CHAPTER I

INTRODUCTION

1.1 Molecular imprinted polymers (MIPs)

During the last decade, research interest in molecular imprinted polymers (MIPs) has strongly increased with potential applications ranging from solid phase extraction materials[1-6] to receptors in sensors[7-10] and immuno-like assays[11-16] (Table 1.1). Due to their highly selective recognition properties, MIPs are often refered to as artificial antibodies. Moreover, MIPs possess several advantages over their biological counterparts such as low cost, ease of production, and good physical and chemical stability.

Molecular imprints have been demonstrated against many classes of compounds. These include drugs, hormones, pesticides, proteins, amino acids, peptides, carbohydrates, coenzymes, nucleotides, nucleotide bases, steroids, and dyes[17].

1.1.1 Theory

Molecular imprinting is a technique for preparation of polymeric materials that have tailor-made selectivity for the templates molecule. The recognition properties of polymers are resulted from functional group interaction between template molecule and functional monomer. The types of interaction can be hydrogen bonds, ion pairing, π-π interaction or driven by the hydrophobic effect. The affinity increases with the number

Table 1.1 Applications of molecular imprinted polymers (MIPs)

Applications of MIPs	Template molecule	References
Stationary phase	Amino pyridine Caffeine	[1] [5,6]
Sensor	Bisphenol A Brucine	[8] [10]
Catalysts	Paraoxon Methylvaline	[18] [19]
Antibody/receptor binding mimics	(*S*)-propranolol Atrazine	[11,12] [15,16]

of interacting groups. However, each individual interaction can be strongly dependent on the properties of the solvent such as protic or aprotic, polarity, dielectric constants, presence of complex forming agents, etc.

The selectivity of MIPs in comparison to non-imprinted polymers (NIPs) -same MIPs procedure without using template molecule- can only occur when the analyte and/or the matrix components of the sample have an increased number of interaction points. Therefore, single and part of the dual point interactions that take place between the template molecule and the polymer will affect the selectivity. Schematic representation of the interaction of template molecule and MIP is shown in Figure 1.1. The most favorable energetic interaction is shown at location A, where three binding sites

result in the most selective (specific) interaction of the template with the polymer. Two-point interactions, which are energetically less favorable, are depicted at locations B, where the template is still allowed to approach the binding sites at more than one way, and therefore is less selective in its interaction with the polymer. Locations C and D show the least selective interaction possible which can lead to non-specific binding and are therefore the least favorable.

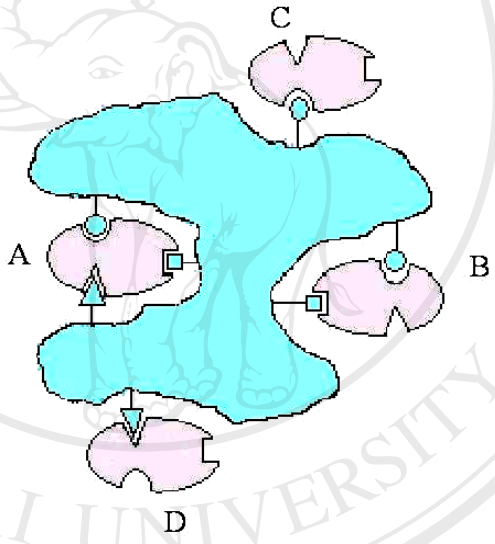


Figure 1.1 Schematic representation of the interaction of the analyte and the synthesized polymer.

1.1.2 Molecular imprinting strategies and procedures

The procedure for synthesis of MIPs have been described in detail[20-24] which generally use the following strategies. During the molecular imprinting process, highly cross-linked co-polymers are formed around analyte molecules acting as cavity-creating templates. The template molecules are then removed, providing binding sites ideally complementary in size, shape and functionality to the templated molecule. Upon re-introduction of the template preferential rebinding within the cavity should occur.

In the pre-polymerization mixture, the dissolved target analyte interacts by covalent, non-covalent, or metal coordination interactions which responsible for localizing the chemically active moieties of the target molecules during copolymerization. Consequently, molecular imprinting is classified into two types according to the type of interactions between functional monomer and target molecules in the pre-polymerization as mixture covalent and non-covalent imprinting (Figure 1.2).

1.1.2.1 The covalent molecular imprinting

The covalent approach to molecular imprinting promises the most homogeneous binding site distribution with largely identical binding pockets. The binding constants for the template molecule are high, since the interaction between template and functional monomer is based on covalent interactions sure to withstand polymerization conditions. A chemical synthesis step is necessary to bind the template to the functional monomer with bond types such as Schiff bases, boronates, ketals, carboxylic amides and esters. However, despite the high affinities of the polymeric material, the range of functional

groups which can be targeted is restricted and removal of the template molecules is difficult (chemical cleavage) and limits the application of covalently prepared MIPs.

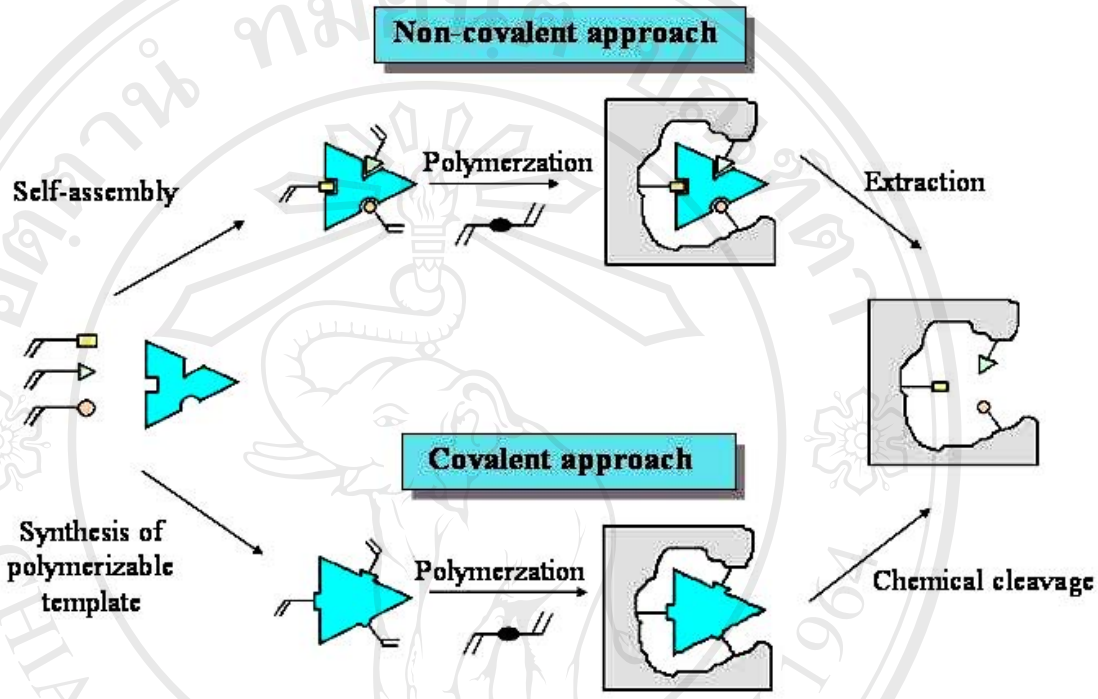


Figure 1.2 Principle of molecular imprinting

1.1.2.2 The non-covalent molecular imprinting

In non-covalent molecular imprinting, complexes assembled by non-covalent interactions are formed in the pre-polymerization mixture. Complexation is achieved by mixing template, functional monomer, and cross-linker in a porogenic solvent matrix. As a consequence, sufficient complex stability is required to enable binding pocket formation during the polymerization process. In contrast to covalent imprinting, the self-assembly approach is characterized by a more heterogeneous binding site distribution.

Resulting, sample overload may occur due to rapid saturation of the comparatively low amount of high-affinity binding sites, which results in a significantly decreased overall polymer performance.

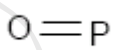
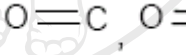
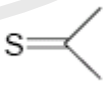
However, non-covalent imprinting compounds is being increasingly adopted for the preparation of MIPs because polymer can be prepared easily and a wide range of imprintable compounds can be used. Therefore, in this study will focus on non-covalent imprinting.

1.1.2.3 Molecular interactions involved in non-covalent imprinting

The main non-covalent interactions responsible for molecular recognition in biomimetic systems are hydrogen bonding, ion-pairing, and π-π interactions. Furthermore, coulombic attraction, charge transfer, induction, dispersion, and exchange-repulsion contribute to the complex formation. The driving forces of ion pairing interactions (ion-ion, dipole-ion, dipole-dipole) are coulombic interactions. Hydrogen bonding is a strong interaction playing an important role in naturally occurring noncovalent interactions. Complexes based on hydrogen bonding typically exhibit comparatively high stability constants. Table 1.2 gives examples of hydrogen bonding donor and acceptor groups. These interactions are favored in weakly polar aprotic solvents such as acetonitrile. In contrast, more polar protic solvents support interactions such as metal-ion coordination of the template molecule. Comparatively weak electrostatic interactions such as π-π stacking may occur between aromatic rings in polar solvents such as water and methanol. Hydrophobic interactions are only facilitated in highly polar solvents or solvent mixtures such as water/methanol. The wide variety of possible interactions implies that molecular recognition of a guest molecule may be

dominated by one mode of molecular interaction or controlled by a combination of different recognition mechanisms, which are enabled or disabled depending on the polarity of the selected protic or aprotic porogen. In general, the combination of two or more interaction modes can be expected.

Table 1.2 Hydrogen bonding donor and acceptor groups

Donor	Acceptor
	
	
	
	
	

1.1.3 Parameters of MIP syntheses [25, 26]

In the processes of MIP synthesis various parameters such as template molecule, function monomers, cross-linkers, porogens and initiators have to be considered in order to create the selective site to the analyte.

1.1.3.1 Template

In the molecular imprinting processes, the template molecule is of central importance in that it directs the organisation of the functional groups pendent to the functional monomers. Unfortunately, not all templates are directly amenable to templating. In terms of compatibility with free radical polymerisation, templates should have following properties:

- should be chemically inert under the polymerisation conditions
- do not contain any polymerisable groups
- have no functionality that could potentially inhibit a free radical polymerisation
- should be stable at initiate temperatures of initiator

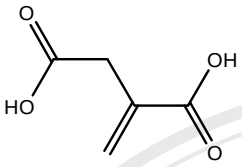
A wide variety of template molecules have been used in various imprinting protocols. A selection of different substances is demonstrated Table 1.3.

Table 1.3 Various print molecules used in imprinting protocols

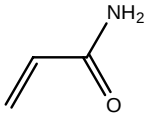
Compound Class	Example	Ref.
Drugs	Theophylline	[27, 28]
	Morphine	[29]
	Ephedrine	[30]
Hormones	Cortisol	[31]
	Epinephine	[32]
	Castasterone	[33]
Pesticides	Atrazine	[15, 34]
	2,4-D	[13, 14]
Amino acids	Phenylalanine	[35, 36]
	L-glutamine	[37]
Carbohydrates	Glucose	[38]
	Lactose	[39]
Enzymes	a-Amylase	[40]
	Lysozyme	[41]
Nucleotide bases	Adenine	[42]
	9-ethyladenine	[43]

1.1.3.2 Functional monomers

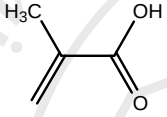
In an imprinted polymer the functional monomers are responsible for the binding interactions with the template molecule for pre-polymerization that this creates the binding sites in the imprinted polymer before the assembled complex is trapped with a cross-linker to form a rigid polymer network. Several functional monomers have been used in molecular imprinting protocols that the most are acrylate-based, acrylamide-based and styrene-based systems. The chemical structures of a selection of the more important functional monomers are shown in Figure 1.3.



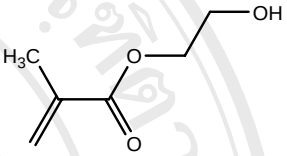
Itaconic acid



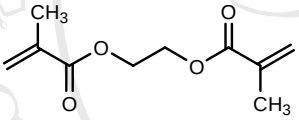
Acrylamide



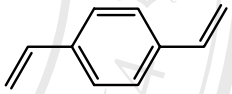
Methacrylic acid (MAA)



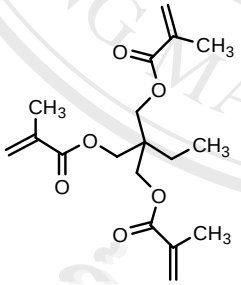
2-hydroxyethyl methacrylate (2-HEMA)



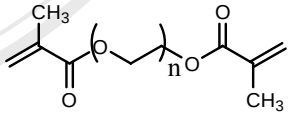
Ethylene glycol dimethacrylate (EGDMA)



p-divinylbenzene (DVB)



Trimethylpropane trimethacrylate (TRIM)



Poly ethylene glycol dimethacrylate (p-EGDMA)

Figure 1.3 The function monomers used in the non-covalent approach.

1.1.3.3 Cross-linkers

Generally, the high degrees of cross-linker (70-90%) are preferred. The cross-linker has three major functions:

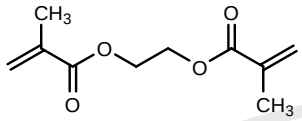
- The cross-linker is important in controlling the morphology of the polymer matrix (gel-type, macroporous or a microgel powder).
- It serves to stabilise the imprinted binding site.
- It imparts mechanical stability to the polymer matrix.

Originally, isomers of divinylbenzene were used for cross-linker of styrene and other functional monomers into polystyrenes. Recently, acrylate or methacrylate-based systems (di-, tri- or tetra-unsaturated) are commonly employed in several systems. The chemical structures of several cross-linkers are shown in Figure 1.4.

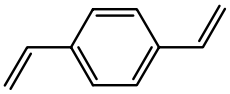
1.1.4.4 Solvents (Porogens)

The solvent plays an important role in the outcome of molecular of molecular imprinted processes. The main responsibilities of solvent are as follows:

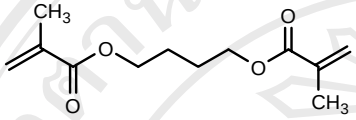
- It brings all the components in the polymerisation, such as template, functional monomer, cross-linker and initiator into one phase.
- It creates the pores in macroporous polymers. For this reason it is quite common to refer to the solvent as the "porogen". When macroporous polymers are being prepared, the nature and the level of the porogen can be used to control the morphology and the total pore volume.
- It is particularly pronounced in self-assembly systems. As porogen in polymerization, the solvent govern the strength of non-covalent interactions.



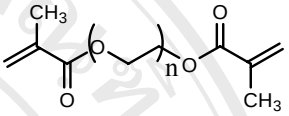
ethylene glycol dimethacrylate (EGDMA)



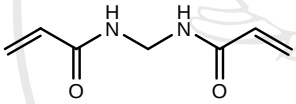
p-divinylbenzene (DVB)



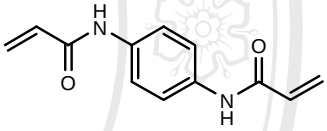
tetramethylene dimethacrylate (TDMA)



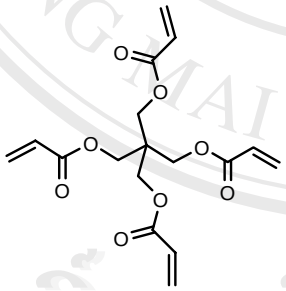
poly ethylene glycol dimethacrylate (p-EGDMA)



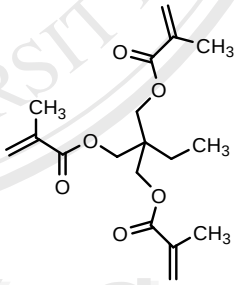
N,N'-methylene bisacrylamide (MDAA)



1,4-phenylene diacrylamide



pentaerythritol tetraacrylate (PETEA)

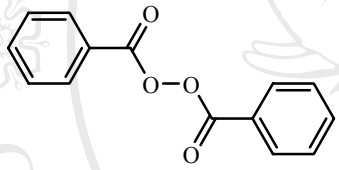


trimethylolpropane trimethacrylate (TRIM)

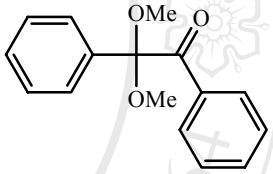
Figure 1.4 The cross-linkers used for molecular imprinting.

1.1.4.5 Initiators

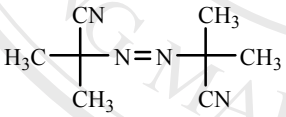
Polymerization of monomers can be done by various types of mechanisms. In MIPs, free radical chemistry is widely used for polymerization. However, in the polymerization reactions, the template molecules are included in the prepolymerize mixture. If the template was unstable under photochemical or thermal reaction, the condition should be triggered thermally and/or photochemically. The chemical structures of selected polymerization initiators are shown in Figure 1.5.



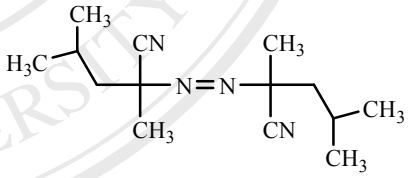
benzoylperoxide (BPO)



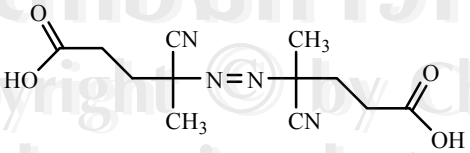
dimethylacetalof benzil



azobisisobutyronitrile (AIBN)



azobisdimethylvaleronitrile (ABDV)



4,4'-azo(4-cyanovaleric acid)

Figure 1.5 Chemical structures of selected chemical initiators

1.2 The molecular imprinted sorbent assay (MIAs)

Recently, molecular imprinted sorbent assays (MIAs) have been developed for determination of analytes in biological and environmental samples. The procedure is similar to the general immunoassay techniques such as radioimmunoassays (RIAs) [44-45] and enzyme immunoassays (ELISAs)[46-51]. However, in MIAs, imprinted polymers have been used for generation of artificial receptor instead of bio-antibodies. Comparison between MIPs and bio-antibodies is shown in Table 1.4.

Table 1.4 Comparison between MIPs and antibodies [52]

MIPs	Antibodies
• Can be used in both aqueous and organic media	• Restricted to aqueous conditions
• Synthetic: no use of animals	• Biological production involving animals
• High chemical, physical and thermal stability	• Very fragile
• Simple storage requirements	• Need to be lyophilised and may denature upon long period storage
• Preparation: fast	• Preparation: time-consuming but monoclonal strategy allows large long-term production once antibody is optimized.
• Require a relatively large amount of template	• Require a relatively small amount of antigen
• Non-covalent approach: no need for derivatisation. Covalent approach: functional monomer–template complex needs to be synthesized.	• Necessary to derivatise small non-immunogenic molecules in order to produce immune response.
• Controllable batch to batch Reproducibility	• Polyclonal antibodies are specific to each animal. Monoclonal antibodies allow batch reproducibility
• Reusable	• Non-reusable

In previous studies[53], there are four kinds of probes used in molecular imprinted sorbent assays (MIAs). These probes include radiolabelled probes, chromophore/fluorophore labelled probes, enzyme labelled probes and non-related probes (see Table 1.5).

The first MIA developed was based on a competitive radioligand-binding measurement reported by Mosbach and co-worker[54]. This format is analogous to solid-phase radioimmunoassay, except that the immobilised antibody is replaced with a molecularly imprinted polymer (MIP). In this assay, MIPs selective to diazepam and morphine were used in competitive radio-MIAs. Other assays developed later have used the same principle and several radioimmunoassay (RIAs) have been studied for several compounds such as theophylline[55], bupivacaine[56].

Imprinted polymer-based assays are conveniently performed using radiolabels, because the labeled analyte has the same structure as the original template. However, this involves the handling of radioactive materials and produces radioactive waste. Thus, alternative assays based on other probes are preferred.

In analogy to immunoassays, several kinds of probes have been introduced in MIAs for detection of compounds of interest. Chromophore labels[57,58], fluorescent labels[59,60] and non-related fluorescent probes[61-63] have been shown to compatible with MIPs. Enzyme labels, on the other hand, although most common with immunoassays, seemed to be less practical in MIAs for two reasons: First, enzymes often only work in aqueous buffers, whereas the use of many imprinted polymers is restricted to organic solvents. Second, the rather hydrophobic nature and highly cross-linked structure of the polymer limits the access of the large protein molecules to the imprinted binding sites.

Table 1.5 Reported MIAs employing several probes

Template	Probe	Assay solvent	Competitors	Ref.
Radiolabelled probes				
Morphine	^{3}H-Morphine	(a) Toluene, (b) citratepH6/EtOH(9:1)	Morphine, related opiates	[64]
Cortisol	^{3}H-Cortisol	THF	Cortisol, related steroids and sterols	[65]
S-Propranolol	^{3}H- *S*-Propranolol	Toluene	*S*-Propranolol, *R*-Propranolol	[66]
2,4-D	^{14}C-2,4-D	Phosphate pH7/Triton X-100 (999:1)	2,4-D, related acids and esters	[13,67]
Caffeine	^{14}C-Caffeine	(a) Heptane/THF (3:1), (b) MeCN	Caffeine, theophylline	[68]
Chromophore-labelled probes				
Chloramphenicol	Chloramphenicol-methyl red	MeCN	Chloramphenicol,chloramphenicol diacetate,thiamphenicol	[57, 69]
Biotin methyl ester	Biotin nitrophenyl ester	MeCN	Biotin methyl ester	[58]
Fluorophore-labelled probes				
Triazine	5-(4,6Dichlorotriazinyl) aminofluoresceine	EtOH	Triazine, atrazine, simazine	[59]
Chloramphenicol	Dansylated chloramphenicol	MeCN	Chloramphenicol,chloramphenicol diacetate,thiamphenicol	[60]

Table 1.5 (continued)

Template	Probe	Assay solvent	Competitors	Ref.
Atrazine	5-(4,6-Dichlorotriazinyl) aminofluoresceine	Water	Atrazine, atraton-d, metribuzin	[70]
Enzyme-labelled probes				
2,4-D	2,4-D-tobacco peroxidase	Phosphate pH7/Triton X-100(999:1)	2,4-D, related acids and esters	[13, 14]
Epinephrine	Norepinephrine-horseradish peroxidase	Phosphate pH6	Epinephrine, related catechols	[70]
Microcystin-*L*,*R*	Microcystin-horseradish peroxidase	Phosphate pH7	Microcystin-*L*,*R*, related peptides	[71]
Unrelated probes				
2,4-D	7-Carboxy-methoxy-4-methylcoumarin	Phosphate pH7/Triton X-100 (999:1)	2,4-D, related aromatics	[72, 73]
Electroactive probes				
2,4-D	Homogentisic acid	Phosphate pH7/MeOH (9:1)	2,4-D	[73, 61]
2-C-4-H	2-C-4-H	Phosphate pH7.4/EtOH (9:1)	2,4-D	[62]

However, during the last few years MIPs that perform well in aqueous solvents have been developed. The format of these assays will be discussed in more details in the next section.

1.3 Enzyme-linked molecularly imprinted sorbent assays

Enzyme labeled probes are used widely in a variety of assay formats because they are detectable down to very low concentrations through the generation of easily visible, colored, fluorescent or luminescent products from neutral substrates.

In MIAs, using enzyme labels probe is a great challenge. The problem of binding site accessibility by a large protein might be circumvented by using imprinted microspheres[13,14,63] and thin film[67,74] of polymers that have binding sites at or close to their surface. ELISA-type assays have recently been developed where the analyte was labeled with the enzyme peroxidase. Colorimetry or chemiluminescence could then be used for the detection.

Piletsky and co-worker[67,74] have reported a colorimetric MIP-assay for epinephrine. The method developed employ polymeric films synthesized in situ in each well of a polystyrene microtiter plate. Aminophenylboronic acid was polymerized in the presence of epinephrine using oxidation of the monomer by ammonium persulfate. This process resulted in the grafting of a thin polymer layer onto the polystyrene surface. The polymer was then used in a competitive enzyme-linked assay using norepinephrine -horseradish peroxidase conjugate.

Surugiu *et al.* used tobacco peroxidase-labelled 2,4-D as a competitive probe for the detection of a herbicide 2,4-D. Two methods were used for measuring the level of enzyme-labelled probe. Firstly, the chemiluminescence assay was adapted to microtiter plates which was coated with polymer microspheres[63] and detection of the bound probe was done via an imaging CCD camera. In the other detection method, the level of unbound probe in supernatant was quantified using colorimetric and chemiluminescence assay [13-14]. In both cases, 2,4-D was able to compete with 2,4-D-TOP conjugate for the binding site in the MIP microsphere.

1.4 Nevirapine (NVP)

Nevirapine (NVP, (Figure 1.8), or 11-cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido-[3,2-b:2',3'-e]) is a non-nucleoside HIV-1 reverse transcriptase inhibitor, manufactured by Boehringer-Ingelheim. It was approved by the U.S. Food and Drug Administration for the treatment of HIV in 1996.

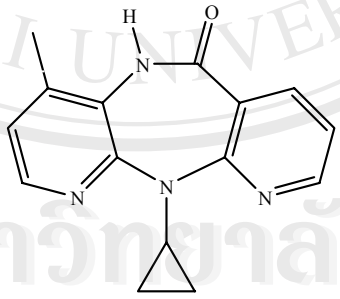


Figure 1.8 Structure of nevirapine (NVP)

The drug functions by binding to viral reverse transcriptase and disrupting the catalytic site to block polymerase activity. The recommended dosage of nevirapine is 200 mg once a day for the first two weeks, and then 200 mg twice a day thereafter. In clinical studies, NVP is readily absorbed after oral administration. After a single 200 mg dose, nevirapine concentrations in plasma can be observed in a concentration range between 3.0 and 10.0 μg/ml [(75)].

HIV seems to get resistant to the effects of NVP very quickly unless the drug is used as part of an effective anti-HIV drug combination. If a person becomes resistant to NVP, they may also be resistant to other NNRTI drugs such as delavirdine (Rescriptor) and efavirenz (Sustiva). The cross-resistance problems could be avoided if the level of NVP is seriously monitored. Currently, many analytical methods are available for determining navirapine in human plasma. These methods include chromatographic techniques such as HPLC[(76-77)], LC-MS[(78)], LC-MS-MS[(79,80)] and GC[(81)], and immunoassay techniques such as ELISA[(82)] assay and immunochromatographic strip test[(75)].

1.5 Aims of this Research

Although HPLC is commonly used in drug monitoring application, the technique often requires high plasma volumes, need specialized technicians and expensive instruments. Therefore, a more rapid and economical method would be beneficial especially when access to the complicate instruments are limited.

Immunoassay[(82)] is an alternative method that can overcome limitations mentioned above. In this technique, antibodies are routinely used for specific binding to the analyte. The binding utilizes the exquisite recognition properties of an antibody

for the antigen, in which the antigen fits exactly into the antibody's binding site, whereas other structurally related compounds are excluded from the site.

Major advantages of the technique include high sensitivity, specificity, and collections of samples can be analyzed simultaneously. Nevertheless, due to the difficulty in antibody preparation and its unstability, many attempts have been made in order to replace antibodies with more stable and easy accessable materials.

One technique that is being increasingly adopted for the generation of such materials is molecular imprinting described earlier. These involve the design and synthesis of biomimetic receptor systems capable of binding target molecules with affinities and specificities.

The aims of this study are therefore to synthesize new molecular imprinted polymers specific to nevirapine and develop new methods of NVP detection with suitable MIPs.