the best rebinding can be achieved at a pH of 5.

Possible elimination of non-specific binding in water can give better specific rebinding performance. Theodoridis et al.\textsuperscript{23} used an alkaline buffer to attain a pH of 9 in which they expected a suppression of non-specific interaction through the masking of reactive acidic moieties on the surface of the polymer. At neutral to high pH, researchers found a good binding response in sensor application.\textsuperscript{12}
Figure 31. Frequency shift of a sensor with adsorption of caffeine to MIP; highest frequency shift implies the best adsorption result and therefore highest specificity.\textsuperscript{13}

![Graph showing frequency shift vs. pH]

Figure 32. PMAA at low pH, binding is contributed by hydrogen bonding interaction and at high pH, binding contributed by electrostatic interaction respectively.\textsuperscript{12}

For binding between caffeine and MAA, both hydrogen bonding and electrostatic interaction plays a role. At low pH, ionization is low and binding is determined by hydrogen bonding only.
Figure 33. Effect of pH on the response of a sensor with a MIP (1) and NIP (2). Frequency shift of a sensor is defined by the difference of stable frequency obtained at background solution to that with the sample solution.\textsuperscript{12}
1.4.3 Gas adsorption on MIP as an adsorbate

1.4.3.1 Absorption and adsorption

Absorption is measured by the concentration of one material or phase within another. In case of a gas and a solid phase, the gas permeates into the solid and moves in between the molecules of solid. On the other hand adsorption is measured by the concentration of one material or phase at the surface of another (Figure 34). In case of similar gas and solid phase the gas molecules accumulate very near the surface of sample solid at a concentration or density greater than that of the space immediately above.

AdSORbate

Figure 34. Gas absorbed into a solid and adsorbed onto a solid surface respectively

Adsorption comes from the attractive forces between the solid surface of the sample and the gas molecules in the space above it. The force is similar to the force in between the molecules of solid often called Van der Waals forces. Van der waal force is strong at close proximity but drops off quickly with distance. Since each molecule in a solid is close enough to its neighbors the attraction is very strong, thus holding the solid together in one piece. Molecules located at the solid surface lack neighbors in one or more direction but still exhibit the attractive force. Fluid molecules
such as water, organic vapors, carbon dioxide or oxygen from the environment come into contact with the solid and get adsorbed. In order to determine the surface property of the solid material those unwanted molecules are removed using a degassing technique with a non-adsorbing gas which is purged in combination with heat.

The clean surface is then exposed to gas molecules under controlled condition and the measurement is made for the amount of gas adsorbed onto the surface at different concentration of gas. The relationship between gas taken up and pressure at a constant temperature is called an isotherm.\textsuperscript{72}

1.4.3.2 Surface area determination

Langmuir equation provides the relationship between gas uptake and pressure in a linear form. The theory supposes a dynamic equilibrium between gas molecules with those which are adsorbed. It is assumed that the adsorbed molecules do not interact and the surface is covered by a monolayer. The adsorption is proportional to the free surface and the exerted pressure, whereas the desorption rate is proportional to the covered surface. At the equilibrium two rates are equal as described in Equation 10. $\theta$ is the fraction of surface coverage of adsorbed molecule and $p$ is the equilibrium pressure and $a$ and $b$ are constants. $\theta$ can be defined by $V/V_m$, where $V$ is the adsorbed volume and $V_m$ is the volume of the monolayer covering the surface. The final expression leads to Equation 11. The slope of $p/V$ gives $V_m$ which allows the determination of the specific area on the condition for the surface occupied by a known single adsorbed molecule of nitrogen.
Equation 10. Langmuir expression for monolayer gas adsorption on solid

\[(1 - \theta)p = b\theta\]

Equation 11. Final form of Langmuir expression

\[\frac{p}{V} = \frac{p}{V_m} + \frac{b}{V_m}\]

In reality, a material shows a little uptake at low pressure, little more at intermediate pressure and a significant uptake at pressure close to which the gas condenses into a liquid free of any surface (Figure 35). In order to account for the extra uptake at higher pressure, BET (developed by Stephan Brunauer, Paul Emmett and Edward Teller)\textsuperscript{52} generalized the Langmuir theory for multilayer adsorption. The attractive force between multilayers is not as strong as for the first layer to the surface, but is significant when the pressure of the adsorptive is increased. The hypothesis for their analysis is that the evaporation and condensation rate of adsorbed molecules is equal in a layer, and except for the first layer, the heat of adsorption in the layers is equal to the liquefication heat of the gas. It is also assumed that at saturation the number of layers is considered infinite.\textsuperscript{73} The resulting equation is as follows (Equation 12). \(P\) is the equilibrium pressure, \(n\) is the adsorbed gas quantity, \(n_m\) is the monolayer gas quantity and \(c\) is the BET constant.

Equation 12. BET expression for multilayer gas adsorption on solid

\[
\frac{P}{n(P_0 - P)} = \frac{1}{n_m c} + \frac{(c - 1)P}{P_0}
\]
While dealing with the adsorption of gas molecules on solid surface, the BET isotherm works well as Langmuir isotherm assumes single layer adsorption (Figure 36). Due to the fact of multilayer formation (Figure 37) of molecules on a surface, Langmuir isotherm looses its validity. This builds an adsorption isotherm which is plotted against a volume of gas adsorbed (cc/g @ STP) versus relative pressure (i.e., sample pressure / saturation vapor pressure) (Figure 38).
Figure 36. Langmuir description of partitioning between gas phase and adsorbed species. The adsorption process between gas phase molecules A, a vacant surface sites S, and the occupied surface sites SA. The equilibrium exists: \( S + A \rightleftharpoons SA \)

Figure 37. Multilayer adsorption explained by the BET isotherm.

In fact surfaces are not flat and there are holes and pores present. Gas adsorbs on the surface inside the pores in a similar fashion as it does for the outside surface. The presence of pores increases the surface area beyond that of a similar sized non-porous solid.

Due to the presence of a porous structure, a good MIP is found to have a significantly larger surface area in comparison to a NIP. In contrast, a MIP with a bound template shows a similar surface area as compared to a NIP. Higher surface area and pore volume in general is common for a well developed MIP structure and increasing
the porogen proportion often contributes to an increase in the surface area. A macroporous resin can possess a wide range of porous structures. The resin having large proportion of macropores possesses a low surface area and a very higher surface area can be found for a resin consists of high proportion of micropores.\(^3\)

Increasing the level of crosslinker yields a shift to smaller pore size. The pore distribution in this case are mesopores ranging from 200-500Å. Surface area also increases with increasing concentration of crosslinker.

Along with the evaluation of surface area, BET gas adsorption study also can mathematically provide information on pore volume and size which allows for MIP performance to be predicted. A plot of pore size versus pore volume gives the total pore volume. Relevant information is found for the pore size distribution, which can be obtained from the plot of pore size versus incremental pore volume.

![Figure 38. Langmuir (red) versus BET (green) isotherm\(^76\)](image-url)

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1.4.3.3 Determination of pore size distribution

Pores in fact have a variety of shapes and sizes. The nuclei of the growing polymer chain fuses to produce the inter-linked porous polymer matrix. Thus typically three size distributions of pores are found; micro, meso and macropores with increasing pore diameter from less than 2 nm to over 50 nm. Very small pores, smaller than 2 nm are called micropores and larger than 50 nm are referred to as macropores. Mesopores have a diameter range of 2 nm to 50 nm. Due to the capillary forces the guest template during the rebinding study has a slow diffusion rate to the micropores. In the case where a non-solvent is used for polymerization, the resulting polymer contains agglomerates of micropores. Compared to the smaller micropores, the sites associated with meso and macropores are expected to have an easier access to the guest binding analyte. The mass transfer for releasing and rebinding of print molecule is facilitated by the formation of a macroporous structure.

In the gas adsorption study the filling of micropores takes place at a very low relative pressure. With increasing pressure the mesopores are filled up with gas. At this stage the attractive forces inside the pore allow gas to condense into liquid at a pressure lower than normal saturation. This phenomenon is called capillary condensation (Figure 35). Capillary condensation and multiple layer adsorptions continue with increasing pressure. With increasing pressure, larger pores fill up eventually. At saturation all the pores are filled. BJH (Barrett, Joyner and Halenda) developed a method where it is possible to look at the amount of gas adsorbed at each point. This starts at the end point and works backwards and it is referred to as BJH pore volume distribution data reduction. The detail calculation is not being discussed here as
it is beyond the scope of this thesis. The pore size distribution can be obtained automatically from the software used for the gas adsorption study.

Physical gas adsorption is a straightforward technique to characterize micro- and mesoporous materials. However, physical phenomena like the tensile strength effect, adsorbate phase transitions, and monolayer formation in a combined micro- and mesoporous materials often lead to extra contributions in the adsorption isotherm. Models for pore size determination in general do not consider these, and assignment to real pores often leads to improper analysis of the adsorption data.\textsuperscript{78}

1.5 Application of MIP

Although MIP has various applications in diverse areas, we are particularly interested in developing a sensor by exploiting its molecular recognition behavior. Our characterization of MIP is targeted to find a suitable formulation to be applicable in a sensor.

![Figure 39. Schematic diagram of a chemical sensor; characterized by a recognition element and a transducer close to each other.\textsuperscript{6}](image)

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1.5.1 MIP as a sensor

A chemical sensor consists of two units, a receptor part and a transducer part. The receptor part consists of the MIPs. The biological sensing element in a biosensor is replaced by MIP in a chemical sensor. Being the key component of the sensor device (Figure 39), a MIP as a recognition element enables the sensor to respond selectively to a particular analyte. To transfer the amount of bound template into a quantified output signal, a transducer is needed. Analytical methods in chemistry have mainly been based on photometric transducers, as in spectroscopic and colorimetric methods. However, most sensors have been developed around electrochemical transducers, because of the simplicity of construction and low cost.

For the sensor part of the project we selected quartz crystal microbalance (QCM) for the transduction technique because of its low cost and portability. In QCM, the oscillator circuit induces the quartz crystal to oscillate at its characteristic frequency. This device can be operated in the range of 5-10 MHz. When the crystal is coated with MIP the frequency is decreased. Thus an increase in mass decreases the frequency and with the adsorption of an analyte a further decrease should be observed. In general the QCM is coated with the imprinted polymer and immersed into the analyte solution. The QCM apparatus shows a frequency change with changes in mass on the electrode.

When binding with template the sensor exhibits a frequency shift. If the MIP has a specific selectivity towards the template, all other substances hardly interfere. For a caffeine imprinted MIP it was reported to have only a slight interference (Figure 40) from its structural analogue theophylline.
Figure 40. Response of a caffeine imprinted MIP sensor to caffeine and theophylline (1&3 respectively) and for a reference sensor to caffeine and theophylline (2&4 respectively).^12

The sensor response is measured by the frequency shift recorded. After achieving a steady resonant frequency in a background solution, a series of standard solution is introduced in an increasing concentration and the corresponding frequency is recorded. At another stable frequency the frequency shift is calculated from the difference from the initial frequency recorded.^12,13

To conclude this background chapter, it can be said that to formulate a successful MIP several factors need to be considered. By physical characterization it is possible to examine the specificity and selectivity of a MIP.

This thesis will consist of the characterization of MIPs prepared in varying condition so that a suitable MIP can be delivered for the application in a field sensor. The sensor development part of this project is not included in this thesis as it has been done in another laboratory. The rest of the thesis is articulated as follows: the experimental procedure that was implemented for the preparation and characterization which is followed by a results and discussions chapter.
CHAPTER II

EXPERIMENTAL PROCEDURE

2 METHOD DEVELOPMENT AND OTHER EXPERIMENTS

2.1 Polymerization and template extraction

2.1.1 Reagents

Table 3. Reagents used in polymerization and template extraction

<table>
<thead>
<tr>
<th>Product</th>
<th>Acronym</th>
<th>Purity</th>
<th>Supplier</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacrylic Acid</td>
<td>MAA</td>
<td>99%</td>
<td>Sigma-Aldrich</td>
<td>Purified over basic alumina</td>
</tr>
<tr>
<td>2-hydroxy ethyl methacrylate</td>
<td>HEMA</td>
<td>99.6%</td>
<td>Sigma-Aldrich</td>
<td>Purified over basic alumina</td>
</tr>
<tr>
<td>Ethylene glycol dimethacrylate</td>
<td>EGDMA</td>
<td>98%</td>
<td>Sigma-Aldrich</td>
<td>Purified over basic alumina</td>
</tr>
<tr>
<td>Glycerol dimethacrylate</td>
<td>GDMA</td>
<td>85%</td>
<td>Sigma-Aldrich</td>
<td>Purified over basic alumina</td>
</tr>
<tr>
<td>N, N’-Methylenediisacrylamide</td>
<td>MBA</td>
<td>99%</td>
<td>Sigma-Aldrich</td>
<td>Used as received</td>
</tr>
<tr>
<td>Product</td>
<td>Acronym</td>
<td>Purity</td>
<td>Supplier</td>
<td>Treatment</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>ACN</td>
<td>99.8%</td>
<td>Sigma-Aldrich</td>
<td>Used as received</td>
</tr>
<tr>
<td>Methyl alcohol</td>
<td>MeOH</td>
<td>99.9%</td>
<td>Sigma-Aldrich</td>
<td>Used as received</td>
</tr>
<tr>
<td>Polyethylene glycol dimethyl ether; Mn~500</td>
<td>PGDME-500</td>
<td>--</td>
<td>Sigma-Aldrich</td>
<td>Used as received</td>
</tr>
<tr>
<td>Water</td>
<td>--</td>
<td>--</td>
<td>Lab supply</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>DMSO</td>
<td>98%</td>
<td>Sigma-Aldrich</td>
<td>Used as received</td>
</tr>
<tr>
<td>Azobis isobutyronitrile</td>
<td>AIBN</td>
<td>98%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>KPS</td>
<td>99%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
<tr>
<td>Caffeine</td>
<td>--</td>
<td>98.5%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
<tr>
<td>Theophylline</td>
<td>--</td>
<td>99%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
<tr>
<td>Guanosine</td>
<td>--</td>
<td>99%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate</td>
<td>SDS</td>
<td>98%</td>
<td>Cambridge Isotope</td>
<td>Used as received</td>
</tr>
<tr>
<td>Cetyl trimethyl ammonium bromide</td>
<td>CTAB</td>
<td>100%</td>
<td>Acros Organics</td>
<td>Used as received</td>
</tr>
</tbody>
</table>
2.1.2 Polymerization

All the reagents were weighed in a round bottomed flask (RBF) according to the formulation (Figure 41) and the solution was then purged with nitrogen gas for 20 minutes. The solution was then placed in a thermostat oil bath and was heated at 65°C for 16 hours. Eventually, a hard monolith polymer was obtained using the bulk polymerization method (Figure 42), which was then ground by mortar and pestle or by mechanical grinder to obtain small sized particles. The particles thus obtained had a broad size distribution in micron range. Particles were then washed thoroughly with methanol.

Typical formulation of MIP in weight percentage

Recognition monomer, 5.85
Crosslinking agent, 38.85
Porogen, 51.62
Template, 3.19
Initiator, 0.49

Figure 41. A starting recipe of MIP by conventional bulk polymerization
We initially concentrated on conventional solution polymerization for preparation of monolith MIPs and for nanoparticles formation, due to the option of using organic medium, we found the precipitation polymerization to be the most suitable one.

For precipitation polymerization all the reagents except the initiator were weighed in a RBF. The solution was purged with nitrogen and heated up to 65°C and initiator solution was introduced at this point to start the polymerization. The polymerization was complete within 2 hours. For aqueous emulsion polymerization, sodium dodecyl sulfate (SDS) was also used as a surfactant and a stable latex was obtained after 2 hours of polymerization.

![Polymerization set up](image)

**Figure 42. Polymerization set up**
2.1.3 Template extraction

Particles obtained using the bulk polymerization method was subjected to template removal by soxhlet extraction (Figure 43) with methanol and water. Methanol was used to remove any excess monomer present and both hot methanol and water were useful to remove caffeine from the MIP. In soxhlet, solvents are heated over their boiling point and after condensation are passed through the sample to wash it. Soxhlet was performed for 24 hours separately for each solvent. For non-imprinted polymer prepared without template, no soxhlet extraction was required. The unreacted monomer was removed by extensive washing with methanol. The wet particles were then dried in a vacuum for 24 hours for further characterization.
Nanospheres obtained by precipitation polymerization were diluted with an aqueous solution of CTAB to obtain stable latex. For particles obtained through emulsion or precipitation polymerization, template extraction was performed by ultra-filtration (UF) (Figure 44). Latex was washed by an aqueous surfactant solution for several hours at room temperature. For particles obtained from emulsion polymerization a dilute
solution of SDS was used and those obtained from precipitation polymerization was washed with a dilute CTAB solution.

Figure 44. Ultrafiltration setup for cleaning latex

2.1.4 Confirmation of template removal

The complete removal of any residual template was examined by HPLC (Figure 45). To do so, a given amount of solid particles was suspended in water and agitated for 24 hours. The supernatant was analyzed by HPLC to check the efficiency of washing. For latex, the filtrate coming out of UF was examined by HPLC to check for any residual template. As long as the concentration of the residual template was below the sensitivity limit of HPLC, it did not affect the future rebinding analysis.
2.1.5 Particle size measurement

The particle size distribution was measured by using a multiangle light scattering technique through laser light diffraction. For micron ranged solid particles, dry mode Microtrac S3000 (Figure 46) was used and for submicron ranged latex particles, a Nanotrac UPA 250 was used for measurement. Solid particles possessed a broad size distribution with an average size of 20-70 micron (Figure 47). A small percentage of material was also found in the 5 micron range as well as in 200 micron range (for particle size distribution of the conventional MIPs see Appendix C). On the other hand, for latex particles the size distribution was quite narrow and was at around 500 nm (Figure 48).
Figure 46. Microtrac S3000 particle size analyzer

Serial Number: S3213
Range: 0.243 - 1408 um

SR Bulk1C
Bulk1C

Summary

Parameter | Value
--- | ---
Size (um) | 88.96
10% | 6.294
60% | 65.37
20% | 19.46
70% | 74.33
30% | 21.11
80% | 212.7
40% | 23.84
90% | 231.9
50% | 57.92
95% | 243.0

Figure 47. Typical particle size range of a ground MIP, as obtained from Microtrac-S3000

Summary

Parameter | Value
--- | ---
Size (um) | 88.96
10% | 6.294
60% | 65.37
20% | 19.46
70% | 74.33
30% | 21.11
80% | 212.7
40% | 23.84
90% | 231.9
50% | 57.92
95% | 243.0

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2.2 Pseudo-equilibrium rebinding experiments

Although rebinding medium could have a significant influence in guest binding, water was pre-determined for our experiments due to the prospective application of the MIPs in a field sensor.

For batch rebinding study, a stock solution of analyte in water was first prepared. In a container (A), 0.4 g of MIP was suspended in 10 ml of water. In another container, only water was there. A 10 μl aliquot of stock solution was introduced in both containers, which were then stirred for 5 minutes. Then, 50 μl of liquid were withdrawn from both vials, and analyzed by HPLC, yielding the concentration of analyte in A and in B. The difference (B-A) yielded the amount of analyte adsorbed onto the MIP. The process was repeated until a close saturation was confirmed by HPLC for the given amount of solid.
particles. For nanoparticles, several vials were prepared with a given amount of latex (solid content matched with the same for polymer prepared in bulk polymerization). Varying aliquot of 10 µl to 150 µl were introduced from stock solution in those vials and then agitated for 5 minutes. Latex was withdrawn from vials, filtered through centrifugal devices (Nanosep 100K omega) and the filtrate was then analyzed by HPLC which yielded the concentration of free analyte. The bound analyte concentration was obtained by subtracting the unbound analyte concentration from the initial concentration loaded.

An increase in experimental temperature can cause the weakening of hydrogen bonding interaction between the recognition monomer and the template and hence can yield a lower adsorption rate (Figure 49). Consequently, the rebinding experiment was done at room temperature.

![Figure 49. Caffeine adsorption amount variation with temperature for a caffeine imprinted MIP](image)

Figure 49. Caffeine adsorption amount variation with temperature for a caffeine imprinted MIP.
2.2.1 Building of adsorption isotherm

2.2.1.1 Characterization by HPLC

The concentration of the analyte was quantified by HPLC. Depending upon the analyte used the optimum static phase, mobile phase and detector were chosen.

2.2.1.2 Choice of static phase, mobile phase and detector

The HPLC column used was silica based reversed phase C18 octadecyl silyl column with 5 μm particle sized packing material\textsuperscript{55} and operated at 30°C. Reversed phase was used as non-polar analyte could be retained on a nonpolar sorbent due to van der Waals forces. Another advantage of using a reversed phase column was that pH does not typically affect the retention of compounds that have a neutral charge, such as caffeine. Acidic pH was used for the silica column as silica gets gradually hydrolyzed at neutral pH or at basic pH. As a mobile phase, water (+1% phosphoric acid) was used. A gradient elution\textsuperscript{57,58} (\textbf{Figure 50}) from water (+1% phosphoric acid) to 50:50 water: acetonitrile was spread over 15 minutes at a flow rate of 1 ml/min. The analyte eluted was then detected by variable wavelength detector (UV wavelength at 254 nm). All the analytes considered in the project (caffeine, theophylline or guanosine) are aromatic compounds and possess one or more double bonds (\pi electrons) or unshared electrons, for example, \textgreater C=O and \textendgraf N=N\textendgraf groups. Thus, they absorb light in ultraviolet region.\textsuperscript{59}
2.2.1.3 Calibration of HPLC for specific analytes

Three types of analytes were used and characterized by HPLC; caffeine, its structural analogue theophylline, and guanosine. All of these can be eluted conveniently with the above mentioned condition. Depending upon the polarity since the analytes eluted at different elusion time it was convenient to analyze those in a mixture. Under stipulated condition, caffeine normally eluted at 12 minutes (Figure 51), theophylline at 8 minutes (Figure 52) and guanosine eluted at around 6 minutes (Figure 53). Depending upon the column brand and running condition, both the elution time of analyte and the width of the chromatogram varied. The chromatogram peak was integrated and the area corresponding to the concentration of analyte was obtained. Absorbance over 1000mAU (mili absorbance unit) was not taken into account for building the calibration curve.
Figure 51. UV chromatogram for caffeine obtained by HPLC analysis

Figure 52. UV chromatogram for theophylline obtained by HPLC analysis

Figure 53. UV chromatogram for guanosine obtained by HPLC analysis
To calibrate the HPLC for a specific analyte a dilute aqueous solution (around 100 mg/lit.) of the analyte was first prepared. Injection volume was varied from 1 µl to 100 µl to obtain the corresponding peak area. With the help of these data, a calibration curve (Figure 54) was plotted against the amount of analyte injected (in gram) with its corresponding area. Based on the calibration curve, any unknown concentration of the analyte can be measured. HPLC was calibrated in frequent time intervals to avoid any possible error.

Figure 54. HPLC calibration curve for caffeine

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2.2.1.4 Data analysis

Data obtained from the pseudo-equilibrium rebinding experiment (section 2.2) were summarized graphically to build the isotherm. The isotherm was built by plotting the free analyte concentration with the bound analyte concentration. Isotherm can be conveniently fitted with non-linear curve fitting by the Langmuir type of adsorption model (Figure 55).

![Figure 55. Isotherm obtained from rebinding experiment (blue) and fitted (red) with bi-Langmuir model](image)

For heterogeneous distribution of sites, a multi-Langmuir model is useful and we chose bi-Langmuir model to explain the isotherm. This was accomplished by using the solver functions for the residual square of bound vs. free analyte ratio in Microsoft excel (Table 4). Solver, the what-if analysis tool was used to find an optimal value for the target cell of sum of Residual Square. Solver adjusted the values in the
adjustable cells and thus optimized four variables using the bi-Langmuir model. Constraints were applied to restrict the values not to collapse. We determined the minimum value of the target cell that can be achieved by solver. Although limited data set for determining four output variables often caused the lack of stability for the optimized values, in most cases we were able to obtain reasonably well output that could be justified theoretically.

The variable parameters used were the specific and non-specific binding coefficient and their corresponding saturation bound analyte concentrations. Association binding constant for an adsorbate is obtained from the product of analyte molecular weight and the binding coefficient as explained in section 1.4.2.2.1 and the number of sites can be calculated from the saturation capacity of the MIP.

Table 4. A sample excel table used to determine the bi-Langmuir variables by using the solver function

<table>
<thead>
<tr>
<th>Caffeine free [g/l]</th>
<th>Caffeine bound exp. [g/l]</th>
<th>bi-Langmuir variables</th>
<th>Caffeine bound theo. [g/l]</th>
<th>residual square</th>
<th>sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.38E-02</td>
<td>3.53E-02</td>
<td>1.04E-01</td>
<td>2.23E-01</td>
<td>1.03E-01</td>
<td>2.53E-04</td>
</tr>
<tr>
<td>2.81E-02</td>
<td>7.06E-02</td>
<td>1.63E-01</td>
<td>2.75E-01</td>
<td>1.68E-01</td>
<td>4.88E-03</td>
</tr>
<tr>
<td>4.53E-02</td>
<td>1.70E-01</td>
<td>3.60E-02</td>
<td>9.70E+00</td>
<td>3.60E-02</td>
<td>5.87E-03</td>
</tr>
<tr>
<td>8.44E-02</td>
<td>2.20E-01</td>
<td>1.03E-01</td>
<td>2.74E-01</td>
<td>1.03E-01</td>
<td>1.03E-04</td>
</tr>
<tr>
<td>1.50E-01</td>
<td>1.50E+00</td>
<td>4.53E-01</td>
<td>5.43E-01</td>
<td>5.43E-05</td>
<td>5.43E-05</td>
</tr>
<tr>
<td>1.08E+00</td>
<td>5.34E-01</td>
<td>6.51E-01</td>
<td>4.93E-05</td>
<td>4.93E-05</td>
<td>4.93E-05</td>
</tr>
<tr>
<td>9.00E-01</td>
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<td>9.24E-01</td>
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<tr>
<td>1.61E+00</td>
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<td>1.03E+00</td>
<td>4.78E-04</td>
<td>4.78E-04</td>
<td>4.78E-04</td>
</tr>
<tr>
<td>1.85E+00</td>
<td>1.10E+00</td>
<td>1.08E+00</td>
<td>6.99E-05</td>
<td>6.99E-05</td>
<td>6.99E-05</td>
</tr>
<tr>
<td>2.11E+00</td>
<td>1.16E+00</td>
<td>1.11E+00</td>
<td>4.72E-04</td>
<td>4.72E-04</td>
<td>4.72E-04</td>
</tr>
<tr>
<td>2.32E+00</td>
<td>1.03E+00</td>
<td>1.16E+00</td>
<td>2.33E-03</td>
<td>2.33E-03</td>
<td>2.33E-03</td>
</tr>
</tbody>
</table>
Two classes of sites were assumed for the prepared MIPs. Sites which are specific to the template have a high binding efficiency and are in low concentration. On the other hand, sites which are non-specific to the template have a low binding efficiency and are present in high concentration. Orwoll et al. referred to these two types of binding sites having different affinities as 'general' and 'special' binding sites. The system behavior dominates with general sites when template species are incorporated in low concentrations and special binding sites are dominant at a high concentration of template species. The general binding sites of a MIP can be considered as the same kind that exists in a NIP.

The binding constant and the saturation capacity also can be estimated graphically from a linearized version of plotting the isotherm in a Scatchard format (Figure 56). The format is implemented by plotting the bound analyte concentration with the ratio of bound and free concentration of analyte. Each linear regression is fitted with a straight line and association binding constant is obtained from the slope. The intercept of the linear fit gives the estimation of the saturation capacity and in turn the number of sites.
This slope does not provide the correct information about specific binding since it accounts for both the specific and non-specific sites.

Figure 56. Scatchard plot of isotherm adhering to Langmuir model

Scatchard plot is a simple way to understand the binding efficiency and site density of a MIP. During rebinding, contribution of specific site is evident at dilute concentration. While fitting with a linear form for specific sites, this ignores the contribution of non-specific sites in that region (Figure 56). Moreover this approach does not take into account the curved portion of the graph, where some contribution of the specific sites could be present. Thus it could produce an erroneous result for describing a specific site. Binding constants were obtained with a curve fit by a bi-Langmuir and for a comparison purpose with the linear fit in Scatchard format.

From the analysis of data between Scatchard format and bi-Langmuir model we found the difference in case of specific sites and the later one was chosen to work with. In scatchard format each linear portion is assumed to have a mono-Langmuir model and data is evaluated likewise. Basically, scatchard format is derived
from the mono-Langmuir model (see appendix B) and it is useful for evaluating a MIP with homogeneous distribution of sites. For the MIPs having one class of non-specific sites and for the NIPs, both the bi-Langmuir and Scatchard format produced comparable results. Since an efficient MIP mostly consist of two types of sites bi-Langmuir fits well because it can provide a pair of binding sites at a time.

2.3 Characterization of MIP by gas adsorption technique

Fully automated TriStar 3000 gas adsorption analyzer from Micromeritics (Figure 57) was used for characterization of the MIPs. From the isotherm analysis (Figure 58), both Langmuir and BET surface area and by an automated theoretical BJH calculation pore size distribution was obtained. The gas adsorption technique is performed at 77K, which is the temperature of liquid nitrogen where inert gases can be physically adsorbed on the surface of the sample. In this context, nitrogen gas is considered to be ideal. This adsorption process is a reversible condensation of gas molecules on the surface of the sample during which heat is evolved. Up to three samples can be run at a time and a good comparison of MIPs prepared in varying condition can be obtained from the data analysis. For more reliable pore size distribution mercury porosimetry can prove good which characterizes a material’s porosity by applying various levels of pressure to a sample immersed in mercury. But our result set (detail result is sited in Appendix G) obtained from gas adsorption technique provided the extent of approximation as desired for a reasonable comparison between MIPs.
Figure 57. Tri-Star 300 gas adsorption analyzer

Figure 58. Isotherm plot obtained from gas adsorption experiment

The next chapter reports the results obtained from the experimental methods sited here. The results will also be supported with discussions.
CHAPTER III

RESULTS AND DISCUSSIONS

3 MIP PERFORMANCE IN VARYING PREPARATION AND CHARACTERIZATION PARAMETERS

The final objective of this project was set to detect saxitoxin as a template. Because the natural concentration of STX in shellfishes is very low (the lethal dose is around 8 μg/kg), the MIP needs to be highly sensitive. This also suggests that during the MIP fabrication, only low amounts of template could be used. The focus of this chapter is based on testing the specificity and selectivity of conventionally prepared MIPs as well as nanoparticles. Due to its reported advantages we had intended to formulate nanoparticles MIPs. Owing to their small size, nanoparticles offer highly specific surfaces (typically around 20 m²/g for a 100 nm nanoparticles). Thus, nanoparticles in theory could present a larger number of active sites per unit mass of a MIP, and the resulting performance would increase.

Because of its toxicity, the manipulation of STX was avoided, and most of the fundamental work was done by using caffeine and guanosine as the templates. Caffeine was selected for building the model as it contains a purine ring system like saxitoxin. Initial research was performed by reviewing a wide range of existing literature on the
detection of caffeine by MIP. We also evaluated guanosine as a template since it possesses a large number of similarities with saxitoxin.

As mentioned in earlier chapters, the characterization was done by a rebinding experiment in an aqueous medium. Since the objective was to develop a field sensor, the environmentally friendly aqueous medium was the obvious choice. This medium also offers the best rebinding reported. From the experimental isotherm and its mathematical fit, the binding constant and the site density of the MIP was examined. The result was supported by the surface area and pore size distribution analysis by the gas adsorption technique. This technique of MIP evaluation was previously pointed out by a few researchers.

We also conducted a measurement of kinetics for a variety of different MIPs to gain an idea of the response time. This could be important for application in a sensor as pointed out by other researchers. For the characterization of various MIPs we kept constant as many experimental conditions as possible. All the rebinding experiments were performed at room temperature to have the best rebinding.

3.1 MIP imprinted with caffeine as a template

We started with MAA as a recognition monomer and EGDMA as a crosslinking agent for the template caffeine, as reported in several research papers. The polymerization was initiated with free radical by using AIBN as described in the experimental section.

We varied the constituents and conditions in different ways (Table 5) from our initial formulation to examine the performance of a MIP. Starting with a higher concentration of template imprinting, which was reported to be better, we eventually
changed the proportion to a lower range and for comparison we also evaluated a non-imprinted polymer (NIP) in each variation employed. For this caffeine-MAA-EGDMA combination, we selected acetonitrile as a starting point for the porogen, which was most commonly used in the domain. We were also interested in seeing the effect of other specific porogens and employed those eventually.

Increasing the porogen proportion in the formulation after a certain point yields nanoparticles instead of a monolith and we were interested in tracking the effect of increasing the amount of porogen on the performance of a MIP. For the crosslinking agent although our work was mostly concentrated on EGDMA, we also evaluated GDMA and a water soluble crosslinker MBA.

Since a sensor response depends upon the pH of the medium, for our rebinding study we varied the pH of the rebinding medium to examine the best condition for guest rebinding. The concentration of a MIP in the medium was also varied to observe the effect, if any. Evaluating the binding constant of a MIP for its template gives an idea of its specificity and for a standardized condition we also judged the selectivity of the MIP towards its template. This was done by a competitive experiment with a structural analogue of the template as reported by many researchers.
Table 5. Scheme of the variation of constituents and conditions for preparation and characterization of a MIP

<table>
<thead>
<tr>
<th>Base formulation of MIP</th>
<th>Variations in conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Template concentration</td>
</tr>
<tr>
<td></td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Porogen type</td>
<td>ACN</td>
</tr>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td></td>
<td>PEGDME</td>
</tr>
<tr>
<td>Porogen proportion in total formulation</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>95%</td>
</tr>
<tr>
<td>Crosslinker type</td>
<td>EGDMA</td>
</tr>
<tr>
<td></td>
<td>GDMA</td>
</tr>
<tr>
<td></td>
<td>MBA</td>
</tr>
<tr>
<td>Crosslinker proportion</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>Very High</td>
</tr>
<tr>
<td>Rebinding experiment</td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>Acidic</td>
</tr>
<tr>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td>Basic</td>
</tr>
<tr>
<td>MIP concentration</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>10%</td>
</tr>
</tbody>
</table>

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3.1.1 Template proportion and porogen effect

A high ratio of target molecule to functional monomer is the favored condition for target specific and selective rebinding. Holdsworth et al. found a ratio of 1:2 as the most favorable. Keeping this fact in mind that a higher proportion of template produces a higher density of binding sites, we started with a formulation of conventional MIP prepared with a high concentration of template (Bulk 1) (Figure 59). We evaluated the MIP in terms of binding constant and site density. As mentioned earlier we also evaluated the MIP by the gas adsorption technique.

A second MIP was prepared by lowering the template concentration (Bulk 4) (Figure 59) for the same constituents. Lower template proportion in the polymerization mixture is supposed to result in a low number of binding sites, and therefore a lower specificity. So while lowering the template concentration, the recognition monomer proportion was increased to see whether or not it could produce some efficient recognition behavior. To keep the monomer: crosslinker molar ratio (0.35) constant, the crosslinker proportion was also increased. The porogen proportion was automatically reduced to keep the formulation at 100%. A NIP (Figure 59) was prepared according to the recipe of Bulk 1 for comparison.
Since the MIP was prepared by exploring the non-covalent interaction, heterogeneous distribution of mainly two types of sites were expected. Only a few are sites are created by the influence of template and most of those are created by other components in the polymerization mixture and by any operation that generates the surface area.

An isotherm was built by plotting the experimental data which was convex upward, hence the type was Langmuirian. To give the answer of the question whether a mono-Langmuir or bi-Langmuir model would fit the best, both models were compared to check the viability (Figure 60). Consideration of Langmuir model to fit the experimentally obtained adsorption data is quite common among the
researchers.\textsuperscript{49,62,63,64,66} Piletsky et al.\textsuperscript{31} used the bi-Langmuir model to fit their experimental isotherm data and two types of binding sites were evaluated for the MIPs they analyzed.

The best isotherm model was selected based on the comparison of the experimental and calculated profiles. Bi-Langmuir model compared to mono-Langmuir model fits better at low concentrations, where the good sites can be found (Figure 60). It is known that, at the low concentration range, the adsorption on the specific binding site is stronger than that on the non-specific binding sites.\textsuperscript{22} Basically mono-Langmuir model takes into account an average of two types of sites that is considered by the bi-Langmuir model. Langmuir-Freundlich model which accounts for the true heterogeneity of sites produces a quite comparable fit with the bi-Langmuir model (Figure 61). The Langmuir-Freundlich model gives the distribution of heterogeneity and since it well corresponds with the bi-Langmuir model, it can be postulated that the assumption of two site model is quite logical. The dissimilarity between the bi-Langmuir and Langmuir-Freundlich model at high concentration (Figure 60) shows the true heterogeneous distribution of sites in a MIP.

For the MIPs where bi-Langmuir model yielded identical result for two types of sites, mono-Langmuir model was applied. The log-log plot (Figure 61) clearly shows the difference between the bi-Langmuir and mono-Langmuir fit.
Figure 60. Experimental isotherm (symbols) of Bulk1 fitted with Langmuir (dashed line), bi-Langmuir (solid line) model and Langmuir-Freundlich model (dotted line)
Figure 61. Log-log plot of experimental isotherm (symbols) of Bulk1 fitted with Langmuir (dashed line) bi-Langmuir (solid line) model and Langmuir-Freundlich model (dotted line)

As pointed out in few studies, Scatchard plots were applied when two types of sites are distinct. The binary model of Langmuir fits particularly well with a Scatchard plot. From the linear regression of the Scatchard plot two types of binding constants and sites were evaluated as in Bulk1. Since using a Scatchard plot involves the manual selection of data points to differentiate between the two types of sites, it was beneficial to move to a more mathematical model (Figure 62). Also the selection of only specific sites from the initial part of a Scatchard plot ignores the reality of the accumulation of guest analytes in the non-specific sites. Keeping this in mind, we found the binary model of Langmuir to be best at providing the binding constant and site density of a MIP. The bi-Langmuir model can differentiate between the two types of sites.
and operates mathematically. In the appendix D the results obtained for both the Langmuir and Scatchard version are given.

![Graph showing comparison of bi-Langmuir (curved fit) and Scatchard format (two linear fits) fitted with the experimentally obtained isotherm (symbols)](image)

Figure 62. Comparison of bi-Langmuir (curved fit) and Scatchard format (two linear fits) fitted with the experimentally obtained isotherm (symbols)

All the results from the rebinding study are thus sited as obtained by the Langmuir model. Bi-Langmuir model was found better to evaluate an efficient MIP as it can produce a distinct difference between the specific and non-specific sites. Langmuir-Freundlich model can give only a heterogeneity index instead with one type of binding constant and site density.

The results from the batch rebinding experiment are summarized in Figure 63 and Table 6. Bulk1 fits the trend of an efficient MIP. It has fewer sites having high binding constant and a large number of sites with lower binding efficiency. The high specificity of a MIP prepared with a high proportion of template was pointed out by Holdsworth et al. and Bulk1 supported those findings.
2000
1800
1600
1400
1200
1000
800
600
400
200
0

151
278
272

specific binding constant

non-specific binding constant

Bulk 1-high template
Bulk 4-Low template
Bulk 12-No template

Template concentration in the polymerization mixture

Figure 63. Influence of template proportion on Binding constant

Table 6. Influence of template proportion on site density while keeping monomer: crosslinker unchanged

<table>
<thead>
<tr>
<th>Sample</th>
<th>Template :Monomer</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>0.55</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk 4</td>
<td>0.008</td>
<td>--</td>
<td>0.09</td>
</tr>
<tr>
<td>Bulk 12</td>
<td>0</td>
<td>--</td>
<td>0.19</td>
</tr>
</tbody>
</table>

When compared to the NIP, the advantage of MIP is evident. The NIP (Bulk12) possesses only one type of site with a low binding constant and has a site density comparable to that of non-specific sites of Bulk1. The sites with a higher binding
constant can be called ‘specific’ to the template or ‘special’ and the sites with lower binding constant are referred to as ‘non-specific’ or ‘general’. Hence Bulk12 can produce only non-specific sites since it was prepared in the absence of template. The general binding sites then can be assigned as the same kind as those found in the NIP.

On the other hand, despite using the template in low concentration, Bulk 4 was not able to produce specific site either or those sites were not possible to extract in our experimental range. Although the apparent conclusion from here was that the lower template concentration gives rise to only non-specific binding site, but this is not always the case. There is an influence of crosslinker and that will be discussed in a later section of this chapter.

From the initial result it can be concluded that acetonitrile as a porogen in general works well. This is due to its good solubility power that helps to form a miscible polymerization mixture. It also has a cyanide group that can stabilize the MAA-caffeine complex. Effective complex formation in turn results in efficient recognition. Xiu-fang et al. employed UV-vis spectroscopic studies to find the most favorable porogen to maximize the interaction between caffeine and MAA. Among methanol, chloroform, benzene and acetonitrile, acetonitrile was found to be the best. During the rebinding, maximum difference in analyte adsorption between a MIP and its corresponding NIP was reported to be the highest in case of acetonitrile.

The Figure 59 summarizes the different experimental conditions to formulate MIP with different template proportion with one porogen ACN. The same type of formulation was implemented for water and polyethylene glycol dimethyl ether (PEGDME, M$_n$ ~500). PEGDME was evaluated as a porogen, since initially we were not
aware of the preparation method for nanoparticles in the conventional solvent acetonitrile. The highest imprinted effectiveness reported for ribonuclease imprinted polymer was achieved by using PEGDME as a monomer. Thus another motivating power of evaluating PEGDME as a porogen was to examine if it could increase the specificity of the MIP. For water and PEGDME as porogens, all other constituents were kept in constant proportions to allow a better comparison.

![Figure 64. Structure of PEGDME](image)

When water was substituted for ACN and all other constituents remained the same it did not produce an efficient MIP. This can be evident from one type of site with low binding constant for Bulk 11 (see appendix E). Other MIPs prepared with water also did not work. There are several possible reasons for this failure, but the fact that water is a polar compound with strong hydrogen bond plays the main role. As we are exploring hydrogen bonding interaction between recognition monomer MAA and the template caffeine, the strong hydrogen bond of water interferes with the complex formation. Hence water prevents the preparation of an efficient imprinting. Another reason is that, water could not make a homogeneous polymerization mixture as it is not a solvent for EGDMA. Efficient imprinting to some extent depends on the uniform distribution of crosslinker and having all the constituents in the same phase during polymerization facilitates this. This phenomenon was discussed by Yan et al. during their research regarding selection of suitable porogen in formation of a MIP.
On the other hand PGDME, a polymeric inert compound, works fairly well (see appendix E) as a porogen. The result is comparable to that of acetonitrile with comparable site density (see appendix E).

The study of polymeric porogen in connection with MIP has not been extensively explored. One literature study reveals that due to the incompatibility between polymers, the phase separation can be achieved at a low level of polymeric porogens. This may be an advantage when nanoparticles are desired. In the meantime, we acquired the technique to prepare nanospheres in acetonitrile and this route was dropped.

Further characterization of the MIPs was done and from the gas adsorption experiment we obtained both Langmuir and BET surface area. Automated BJH calculation yields the pore size distribution. We are considering only the BET surface area and the average pore diameter for the discussion, as those are sufficient to evaluate a MIP. The result in graphical form is sited in Figure 65 and Figure 66.
Figure 65. Surface area (m$^2$/gm) of MIPs as obtained from gas adsorption experiment

Figure 66. Average pore diameter of MIPs as obtained from gas adsorption experiment
For varying porogen and template proportion in general it can be concluded that MIPs that respond well in the rebinding study, possess a large surface area. Bulk 1 and MJB 40 follow this trend and have a relatively high surface area. The reverse is not true as we found some MIPs with a high surface area that did not rebind well. By comparing the pore diameter of an efficient MIP with an inefficient NIP, we can conclude that for the efficient imprinting of the template caffeine the average pore diameter could be in the range of 6-7 nm. Water did not work at all and the BET results also support this fact. There was hardly any difference in specificity between the MIPs prepared in water with varied proportion of template (Figure 67). Although in general a NIP should possess a low surface area, with acetonitrile as a porogen it is greater than a MIP. This could be explained from the fact that acetonitrile as a porogen works very well in forming a macroporous polymer. The lower surface area of MIP over its NIP may be due to the trapped template molecules in the crosslinked structure of Bulk1 that could not be recovered during template removal.

Figure 67. Isotherm plot obtained from BET for MIP prepared in water with varied proportion of template (black for Bulk 11-high template, red for Bulk 2-low template and blue for Bulk 3-no template)

100

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3.1.2 Influence of crosslinking agent

MAA as a recognition unit and EGDMA as a crosslinking agent have been used for caffeine imprinting for decades. To check the necessity of these constituents, a MIP was prepared without any recognition monomer (Bulk 13) and Glycerol dimethacrylate (GDMA) was substituted for EGDMA (Bulk 14); both following the base recipe of Bulk 1 (Figure 68). In both cases the MIP had poorer recognition over the traditionally derived one and showed only one class of sites (Figure 69 & Table 7). BET measurements were performed with Bulk 13 and 14 and the results are sited graphically in Figure 70 and Figure 70.

![Figure 68. Formulation for examining the effect of crosslinking agent](image-url)
Figure 69. Effect of crosslinker on Binding constant; the template, porogen type and their proportions kept constant

Table 7. Effect of crosslinker on Site density; the template, porogen type and their proportions kept constant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Monomer and crosslinker combination</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>MAA/ EGDMA</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk 13</td>
<td>EGDMA only</td>
<td>--</td>
<td>0.24</td>
</tr>
<tr>
<td>Bulk 14</td>
<td>MAA/ GDMA</td>
<td>--</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The MIP prepared without recognition monomer showed very large surface area (Figure 70) with an average pore diameter (Figure 71) comparable to the one of an efficient MIP. This is an example that high surface area does not necessarily indicate an efficient MIP. The porous structure could be obtained from the hydrogen bonding.
interaction of caffeine and EGDMA in presence of a suitable porogen. But the monolith was not suitable to rebind its template. As we were interested in the rebinding characteristics of MIPs, this would not serve the purpose. GDMA did not work in any regards and the inefficiency of rebinding was supported by its higher average pore diameter. The surface area of the MIP prepared with GDMA was lower than a NIP prepared in ACN (Figure 72.).

![Figure 70. Surface area (m$^2$/gm) of MIPs as obtained from gas adsorption experiment](image)

Figure 70. Surface area (m$^2$/gm) of MIPs as obtained from gas adsorption experiment
Figure 71. Average pore diameter of MIPs as obtained from gas adsorption experiment

Figure 72. Isotherm data obtained from BET gas adsorption experiment (blue for Bulk 13-prepared with EGDMA w/o MAA; red for Bulk 12-a NIP by MAA/EGDMA; black for Bulk 14 by MAA/GDMA)

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We tried to evaluate N, N’ methylene bisacrylamide (MBA) in this regard as it is a water soluble monomer and produced a miscible pre-polymerization mixture. We used potassium persulfate as an initiator in complete aqueous medium polymerization. Due to the low solubility of MBA in water the required monomer: crosslinker ratio could not be reached and insufficient crosslinking did not yield a hard monolith. This was the effect of using low concentration of crosslinking agent as reported. As the product swelled while performing the washing operation, no characterization was performed with the product.

![Graphical comparison of formulation of Bulk 1, Bulk 4 & Bulk 21](image)

**Figure 73. Graphical comparison of formulation of Bulk 1, Bulk 4 & Bulk 21**

We found that Bulk 1 has an effective combination ratio for monomer, crosslinking agent and porogen ACN. For low concentration template imprinting (Bulk 4) the porogen: crosslinker (w/w) ratio was deviated (Figure 73) which showed hardly a
difference in recognition behavior over the non-imprinted one. Keeping the ratio of monomer, crosslinker and the porogen (acetonitrile) proportion close to that of Bulk 1 (Figure 73); we found that the imprinting with low template concentration also produces to have a good recognition behavior as seen in Bulk 21 (Figure 74 & Table 8). Although we found the similar site density for the specific sites for both high and low template incorporation, the confidence interval (CI) should be considered before to conclude. The CI of the experiment with Bulk 21 suffered from a higher CI (34%) in comparison with the experiment done with Bulk 1 (16%). Detail calculation of CI has been sited in Appendix F.

**Figure 74. Influence of crosslinker proportion on Binding constants**
Table 8. Effect of crosslinker proportion on site density

<table>
<thead>
<tr>
<th>Sample</th>
<th>Porogen: Crosslinker</th>
<th>Template :Monomer</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>1.33</td>
<td>0.55</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk 4</td>
<td>0.58</td>
<td>0.008</td>
<td>--</td>
<td>0.09</td>
</tr>
<tr>
<td>Bulk 21</td>
<td>1.32</td>
<td>0.005</td>
<td>0.02</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Both reduction of porogen and increase in crosslinker adversely affect the rebinding capability of Bulk 4. After achieving the correct ratio in Bulk 21, it can be concluded that it is possible to build an efficient MIP with a low concentration of template incorporation. A high concentration might lead to an aggregation of the templates and most of the templates do not participate in the self assembled complex. This rebinding result was also supported by BET measurement which showed a higher surface area (Figure 75) over the initial formulation of Bulk 1. The higher surface area could be due to more efficient extraction of templates for the MIP prepared with low proportion of analyte. However the pore diameter of Bulk 4 and Bulk 21 was found to be similar (Figure 76) and both were larger than Bulk 1.
Figure 75. Surface area (m$^2$/gm) of MIPs as obtained from gas adsorption experiment

Effect of monomer:crosslinker ratio with template proportion in the formulation

Figure 76. Average pore diameter of MIPs as obtained from gas adsorption experiment

Effect of monomer:crosslinker ratio with template proportion in the formulation
3.1.3 Examining the selectivity of MIP

To judge the selectivity of the caffeine imprinted MIP BULK1, a rebinding experiment was done with the structural analogue (Figure 77) theophylline (Figure 78 & Table 9) in the same experimental conditions. A competitive experiment (Figure 78 & Table 9) with caffeine and theophylline together was also performed to examine the competitive adsorption of the caffeine imprinted MIP.

![Caffeine and Theophylline Structures](image)

Figure 77. Structure of caffeine and theophylline

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Figure 78. Binding constant for co-adsorption of MIP prepared in ACN

Table 9. Rebinding experiment for co-adsorption of Bulk 1 at 1% MIP concentration

<table>
<thead>
<tr>
<th>MIP</th>
<th>Adsorbate</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
<th>Co-adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>Caffeine</td>
<td>0.01</td>
<td>0.12</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>0.003</td>
<td>0.16</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Caffeine</td>
<td>0.01</td>
<td>0.2</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>0.02</td>
<td>0.05</td>
<td>Y</td>
</tr>
</tbody>
</table>
From the binding constant data sited in Figure 78 it is evident that caffeine imprinted MIP is more selective to caffeine compared to its structural analogue theophylline, which differs from caffeine by only one methyl group (Figure 77). From the co-adsorption it was found that although caffeine binding efficiency was reduced, the theophylline binding efficiency was much lower than caffeine. The reduction may be due to competition between caffeine with its structural analogue (Figure 77); which might fit well into the imprinted cavity due to very similar structure. For the co-adsorption experiment the binding constant of theophylline as a guest template for specific and non-specific sites was found to be close unlike caffeine where there is a notable difference. This also explains the high selectivity of the caffeine imprinted MIP.54

Although expected to be higher, the lower concentration of non-specific sites of theophylline co-adsorption could have been resulted from the CI of the experiment which was suffered with a higher percent over caffeine co-adsorption (sited in Appendix F).

We also evaluated the MIP for selectivity which did not show specificity (for its analyte, here caffeine). Bulk 2 having one class of site was also subjected to a competitive experiment (Figure 79 & Table 10) for theophylline and its template caffeine. This non-specific MIP also showed some sort of selectivity which can be concluded from the lower binding constant of theophylline.
Figure 79. Binding constant for co-adsorption of MIP prepared in water

Table 10. Rebinding experiment for co-adsorption of Bulk 2; MIP prepared in water as a porogen

<table>
<thead>
<tr>
<th>MIP</th>
<th>Adsorbate</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
<th>Co-adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>Caffeine</td>
<td>--</td>
<td>0.06</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Theophylline</td>
<td>--</td>
<td>0.08</td>
<td>Y</td>
</tr>
</tbody>
</table>
3.1.4 Influence of solid content in rebinding experiment

An increase in solid content of a MIP in the rebinding experiment also increases the binding constant. We initially started with 1 gm. of solid suspended in 10 ml. of water for the experiment. The result is sited in Figure 80 and Table 11.

![Graph showing influence of solid content on binding constant](image)

Figure 80. Influence of solid content on Binding constant of MIP

Table 11. Influence of solid content on site density while keeping monomer: crosslinker unchanged

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solid content (mmol/gm)</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>4%</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>0.01</td>
<td>0.12</td>
</tr>
</tbody>
</table>

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The rest of the experiments were carried out at a fixed solid content of 4%, except when otherwise mentioned. Now from the comparison it is an apparent conclusion that increasing the solid content helps increase the binding constant.

Although specific site density is not dependent on solid content of the MIP as described in its measurement unit (mmol/gm), we found the dependency. This can be explained from the poor linearity of the specific region and very high confidence interval of the rebinding experiment with 10% solid content ($r^2=0.59$; CI 61%) over the same done with 4% solid content ($r^2=0.967$; CI 16%). From the comparative isotherm sited in Appendix G it can only be concluded that the increasing solid content it is possible to find more sites in the experimental range.

3.1.5 Influence of pH in rebinding study

Recognition behavior was examined for a MIP at different pH levels (Figure 81 & Table 12); acidic, basic and neutral. The pH of the washed conventional caffeine imprinted MIP was usually 5 when suspended in water. The best recognition behavior was observed at that pH.
The difference in adsorption behavior is due to the fact that the functional sites are created by the organic acid MAA. Two types of interactions mainly contribute to the binding interaction between the recognition monomer and the template. Electrostatic interaction is dominant at higher pH and hydrogen bonding interaction plays the major role.
role for binding at lower acidic pH. Since MAA is a weak acid and caffeine is a weak base, depending upon the pKa values of both there should exist an optimum pH, where both types of interactions are the most favorable.

From the pH plot shown in Figure 82 it is found that pH 7 could be the most favorable to extract the ionic interaction between caffeine and MAA. Although for higher pH we found a lower binding constant, the site density is higher. Since at higher pH all the acid groups exist in ionic form it could have the more accessibility over the hydrogen bonding. We found pH 5 is the best for extracting the highest specific binding constant. That indicates the fact that mostly hydrogen bonding plays the interaction between MAA and caffeine, but there exist some ionic forms of MAA in the cavity that can form ionic interaction with caffeine-H+.

The efficiency of a MIP is a balance between the binding constant and site density and the difference in rebinding, exploited at various pH changes within a narrow range. We chose pH 5 for all of our rebinding experiments due to the high accuracy limit (see Appendix G) at that pH and having the best binding constant extracted.

Work of Anderson91 supported the fact since he found the maximum difference in rebinding between a MIP and its corresponding NIP at that pH. Anderson adjusted the medium with a buffer that he argued to be helpful in suppressing the non-specific rebinding. The MIP we used did not demand any buffer to attain the specific pH of the medium and in a deionized water, reported as superior,17 we found reasonably well rebinding performance.

Although previous publications showed that highest frequency shift in a sensor occurred at a pH 8,13 our evaluation showed that the optimum condition for the
best rebinding strength can be achieved at a slight acidic pH of 5. The increasing site density at an alkaline pH could be the reason of highest sensitivity found in the sensor as reported.\textsuperscript{10,12,13}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{pH_curve.png}
\caption{Approximate pH curve for the interaction of caffeine and MAA}
\end{figure}

Since we explored a non-covalent interaction between the recognition site and the guest template, strong hydrogen bonding of water could have weakened the rebinding.\textsuperscript{71,69,92} The rebinding condition was not that much worse and we have evaluated some relatively high binding capacity for some of the MIPs in the aqueous medium. This shows a potential application of the MIPs in a field sensor using an environmentally friendly aqueous medium.
3.1.6 Influence of particle size in rebinding

The series of conventional MIPs prepared were of varying particle size. Since we aimed at preparing nanoparticles MIPs we tried to examine the pathway of micron to submicron range in terms of binding constant. Bulk1A to Bulk 1D MIPs (Table 13) were prepared by diluting Bulk1 with an excess amount of porogen. Although Bulk1A and Bulk 1B had low average particle diameter (Figure 83), they did not rebind well due to the reduced amount of template incorporated during imprinting. For Bulk 1C and Bulk 1D the template percentage in the polymerization mixture was adjusted to same as the Bulk 1 after dilution. All these counter formulations yielded a fluffy solid material instead of a hard monolith. The binding constants and site densities are shown in Figure 84 & Table 14. Although Bulk 1C and Bulk 1D yielded a pair of binding constants and site densities, Bulk 1A and Bulk 1B followed the single site model. Both smaller average particle size and high proportion of template imprinting showed a significant increase in binding constant. In this context, the high binding efficiency of Bulk 21 prepared with a low concentration of template imprinting can also be explained by the high percentage of smaller sized particles.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Bulk1</th>
<th>Bulk 1A</th>
<th>Bulk 1B</th>
<th>Bulk 1C</th>
<th>Bulk 1D</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA</td>
<td>5.85</td>
<td>2.75</td>
<td>1.55</td>
<td>3</td>
<td>1.7</td>
</tr>
<tr>
<td>EGDMA</td>
<td>38.85</td>
<td>18.3</td>
<td>10.3</td>
<td>18</td>
<td>10.1</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3.2</td>
<td>1.5</td>
<td>0.85</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>51.6</td>
<td>77.2</td>
<td>87.2</td>
<td>75.2</td>
<td>84.5</td>
</tr>
</tbody>
</table>

Table 13. Conventional MIP formulations with dilution of solvent
Figure 83. Particle size distribution of Bulk 1 and its counter formulations

Figure 84. Influence of dilution in Binding constants of MIP

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Table 14. Influence of dilution to site density of MIP

<table>
<thead>
<tr>
<th>Sample</th>
<th>Porogen proportion in total</th>
<th>Template proportion in total</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk1</td>
<td>~50%</td>
<td>3.2%</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk1C</td>
<td>~75%</td>
<td>3.3%</td>
<td>0.0007</td>
<td>0.066</td>
</tr>
<tr>
<td>Bulk1D</td>
<td>~85%</td>
<td>3.2%</td>
<td>0.0008</td>
<td>0.12</td>
</tr>
<tr>
<td>Bulk21</td>
<td>~50%</td>
<td>0.03%</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>Bulk1A</td>
<td>~75%</td>
<td>1.5%</td>
<td>--</td>
<td>0.09</td>
</tr>
<tr>
<td>Bulk1B</td>
<td>~85%</td>
<td>0.8%</td>
<td>--</td>
<td>0.08</td>
</tr>
</tbody>
</table>

From the above listing it is evident that diluted monomer solution creates very few specific sites with a high binding constant. Smaller particle size also yields greater surface area and helps to rebind more in specific sites. Moving towards more dilution from Bulk1C to Bulk 1D increases the site density but decreases the binding efficiency. This could result from the adverse effect of the presence of more porogen in the system and we might expect a decrease in efficiency of nanoparticles as the dilution is higher during polymerization. This result is in accordance with the work sited by Mosbach et al.19

BET gas adsorption experiment was performed (Figure 85 & Figure 86). Bulk 1A and Bulk 1B, which were produced with a lower template concentration did not rebind well. This finding was supported by their larger pore diameter. The conclusion can be further supported by comparing Bulk 21 with Bulk 1A and Bulk 1B. Although Bulk 21 was prepared with a low template concentration, it was not from a diluted
polymerization mixture and showed a more efficient imprinting than Bulk 1A and Bulk 1B (Figure 87). Bulk 1C and 1D was successfully imprinted as evident from the pore size, although the surface area found was somewhat lower than Bulk 1. The lowering of guest analyte binding from Bulk 1C to Bulk 1D was supported by the lowered surface area as indicated by BET gas adsorption (Figure 88).

![BET Surface area](image)

**Figure 85.** Influence of dilution in Surface area (m²/gm) of MIPs as obtained from gas adsorption experiment

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Figure 86. Influence of dilution in Average pore diameter of MIPs as obtained from gas adsorption experiment

Figure 87. Gas adsorption isotherm as obtained from BET experiment for imprinting with a low concentration of template (Blue is for Bulk 21-prepared with 50% porogen; red is for Bulk 1A-prepared with 75% porogen; black is for Bulk 1B-prepared with 85% porogen)
3.1.7 Time dependent rebinding study

A kinetics study for the rebinding of analyte was performed. An adsorption kinetics measurement of a conventional MIP was performed by suspending a given amount of solid in water and introducing 100 µl aliquot of analyte stock solution in it. The mixture was subjected to constant agitation and the supernatant was taken out after several time intervals. The concentration of the bound analyte was calculated from the free analyte concentration as obtained from the HPLC detection.

The response time of conventional MIPs to reach saturation varied depending on its particle size and to some extent its recognition capability. Bulk 1, which proved to have a fair recognition, had a response time of around 5 hours; whereas Bulk 2 having a poor recognition had a response time of around 1 day (Figure 89). Both Bulk 1

Figure 88. Gas adsorption comparative isotherm obtained from BET (red for Bulk 1C-MIP prepared in 75% porogen and blue for Bulk 1D-MIP prepared in 85% porogen)
and bulk 2 possessed a similar distribution of particle size though. Thus better specificity could give rise to a faster response.

An efficient MIP with a finer distribution of particle size can be expected to have a faster response, as reported. Bulk 1C having a high binding constant had a response time of few minutes (Figure 89). This phenomenon can be attributed to the finer particle size distribution of Bulk 1C (Figure 83). Due to further lowering of particle size, the response time for nanoparticles (Figure 89) was in between few minutes as expected.

![Graph showing time dependent adsorption of caffeine imprinted MIPs](image)

**Figure 89. Time dependent adsorption of caffeine imprinted MIPs**

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3.1.8 Formulation of nanoparticles MIP

A high concentration of crosslinking monomer is required for conventional monolith preparation. The phase separation occurs during the cross-linking process and the growing polymer chain phase separates out when it reaches the limit of solubility in the reaction medium. If the amount of the solvent is gradually increased in the formulation, it causes the growing chain to be unable to occupy the entire reactor volume and a dispersion of macrogel particles is obtained. Further dilution leads to a decrease in the size of the gel particles and discrete microgels can be formed (Figure 90).22,39,46,48

Based on the convincing imprinting effect we obtained via the conventional method, we initiated the preparation of the nanoparticles MIPs in a similar polymer system and against the same template caffeine. For the rebinding study the microspheres thus obtained were centrifuged and dried as reported by others.39,48

Figure 90. A scheme showing the change of polymer structure from macroporous monolith to microgel by dilution of the reaction solution22

The microgel (MIP nanoparticles) was produced by diluting the polymerization mixture with the porogen acetonitrile, which as reported can produce satisfactory microspheres with high yield.39 The nanoparticles were prepared using the same molar ratio of MAA: EGDMA similar to monolith Bulk 1, except that porogen
proportion was increased to 95% of the total constituents.\textsuperscript{19,22} MIPs were prepared by using both the high and low concentration of the template caffeine (Table 15). Starting from this diluted monomer solution stable latex was obtained in acetonitrile and by ultrafiltration technique, template was removed by replacing the medium with water. CTAB (Cetyl Trimethyl Ammonium Bromide), at a concentration below its CMC was used to stabilize the nanoparticles in water. Two types of rebinding experiment were performed. In one method, particles were dried and cleaned by soxhlet where no CTAB was used. Dried particles were then treated as a conventional MIP for characterization. In another method stable latex in water with surfactant CTAB was analyzed by rebinding experiment as stated in the experimental section.

Table 15. Formulation of nanoparticles latex against conventional MIP

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Bulk 1</th>
<th>MJB 58</th>
<th>MJB 68</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAA</td>
<td>5.85</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>EGDMA</td>
<td>38.85</td>
<td>2.16</td>
<td>2.11</td>
</tr>
<tr>
<td>Caffeine</td>
<td>3.19</td>
<td>0.97</td>
<td>3.26</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>51.62</td>
<td>96.4</td>
<td>94.19</td>
</tr>
</tbody>
</table>

Rebinding experiment (Figure 91 & Table 16) apparently suggests that increasing porogen influence after a certain limit (as in Bulk 1C) hinders the efficient imprinting. These binding constants were evaluated at the same experimental condition and same concentration ranges of guest analyte. Prepared with same proportion of template, increasing porogen beyond the proportion used in Bulk 1C decreases the binding constant for the specific sites created. Increasing the proportion of diluent from
Bulk 1C to Bulk 1D and then to MJB 68 (formed as latex) this lowering of binding constant was evident. For MJB 58 which was prepared with a low concentration of template, two classes of sites can be found but with a lower binding constant than MJB 68. MIP prepared with a low template concentration (as in MJB 58) produces a very high concentration of non-specific sites with a very low binding constant when compared to the MIP prepared with high proportion of template. Thus excess amount of porogen forms high density of non-specific site and only a very few specific sites. Although we need to consider the confidence interval of the rebinding experiment of MJB 58, which due to the poor linearity of the experimental points in the specific region, was not feasible to estimate.

Increasing the template proportion in the polymerization mixture increases the specific site density and decrease the non-specific type. The same phenomenon was reported by Mosbach et al.\textsuperscript{19} for their microsphere manipulation.
Figure 91. Binding constant comparison for conventional MIP with latex in dried form

Table 16. Site density comparison for conventional MIP with latex

<table>
<thead>
<tr>
<th>Sample</th>
<th>Porogen proportion in total</th>
<th>Template proportion in total</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk1</td>
<td>~50%</td>
<td>3.2%</td>
<td>0.02</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk1C</td>
<td>~75%</td>
<td>3.3%</td>
<td>0.0007</td>
<td>0.066</td>
</tr>
<tr>
<td>Bulk1D</td>
<td>~85%</td>
<td>3.2%</td>
<td>0.0008</td>
<td>0.12</td>
</tr>
<tr>
<td>MJB 68</td>
<td>~95%</td>
<td>3.3%</td>
<td>0.01</td>
<td>0.24</td>
</tr>
<tr>
<td>MJB 58</td>
<td>~95%</td>
<td>0.97%</td>
<td>0.003</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Although both for Bulk 1C and Bulk 1D we determined a high binding constant, the site density for the specific was found in a low concentration. Keeping this fact in mind that efficient MIP should possess a high binding constant for relatively high concentration of site density, Bulk 1 could be considered so far to be the best one due to the combination of both. Now if we compare MJB 68 with Bulk 1, we can justify the advantages of forming nanoparticles to some extent. MJB 68 (nanoparticles) possesses a higher binding constant than Bulk 1 (conventional monolith) for half of the site density of Bulk 1. Even MJB 58, which was prepared with a low concentration of template, showed a higher binding efficiency over Bulk 1. The site density was reduced though. This lowering of site density was contrasting with the previous studies reported.39,46

In comparison with a monolith MIP, which was prepared with a very low concentration of template, we found a site density compared to the MIP prepared with high concentration of template. Nanoparticles did not show this advantage although expected. The low concentration of template that was dispersed in excess proportion of porogen was unable to imprint sufficiently.

The above result was sited when the latex was separated from its medium and treated as a conventional MIP for characterization. The latex stabilized with CTAB when subjected to rebinding experiment showed an inferior result. Although two classes of sites were extracted from the analysis, the binding constant for the specific sites was in a very low range (Figure 92 & Table 17). This variation of result could be from the influence of CTAB adsorption in the sites or due to experimental constraint. Using the surfactant in a lower concentration than its critical micelle concentration (CMC) might discard the first assumption, but we need further investigation to confirm the fact.
Experimentally for a rebinding analysis we were supposed to operate the condition in a constant volume to keep the MIP concentration identical throughout the analysis. Since in most of the cases the introduced volume of the analyte solution and the volume taken out were not identical, that made the MIP concentration varied. Although the variation was in a low range during manipulation with a conventional MIP suspended in water, it was in a long range for a latex with low solid content. Consequently, the eventual reduction of the MIP concentration in a latex might have produced some erroneous result.

![Bar chart showing specific and non-specific binding constants for MJB 58-low template and MJB 68-High template.](image)

**Figure 92. Binding constant of latex characterized with individual latex form**

**Table 17. Site density of latex characterized with individual latex form**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Porogen proportion in total</th>
<th>Template proportion in total</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MJB 68</td>
<td>~95%</td>
<td>3.3%</td>
<td>0.008</td>
<td>0.02</td>
</tr>
<tr>
<td>MJB 58</td>
<td>~95%</td>
<td>0.97</td>
<td>0.0003</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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The latex MIP prepared with high proportion of template was dried and surface area measurement was performed by BET gas adsorption. Since nanoparticles without its medium often looses its integrity, this experiment might be argued as a crude assumption. Still a general idea can be obtained for discussion. Two types of samples were analyzed; one was before template removal, and the other one was after removal of the template. The template was removed by two techniques; one was by ultrafiltration operation where CTAB was used for stabilization and the other one was by soxhlet operation for dried particles without any additional stabilizer. A low surface area (21 m$^2$/gm) and a high pore diameter (21 nm) were obtained for the particles with template as expected. But after the template removal the surface area did not show a significant increase. The surface area was measured as 64.5 m$^2$/gm with a 20 nm pore size. The low surface area and no change in pore size distribution before and after removal of the template might point out the less efficient imprinting in excess of porogen compared to a conventional monolith. In contrast, similar range of surface area for imprinted microspheres with a high specificity and selectivity was reported in literatures.$^{39,40}$ In the experimental range no surface area was determined for the particles combined with CTAB. The visual comparison of isotherm implies the fact that the surface area is lower than the particles with template (Figure 93). This operation might generate the idea of the adverse effect of CTAB as a stabilizer.
Figure 93. Isotherm plot for MJB 68 as obtained from BET gas adsorption experiment for dried nanoparticles (black for MIP particles with template; red for MIP particles cleaned by soxhlet; blue for MIP particles cleaned by ultrafiltration with CTAB)

All the results from rebinding experiments were supported by possible explanations. Except for the few cases where the critical discussions have not been provided, can be explained by the higher confidence interval of the experiment as sited in Appendix F calculated at 95% interval.

The variation of the specific site density with binding constant is shown (Figure 94) for the well developed MIP structure. The single reported data from rebinding experiment as sited by Wang et al. 49 follows our data trend. The trend as shown by its fit following a relation of \( y = A \left( \frac{1}{x} \right) \) \{where, \( A \) is a constant\} also indicates that for an efficient MIP there is always a balance between the specific binding constant and site density.
Figure 94. Trend of specific binding constant with its specific site density for well developed MIP structure along with the data reported in literature. Error bars are shown for both parameters as calculated for confidence interval at a 95% confidence limit.
3.2 **MIP imprinted with guanosine as a template**

Based on the initial work on caffeine imprinted MIP the same technique was applied to prepare guanosine imprinted MIP. Due to the similarity in structure with saxitoxin (Figure 95), guanosine was selected.

![Saxitoxin and Guanosine Structures](image)

**Figure 95. Comparison of structure of saxitoxin and guanosine**

Before starting the manipulation with the potent neurotoxin, we chose guanosine as a template for producing a MIP.\(^{105}\) Caffeine is soluble in wide range of solvents and that makes easier to select its porogen. On the contrary, guanosine is soluble in water but at a low concentration. Later on we found DMSO as an alternative solvent. On the other hand guanosine was found only sparingly soluble in the well proved porogen acetonitrile.

Although wide range of literature exists on caffeine imprinted MIPs and that helped us to choose its recognition monomer; for guanosine it was a challenge. We worked with different functional monomers and porogens to make a successfully imprinted MIP against guanosine. The template from the imprinted conventional MIP was washed by Soxhlet extraction with water and methanol respectively.

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3.2.1 Choice of functional monomer and porogen

3.2.1.1 Water as a porogen

We started our manipulation of guanosine imprinted MIP with the well known solvent of guanosine, which is water. Based on this, we prepared few MIPs exploring the non-covalent interaction between various recognition monomer and guanosine. Sreenivasan et. al.\textsuperscript{93} evaluated the imprinting of creatinine (Figure 96) with the functional monomers MAA, N-vinyl pyrrolidine and HEMA in a mixture of water and methanol as porogen. EGDMA was used as a crosslinking agent and the MIP imprinted with HEMA was found to have a high selectivity over creatine (Figure 96). Finding some similarity in structure with creatinine, following the formulation they sited guanosine was subjected to imprint with HEMA. The porogen proportion was adjusted (Table 18) such that a miscible polymerization mixture\textsuperscript{41} can be formed.

Figure 96. Structure of creatinine and creatine respectively

\[ \text{Figure 96. Structure of creatinine and creatine respectively} \]
Table 18. Formulation of guanosine imprinted conventional MIP in water

<table>
<thead>
<tr>
<th>Recipe in %</th>
<th>Bulk 5</th>
<th>Bulk 7</th>
<th>Bulk 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEMA</td>
<td>7.5808</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>--</td>
<td>8.66</td>
<td>--</td>
</tr>
<tr>
<td>Crown Ether</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Modified Cytidine</td>
<td>--</td>
<td>--</td>
<td>6.69</td>
</tr>
<tr>
<td>EGDMA</td>
<td>89.5922</td>
<td>56.39</td>
<td>49.55</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.0014</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Water: Methanol</td>
<td>1.723</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(1:1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>--</td>
<td>32.70</td>
<td>42.15</td>
</tr>
</tbody>
</table>

3.2.1.1.1 Crown ether as a recognition unit

We exploited the fact that STX is an ion-channel disruptor and can be recognized by crown ethers. Thus an acrylamide monomer bearing crown ether was synthesized to implement as a recognition unit for saxitoxin (Figure 97) and was first applied against guanosine (Table 18).

Figure 97. Saxitoxin – crown ether complex, molecular model using MM2 force field
Synthesis of N-acrylamido aza-18-crown-6-ether

The reaction scheme is sit in Figure 98. Anhydrous K$_2$CO$_3$ was desiccated in vacuum at 80°C for 20 hours and used immediately after. Toluene was dried by distillation over calcium hydride. A solution of acryloyl chloride (0.33g, 3.63 mmol) in15 ml of dried toluene is added drop-wise to a stirred mixture of aza-18-crown-6 (0.96g, 3.63mmol) and powdered K$_2$CO$_3$ (1.00g, 7.26 mmol) in 50 ml. of dried toluene. It was then stirred for 16 hours with a magnetic stir bar, the mixture was filtered on suction pump by using fritted-glass filter “C” and the filtrate was concentrated by evaporation by using high-vacuum rotary evaporator at a pressure of 10 torr and at a temperature of 15°C to get an oily liquid. The oil was kept in the refrigerator (it can auto-polymerize). Yield: 1.1500g = 100 %. 1H NMR (C$_6$D$_6$, 360MHz ): 3.2-3.8 (m, NCH$_2$CH$_2$O, OCH$_2$CH$_2$O); 5.3 - 5.4 -CH-CO-( J= 9.5Hz); 6.4-6.6 CH$_2$=C- (dd J= 9.5 Hz, J= 2-3 Hz ) 13C NMR (C$_6$D$_6$, 100 MHz ); 167.02 (-NCO-); 127.26 ( CH$_2$ = ) 128.88 (-CH= ) ; 71.45, 71.19, 71.06, 70.83, 70.42 (NCH$_2$CH$_2$O, OCH$_2$CH$_2$O); 49.41, 48.03 (NCH$_2$CH$_2$O).

Figure 98. Synthesis of the acrylamide crown ether monomer as recognition unit of guanosine and STX.
3.2.1.1.2 Cytidine as a recognition unit

It was thought\textsuperscript{105} that the perfect molecule to imprint guanosine could be cytidine. It was believed that this recognition unit could be capable of competing with water for its hydrogen bonds. The recognition unit based on cytidine was synthesized in presence of acryloyl chloride. The MIP was prepared with EGDMA as a crosslinking agent in the water as a porogen. The polymerization mixture was not homogeneous similar to the solution where crown ether was used as a recognition monomer.

Similar to a caffeine imprinted polymer, guanosine imprinted conventional MIPs were ground to yield smaller sized particles. Following the same experimental set up as mentioned earlier, those were characterized by a rebinding study (Figure 99 & Table 19) and was also evaluated by the gas adsorption technique (Figure 100).

![Figure 99. Binding constant for guanosine imprinted MIP prepared in the porogen water with varied type of recognition monomer](image-url)

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Table 19. Site density for guanosine imprinted MIP prepared in the porogen water with varied type of recognition monomer

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recognition monomer</th>
<th>Template proportion in total</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 5</td>
<td>HEMA</td>
<td>0.001%</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Bulk 7</td>
<td>Crown ether</td>
<td>0.03%</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Bulk 9</td>
<td>Cytidine</td>
<td>0.04%</td>
<td>--</td>
<td>0.1</td>
</tr>
</tbody>
</table>

None of the MIPs worked where water was used as a porogen, which was evident from the low binding constant values. Only one class of sites was created and based on the previous theoretical discussions those were referred as non-specific sites. Hence a mono-Langmuir model was fitted with the experimentally obtained isotherm and binding constant was determined as explained before. The water having a strong hydrogen bond hindered the non-covalent interaction between a recognition monomer and the template. Thus the MIPs thus produced were not found efficient. For HEMA as a monomer, very low porogen proportion would have yielded hard structure, which prevented the rebinding. For crown ether as a recognition unit although electrostatic interaction was explored, a heterogeneous polymerization mixture affected adversely. Modified cytidine worked better over other two, but the binding constant was too low to be assigned as an efficient MIP.

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Figure 100. Result from gas adsorption experiment for MIP prepared in water

For the MIPs prepared in water the result from the rebinding study was supported by the gas adsorption result. The surface area of the MIPs were negligible similar to a non-porous material. The large pore diameter did not also imply efficient imprinting. The gas adsorption technique was not able to evaluate Bulk 7 due to some unknown reason.

3.2.1.2 Acetonitrile as a porogen

Since acetonitrile worked well in our initial work with caffeine imprinted MIP, we tried to evaluate the same for guanosine as a template. Negligible solubility of guanosine was the problem. To obtain a homogeneous mixture before polymerization low concentration of template was chosen with HEMA, MAA and
acrylamide crown ether as a separately used recognition monomer. The monomer:
crosslinker ratio was kept constant similar to the caffeine imprinted MIP Bulk1. The
porogen proportion was increased to obtain a significant amount of template soluble.
Identical formulation (Table 20) was employed for all the recognition monomers chosen
and following the standard experimental techniques, as described previously, the MIPs
were characterized.

Table 20. Formulation of guanosine imprinted conventional MIP in ACN

<table>
<thead>
<tr>
<th>Ingredients in %</th>
<th>Bulk 8</th>
<th>Bulk 10</th>
<th>Bulk 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEMA</td>
<td>--</td>
<td>4.33</td>
<td>--</td>
</tr>
<tr>
<td>Acrylamide Crown Ether</td>
<td>4.33</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>MAA</td>
<td>--</td>
<td>--</td>
<td>4.33</td>
</tr>
<tr>
<td>EGDMA</td>
<td>28.20</td>
<td>28.20</td>
<td>28.20</td>
</tr>
<tr>
<td>Guanosine</td>
<td>0.003</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>66.36</td>
<td>66.36</td>
<td>66.36</td>
</tr>
</tbody>
</table>

Only one class of non-specific sites was obtained from the rebinding study (Figure 101 & Table 21). This might suggest that a very low concentration of template is not sufficient for a successful imprinting. MAA worked better among others but only one class of sites was found in the experimental range.
Figure 101. Binding constant for guanosine imprinted MIP prepared with varied type of recognition monomer in the porogen ACN but keeping other constituents and proportions constant

Table 21. Site density for guanosine imprinted MIP prepared with varied type of recognition monomer in the porogen ACN but keeping other constituents and proportions constant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recognition monomer</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 8</td>
<td>Crown ether</td>
<td>--</td>
<td>0.06</td>
</tr>
<tr>
<td>Bulk 10</td>
<td>HEMA</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Bulk 18</td>
<td>MAA</td>
<td>--</td>
<td>0.03</td>
</tr>
</tbody>
</table>

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Reasonably large surface areas were obtained from the gas adsorption experiment (Figure 102) especially for both the MIPs prepared with the recognition monomer HEMA and MAA. This could be an influence of the effective porogen acetonitrile. This can be explained due to the fact of producing similar phenomenon for a NIP with higher surface area than an efficient caffeine imprinted MIP. A 6-7 nm of average pore diameter for these guanosine imprinted MIPs might suggest a good imprinting. Thus it can be concluded that imprinting occurred in presence of acetonitrile as the porogen, but that could be with very few sites and it was not possible to extract these data in our experimental range.

Figure 102. Result from gas adsorption experiment for MIP prepared in ACN
3.2.1.3 DMSO as a porogen

DMSO was found to be a good solvent for guanosine and we tried to evaluate (Table 22) whether it could act as an efficient porogen or not. With the functional monomer MAA, HEMA and acrylamide crown ether, MIPs with a higher proportion of template was prepared by following the formulation of the caffeine imprinted conventional MIP Bulk1. MIP with a lower template concentration (Bulk 16) was also prepared by keeping the other ingredient ratio similar to Bulk 1. A NIP (Bulk 17) was also prepared for comparison.

Table 22. Formulation of guanosine imprinted conventional MIP in DMSO

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MJB 48</td>
</tr>
<tr>
<td>MAA</td>
<td>5.85</td>
</tr>
<tr>
<td>HEMA</td>
<td>--</td>
</tr>
<tr>
<td>Crown ether</td>
<td>--</td>
</tr>
<tr>
<td>EGDMA</td>
<td>38.85</td>
</tr>
<tr>
<td>Guanosine</td>
<td>3.2</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>51.62</td>
</tr>
</tbody>
</table>

From the rebinding study (Figure 103 & Table 23) it was quite evident that DMSO did not work at all as a porogen. This phenomenon observed for DMSO was also supported by Turner et al. during their study on MIP beads. The result from the rebinding study as obtained for the MIPs prepared with the porogen DMSO was

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erratic in nature. Crown-ether worked to some extent over other monomers but still it had only one class of non-specific sites.

Figure 103. Binding constant comparison for guanosine imprinted MIP prepared in DMSO with varied type of recognition monomer and with varied proportion of template

Table 23. Site density comparison for guanosine imprinted MIP prepared in DMSO with varied type of recognition monomer and with varied proportion of template

<table>
<thead>
<tr>
<th>Sample</th>
<th>Recognition monomer</th>
<th>Template proportion in total</th>
<th>Specific sites (mmol/gm)</th>
<th>Non-specific sites (mmol/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk15</td>
<td>HEMA</td>
<td>3.2%</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Bulk20</td>
<td>Crown ether</td>
<td>3.2%</td>
<td>--</td>
<td>0.01</td>
</tr>
<tr>
<td>MJB48</td>
<td>MAA</td>
<td>3.2%</td>
<td>--</td>
<td>0.04</td>
</tr>
<tr>
<td>Bulk16</td>
<td>MAA</td>
<td>0.08%</td>
<td>--</td>
<td>0.08</td>
</tr>
<tr>
<td>Bulk17</td>
<td>MAA</td>
<td>--</td>
<td>--</td>
<td>0.85</td>
</tr>
</tbody>
</table>
Although rebinding of an analyte for the MIPs prepared in DMSO merely worked, but the surface area as found from the gas adsorption experiment (Figure 104) contradicts the result. All the MIPs as well as the NIP prepared in DMSO had a high surface area. DMSO might have created a porous polymer but for the rebinding study we can not assign those as efficient imprint. The comparable surface area of all the products may suggest the fact that the surface area was generated from the influence of the porogen and the template did not have any influence on that.

![Figure 104. Gas adsorption experiment for MIP prepared in DMSO](image)

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3.2.1.4 DMSO-Acetonitrile a mixture of porogen

Based on the previous experiments, we tried to explore the effective porogenic property of acetonitrile along with the good solubility power of DMSO. We assumed a mixture of both (Figure 105) could work well for guanosine imprinted polymer with a recognition monomer MAA.

![Guanosine imprinted MIP in mixture of porogen](image)

**Figure 105. Formulation of Guanosine imprinted conventional MIP Bulk19**

Characterization by rebinding study (Table 24) could not extract any specific site though. From the gas adsorption technique (Table 25) the resulting MIP was found to have a low surface area.

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Table 24. Results from rebinding experiment of Bulk 19

<table>
<thead>
<tr>
<th>MIP with porogen mixture of ACN &amp; DMSO</th>
<th>Recognition monomer</th>
<th>Guanosine as an adsorbate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 19</td>
<td>MAA</td>
<td>346</td>
</tr>
</tbody>
</table>

Table 25. Result from gas adsorption experiment of Bulk 19

<table>
<thead>
<tr>
<th>MIP with ACN+DMSO</th>
<th>Template concentration</th>
<th>BET Surface area (m²/gm.)</th>
<th>Adsorption Average Pore Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 19</td>
<td>Low</td>
<td>69.5</td>
<td>11.8</td>
</tr>
</tbody>
</table>

3.2.2 Time dependent adsorption study

The time dependent adsorption study of guanosine imprinted MIPs did not show any specific trend in their response. This could be due to the unsuccessful imprinting. The response time varied from a few to several hours (Figure 106) for comparable size distribution in micron range (see Appendix C for the particle size distribution).

Imprinting of guanosine still remained a challenge due to the absence of a suitable porogen. Finding a good porogen can resolve the issue and testing can be done by selecting an appropriate recognition monomer. Careful consideration should be given to the rebinding study. Since the guest analyte guanosine has a low solubility in water, during introducing an incremental amount of the analyte the concentration of the MIP solution could be varied in a long range. This could produce an erroneous result.
Figure 106. Kinetics measurement of guanosine imprinted polymers
Based on the experimental work with caffeine as a template we built a non-linear mathematical model. A Neural Network (NN) can forecast a set of output variables based on a set of input variables. We intended using the NN to identify a formulation of caffeine imprinted MIP having a better binding constant in our experimental range with a higher concentration of site density. In our earlier experimental work we mostly found high specific site densities with a relatively low binding constants (Bulk 1 in chapter 3.1.1) and higher binding constant with lower site densities (Bulk 1C or Bulk 1D in chapter 3.1.6). Here we investigate if both parameters could be reached to a high limit. To do so, we selected those MIPs having specific binding sites and built a ‘Neural Network’ based on these results. The MIP formulations having only ‘general’ binding sites, like the MIPs with only non-specific types of sites and the control polymers, i.e. the NIPs were excluded from the analysis. The MIPs having ‘specific’ binding sites were the prime focus.

In order to build a NN, a large set of experimental results are need to ‘train’ the NN. ‘Training’ is the actual determination of all the non-linear parameters of the NN. We excluded experimental results where only a single input variable change was done. For example, we could not include the adsorption experiment where a single experiment was run in higher solid content and it also excluded the single efficient MIP obtained in presence of PEGDME as the porogen. This restricted our training data set to the MIPs prepared in the porogen acetonitrile, where a variety of conditions were employed. The constant elements conditions for the MIPs considered in the NN training are listed in Table 26.
Table 26. Constant conditions for the MIPs considered in the network

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator</td>
<td>AIBN</td>
</tr>
<tr>
<td>Recognition monomer</td>
<td>MAA</td>
</tr>
<tr>
<td>Crosslinker</td>
<td>EGDMA</td>
</tr>
<tr>
<td>Porogen</td>
<td>ACN</td>
</tr>
<tr>
<td>Host and guest template</td>
<td>Caffeine</td>
</tr>
<tr>
<td><strong>Solid content in rebinding</strong></td>
<td>4%</td>
</tr>
<tr>
<td><strong>experiment</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Operating temperature of rebinding</strong></td>
<td>25°C</td>
</tr>
</tbody>
</table>

4.1 Neural network

A neural network is inspired by the way the biological nervous systems, such as the brain, process information. An artificial neural network (ANN) or simply just neural network (NN) is a group of interconnected artificial neurons and can process information by using a mathematical or computational model. It is a non-linear statistical data modeling tool and is used to build complex relationships between the inputs and the outputs or to find a pattern of the data. It operates in a mode of many inputs and one output. The most common type of ANN consists of three neuron layers. The input layer of elements is connected to a hidden layer of elements which in turn is connected to the output layer of neuron elements (Figure 107).
4.1.1 Application of Neural network model

One major factor in the consideration of this model is its ability to forecast.

Since our experimental data was fitted with a bi-Langmuir model to obtain the binding constant \( b \) and the site density \( q_s \) of the MIPs, we would like to use a NN to predict \( b \) and \( q_s \) for other MIP formulations.

We had a limited amount of data to form a successful network, but we had an attempt to forecast a formulation based on the given conditions.

We identified four independent factors that affect the binding constant and the site density of a specific MIP. These four factors are the four inputs for our neural network. Three of those are the different constituent proportions with which the MIPs were prepared: percentage of the template, porogen and the crosslinker. The other one is one of the experimental conditions in which the rebinding experiment was performed, the
pH of the medium. All four inputs for different samples are listed. For an easy reference the chapter number of the samples is also mentioned.

Table 27. Neural network matrix

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Sample</th>
<th>Template (%)</th>
<th>Porogen (%)</th>
<th>Cross-linker (%)</th>
<th>pH</th>
<th>Specific K_{sp} (l/mol)</th>
<th>fraction of specific sites</th>
<th>bq_s</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1</td>
<td>Bulk1</td>
<td>3.20</td>
<td>51.60</td>
<td>38.85</td>
<td>5</td>
<td>1874</td>
<td>0.232</td>
<td>1.67</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Bulk2</td>
<td>0.03</td>
<td>53.00</td>
<td>40.30</td>
<td>5</td>
<td>1095</td>
<td>0.11</td>
<td>1.045</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Bulk1</td>
<td>3.20</td>
<td>51.60</td>
<td>38.85</td>
<td>3</td>
<td>1010</td>
<td>0.147</td>
<td>1.66</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Bulk1</td>
<td>3.20</td>
<td>51.60</td>
<td>38.85</td>
<td>7.5</td>
<td>576</td>
<td>0.235</td>
<td>0.87</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Bulk1</td>
<td>3.20</td>
<td>51.60</td>
<td>38.85</td>
<td>9.5</td>
<td>518</td>
<td>0.25</td>
<td>0.94</td>
</tr>
<tr>
<td>3.1.6</td>
<td>Bulk1C</td>
<td>3.30</td>
<td>75.20</td>
<td>18.00</td>
<td>5</td>
<td>31500</td>
<td>0.007</td>
<td>0.946</td>
</tr>
<tr>
<td>3.1.6</td>
<td>Bulk1D</td>
<td>3.2</td>
<td>84.5</td>
<td>10.1</td>
<td>5</td>
<td>21956</td>
<td>0.007</td>
<td>0.72</td>
</tr>
<tr>
<td>3.1.8</td>
<td>MJB58</td>
<td>0.97</td>
<td>96.40</td>
<td>2.20</td>
<td>5</td>
<td>2494</td>
<td>0.0006</td>
<td>0.33</td>
</tr>
<tr>
<td>3.1.8</td>
<td>MJB68</td>
<td>3.30</td>
<td>94.20</td>
<td>2.10</td>
<td>5</td>
<td>4170.6</td>
<td>0.04</td>
<td>1.69</td>
</tr>
</tbody>
</table>

We used the neural network software FORECASTER XL™ from ALYUDA Research, Inc. This is a MS Excel add-in program that uses neural network method for forecasting and data analysis. By using the program we formed independently two NNs to forecast the specific binding constant ($K_{sp}$ in l/mol) and the fraction of the specific sites [$q_{sp}/(q_{sp}+q_{non-sp})$] with respect to the total sites. We also attempted to forecast the combination of the specific binding constant and site density.
with the term $bq$, which basically is the initial slope of the bi-Langmuir isotherm plotted. An efficient MIP should have a combination of high binding constant with a high site density and we tried to account for that possibility in our forecast. In this way, we have four inputs for nine sets of data and three outputs. However, each output was evaluated independently. The program recommends the data sets should be at least three times larger than the number of inputs ($3 \times 4 = 12$), thus we were below that limit (9). Due to the lack of data set we often faced the problem of bad forecasts. Thus we attempted to combine all the patterns obtained for different outputs, and from a manual observation we examined the feasibility of formulating a better MIP.

4.1.2 Neural network model analysis

The only task for the modeling is to assemble the input and output matrix. All calculations (training and evaluation) are performed automatically by excel and forecaster. For each NN trained, the program gave a report.

4.1.2.1 Neural Network Model analysis for specific binding constant

For the prediction of specific binding constant the deviation from the actual to forecasted value (Figure 108) was acceptable except for the initial rows. Within the limitation of a meager experimental data set this was a reasonable network model for the prediction of specific binding conditions.

Figure 109 shows the influence of the input parameters on specific binding constant and both porogen and crosslinker proportions are found to have significant influence on that. The pH and template proportion in the polymerization mixture did not show any effect.

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Figure 108. The actual vs. forecast for the neural network for predicting specific binding constant

Figure 109. Input importance for each parameter in the network for predicting specific binding constant
Neural networks are non-linear mapping structures suitable for forecasting because of their approximation ability for generalization. We attempted to identify forecast trends by changing one variable while keeping others constant. We forecasted the increasing template fraction (Figure 110) in a MIP formulation and it showed an increase in binding constant. Because the importance level of this input is low, the variation has a narrow range.

![Figure 110. Forecasted effect of increasing template proportion in a MIP formulation for specific binding constant](image)

Forecasting for the increasing porogen proportion (Figure 111) in a MIP formulation showed a significant effect on specific binding constant. There is an optimum proportion (around 75%) and above that the output value showed a gradual reduction.
The crosslinking agent, which has a significant effect on specific binding constant, forecasted a gradual reduction (Figure 112) after a certain percentage (around 10%). The pattern implies that the lower the proportion of the crosslinking agent the greater the binding constant.

![Graph showing forecasted effect of increasing porogen proportion in MIP formulation for specific binding constant.]

**Figure 111.** Forecasted effect of increasing porogen proportion in MIP formulation for specific binding constant
Figure 112. Forecasted effect of increasing crosslinker proportion in MIP formulation for specific binding constant

Figure 113. Forecasted effect of pH in rebinding experiment for specific binding constant
A pH forecast did not show any significant importance in determining binding constant, the forecasted pattern varies only in a narrow range and shows a gradual decrease with increasing pH.

**4.1.2.2 Neural Network Model analysis for fraction of specific sites**

In order to predict the fraction of specific sites by the neural network the program also visualized the variation of forecasted value from the actual one (Figure 114). The network formed was once again reasonable with a few variations only.

The forecasting of the fraction of specific sites (Figure 115) showed that the percentage of the template and porogen in the MIP formulation was similar in importance to that of the pH of the rebinding medium. The crosslinker proportion was found to be the most important in the formation of the specific binding sites.

Based on the network for the output of fraction of specific sites, we attempted to forecast the pattern of the inputs. The graphs for the patterns are listed in the appendix G section. All of the forecasted patterns showed a well maintained trend. One condition was varied at a time, keeping other factors constant. Increase in the template and crosslinker proportion in a MIP formulation and pH of the medium in rebinding experiment showed an increase in the specific site density. But increasing porogen proportion indicated a reduction in the fraction of specific sites.
Figure 114. The actual vs. forecast for the neural network for predicting fraction of specific sites

Figure 115. Input importance for each parameter in the network for predicting fraction of specific sites

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4.1.2.3 Neural Network Model analysis for \( b_{qs} \)

The deviation from the actual and forecasted value for the combination of the binding constant and site density for the specific sites is shown in Figure 116. We found some deviations in results but also found some close matches.

The input importance for forecasting \( b_{qs} \) (Figure 117) showed a similar pattern as obtained for site density. Crosslinker proportion had the foremost importance and other inputs have a close ranged significance.

![Actual vs. Forecasted](image)

*Figure 116. The actual vs. forecast for the neural network for predicting \( b_{qs} \)*
Figure 117. Input importance for each parameter in the network for predicting $bq_s$

From the network obtained we built the pattern by which the output $bq_s$ varies with the variation of a single input. The patterns in a graphical form are listed in the appendix G section. Gradual reduction of $bq_s$ observed with increasing porogen proportion. For other inputs a range of optimum condition was obtained. At a template concentration of 1% or higher, the output parameter is close to saturation. For the crosslinker proportion it showed an optimum level of 30% where the maximum $bq_s$ was forecasted. The maximum output value was obtained at a pH of 3.5-4 of the rebinding medium.
Since the neural network works in a given experimental range it can predict the optimum condition only in that range. From the summary of the forecasts (Figure 118) for these three outputs we attempted to formulate an optimum formulation for a caffeine imprinted MIP. We had some contradictions in the level of optimization in different outputs; hence we optimized the combination (Figure 119) applying our knowledge along with the forecast. During optimizing we closely followed the output forecast obtained for $bq_s$ as this combines both the binding constant and the site density. This formulation deviated from the monomer: crosslinker ratio adopted for most of the formulation previously employed and the ratio was reduced from around 1:6 to 1:2. As the input pattern of template proportion showed an increasing trend for all forecasted output, we tried a higher limit than formerly applied. It was also reported$^9$ in previous studies that higher proportion of template and recognition monomer could prove beneficial; although no upper limit was mentioned. The rebinding medium was optimized at a pH of 4 since the NN forecasted that it could produce a larger $bqs$. 

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Figure 118. Summary of forecasted optimized formulation

- MAA 
- EGDMA 
- Caffeine 
- Acetonitrile

Figure 119. Optimized formulation for caffeine imprinted MIP (Bulk NN) obtained from neural network forecast

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This optimized MIP was prepared using the formulation extracted from the neural network (Figure 119) and was characterized with a rebinding experiment to examine the specificity. The result was not encouraging as we could not identify any specific sites for this MIP. The large above the range deviation of the template and recognition monomer might have affected adversely. The non-associated proportion of the recognition monomer produced mostly the non-specific sites. Since the non-specific elements dominated, any selectivity presented by the imprints remained undetected. The pH of the rebinding medium was adjusted both for 4 and 5 and the adsorption trend was found comparable (Figure 120).

Figure 120. Scatchard isotherm of the binding characteristics of Bulk NN at pH 4. The expected specific binding (not identified) is marked.
Although neural network practically worked in some instances\textsuperscript{97}, we could not manipulate it to work for a good formulation forecast and the probable reason was our limited data set. Nevertheless, the mathematical trend of the input on a specific output was worthy and can be used as guidance in the future research work on MIP.
CONCLUSION

In this thesis, two major sections of works were presented. First, the synthesis of a wide variety of MIPs was reported, and second the rebinding properties of those MIPs in aqueous solution were investigated.

Rebinding experiments were used to evaluate the binding constant and site density of the MIPs and the bi-Langmuir model was found to be the best to fit the experimental binding isotherm and for the comparison between MIPs.

An efficient MIP is found to have a heterogeneous distribution of sites with low site density of the specific sites and a higher site density for the non-specific ones. We found that the MIP having only general type non-specific sites are as inefficient as of a non-imprinted polymer.

We established that water is a good rebinding medium which was able to distinguish sufficiently between an efficient MIP from an inefficient one. The pH has a significant influence in rebinding and at a pH of 5, the highest binding affinity was determined.

Caffeine imprinted MIP whether highly specific or not shows a high selectivity towards its analyte compared to its structural analogue theophylline. For the co-adsorption experiment of an efficient caffeine imprinted MIP the factor of selectivity in terms of specific binding constant is found 2.3.
We initiated our work on conventional monolith MIPs and several variations were employed in formulations and characterizations. We found acetonitrile was found to be the best porogen and also conclude that using an aprotic porogen is best. A polymeric porogen poly (ethylene glycol dimethyl ether) was found to produce a MIP with similar rebinding efficiency as acetonitrile. Water being a polar protic porogen inhibits an efficient imprinting.

We investigated the effect of a low concentration (0.03 wt. percent) of template incorporation and was able to achieve a reasonably efficient conventional MIP (specific binding constant 1095 l/mol and specific site density 0.02 mmol/gm) compared to the one prepared with high concentration (3.2 wt. percent) of template (specific binding constant 1874 l/mol and specific site density 0.02 mmol/gm).

The rebinding response was found to be faster with reduction of particle size from microns to nanometer range. For efficient MIPs, the micron sized particle reached equilibrium in 5 hours while sub-micron ranges particles within 2 minutes.

Nanoparticles MIPs were prepared in acetonitrile and by examining the increasing amount of porogen we were able to formulate MIPs with a relatively higher binding constant (specific binding constant 31500 l/mol). Nanoparticles MIPs when using a high concentration of template were found to have a high binding affinity (4170 l/mol) but with a modest specific site density (0.01 mmol/gm). The rebinding performance for nanoparticles is comparable to the performance of an efficient bulk monolith.

From our research it became evident that there is a balance between binding constant and site density for the specific types of sites and achieving both at their optimum range is a challenge (Table 27). Although we applied various changes in our formulations, the

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product of equilibrium binding constant and site density for specific sites \((bq_s)\) only varied in a narrow range and within a factor of 5 (0.33-1.69). On the contrary binding constant varies within two orders of magnitude (518-31500 l/mol) and fraction of specific sites within 3 orders of magnitude (0.0006-0.23). This result suggests the limitation of increasing the efficiency of a MIP in terms of specific binding. Nevertheless, for the specific sites the highest binding constant was found for a MIP was 31500 l/mol. and the highest site density was achieved at 0.02 mmol/gm. A reasonably good balance was found for nanoparticles (MJB 68) and it was the highest \((bq_s = 1.69)\) among all the MIPs formulated.

In order to investigate the impact of the constituents a statistical technique was approached. From the forecasting of a neural network it was found that for producing a high specific binding constant the porogen proportion is the important factor. On the contrary, in producing a high density of the specific sites, the impact of crosslinking agent is the most important. For an optimal combination of both the specific equilibrium binding constant and specific site density \((bq_s)\) we found that both the porogen and crosslinker are of equal importance.

A gas adsorption experiment was performed to analyze the surface area and pore size distribution. We identified that MIPs with a high binding affinity for its specific site possess a high surface area.

The best MIP formulation is a nanoparticles MIP (MJB 68) with \(bq_s = 1.69\), with very fast adsorption kinetics.
RECOMMENDATIONS

More focus is needed for the formation of nanoparticles, because like our predecessor researchers we found the prospect of employing nanoparticles in the field of MIPs reasonably encouraging. Working in a lower concentration range of the analyte in the rebinding experiment might enable us to get a better binding constant for nanoparticles MIP as reported by Wang et al.\textsuperscript{49} Employing a tri-functional crosslinking agent could be beneficial as reported by Mosbach et al.\textsuperscript{19} Since trimethylolpropane triacrylate (TRIM) has one more vinyl group over EGDMA, lower proportion is required to ensure the same backbone rigidity that contributes the specificity of a MIP. As we found from our experimental result, crosslinker hardly contributes to the binding affinity to the template and as little as of that would be suitable.

Water as a porogen is remained a challenge and by employing conventional non-covalent interaction we were far beyond to develop a sensitive MIP. Exploring an ionic bonding interaction instead, could be a solution to overcome the problem.\textsuperscript{45}
The heat of adsorption can provide a direct measurement of the binding strength of the adsorbate to the adsorbent. To better understand the rebinding of a guest analyte, an Isothermal Titration Calorimetric study could be useful which can evaluate the binding enthalpy. In order for a significant binding to occur, the free energy change of adsorption should be negative which requires an exothermic heat of adsorption. The specific sites for the template should have a higher binding enthalpy than the non-specific sites and the adsorption towards the two classes of sites can be clearly differentiated from this calorimetric study.
APPENDICES
APPENDIX A

PARALYTIC SHELLFISH POISONING

A.1 Background

While some algae are harmless and useful in foods and drug applications, there are some others which produce toxins. Marine animals like shellfish eat the algae, which are a common food source for marine creatures, store the toxins. This leads to potentially fatal poisoning not only for the shellfishes, but also any creatures that consume the contaminated shellfish, particularly human beings. Depending upon the species of algae the poisoning symptoms can be classified as follows:

1. Nervous system damage (Paralytical Shellfish Poisoning) (PSP)
2. Memory Loss (Amnesic Shellfish Poisoning) (ASP)
3. Phenomenons of Neurotoxicity (Neurotoxic Shellfish Poisoning) (NSP)
4. Diarrheic Shellfish Poisoning (DSP)

The most prevalent one is the paralytic shellfish poisoning (PSP), which causes both gastrointestinal and neurological symptoms. Human nerves are especially very sensitive to this toxin and there is no specific antidote discovered yet. Saxitoxin, the most well known, major component of the PSP associated neurotoxins, is produced by dinoflagellate algal blooms such as *Alexandrium tamarense*, *Gymnodinium catenatum*, and *Pyrodinium bahamense*. 

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A scanning electron micrograph of two *Pyrodinium bahamense* cells.\textsuperscript{106} Scale bar = 20 μm.

Saxitoxin (STX) is a low molecular weight, water soluble, nitrogen containing compound which is stable in heat and acid.\textsuperscript{107} It is considered one of the most potent toxins known so far. Although PSP is a worldwide problem, it is particularly a concern on both the east and west coast of North America during summer months.\textsuperscript{108} Especially New England has a long history of shellfish poisoning associated with dinoflagellate organisms. Shellfish feeds on this organism and the neurotoxin from there eventually are accumulated in the tissues of shellfish. When the contaminated shellfish are eaten it causes illness.
Comparison of deadliness for common toxins$^{109}$

Death of human by Micrograms per kilograms per body weight
World PSP toxin map as of 2004\textsuperscript{110}

PSP events in the United States of America

Frequency of PSP outbreaks in coastal area of USA\textsuperscript{110}
These toxins block the opening of Na+ channel and prevent the pathway of sodium cation from the cell in body.\textsuperscript{100} This eventually interrupts the transmission of nerve impulses and causes paralysis. For mice, the LD\textsubscript{50}\textsuperscript{111} of saxitoxin is 3-10\textmu g/kg of body weight and orally it is 263 \textmu g/kg. The oral dose for human death is 1 to 4 mg (5,000 to 20,000 mouse units) depending upon the condition of patient.\textsuperscript{102} Numbness and respiratory arrest can occur with as small an amount as 0.5-1.0 \textmu g when orally ingested.\textsuperscript{112} The maximum acceptable limit for PSTs in shellfish set by The Food and Drug Administration is 400 MU (mouse unit) or 80 microgram/100 gram of tissue.\textsuperscript{109}

Structurally saxitoxin has a purine ring system with two guanidinium moieties. It has a carbamoyl moiety (-O-CO-NH-) and like most other carbamate derivative saxitoxins are toxic to human beings.\textsuperscript{113} There are varieties of saxitoxins depending upon the chemical substitution and origin. The chemical properties of those are similar and that in turn makes accurate differentiation and quantification difficult.\textsuperscript{102} Thus, affected beach areas demand constant monitoring of shellfish due to this unpredictable pattern of toxicity.
Saxitoxin (consisted of purine ring) and its related toxins. STX = saxitoxin, NeoSTX = neosaxitoxin, GTX = Gonyautoxin.

A.2 Present method of detection of Saxitoxin

Several efforts have been made over the past 70 years to detect and quantify this toxin. The following table will provide an overview of the detection method for marine biotoxins. The first conventional method of detection was by bioassay and eventually chemical assays were developed. However, the bioassay is still the most common.
### Overview of common detection methods for marine biotoxins

<table>
<thead>
<tr>
<th>Detection Method</th>
<th>Short description</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse Bioassay</strong></td>
<td>Considered positive when 2 out of 3 mice are dead within a day.</td>
<td>Biological test directly demonstrates presence of unknown toxin.</td>
<td>Not very sensitive and conflict with Animal Welfare Directive.</td>
</tr>
<tr>
<td><strong>Enzyme Immunoassay</strong></td>
<td>Detection by binding to specific antibodies.</td>
<td>Rapid detection and more sensitive than mouse bioassay.</td>
<td>For each group a different test is required and cross reactions are possible with the same groups of toxin.</td>
</tr>
<tr>
<td><strong>Analytical Methods</strong></td>
<td>Detection by analytical instruments.</td>
<td>Rapid quantitative identification and more sensitivity and selectivity.</td>
<td>In general detection is not possible for structurally unrelated unknown toxins.</td>
</tr>
</tbody>
</table>

**A.2.1 Bioassay**

Bioassay is the appraisal of any biological activity of a substance by testing its effect on an organism and comparing the result with some agreed standard. There exist several bioassays to detect saxitoxin with varying detection limit.
A.2.1.1 Mouse Bioassay

Universally 'mouse mean death time bioassay' is used to detect the level of PSP toxins in shellfish. The basis of this assay was developed by Sommer and Meyer (1937)\textsuperscript{116}. The amount of toxin present in shellfish is determined by the time of death of mice. The detection limit of mouse bioassay is 37µg/100gm of shellfish with a precision of ±20% and this bioassay demands a supply of proper sized mice.\textsuperscript{108} Although low sensitivity, high cost and regulatory issues regarding uses of animals have been a problem, it is still the most reliable way of detection and quantification for PSP toxins. But this assay can not give the information about the composition of the toxin and therefore it is unable to differentiate between PSPs or differentiate PSPs from other neurotoxins.\textsuperscript{117}

A.2.1.2 Commercial Bioassay

Commercially there exists competitive enzyme-linked immunosorbent assay (ELISA)\textsuperscript{112} for the rapid quantitative analysis of saxitoxin. This analysis is sensitive with a detection limit of 50 ppb (µg/kg) and has a high reproducibility. The specificity of this test is established upon analyzing the cross-reactivity of saxitoxin towards its corresponding toxins. Antigen-antibody reaction is the basis of this test. This reaction occurs through the competitive binding of free saxitoxin and saxitoxin enzyme conjugate, for the saxitoxin antibody binding sites. Then the measurement is done photometrically at 450 nm and the measured absorbance is inversely proportional to the saxitoxin concentration in the sample. Any unknown concentration in a 5-20 µg/kg (ppb) range corresponding to the absorbance can be determined from a linear calibration curve.
Schematic representation of the basics of ELISA$^{118}$

The commercialized antibody mixture in the rapid test kit$^{119}$ can detect all the members of PSP family in the contaminated shellfish tissue in varied efficacy. It only needs a test strip to work with, thus has the benefit of durability for field-use. In brief, it is simple to use, cost effective and excludes the requirement of any specific laboratory equipment. However, this rapid 35 minutes diagnostic test can give only a yes or no answer with a detection limit of 40μg/100gm of shellfish.
Mix buffer and extract together by inserting the dispenser in the buffer tube and squeezing dispenser bulb three (3) times.

Fill dispenser up to the black line.

Empty dispenser contents into the sample hole.

Wait 35 min for results.

Rapid test for PSP: easy to use

A.2.1.3 Other biosensor

Cheun et al. developed a tissue biosensor which was applied to detect low amount of STX and other Na⁺ channel blockers. The sensor consists of a frog bladder covered Na⁺ electrode integrated in a flow cell. The frog bladder acts as a membrane and provides active transport of Na⁺ from the internal to external face. The response for different toxins can then be recorded. By determining a good correlation...
coefficient between the developed tissue biosensor and the conventional mouse bioassay they argued that they had a highly sensitive biosensor for marine environment.

![Schematic diagram of HPLC study](image)

1. Degassing units
2. Low-pressure gradient unit
3. Solvent delivery units
4. Mixer
5. Auto injector
6. Column oven
7. Analysis column
8. Chemical reaction tank
9. Blender
10. Fluorescent detector
11. Reaction coils
12. M1, M2 Mobile phases
13. R1, R2 Reaction liquids

**Schematic diagram of HPLC study**

### A.2.2 Chemical Assays

Several chemical assays have been developed overtime with the help of analytical measuring instruments, where separation is based on the physiochemical properties of the molecule. Among those, a continuous-flow fast-atom bombardment mass spectroscopy was developed by Mirocha et al. This technique is able to analyze saxitoxin in urine with sensitivity in nanogram unit.

High performance liquid chromatography (HPLC) was explored in this area and with conjunction of fluorometric detection it has proved promising. Chemical assay
based on high performance liquid chromatography (HPLC) has been developed to detect individual PSP with a detection limit of 0.2 ppm.\textsuperscript{124}

![Chromatogram of standard PSP toxin solution (B is for saxitoxin with a concentration of 9.52 ng/ml.)\textsuperscript{125}]

A.2.2.1 High Performance Liquid Chromatography

High sensitivity and selectivity can be obtained from the liquid chromatographic method. Following the column resolution toxins can be visualized as a highly fluorescent derivative by post column oxidation in alkaline condition.\textsuperscript{126} Post column derivatization\textsuperscript{127} eliminates the need to separate and identify several oxidation products that is required for pre-column oxidation. In the pre-column operation toxin proportion changes with pH, temperature and time gap between oxidation and injection. On the other hand in the post-column process, the parent compounds are separated into individual toxins during elution. The toxins are then oxidized and quantified by fluorescent detection. Several efforts have been sited in the literature (Error! Reference source not found.).
HPLC determination of PSP toxins (Saxitoxin)

<table>
<thead>
<tr>
<th>Literature</th>
<th>Column used</th>
<th>Post-column derivatization by</th>
<th>Detector</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rubinson et al.(^{128}) (1982)</td>
<td>C18 column</td>
<td>--</td>
<td>Differential-refractive-index detector</td>
<td>Accuracy of ±10%</td>
</tr>
<tr>
<td>Sullivan et al.(^{108}) (1983)</td>
<td>Bonded phase cyan column</td>
<td>NH(_4^+) and periodic acid</td>
<td>Fluorescence</td>
<td>--</td>
</tr>
<tr>
<td>Nagashima et al.(^{17,129}) (1987, 88)</td>
<td>Octadecyl silica (ODS) column</td>
<td>periodic acid</td>
<td>Fluorescence with excitation at 336nm and emission at 390nm</td>
<td>Detection limit is up to 0.5 mouse unit.</td>
</tr>
<tr>
<td>Oshima et al.(^{126}) (1995)</td>
<td>C8 column</td>
<td>periodic acid</td>
<td>Fluorescence</td>
<td>Results within 5% error</td>
</tr>
<tr>
<td>Lawerence et al.(^{127}) (1996)</td>
<td>Polymeric reverse phase column (Hamilton PRP-1)</td>
<td>MnO(_2) as a solid phase oxidant</td>
<td>Fluorescence with excitation at 330nm and emission at 400nm</td>
<td>Result with a standard deviation of ≤10%</td>
</tr>
<tr>
<td>Zhi He et al.(^{130}) (2005)</td>
<td>NH(_2) column</td>
<td>periodic acid</td>
<td>Fluorescence with excitation at 330nm and emission at 390nm</td>
<td>Recovery range of almost 100%</td>
</tr>
</tbody>
</table>
A.2.2.2 Detection by Fluorescence sensor

STX is an ion-channel disruptor, and, as such, it can be viewed as a Na⁺ or K⁺ mimic. These cations are recognized by crown ethers.131 Several crown ethers have been developed131,132 overtime to act as fluorescence sensors for saxitoxin. Monoazo and diazo-crown ethers both are effective and can bind STX in a stoichiometric ratio of 1:1. Based on this ratio a crown ether sensor could be designed. The developed sensor has a higher selectivity for STX than it does for sodium and potassium ions in water or compounds having a guanidium moiety or a purine ring.

Because the development of a fluorophor from saxitoxin has been successful, a few other systems have been developed using the same principles. Two such procedures are the solution fluorimetric133 and the fast flurometric134, which work well and produce similar results to those obtained from the conventional mouse bioassay.

In summary, the above described assay systems could offer an alternative to mouse bioassay that sometimes could provide a higher sensitivity. But there still does not exist any quantitative test which is simple, that is also sensitive, portable and fast. In this regard a chemical sensor could provide a viable option. In the sensor application, MIPs have already been recognized as valuable recognition elements.13 Thus we were interested in preparing MIP which could be used as sensors for the detection of STX.2
Henry’s Law

This is the experimentally observed limiting law that applies to all solutions, called Henry’s law for the solute. Solute is assumed as component 2 and the Henry’s law is written as:

\[ \lim_{x_2 \to 0} a_2 = \gamma_2^0 x_2 \]

The coefficient \( \gamma_2^0 \) called the Henry’s law constant. This is the activity coefficient for the solute and in the dilute range it is independent of composition.

For the solute, the activity approaches zero along a line that has a slope of 1, which equals to Henry’s law constant for that solution.\(^{135}\)

Variation of activity with composition for solute in a dilute solution

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► pKa

The pKa is the negative logarithm of the acid dissociation constant, Ka. It describes the acid or basic properties of a substance.\textsuperscript{136}

- pKa <2 means strong acid
- pKa >2 but <7 means weak acid
- pKa >7 but <10 means weak base
- pKa >10 means strong base

► Association and dissociation binding constant

The binding constant is a special case of the equilibrium constant. This is the equilibrium state of molecular binding where after an infinite reaction time, there is a balance between the binding and dissociation process. This can be formalized as the unbound reactants transformed into a complex.

This direction refers to the association binding constant $K_a$ in a dimension of 1/mol

The reverse direction defines the dissociation binding constant and defined as $K_d$ in a dimension of mol/l.\textsuperscript{137}

\[ [C] + [S] \leftrightarrow [CS] \]

- [C] = concentration of free analyte
- [S] = concentration of free sites
- [CS] = sites occupied with analyte = bound analyte

\[ K_a = [CS]/ ([C] [S]) \]

\[ K_d = ([C] [S])/ [CS] \]
Derivation of Langmuir expression

\[ [C] + [S] \leftrightarrow [CS] \]

\([C]\) = concentration of free analyte

\([S]\) = concentration of free sites

\([CS]\) = sites occupied with analyte = \(q\) = bound analyte

Equilibrium constant \(b_1 = \frac{[CS]}{([C] [S])}\)

\([S] + [CS] = [S_0] = \text{total available sites} = \text{saturation capacity } q_s\)

\(b_1 = \frac{[CS]}{([C] ([S_0]-[CS]))}\)

\(= \frac{q}{([C] (q_s - q))}\)

\(\Rightarrow b_1[C] \left( \frac{q_s}{q} - 1 \right) = 1\)

\(\Rightarrow b_1[C] \frac{q_s}{q} = 1 + b_1[C]\)

\(\Rightarrow q = b_1 \frac{q_s [C]}{1 + b_1[C]}\)

\(= \frac{a_1 [C]}{1 + b_1[C]}\)
Derivation of Scatchard expression from Langmuir expression:

Langmuir expression:

\[
C_{\text{bound}} = \frac{a_1 C_{\text{free}}}{(1 + b_1 C_{\text{free}})} \quad \text{--- 1}
\]

or, \( C_{\text{bound}} + C_{\text{bound}} b_1 C_{\text{free}} = a_1 C_{\text{free}} \)

or, \( C_{\text{free}} = \frac{C_{\text{bound}}}{(a_1 - C_{\text{bound}} b_1)} \quad \text{--- 2} \)

From 1,

\[
\frac{C_{\text{bound}}}{C_{\text{free}}} = \frac{a_1}{(1 + b_1 C_{\text{free}})} \quad \text{--- 3}
\]

Combining 2 & 3,

\[
\frac{C_{\text{bound}}}{C_{\text{free}}} = \frac{a_1}{[1 + b_1 \{C_{\text{bound}}/(a_1 - C_{\text{bound}} b_1)\}]} \]

= \(a_1 (a_1 - C_{\text{bound}} b_1)/(a_1 - C_{\text{bound}} b_1 + C_{\text{bound}} b_1)\)

= \(a_1 - C_{\text{bound}} b_1 \quad \text{--- 4} \)

Again, \( q_{s1} = a_1 / b_1 \quad \text{--- 5} \)

Combining 4 & 5,

\[
\frac{C_{\text{bound}}}{C_{\text{free}}} = - C_{\text{bound}} b_1 + b_1 q_{s1}
\]

or, \( q/C = -bq + bq_{s} \) (Scatchard expression)

i.e. \( y = mx + C \)

\( m = \text{slope} = -b \)

\( C = \text{Intercept} = bq_{s} = a \)
APPENDIX C

LIST OF PARTICLE SIZE DISTRIBUTION FOR THE BULK MIPS

⇒ Caffeine imprinted MIPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chapter</th>
<th>Particle Diameter in μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>3.1.1</td>
<td>270(76%) 63(23%) 16(1%)</td>
</tr>
<tr>
<td>Bulk 1A</td>
<td>3.1.6</td>
<td>102(43%) 26(36%) 5(15%) 3(6%)</td>
</tr>
<tr>
<td>Bulk 1B</td>
<td>3.1.6</td>
<td>186(53%) 48(34%) 13(11%) 4(2%)</td>
</tr>
<tr>
<td>Bulk 1C</td>
<td>3.1.6</td>
<td>224(29%) 64(27%) 21(32%) 6(12%)</td>
</tr>
<tr>
<td>Bulk 1D</td>
<td>3.1.6</td>
<td>233(33%) 69(31%) 21(26%) 5(10%)</td>
</tr>
<tr>
<td>Bulk 2</td>
<td>3.1.1</td>
<td>199(82%) 57(18%)</td>
</tr>
<tr>
<td>Bulk 3</td>
<td>3.1.1</td>
<td>269(100%)</td>
</tr>
<tr>
<td>Bulk 4</td>
<td>3.1.1</td>
<td>161(70%) 58(25%) 8(5%)</td>
</tr>
<tr>
<td>Bulk 11</td>
<td>3.1.1</td>
<td>133(74%) 49(26%)</td>
</tr>
<tr>
<td>Bulk 12</td>
<td>3.1.1</td>
<td>147(82%) 49.5(18%)</td>
</tr>
<tr>
<td>Bulk 13</td>
<td>3.1.4</td>
<td>137(58%) 55(36%) 12(6%)</td>
</tr>
<tr>
<td>Bulk 14</td>
<td>3.1.4</td>
<td>162(78%) 64(22%)</td>
</tr>
<tr>
<td>MJB 40</td>
<td>3.1.1</td>
<td>223(44%) 66(21%) 17(19%) 4.5(16%)</td>
</tr>
<tr>
<td>MJB 41</td>
<td>3.1.1</td>
<td>220(62%) 58(21%) 13(10%) 3.5(7%)</td>
</tr>
<tr>
<td>MJB 46</td>
<td>3.1.1</td>
<td>229(76%) 67(20%) 13(4%)</td>
</tr>
<tr>
<td>Bulk 21</td>
<td>3.1.4</td>
<td>186(13%) 57(20%) 16(37%) 3(30%)</td>
</tr>
</tbody>
</table>
### Guanosine imprinted MIPs

<table>
<thead>
<tr>
<th>MIP</th>
<th>Chapter</th>
<th>Particle Diameter in µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 5</td>
<td>3.2.1.1</td>
<td>171(81%) 50(19%)</td>
</tr>
<tr>
<td>Bulk 7</td>
<td>3.2.1.1</td>
<td>283(100%)</td>
</tr>
<tr>
<td>Bulk 8</td>
<td>3.2.1.2</td>
<td>128(85%) 42(15%)</td>
</tr>
<tr>
<td>Bulk 10</td>
<td>3.2.1.2</td>
<td>143(86%) 52(14%)</td>
</tr>
<tr>
<td>Bulk 15</td>
<td>3.2.1.3</td>
<td>257(67%) 57(27%) 15(6%)</td>
</tr>
<tr>
<td>MJB 48</td>
<td>3.2.1.3</td>
<td>187(43%) 57(26%) 20(25%) 5(6%)</td>
</tr>
<tr>
<td>Bulk 16</td>
<td>3.2.1.3</td>
<td>212(59%) 65(29%) 15(11%) 4(1%)</td>
</tr>
<tr>
<td>Bulk 17</td>
<td>3.2.1.3</td>
<td>182(75%) 55(23%) 13(2%)</td>
</tr>
<tr>
<td>Bulk 18</td>
<td>3.2.1.2</td>
<td>237(36%) 62(31%) 17(22%) 4(11%)</td>
</tr>
<tr>
<td>Bulk 19</td>
<td>3.2.1.4</td>
<td>228(76%) 67(23%) 15(1%)</td>
</tr>
<tr>
<td>Bulk 20</td>
<td>3.2.1.3</td>
<td>152(75%) 39(14%) 12(11%)</td>
</tr>
</tbody>
</table>
## APPENDIX D

**BINDING CONSTANT AND SITE DENSITY COMPARISON BETWEEN SCATCHARD FORMAT AND BI-LANGMUIR MODEL FOR CAFFEINE IMPRINTED MIPS**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chapters</th>
<th><strong>Scatchard format</strong></th>
<th><strong>Bi-Langmuir</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ka (b*MW) (l/mol)</td>
<td>sites (mmol/gm)</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>3.1.5</td>
<td>1032.8</td>
<td>0.06</td>
</tr>
<tr>
<td>pH ~ 5</td>
<td></td>
<td>136</td>
<td>0.24</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>3.1.5</td>
<td>667</td>
<td>0.1</td>
</tr>
<tr>
<td>pH 3</td>
<td></td>
<td>190</td>
<td>0.26</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>3.1.5</td>
<td>515</td>
<td>0.1</td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td>202.7</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>3.1.5</td>
<td>649</td>
<td>0.09</td>
</tr>
<tr>
<td>pH 9.5</td>
<td></td>
<td>224</td>
<td>0.2</td>
</tr>
<tr>
<td>Bulk 1A</td>
<td>3.1.6</td>
<td>403</td>
<td>0.08</td>
</tr>
<tr>
<td>Bulk 1B</td>
<td>3.1.6</td>
<td>224.6</td>
<td>0.09</td>
</tr>
<tr>
<td>Bulk 1C</td>
<td>3.1.6</td>
<td>3305</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33</td>
<td>0.19</td>
</tr>
<tr>
<td>Bulk 1D</td>
<td>3.1.6</td>
<td>2390</td>
<td>0.016</td>
</tr>
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<td></td>
<td></td>
<td>74</td>
<td>0.22</td>
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<tr>
<td>MJB 58</td>
<td>3.1.8</td>
<td>139.84</td>
<td>0.05</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MJB 68</td>
<td>3.1.8</td>
<td>2034</td>
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<td></td>
<td></td>
<td>103</td>
<td>0.2</td>
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<tr>
<td>Bulk 2</td>
<td>3.1.1</td>
<td>43.5</td>
<td>0.06</td>
</tr>
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<td>Bulk 3</td>
<td>3.1.1</td>
<td>21</td>
<td>0.03</td>
</tr>
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<td>Bulk 4</td>
<td>3.1.1</td>
<td>235</td>
<td>0.09</td>
</tr>
<tr>
<td>Bulk 11</td>
<td>3.1.1</td>
<td>144</td>
<td>0.06</td>
</tr>
<tr>
<td>Bulk 12</td>
<td>3.1.1</td>
<td>242</td>
<td>0.2</td>
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<tr>
<td>Bulk 13</td>
<td>3.1.4</td>
<td>240</td>
<td>0.24</td>
</tr>
<tr>
<td>Bulk 14</td>
<td>3.1.4</td>
<td>142</td>
<td>0.13</td>
</tr>
<tr>
<td>MJB 40</td>
<td>3.1.1</td>
<td>787.4</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>172</td>
<td>0.2</td>
</tr>
<tr>
<td>MJB 41</td>
<td>3.1.1</td>
<td>85.4</td>
<td>0.06</td>
</tr>
<tr>
<td>MJB 46</td>
<td>3.1.1</td>
<td>215</td>
<td>0.07</td>
</tr>
<tr>
<td>Bulk 21</td>
<td>3.1.4</td>
<td>2927</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>161</td>
<td>0.23</td>
</tr>
</tbody>
</table>
APPENDIX E

BINDING CONSTANT AND SITE DENSITY COMPARISON FOR CAFFEINE IMPRINTED MIPS PREPARED WITH VARYING POROGEN AND TEMPLATE PROPORTION

![Graph showing binding constants](image)

Variation of porogen along with template proportion.

**Binding constants (l/mol) from rebinding experiment with different porogen and varying proportion of template**

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Site density (mmol/gm) from rebinding experiment with different porogen and varying proportion of template
APPENDIX F

CALCULATION OF CONFIDENCE INTERVAL AT A CONFIDENCE LEVEL OF 95% FOR THE SAMPLES EVALUATED TO HAVE SPECIFIC TYPES OF SITES

<table>
<thead>
<tr>
<th>Sample</th>
<th>Main features</th>
<th>$K_a$</th>
<th>$q_5$</th>
<th>$r^2$</th>
<th>Variance</th>
<th>Std deviation</th>
<th>Std error</th>
<th>t value</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>caffeine-10% solid</td>
<td>5648</td>
<td>0.01</td>
<td>0.59</td>
<td>0.2420</td>
<td>0.4919</td>
<td>0.2200</td>
<td>2.78</td>
<td>61.08%</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>theophylline-10%</td>
<td>4579</td>
<td>0.003</td>
<td>0.707</td>
<td>0.2074</td>
<td>0.4554</td>
<td>0.1859</td>
<td>2.57</td>
<td>47.79%</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>co-caffeine-10%</td>
<td>1851</td>
<td>0.01</td>
<td>0.635</td>
<td>0.2317</td>
<td>0.4813</td>
<td>0.1702</td>
<td>2.36</td>
<td>40.24%</td>
</tr>
<tr>
<td>Bulk 1</td>
<td>co-theophylline-10%</td>
<td>809</td>
<td>0.02</td>
<td>0.611</td>
<td>0.2378</td>
<td>0.4876</td>
<td>0.1991</td>
<td>2.57</td>
<td>51.17%</td>
</tr>
<tr>
<td>Bulk1</td>
<td>pH3</td>
<td>1010</td>
<td>0.05</td>
<td>0.872</td>
<td>0.1119</td>
<td>0.3345</td>
<td>0.1264</td>
<td>2.45</td>
<td>30.94%</td>
</tr>
<tr>
<td>Bulk21</td>
<td>ACN-low template</td>
<td>1095</td>
<td>0.02</td>
<td>0.789</td>
<td>0.1667</td>
<td>0.4083</td>
<td>0.1444</td>
<td>2.36</td>
<td>34.13%</td>
</tr>
<tr>
<td>Bulk1</td>
<td>pH5</td>
<td>1874</td>
<td>0.02</td>
<td>0.967</td>
<td>0.0324</td>
<td>0.1799</td>
<td>0.0680</td>
<td>2.45</td>
<td>16.64%</td>
</tr>
<tr>
<td>Bulk1</td>
<td>pH7.5</td>
<td>576</td>
<td>0.04</td>
<td>0.863</td>
<td>0.1186</td>
<td>0.3444</td>
<td>0.1302</td>
<td>2.45</td>
<td>31.85%</td>
</tr>
<tr>
<td>Bulk1</td>
<td>pH9.5</td>
<td>518</td>
<td>0.05</td>
<td>0.769</td>
<td>0.1777</td>
<td>0.4216</td>
<td>0.1593</td>
<td>2.45</td>
<td>38.99%</td>
</tr>
<tr>
<td>Bulk1C</td>
<td>75% ACN</td>
<td>31500</td>
<td>0.0007</td>
<td>0.984</td>
<td>0.0159</td>
<td>0.1262</td>
<td>0.0631</td>
<td>3.18</td>
<td>20.09%</td>
</tr>
<tr>
<td>Bulk1D</td>
<td>85% ACN</td>
<td>21956</td>
<td>0.0008</td>
<td>0.945</td>
<td>0.0521</td>
<td>0.2282</td>
<td>0.1020</td>
<td>2.78</td>
<td>28.33%</td>
</tr>
<tr>
<td>MJB68</td>
<td>Nanoparticles</td>
<td>4170.6</td>
<td>0.01</td>
<td>0.781</td>
<td>0.1709</td>
<td>0.4134</td>
<td>0.1688</td>
<td>2.57</td>
<td>43.38%</td>
</tr>
<tr>
<td>MJB 40</td>
<td>PEG</td>
<td>1694</td>
<td>0.2</td>
<td>0.769</td>
<td>0.1777</td>
<td>0.4216</td>
<td>0.1593</td>
<td>2.45</td>
<td>38.99%</td>
</tr>
</tbody>
</table>

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APPENDIX G

LANGMUIR MODEL FIT FOR THE EXPERIMENTAL ISOTHERM BUILT FOR MIPS

⇒ Caffeine imprinted MIPs

For all sited isotherms BC is binding constant for the specific sites in 1/mol and SC is site density for the specific sites in mmol/gm. CI is the confidence interval shown in percentage at a confidence limit of 95%.

Experimental isotherm (symbols) of Bulk1 (operated at different pH) fitted with bi-Langmuir (line) model. The linearity of specific site region is shown.
Competitive experimental isotherm (symbols) of Bulk1 fitted with bi-Langmuir (line) model. The linearity of specific site region is shown.

Experimental isotherm (symbols) of conventional MIPs prepared with high concentration of analyte; Bulk1 (ACN), MJB 40 (PEGDME), water (Bulk 11) fitted with bi-Langmuir (line) model.
Experimental isotherm (symbols) of Bulk1 (at different solid content in rebinding experiment) fitted with bi-Langmuir (line) model. The linearity of specific site region is shown.

Experimental isotherm (symbols) of conventional MIPs prepared in ACN with high concentration of analyte Bulk1 and with low concentration of analyte Bulk 21; both fitted with bi-Langmuir (line) model.
Experimental isotherm (symbols) of conventional MIPs prepared in 75% ACN with high concentration of analyte (Bulk 1C) and with low concentration of analyte (Bulk 1A). Experimental isotherm (symbols) of conventional MIPs prepared in 85% ACN with high concentration of analyte (Bulk 1D) and with low concentration of analyte (Bulk 1B). Bulk 1C and 1D fitted with bi-Langmuir (line) model. Bulk 1A and 1B fitted with Langmuir (line) model. The linearity of specific site region of Bulk 1C and Bulk 1D is shown.
Log plot of experimental isotherm (symbols) of latex MIPs prepared in 95% ACN with high concentration of analyte MJB 68 and with low concentration of analyte MJB 58; both fitted with bi-Langmuir model. The linearity of specific site region is shown.
APPENDIX H

BET GAS ADSORPTION DATA FOR THE BULK MIPS PREPARED

⇒ Caffeine imprinted MIPS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chapter</th>
<th>BET Surface area (m²/gm)</th>
<th>Langmuir Surface Area (m²/gm)</th>
<th>BJH cumulative pore area (m²/gm)</th>
<th>BJH cumulative pore vol (cm³/g)</th>
<th>BET av. pore dia. (nm)</th>
<th>BJH av. pore dia. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 1</td>
<td>3.1.1</td>
<td>236</td>
<td>363</td>
<td>137.5</td>
<td>0.28</td>
<td>5.77</td>
<td>8.16</td>
</tr>
<tr>
<td>Bulk 1A</td>
<td>3.1.6</td>
<td>74.5</td>
<td>115</td>
<td>54.5</td>
<td>0.52</td>
<td>28.6</td>
<td>38</td>
</tr>
<tr>
<td>Bulk 1B</td>
<td>3.1.6</td>
<td>158.5</td>
<td>241.7</td>
<td>85.3</td>
<td>0.25</td>
<td>7.5</td>
<td>12</td>
</tr>
<tr>
<td>Bulk 1C</td>
<td>3.1.6</td>
<td>196</td>
<td>302</td>
<td>118</td>
<td>0.23</td>
<td>5.74</td>
<td>7.89</td>
</tr>
<tr>
<td>Bulk 1D</td>
<td>3.1.6</td>
<td>132</td>
<td>201</td>
<td>70.5</td>
<td>0.13</td>
<td>5</td>
<td>7.47</td>
</tr>
<tr>
<td>Bulk 2</td>
<td>3.1.1</td>
<td>0.23</td>
<td>0.32</td>
<td>0.2</td>
<td>0.003</td>
<td>52</td>
<td>56</td>
</tr>
<tr>
<td>Bulk 3</td>
<td>3.1.1</td>
<td>0.003</td>
<td>0.0035</td>
<td>0.0235</td>
<td>0.00041</td>
<td>578</td>
<td>70</td>
</tr>
<tr>
<td>Bulk 4</td>
<td>3.1.1</td>
<td>49</td>
<td>79</td>
<td>41.3</td>
<td>0.12</td>
<td>10.6</td>
<td>11.6</td>
</tr>
<tr>
<td>Bulk 11</td>
<td>3.1.1</td>
<td>0.17</td>
<td>0.22</td>
<td>0.24</td>
<td>0.0008</td>
<td>21.2</td>
<td>14</td>
</tr>
<tr>
<td>Bulk 12</td>
<td>3.1.1</td>
<td>269.84</td>
<td>420.14</td>
<td>165.95</td>
<td>0.4455</td>
<td>7.5757</td>
<td>10.7380</td>
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<tr>
<td>Bulk 13</td>
<td>3.1.4</td>
<td>438.88</td>
<td>670.49</td>
<td>268.85</td>
<td>0.5921</td>
<td>6.2817</td>
<td>8.8098</td>
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<tr>
<td>Bulk 14</td>
<td>4.1.4</td>
<td>142.48</td>
<td>228.87</td>
<td>127.3</td>
<td>0.46</td>
<td>13.6265</td>
<td>14.451</td>
</tr>
<tr>
<td>MJB 40</td>
<td>3.1.1</td>
<td>280</td>
<td>435</td>
<td>180</td>
<td>0.33</td>
<td>5.7</td>
<td>7.4</td>
</tr>
<tr>
<td>MJB 41</td>
<td>3.1.1</td>
<td>5.8</td>
<td>9.3</td>
<td>6.04</td>
<td>0.07</td>
<td>49</td>
<td>46</td>
</tr>
<tr>
<td>MJB 46</td>
<td>3.1.1</td>
<td>223</td>
<td>351</td>
<td>161.3</td>
<td>0.55</td>
<td>10.7</td>
<td>13.7</td>
</tr>
<tr>
<td>Bulk21</td>
<td>3.1.4</td>
<td>297</td>
<td>464.5</td>
<td>192.5</td>
<td>0.65</td>
<td>9.65</td>
<td>13.5</td>
</tr>
</tbody>
</table>
### Guanosine imprinted MIPs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chapter</th>
<th>BET Surface area (m²/gm)</th>
<th>Langmuir Surface Area (m²/gm)</th>
<th>BJH cumulative pore area (m²/gm)</th>
<th>BJH cumulative pore vol (cm³/g)</th>
<th>BET av. pore dia. (nm)</th>
<th>BJH av. pore dia. (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk 5</td>
<td>3.2.1.1</td>
<td>0.11</td>
<td>0.14</td>
<td>0.13</td>
<td>0.0019</td>
<td>72</td>
<td>55</td>
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<tr>
<td>Bulk 8</td>
<td>3.2.1.2</td>
<td>138</td>
<td>215</td>
<td>98</td>
<td>0.23</td>
<td>7.4</td>
<td>9.2</td>
</tr>
<tr>
<td>Bulk 9</td>
<td>3.2.1.2</td>
<td>0.1868</td>
<td>0.2611</td>
<td>0.1828</td>
<td>0.001773</td>
<td>39.8712</td>
<td>38.7879</td>
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<tr>
<td>Bulk 10</td>
<td>3.2.1.3</td>
<td>286</td>
<td>443</td>
<td>188</td>
<td>0.41</td>
<td>6.7</td>
<td>8.8</td>
</tr>
<tr>
<td>Bulk 15</td>
<td>3.2.1.3</td>
<td>260.828</td>
<td>403.417</td>
<td>170.2970</td>
<td>0.202444</td>
<td>--</td>
<td>4.7551</td>
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<tr>
<td>MJB 48</td>
<td>3.2.1.3</td>
<td>261.2476</td>
<td>407.6270</td>
<td>191.4357</td>
<td>0.496805</td>
<td>8.4842</td>
<td>10.3806</td>
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<tr>
<td>Bulk 16</td>
<td>3.2.1.3</td>
<td>213.7696</td>
<td>335.3217</td>
<td>107.6617</td>
<td>0.073770</td>
<td>--</td>
<td>2.7408</td>
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<tr>
<td>Bulk 17</td>
<td>3.2.1.2</td>
<td>112.5</td>
<td>179.6</td>
<td>91</td>
<td>0.26</td>
<td>9.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Bulk 18</td>
<td>3.2.1.4</td>
<td>208</td>
<td>325.3</td>
<td>139</td>
<td>0.3</td>
<td>6.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Bulk 19</td>
<td>3.2.1.3</td>
<td>69.5</td>
<td>110</td>
<td>57.2</td>
<td>0.19</td>
<td>11.8</td>
<td>13.4</td>
</tr>
<tr>
<td>Bulk 20</td>
<td>3.2.1.1</td>
<td>221.2</td>
<td>345.5</td>
<td>170.6</td>
<td>0.5</td>
<td>9.8</td>
<td>11.6</td>
</tr>
</tbody>
</table>
APPENDIX I

FORECASTED PATTERN OF THE INPUTS BY NEURAL NETWORK

⇒ For specific sites

Forecasted effect of increasing template proportion in MIP formulation for specific sites

Forecasted effect of increasing porogen proportion in MIP formulation for specific sites

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Forecasted effect of increasing crosslinker proportion in MIP formulation for specific sites

Forecasted effect of pH in rebinding experiment for specific sites
For $bq_s$,

Forecasted effect of increasing template proportion in MIP formulation for $bq_s$.

Forecasted effect of increasing porogen proportion in MIP formulation for $bq_s$. 

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Forecasted effect of increasing crosslinker proportion in MIP formulation for $bq_s$.

Forecasted effect of pH in rebinding experiment for $bq_s$.

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