

Chapter 3
Experimental

EXPERIMENTAL

3.1. General

3.1. A Source of chemicals

N-tert-butoxycarbonyl-L-phenylalanine (Boc-L-Phe-OH), N-tert-butoxycarbonyl-D-phenylalanine (Boc-D-Phe-OH), N-tert-butoxycarbonyl-L-tryptophan (Boc-L-Trp-OH) and N-tert-butoxycarbonyl-D-tryptophan (Boc-D-Trp-OH) were purchased from Fluka, Switzerland. L-mandelic acid (L-MDA), D-mandelic acid (D-MDA), and L-phenylalanine (L-Phe) were from SRL, Mumbai. Ethylene glycol dimethacrylate (EGDMA) and divinylbenzene (DVB) were from Sigma-Aldrich, USA. 4-Vinylpyridine (4-VP) was from Alfa Acer and 2,2'-azo-bis-isobutyronitrile (AIBN) was from Merck, Germany. Chloroform and methanol were from Merck, India. Other chemicals used were analytical grade and obtained from commercial sources.

3.1.B.1 Purification of chemicals

The vast majority of monomers especially liquid monomers are normally supplied with polymerisation inhibitor present to suppress on-shelf degradation. Whilst it is certainly possible to polymerize the monomer in presence of inhibitor especially when the levels of inhibitor are low, to ensure good batch-to-batch reproducibility of experiments it is probably advisable to remove the polymerisation inhibitors from monomers with a second purification step like distillation. 4-Vinyl pyridine (4-VP) was stabilised with hydroquinone and was destabilised by distillation under reduced pressure prior to use. EGDMA was also

distilled under reduced pressure. Boc-L-Phe-OH, Boc-L-Trp-OH and D-MDA being solid were used as such.

Since divinylbenzene (DVB) was stabilised with 4-tert-butylcatechol it was destabilised by washing with NaOH solution (1%) in a separating funnel till the alkali layer turns colourless. The organic layer was washed with distilled water till it was free of alkali. Finally it was dried over anhydrous sodium sulphate. DVB being a mixture of isomers and since it contains only 50% of the required isomer double the volume of required quantity was taken. Doubly distilled water was used throughout.

2 Purification of solvents

Acetonitrile (CH₃CN): Water, acetamide, ammonium acetate and ammonia are the contaminants present in it. Drying by shaking with Linde 4A^o sieves removed water in acetonitrile. It was then distilled over P₂O₅.

Methanol (CH₃OH): Water present as impurity was removed by fractional distillation and purified by passing through Linde type 4A^o molecular sieves.

Chloroform (CHCl₃): Refluxed with calcium chloride for 2 h, distilled out and kept over anhydrous calcium chloride. Kept in dark to avoid photochemical oxidation to phosgene.

Dichloromethane (CH₂Cl₂): Decomposes on exposure to air and light producing hydrogen chloride. It was purified by keeping over potassium hydroxide pellets overnight and distilled over P₄O₁₀. Distilled dichloromethane was kept in dark coloured bottles with Linde type 4A^o molecular sieves.

3.1.C Analysis and characterization

The instruments used for the analysis and characterization of various polymers are listed below.

FT-IR Spectrophotometer: Fourier-Transform Infra-red Spectrophotometer 8400 S (DIN 206-72400) Shimadzu, Japan was used to record FT-IR spectra of pre-polymerisation complex solutions as well as for characterizing the MIP and NIP using KBr pellets.

UV-vis Spectrophotometer: UV-vis spectral measurements were done to follow the binding studies on a Shimadzu UV-vis Spectrophotometer model 2450.

¹H NMR spectrometer: ¹H NMR spectra of the pre-polymerisation complex in CDCl₃ were recorded on a BRUKER AMX-400 spectrometer operating at 500 MHz at 298K.

Scanning Electron Microscope: Scanning electron micrographs of the imprinted and non-imprinted polymers were taken on JEOL - JSM - 840A scanning electron microscope.

3.2. Synthesis of Molecular Imprinted (MIPs) and Non-imprinted Polymers (NIPs)

3.2.A Molecular Imprinting of Boc-L-Tryptophan

3.2.A.1 Synthesis of EGDMA- and DVB-crosslinked MIPs and NIPs with varying composition of Boc-L-Trp-OH and functional monomer

EGDMA- and DVB-crosslinked Boc-L-Trp-OH imprinted polymers with 1:2, 1:4, and 1:8 ratios of Boc-L-Trp-OH, and 4-VP were

synthesized. The ratio between the functional monomer and crosslinking agent was kept constant (40%) in all these polymers. The composition of the functional monomer (4-VP), crosslinking agent (EGDMA/DVB), and template (Boc-L-Trp-OH) used is given in Tables III. 1-2. The relative amounts of template, functional monomer and the crosslinker are given in units of millimoles.

The polymers were prepared according to the method described by Ramstrom et al. Appropriate amounts of print molecule (Boc-L-Trp-OH) and the functional monomer (4-VP) were weighed in 25 ml borosilicate glass tube and dissolved in 15 ml chloroform. The crosslinker (EGDMA/DVB), and the initiator AIBN (0.32 mmol) were then added to the solution. Oxygen gas retards free radical polymerizations, thus in order to maximize the rates of monomer propagation removal of the dissolved oxygen from monomer solutions immediately prior to polymerisation is advisable. Removal of dissolved oxygen can be achieved simply by ultrasonication or by spraying of the monomer solution by nitrogen. The tubes were then sealed and inserted in a thermostated water bath for 4 h at 65°C. The resulting polymers were crushed and ground in a mechanical mortar, wet-sieved in acetone through a 25 µm sieve. Grinding and sieving were repeated on the coarse particles until all polymer passed through the sieve. The fines were removed by repeated floatation in acetone. Corresponding non-imprinted polymers without Boc-L-Trp-OH were also prepared. The imprinted and the non-imprinted polymers were washed, dried and weighed.

Table III. 1. Synthesis of EGDMA-crosslinked (40%) MIPs and NIPs with varying composition of Boc-L-Trp-OH and functional monomer

T/FM ratio (mmol)	Boc-L-Trp-OH (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:2	0.3044	0.21	0.27	76	77
1:4	0.3044	0.43	0.53	82	84
1:8	0.3044	0.87	1.04	84	77

T: template FM: functional monomer CLA: crosslinking agent

Table III. 2. Synthesis of DVB-crosslinked (40%) MIPs and NIPs with varying composition of Boc-L-Trp-OH and functional monomer

T/FM ratio (mmol)	Boc-L-Trp-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:2	0.3044	0.21	0.41	43	41
1:4	0.3044	0.43	0.78	77	72
1:8	0.3044	0.87	1.57	78	73

Extraction of template from the imprinted polymers

The polymer particles were washed first with the porogen (chloroform) and with a mixture of methanol-acetic acid (8:2 v/v) for 2 h followed by Soxhlet extraction with methanol and with chloroform until

the template could no longer be detected under UV (λ_{max} : 279nm). Modifiers like acetic acid are able to interfere with the ion pair formation between the 4-VP and the –COOH group thereby facilitating the release of the template molecule. The washed particles were suspended in acetone for 4 h and allowed to settle. The solvent was removed by centrifugal separation, and the particles were dried to constant weight at 60°C in vacuum. Both sets of polymers were subjected to the same template removal procedure.

3.2.A.2. Synthesis of Boc-L-Trp-OH imprinted and non-imprinted polymers with varying extents of EGDMA and DVB crosslinking

In EGDMA-crosslinked polymers though the binding by the imprinted and non-imprinted polymers of the 1:8 system is higher than the 1:4 system their specificities are comparable. For a further understanding of the effect of the crosslink density on the binding properties, polymers having varying percentage of crosslinking (40-80 mmol%) keeping the T/FM ratio 1:4 were prepared (Table III. 3-4). In DVB-crosslinked system polymers having 1:4 T/FM ratio possess the highest specific binding. In this system though 1:4 polymer possess high specificity for the template molecule its enantioselectivity was poor. So different polymers of the 1:2 category with a different set of crosslinking percentage (25%, 40%, and 50%) were prepared (Table III. 5) and their binding studies were conducted.

1. **Synthesis of Boc-L-Trp-OH imprinted and non-imprinted polymers with varying extents of EGDMA and DVB crosslinking having template to functional monomer ratio 1:4**

Table III.3. Preparation of MIPs and NIPs of Boc-L-Trp-OH with varying extents of EGDMA crosslinking (1:4)

T:FM:CLA(mmol) (Crosslinking %)	Boc-L-Trp-OH (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:4: 2.75 (40)	0.3044	0.43	0.52	73	83
1:4: 5.0 (55)	0.3044	0.43	0.94	79	82
1:4:7.5 (65)	0.3044	0.43	1.41	89	83
1:4:10 (70)	0.3044	0.43	1.88	77	72
1:4:20 (80)	0.3044	0.43	3.77	69	79

Table III. 4. Preparation of MIPs and NIPs of Boc-L-Trp-OH with varying extents of DVB crosslinking (1:4)

T:FM:CLA(mmol) (Crosslinking %)	Boc-L-Trp-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:4:2.75 (40)	0.3044	0.43	0.78	73	77
1:4: 5 (55)	0.3044	0.43	1.42	79	72
1:4:7.5 (65)	0.3044	0.43	2.14	78	78
1:4:10 (70)	0.3044	0.43	2.86	71	69
1:4:20 (80)	0.3044	0.43	5.70	72	68

2. Synthesis of Boc-L-Trp-OH imprinted and non-imprinted polymers with varying extents of DVB crosslinking with 1:2 T/FM ratio

Polymers with varying extents of DVB crosslinking were prepared in 1:2 template-functional monomer ratio. The composition of the ingredients and the yield obtained are given in Tables III. 5.

Table III. 5. Preparation of MIPs and NIPs of Boc-L-Trp-OH with varying extents of DVB crosslinking (1:2)

T:FM:CLA(mmol) (Crosslinking %)	Boc-L-Trp-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:2:0.68 (25)	0.3044	0.21	0.20	47	66
1:2:1.25 (40)	0.3044	0.21	0.36	70	69
1: 2: 1.9 (50)	0.3044	0.21	0.53	74	73

3.2.B Molecular imprinting of Boc-L-Phenylalanine

3.2.B.1. Synthesis of EGDMA-, DVB- and NNMBA-crosslinked MIPs and NIPs with varying composition of Boc-L-Phe-OH and functional monomer

EGDMA-, DVB- and NNMBA-crosslinked Boc-L-Phe-OH imprinted polymers with 1:2, 1:4, 1:8, and 1:16 ratios of Boc-L-Phe-OH and 4-VP were synthesized. The percentage of crosslinking was kept constant (40%) in all these polymers. The composition of template Boc-L-Phe-OH,

functional monomer (4-VP) and crosslinking agents (EGDMA/DVB/NNMBA) used are given in Tables III. 6 - 8.

For each polymerization, 1mmol (0.2653 gm) of Boc-L-Phe-OH and functional monomer (4-VP) were dissolved in 15 ml chloroform in a glass tube. The crosslinker and the initiator were then added to the solution and the tubes were purged thoroughly with nitrogen before polymerization. After nitrogen gas being flushed into the mixture for 30 min. the sealed tube was kept in a thermostated water bath at 65°C which yielded the imprinted polymer. The obtained bulk polymer was dried under vacuum and ground into particles of uniform size. To compare the binding capacity of the imprinted polymer, non-imprinted polymers without Boc-L-Phe-OH were also prepared, ground and sieved.

Table III. 6. Synthesis of EGDMA-crosslinked (40%) MIPs and NIPs with varying composition of Boc-L-Phe-OH and functional monomer

T/FM ratio mmol	Boc-L-Phe-OH (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:2	0.2653	0.21	0.26	63	66
1:4	0.2653	0.43	0.5 2	82	78
1:8	0.2653	0.86	1.05	79	82
1:16	0.2653	1.72	2.07	82	75

Table III. 7. Preparation of DVB-crosslinked (40%) MIPs and NIPs with varying composition of Boc-L-Phe-OH and functional monomer

T/FM ratio (mmol)	Boc-L-Phe-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:2	0.2653	0.21	0.39	73	72
1:4	0.2653	0.43	0.78	80	70
1:8	0.2653	0.86	1.57	76	82
1:16	0.2653	1.72	3.13	79	86

Table III. 8. Synthesis of NNMBA-crosslinked (40%) MIPs and NIPs with varying composition of Boc-L-Phe-OH and functional monomer

T/FM ratio mmol	Boc-L-Phe-OH (gm)	4-VP (ml)	NNMBA (gm)	Yield (%)	
				MIP	NIP
1:4	0.2653	0.43	0.43	78	63
1:8	0.2653	0.86	0.85	77	76
1:16	0.2653	1.73	1.70	69	74

3.2. B.2 Synthesis of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of EGDMA and DVB crosslinking

1. Synthesis of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of EGDMA crosslinking having 1:4 template to functional monomer ratio

Since the specificities of EGDMA and DVB crosslinked MIPs with 1:4 template-functional monomer ratio was higher, polymers with different extents of crosslinking were synthesized in this ratio. NNMBA system is found to be nonspecific and hence it's further studies were not conducted. For the preparation of EGDMA- and DVB-crosslinked Boc-L-Phe-OH imprinted polymer, the template Boc-L-Phe-OH (1 mmol), 4-VP (4 mmol), required amount of crosslinking monomer EGDMA or DVB and initiator AIBN (0.32 mmol) were weighed into a 25 ml glass tube and dissolved in chloroform. The solution was degassed with nitrogen gas for 20 min., sealed and kept in a water bath at 65°C for 24 h. The resulted bulk polymers were ground and wet sieved to pass through 25 µm sieve. Non-imprinted polymers without the template Boc-L-Phe-OH were also synthesized, ground and wet sieved. The imprinted and non-imprinted polymers were washed as described earlier. This procedure was repeated several times until the imprint molecule in the filtrate could not be detected by a UV spectrometer. The composition of the polymerisation mixture and the yield obtained are given in Tables III. 9 -10.

Table III. 9. Preparation of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of EGDMA crosslinking having 1:4 template to functional monomer ratio

T:FM:CLA Mmol (crosslinking %)	Boc-L-Phe-OH (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:4:2.75 (40)	0.2653	0.43	0.52	42	31
1:4:5 (55)	0.2653	0.43	0.94	83	74
1:4:7.5 (65)	0.2653	0.43	1.41	77	64
1:4:10 (70)	0.2653	0.43	1.88	64	65
1:4:20 (80)	0.2653	0.43	3.77	64	66

Table III. 10. Preparation of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of DVB crosslinking having 1:4 template to functional monomer ratio

T:FM:CLA mmol (crosslinking%)	Boc-L-Phe-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:4:2.75 (40)	0.2653	0.43	0.78	67	36
1:4:5 (55)	0.2653	0.43	1.42	61	68
1:4:7.5 (65)	0.2653	0.43	2.14	66	72
1:4:10 (70)	0.2653	0.43	2.85	63	61
1:4:20 (80)	0.2653	0.43	5.70	62	61

2. Synthesis of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of DVB crosslinking having 1:2 template to functional monomer ratio

The DVB-crosslinked 1:4 system though it is specific to the template molecule its enantioselectivity is negligible and therefore polymer of the 1:2 category having different crosslinking percentage were prepared. The composition of the polymerisation mixture and the yield obtained are given in Tables III .11

Table III. 11. Preparation of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of DVB-crosslinking having 1:2 template to functional monomer ratio

T:FM:CLA mmol (DVB %)	Boc-L-Phe-OH (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:2: 0.68 (25)	0.2653	0.21	0.20	77	64
1:2: 1.25 (40)	0.2653	0.21	0.37	74	68
1: 2: 1.87 (50)	0.2653	0.21	0.53	67	70
1:2: 3 (60)	0.2653	0.21	0.85	77	63

3. 2.C Cocktail polymerisation

In order to check whether the selectivity of the imprinted polymer could be improved by using two different functional monomers simultaneously, cocktail polymerisation using acrylamide and 4-VP was carried out.

3. 2.C.1 Synthesis of EGDMA-crosslinked Boc-L-Phe-OH imprinted and non-imprinted polymers with varying composition of Boc-L-Phe-OH and functional monomers

For the preparation of polymer the required amounts of Boc-L-Phe-OH, 4-VP and acrylamide (functional monomers), EGDMA (crosslinker) and AIBN (initiator) were weighed into a 25 ml glass tube and dissolved in 15 ml of chloroform. After nitrogen gas is purged into the mixture for 30 min, the flask was sealed and kept in a thermostated water bath at 65°C for 24h. The resulted bulk polymer was ground to pass through 25 µm sieve. The polymer particles were Soxhlet extracted with a mixture of methanol-acetic acid (8:2 v/v) for 2 h and with methanol and with chloroform until the template could no longer be detected under UV (λ_{max} : 259nm). Then the washed particles were suspended in acetone and allowed to settle for 4 h. The solvent was removed by centrifugal separation and the fine particles were dried to constant weight at 60⁰ C in vacuum. A non-imprinted polymer (NIP) was prepared in the same way as described above but without template. The composition of the functional monomer 4-VP and acrylamide, crosslinking agent EGDMA, template Boc-L-Phe-OH and the yield obtained are given in Table III.12.

3. 2.C.2 Synthesis of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of EGDMA crosslinking having template to functional monomer ratio 1:8:8

Since polymers having 1:8:8 T/FM has the highest specific binding EGDMA-crosslinked poly(4-VP-co-acrylamide) with different percentage of EGDMA crosslinking in this ratio were synthesized. For

this Boc-L-Phe-OH (1mmol), 4-VP (8mmol), acrylamide (8mmol) and EGDMA were mixed with AIBN and dissolved in chloroform. Heating at 65°C under nitrogen atmosphere in a water bath for 24 h afforded the imprinted polymer. The solid polymer was ground and sieved. Non-imprinted polymer without the template Boc-L-Phe-OH was also prepared. The composition of the functional monomers 4-VP and acrylamide, crosslinking agent EGDMA, template Boc-L-Phe-OH and the yield obtained are given in Table III .13.

Table III. 12. Synthesis of EGDMA-crosslinked Boc-L-Phe-OH imprinted and non-imprinted polymers with varying composition of Boc-L-Phe-OH and functional monomers

T/FM ratio (mmol)	Boc-L-Phe-OH (gm)	AAM (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
					MIP	NIP
1:2:2	0.27	0.14	0.21	0.52	56	59
1:4:4	0.27	0.28	0.43	1.04	71	79
1:8:8	0.27	0.57	0.86	2.07	76	74

Table III. 13. Synthesis of Boc-L-Phe-OH imprinted and non-imprinted polymers with varying extents of EGDMA crosslinking having template to functional monomer ratio 1:8:8

EGDMA (%)	Boc-L-Phe-OH (gm)	AAM (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
					MIP%	NIP%
41	0.27	0.57	0.86	2.07	60	59
55	0.27	0.57	0.86	3.77	72	74
65	0.27	0.57	0.86	5.66	79	74
70	0.27	0.57	0.86	7.54	77	70
80	0.27	0.57	0.86	15.09	76	69

3.2. D Molecular Imprinting of D-Mandelic Acid

3.2. D.1. Synthesis of EGDMA- and DVB-crosslinked MIPs and NIPs with varying composition of D-mandelic acid and functional monomer

EGDMA- and DVB-crosslinked polymers with 1:2 and 1:4 and 1:8 ratios of D-mandelic acid and 4-VP were synthesized. The composition of the template, functional monomer and crosslinking agents are given in Tables III. 14-15. The required amount of template, functional monomer, crosslinker (EGDMA/DVB), and initiator (AIBN) were dissolved in acetonitrile in glass tubes. They were degassed in a sonicating water bath and saturated with nitrogen for 5 min. The tubes

were then sealed with parafilm and polymerized at 65°C for 4 h. After the polymerisation had completed, the polymers were collected and ground in a mechanical mortar. The polymers were wet sieved with acetone through a 25- μ m sieve, the particles which passed the sieve were collected, and those of size bigger than 25 μ m were dried, ground, and sieved again. This process was repeated several times until all the particles passed through the sieve. The fines were removed by repeated sedimentation in acetone. Non-imprinted polymers without D-mandelic acid were also prepared.

Table III. 14. Synthesis of EGDMA-crosslinked (40%) D-MDA imprinted and non-imprinted polymers with varying composition of D-MDA and functional monomer

T/FM ratio (mmol)	D-MDA (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:2	0.152	0.21	0.26	20	22
1:4	0.152	0.42	0.52	34	26
1:8	0.152	0.86	1.04	50	54

Table III.15. Preparation of DVB-crosslinked (40%) D-MDA imprinted and non-imprinted polymers varying composition of D-MDA and functional monomer

T/FM ratio (mmol)	D-MDA (gm)	4-VP (ml)	DVB (ml)	Yield (%)	
				MIP	NIP
1:2	0.152	0.21	0.39	34	32
1:4	0.152	0.43	0.78	36	33
1:8	0.152	0.86	1.57	52	51

3.2.D.2 Synthesis of D-mandelic acid imprinted and non-imprinted polymers with varying extents of EGDMA crosslinking

Similar to Boc-L-Phe-OH imprinted polymers, maximum specificity was obtained for the polymers with 1:4 D-mandelic acid to 4-VP ratio. Hence EGDMA-crosslinked D-mandelic acid imprinted and

Table III. 16. Preparation of MIPs and NIPs of D-mandelic acid with varying extents of EGDMA crosslinking

T:FM:CLA (mmol) (EGDMA %)	D-MDA (gm)	4-VP (ml)	EGDMA (ml)	Yield (%)	
				MIP	NIP
1:4:2.75 (40)	0.152	0.43	0.52	34	26
1:4:5 (55)	0.152	0.43	0.94	69	57
1:4:7.5 (65)	0.152	0.43	1.41	72	61
1:4:10 (70)	0.152	0.43	1.88	61	65
1:4:20 (80)	0.152	0.43	3.77	79	68

non-imprinted polymers with 1:4 template-functional monomer ratio and having different extents of crosslinking were prepared by using the required amounts of the template, monomer and initiator and by following the routine method of synthesis. The bulk polymer monolith obtained were ground and sieved to get particles of uniform size. The non-imprinted polymers were prepared in the absence of the template. All the different set of polymers were powered, washed and weighed. The compositions of various components and the yield are exhibited in Table III.16.

3.3 Swelling studies

Polymer particles (50mg) were packed into a sintered crucible and were filled with the solvent. After 24 h of equilibration, the excess solvent was removed from the polymer by applying reduced pressure for 1 min. and the weight of the swollen particles was measured. The swelling ratio (S_R) of the polymer was calculated from the following equation

$$S_R = (m_s - m_o) / m_o$$

where m_s the mass of the swollen polymer and m_o is the weight of the dry polymer.

3.4 Binding of template: General procedure

The template desorbed polymers contains imprints that exhibit a 'memory' in terms of complementarities of both shape and chemical functionality for the original imprint molecule. In order to investigate the binding ability, the template desorbed polymer was treated with solution of the print molecule and the extent of binding was followed by UV-vis spectroscopy.

3.4.A Binding capacity

The investigation of the specific binding of template by the imprinted polymer is calculated based on a reference, which can be an internal standard or the binding to a non-imprinted polymer. Fixed amount of the MIP and NIP were incubated with known concentrations of the solution of template in chloroform (7 ml) in sample tubes, sealed, and shaken for 3h in a thermostatted shaker kept at 30°C. The concentrations of the template solution before and after binding were followed by UV measurements at their respective λ_{\max} (Boc-L-Trp-OH: 279 nm, Boc-L-Phe-OH: 259nm, and D-mandelic acid: 258nm). The difference in binding between the MIP and NIP is the specificity in binding.

3.4.B Parameters affecting specific binding

1 Extent of crosslinking

In order to study the effect of crosslinking on specific binding equal amounts of MIP and NIP of varying degrees of crosslinking were introduced into equal volume of template solutions (7ml) of known concentrations for same interval of time. The specificity in binding was followed spectrophotometrically as mentioned earlier.

2 Concentration of template solution

The batch wise guest binding experiments were used to evaluate the influence of concentration on imprinting efficiency. Definite weight of the polymer particles were put into sealed sample tubes and the template solution of different concentration were introduced. The resulting suspension was shaken for 3h. After this incubation, the polymer particles were centrifuged, and the remaining concentration of the template was

determined spectrophotometrically. The quantity of template bound by the polymer was determined from the difference in concentrations of the template solution before and after incubation. From the results of the binding experiments at various concentrations of template, the binding isotherms were obtained and the data were analyzed employing the Scatchard method.

3 Solvent

Equal amounts of the MIP and NIP were introduced into solutions of the templates (7 ml) in different solvents for equal interval of time and extent of template bound were determined spectrophotometrically. The solvent with maximum binding specificity was used for further investigations.

4 Time

To investigate the time taken for saturation of binding sites definite weight of the MIP and NIP were incubated in template solution of definite concentration at 30°C. The extent of template binding at regular intervals of time was followed spectrophotometrically.

5 Mass of polymer

Different masses of MIP and NIP were introduced into template solutions of fixed concentration for fixed time interval. The variation of the binding capacity with mass was followed as described earlier.

3.4.C Selectivity studies

To the template desorbed polymer equal volumes (7ml) of the solutions of template and of structural analogues having same

concentration were added and the difference in the extent of binding was estimated spectrophotometrically. The imprinting factor or separation factor (α) which represented the effect of the imprinting process was the ratio of the amount of substrate bound by the MIP to that bound by the corresponding NIP. The selectivity of the imprinted polymers towards the template was calculated in terms of selectivity factor

$$\text{Separation factor } (\alpha_{\text{Temp}}) = K_{\text{MIP}} / K_{\text{NIP}}$$

$$K = \frac{\text{Template}_{\text{Bound}}}{\text{Template}_{\text{Free}}}$$

$$\text{Selectivity factor} = \alpha_{\text{Template}} / \alpha_{\text{Analogue}}$$