CHAPTER II

SYNTHESIS AND EVALUATION OF MOLECULARLY IMPRINTED POLYMERS FOR THE BINDING AFFINITY TO NVP

2.1 Introduction

Molecular imprinting is a powerful technique for preparing recognition sites in a synthetic polymer. One advantage of the technique is simplicity of preparing different MIPs with a predetermined selectivity for various compounds and the binding ability of the template molecule from the sample solution may occur by diffusion into the size.

However, sicnce MIPs are prepared by using the target analyte as template molecule, the leakage of a trace amount of the imprinted molecules remaining in the MIPs can hindered the accuracy and precision assay of the assay for the target analyte. One strategy to solve this problem is the utilization of an analyte analog or so called "dummy template", instead of the analyte itself as template during polymerization. With this approach, any leakage of the dummy template will not interfere with the analysis (provided that the physical properties of dummy template and the analyte are not equally the same). Ideally, when using dummy template in preparation of MIP, the obtained MIP should preferentially bind to target analyte than the dummy corresponding template to avoid interference if residue of dummy template was presented.

Recently, there are many reports using dummy template in MIPs study. MIPs selective to herbicide were synthesis using phenoxyacetic acid (PA) as a dummy template molecule were synthesized and em ployed with phenoxyacetic herbicides (benzoic acid (BA), PA, 2-methyl-4-chlorophenoxyacetic acid (MCPA), 4-chlorophenoxyacetic acid (4-CPA), and 2,4-dichlorophenoxyacetic ac(2,4-D)) by H. Zhang et. al [81]. The obtained MIP was packed in the column to investigated the retention behaviors of 2, 4-D. The retention behavior on this MIP column indicated that this material can selectively retain phenoxyacetic herbicides and this kind of polymers could be useful as stationary phases to extract 2, 4-D, 4-CPA or MCPA and avoid leakage of a trace amount of target analyte remaining in the MIPs.

Moreover, G. Theodoridis et. al. [82] were preparation of a molecularly imprinted polymer for the solid-phase extraction of scopolamine with hyoscyamine as a dummy template molecule. The best performance was observed after loading the analyte in aqueous environment facilitating retention on the MIP by non-selective hydrophobic interactions and the recoveries up to 79% were achieved for the analyte of interest from biological samples.

In the study described here, the NVP structurally related compounds, nicotinamide (NAM) and benzamide (BZM), were chosen as dummy templates to prepare molecularly imprinted polymers. Their binding efficiency were evaluated by comparison with MIPs prepared with NVP and benzophenone (BZP) as the template as a positive and negative control, respectively.

2.2 Experimental

2.2.1 Chemicals

Nevirapine (NVP), $C_{15}H_{14}N_4O$, Government Pharmaceutical Organization (GPO) Thailand.

Plasma sample, Research Institute for Health and Sciences, Chiang Mai University, Thailand.

Nicotinamide (NAM), $C_6H_6N_2O$, assay 98.5%, BDH, England

Benzamide (BZM), $C_7H_{16}NO$, assay 98.5%, Aldrich, U.S.A.

Methacrylic acid (MAA), $C_4H_6O_2$, assay 98%, Fluka, Switzerland

Benzophenone (BZP), $C_{13}H_{10}O$, assay 98.5%, Aldrich, U.S.A.

Trimethylopropane trimethacrylate (TRIM), $C_{18}H_{26}O_6$, Aldrich, U.S.A.

Benzoyl peroxide, $C_{14}H_{10}O_4$, Janssen Chemica, Belgium

Potassum hydrogen phosphate, K_2HPO_4, Fluka, Switzerland

Potassium dihydrogen phosphate, KH_2PO_4, Fluka, Switzerland

Tetrahydrofuran, C_4H_4O, Fluka, Switzerland

Polyoxyethylene, Tween 20, BDH, England

Dimethylsilfoxide, C_2H_6SO, Fluka, Switzerland

Acetic acid, $C_2H_4O_2$, Carlo Erba, Italy

Methanol, CH_4O, Fluka, Switzerland

Acetonitrile, C_4H_3N, Fluka, Switzerland

Methanol, CH_3OH, HPLC grade, Fisher, England

Acetonitrile, CH_3CN, HPLC grade, Fisher, England

2.2.2 Instrumental

UV-Vis spectrophotometer (Perkin Elmer, Lambda25), U.S.A.

Centrifuge (BECKMAN COULTER, Allergra), U.S.A.

Rocking table (Specimen mixer, BCT-33)

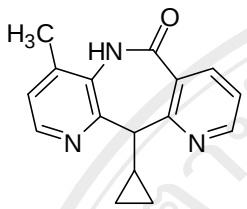
High performance liquid chromatography (HPLC) (Aligent), U.S.A.

C_{18} column 4.0×250 mm, 5µm (Hewlett-Packard) Germany

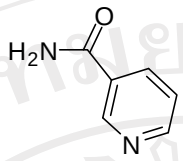
2.2.3 Synthesis of MIPs

The appropriate amounts of template (NVP, NAM BZM and BZP, 0.25 mmol) and MAA (1 mmol) were dissolved in 15 ml THF-MeOH-H_2O (5:4:1, v/v) in round bottom flask and incubated for 10 min. TRIM (1 mmol) and benzoyl peroxide (0.125 mmol) were then added and the flask was sealed with rubber cap. After the flask was purged with nitrogen for 10 min, the polymerization was carried out at 60ºC in oven for 24 h. The template was removed by extraction of the obtained polymers with MeOH-acetic acid (9:1, v/v) using a Soxhlet extractor and then the particles were washed with acetonitrile to remove residual of acetic acid and MeOH in extraction procedure. The particles were finally dried at 50 ºC in oven before used. Non-imprinted polymer (NIP) was prepared using the same procedure but without using template. The structure of template, functional monomer, cross-linker and initiator used for synthesis MIPs are shown in Figure 2.1.

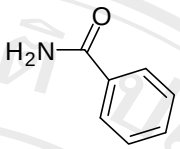
Template molecules



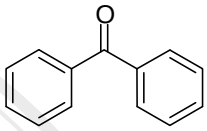
NVP



NAM

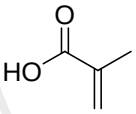


BZM



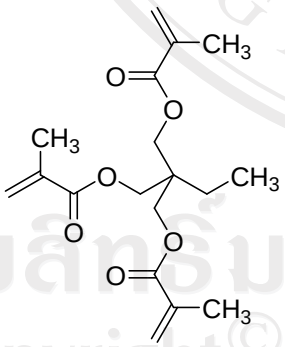
BZP

Functional monomer



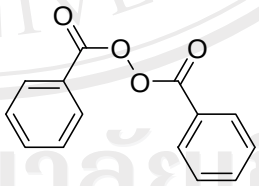
MAA

Cross-linker



TRIM

Initiator



Benzoyl peroxide

Figure 2.1 Structure of templates, functional monomer, cross-linker and initiator used in MIP synthesis.

The conditions used to synthesized MIPs and NIP were summarized as shown in Table 2.1.

Table 2.1 Conditions of MIPs preparation.

Polymers	**Templates** **0.25 mmol**	**Functional monomers** **1 mmol**	**Cross-linkers** **1 mmol**	**Porogen**
P(NVP)	NVP	MAA	TRIM	THF-MeOH-H_2O
P(NAM)	NAM	MAA	TRIM	THF-MeOH-H_2O
P(BZM)	BZM	MAA	TRIM	THF-MeOH-H_2O
P(BZP)	BZP	MAA	TRIM	THF-MeOH-H_2O
NIP	-	MAA	TRIM	THF-MeOH-H_2O

2.2.4 Equilibrium binding study of MIPs with the corresponding templates

The binding efficiency of imprinted polymers was determined by rebinding study using UV-Vis spectrophotometer. The absorbent measurements and spectrums were recorded on a Lambda 25 UV/Vis spectrophotometer with 1 cm quartz cells. The appropriate amount of MIP was added into a solution substrate (0.2 mM) in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20. The samples were incubated on a rocking table for overnight at room temperature. After that the polymers were centrifuged and the clearly supernatant was analyzed by UV-Vis spectrophotometer at λ 281, 262, 272 and 258 nm for NVP, NAM, BZM and BZP, respectively. Percentage bound (%bound) of the analyte was calculated according to this equation;

$$\%\text{Bound} = (Q/Q_{initial}) \times 100$$

When Q is the amount of analyte bound to polymer, was calculated by subtracting the concentration of free analyte from the initial analyte loading, $Q_{initial}$ is the amount of analyte unbound to polymer. This experiment was done in triplicate for each polymer.

The imprinting factor (α) used as a measurement of the strength of interaction between the template and MIPs were calculated according to the following equation:

$$\alpha = \frac{MIP_{(b)}}{NIP_{(b)}}$$

Where $MIP_{(b)}$ is the %bound of the MIP, while $NIP_{(b)}$ is the %bound of the NIP.

2.2.5 Equilibrium binding study of MIPs with NVP

In this study, all polymers except P(NVP) were incubated in a solution of NVP (0.2 mM) in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20. the UV analysis were performed using the previously described procedure at λ 281 nm.

To evaluate the selectivity of the polymers to NVP, the NVP selectivity factors (α') were used to measure the strength of binding interaction of MIPs with NVP comparing with the binding to their corresponding template. The selectivity factors were calculated according to the following equation:

$$\alpha' = \frac{B_{(NVP)}}{B_{(template)}}$$

Where $B_{(NVP)}$ is the %bound of the MIPs with NVP, while $B_{(template)}$ is the %bound of the MIPs with corresponding template

2.2.6 Competitive binding study

To study the effect of NAM on binding ability of NVP, the competitive binding studies were investigated. Stock solution containing NAM: NVP at concentration of 0.2:0.2, 1:0.2 and 2:0.2 mM in 0.1 M phosphate buffer pH 7 containing 0.05% Tween 20 were prepared. After that P(NAM) 5 mg was added into a 1 ml of the prepared solution. After the equilibrium binding (24 h) as described above, the supernatants were isolated by centrifugation and the supernatant were analyzed by HPLC.

2.2.7 Chromatographic conditions

HPLC analysis were performed using Agilant technologies (USA) HPLC system employing a model 1100 quaternary gradient pump and variable-wavelength detector. The injection volume was 20 µl throughout the study. Separations were carried out on 4.0×250 mm, 5 µm Hewlett-Packard (Germany) C_{18} column. The gradient mobile phases consist of 15mM of phosphate buffer pH 7.2 as solvent A and pure acetonitrile as solvent B. The flow rate used is 1.0 ml/min with gradient system. The chromatographic conditions for analysis were shown in Table 2.2.

Table 2.2 Chromatographic conditions for analysis.

Substrate	Gradient system	λ(nm)
NVP	10-90% solvent B, 10 min	281
Mixed of NVP and NAM	10-90% solvent B, 10 min	254

2.3 Results and discussions

2.3.1 Synthesis of MIPs

Since NVP is quite expensive and not easily available, it is impractical to use this compound as a template in MIP preparation for MISPE application. Moreover, to avoid interference of the residual template when leak out of the MISPE column, the polymer sorbent should be prepared using other structurally related compounds of NVP. In this study, NAM and BZM were selected as dummy template due to their structural similarity to NVP. Since NAM and BZM are less expensive and wildly distribute, both of them was chosen to be used to prepare P(NAM) and P(BZM), respectively. And the biding affinity with the polymers with their corresponding template and NVP were evaluated in comparison with P(NVP), a polymer imprinted with NVP. P(BZP) was also prepared from a non-related structure to be used as a negative control. Under the same polymerization conditions these MIPs were synthesized using MAA as a functional monomer and TRIM as a cross-linker. MAA is a commonly used functional monomer that has a good interaction with all above templates via hydrogen bonding. TRIM is commonly used to generate highly cross-link that show a good ability to controlling morphology and stabilize the imprinting binding site. All MIPs were synthesized by precipitation polymerization in THF-MeOH-water and used benzoyl peroxide as an initiator. NIP was also prepared under the same condition without the present of template. All polymers were obtained as white powder with percentage yield ranging from 85-95%.

2.3.2 Equilibrium binding study of MIPs with the corresponding templates

The binding abilities of all MIPs with the corresponding template in phosphate buffer pH 7 containing 0.05% Tween 20 and the imprinting factors of MIPs were shown in Figure 2.2. In Figure 2.2 (a), NVP showed high percent bound (97.53±1.42) to P(NVP). Highest imprinting factor (2.54) was also obtained suggesting that the polymer have a good capacity to recognize to NVP molecule. Due to the structure of NVP containing two pyridyl rings fused with the amide and amine bound, therefore, it gives the three binding sites in molecule that can made self-assembly with the functional monomer (MAA) and this property lead to the selectivity of P(NVP). Schematic representation of the synthesis of P(NVP) is shown in Figure 2.3.

From Figure 2.2 (b)-(d), P(NAM) showed the lowest percent NAM bound (20.42±1.67), follow by P(BAM) (51.67±6.29), whereas P(BZP) showed the highest percent bound (114.74±2.16) with the corresponding template. Nevertheless, the imprinting factor of all three polymers were close to unity, 0.99, 0.87 and 1.01 for P(NAM), P(BZM) and P(BZP), respectively, suggesting that there is no difference between imprinted and non-imprinted polymer when the binding study was performed at the polymer concentration of 5 mg/ml. In the case of P(BZP) it can be seen that the over 100 of %bound was observed. This may be due to the interference of the template bleeding that can be observed when template was not completely removed in the template extraction step.

From the results, P(NVP) has better binding performance than other polymers. There are many reasons that can explain this observation. Firstly, comparing NVP and

other molecule, NVP contained three possible binding sites (Figure 2.3) whereas the rest of the other templates except NAM have only one binding site to interact with the polymers. When using NVP as template, more specific binding site should be expected. Secondly, when considering aqueous solubility of all template molecules, NAM is the most polar compound, whereas BZP is the most hydrophobic followed by NVP and BZM, respectively. Therefore, binding of the more hydrophobic molecules to their corresponding MIPs in aqueous media can be facilitated by hydrophobic interaction causing higher percent bound observed in P(BZP), P(NVP) and P(BZM), respectively. On contrary, NAM is more soluble in aqueous media, the compound thus has less potential to bound to P(NAM). From the imprinting factor, only P(NVP) showed specific binding whereas the rest of the MIPs bind with the corresponding template non-selectively. Therefore, at this polymer concentration, it can only be concluded that specific interaction between template and polymer for P(NAM), P(BZM) and P(BZP) has not yet been observed.

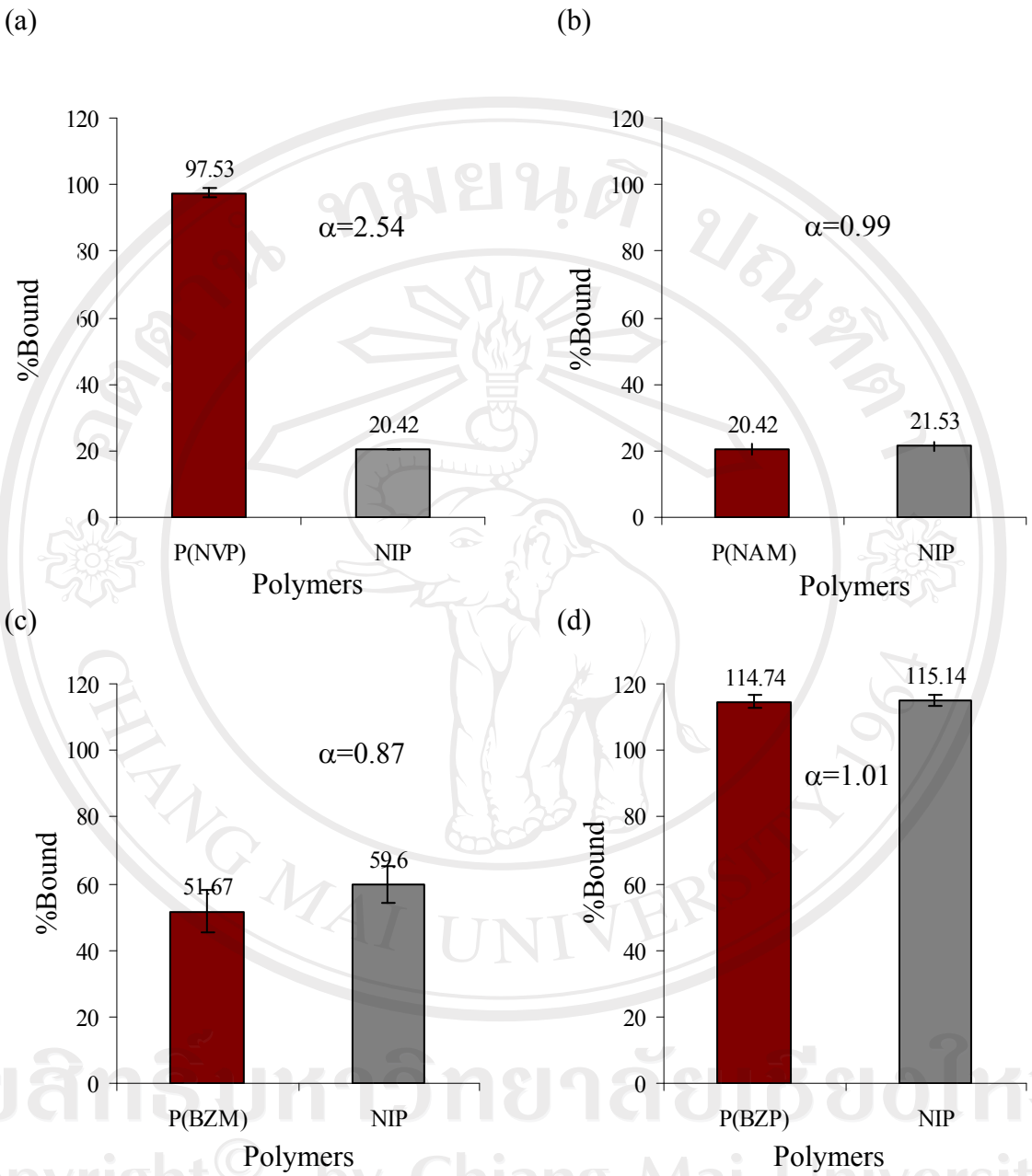


Figure 2.2 The imprinting factor and the percentage bound of polymers; (a) binding of P(NVP) and NIP in NVP solution (b) binding of P(NAM) and NIP in NAM solution (c) binding of P(BZM) and NIP in BZM solution and (d) binding of P(BZP) and NIP in BZP solution.

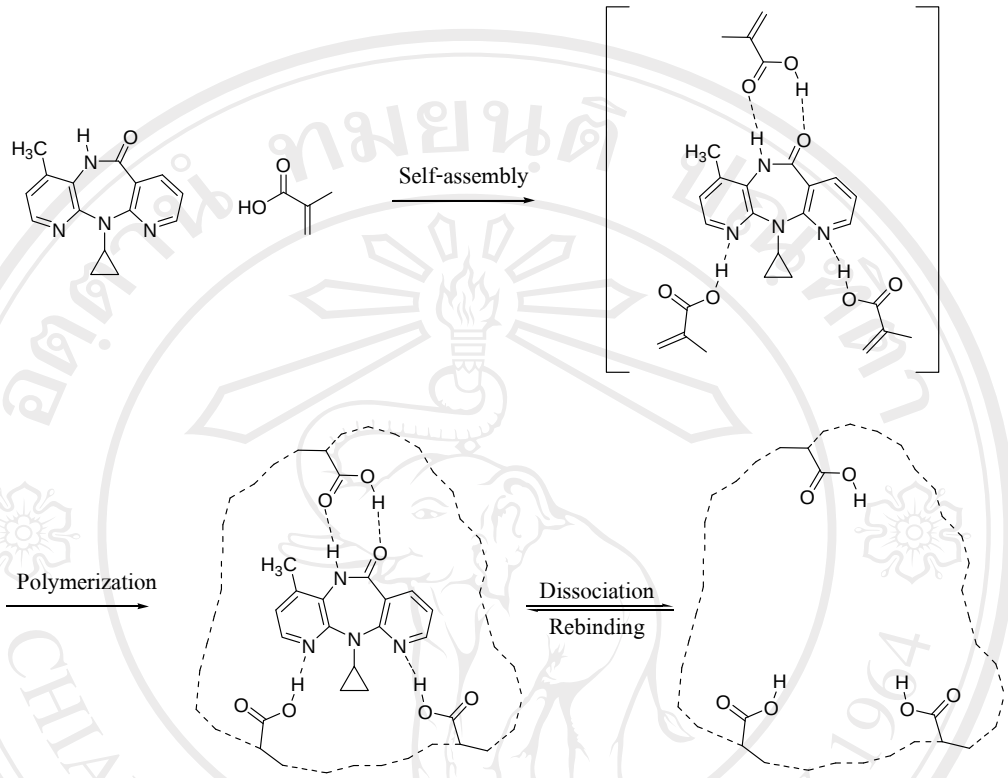


Figure 2.3 Schematic representation of the synthesis of P(NVP).

2.3.3 Equilibrium binding study of MIPs with NVP

To evaluate NVP binding performance of the MIPs prepared by dummy templates, P(NAM) and P(BZM), the above experiment was performed using P(NVP), P(NAM), P(BZM) and P(BZP). P(NVP) and P(BZP) were used as a positive and negative control, respectively. The results of the binding study of P(NVP), P(NAM), P(BZM), P(BZP) and NIP to NVP were shown in Figure 2.4. The NVP selectivity factors that measure of the binding ability between the target analyte (NVP) and corresponding template in each of MIPs were shown in Table 2.4. It was found that P(NAM) showed a high binding efficiency with NVP in comparative to P(NVP) (98.00±0.69). Moreover, the highest NVP selectivity factor was also observed in this polymer (4.80). These results indicated that P(NAM) can selectively bind with NVP efficiently. Since NAM is a structurally related compound of NVP that has one pyridyl ring and amide bound at exactly the same position as those in NVP structure, therefore, P(NAM) showed a good binding affinity to NVP. Schematic presentation of interaction between NAM and P(NAM) was shown in Figure 2.5.

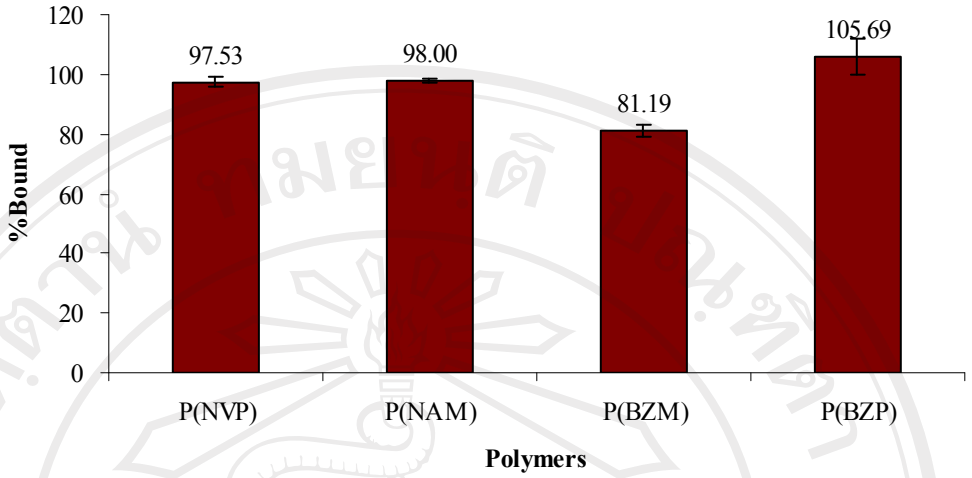


Figure 2.4 The percentage bound of P(NVP), P(NAM), P(BZM) and P(BZP) to NVP (0.2 mM) in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20.

Table 2.3 The NVP selectivity factors of all polymers in 0.2 mM NVP in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20.

Polymers	NVP selectivity factor (α')
P(NVP)	1.00
P(NAM)	4.80
P(BZM)	1.57
P(BZP)	0.92

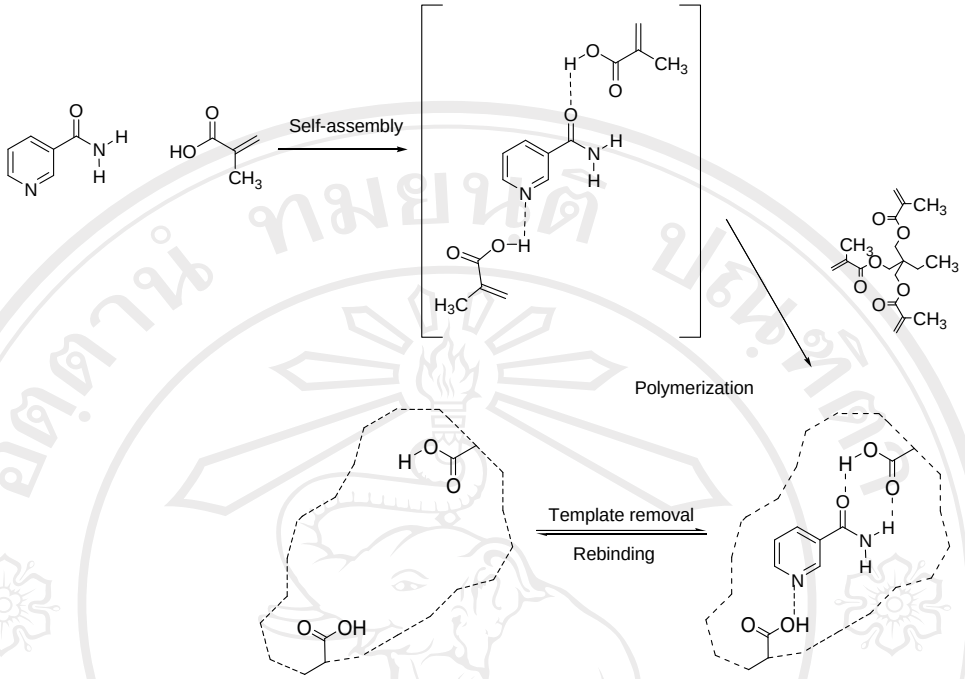


Figure 2.5 The schematic presentation the P (NAM) synthesis: (1) self-assembly (2) polymerization (3) extraction of template.

For the binding ability of P(BZM) to NVP (Figure 2.4), the 81.19±2.13 %bound was observed. The lower percent bound of P(BZM) comparing with those of P(NAM) is probably due to the structure of BZM which contain only one amide bond that can provide only one binding site interaction. Therefore, NVP showed less affinity to this polymer. For P(BZP) prepared by NVP structurally non-related template, the highest %bound with NVP (105.69±5.98) was obtained (Figure 2.4). However, the NVP selectivity factor of this polymer is closed to one (Table 2.3), indicating that this polymer

can not differentiate the binding of NVP and BZP. In other words, the result suggested that the binding interaction between NVP and this polymer is highly non-specific.

From all binding study results, it can be concluded that P(NAM) has the best binding performance to NVP as indicated from its high percent bound of NVP and selectivity factor. Because of the fact that the binding ability of P(NAM) is comparable to P(NVP) and that NAM is less expensive and easily available, P(NAM) was selected for being used as solid sorbent in place of P(NVP) in further MISPE experiments.

2.3.4 The effect of amount of polymer to the binding efficiency of NAM to P(NAM)

From the previous result, the percent bound of NAM to P(NAM) was very low and the imprinting factor was less than one when the concentration was at 5 mg/ml. this data suggest that under this condition, there is no difference between P(NAM) and NIP in the binding of NAM to P(NAM) and NVP as indicative of non-specific binding. In this study, to examined whether specific binding between NAM and P(NAM) can actually occurred, fixed amount of NAM was allowed to bind with increasing amount of P(NAM) in comparison with NIP. The 5, 10 and 20 mg of P(NAM) were incubated in 0.2 mM NAM in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20 and the binding experiment were done as describe above. The results of percent bound of NAM to P(NAM) and NIP was shown in Figure 2.6.

From Figure 2.6, when the polymer concentration was increased from 5, 10 and 20 mg/ml, the percentage bound of NAM solution were 19.97±0.28, 26.79±5.58 and 37.99±4.97 respectively. It can be seen that the binding affinity as well as imprinting

factors (Table 2.4) of NAM to P(NAM) were gradually increased with increasing the amounts of polymer. This data suggested that specific binding of NAM to P(NAM) was occurred when the concentration of the polymer was at the optimum concentration. In case of the binding with NVP, lower amount of polymer can be used because NVP is less polar than NAM and has higher tendency to bind with the polymer.

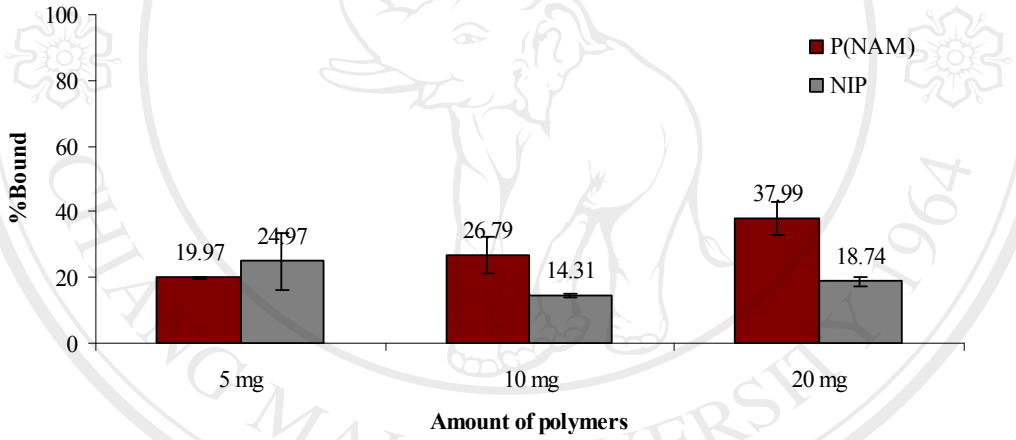


Figure 2.6 The effect of amount of P(NAM) to the binding affinity with corresponding template.

Table 2.4 The imprinting factors of P(NAM) in 0.2 mM NVP in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20.

Amount of P(NAM)	Imprinting factor (α)
5 mg	0.80
10 mg	1.87
20 mg	2.03

2.3.5 The competitive binding study

The competitive binding study was also investigated to study the effect of NAM concentration on the binding of NVP to P(NAM). In this study, fixed amount of NVP were mixed with increasing amount of NAM in 0, 0.2, 1 and 2 mM. After incubated with P(NAM) for 24 h., the amount of NVP in the solution was monitored by HPLC. The result of this experiment was shown in Figure 2.7. The percentage bound of P(NAM) to NVP were 97.53±1.42, 86.21±1.44, 82.20±1.88 and 73.78±1.91, respectively. It can be seen that when the amount of NAM was increased to ten times the amount of NVP, the binding ability of NVP was decreased by 23.75%. This data suggesting that competition between NAM and NVP can be occurred but not significantly interfere with the percent bound with NVP. Moreover, the level of NAM generally found in biological sample was generally lower than 0.2 mM [83], therefore, the corresponding amount of NAM should not affect the ability of P(NAM) in MISPE experiment for NVP.

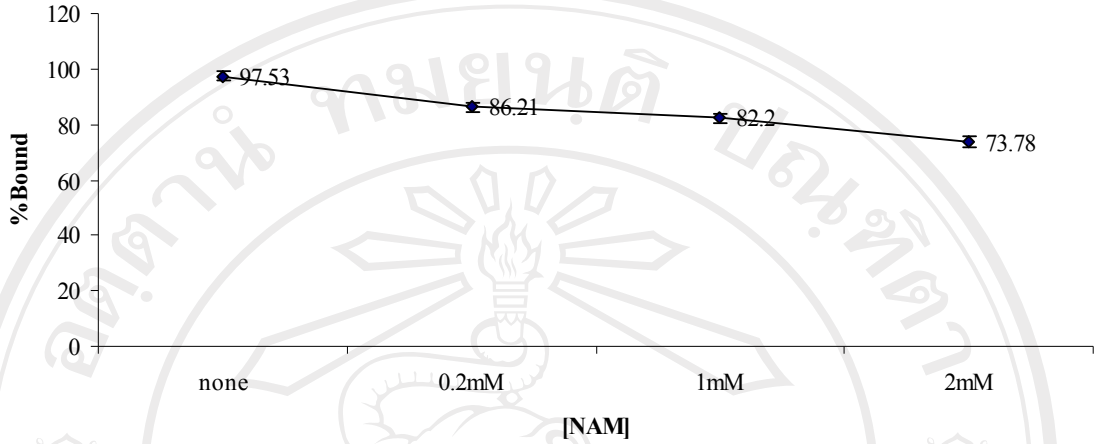


Figure 2.7 The competitive binding study of P(NAM) with mix solution of NAM:NVP in 0.01 M phosphate buffer pH 7 containing 0.05% Tween 20 with increased concentration of NAM in 0, 0.2, 1 and 2 mM