CHAPTER II

EXPERIMENTAL

2.1 Chemicals

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

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

2-Amino pyridine (2-Apy), $C_5H_6N_2$, assay 98%, Merck, Germany

3-Amino pyridine (3-Apy), $C_5H_6N_2$, assay 98%, Fluka, Switzerland

4-Amino pyridine (4-Apy), $C_5H_6N_2$, assay 98%, Fluka, Switzerland

Pyridine, C_5H_5N, assay 98%, Fluka, Switzerland

Aniline, $C_6H_5N_2$, assay 98.5%, BHD, England

o-phenylenediamine (OPD), $C_6H_8N_2$, assay 99%, Fluka, Switzerland

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

Acrylamide (ACA), C_3H_5NO, assay 99%, Sigma, U.S.A.

2-hydroxyethyl acrylate (2-HEA), $C_5H_7O_2$, assay 98%, Aldrich, U.S.A.

Itaconic acid (ITA), $C_5H_6O_4$, assay 99%, Aldrich, U.S.A.

Ethylene glycol dimethacrylate (EGDMA), $C_{10}H_{10}O_4$, assay 98%, Aldrich, U.S.A.

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

p-divinylbenzene (DVB), $C_{10}H_{10}$, assay 50%, Fluka, Switzerland

Polyethylene glycol dimethacrylate (p-EGDMA), Mn 700, Aldrich, U.S.A.

Benzoylperoxide, $C_{14}H_{10}O_4$, assay 97%, Fluka, Switzerland

Methyl 5-bromovalerate, $C_6H_{11}O_2Br$, assay 97%, Aldrich, U.S.A.

Potassium carbonate, K_2CO_3, assay 99%, Aldrich, U.S.A.

Lithium hydroxide, $LiOH.H_2O$, assay 99%, Aldrich, U.S.A.

N-hydroxy succinamide, $C_4H_5NO_3$, assay 97%, Aldrich, U.S.A.

N,N'-diisopropylcarbodiimide, $C_7H_{14}N_2$, Sigma, U.S.A.

N,N'-Dimethylformamide, C_3H_7NO, assay 99.8%, Aldrich, U.S.A.

Diisopropyl ethylamine, $C_8H_{19}N$, assay 99.5%, Sigma-Aldrich, U.S.A.

Horseradish peroxidase (HRP), Sigma, U.S.A.

Dimethylsulfoxide (DMSO), $C_{14}H_{10}O_4S$, assay 99.5%, Fluka, Switzerland

TMB microwell peroxidase substrate, KPL

2.2 Instruments

UV/Vis spectrophotometer (Perkin Elmer, Lambda 25), U.S.A.

Scanning Electron Microscope (SEM) (JEOL, 6335F), Japan

NMR spectrometer (Bruker, Avance), Germany

Fourier transform infrared (FT-IR) spectrometer (Bruker, TENSOR 27), Germany

Centrifuge (BECMAN COULTER, Allergra 64R), U.S.A.

Rocking table (Specimen mixer, BCT-33)

2.3 General method for synthesis of molecular imprinted polymers (MIPs)

2.3.1 Bulk polymerization

Appropriate amounts of template molecule and functional monomer were dissolved in solvent (porogens) and incubated for 10 min. The cross-linker and initiator (benzoyl peroxide) were then added. The flask was sealed with rubber cap and was purged with nitrogen for 10 min. The polymerization was then carried out at 60 ^{0}C in oven for 24 h. The resulting bulk rigid polymers were ground into fine powder. Template removal was done by extracting the obtained polymers with methanol/acetic acid (9/1, v/v) using soxhlet extractor and washed with methanol until the template could no longer be detected by UV spectrophotometer. Then the particles were washed with the acetonitrile to remove residual acetic acid and methanol. The particles were dried at 50 ^{0}C in oven and under vacuum. Non-imprinted polymers (NIPs) were prepared by same procedure but without using template.

2.3.2 Precipitation polymerization

Appropriate amounts of template molecule and functional monomer were dissolved in porogens under very dilute conditions and incubated for 10 min. The cross-linker and initiator (benzoyl peroxide) were then added. The flask was sealed with rubber cap. After the flask was purged with nitrogen for 10 min, the polymerization was carried out at 60 ^{0}C in oven for 24 h. The microspheres polymers were extracted with methanol/acetic acid (9/1, v/v) using soxhlet extractor and washed with methanol solvent until the template could no longer be detected by UV spectrophotometer. Then the particles were washed with the acetonitrile to remove residual acetic acid and methanol.

The particles were dried at 50 ^{0}C in oven and under vacuum. Non-imprinted polymers (NIPs) were prepared by same procedure but without using template.

The compositions and conditions for preparation of MIPs using nicotinamide (NAM) as template (P1-P11) and their corresponding NIPs (P1'-P11') are shown in Table 2.1.

Table 2.1 Composition of MIP preparation for NAM

Polymers	NAM (mmol)	Functional monomers (mmol)				Cross-linkers (mmol)				Porogens
		MAA	ACA	HEA	ITA	EGDMA	TRIM	DVB	p-EG	
P1	0.25	-	1.0	-	-	5.0	-	-	-	CH_2Cl_2
P1'	-	-	1.0	-	-	5.0	-	-	-	CH_2Cl_2
P2	0.25	-	-	1.0	-	5.0	-	-	-	CH_2Cl_2
P2'	-	-	-	1.0	-	5.0	-	-	-	CH_2Cl_2
P3	0.25	-	-	-	1.0	5.0	-	-	-	THF
P3'	-	-	-	-	1.0	5.0	-	-	-	THF
P4	0.25	1.0	-	-	-	5.0	-	-	-	CH_2Cl_2
P4'	-	1.0	-	-	-	5.0	-	-	-	CH_2Cl_2
P5	0.25	1.0	-	-	-	-	5.0	-	-	CH_2Cl_2
P5'	-	1.0	-	-	-	-	5.0	-	-	CH_2Cl_2
P6	0.25	1.0	-	-	-	-	-	5.0	-	CH_2Cl_2
P6'	-	1.0	-	-	-	-	-	5.0	-	CH_2Cl_2
P7	0.25	1.0	-	-	-	-	-	-	5.0	CH_2Cl_2
P7'	-	1.0	-	-	-	-	-	-	5.0	CH_2Cl_2
P8	0.25	1.0	-	-	-	-	1.0	-	-	CH_2Cl_2
P8'	-	1.0	-	-	-	-	1.0	-	-	CH_2Cl_2
P9	0.25	10	-	-	-	-	1.0	-	-	CH_2Cl_2
P9'	-	1.0	-	-	-	-	1.0	-	-	CH_2Cl_2
P10	0.25	1.0	-	-	-	-	1.0	-	-	MeOH:H_2O
P10'	-	1.0	-	-	-	-	1.0	-	-	MeOH:H_2O
P11	0.25	1.0	-	-	-	-	1.0	-	-	T:M:H
P11'	-	1.0	-	-	-	-	1.0	-	-	T:M:H

ratio of MeOH:H_2O = 4:1 (v/v), ratio of T:M:H (THF:MeOH:H_2O) = 5:4:1 (v/v)

P1-P8 were prepared by bulk polymerization, porogen 1.5 ml

P9-P11 were prepared by precipitation polymerization, porogen 15 ml

Conditions for synthesis of MIPs using NVP as a template (P12-P16) and their corresponding NIPs (P12'-P16') are listed in Table 2.2.

Table 2.2 Composition of MIP preparation for NVP

Polymers	NVP (mmol)	Functional monomers (mmol)		Cross-linkers (mmol)		Porogens
		MAA	ITA	EGDMA	TRIM	
P12	0.25	1.0	-	5.0	-	CH_2Cl_2
P12'	-	1.0	-	5.0	-	CH_2Cl_2
P13	0.25	1.0	-	-	1.0	CH_2Cl_2
P13'	-	1.0	-	-	1.0	CH_2Cl_2
P14	0.25	1.0	-	5.0	-	THF:MeOH:H_2O
P14'	-	1.0	-	5.0	-	THF:MeOH:H_2O
P15	0.25	1.0	-	-	1.0	THF:MeOH:H_2O
P15'	-	1.0	-	-	1.0	THF:MeOH:H_2O
P16	0.25	-	1.0	-	1.0	THF:MeOH:H_2O
P16'	-	-	1.0	-	1.0	THF:MeOH:H_2O

ratio of THF:MeOH:H_2O = 5:4:1 (v/v)

P12-P13 were prepared by bulk polymerization, porogen 1.5 ml

P14-P16 were prepared by precipitation polymerization, porogen 15 ml

2.4 General methods for UV rebinding

The recognition ability of the imprinted polymers was examined by batch rebinding using UV spectrophotometer. In a typical binding assay, an appropriate amount of powder polymer was added to a solution of analyte in ACN or 10% EtOH in 0.01 M phosphate buffer pH 7. The samples were incubated on a rocking table for overnight at room temperature. After that the polymers were centrifuged and then filtered off by HPLC filter. The filtrate was analyzed by UV spectrometer and the quantity of analyte in the solution was determined by reference to a calibration curve. The amount of analyte bound to the polymer, Q, was calculated by subtracting the concentration of free analyte from the initial analyte loading. % Bound of analyte was calculated according to the equation, % Bound = $(Q/Q_{initial})$ x 100. This experiment was done in triplicate for each polymer.

Table 2.3 UV binding conditions of obtained polymers

Conditions	polymers	Analyte (mM)	Solvent	polymer (mg/ml)	λ_{max}
1	P1-P11	NAM (0.5)	ACN	40	262
2	P1-P11	NAM (0.5)	Buffer pH 7	40	262
3	P1-P11	NAM (0.5)	Buffer pH 7	20	262
4	P12-P16	NVP (0.2)	ACN	20	281
5	P12-P16	NVP (0.2)	Buffer pH 7	20	281
6	P12-P16	NVP (0.2)	Buffer pH 7	5	281

2.5 Scanning electron microscopy (SEM)

The morphologies of the polymers were studied using a JEOL scanning electron microscope (SEM). SEM specimens were prepared by diluting the particle dispersions with acetone and placing one drop each on a stub. The drops were allowed to dry at room temperature and then sputter coated with gold prior to imaging.

2.6 Fourier transform infrared (FT-IR)

FT-IR was used for characterization of MIP and NIP polymers. The samples for FT-IR analysis were prepared in form of KBr discs**.**

2.7 Selectivity determination

The selectivity tests of P11 and P15 and their corresponding NIPs were carried out using a series of NVP structurally related compounds including NAM, BAM, 2-Apy, 3-Apy, 4-Apy, pyridine, aniline and OPD. The amounts bound to the polymers were determined by batch rebinding. To the solution of test compounds (0.2 mM, 2.0 ml in 0.01 M phosphate buffer pH 7 containing 0.05% tween 20) were added with the polymer (10 mg). The samples were incubated on a rocking table for 1 h. at room temperature. The polymer particles were then filtered off by centrifugation and the filtrate was analysed by UV spectroscopy at λ_{max} of each compounds. The amount of bound to the polymer was calculated by subtracting the concentration of free from the initial loading. This experiment was done in triplicate for each polymer.

2.8 Synthesis of NVP–linked horseradish peroxidase

The NVP-HRP conjugate was synthesized according to literature procedure[75].

2.8.1 Synthesis of NVP linked methyl ester (compound 1)

A solution of methyl 5-bromovalerate (60 μl, 0.37 mmol) in DMF (1.5 ml) was added to a mixture of NVP (100 mg, 0.37 mmol) and potassium carbonate (149.3 mg, 1.08 mmol) in DMF (1.5 ml). The resulting mixture was then stirred and refluxed overnight at reflux before the solvent was removed *in vacuo*. The crude residue was purified by chromatography on a silica gel (hexane-ethyl acetate 60/40, R_f =0.37) to generate NVP linked methyl ester (1) as yellow oil (78.1 mg, 52%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (dd, *J* = 2, 4.8 Hz, 1H), 8.05 (d, *J* = 4.8 Hz, 1H), 7.75 (dd, *J* = 2, 7.6 Hz, 1H), 6.97 (dd, *J* = 7.6, 4.8 Hz, 1H), 6.92 (d, *J* = 5.2, 1H), 4.38 (t, 2H), 3.66 (s, 3H), 3.65 (m, 1H), 2.42 (t, 2H), 2.34 (s, 3H), 1.87 (m, 4H), 0.96 (m, 2H), 0.47 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.7, 162.9, 159.0, 152.8, 151.3, 143.8, 143.5, 137.3, 132.7, 122.0, 119.6, 118.4, 51.5, 33.7, 29.0, 28.2, 21.7, 18.0, 8.7, 8.6.

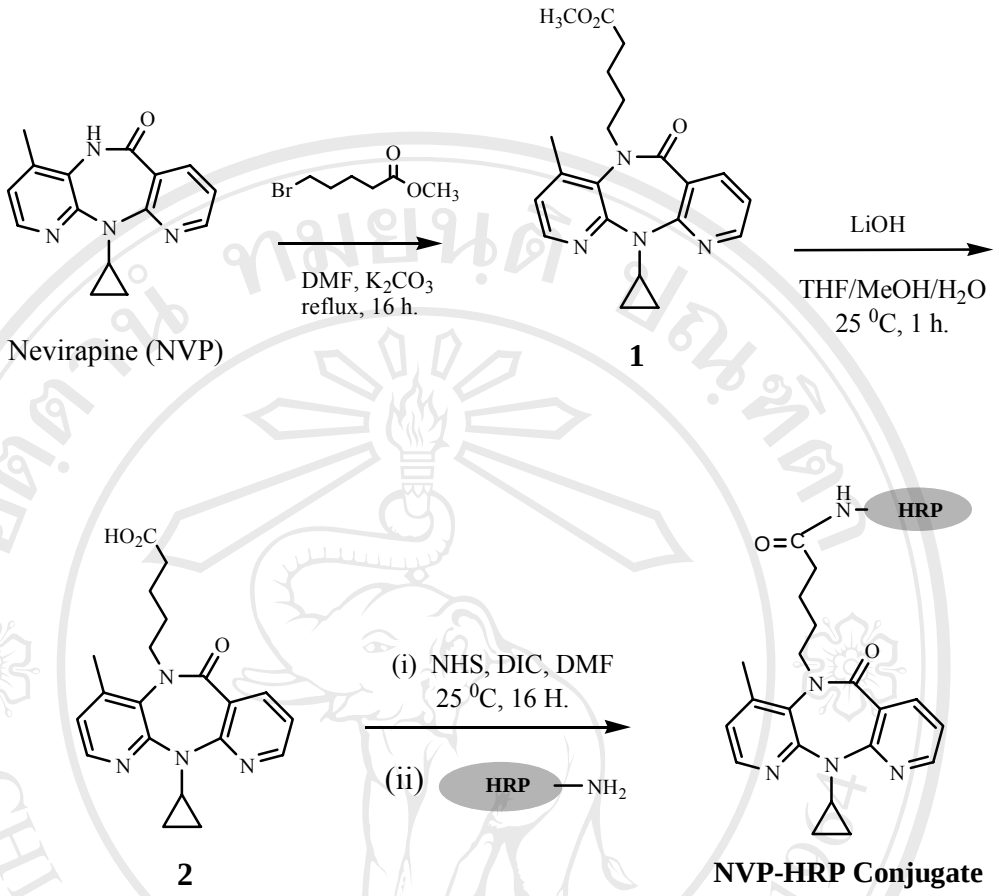
2.8.2 Synthesis of NVP linked carboxylic acid (compound 2)

Compound **1** (78.1 mg, 0.20 mmol) was dissolved in a solution of THF/ MeOH/ H_2O 3:2:1 (2 mL). $LiOH.H_2O$ (16.7 mg, 0.40 mmol) was then added and the mixture was stirred for 1 hour at 25 °C. After following concentration of the mixtureed *in vacuo*, the crude residue was diluted with water and neutralized with 1 N M HCl. The resulting solution was extracted with 2 x 2 ml EtOAc, dried with anhydrous Na_2SO_4 and concentrated *in vacuo* to yield product compound **2** as yellow oil (62.3 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.49 (dd, *J* = 2, 4.8 Hz, 1H), 8.10(d, *J* = 4.8 Hz, 1H), 7.78

(dd, *J* = 2, 7.6 Hz, 1H), 6.99 (dd, *J* = 7.6, 4.8 Hz, 1H), 6.90 (d, *J* = 5.2 Hz, 1H), 4.40 (t, 2H), 3.66 (m,1H), 2.42 (t, 2H), 2.32 (s, 3H), 1.87 (m, 4H), 0.96 (m, 2H), 0.47 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.9, 162.8, 159.0, 152.6, 151.4, 144.0, 143.7, 137.4, 132.8, 122.0, 119.6, 118.5, 33.6, 29.0, 28.1, 21.0, 18.1, 14.1, 8.9, 8.7.

2.8.3 Synthesis of NVP-HRP conjugate

Compound **2** (33 mg, 0.087 mmol) was reacted with NHS (12.0 mg, 0.1 mmol) in the presence of 1,3-diisopropylcarbodiimide (23.4 mg, 0.11 mmol) in anhydrous DMF (3.6 ml) for 16 hours at 25 °C. The mixture was then added dropwise to a stirred solution of HRP in 5 ml of 0.05 M carbonate/bicarbonate buffer pH 8.2 at 0 °C. The solution was stirred further for an hour at 25 °C and then for 24 hours at 4 °C. The synthesized enzyme conjugate was then dialyzed with 0.1 M phosphate buffer saline (pH 7.4). The resulting solution was kept frozen at -20 °C until it was used.



Scheme 2.1 Synthesis of NVP-HRP conjugate

2.9 Characterization of NVP-HRP conjugate by competitive ELISA

Assays were performed in 96-well microtiter plates coated with rabbit anti-NVP antibodies (1:2000 dilution in coating buffer 0.05 M carbonate pH 9.6, 50 µl/well) at 4 ^{0}C for over night, and then blocked within PBS buffer containing 2% skim milk (200 µl/well) at room temperature for 2 h, followed by three washes in 0.05% tween 20/PBS. The well were added with mixture of NVP-HRP (1:4000 dilution in 2% skim milk/PBS) and NVP (10, 1.0, 0.1, 0.01 µg/ml and non-inhibition) in 2% skim milk/PBS (50 µl/well) at room temperature. After 1 h., the plates were washed with 0.05% tween 20/PBS for 3

time, and 100 µl of TMB/H_2O_2 substrate was added to each well. After incubation for 20 min, the reaction was stopped by adding 1 N HCl (50 µl/well). Then read absorbances at appropriate wavelength (λ_{max} = 450 nm)

The results are expressed in terms of $B/B_0 \times 100$ as a function of the concentration (logarithmic scale); B and B_0 represent the bound enzymatic activity in the presence and absence of competitor, respectively. A linear log-logit transformation was used to fit the calibration curve.

2.10 Development of enzyme linked sorbent assay

The polymers used in this study were P11, P15 and their corresponding NIPs. All experiments were done in triplicate for each polymer.

2.10.1 Studies the effect of additive surfactant

The polymers (0.8 mg) was incubated with a solution (1.00 ml) of NVP-HRP conjugate (1:500,000 dilution) in 0.01 M phosphate buffer, pH 6 containing 0.05% tween 20 and without addition of tween 20 in the system. The mixtures were incubated on a rocking table for 1.0 h. Then the particles were removed by centrifugation for 5 min at 16000 rpm. For colorimetric measurements, 200 µl of supernatant was withdrawn and added to 200 µl of colorimetric substrate solution (TMB/H_2O_2 substrate). After incubation for 5 min, the reaction was stopped by adding 1 N HCl (200 µl). The absorbance was measured at appropriate wavelength (λ_{max} = 450 nm).

2.10.2 Studies concentration of polymer (IC_{50}) to be use in assays at pH 6, 7 and 8

Various amount of polymers (0.4-15 mg) were incubated with a solution (1.00 ml) of NVP-HRP (1:500,000 dilution) in 0.01 M phosphate buffer containing 0.05% tween 20 at pH 6, 7 and 8. The samples were incubated on a rocking table for 1.0 h, after which the particles were removed by centrifugation for 5 min at 16000 rpm. For colorimetric measurements, 200 μl of supernatant was withdrawn and added to 200 μl of colorimetric substrate solution (TMB/H_2O_2 substrate). After incubation for 5 min, the reaction was stopped by adding 1 N HCl (200 μl). Then the absorbance was measured at appropriate wavelength (λ_{max} = 450 nm).

2.10.3 Binding studies of NVP in 0.01 M phosphate buffer pH 6

The polymers (0.8 mg) was incubated with 1.00 ml of various concentration of NVP (25-500 μg/ml) in 0.01 M phosphate buffer, pH 6 containing 0.05% tween 20 for 1.0 h. Then, the mixture was centrifuged for 5 min at 16000 rpm, and the concentration of free substrate in the solutions was determined using a spectrophotometer (λ_{max} = 281 nm). The amount of substrates bound to the polymer, Q, was calculated by substracting the concentration of free substrate from the initial substrate concentration.

2.10.4 The competitive ELISA for NVP determination

The polymers were added with solution (0.50 ml) of NVP (0, 0.2, 2, 20, 200 and 1,000 μg/ml) in 0.01 M phosphate buffer pH 6 containing 0.05% tween 20. Each mixture was incubated for 1.0 h. and then added with 0.50 ml solution of NVP-HRP conjugate to give a total volume of 1.00 ml. The samples were incubated for additional 1.0 h on a rocking table, after which the particles were removed by centrifugation for 5 min at 16000 rpm. The supernatant (200 μl) was withdrawn and added to 200 μl of colorimetric

substrate solution (TMB/H_2O_2 substrate). The reaction was developed for 5 minutes and was stopped by adding 200 µl of 1 N HCl. Then absorbances were read at λ_{max} = 450 nm.

When using 1:500,000 dilution of enzyme probe and polymer 0.8 mg, the results are expressed in terms of percent B/B_0 as a function of the NVP concentration (logarithmic scale); B and B_0 represent the bound enzymatic activity in the presence and absence of competitor, respectively. The amount of NVP-HRP conjugate bound to the polymer was calculated by substracting the concentration of unbound NVP-HRP in the supernatant from the initial NVP-HRP concentration.

When 1:20,000 dilution of enzyme probe and polymer 4.0 mg was used, the results are expressed in terms of ΔAbs as a function of the NVP concentration. ΔAbs was calculated by subtracting the absorbance value obtained from the supernatant in the presence and absence of NVP.