SYNTHESIS OF MOLECULARLY IMPRINTED POLYMER FOR SELECTIVE SOLID-PHASE EXTRACTION OF SALBUTAMOL FROM URINE SAMPLES

by

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LIST OF ABBREVIATION

%	Percent
%, v/v	Percent volume per volume
℃	Degree Celsius
℃/min	Degree Celsius per minute
Å	Angstrom
µL	Microliter
µm	Micrometer
µg/L	Microgram per liter
µg/mL	Microgram per milliliter
ACN	Acetonitrile
AIBN	Azobisisobutyronitrile
AMVN	2,2'-azobisdimethyl valeronitrile
AR	Analytical reagent
BIP	Bamethane imprinted polymer
CD_3CN	Deuterated acetonitrile
CDER	Centre for Drug Evaluation Research
CD_3OD	Deuterated methanol
CH_3COONa	Sodium acetate
$CH_3COONa.3H_2O$	Sodium acetate trihydrate
CIP	Methyl-salbutamol imprinted polymer
cm^{-1}	Wave length in centimeter unit
CV	Coefficient of variation
CVM	Center for Veterinary Medicine
DCM	Dichloromethane
DVB	Divinylbenzene

EGDMA	Ethylene glycol dimethacrylate
ELISA	Enzymed-linked immunosorbents assay
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography coupled to mass spectrometry
GR	Grade
HAA	Haloacetic acid
^{1}H NMR	Proton nuclear magnetic resonance
HAc	Acetic acid
HBr	Hydrobromic acid
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICH Q2 (R1)	Validation of analytical procedures: Text and Methodology
IR	Infrared
K_2HPO_4	Dipotassium phosphate
L	Liter
LC	Liquid Chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of Detection
LOQ	Limit of quantitation
log *P*	Log of octanol-water partition coefficient
M	Molar
m/z	Mass to charge ratio
MAA	Methacrylic acid
MAE	Microwave assisted extraction
MeOH	Methanol

mg/mL	Milligram per milliliter
mg	Milligram
MHz	Megahertz
min	Minute
MIP(s)	Molecularly imprinted polymer(s)
MIPSPE	Solid-phase extraction based on molecularly imprinted polymer
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mM	Millimolar
mmHg	Milliliter of mercury
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide
MTBE	*Tert*-butyl dimethylsilyl
N	Normal
NaOH	Sodium hydroxide
ng	Nanogram
ng/mL	Nanogram per milliliter
NH_4I	Ammonium iodide
NIP	Non-imprinted polymer
NIST	National Institution of Standards and Technology
nm	Nanometer
nM	Nanomolar
pKa	Log of acidity constants
r^2	Correlation of coefficient
rpm	Revolutions per minute
Rt	Retention time
SD	Standard deviation

SIM	Selected ion monitoring
SPE	Solid-phase extraction
T: M: X	Ratio of template: monomer: cross-linking agent
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TRIM	Trimethylpropane trimethacrylate
UV	Ultraviolet
v/v	Volume per volume

LIST OF SYMBOLS

>	Greater than
<	Less than
+	Plus
±	Plus minus
Δ	Chemical shift
Δ δ	Difference in chemical shift

SINTESIS POLIMER TERTERA MOLEKUL BAGI PENGEKSTRAKAN SELEKTIF FASA PEPEJAL UNTUK SALBUTAMOL DARIPADA SAMPEL AIR KENCING

ABSTRAK

"Molecularly imprinted polymers" (MIPs) telah disediakan bagi mengekstrak salbutamol dalam sampel urine dengan mengguna molekul yang mempunyai struktur analog sebagai templat. MIPs telah disediakan dengan mengguna bamethane (BIP) dan metil-salbutamol (CIP), dua molekul yang berstruktur analog dengan analyte, salbutamol, sebagai templat Pempolimeran radikal bebas secara bukan kovalen dengan menggunakan asid metacrilik (MAA) sebagai monomer dan etilena glikol dimetakrilat (EGDMA) sebagai bahan silang. Proses pempolimeran dimulakan dengan menambah 2,2'-azobisdimetil valeronitril dan pempolimeran berlaku pada 50 ℃ selama 24 jam. Nisbah molar bagi molekul templat: monomer: bahan silang ditentukan pada 1:4:20. Monolit polimer berliang yang dihasilkan perlu dikisar dan ditapis untuk mendapat partikel bersaiz 40 hingga 75 μm. Partikel yang diperolehi diestrak dengan kaedah pengestrakan Soxhlet untuk menyingkirkan templat supaya tapak pengecaman dapat dibentuk. Pada masa yang sama, proses pempolimeran yang sama bagi menghasilkan "non-imprinted polymer" dijalankan tanpa menggunakan molekul templat. Dalam esperimen pemilihan, perbezaan yang signifikan diperhatikan dalam profil elusi polimer-polimer yang dihasilkan. Pengikatan analit yang spesifik berlaku dalam polimer-polimer "imprinted". Manakala bagi polimer "non-imprinted", hanya interaksi yang lemah dan tidak spesifik berlaku. Kajian reaktiviti silang menunjukkan polimer-polimer "imprinted" mempunyai cirri pengecaman molekul terhadap sebatian yang mempunyai struktur analog dengan salbutamol. Analisis IR and ^{1}H NMR telah dilakukan bagi mengkaji interaksi antara analit dan monomer. Ikatan hidrogen telah dibukti wujud di antara analit dan monomer. MIP digunakan dalam pengestrakan fasa pepejal (SPE)

bagi urin manusia sebelum analisis GC-MS. Kajian pengesahan bagi kaedah yang dicadangkan menunjukkan kelinearan, ketepatan, kepersisan, pengulangan dan pemilihan yang bagus dalam rangkum kepekatan 1 hingga 10 ng/mL dengan BIP sebagai fasa pegun. Selain itu, kaedah yang dicadangkan menunjukkan ketepatan, kepersisan dan pemulihan yang lebih baik berbanding dengan kaedah rujukan (SPE fasa campuran dan MIPSPE yang komersil). Oleh kerana CIP menunjukkan kelinearan yang kurang baik, kajian pengesahan yang selanjutnya tidak dijalankan. Selain itu, beberapa rawatan pasca pempolimeran ,seperti sonikasi, refluks dan kombinasi rawatan-rawatan yang sedia ada, telah dijalankan ke atas CIP dan BIP untuk menilai keadaan pendarahan molekul templat. Selepas rawatan dijalankan, templat dianggarkan dari jumlah molekul templat yang dibebaskan dalam eluen (acnetonitril: asid asetik glasier, 90:10%, v/v). Kombinasi pengestraka Soxhlet, sonikasi dan refluks menunjukkan paras pendarahan templat yang terendah di antara rawatan-rawatan pasca pempolimeran. Paras pendarahan templat dari BIP dan CIP adalah 100 kali dan 15 kali lebih rendah daripada polimer yang belum dirawat. BIP yang disediakan mempunyai potensi digunakan secara rutin dalam analisus yang mengesahkan salbutamol dan β-agonists.

SYNTHESIS OF MOLECULARLY IMPRINTED POLYMER FOR SOLID-PHASE EXTRACTION OF SALBUTAMOL IN URINE SAMPLES

ABSTRACT

High selective molecularly imprinted polymers were synthesized to extracted salbutamol from human urine samples using structurally analogue template molecules. Molecularly imprinted polymers (MIPs) were prepared by using bamethane (BIP) and methylated salbutamol (CIP), two structural analogue of the targeted analyte, salbutamol as template. Free radical polymerization was conducted in a non-covalent approach using methacrylic acid (MAA) as functional monomer and ethylene glycol methacrylate (EGDMA) as cross-linking agent. The polymerization process was initiated by 2,2'-azobisdimethyl valeronitrile and conducted at 50 ℃ for 24 hours. Molar ratio of template molecule: functional monomer: cross-linking agent was fixed at 1:4:20. A macroporous monolith polymer was formed, grinding and sieving was required to obtain the particles with size between 40 and 75 μm. The particles obtained were subjected to Soxhlet extraction for the removal of template in order to create recognition sites. An equivalent non-imprinted polymer was synthesized simultaneously using the same procedure but in the absence of template molecule. In selectivity experiments, significant differences in the elution profile between the polymers were observed. With the imprinted polymers, specific binding toward targeted analyte occurred. However, with the blank polymer, only weak and non-specific interaction occurred. Cross-specificity studies showed that the MIPs had molecular recognition properties towards other compounds that are structurally related to salbutamol, such as clenbuterol, fenoterol, isoxsuprine, metoprolol, ractopamine and terbutaline. IR and ^{1}H NMR analysis were done to study the interaction between analytes and monomer. Hydrogen bonding was proven to exist in between analytes and monomer. The MIPs were used in the solid phase extraction of spiked human urine samples prior to GC-MS. The

validation results of proposed method demonstrated good linearity, accuracy, precision, repeatability and selectivity in the concentrations range of 1 to 10 ng/mL with BIP as the sorbent. Moreover, the proposed method showed better accuracy, precision and recovery as compared with reference methods (SPE mixed mode and commercial MIPSPE). In contrast, CIP showed poorer linearity, thus further validation was not done. Besides, different post-polymerization treatments were applied on CIP and BIP to assess the template bleeding condition, such as sonication, reflux and combination of all available post-polymerization treatments. After treatment, the residual template was estimated by the amount of template released in the eluent (acetonitrile: glacial acetic acid, 90:10%, v/v). The combination of Soxhlet extraction, sonication and reflux showed the lowest bleeding level among the post-polymerization treatments. The bleeding levels from BIP and CIP are 100 fold lower and 15 folds lower than untreated imprinted polymers. The synthesized BIP has potential to be used routinely in confirmatory analysis of salbutamol and β-agonists.

CHAPTER ONE

INTRODUCTION TO MOLECULARLY IMPRINTED POLYMER (MIP)

1.1 Introduction

Molecular imprinting is a useful technique for the production of polymeric artificial receptors with molecular recognition properties. It uses a template to build its recognition site in a synthetic polymer. Such a technique allows highly specific binding of structurally related compounds due to its 'biomimetic' properties (Haupt and Mosbach, 1998). Due to the high selectivity and stability of molecularly imprinted polymers (MIP), this technology has a wide range of applications in analytical science, such as bioanalysis, pharmaceutical analysis, food analysis and environmental analysis (Mohamed et al., 2008, Owens et al., 1999, Pereira and Rath, 2009, Ramstrom et al., 2001, Schirmer and Meisel, 2008, Sellergren, 1999, Svenson and Nicholls, 2001, Zurutuza et al., 2005, Mahony et al., 2005, Masque et al., 2001, Xu et al., 2004). In these examples, the MIP acts as an artificial antibody showing strong binding to the targeted analyte. The advantages of MIP for analytical applications compared to conventional antibodies are ease in preparation, chemical and thermal stability and cost effectiveness since the materials involved are relatives inexpensive and readily available (Mahony et al., 2005, Svenson and Nicholls, 2001). MIP appears in different formats such as solid phase extraction (SPE) cartridges (Andersson, 2001, Caro et al., 2004, Cormack et al., 2000, Haginaka, 2004, He et al., 2007, Hu et al., 2005b, Jiang et al., 2009, Mahony et al., 2005, Zhu et al., 2009), in-line extraction devices (Dias et al., 2008, Hantash et al., 2007, Huang et al., 2004, Liu et al., 2005, Yano and Karube, 1999, Zhang et al., 2009, Zhuang et al., 2007), liquid chromatography column (Schweitz et al., 1997, Vallano and Remcho, 2000), sensors and membranes (Haupt, 2001, Kriz et al., 1995, Sergeyeva et al., 2001, Sergeyeva et al., 1999, Suedee et al., 2004). A summary of advantages and disadvantages of MIP is given in Table 1.1.

Table 1.1 Summary of advantages and disadvantages of MIP for analytical applications. (Adapted from: Mahony et al., 2005)

Advantages	Disadvantages
• Cost-effective compared with biomolecule-based recognition • Easy in preparation, thermally and chemically more stable than antibodies • According to the need of application, MIP can be prepared in different formats (bead/block/thin film) • Can be stored for a long period without losing the affinity toward targeted analyte.	• Lower catalytic capabilities than biological counterparts • Unfavorable adsorption isotherm and slow mass transferring the polymer matrix • Bleeding of template requires suitable template analogue and affects the quantitative applications. • High losses and labor-intensive in grinding and sieving of bulk polymer for the application of SPE/LC

1.2 General Principle of Molecular Imprinting

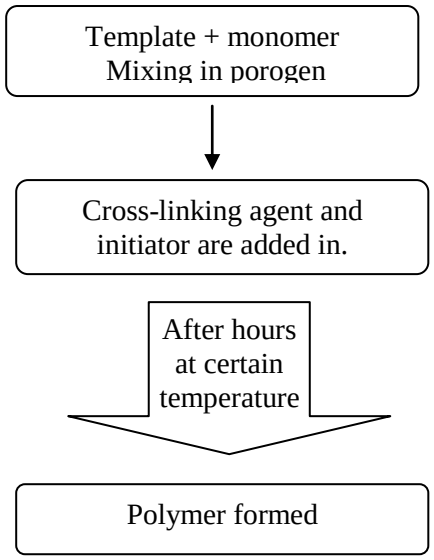


Figure 1.1 Flow chart of the general process of polymerization of imprinted polymer.

Generally, polymerization of imprinted polymer involves template molecule, monomer, cross-linking agent, initiator and porogen which serves as solvent that bring all components into one phase (Figure 1.1). The formation of MIPs involve the polymerization

of monomer in the presence of an appropriate template molecule. An appropriate combination of monomer, template, cross-linking agent will allow optimum guest-binding sites. The process of synthesizing MIPs starts with mixing the template and monomers in a porogen. Monomer and template either connected covalently and formed monomer-template conjugate or being placed nearby and through non-covalent interactions forming monomer-template adduct. A cross-linking agent is added to connect the functional molecules acting as a polymer backbone, in order to maintain the alignment of functional groups which are optimally set for the binding of template molecule. An initiator is added into the mixture and allows polymerization process at certain temperature for a certain period to happen. The structure of conjugates or adducts are frozen in a three dimensional polymer network.

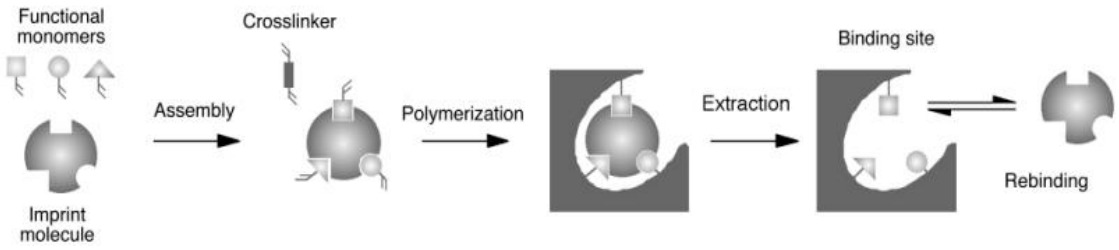


Figure 1.2 Schematic representation of the molecular imprinting principle. (Adapted from: Haupt, 2001)

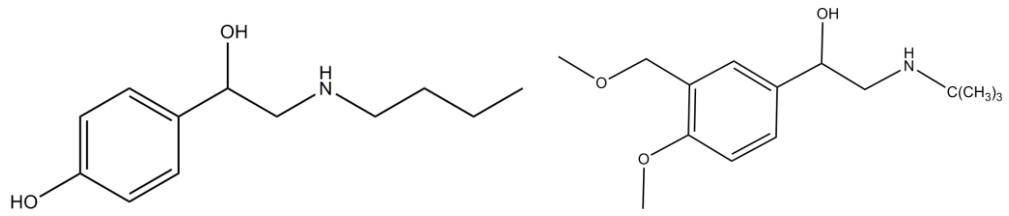
After the polymer is formed, the template needs to be removed from the polymer network. Removal of template allows the space that was originally occupied by template to be left as a cavity that is complementary to the template in their size and shape, and in the arrangement of functional groups. Under appropriate conditions, the cavities will remember the size, structure and other physiochemical properties of the template and rebind the molecule or its analog efficiently and selectively (Haupt and Mosbach, 1998, Komiyama et al., 2003, Takeuchi et al., 1998).

1.3 Polymerization Reagents

In the process of synthesizing MIP, the chemicals required are template molecule, functional monomer, cross-linking agent, initiator and solvents that serve as porogen.

1.3.1 Template

The template is of central importance in all imprinting processes. It directs the organization of functional groups on functional monomers. Template molecules must chemically inert under polymerization conditions. Since all polymerization involves free radicals, template molecules must not participate in the radical reaction, and should be stable upon the exposure to UV radiation of high temperature (Cormack and Elorza, 2004). After MIP is formed, the polymer is subjected to extraction, reflux or sonication to remove the template molecule. To date, MIP still encounters template leakage problems. The difficulty in extracting 100% of the template molecule from imprinted polymer has long been recognized even after an extensive washing and extracting procedures. Recently it was widely believed that the few percent of template molecule remaining within the polymer network was permanently entrapped (Cormack and Mosbach, 1999). Rather than take steps to minimize leakage, the alternative is to use a close structural analogue of target analyte as template (Andersson et al., 1997, Cormack et al., 2000) to overcome bleeding problems. In both the study of Blomgren et al., 2002, Crescenzi et al., 2001, they used bromoclenbuterol as the template in the MIP for solid phase extraction for the extraction of clenbuterol from calf urine and bovine liver respectively. Andersson et al., 1997 used a close structural analogue of sameridine as template molecule in producing an imprinted polymer which still had strong affinity for sameridine. In this study, bamethane and methylated salbutamol were used as the template molecules to synthesize imprinted polymers. These two compounds were used as template due to their structure similarity with targeted analyte, salbutamol.



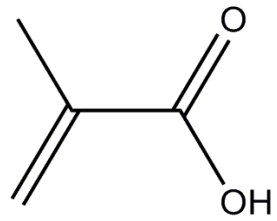
(a) Bamethane (b) Methylated salbutamol

Figure 1.3 Chemical structures of template molecules: (a) bamethane, and (b) methylated salbutamol

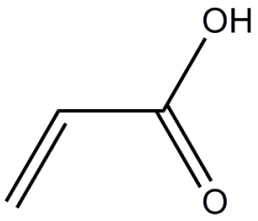
1.3.2 Functional Monomer

Functional monomers are responsible for the binding interactions in the imprinted binding sites. Generally in non-covalent molecular imprinting procedure, functional monomers are used in excess relative to the amount of template (in moles) to stabilize the formation of template-monomer assemblies. The ratio of template to functional monomer of 1:4 and upwards are rather common in non-covalent imprinting approach. The functional groups on template molecules and functional groups on monomers must match in a complementary fashion in order to obtain maximum complex formation. Functional monomers can be divided into acidic, basic and neutral monomers. Methacrylic acid is the most common acidic monomer and widely used by various research groups (Dirion et al., 2002, Jiang et al., 2009, Mohamed et al., 2008, Theodoridis and Manesiotis, 2002, Zurutuza et al., 2005). For acidic template molecules, vinylpyridine and acrylamide can be used as functional monomers (Hu et al., 2005b, Möller et al., 2004, Simon and Spivak, 2004). Figure 1.3 shows the structure of some functional monomers use in non-covalent imprinting.

(a) Acidic monomers

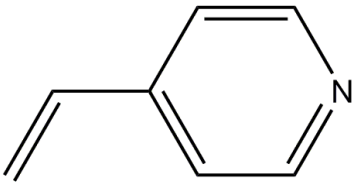


Methacrylic acid

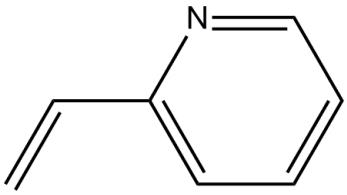


Acrylic acid

(b) Basic monomers

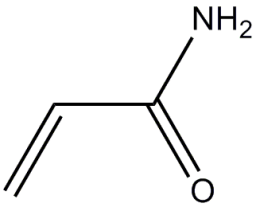


4 – Vinylpyridine

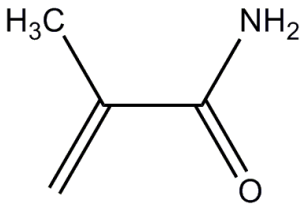


2 – Vinylpyridine

(c) Neutral monomers



Acrylamide



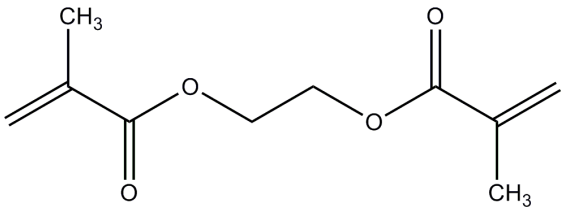
Methacrylamide

Figure 1.4 Chemical structures for some monomers used in non-covalent imprinting approach. (a) Acidic monomers, (b) basic monomers, (c) neutral monomers.

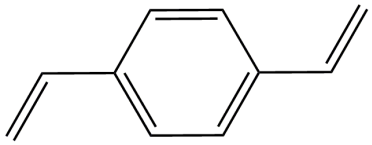
1.3.3 Cross-Linking Agent

In polymerization processes, a cross-linking agent is added to fulfill three major functions. Cross-linking agents control the morphology of the polymer network, stabilize the imprinted binding sites and contribute to the mechanical stability of the polymer matrix. High cross-linking ratios (70-90%) are generally preferred in order to retain the shape of the binding sites and generate polymers with adequate stability (Cormack and Elorza, 2004, Masque et al., 2001). In order to ensure the polymerization takes place sufficiently, the

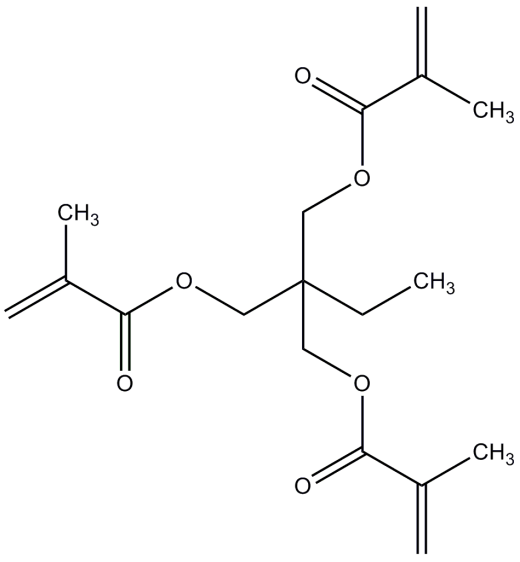
reactivity of cross-linking agent should be similar to the reactivity of the functional monomer to ensure smooth incorporation of the monomer and cross-linking agent. Subsequently, the functional residues (derived from functional monomers) can uniformly distribute in the polymer network. Figure 1.4 shows several of well-known and commercially available cross-linking agents such as ethylene glycol dimethacrylate (EGDMA), divinylbenzene (DVB) and trimethylpropane trimethacrylate (TRIM).



Ethylene glycol dimethacrylate (EGDMA)



Divinylbenzene (DVB)



Trimethylpropane trimethacrylate (TRIM)

Figure 1.5 Selection of cross-linking agents commercially available and commonly used for molecular imprinting.

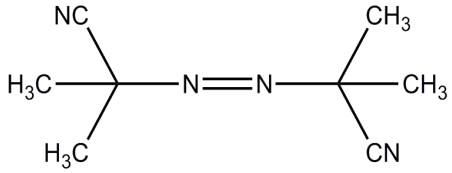
1.3.4 Porogen

The porogen is the solvent that brings all the components in polymerization into one phase. Through the nature and volume of porogen used in polymerization, the morphology and total pore volume can be controlled in macroporous polymers. The porogen promotes the rates of analyte binding and release. Thermodynamically good solvents will tend to lead to polymers with well-developed pore structure and high specific surface areas. Alternatively, thermodynamically poor solvents will lead to polymers with poorly developed pore structures and low specific surface areas. Excess chemical potential of mixing is defined as the difference between the chemical potential polymer and solvent. When the excess chemical potential of mixing between polymer and porogen is negative, it shows the solvent is thermodynamically good solvent. Selection of porogen is dependent on the kind of imprinting (Cormack and Elorza, 2004). In covalent imprinting, solvents are used as long as all the components are dissolved. Besides acting as pore forming agent, solvents in non-covalent imprinting also promote the formation of template-monomer adducts and subsequently enhance the imprinting efficiency (Komiyama et al., 2003). Generally, aprotic and non-protic solvents such as toluene, chloroform and acetonitrile are used to stabilize hydrogen bonds (Chassaing et al., 2004). If hydrophobic forces are needed to drive the complexation of template-monomer, then water could be the solvent of choice (Caro et al., 2004).

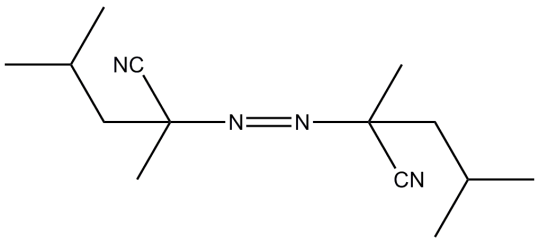
1.3.5 Initiator

In the imprinting systems, initiators are used to initiate free radical polymerization. Initiators are triggered either by heat, UV radiation or chemicals. After the initiator is triggered, the carbon centered free radicals will form and these unpaired electrons will react with other monomers or cross-linking agent in order to propagate into longer chains.

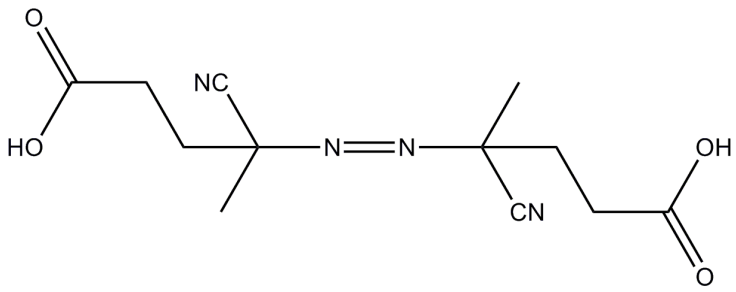
Polymerization stops when two free radicals react with each other. Relatively, the amount of initiator added into polymerization process is low as compared to the amount of monomer. Azo initiators are the most common initiator that widely used by various research groups (Bereczki et al., 2001, Claude et al., 2008, Khorrami and Mehrseresht, 2008, O'Shannessy et al., 1989). Azo initiator such as azobisisobutyronitrile (AIBN) can be conveniently decomposed by UV or thermolysis. Figure 1.5 shows the chemical structure of some azo initiators.



Azobisisobutyronitrile (AIBN)



2,2’-Azobis(2,4-dimethylvaleromitrile) (AMVN)



4,4’-azo(4-cyanovaleric acid)

Figure 1.6 Chemical structure of some azo initiators.

1.4 Molecular Imprinting Approaches

Techniques for molecular imprinting can be classified into two approaches, covalent approach and non-covalent approach. This classification depends on the chemical bonding involved between the monomer and template.

1.4.1 Covalent Approach

The covalent approach was first introduced by Wulff and co-workers (Wulff, 1995, Wulff and Poll, 1987) followed by Shea and co-workers (Shea and Dougherty, 1986, Shea and Sasaki, 1989, Shea and Sasaki, 1991). In covalent systems, a template-monomer complex is formed through reversible covalent bonding and usually the template is a polymerizable derivative of the target analyte. Due to the stability of covalent bonding, a homogenous population of binding sites is obtained (Takeuchi et al., 1998, Turiel and Martin-Esteban, 2004). After polymerization, in order to remove the template, the covalent bonds are chemically cleaved and subsequently reformed during the rebinding of target analyte (Haupt and Mosbach, 1998).

Boronic acid monomers are suitable for the covalent approach. A lot of diols have been used as template-forming boronic acid esters with 4-vinylphenylboronic acid which are relatively stable. After polymerization, the covalent bonds are cleaved through hydrolysis. Boronic groups are arranged suitably for guest binding. These conjugates are useful in the molecular imprinting of carbohydrates and its derivatives (Wulff et al., 1997, Wulff and Schauhoff, 1991). Ketone and aldehyde forming ketal and acetal bonding with 1,3-diol compounds which are useful to use as functional monomers (Shea and Dougherty, 1986, Shea and Sasaki, 1989, Shea and Sasaki, 1991). In principle, Schiff bases are also suitable for imprinting. Reaction of aldehyde with amine compounds will produce Schiff base

compound can be used in molecular imprinting (Shea et al., 1990, Wulff et al., 1986). Metal coordination involves the metal ions interacting with functional monomer produces polymerizable metal complexes which act as functional monomers to bind with appropriate ligand (template) through covalent binding (Chen et al., 1997, Matsui et al., 1998, Matsui et al., 1996).

1.4.2 Non-Covalent Approach

Mosbach and co-workers introduced a non-covalent approach in imprinting techniques which utilize non-covalent forces such as hydrogen bonds, van der Waals forces, ionic interactions and hydrophobic effects (Arshady and Mosbach, 1981, Ekberg and Mosbach, 1989, Mosbach, 1994, Mosbach and Ramstrom, 1996, Vlatakis et al., 1993, Sellergren et al., 1988). The non-covalent approach in molecular imprinting is the simplest method in producing selective polymers. Complexation is done by mixing template and functional monomers and copolymerized with cross-linking agent without needing to synthesize covalent conjugates before polymerization. Furthermore, the template can easily be removed from the polymer network under mild conditions. But non-covalent bonding may not be strong enough to maintain template-functional monomer complexes. Thus, excess functional monomers are usually added to ensure as many interactions as possible occur to stabilize template-functional monomer complexes during polymerization. This leads to heterogeneous binding sites (Mosbach, 2008, Takeuchi and Haginaka, 1999, Wulff, 1995).

Many important molecules such as pharmaceutical substances, herbicides, biologically active substances and environmental contaminants have polar groups such as hydroxyl, carbonyl, amino and amide groups which are suitable for non-covalent interactions. The most appropriate non-covalent bonding for precise molecular recognition is hydrogen bonding. Hydrogen bonding is highly dependent on the distance and direction

between monomers and templates. For electrostatic interactions, strong acids and strong bases are unfavorable since they are less dependent on the distance and direction. A proton is transferred completely from acid to base. For combinations that consist of intermediate acid and base strength, efficient imprinting will be achieved since hydrogen bonding will be dominant. Interactions of weak acids and weak bases are weak, which are inappropriate for imprinting. Due to the simplicity, ease and versatility of non-covalent approach molecular imprinting, this technique has been widely used (Anderson et al., 1995, Bereczki et al., 2001, Jiang et al., 2009, Muldoon and Stanker, 1997, Zander et al., 1998). For example, Pereira and Rath, 2009 used the molecularly imprinted solid-phase extraction which prepared using non-covalent approach to extract fenitrothion from tomatoes. Schirmer and Meisel, 2008 prepared non-covalent imprinted polymer to use as solid phase extraction of chloramphenicol from honey samples. Vallano and Remcho, 2000 prepared a highly selective MIP column for capillary electrochromatography.

In a study by Whitcombe et al., 1995, a combination of covalent and non-covalent approach has been reported for cholesterol imprinting. Template-monomer complexes are covalently bound and used in polymerization process. The template is easily and efficiently cleaved in hydrolysis, leaving complementary binding sites, bearing a phenolic residue, capable of interacting with the template (ligand) through hydrogen bonding, resulting in more precise, homogenous and high specificity binding sites.

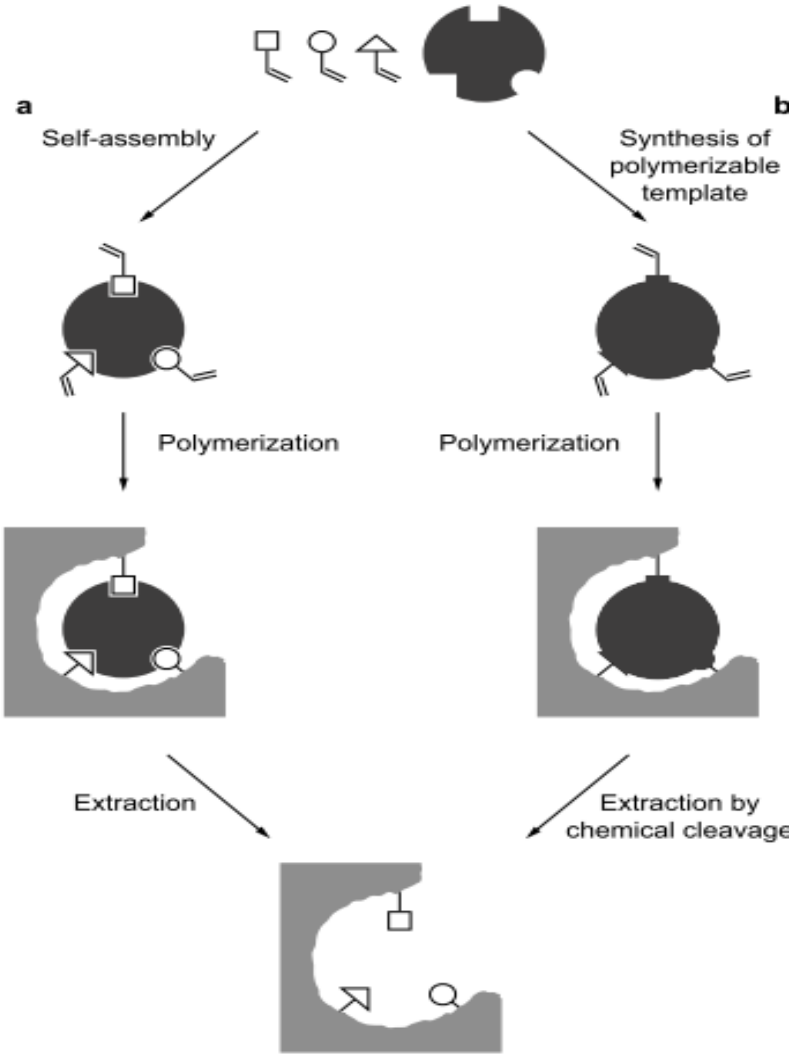


Figure 1.7 Schematic diagram of molecularly imprinted polymer. (a) Self-assembly (non-covalent) approach and (b) covalent approach. (Adapted from: Haupt and Mosbach, 1998).

1.4.3 Advantages and Disadvantages of Covalent and Non-Covalent Imprinting

Both covalent and non-covalent imprinting are significantly different from each other. Generally, non-covalent imprinting is easier to achieve and applicable to a wider range of templates. Covalent imprinting is always superior according to the strictness of imprinting (Komiyama et al., 2003, Mosbach, 2008, Takeuchi and Haginaka, 1999). Table 1.2 summarizes the advantages and disadvantages of covalent and non-covalent imprinting.

1.4.3.1 Advantages of Covalent and Non-Covalent Imprinting

For covalent imprinting, the template-monomer conjugates are stable and stoichiometric, resulting in more specific and homogenous binding sites. Due to the stability of template-monomer conjugates, a wide variety of polymerization conditions could be applied (Komiyama et al., 2003).

In non-covalent imprinting, the synthesis of monomer-template conjugate is unnecessary. Since the template is only bound weakly to the monomer through non-covalent interactions, the template can be easily removed from the polymer network under mild conditions. Taking the advantage of non-covalent interactions, the guest binding and releasing from the polymer network are fast.

1.4.3.2 Disadvantages of Covalent and Non-Covalent imprinting

In covalent systems, the synthesis of template-monomer conjugate is always troublesome and uneconomical. The option for reversible covalent linkages available is limited. Severe conditions are required for the cleavage of covalent linkages to remove template. Since the guest binding and guest releasing involve formation and breakdown of covalent bonding, thus, the processes are rather slow (Komiyama et al., 2003).

One disadvantage of non-covalent imprinting is that the imprinting process is less clear-cut, the template-monomer adduct is labile and not stoichiometric. Due to the non-covalent bonding between template-monomer, the polymerization conditions must be carefully chosen to maximize the formation of adduct in the mixtures. Addition of excess amount of monomer into the mixtures in order to stabilize the template–monomer adducts will result in non-specific binding sites and diminishes the binding selectivity.

Table 1.2 Advantages and disadvantages of covalent and non-covalent imprinting. (Adapted from: Komiyama et al., 2003)

Type of imprinting	Covalent	Non-covalent
Synthesis of template-monomer conjugate	Necessary	Unnecessary
Polymerization Conditions	Wide variety	Restricted
Removal of template after polymerization	Difficult. Severe conditions needed.	Easy. Can be done under mild conditions.
Guest binding and guest release	Slow	Fast
Structure of guest binding site	More specific and homogeneous	Non-specific

1.5 Synthesis of Molecularly Imprinted Polymer

Imprinted polymers, in the form of particles, are made by various polymerization methods. Each of the methods are developed to suit different target and applications. To date, MIPs have been prepared by bulk, suspension, two-step swelling, precipitation and emulsion core-shell polymerization. Other less common methods are such as film synthesis, aerosol polymerization and polymerization on silica particles. Each of these procedure of polymerization involves the control of different parameters during the synthesis, thus producing polymers with different properties (Perez-Moral and Mayes, 2004).

1.5.1 Polymerization Method

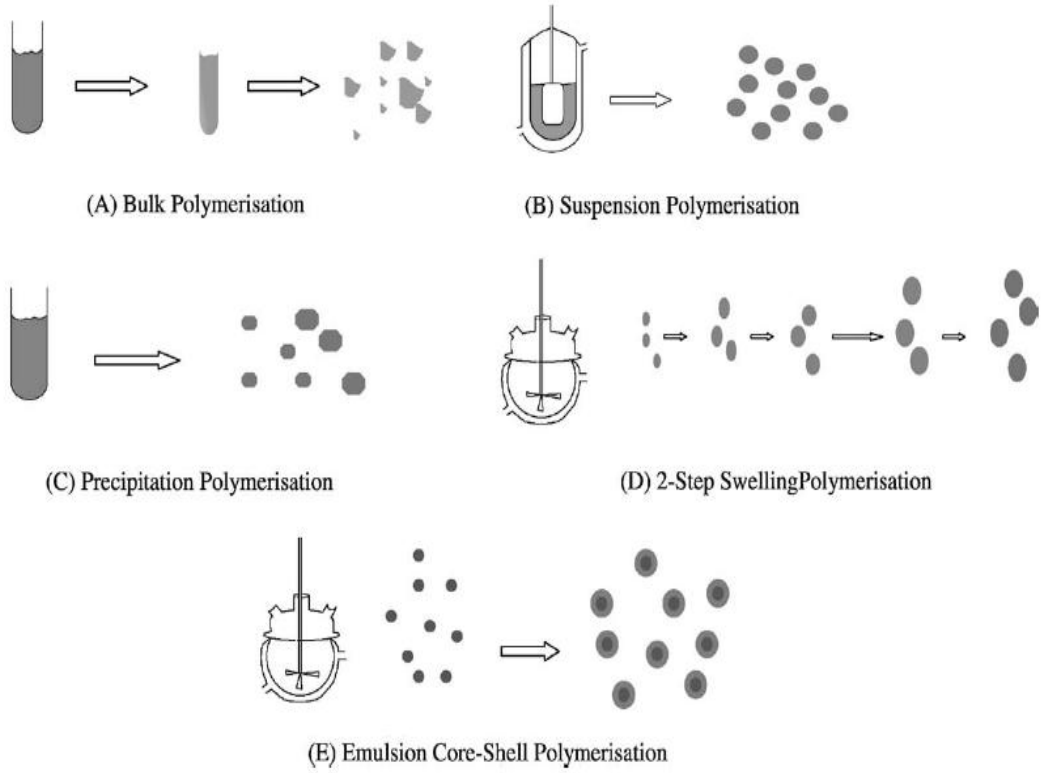


Figure 1.8 Schematic representation of the synthetic step in different polymerization procedures. (Adapted from: Perez-Moral and Mayes, 2004)

Table 1.3 Comparison of different polymerization methods. (Adapted from: Mayes and Mosbach, 1997)

Polymerization Methods		Complexity	Product	Advantages	Disadvantages
Bulk	In block	Very straight forward	Random fragment after grinding	Simple, imprinting not affected by method derived factors.	Tedious processing, wasteful, poor particle shape for HPLC.
	In columns	Moderate	Solid block filling column	Simple, no column packing required.	High back-pressure, poor peak shape.
Suspension	In water	Complex	Spherical beads, polydisperse	Highly reproducible results, large scale possible, high quality beads	Usage of water only possible for some covalent and metal chelate based processes.
	In perfluorocarbon	Complex	Spherical beads, polydisperse	Dispersant does not interfere with all methods, good quality beads	Expense of liquid fluorocarbons, specialist surfactant required.
Two-step swelling		Very Complex	Monodisperse beads	Excellent packing for HPLC	Need for aqueous emulsions, rules out many imprinting processes.

1.5.1.1 Bulk Polymerization

The first and most common polymerization method applied for MIP synthesis is based on bulk polymerization. Due to its simplicity and versatility, this method is by far the most widely used in imprinting work. All the polymerization ingredients such as template molecule, monomer, cross-linking agent, initiator, are mixed well in organic solvents (porogen) and then polymerized. The outcome of this method is a macroporous monolith polymer block that needs to be crushed and ground to obtain particles of irregular shape and size. The ground particles are sieved to obtain the required particle size. The process to grind

the polymer block is laborious and time consuming. During the grinding and sedimentation process, a lot of the produced polymer is wasted. Lack of control of the polymerization process, particularly when UV initiation is used may result in producing areas of heterogeneity in the polymer matrix (Mosbach and Ramstrom, 1996, Perez-Moral and Mayes, 2004).

1.5.1.2 Suspension Polymerization

Mayes and Mosbach (Mayes and Mosbach, 1996) are the first to describe suspension in a fluorocarbon solvent. This is a fast and reliable methodology. The particles of polymer are synthesized by UV radiation in less than 2 hours. Depending on the speed of stirring during the polymerization and the amount of surfactant, the diameter of beads obtained can vary from 5 to 50 μm. The usage of perfluorocarbon solvent (perfluoro-(1,3-dimethyl-cyclohexane) in the continuous phase allows the establishment of the same interactions as in bulk polymerization. The fluorocarbon suspending medium can be recycled by distillation. This method offers a simple one-step route to high quality beads with a quantitatively yield of product and is an attractive alternative to labor and time consuming grinding and sieving, especially for chromatographic applications. In the polymerization, coupled with the use of TRIM (trimethylpropane trimethylacrylate) as cross-linking agent, beads produced have high load capacities and show excellent separations, where as ground and sieved polymers perform poorly in this aspect (Mayes and Mosbach, 1997).

1.5.1.3 Precipitation Polymerization

The precipitation polymerization method can provide particles in the submicron scale (0.3-10 μm) with the desired characteristics. In this method, the polymerization of the

mixture, including the template molecule, monomer and cross-linking agent, occurs in the presence of a higher amount of porogen than that typically used in bulk polymerization method. Precipitation polymerization is based on the precipitation of the polymeric chains out of the solvent in the form of particles as they grow more and more insoluble in an organic continuous medium. In this method, extra stabilizer is not needed since the particles are prevented from coalescence by the rigidity obtained from the cross-linking of the polymer. In a study by Cacho et al., 2004, they produced polymers with a more homogenous binding sites distribution and higher affinity constant compared to polymers obtained by bulk polymerization using two different propazine-imprinted methacrylic based polymers. The group of Ye et al., 1999 successfully obtained two models of molecularly imprinted monodisperse microspheres which are highly specific and very stable. The average particle diameter of 17β-estradiol-imprinted microspheres is 0.3 μm and theophylline-imprinted microspheres is 0.2 μm. Tamayo et al., 2003 produced a fenuron methacrylic acid based polymer which possessed homogeneous binding site distribution and in spherical form (~ 1 μm) through precipitation polymerization. They developed MIPSPE procedure using the produced microspheres for the trace-enrichment and clean-up of fenuron from plant sample extracts.

1.5.1.4 Two-Step Swelling Polymerization

Two-step swelling polymerization was developed with MIP by Hosoya et al., 1994. Several swelling steps on the initial particles are required with the imprinting mixture before polymerization proceeds. Water dispersion polystyrene seed particles was mixed with microemulsion and water by sonicating. The mixture was stirred for the first step swelling until oil microdrops vanished. Template, monomer and cross-linking agent and porogens in water contained dispersion stabilizer were added into the swollen particles and stirred for the second step swelling. Polymerization started under a flow of argon atmosphere at 70-75 ℃

with slow stirring after second swelling step was completed (Hosoya and Frechet, 1993, Hosoya et al., 1996). Two-step swelling polymerization method is a kind of suspension polymerization method which water is used as continuous phase where the interactions between template molecules and monomers are hampered (Haginaka, 2004). Monodisperse particles in the micron size range (2-50 μm) are produced with the good control of the final size and number of the particles (Perez-Moral and Mayes, 2004). These uniformly sized and monodispersed particles could be easily prepared and in-situ modification could be performed. The resulting monodisperse micron size particles are suitable for HPLC packing materials or SPE materials. The preparation of monodisperse polymer particles based separation media in HPLC system provide excellent column efficiency with very low column pressure drops (Ellingsen et al., 1990, Kulin et al., 1990). Hosoya et al., 1994 prepared uniform-size macroporous polymer based imprinted polymers for the separation of naphthalene enantiomers. In 1997, Haginaka et al. developed a molecularly imprinted uniform-sized polymer based stationary phase for the separation of naproxen enantiomers.

1.5.1.5 Emulsion Core-Shell Polymerization

This emulsion polymerization method will produce core-shell particles. These particles have a structured morphology that allows the incorporation of any added property into the core of the particle without interfering with the imprinted shell. Water is used as continuous medium during polymerization. In this method, particles produced are monodisperse with colloidal size in the range of 0.05-2 μm. In core-shell method, the final number, size and surface area of imprinted monodisperse particles can be efficiently controlled by the composition, structure of monomers and the reaction conditions used. The specific properties such as fluorescence or magnetism can be introduced into the cores as well (Pérez-Moral and Mayes, 2002). This polymerization method starts with the preparation of seed latex which can be prepared from a wide variety of materials such as styrene,

divinylbenzene, alkyl acrylate and methacrylate ester. Emulsion polymerization is used to produce seed latex, which droplets of monomer are emulsified with surfactant in a continuous phase of water in a reactor. These seed latex monodisperse particles have diameter size range from approximately 30 nm to greater than 1 μm. In second stage, the seed is mixed with cross-linker monomer or mixture of monomers and template in a reactor before surfactant and deionized water were added. For the case of a semi-continuous process in the second stage, the monomer is fed into a reactor which contains the seed particles under carefully controlled conditions (Pérez-Moral et al., 2000). In the research of Pérez-Moral et al., 2000, they developed a cholesterol-imprinted ethylene glycol dimethacrylate shell which displayed binding properties similar to that of analogous bulk polymers prepared by bulk polymerization techniques.

1.5.2 Factors to Consider for the Synthesis of Selective MIP

1.5.2.1 Molar Ratio of Template: Monomer: Cross-Linking Agent (T:M:X)

Homogeneity and the efficiency of the MIP recognition sites are highly dependable on the molar relationship between template and functional monomer. The typical molar ratio of template molecule, monomer and cross-linking agent (T:M:X) to synthesis imprinted polymer is 1:3-5:20-30 (Komiyama et al., 2003). High molar ratio of template: monomer (T:M) affords less than optimal complexation due to insufficient amount of functional monomer (Andersson et al., 1999). A low molar ratio of T:M causes non-specific binding that arises from random functional monomer residue orientation (Nicholls, 1995). In the results of Andersson et al., 1999 study show that an excess of either functional monomer or template in polymerization is unfavorable with regard to selectivity. In their study, Andersson and co-worker prepared a series of polymer with different molar ratios of T:M for selectivity test. As shown in their results, polymers with a ratio T:M of 1:4 showed the best

selectivity properties as compared to other polymers. Polymers prepared at T:M = 1:15 and 1:20 showed limited molecular recognition effect. In an experiment done by (Baggiani et al., 2004), the imprinted polymers prepared at T:M = 1:15 and 1:20 do not clearly discriminate them from corresponding non-imprinted polymers. Research by Theodoridis et al., 2004 showed that at higher template molar concentrations, agglomeration of template occurred on the organic solvent environment. Thus, polymers prepared at the ratio of 1:4.6:23 and 1:46:230 showed better recognition properties than polymers synthesized at the ratio of 1:2.7:13.4. Other research groups such as Caro et al., 2004, Mena et al., 2002, Muldoon and Stanker, 1997, Urraca et al., 2006 and Zander et al., 1998 prepared polymers at the ratio of 1:4:20. But according to the research of Davies et al., 2004, through a chemometric approach, the optimum predicted ratio for T:M:X were 1:10:55 and 1:10:10.

1.5.2.2 Stability of Monomer-Template Assemblies

The functional monomer-template assemblies must be stable to achieve a high yield of imprinted binding sites (Sellergren, 1999). The choices of functional monomer and porogen are important to produce stable monomer-template assemblies. As mentioned in Section 1.3.2 that the functional groups of the selected monomer should be able to match with functional group of template molecule forming covalent or non-covalent interactions. The most suitable solvents to serve as porogen will be aprotic and non-protic solvents which have poor hydrogen binding capacity and low dielectric constants. Solvents with low dielectric constants are likely to lead to a large interaction energy between template molecule and functional monomer, resulting in better affinity and selectivity on MIP (Wu et al., 2005). Work from Wu et al., 2005 showed that small dielectric constant solvents were in the favor of forming higher concentration of monomer-template assemblies, thus producing a MIP with high affinity and selectivity. Figure 1.6 shows the factors that influence the recognition sites related to the monomer-template assemblies.

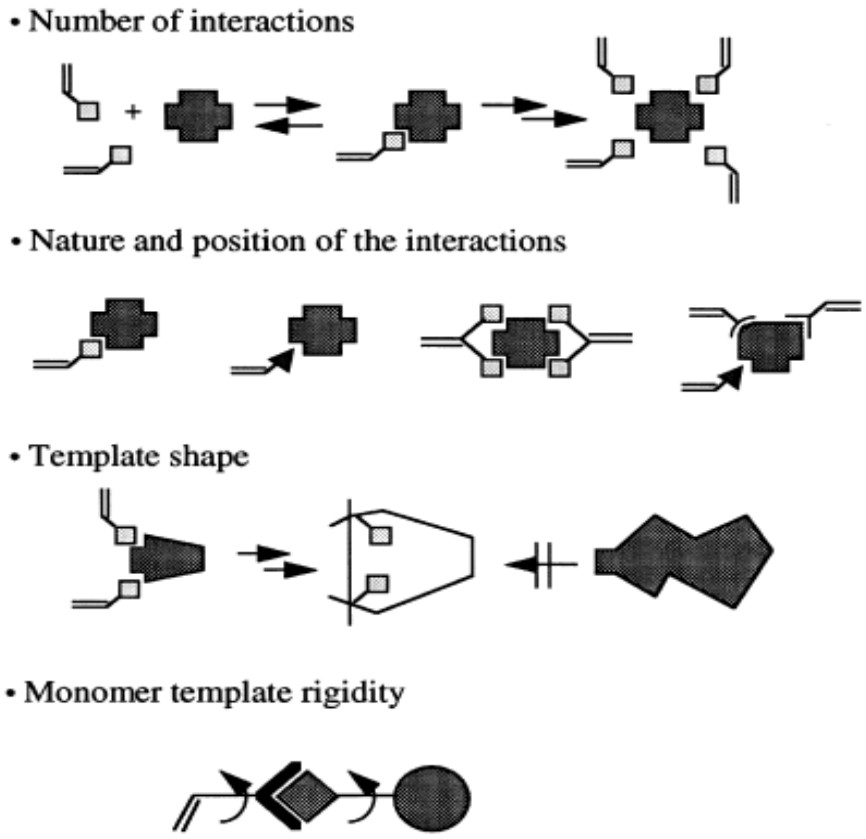


Figure 1.9 Factors influencing the recognition sites related to the monomer-template assemblies. (Adapted from: Sellergren, 1999)

1.5.2.3 Polymerization Temperature

Temperature affects the polymerization process and polymer structure, which in turn influences the quality and the quantity of the recognition sites. At lower temperatures, polymerization of MIP forms polymers with greater selectivity as compared with polymers synthesized at elevated temperatures. Usually lower temperatures will stabilize the monomer-template assemblies since it leads to a greater strength of electrostatic interactions (Lu et al., 2004, Spivak, 2005). In the work of Lu et al., 2004, they prepared 3-L-Phenylalanylaminopyridine imprinted polymers at various temperatures of 10, 40 and 60 ℃ respectively. Imprinted polymers prepared at 40 ℃ showed the highest enantioselectivity and largest load capacity as compared to the other polymers prepared at 10 and 60 ℃. In the

research work of Wei et al., 2005, it was found that imprinted polymers prepared at 4 ℃ have higher capacity factors than imprinted polymers prepared at 60 ℃. In the experiment carried out by Piletsky et al., 2002, the results suggest that the polymer is able to memorize the polymerization temperature in a manner similar to memory effects for the template and polymerization solvent.

1.6 Polymer Structure and Morphology

The structural integrity of the monomer-template assemblies has to be preserved during polymerization in order to allow the functional groups that on the monomer to be fixed in space in stable arrangement. Thus removal of the template from the polymer network will generate a structure complementary to the template or an analogue structure. The polymer matrix not only contains binding sites in a stable form but provides porosity allowing easy access for the template to all sites. Porosity in polymer network is achieved with the presence of porogen and the stability of the binding sites achieved by applying high levels of cross-linking agent.

1.6.1 Types of Pores

A wide distribution of pore sizes appears in most of the cross-linked imprinted polymer network associated with various degrees of diffusional mass transfer limitations and swelling. The pore sites can be classified into different types according to the criteria of site accessibility, integrity, and stability. Typically, there are three types of pores which are mesopores, macropores and micropores. Mesopores and macropores (site A) which with the pore size larger than 20 Å are expected to be easily accessible. On the other hand, micropores of < 20 Å (site B) have slow diffusion but are higher in number since the surface