Mechanistic Aspects of Molecular Imprinting by Precipitation Polymerisation

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Declaration

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision.

The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University’s Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

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Publications and Conference Presentations


Table of Contents

Chapter 1
1.1. Synthesis and Formats of Molecularily Imprinted Polymers ................................................ 3
1.2. Factors affecting the efficiency of MIPs .................................................................................. 7
  1.2.1. Solvents/Diluents/Porogens .......................................................................................... 8
  1.2.2. Method of initiation and concentration of initiator ..................................................... 10
  1.2.3. Cross-linker ................................................................................................................ 10
  1.2.4. Functional monomer ................................................................................................... 11
1.3. Objectives .......................................................................................................................... 13

Chapter 2
2.1. Materials and Reagents ....................................................................................................... 16
2.2. Synthesis of Polymers ........................................................................................................ 18
2.3. Determination of the Polymer Composition using NMR ................................................... 20
2.4 Rebinding Studies ............................................................................................................... 22
  2.4.1. CAF and THP system ................................................................................................. 22
  2.4.2. Phenolic templates system ......................................................................................... 23
  2.4.3. Imide-containing templates ....................................................................................... 24
  2.4.4. PNL system .............................................................................................................. 25
  2.4.5. Binding isotherm analyses ......................................................................................... 25
2.5. Template-Monomer Interaction Studies ............................................................................ 26
  2.5.1. Phenolic templates .................................................................................................... 26
  2.5.2. Imide-containing templates ....................................................................................... 26
2.6. Selectivity Studies ............................................................................................................. 26
  2.6.1. Phenolic templates .................................................................................................... 26
  2.6.2. Imide-containing templates ....................................................................................... 27
2.7. Determination of Degree of Crosslinking ........................................................................... 27
2.8. Sample Morphology .......................................................................................................... 29
2.9. Particle Size Analyses ....................................................................................................... 30
2.10. Thermal Analysis ............................................................................................................ 30
2.11. X-Ray Diffraction analyses ............................................................................................. 30
2.12. Specific Surface Area and Porosity (Brunauer-Emmett-Teller) ........................................ 31

Chapter 3
3.1. Introduction ....................................................................................................................... 32
3.2. Results and Discussion ..................................................................................................... 34
  3.2.1. Variation of template to functional monomer ratios (T:FM) ........................................ 39
3.2.1.1. Particle size and morphology ................................................................. 39
3.2.1.2. Polymer composition ........................................................................... 41
3.2.1.3. Template incorporation ...................................................................... 43
3.2.1.4. Template rebinding studies ................................................................. 45
3.2.2. Variation of functional monomer to cross-linker ratio (FM:XL) .............. 48
  3.2.2.1. Particle size and morphology ............................................................. 48
  3.2.2.2. Polymer composition ......................................................................... 50
  3.2.2.3. Template incorporation .................................................................... 52
  3.2.2.4. Template rebinding studies ............................................................... 53
3.2.3. Variation of amount of initiator to total monomer ratio (I: TM) .............. 57
  3.2.3.1. Particle size and morphology ............................................................. 57
  3.2.3.2. Polymer composition ......................................................................... 61
  3.2.3.3. Template incorporation .................................................................... 63
  3.2.3.4. Template rebinding studies ............................................................... 64
3.2.4. Polymer Surface area .............................................................................. 67
3.3. Summary ..................................................................................................... 68

Chapter 4
4.1 Introduction ................................................................................................. 71
4.2. Results and Discussion ............................................................................. 72
  4.2.1. Thermally-synthesized imprinted microspheres ..................................... 73
    4.2.1.1. 3-5, Dimethylphenol MIPs (T-1OH) ............................................... 73
    4.2.1.2. 5-Methylbenzene-1,3-diol MIPs (T-2OH) ..................................... 84
    4.2.1.3. Benzene-1,3,5-triol MIPs (T-3OH) ............................................... 93
    4.2.1.4. Comparison of the thermally-synthesized MIP systems .................. 102
    4.2.1.5. Cross-reactivity studies ................................................................. 107
    4.2.1.5. Molecular modelling and $^1$H NMR Interaction Studies ............... 109
    4.2.1.6. Polymer surface area and porosity ............................................... 119
  4.2.2. Photochemically synthesized imprinted microspheres .......................... 126
    4.2.2.1. 3,5-dimethylphenol MIPs (P-1OH) ............................................... 126
    4.2.2.2. 5-Methylbenzene-1,3-diol MIPs (P-2OH) ..................................... 133
    4.2.2.3. Benzene-1,3,5-triol polymers MIPs (P-3OH) ............................... 139
    4.2.2.4. Comparison of the photochemically synthesized polymers .......... 145
  4.2.3. Comparison of Thermal vs Photochemical initiation method ............... 148
  4.3. Summary .................................................................................................. 152
Chapter 5
5.1. Introduction ..................................................................................................................... 154
5.2. Results and Discussion ................................................................................................... 156
5.2.1. Synthesis of MIPs ........................................................................................................ 157
  5.2.1.1. Bulk Polymerisation .......................................................................................... 157
  5.2.1.2. Precipitation Polymerisation .............................................................................. 162
5.2.2. TAU-BAAPy Interaction Studies ................................................................................ 163
5.2.3. Template rebinding studies ........................................................................................ 170
  5.2.3.1. TAU rebinding efficiency .................................................................................. 171
  5.2.3.2. Characterisation of Binding Sites: Binding Isotherms ...................................... 172
5.2.4. Selectivity Studies ........................................................................................................ 175
5.2.5. Other Factors ................................................................................................................ 178
  5.2.5.1. Effect of Initiator Concentration ........................................................................ 178
  5.2.5.2. Effect of Agitation ............................................................................................. 181
5.3. Summary ......................................................................................................................... 183

Chapter 6
6.1. Introduction ..................................................................................................................... 186
6.2. Results and discussion .................................................................................................... 189
6.2.1. Synthesis of Polymers .................................................................................................. 191
6.2.2. Polymer composition ................................................................................................... 191
6.2.3. Template Rebinding Studies ....................................................................................... 198
6.2.4. Other Physical Characterisation .................................................................................. 201
  6.2.4.1. X-ray diffraction ................................................................................................ 201
  6.2.4.2. Differential Scanning Calorimetry .................................................................... 202
  6.2.5. Characterization of the ionic liquid dispersible fraction from IL-PP polymer (IL-PP-2) ......................................................... 204
6.3. Summary ......................................................................................................................... 206

Chapter 7
7.1. Summary ......................................................................................................................... 209
7.2. Recommendations for Future Work ................................................................................ 218
List of Figures

Chapter 1

Figure 1. 1. Overview of molecular imprinting process. ................................................................. 2

Chapter 2

Figure 2. 1. Structures of the components of the polymerisation feed. The internal standard used for the 1H NMR analyses, 1,4-dioxane (STD), cross-linkers employed: ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRM), functional monomers used: 2,6-bis(acryl)amidopyridine (BAAPy) and methacrylic acid (MAA), the templates and analogues used: theophylline (THP), caffeine (CAF), 3,5-dimethylphenol (1OH), 5-methylbenzene-1,3-diol (2OH), benzene-1,3,5-triol (3OH), 2,3,5-tri-O-acetyltidine, 2,3,5-tri-O-acetyltidine (TAC), uridine (Urd) and (+) Propanolol (PNL). Labelled atoms correspond to nuclei used for NMR analysis ........................................................................................................ 17

Figure 2. 2. 1H NMR calibration curves of EGDMA (A) and MAA (B) for the determination of the amount of each component incorporated in the polymers. The following calibration curves were prepared using the following range of standards: 0.010-20.0 mM for EGDMA, 0.002 – 40 mM for MAA and 1.0 mM dioxane in DMSO-d6 as reference standard S. NOTE: I(S)/I(EGDMA) = ratio of the peak integrations of the dioxane standard to EGDMA, [S]/[EGDMA] = ratio of the concentration of dioxane standard to the concentration of EGDMA, I(S)/I(MAA) = ratio of the peak integrations of the dioxane standard to MAA, [S]/[MAA] = ratio of the concentration of dioxane standard to the concentration of MAA ............................................................................... 21

Figure 2. 3. An example of a 1H NMR spectrum of the caffeine pre polymerization solution. Peaks of the components are as follows; cross-linker (EGDMA, O-CH2 signal) at 4.32 ppm, functional monomer (the combination of the signals of -CH2=CH2- of MAA and EGDMA) at 5.96 and 6.39 ppm and for caffeine at 4.23 ppm (-N-CH3) ................................................................................. 22

Figure 2. 4. An example of a partial infrared spectrum of MAA:EGDMA (1:5) solution overlayed with spectrum of DMP-1:100-T MIP using Perkin Elmer Infrared Spectroscopy-Two based on a Universal Attenuated Total Reflectance sensor (UATR-FTIR). The amount of cross-linking in the polymers were determined using the peaks of -C=O and -C=C- at 1730 and 1650 cm⁻¹ respectively. .................................................................................................................................. 29

Chapter 3

Figure 3. 1. Structures of caffeine (CAF) and theophylline (THP). .................................................... 36

Figure 3. 2. SEM images of microspheres synthesized at various template: functional monomer (T:FM) ratios. TM2-N (A), TM2-C (B ) and TM2-T (C ), TM4-N (D), TM4-C (E ) and TM4-T (F), TM6-N ( G), TM6-C (H) and TM6-T (I), TM8-N ( J), TM8-C (K) and TM8-T (L). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS................................................................................................................................ 40

Figure 3. 3. Hydrodynamic sizes, \(d_{\text{H}}\), of microspheres synthesized in various T:FM ratios. Measurements were conducted using Dynamic Light scattering (DLS) and acetonitrile as the dispersant ................................................................................................................................ 41

Figure 3. 4. The degree of cross-linking in the polymers synthesized at various T:FM ratios. Residual double bonds in the polymer were quantified using infrared spectroscopy and compared to the double bonds of the EGDMA monomer ........................................................................................................ 43
Figure 3. 5. Percentages of CAF and THP incorporated in the polymers in various T:FM ratios measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. 45

Figure 3. 6. CAF incorporation, MIP and NIP binding performance of polymers synthesized at various T:FM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution with 70-90% binding from the rebinding solution. Post rebinding solutions were analyzed by HPLC. Template incorporation in μmol/g is also shown for comparison purposes. 46

Figure 3. 7. THP incorporation, MIP and NIP binding performance of polymers synthesized at various T:FM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution with 70-90% binding from the rebinding solution. Post rebinding solutions were analyzed by HPLC. 47

Figure 3. 8. Hydrodynamic sizes, $d_H$, of microspheres synthesized in various FM:XL ratios. Measurements were conducted using Dynamic Light Scattering (DLS) and used ACN as the dispersant. 49

Figure 3. 9. SEM images of polymers synthesized in various FM:XL ratios. MX2- N (A), MX2-C (B), MX2-T (C), MX5- N (D) MX5-C (E), MX5-T (F) and MX10- N (G) MX10-C (H) and MX10-T (I). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS. 50

Figure 3. 10. Degree of cross-linking in the polymers synthesized at various FM:XL ratios. The residual double bonds in the polymers were quantified using Infrared spectroscopy and compared to the double bonds of the EGDMA monomer. 51

Figure 3. 11. Percentages of CAF and THP incorporated in the polymers in various FM:XL ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. 53

Figure 3. 12. Comparison of the amount of CAF incorporated, rebound by the MIPs and NIP prepared in various FM:XL ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analyzed by HPLC. 55

Figure 3. 13. Summary of the THP incorporation and rebinding performance of the MIPs and NIPs prepared in various FM:XL ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analysed by HPLC. 56

Figure 3. 14. Hydrodynamic sizes, $d_H$, of microspheres synthesized at various I:TM ratios. Measurements were conducted using Dynamic Light Scattering (DLS) and using ACN as the dispersant. 59

Figure 3. 15. SEM images of microspheres synthesized using different initiator : total monomer ratios (I:TM). IM1000-N (A), IM1000-C (B) and IM1000-T (C), IM500-N (D), IM500-C (E) IM500-T (F), IM100-N (G), IM100-C(H) IM100-T (I), IM10-N (J), IM10-C (K) IM10-T (L), and IM5-N (M) IM5- C(N) and IM5-T (O). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS. 60

Figure 3. 16. The degree of cross-linking in the polymers synthesized at various I:TM ratios. Residual double bonds were quantified using Infrared Spectroscopy and compared with the double bonds of EGDMA. 63

Figure 3. 17. CAF and THP incorporation in the polymers at various I:TM ratios measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. 64

Figure 3. 18. Comparison of the amount of CAF incorporated, rebound by the MIP and the NIP polymers synthesized at various I:TM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analyzed by HPLC. 65

Figure 3. 19. Comparison of the amount of THP incorporated in the polymers, rebound by the imprinted and non-imprinted polymers synthesized in various I:TM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analyzed by HPLC. 66
Chapter 4

Figure 4. 1. Percentages of 1OH incorporated in the polymers in various I:TM ratios. Measured by $^1\text{H}$ NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. ................................................................. 76

Figure 4. 2. SEM images of T-1OH microspheres in varying amount of initiator in the polymerization feed. T-1OH-1:100 MIP (A) and NIP (B), T-1OH-1:50 MIP (C) and NIP (D), T-1OH-1:25 MIP (E) and NIP (F), T-1OH-1:10 MIP (G) and NIP (H), T-1OH-1:5 MIP (I) and ........................................ 78

Figure 4. 3. Time binding experiment of 1OH imprinted polymers (P-1OH-1:50) and non-imprinted counterpart. 10.0 mg polymers were incubated at different time periods using 100 μM DMP solution................................................................. 79

Figure 4. 4. 1OH incorporation, MIP and NIP binding performance of polymers synthesized in various I:TM ratios. Polymers were incubated for 60 mins in 100μM template rebinding solution with 40-70% binding from the rebinding solution. Post rebinding solutions were analyzed by $^1\text{H}$ NMR. Template incorporation in μmol/g is also shown for comparison purposes. ...................... 81

Figure 4. 5. Binding isotherms for T-1OH-1:100 polymers fitted to (A) Non-linear Langmuir model (B) Linearmised Langmuir isotherm................................................................. 83

Figure 4. 6. Percentages of 2OH incorporated in the polymers in various I:TM ratios. Measured by $^1\text{H}$ NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. .................................................. 86

Figure 4. 7. SEM images of T-2OH-imprinted microspheres in varying amount of initiator in the polymerization feed. T-2OH-1:100 MIP (A) and NIP (B), T-2OH-1:50 MIP (C) and NIP (D), T-2OH-1:25 MIP (E) and NIP (F), T-2OH-1:10 MIP (G) and NIP (H), T-2OH-1:5 MIP (I) and NIP (J). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM................................................................. 88

Figure 4. 8. Time binding tests using 10.0 mg T-2OH microspheres incubated in 1.00 mL of 100 μM 2OH solution. Amount of template bound to the polymers were analyzed using $^1\text{H}$ NMR spectroscopy using the peak signal at 6.47 ppm for 2OH-H2................................................................. 89

Figure 4. 9. Amount of 2OH incorporation in the polymers and the rebound of 2OH-MIPs and NIPS in different I:TM experiments analysed by $^1\text{H}$ NMR monitoring the 2OH peak at 6.47 ppm and the peak at 3.57 ppm for the standard1,4-dioxane in DMSO-$d_6$ ................................................................. 90

Figure 4. 10. Binding isotherms for T-2OH-1:100 polymers fitted to (A) Non-linear Langmuir model (B) Linearised Langmuir isotherm................................................................. 92

Figure 4. 11. Percentages of 3OH incorporated in the polymers in various I:TM ratios. Measured by $^1\text{H}$ NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard................................. 95

Figure 4. 12. SEM images of T-3OH polymers produced in varying I:TM ratios T-3OH-1:100 MIP (A) NIP (B), T-3OH-1:50 MIP (C) NIP (D), T-3OH-1:25 MIP (E) NIP (F), T-3OH-1:10 MIP (G) NIP (H), T-3OH-1:5 MIP (I) NIP (J). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM. ......................... 97

Figure 4. 13. Time binding experiment for T-3OH polymer (T-3OH-1:50). Using 10.0 mg polymers and 100 uM 3OH rebinding solution. $^1\text{H}$ NMR results were quantified using the-CH=C- signal of 3OH at 6.11 ppm and using 1,4-dioxane as the reference standard in DMSO-$d_6$ ................................................. 98

Figure 4. 14. Amount of template rebound by theT-3OH MIPs and the NIPs at varying I:TM ratios. Unreacted components remaining in the post polymerisation solution was quantified by $^1\text{H}$ NMR using the-CH=C- peak of T-3OH at 6.11 ppm and 1,4-dioxane in DMSO-$d_6$ at 3.57 ppm as the reference standard. ................................................................. 100

Figure 4. 15. Binding properties of the T-3OH-1:100 polymers in different binding models, Non-linear Langmuir model (A) and Linearized Langmuir model (B) .................................................. 101
Figure 4. 16. Dimerisation reaction of 2OH in the presence of a radical. 104
Figure 4. 17. Comparison of the bound templates and two other analogues investigated for non-
competitive studies of the three different imprinted polymers MIP (A) and NIP (B) 108
Figure 4. 18. Electrostatic potential maps, molecular interactions and corresponding distances in
1OH-4MAA cluster between the template 1OH (A), and the analogues 2OH (B) and 3OH (C)
measured by Spartan ’14 v1.1.8. Intermonomer distances observed (data not shown) were also
observed with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding
interactions........................................................................................................................................ 111
Figure 4. 19. Chemical shifts of protons (A) of MAA and each of the three template monitored by 1H
NMR and carbons (B) monitored by 13C NMR. Molecular structures of the template; 1OH R1 and
R2 = CH3, 2OH R1 = OH and R2 = CH3 and 3OH R1 and R2 = OH, and the functional monomer
MAA with the atom labelling for the NMR interaction studies..................................................... 112
Figure 4. 20. Electrostatic potential maps, molecular interactions and corresponding distances in
2OH-4MAA cluster between the template 2OH (A), and the analogues 1OH (B) and 3OH (C)
measured by Spartan ’14 v1.1.8. Intermonomer distances observed (data not shown) were also
observed with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding
interactions........................................................................................................................................ 114
Figure 4. 21. Electrostatic potential maps, molecular interactions and corresponding distances in
3OH-4MAA cluster between the template 3OH (A), and the analogues 1OH (B) and 2OH (C)
measured by Spartan ’14 v1.1.8. Inter-monomer interactions were also observed (data not shown)
with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding interactions. .... 116
Figure 4. 22. Electrostatic potential maps, molecular interactions and corresponding distances in
between the 2OH dimer and 4 MAA measured by Spartan ’14 v1.1.8. Inter-monomer interactions
were also observed (data not shown) with distances ranging from 2.0-2.4 Å which corresponds to
weak H-bonding interactions........................................................................................................... 119
Figure 4. 23. Incremental pore volume versus pore width plots for MIPs of T-1OH-1:100 and 1:5. 122
Figure 4. 24. Comparison of the binding capacity of the T-1:100 MIP systems using different
phenolic templates normalized with respect to mass (A) and surface area (B). ......................... 124
Figure 4. 25. Comparison of the binding capacity of T-1OH polymers in different concentrations of
initiator normalized with respect to mass (A) and surface area (B).............................................. 125
Figure 4. 26. Percentages of 1OH incorporated in the photo-chemically synthesized polymers in
various I:TM ratios. Measured by 1H NMR using 1,4-dioxane in DMSO-d6 as the reference
standard. ........................................................................................................................................ 129
Figure 4. 27. SEM images of photochemically synthesized polymers. P-1OH-1:100 MIP (A) and NIP
(B) and P-1OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres
with the corresponding polydispersity indexes (PDI) measured by DLS and SEM. ..................... 131
Figure 4. 28. Comparison of the amount of template incorporated and binding performance by P-1OH
polymers produced in different I:TM ratios measured by 1H NMR using 1,4-dioxane in DMSO-
d6 as the reference standard. 10.0 mg polymers incubated in 100 µM 1OH rebinding solution for
60 mins........................................................................................................................................... 133
Figure 4. 29. Percentages of 2OH incorporated in the photo-chemically synthesized polymers in
various I:TM ratios. Measured by 1H NMR using 1,4-dioxane in DMSO-d6 as the reference
standard. ........................................................................................................................................ 136
Figure 4. 30. SEM images of photochemically synthesized polymers. P-2OH-1:100 MIP (A) and NIP
(B) and P-2OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres
with the corresponding polydispersity indexes (PDI) measured by DLS and SEM............ 137
Figure 4. 31. Comparison of the template incorporation and the binding performance of the MIP and NIP of P-2OH polymers synthesized in various I:TM experiments. Amount of 2OH were quantified by $^1$H NMR using 1,4 dioxane in DMSO-$d_6$ as reference standard and monitoring the $\text{-CH=CH-}$ peak at 6.47 ppm of 2OH. 139

Figure 4. 32. SEM images of the photochemically synthesized P-3OH polymers. P-3OH-1:100 MIP (A) and NIP (B) and P-3OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM. 143

Figure 4. 33. Comparison of the Template incorporation and binding performances of P-3OH microspheres produced in different I:TM ratios with respect to the initial amount(4.20 μM). Quantitative analyses were performed by $^1$H NMR using 1,4 dioxane in DMSO-$d_6$ as the reference standard. 145

Figure 4. 34. Comparison of the template incorporation (A), MIP (B) and NIP (C) binding performance of the MIP and NIP of the photochemically synthesized polymers. Amount of each templates were quantified by $^1$H NMR using 1,4 dioxane in DMSO-$d_6$ as reference standard. 146

Figure 4. 35. Proposed dimerisation reaction of 1OH (A) and 3OH (C) under UV irradiation with the partial NMR spectra of initial (bottom spectra) and post polymerisation (top spectra) solutions of P-1OH (B) and P-3OH (D). 150

Chapter 5

Figure 5. 1. Illustration of the DAD/ADA hydrogen bonding array of the trans-amide group of a bis-acylamidopyridine-based compound and an imide functionality. 155

Figure 5. 2. SEM images of ground monolithic MIP (A) and NIP (B) in acetonitrile porogen (BP-1:1- A); and MIP (C) and NIP (D) in chloroform porogen (BP-1:1-C) in 30k magnification. 158

Figure 5. 3. SEM images of precipitation polymers PP-1:1-A MIP (A) and NIP (B). 162

Figure 5. 4. The predominant hydrogen bonding interaction points (distances 2.2 ≥ 2.5 Å) between BAAPy and TAU measured by Spartan ‘14 v1.1.8 in a 1:1 (A) and 1:3 (B) TAU:BAAPy clusters. 164

Figure 5. 5. Partial $^1$H NMR spectra of a pure BAAPy (A), pure TAU (B) 1:1 mole ratio of TAU:BAAPy (C) and 1:3 mole ratio of TAU:BAAPy (D), measured at 60°C in d-DMSO, showing marked chemical shift movements of protons involved in TAU-BAAPy interactions. 167

Figure 5. 6. Partial $^1$C NMR spectra of a pure BAAPy (A), pure TAU (B) 1:1 mole ratio of TAU:BAAPy (C) and 1:3 mole ratio of TAU:BAAPy (D), measured at 60°C in d-DMSO, showing marked chemical shift movements of protons involved in TAU-BAAPy interactions. 168

Figure 5. 7. Chemical shifts of selected protons (A) carbons (B) of TAU and BAAPy measured by $^1$H and $^{13}$C NMR respectively. Note that $\Delta$ chemical shift = chemical shift of the mixture - chemical shift of the pure solution of TAU or BAAPy. 169

Figure 5. 8. In-situ time-binding tests using 10.0 mg of PP-1:1-A microspheres incubated in 0.5 mL of 100 µM TAU solution. 170

Figure 5. 9. TAU incorporation and binding efficiency of PP-1:1-A microspheres and bulk polymers BP-1:1-A and BP-1:1-C. 10.0 mg of polymers were incubated in 0.500 mL of 100 µM TAU solution for 1 hour prior to quantitative $^1$H NMR analysis. 171

Figure 5. 10. Binding isotherms of BP-1:1-A and PP-1:1-A polymers fitted to non-linear (A) and linearized (B) Langmuir models. Isotherms obtained using 10 mg polymer incubated for 1 hr in 0.500 mL of TAU solution. Free TAU was measured by in-situ quantitative $^1$H NMR spectroscopy. 173
Figure 5. 11. Binding capacities of PP-1:1-A MIP in non-competitive cross-binding and competitive assays against Urd (Ur) and 2',3',5'-tri-O-acetyl-cytidine (TAC). 10.0 mg of polymers were incubated for 1 hour prior to $^1$H NMR analysis using 0.500 mL of 100 μM of analyte for non-competitive rebinding and equimolar concentration (50 μM) of TAU and analogue for competitive rebinding. ................................................................. 176

Figure 5. 12. Urd:BAAPy (A) and TAC:BAAPy (B) 1:3 clusters showing interaction points and their corresponding distances measured by Spartan '14 v1.1.8. The BAAPy cluster was generated using TAU as template (see Figure 5.4. B) and frozen in place. ...................................................... 177

Figure 5. 13. SEM images of PP-1:1 microspheres produced by varying the initiator:total monomer (I:TM) ratios in acetonitrile at 60°C: 1:50 (PP-I-1:50) MIP (A) and NIP (B); 1:131 (PP-1:1-A) MIP (C) and NIP (D); 1:200 (PP-I-1:200) MIP (E) and NIP (F). Refer to Table 5.1 and 5.2 for additional details of their synthesis. .................................................................................... 179

Figure 5. 14. Comparison of the template (TAU) incorporation (i.e. imprinted) and binding capacities of PP-1:1 microspheres produced by varying initiator(AIBN):total monomer (I:TM) ratios in acetonitrile at 60°C: 1:50 (PP-I-1:50), 1:131 (PP-1:1-A) and 1:200 (PP-I-1:200). Refer to Table 5.1 and 5.2 for additional details of their synthesis................................................................. 180

Figure 5. 15. SEM images of microspheres of PP-1:1-A-Rd MIP (A) and NIP (B), PP-1:1-A-St MIP (C) and NIP (D). See Figure 5.3 for SEM images of PP-1:1-A. ................................................................. 182

Figure 5. 16. Comparison of the template uptake, template bound by the MIP and the NIP of the polymers obtained from PP-1:1-A (no agitation), PP-1:1-A-Rd (rolled at ~9.5 rpm) and PP-1:1-A-St (stirred at ~130 rpm)................................................................. 183

Chapter 6

Figure 6. 1. Structure of an imidazolium ion ...................................................................................... 188

Figure 6. 2. SEM micrographs of ACN- PP MIP (A) and NIP (B), and IL-PP MIP (C) and NIP (D). Insets are the particle sizes ($d_H$) measured by dynamic light scattering in nm. ......................... 195

Figure 6. 3. Particle size distribution of IL-PP MIP (A) and NIP (B). ............................................. 196

Figure 6. 4. Comparison of the amount of templates incorporated and bound by the ACN-PP and IL-PP normalized with respect to mass, μmol/g (A) and surface area, μmol/m$^2$ (B)........... 200

Figure 6. 5. X-ray diffraction patterns of ACN-PP, IL-PP polymers and IL-PP polymers after further dialysis. ................................................................................................................. 202

Figure 6. 6. DSC curves of ACN-PP MIP and NIP, IL-PP MIP and NIP and the second fraction of IL-PP (IL-PP-2) NIP using DSC with a maximum temperature of 250 °C and a heating rate of 30°C/ min................................................................. 203

Figure 6. 7. $^1$H NMR spectrum of the second fraction (IL-PP-2) in CDCl$_3$ after second dialysis against water and washed with methanol .............................................................................. 205

Figure 6. 8. Overlaid IR (UATR) spectra of IL-PP- NIP and IL-PP-2 NIP ................................. 205
List of Tables

Table 1. 1. A summary of the Molecular Imprinting process.9 .......................................................... 5
Table 2. 1. Polymer feed composition of all systems investigated. .................................................. 19
Table 3. 1. Imprinting results of polymers synthesized in various template: functional monomer (TM),
functional monomer: cross-linker (MX) and initiator : total monomer (IM) ratios. .................. 37
Table 3. 2. BET surface area (m²/g) data for selected CAF and THP MIPs and NIPs. ..................... 68
Table 4. 1. Composition and degree of cross-linking of thermally-synthesized 3,5-dimethylphenol (T-1OH)
imprinted and non-imprinted polymers. .................................................................................. 75
Table 4. 2. Affinity constants ($K_a$) and number of binding sites ($N$) of T-1OH polymers determined by the non-
linear Langmuir (NLL) and linearized Langmuir (LL) models. ........................................... 82
Table 4. 3. Composition and degree of cross-linking of thermally-synthesized 5-methylbenzene-1,3-diol (2OH)
imprinted and non-imprinted polymers. .................................................................................. 85
Table 4. 4. Affinity constants ($K_a$) and number of binding sites ($N$) of T-2OH polymers determined by the non-
linear Langmuir (NLL) and linearized Langmuir (LL) models. ........................................... 91
Table 4. 5. Composition and degree of cross-linking of thermally-synthesized Benzene-1,3,5-triol (3OH)
imprinted and non-imprinted polymers. .................................................................................. 94
Table 4. 6. Composition and degree of cross-linking of thermally-synthesized 3,5-dimethylphenol (T-1OH)
imprinted and non-imprinted polymers. .................................................................................. 102
Table 4. 7. Composition and degree of cross-linking of thermally-synthesized 5-methylbenzene-1,3-diol (2OH)
imprinted and non-imprinted polymers. .................................................................................. 106
Table 4. 8. Composition and degree of cross-linking of thermally-synthesized Benzene-1,3,5-triol (3OH)
imprinted and non-imprinted polymers. .................................................................................. 110
Table 4. 9. BET surface areas (m²/g), average pore volumes (cm³/g) and average pore sizes (Å) of selected
microspheres. .......................................................................................................................... 112
Table 4. 10. Composition and degree of cross-linking for photo-chemically-synthesized 3,5-dimethylphenol (P-
1OH) polymers. ...................................................................................................................... 128
Table 4. 11. Imprinting results of photochemically synthesized 5-Methylbenzene-1,3-diol polymers (P-2OH)
polymers in various I:TM ratios. ............................................................................................. 135
Table 4. 12. Polymer composition and cross-linking density in the photochemically synthesized Benzene-1,3,5-
triol (P-3OH) polymers. .......................................................................................................... 141
Table 5. 1. TAU imprinting results for bulk (BP) and precipitation (PP) polymers. ......................... 160
Table 5. 2. Feed and polymer composition for bulk (BP) and precipitation (PP) MIPs and NIPs. .......... 161
Table 5. 3. Binding affinity constants ($K_a$) and number of binding sites ($N$) for BP-1:1-A and PP-1:1-A estimated
from non-linear (NL) and linearised (LL) Langmuir curves given in Figure 5.10. ................. 174
Table 6. 1. Polymer composition of An-PP and IL-PP MIP and NIP. ............................................... 192
Table 6. 2. PET surface area data, pore volume and sizes of ACN-PP polymers and IL-PP polymers. ... 198
Table 6. 3. Glass transition temperatures $T_g$ of the ACN-PP and IL-PP polymers observed using DSC. ... 203
Table 7. 1. Comparison of imprinting efficiencies and binding parameters of the MIP systems included in this
study. ........................................................................................................................................... 217
Abstract

This study aims to further understand the mechanistic aspects of the non-covalent approach of molecular imprinting produced by precipitation polymerisation, with emphasis on the effect of the nature of template, temperature, the formulation and nature of porogen on the properties and binding performance of the microspheres as literature has demonstrated that precipitation imprinting is susceptible to changes in polymerisation conditions. Additionally, we have successfully utilized NMR as the main tool for quantitative measurements, which, to the best of our knowledge the first time that NMR has been applied to the full extent for MIP characterisation. Throughout the course of this study, azobisisobutyronitrile (AIBN) and acetonitrile (ACN) were employed as initiator and porogen, respectively, with the porogen:monomer ratio for all precipitation imprinting experiments maintained at 10-12 mL per mmol of total monomer. Reaction temperature was set at 60°C except during photochemical initiation when it was recorded to be 27-35°C.

The effects of the feed formulation, i.e. template:functional monomer (T:FM) ratio, functional monomer: cross-linker (FM:XL) ratio and initiator:total monomer (I:TM) ratio, as well as temperature (60°C vs 27-35°C) were exemplified by investigating microspheres imprinted with xanthine derivatives caffeine (CAF) and theophylline (THP) and phenolic targets 3,5-dimethylphenol (1OH), 5-methylbenzene-1,3-diol (2OH) and 1,3,5-benzenetriol (3OH). Our results suggest that FM:XL ratios should be maintained between 1:5-1:10 in order to get rigid MIPs that can favourably form higher number of template-functional monomer (T-FM) complexes during the imprinting process but lower NIP binding. Interestingly, regardless of the T:FM ratio and level of template incorporation, comparable template rebinding was obtained for MIPs as well as for NIPs resulting in comparable imprinting factors. Our results yielded no preference to T:FM ratios between 1:2 to 1:8 for MIP synthesis by precipitation polymerisation.

Higher concentration of initiator (I:TM = 1:5), and hence, faster rate of polymerisation, was found to favour template incorporation and rebinding of both CAF and THP when the FM:XL ratio was kept at 1:5 while moderate level of initiator (I:TM = 1:100) was sufficient at higher concentration of crosslinker (FM:XL = 1:10). In contrast with the xanthine derivatives, higher template incorporation, rebinding and IF (due to comparable NIP binding across all tested I:TM ratios) were observed with MIPs for phenolic targets 3,5-dimethylphenol (1OH) and 1,3,5-benzenetriol (3OH) synthesized at lower concentration of initiator (I:TM = 1:100) and equivalent FM:XL ratio of 1:5. These contrasting results indicate that various templates require different times to reach optimum equilibrium concentration of T-FM complex and should be a primary consideration for precipitation imprinting. It would seem that, overall, MIP performance can improved by keeping the following feed formulation: T:FM ratios of 1:2 to 1:8, FM:XL ratios of 1:5 to 1:10 and I:TM ratios of 1:5 to 1:100.

In all cases, the incorporation and binding efficiency of THP are higher than CAF signifying a stronger interaction with the functional monomer. Template incorporation for
these systems were 20-75% but, interestingly, this has not been efficiently translated to high fidelity binding sites showing only 5-10% binding conversions. Both 1OH and 3OH MIPs gave high IF’s (1.8) and N (2.3 µmol/g). Cross-reactivity studies, however, demonstrated that 3OH MIP is the most selective towards its template giving selectivity indices of 0.58 and 0.67 for 1OH and 2OH, respectively, indicating the formation of higher fidelity binding sites due to stronger 3OH-MAA interaction. While thermal initiation at 60°C only showed evidence of dimerisation for 2OH, in photochemical initiation (27-35°C), all three phenolic templates demonstrated evidence of dimerisation. Thus, while photochemical initiation at lower temperature has the potential to yield better performing MIPs, potential side reactions limit its applicability.

Particle sizes, surface areas and porosities of the microspheres imprinted with the xanthine derivatives (CAF and THP) and phenolic templates (1OH, 2OH and 3OH) was demonstrated to be affected by the nature of the template. Results obtained for selected phenolic templated MIPs also provided evidence of the effect of the concentration of initiator on particle size, surface area and porosity of the microspheres. Further investigation is warranted in order to deduce a more evident correlation.

With the use of a stoichiometric functional monomer, 2,6-bis(acryl)amido pyridine (BAAPy), capable of forming an array of H-bond interactions (donor-acceptor-donor/acceptor-donor-acceptor, DAD/ADA) with an imide-containing template, 2’,3’,5’-tri-O-acetyl uridine (TAU) in this study, more binding efficient polymers were produced, exhibiting IFs (3.0) and N higher than those generated using the non-stoichiometric functional monomer MAA. Interestingly, the stoichiometric 1:1 T:FM ratio, observed in bulk imprinting, has not been maintained in precipitation polymerisation and an optimal TAU:BAAPy ratio of 1:2.5 was obtained due to the additional hydrogen bond interaction with the acetyl groups of TAU. While a moderate initiator concentration, (I:TM ratio of 1:131), resulted in high template incorporation (60%), template rebinding was only 3-4% of the incorporated template.

The application of a room temperature ionic liquid (IL) 1-butyl-3-imidazolium hexafluorophosphate (bmimPF6) as porogen in precipitation imprinting of propranolol (PNL) was further explored using TRIM as cross-linker, MAA as functional monomer, and a T:FM:XL ratio of 1:1.5:4. Both ACN and IL gave comparable template incorporation (64%) while IF for IL, with respect to mass, was lower due to high IL NIP binding which could be attributed to its surface area, average pore volume and size being significantly higher that the IL MIP. Binding capacities normalised with respect to surface area, however, were comparable for both ACN and IL microspheres resulting in equivalent IFs (~2.5). Both ACN and IL systems exhibited similar microstructure while the glass transition temperatures ($T_g$’s) of their MIPs were observed to be lower than those of their NIPs as an expected consequence of an efficient imprinting process. Molecular imprinting technology is becoming a widespread technology
Chapter 1
Introduction

Molecularly imprinted polymers or MIPs are synthetic receptors with predetermined molecular recognition capabilities specific for its target molecule.\textsuperscript{1-4} Widespread applications of these materials are due to their selectivity and specificity which are comparable with biomolecules (e.g. enzymes and antibodies) but without the associated with issues.\textsuperscript{5-8} MIPs are stable in high or low pH, temperature and pressure. These materials are more economical to use than biomolecules since these materials can be reused and have longer degradation times. Due to this advantage over biomolecules, MIPs have been widely used in more applications and continuously being studied for more applications, such as sensors, separation chemistry, and chromatography.\textsuperscript{6,9-13}

The process of molecular imprinting requires a template, usually the target molecule or an analogue, a porogenic solvent, and a functional monomer. The choices of the appropriate formulation (identity and the amount of the components) are traditionally determined by trial and error, however, several approaches (NMR studies, Section 1.2.5.2. and Molecular modelling, Section 1.2.5.1), have been developed to assist in assessing the most efficient formulation. The template molecule, by virtue of its functional groups or association sites, forms a loosely associated pre-polymerisation cluster with the functional monomer. Subsequently, this cluster will be joined together in a three dimensional framework by the reaction of the cross-linker and functional monomer, which is usually a free radical polymerisation process. The template is then removed from the imprinted polymers, leaving a cavity that is complimentary in shape and functionality with the template. A schematic representation of the molecular imprinting process is shown in Figure 1.1.
Molecular imprinting was first introduced by Wullf et al. through covalent (Figure 1, C), or often referred to as pre-organized approach. It involves formation of a reversible covalent bond between the template and the functional monomer. This bond is cleaved during the template extraction and reformed during the rebinding process. Due to this distinct interaction between the target template and that of the functional monomer, an exact stoichiometric ratio between the template and the functional monomer is ensured, producing a more selective material. This bond also reduces the number of non-selective binding sites or sites formed that are not due to the interactions of the template and functional monomer. The major drawback, however, lies on the limited number of materials that can be used. The suitability of the binding site monomers is constrained in few numbers of templates, and in most cases the template-monomer complexes are too stable to be cleaved, thus the reaction consumes time for the template removal.

The second approach of molecular imprinting and the most widely used is the non-covalent or self-assembly approach. Unlike with the covalent imprinting, non-covalent
approach does not rely on bond formation but with non-covalent interactions such as hydrophobic, ionic and hydrogen-bonding interaction.\textsuperscript{6, 15, 19-20} These type of bonding interactions are typically weaker than those used in the covalent approach, thus simplify the process of producing MIPs. This is commonly a straight-forward, one-pot synthesis method; all of the components (the functional monomer, template, cross-linker, initiator and porogen) are usually made to react in one container with the pre-polymerisation cluster allowed to form prior to polymerisation.\textsuperscript{6, 20} The major drawback with this approach is the production of heterogeneous binding sites, which leads to non-specific binding.\textsuperscript{12, 17} Despite of the drawback, this is still the most commonly used approach in molecular imprinting.

The third method of molecular imprinting is called semi-covalent, and it is the hybrid of the two previously mentioned approaches. This combines the advantages of both the covalent and non-covalent methods but suffers the disadvantages of the two methods. This imprinting process is similar to the covalent approach where the functional monomer and the template forms covalent bond during the polymerisation process.\textsuperscript{12, 17} Similarly, with the problems related to covalent imprinting, there is only limited number of templates that can form reversible covalent bond with commercially available binding site monomers (e.g. Schiff’s bases).

1.1. Synthesis and Formats of Molecularly Imprinted Polymers

There are few polymerisation methods that can be applied, but the most conventional is through free radical polymerisation (FRP). Most of the high fidelity MIPs studied were produced through FRP. Imprinted polymers prepared via free radical polymerisation are found to be more stable even in harsh conditions and its preparation is less time consuming compared to the other processes.\textsuperscript{17, 21} Thus, more research aimed in MIPs synthesized by free
radical polymerisation is required, which is the polymerisation method employed in this research.

The applications of MIPs are shown to be dependent on the polymerization method used (Table 1.1). Different methods can produce materials with different morphologies, binding characteristics and particle sizes. MIPs were first prepared via bulk polymerisation, wherein the initiator is added to the monomer solution (cross-linker and functional monomer) forming monoliths in the presence of a low amount of solvent/porogen. The resulting monoliths were then ground and sieved, making it a laborious and time consuming. The most important disadvantage of bulk polymerisation method is the reduction in the binding capacity because of the damaged binding cavity due to grinding and sieving of the polymers. Additionally, incomplete removal of template from the monoliths due to the trapped templates located in the bulk polymeric structure, may cause bleeding during the rebinding analyses and ultimately lead to inaccuracy in the calculation of the imprinting efficiency. The grinding process also produces irregularly shaped particles, making monolithic MIPs less suitable as a packing material for chromatographic applications.
Table 1. A summary of the Molecular Imprinting process.

<table>
<thead>
<tr>
<th>Process</th>
<th>Format</th>
<th>Advantages</th>
<th>Limitations</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk polymerization</td>
<td>Monoliths</td>
<td>- Uses less or absence of solvent in the synthesis</td>
<td>- Produce irregularly-shaped particles with poor binding performance microspheres</td>
<td>-For chromatographic and biological assay applications³¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Ease in preparation</td>
<td>- Tedious procedure</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Different types of functional monomers can be used</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>- No sophisticated instrumentations are required</td>
<td></td>
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</tr>
<tr>
<td>Suspension polymerization</td>
<td>Microspheres</td>
<td>- Produce uniform spherical particles</td>
<td>- Surfactants used also acts as an impurity</td>
<td>-For chromatographic and biological assay applications³²-³³</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Reproducible results</td>
<td>- Complicated procedure</td>
<td>-Drug delivery³⁴</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- High yield</td>
<td>- Solvent is incompatible with other components in the mixture</td>
<td></td>
</tr>
<tr>
<td>Emulsion polymerization</td>
<td>Microspheres</td>
<td>- High yield</td>
<td>- Usage of water lower the binding efficiency of the MIPs</td>
<td>-Drug delivery</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Produced mono-dispersed microspheres</td>
<td>- Emulsifying agents act as an impurity¹²</td>
<td>-For chromatography and assays.¹³</td>
</tr>
<tr>
<td>Multi-step swelling</td>
<td>Microspheres</td>
<td>Yields uniformly-sized particles</td>
<td>Complicated procedure and conditions</td>
<td>-For chromatographic applications ³⁵-³⁸</td>
</tr>
<tr>
<td>Precipitation polymerization</td>
<td>Microspheres</td>
<td>Produces uniformly-sized microspheres with high yield</td>
<td>Requires large amount of organic solvents and template</td>
<td>-For chromatography applications³⁹-⁴⁰</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Sample preparation³¹</td>
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<td></td>
<td></td>
<td>- Enantioselective separation²²-²³</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Drug delivery⁴⁴</td>
</tr>
</tbody>
</table>
Due to several drawbacks of bulk polymerisation, new and improved methods have been employed. Such methods include: emulsion polymerisation, suspension polymerisation, multi-step swelling and precipitation polymerisation. These processes involve soluble monomers (cross-linker and functional monomer) and initiator, but produced insoluble polymeric chains in the porogen used. These processes differ from bulk polymerisation due to usage of high amount of non-solvent (>95%). In these processes, polymer formation is due to the continuous addition of monomer to the growing polymeric chain forming short chain of oligomers or nucleation. These chains will continue to add monomer units (polymerisation) until they become insoluble in the porogen. The particles produced ranges in size from nanometer to micrometer in sizes with spherical shapes, hence the term microspheres.

One of the first alternative methods tested is with the use of suspension polymerization. All of the components (initiator, cross-linker and functional monomer) are suspended in the aqueous dispersing phase in the presence of an organic surfactant. Insoluble particles are produced and the sizes of the particles are dependent on the agitation during this process. Subsequently, polymerisation will follow by heating the solution in the desired temperature. Homogenously-sized particles, in the range of 5-100 μm in diameter, are produced from the aqueous solution of the monomers. The major disadvantage of this method lies on its usage of aqueous solution. Polar compounds were found to disrupt the molecular interactions of the template and the functional monomer, thereby lowering the binding efficiency and selectivity of the MIPs produced. Aqueous solvents also prohibits of using other possible compounds for the synthesis of MIPs, since most of the monomers available are insoluble in aqueous solvent. Another limitation of this method is that this can only produce large beads which could not be made smaller than a micrometer.
Emulsion polymerisation is another method that can be used for the synthesis of MIPs. Unlike suspension polymerisation, the monomers (functional monomers and cross-linker) are insoluble with the medium used in the process, but is emulsified by a surfactant.\textsuperscript{48} The most common emulsion is an oil/water emulsion solution. This method also suffers the same problems encountered by suspension polymerisation, since this method also uses water as a component of the synthesis. \textsuperscript{13, 48}

Among the methods used to prepare MIP microspheres, precipitation polymerisation is the most widely used because of its convenient one-pot preparation of polymers.\textsuperscript{6, 9, 12, 28, 49} The microspheres produced by precipitation polymerisation are more uniform in size and shape than microspheres produced from other solution polymerisation methods (suspension, emulsion and multi-step swelling). Most of the MIPs produced by precipitation polymerisation use organic compounds as the solvent or porogen in the polymerisation process. Despite its potential applications, previous studies have demonstrated that this method is sensitive to changes in the polymerization conditions: identity and volume of solvent used the formulation (i.e. the composition of the polymerisation feed), the template and temperature, which affect the properties of the imprinted polymers including the binding efficiency.\textsuperscript{5, 11, 41, 50-53}

1.2. Factors affecting the efficiency of MIPs.

Molecular imprinting has been developed in order to mimic the molecular recognition capabilities of biological molecules \textsuperscript{3-4, 14, 16, 27, 46, 54-55} and there have been continuous developments ever since its introduction. The application of the molecularly imprinted polymers (MIP) is what dictates the format of the material, film or particles, to be used.\textsuperscript{9} Regardless of the format of the MIPs, it is an accepted dogma in molecular imprinting technology that the strength of interaction between the template and functional monomer defines the efficiency of the imprinted polymers.\textsuperscript{56} Previous research focused on the
determination of factors that can increase the concentration and enhance the stability of the template-functional monomer (T-FM) complex with the aim of enhancing the binding performance of the resulting MIPs. The reaction of the template and the functional monomer is an equilibrium governed reaction, therefore, increasing either the amount of template or the functional monomer, or changing the system temperature would affect the formation of more T-FM complex that can affect the number of binding sites and ultimately leading to more binding efficient MIPs. 18-19, 21, 46, 57-58

MIPs, especially in the microsphere format, produced via the non-covalent approach increased exponentially from the time it was introduced, because of its simplicity and versatility. Despite the simplicity that non-covalent approach offers, the synthesis is still a complex mixture of compounds, each of which has its own role in the final property of the imprinted polymers.

The following sections focus on several factors that can affect the physical properties and binding performances of MIPs with non-covalent approach of molecular imprinting.

1.2.1. Solvents/Diluents/Porogens

Several terms are used for the compounds that are used to dissolve and bring all of the other components in the MIPs synthesis to a homogeneous phase: solvents, diluents and porogens as they also influence the porosity and surface area of the imprinted polymers. Surface area and porosity of the material are related to the solubility of the growing polymeric chain with the porogen/solvent. Low solubility porogens have been linked to MIPs with larger pores (higher surface area) because of early polymer formation during the synthesis. Conversely, high solubility porogens produces MIPs and NIPs with smaller pores or materials with a higher surface area. Moreover, some studies have reported that
the solvents can also affect the template-functional monomer complex formation, \cite{12, 60-62} and that the nature of the solvents can either enhance or disrupts the complex formation. \cite{61, 12, 21}

Most conventional porogens used in molecular imprinting are volatile organic solvents since it fulfils the solvency requirements of most of the components in the process. \cite{12, 18, 60} Organic solvents such as methanol, tetrahydrofuran (THF), dichloromethane, chloroform and acetonitrile (ACN) were assessed to enhance binding efficiencies of the resulting polymers. \cite{18, 62-65}.

More recently, the usage Room Temperature Ionic Liquids (RTILs) as a class of a novel and interesting porogen in molecular imprinting has been published. RTILs are organic salts that are in liquid form at room temperature with low melting point, thermally stable, high viscosity and negligible vapour pressure. They are typically composed of a poorly coordinated large organic cation and a small organic or inorganic anion so they are highly polar but considered as a non-coordinating solvent. Due to all of these properties, ILs garnered attention as a “green” alternative solvent for organic reactions. The effects of using RTILs in polymerisation reactions were initially investigated in polymerisation reactions and demonstrated its ability to accelerate the rate of polymerisation\cite{66-68} and improve the reaction conversion\cite{69}. Due to several advantages of using RTILs as a polymerisation solvent, its application as a porogen in molecular imprinting was later explored by Booker et al.\cite{70-73} Imidazolium based RTILs were reported to facilitate the polymerisation reaction even at low temperatures which was not observed in volatile organic compounds (VOCs). In addition, the selectivity of the RTILs-synthesized polymers is at par with VOC-synthesized polymers. \cite{72}
1.2.2. Method of initiation and concentration of initiator

The synthesis of MIPs by free radical polymerisation has conventionally been conducted via thermal initiation due to method practicality. Aside from thermal initiation, there are other initiation methods that were recently investigated and explored: photo-chemical \(^74-78\), microwave \(^79-83\) and redox \(^84\).

It has been demonstrated that the heat in the thermal initiation disrupts the formation of template-monomer complex, thus thermally-synthesized MIPs performed less efficiently than MIPs produced in other methods of initiation.\(^74, 76, 78, 85-89\) This is due to the formation of the template-functional monomer association complex (T-FM) being an exothermic reaction. Thus, additional heat to the system will favour the reverse reaction, breaking the association of the template and the functional monomer resulting to less binding efficient MIPs. \(^78\)

Azo initiators can be used for both thermal and photo-chemical initiation with azo-bis-isobutyronitrile (AIBN) being the standard. AIBN can undergo photolytic homolysis when irradiated with a UV source at 366 nm \(^90-91\) and has a life of 8 hours at 0 °C when irradiated at 366 nm \(^76\). It was initially assumed that the concentration and identity of initiator does not have an effect in the physical properties and rebinding performance of polymers. However, a recent work has illustrated that the concentration of initiator also plays a crucial role in the imprinting process. Mijangos et al. demonstrated that poor binding efficient monoliths were produced in higher concentration of initiators due to the formation of additional heat from the exothermic decomposition of the initiator. \(^77\)

1.2.3. Cross-linker

The major functions of a cross-linker are (a) morphological control of the polymeric network during the imprinting process \(^92-93\) (b) to provide structural rigidity to the cavity
formed from template association, and lastly is to (c) secure the three dimensional arrangement of the functional monomer to preserve the template specific cavity of the network. Therefore, it can be concluded that cross-linkers mainly affects the physical attributes of the polymers. However, Muhammad et al. suggested that the proper cross-linker should display a lower interaction strength with the template thereby strengthening the template-monomer interaction.

1.2.4. Functional monomer

Previous studies deduced that the stability of the template-functional monomer cluster and the correct three dimensional positioning of the functional groups in the cavity formed affect the molecular recognition of the MIPs. The most appropriate functional monomer and its optimum concentration for a given template can be chosen by NMR titration and molecular modelling, which are discussed in the succeeding section of this chapter.

For non-covalent approach, the convention is with the use of a single functional monomer, and is usually added in excess with respect to the concentration of the template. It is however suggested that the appropriate template: functional monomer (T:FM) should be within the range of 1:2-1:4, based from the results of several published documents.

The ratio of functional monomer: cross-linker was also correlated to the rebinding performance of the microspheres. Yoshimatsu suggested that excessively high and low concentration of functional monomer (equal changes in the concentration of cross-linker) in the feed both results with a decrease in binding efficiency of the microspheres: low concentration of functional monomer results in an insufficient T-FM complexes, while high concentration of functional monomer (equal decrease in the concentration of cross-linker)
results in lower mechanical stability of the microspheres providing an inability to preserve the imprinted memory of the cavities.\textsuperscript{99-100} Thus, a correct balance in the concentration of FM and XL in the feed should be observed and currently, the widely accepted FM:XL used is within the range of 1:3-1:6\textsuperscript{21}.

In order to enhance the selectivity and the binding affinity of the polymers to the template, custom-designed functional monomers were synthesized, which were capable of forming multiple hydrogen bonding with the templates.\textsuperscript{101-104} Moreover, using a custom designed functional monomer reduces the usage of excess functional monomer since it is a stoichiometric reaction (1:1 mole), lowering the formation of non-specific binding.\textsuperscript{105-107} An example of a custom designed functional monomer is 2, 6-bis-(acrylamido) pyridine (BAAPy), and is capable of forming an array of hydrogen bond (donor-acceptor-donor, DAD) interactions with an imide-containing templates. Polymers synthesized using BAAPy as a functional monomer displayed a high affinity constant that resulted in improvement in the selectivity and efficiency of the polymers.\textsuperscript{102-103, 108-113}

The information regarding nature and strength of the interaction between the template and the functional monomer can be determined by spectroscopic methods.\textsuperscript{114-115} UV-vis, FTIR, NMR studies and molecular modelling. The last two techniques are the most commonly applied as they provide an insight to the possible T-FM complex formation.

\textbf{1.2.5.1. Template-Monomer Interaction studies.}

The introduction of molecular modelling software made MIP synthesis easier and labour-friendly. It has been found that successful imprinting of molecules is governed by the energy and thermodynamic properties of the system, particularly the energy of the interactions associated with the template-functional monomer complex and this can be determined through molecular modelling.\textsuperscript{96-97} Results from molecular modelling experiments
showed that in preparing successful MIP design with efficient binding capacities, there should be an adequate interaction and energy with each component, most especially between the functional monomer and the template. The rest of the components are of secondary priority for the consideration, since the template-functional monomer interaction creates the cavity, in which the efficiency of the MIPs is mostly dependent on. 50, 92, 116-119

NMR spectroscopy is one of the most preferred methods in determining the interactions of the functional monomer and the template, because of its simplicity and sensitivity. 120 In addition to this, a plot of the concentration of the template against the changes in chemical shift of the functional monomer could be obtained. From this plot, the optimum ratio of the functional monomer and the template to be used in the MIP synthesis could also be obtained from the titration data.120 This method has been used prior to the actual synthesis of the MIPs to save time and avoid laborious experiments in determining the optimum parameters and conditions of the synthesis. NMR titration has been used in MIP synthesis of the following templates: phenylalanine anilide 120-121, atrazine 122 and 4-(4-vinylphenyl)pyridine 118

1.3. Objectives

The increasing number of possible applications of MIPs prepared through precipitation polymerisation is gearing MIP researchers towards producing more efficient MIPs, in terms of selectivity and binding capacity. The ease in the preparation of microspheres by precipitation polymerisation makes it the preferred choice than the traditional bulk polymerisation. In addition, some studies demonstrated that microspheres are more binding efficient than their bulk counterparts. 12, 22-24, 26, 123-125 Literature indicates that the quality and properties of the MIP microspheres produced from precipitation polymerisation is dependent on the association of the functional monomer and the template and the polymerisation
process (particularly the nucleation stage). The template-monomer association and particle nucleation processes are critical to the formation of imprints and its efficiency, respectively. These processes have been observed to be susceptible to template effects, which are noticeable in differences in sizes between different MIPs and NIPs. In line with this, the main objective of this research is to further understand further the mechanistic aspects of molecular imprinting prepared by precipitation polymerisation, specifically the formation of the association complex between the template and the functional monomer and how this is influenced by the formulation. Polymers synthesized in every system were assessed based on their physical properties and those were correlated to their corresponding binding performance.

Chapter 3 compares the binding efficiencies of caffeine and theophylline MIPs prepared by thermal precipitation polymerisation using acetonitrile as porogen and how these are affected by varying the formulation. The aim is to obtain optimal feed formulations by investigating the correlation between binding performance with varying template: functional monomer (T:FM), functional monomer: cross-linker (FM:XL) and initiator: total monomer (I:TM) ratios. This study also investigates the correlation between template incorporation and rebinding employing two templates: caffeine (CAF) and theophylline (THP), with varying capability for interaction with the functional monomer, MAA in this case.

Chapter 4 seeks to further understand template effects by focusing on the effect of initiation: concentration (using an azo initiator) and method and temperature (thermal vs photochemical) on the physical properties and binding performance of precipitation MIPs by employing three phenolic templates with varying number of interaction points: 3,5-dimethylphenol (1OH), 5-methylbenzene-1,3-diol (2OH) and benzene-1,3,5-triol (3OH).
Chapter 5 assesses the mechanistic aspects of stoichiometric precipitation imprinting using a pyridine-based functional monomer, 2,6-bis-(acrylamido) pyridine, and the binding performance of the resulting MIPs in comparison to MIPs synthesized by bulk polymerisation. As with Chapter 4, this study also delved into the effect of the concentration of the initiator on the properties and binding performance of resulting MIPs. Additionally, the effect of agitation on T:FM interaction and binding and physical properties of the imprinted microspheres have been investigated.

Chapter 6 is an extension of the previous work of our group and provides additional information on the imprinting efficiency and further understanding of the application of room temperature ionic liquids (RTILs) as porogens in precipitation molecular imprinting compared to VOC-synthesized MIPs.

This study also includes the development and application of the quantitative NMR method for MIP characterisation and allows for accurate measurement of the concentrations of the components in the polymerisation feed, particularly template incorporation. This method has also been successfully employed to measure unbound analyte in solution without the need to separate the solid MIP microspheres.

While recognising that all MIP systems suffer from drawbacks, the candidate’s research has only focused on factors affecting, and challenges associated with, the synthesis and performance of MIP microspheres from precipitation polymerisation by the non-covalent approach.
Chapter 2
Experimental

2.1. Materials and Reagents

Methacrylic acid (MAA, Sigma), ethylene glycol dimethacrylate (EGDMA, Sigma) and trimethylolpropane (TRM) were freed from polymerization inhibitor by passing through a basic aluminum oxide column. 2,6-Bis(acryl)amino pyridine (BAAPy) was initially provided by Dr. Andrew Hall and later synthesised according to standard procedure briefly described below. 2,6-Diamino pyridine, acryloyl chloride and triethylamine were purchased from Sigma and was used as received. Acryloyl chloride (96%) was purchased from Aldrich and was used as received. Caffeine (CAF) and theophylline (THP) were purchased from Sigma Aldrich and used as received. 3,5-dimethylphenol (1OH) was purchased from Sigma Aldrich and recrystallized in methanol prior to use. 5-methylbenzene-1,3-diol (2OH) and benzene-1,3,5-triol (3OH) were purchased from Sigma Aldrich and were used as received. 2’,3’,5’-tri-O-Acety luridine (TAU, Sigma-Aldrich) was used as received. 2’3’5’-tri-O-Acetylcytidine (TAC) was obtained by neutralising acetylcytidine hydrochloride (Sigma-Aldrich) with NaHCO₃, extracted in dichloromethane and dried in vacuo. Uridine (Urd) was recrystallized in methanol prior to use. (±)-Propanolol hydrochloride was purchased from Sigma Aldrich and was converted to free base by addition of NaHCO₃ filtered, dried with methanol and further dried under vacuum at 40 °C overnight to afford propanolol (PNL). 2,2’-Azobisisobutyronitrile (AIBN, Dupont Chemicals) was recrystallized from methanol prior to use. Acetonitrile, methanol, chloroform and diethyl ether (VWR Chemicals) were of analytical grade and used as received. 1,4-Dioxane was purchased from
Acros and used as received. DMSO-\textit{d}_6 was purchased from Cambridge Laboratories. Figure 2.1 shows the structures of the chemicals used in this research project.

\textbf{Figure 2.1.} Structures of the components of the polymerisation feed. The internal standard used for the 1H NMR analyses, 1,4-dioxane (STD), cross-linkers employed: ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRM), functional monomers used: 2,6-bis(acryl)amidopyridine (BAAPy) and methacrylic acid (MAA), the templates and analogues used: theophylline (THP), caffeine (CAF), 3,5-dimethylphenol (1OH), 5-methylbenzene-1,3-diol (2OH), benzene-1,3,5-triol (3OH), 2,3,5-tri-O-acetylarabinose, 2,3,5-tri-O-acetylcytidine (TAC), uridine (Urd) and (±) Propanolol (PNL). Labelled atoms correspond to nuclei used for NMR analysis.
2.2. Synthesis of Polymers

The polymerization feed for each system was prepared according to Table 2.1 prior to purging with nitrogen gas for 15 mins. Subsequently, the polymerisation solutions were polymerized either thermally at 60 °C for 24 hours in a water bath (Julabo F12-ED Refrigerated/Heating Circulator) with (PP-1:1-A-St and PP-1:1-A-Rd) or without agitation or photochemically by placing the mixture in a water bath in a photochemical reactor cabinet and UV irradiated (200-400 nm) using a 450W medium pressure mercury vapour, quartz UV lamp (Ace glass, No. 7825-34) with the temperature kept between 22 and 27 °C during the reaction. Post polymerisation solutions were isolated from the microspheres by centrifugation (90 mins) at 2500 rpm and the supernatant collected, filtered through 0.25 μm syringe filters and stored at <10 °C before analysis by 1H NMR.

Templates were extracted by washing the microspheres overnight with 3 mL methanol:acetic acid mixture (90:10) and washed three times with 3.0 mL methanol. This procedure was repeated until no template was observed in the centrifugant as per 1H NMR. The microspheres were then washed with diethyl ether and placed in a vacuum oven at 40 °C for further drying. The same procedure was carried out for the non-imprinted counterparts in the absence of the template.
Table 2.1. Polymer feed composition of all systems investigated.

<table>
<thead>
<tr>
<th>EXPT/Polymer</th>
<th>[template] (mM)</th>
<th>[FM] (mM)</th>
<th>[XL] (mM)</th>
<th>[initiator] (mM)</th>
<th>Porogen volume (mL)</th>
<th>T:FM:XL</th>
<th>I:TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1:1000-T/C</td>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
<td></td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>I-1:500-T/C</td>
<td></td>
<td></td>
<td></td>
<td>0.20</td>
<td></td>
<td>1:500</td>
<td></td>
</tr>
<tr>
<td>I-1:1000-T/C, MX5-T/C</td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
<td>1:1000</td>
<td>1:100</td>
</tr>
<tr>
<td>T/P-1OH/2OH/3OH-1:100</td>
<td>4.16</td>
<td>16.66</td>
<td>83.34</td>
<td>1.00</td>
<td></td>
<td>1:4:20</td>
<td></td>
</tr>
<tr>
<td>I-1:10-T/C, T/P-1OH/2OH/3OH-1:10</td>
<td></td>
<td></td>
<td></td>
<td>10.00</td>
<td></td>
<td>1:2:10</td>
<td>1:100</td>
</tr>
<tr>
<td>I-1:5-T/C, T/P-1OH/2OH/3OH-1:5</td>
<td></td>
<td></td>
<td></td>
<td>20.00</td>
<td></td>
<td>1:4:8</td>
<td>1:5</td>
</tr>
<tr>
<td>MX2-T/C</td>
<td>8.40</td>
<td>33.40</td>
<td>66.60</td>
<td>5.00</td>
<td>1:4:8</td>
<td></td>
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<tr>
<td>MX10-T/C</td>
<td>2.80</td>
<td>9.00</td>
<td>91.00</td>
<td></td>
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<tr>
<td>TM2-T/C</td>
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<td></td>
<td></td>
<td>1.00</td>
<td></td>
<td>1:2:10</td>
<td>1:100</td>
</tr>
<tr>
<td>TM6-T/C</td>
<td>4.20</td>
<td>16.60</td>
<td>83.40</td>
<td></td>
<td></td>
<td>1:4:20</td>
<td></td>
</tr>
<tr>
<td>TM8-T/C</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>1:8:40</td>
<td></td>
</tr>
<tr>
<td>T/P-1OH/2OH/3OH-1:50</td>
<td>4.16</td>
<td>16.66</td>
<td>83.34</td>
<td>2.00</td>
<td></td>
<td>1:2:10</td>
<td>1:50</td>
</tr>
<tr>
<td>T/P-1OH/2OH/3OH-1:25</td>
<td></td>
<td></td>
<td></td>
<td>4.00</td>
<td></td>
<td>1:4:20</td>
<td>1:25</td>
</tr>
<tr>
<td>PP-1:1-A-150</td>
<td></td>
<td></td>
<td></td>
<td>2.00</td>
<td></td>
<td>1:1:20</td>
<td>1:50</td>
</tr>
<tr>
<td>PP-1:1-A-1200</td>
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<td></td>
<td></td>
<td>0.50</td>
<td></td>
<td>1:131.25</td>
<td></td>
</tr>
<tr>
<td>BP-1:1-A, BP-1:1-C</td>
<td>179</td>
<td>179</td>
<td>3.56 X10^3</td>
<td>28.6</td>
<td></td>
<td>1:131.25</td>
<td></td>
</tr>
<tr>
<td>ACNPP/IL-PP</td>
<td>13.32</td>
<td>33.34</td>
<td>50.00</td>
<td>4.16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3. Determination of the Polymer Composition using NMR

Analyses of the polymer composition was conducted by calculating the amount of the components left in the post-polymerisation solution and comparing those with the initial amount in the pre-polymerisation solution. This was achieved by $^1$H NMR using Bruker Avance III on a 5mm probe coaxial insert with 1,4-dioxane in DMSO-$d_6$ as the reference standard. The following peaks were chosen for each of the components in the polymerisation feed for the preparation of calibration curves used for quantitative analyses: O-$\text{CH}_2$ signal at 4.32 ppm for EGDMA, O-$\text{CH}_2$ signal of TRIM at 4.46 ppm, CH$_2$=C- signal at 5.10 and 5.65 ppm for MAA and EGDMA and CH$_2$=C- less the O-$\text{CH}_2$(EGDMA) peak for MAA, CH= C- at 8.25 ppm for BAAPy (BAAPy-H3), N-CH$_3$ signal of CAF (CAF-H1) at 4.23 ppm, N-CH signal of THP (THP-H1) at 3.84 ppm, -CH=CH- signal of TAU (TAU-H5) at 6.22 ppm, -CH=C- (hydrogen in position 2, i.e. 1OH-H2) at 6.75 ppm, for 1OH (1OH-H2), -CH=C- signal at 6.47 ppm for 2OH (2OH-H2) and -CH=C- at 6.11 ppm for 3OH (3OH-H2) and the –CH=CH- signal of PNL (PN-H1) at 8.15 ppm. An example of a calibration curve of EGDMA and MAA is shown in Figure 2.2 and a partial $^1$H NMR spectrum of a pre-polymerisation solution of 1OH is shown in Figure 2.3.
Figure 2.2. $^1$H NMR calibration curves of EGDMA (A) and MAA (B) for the determination of the amount of each component incorporated in the polymers. The following calibration curves were prepared using the following range of standards: 0.010–20.0 mM for EGDMA, 0.002 – 40 mM for MAA and 1.0 mM dioxane in DMSO-$d_6$ as reference standard S. NOTE: 

$I(S)/I(EGDMA) =$ ratio of the peak integrations of the dioxane standard to EGDMA, 

$[S]/[EGDMA] =$ ratio of the concentration of dioxane standard to the concentration of EGDMA, 

$I(S)/I(MAA) =$ ratio of the peak integrations of the dioxane standard to MAA, 

$[S]/[MAA] =$ ratio of the concentration of dioxane standard to the concentration of MAA.
Figure 2.3. An example of a $^1$H NMR spectrum of the caffeine pre polymerization solution. Peaks of the components are as follows; cross-linker (EGDMA, O-CH$_2$ signal) at 4.32 ppm, functional monomer (the combination of the signals of -CH$_2$=CH$_2$- of MAA and EGDMA) at 5.96 and 6.39 ppm and for caffeine at 4.23 ppm (-N-CH$_3$)

2.4 Rebinding Studies

2.4.1. CAF and THP system

The template rebinding experiments of the CAF and THP system, 10.0 mg polymers were incubated in 1.00 mL of 100 $\mu$M rebinding solution in acetonitrile for 18 hours. The post-rebinding solution was collected by centrifugation and the amount of template left in the solution was quantified using a Shimadzu LC-20AD HPLC instrument equipped with an Econosphere™ C18, 5$\mu$m column (Grace), LC-20 AD pump, an SPD-20A UV detector and SIL-20A/20AC injector operated with SIL-20A autosampler. Using two solvent gradient elution consisting of 25% of a mixture of acetonitrile: water 70:30 with 10 mM trimethylamine and 75%
of a 50 mM aqueous phosphate buffer (pH= 3.5) with a run time of 10 mins at a flow rate of 1.0 mL/min and detection wavelength of 270 nM. A calibration curve was generated for every batch binding analyses conducted using 7 standard solutions of CAF and THP in the range of 10-1000 μM.

2.4.2. Phenolic templates system

The time binding experiment was conducted using 10.0 mg of MIP and NIP of the phenolic system (both thermally synthesized and photochemically synthesized), incubated with 1.00 mL of 100 μM rebinding solution at different times: 5, 15, 30, 60, 90, 120 mins, 4 hours, 6 hours and 24 hours, while shaking with the Intelli-mixer RM-2.

For the time rebinding experiment of 1OH, the 1OH left in the solution after a period of time was quantified using a Shimadzu LC-20AD HPLC instrument equipped with an EconosphereTM C18, 5μm column (Grace), LC-20 AD pump, an SPD-20A UV detector and SIL-20A/20AC injector operated with SIL-20A autosampler. Using acetonitrile: 2% acetic acid in water (10:90 v/v, with a pH of 4.5), as the mobile phase, a 1 μL injection volume was used with a run time of 6 min at a flow rate of 1 mL/min and detection wavelength of 280 nm. A calibration curve was generated by monitoring the peak at 4.13 mins and using 7 standard solutions with concentrations ranging from 10 to 100 μM.

2OH and 3OH were quantified by 1H NMR using Bruker Avance III 600 MHz-NMR on a 5-mm probe at 35 ºC by monitoring the proton peak of 2OH (H2 of 2OH, i.e. 2OH-2) at 6.47 ppm for 2OH and 6.11 ppm for 3OH (3OH-2) and using the proton peak at 3.57 ppm of 100 μM 1,4-dioxane (STD-1) internal standard in DMSO-d6 contained in a co-axial insert. The data obtained were processed using Bruker Topspin 3.2 software.
For subsequent experiments, 10.0 mg of polymers were incubated in 1.00 mL of 500 nM rebinding solution in acetonitrile for 60 mins for 1OH, 45 mins for 2OH and 75 mins 3OH including centrifugation. The post-rebinding solution was collected and analyzed by $^1$H NMR using 100 µM 1,4-dioxane in DMSO-$d_6$ as the reference standard contained in a coaxial insert. Binding isotherms were generated by incubating polymers with various concentrations of template solutions ranging from 5 to 75 µM.

Binding isotherms were obtained for the MIP systems synthesized in I:TM of 1:100 by incubating, with shaking, 10.0 mg of polymers for the required equilibration time for each system 1.00 mL of various concentrations of each template ranging from 5 µM to 75 µM. Post-rebinding solutions were collected after centrifugation, filtered and analyzed by $^1$H NMR as with the time binding experiments.

2.4.3. Imide-containing templates

Time-binding experiments were conducted in situ by $^1$H NMR at 35 °C by incubating 10.0 mg of polymer PP-1:1-A (both NIP and MIP) in 0.50 mL of 50.0 µM TOAU in acetonitrile in a 5 mm NMR tube at various times from 15 to 180 min. The amount of TOAU remaining in solution was quantified by monitoring the peak at 6.22 ppm corresponding to proton 5 of TAU (TAU-H5) with respect to the peak, at 3.57 ppm, of 100 µM 1,4-dioxane in DMSO-$d$ internal standard contained in a co-axial insert.

For subsequent batch rebinding experiments of this system, 10.0 mg of polymers were incubated (with shaking) in 0.500 mL of 100 µM TOAU rebinding solution in acetonitrile in 5 mm NMR tubes and shaken (Intelli mixer RM-2) for 1 hour. The suspensions were then subjected to in-situ NMR analyses as with the time binding experiments.
Binding isotherms were obtained for PP-1-A and BP-1-A by incubating, with shaking, 10.0 mg of polymers for 60 min in 0.500 mL of various concentrations of TAU ranging from 1 µM to 100 µM. Post-rebinding solutions were collected after centrifugation, filtered and analyzed by \(^1\)H NMR as with the time binding experiments.

**2.4.4. PNL system**

The saturation period of 24 hrs for the PNL in this system has already been determined based upon the previous study.\(^7\)\(^1\) Thus the following template rebinding experiments were conducted over a 24 hr period. PNL was quantified by \(^1\)H NMR using Bruker Avance III 600 MHz-NMR on a 5-mm probe at 35 °C by monitoring the proton peak of PNL (H1 of PNL, i.e. PNL-H1) at 8.11 ppm and the proton peak of the reference standard at 3.57 ppm (100 µM) contained in a co-axial insert. The data obtained were processed using Bruker Topspin 3.2 software. 10.0 mg polymers were incubated in 1.00 mL of 500 µM rebinding solution in acetonitrile for 24 hours. The post rebinding solution was collected by centrifugation and analysed by \(^1\)H NMR.

**2.4.5. Binding isotherm analyses**

Binding parameters \(K\) (association constants) and \(N\) (number of binding sites) were calculated directly from the non-linear binding isotherms using the one-site parabola model of GraphPad Prism 5 based on Equation 2.1 and from the linearized curves generated using Equation 2.2 according to the Langmuir model.

\[
Y = \frac{B_{\text{max}} \cdot X}{K_d + X} \tag{Equation 2.1}
\]

Where \(X\) is the concentration of the template and \(Y\) is the specific binding.

\[
\frac{F}{B} = \frac{1}{N} F + \frac{1}{NK} \tag{Equation 2.2}
\]
where the slope is equal to 1/N, y-intercept equal to 1/NK, B is the concentration of the bound template and F is the unbound/free template.

2.5. Template-Monomer Interaction Studies

In order to study the potential interactions between the three phenolic templates (1OH, 2OH and 3OH) and MAA, and between TAU and BAAPy. Molecular modelling simulation software Spartan '04 (Wavefunction, Inc. USA) was employed. $^1$H and $^{13}$C NMR titration experiments were conducted using Bruker Avance III 600 MHz-NMR on a 5-mm probe at 30° and 60°C (for TAU and BAAPy) and processed using Bruker Topspin 3.2 software.

2.5.1. Phenolic templates

The changes in the chemical shifts of the nuclei of the templates and MAA were monitored after mixing 600 µL of 4.16 mM (0.02083 mmol) template solution with 50 µL of 16.67 mM (0.08333 mmol) MAA, following the reaction feed ratios.

2.5.2. Imide-containing templates

Increasing the amount of BAAPy (from a 50.0 mM in DMSO-$d$) ranging from 1.00 to 10.00 mmol in 20.0 µL (1.00 mmol) increments was added to 1.00 µmol (370.31 µg) of TOAU in 0.50 mL of DMSO-$d$. The complexation-induced shifts of the carbons and the protons of both BAAPy and TOAU were observed.

2.6. Selectivity Studies

2.6.1. Phenolic templates

The affinity of the best performing thermally-synthesized polymers (T-1OH-1:100, T-2OH-1:5 and T-3OH-1:50) of each system were investigated against the other phenolic
compounds as analogues (e.g. 1OH imprinted polymers using 2OH and 3OH as analogues). Triplicate samples of 10.0 mg of the polymers (MIPs and NIPs) of each system were incubated in 100 µM solution of the analogue for the saturation period determined by time-binding experiments (60 mins for 1OH, 45 mins for 2OH and 75 mins 3OH). The post-rebinding solution was collected by centrifugation and analyzed by $^1$H NMR using 100 µM 1,4-dioxane in DMSO-$d_6$ as the reference standard contained in a coaxial insert.

2.6.2. Imide-containing templates

The affinity of the TAU-imprinted microspheres towards the two analogues: TAC and Urd was tested using PP-1-A by incubating 10.0 mg of polymers in 0.500 mL of 50 uM solution of TOAC or Urd in 5-mm NMR tubes and shaken for 1 hour. The suspensions were then subjected to in-situ NMR analyses using 10 µM 1,4-dioxane internal standard by monitoring the 4.137 (H5’) and 6.077 (H5) ppm peaks for TAC and Urd, respectively.

Selectivity of the TAU-imprinted microspheres against TAC was tested using PP-1:1-A by incubating 10.0 mg of polymers in a mixed solution of 0.250 mL of 50 uM solution of TAU and 0.250 mL of 50 uM solution TAC (3) in 5-mm NMR tubes and shaken for 1 hour. The suspensions were then subjected to in-situ NMR analyses as with the non-competitive affinity tests. The procedure was repeated using 0.250 mL of 50 uM solution of TAU and 0.250 mL of 50 uM solution of uridine.

2.7. Determination of Degree of Crosslinking

The degree of cross-linking in the polymers can be correlated to the amount of reacted double bonds in the polymers which is the difference between the initial (before polymerisation) and the residual double bonds in the polymers (Equation 2.3). The residual double bonds were determined by infrared spectroscopy using Perkin Elmer Two based on a Universal Attenuated
Total Reflectance sensor (UATR-FTIR). Approximately 1 mg of the dried polymers was analysed and the data were processed using the Spectrum software. The residual double bonds were calculated using the ratio of the peak height of -C=O (1730 cm\(^{-1}\)) and -C=C- (1650 cm\(^{-1}\)) in the polymers with respect to the ratio of the reference compound, pure EGDMA. The contribution of the signals from MAA was not considered in the calculation since it was only minimal giving a difference of only 0.5-1 % in the calculated cross-linking in using pure EGDMA.

\[
\text{degree of cross – linking} = 100 \times \left[ 1 - \frac{(H_{C=O})_{\text{polymer}}}{(H_{C=O})_{\text{monomer}}} \right]
\]

*where: HC=C and HC=O are the peak height of C=C at 1750 cm\(^{-1}\) and C=O at 1690 cm\(^{-1}\) respectively.*
Figure 2.4. An example of a partial infrared spectrum of MAA:EGDMA (1:5) solution overlayed with spectrum of DMP-1:100-T MIP using Perkin Elmer Infrared Spectroscopy-Two based on a Universal Attenuated Total Reflectance sensor (UATR-FTIR). The amount of cross-linking in the polymers were determined using the peaks of \(-\text{C}=\text{O}\) and \(-\text{C}=\text{C}^-\) at 1730 and 1650 cm\(^{-1}\) respectively.

2.8. Sample Morphology

Scanning electron microscopy (SEM) imaging was conducted using Zeiss SEM Gemini. Dried microspheres were gold coated thrice using a SPI- Module sputter coater; twice at an 45-degree angle and once lying flat, prior to SEM imaging. Images of the particles were obtained using a magnification of 10,000-30,000 kx, and were analyzed using the Zeiss Zen lite 2012 software.
2.9. Particle Size Analysis

Dynamic Light Spectroscopy (DLS) measurements were carried out using a Malvern Zetasizer Nano ZS with DTS Version 5.03 software package (Malvern Instruments Ltd., Worcestershire, UK). Approximately 0.1 mg of the sample was suspended in ~0.5 mL of acetonitrile and sonicated using a benchtop ultrasonicator for ten seconds to minimize particle aggregation. Three measurements were carried out for each sample and average sizes are expressed in terms of the intensity weighted size distributions based on hydrodynamic diameters ($d_H$).

2.10. Thermal Analysis

Thermal stability of a representative polymer was carried out to observe the first stage of degradation using Perkin Elmer Diamond TG/DM Thermogravimetric/ Differential Thermal Analyzer with a heating rate of 10°C within the temperature range of 30-600°C per min and 2.0 mg sample. Glass transition determination experiments were performed by Shimadzu DSC-60A differential scanning calorimetry using a heating rate of 30°C/min and ~3.0 mg sample.

2.11. X-Ray Diffraction analyses

X-ray diffraction experiment was carried out using a Panalytical Xpert PRO MPD XRD equipped with a Cu anode tube, 4 degree anti scatter slit on incident side and a 10mm mask on incident side. The energy of monochromatic radiation is 40 kV (40 mA). Scattered intensities were recorded by PixCel 1d detector. Samples were back loaded into standard sample holders and analysed with rotation using the sample spinner.
2.12. Specific Surface Area and Porosity (Brunauer-Emmett-Teller)

Gas adsorption analyses were carried out using Micromeretics ASAP 2020 Accelerated Surface area and Porosity Instrument (Norcross, GA, USA). 100 mg samples were degassed under vacuum at 110 C. The adsorption isotherm was then measured using nitrogen gas as the adsorbate covering the partial pressure range of 1X10-6 to 0.01. The specific surface area of each sample was then determined from the adsorption data using the linearised BET equation.
Chapter 3
Precipitation imprinting of caffeine and theophylline: Effect of formulation in imprinting efficiency

3.1. Introduction

Only a few studies have investigated the effects and correlation of feed formulation to the binding performance of molecularly imprinted microspheres prepared by precipitation polymerisation. At low template concentrations, i.e. T:FM < 1:60\textsuperscript{126} and T:FM < 1:12\textsuperscript{100}, a decrease in the number of high-affinity binding sites in the microspheres was observed. Additionally, lowering the template concentration was found to increase the non-selective binding of the MIPs.\textsuperscript{21,116}

Varying the concentration of FM:XL in the feed was found to greatly affect the binding efficiency of the polymers as was presented by the study conducted by Yoshimatsu \textit{et al.} It was observed that higher concentration of MAA (decrease in the cross-linker) in the feed results in lower binding capacity (~2 times lower or halved) due to loss of rigidity and selectivity of the polymers\textsuperscript{99}. Similarly, lowering the amount of MAA (MAA:TRIM = 1:2.3) in the feed also gave a similar effect due to insufficient concentration of the T-FM complex formed during association stage.\textsuperscript{99} Thus, there should be a balance between the concentration of cross-linker, that provides the rigidity of the microspheres, and the functional monomer, that dictates the amount of T:FM complexes formed, to be able to determine the optimized FM:XL\textsuperscript{97} for a particular system. The
most binding efficient polymers according to published results are the polymers synthesized in FM:XL between 1:4-1:6.\textsuperscript{21,50}

In the case of the concentration of initiator, Mijangos \textit{et al.} recommended the use of low concentration of initiator since it enhances the selectivity of the microspheres due to an increase in the stability of the T-FM complex. Mijangos proposed that the heat formed due to the decomposition of high concentration of initiator could disrupt the formation of a stable T-FM complex.\textsuperscript{77} Another factor that has an effect on the binding efficiency of the MIPs is the nature of the template, particularly the number of complementary interaction points of the template. It has been established that higher number of complementary interaction points (functionality) increases the binding strength and fidelity of the recognition capabilities of MIPs.\textsuperscript{57,119,127-129} It is not only the synthesis of MIPs that needs to be considered but also its NIP counterparts since the efficiency of MIPs is always compared with the NIPs. Therefore, it is important that the physical properties (surface area, particle size, porosity) of the NIPs and the MIPs are comparable; however, few reports mention and emphasize the differences\textsuperscript{24,49,99,130-136} and this imposes difficulty in the determination of the actual efficiency of the MIPs.

Examining previous studies, it appears that the optimal conditions and formulations vary for every system. Thus in this study, we aim to determine the optimized formulation of the polymerization feed suitable for two MIPs system: caffeine (CAF) and theophylline (THP) by varying the following ratios in the feed: initiator:total monomer (I:TM), functional monomer:cross-linker (FM:XL) and template:functional monomer (T:FM). In addition, this chapter also reports on the effects of the nature of the template on the properties of the microspheres such as particle sizes, polymer composition and surface area and correlate them to the binding efficiency of the polymers.
3.2. Results and Discussion

Determining which component of the imprinting system predominantly affects the binding performance of MIP has been the subject of previous studies. Similar and contradicting results regarding the effects of the ratios of the components of the polymerisation feed have been presented using different systems. Both Sellergren and Yoshimatsu demonstrated that a loss in selectivity and binding efficiency is observed in the microspheres in high amount of functional monomer (above 35% functional monomer with respect to the total monomer). Sellergren studied L-phenylalanine anilide bulk MIP while Yoshimatsu investigated the efficiency of propranolol imprinted microspheres prepared by precipitation polymerisation. This however, is in contrast with the results obtained by Yilmaz et al. wherein they illustrated that monoliths synthesized in high concentration of functional monomer (0.2 % or T:FM = 1:500) recorded a higher yield of high affinity binding sites. In terms of the amount of cross-linker in the feed, contradicting results were presented by Yilmaz and Yoshimatsu. Yilmaz et al. demonstrated that even at low concentration of cross-linker (<20% or FM:XL = 1:5) cross-linker with respect to total monomer in the feed), the efficiency of the polymers were still comparable with MIPs synthesized in higher concentration of cross-linker (>50% or FM:XL = 1:2). This is due to the flexibility of the resulting polymers that can allow the access of the templates in the cavity that increased the MIP binding observed. Yoshimatsu suggested that lower concentration of cross-linker resulted to the loss of rigidity of the microspheres. Similar and contradicting results from published studies seem to suggest that the effect of different parameters is not only dependent on the MIP formats but also on the template used in the system. In this study, CAF and THP were utilised as model templates to determine the effects of the various components on the
performance of the MIPs. Three different experiments which involve varying the ratio of different components in the polymerisation feed; initiator: total monomer (I:TM, Section 1.3.1), functional monomer:cross-linker (FM:XL, Section 1.3.2) and template: functional monomer (T:FM, Section 1.3.3) were performed and assessed. The results are summarised in Table 3.1.

The effect of the interaction of the template with the functional monomer was also correlated with the properties investigated (polymer composition, particle size, morphology and binding performance). The only difference in the structures of caffeine and theophylline (Figure 3.1), the templates investigated in this study, is the number of possible interaction points. THP has the ability to simultaneously donate and accept electrons (H-bonding) from MAA while CAF can only donate electrons. In addition, it was also demonstrated in previous studies that the additional interaction of THP with MAA made the binding energy higher, i.e. more favourable interaction, compared to CAF as was calculated by molecular modelling. CAF and THP are also one of the commonly used templates in molecular imprinting due to availability and non-toxic nature with high association constants. The association constants for caffeine was determined within the range of 1.62- 5.43 x 10^3 M^{-1} and N between 16.98 to 8.39 X 10^3 μmol/g which was calculated by Scatchard model. In the case of theophylline, the association constants range from 1.53 X10^4 to -1.0 X10^8.
Figure 3. Structures of caffeine (CAF) and theophylline (THP).
Table 3. Imprinting results of polymers synthesized in various template (TM), functional monomer: cross-linker (MX) and initiator : total monomer (IM) ratios.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Incorporated components in the polymers</th>
<th>% incorporation (μmol/g)</th>
<th>Polymer Composition</th>
<th>Degree of Crosslinking</th>
<th>Hydrodynamic size, $d_H$ (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGDMA</td>
<td>MAA</td>
<td>Template</td>
<td>T :FM : XL</td>
<td></td>
</tr>
<tr>
<td><strong>TM2</strong> MIP</td>
<td>90 ± 1</td>
<td>79 ± 1</td>
<td>70 ± 1</td>
<td>0.44:1:6</td>
<td>68.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(375)</td>
<td>(66)</td>
<td>(29)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>92 ± 1</td>
<td>75 ± 1</td>
<td>76 ± 1</td>
<td>0.51:1:6</td>
<td>62.3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(382)</td>
<td>(63)</td>
<td>(32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td>83 ± 7</td>
<td>75 ± 2</td>
<td>1:5.58</td>
<td>70.3 ± 0.06</td>
<td>104 ± 1</td>
</tr>
<tr>
<td></td>
<td>(347)</td>
<td>(62)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TM4/MX5/IM100</strong> MIP</td>
<td>89 ± 2</td>
<td>80 ± 2</td>
<td>18 ± 1</td>
<td>0.06:1:5.53</td>
<td>71.0 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(369)</td>
<td>(67)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>85 ± 2</td>
<td>77 ± 1</td>
<td>49 ± 2</td>
<td>0.16:1:5.10</td>
<td>69.7 ± 0.01</td>
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<tr>
<td></td>
<td>(359)</td>
<td>(64)</td>
<td>(10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td>92 ± 2</td>
<td>80 ± 1</td>
<td>1:5.77</td>
<td>71.0 ± 0.01</td>
<td>93 ± 1</td>
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<tr>
<td></td>
<td>(384)</td>
<td>(67)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>TM6</strong> MIP</td>
<td>87 ± 2</td>
<td>74 ± 3</td>
<td>35 ± 1</td>
<td>0.08:1:5.85</td>
<td>63.3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(362)</td>
<td>(62)</td>
<td>(5)</td>
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<tr>
<td>MIP</td>
<td>90 ± 2</td>
<td>77 ± 2</td>
<td>49 ± 1</td>
<td>0.11:1:5.82</td>
<td>60.6 ± 0.01</td>
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<td></td>
<td>(373)</td>
<td>(64)</td>
<td>(7)</td>
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<td>NIP</td>
<td>92 ± 2</td>
<td>88 ± 4</td>
<td>1:5.24</td>
<td>63.3 ± 0.01</td>
<td>93 ± 1</td>
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<td></td>
<td>(382)</td>
<td>(73)</td>
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<tr>
<td><strong>TM8</strong> MIP</td>
<td>93 ± 1</td>
<td>87 ± 1</td>
<td>64 ± 1</td>
<td>0.09:1:5.36</td>
<td>62.3 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>(387)</td>
<td>(72)</td>
<td>(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>93 ± 2</td>
<td>82 ± 1</td>
<td>77 ± 1</td>
<td>0.12:1:5.63</td>
<td>61.0 ± 0.01</td>
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<td>(386)</td>
<td>(69)</td>
<td>(8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td>86 ± 2</td>
<td>79 ± 1</td>
<td>1:5.57</td>
<td>68.7 ± 0.01</td>
<td>89 ± 1</td>
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<td>(360)</td>
<td>(65)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>MX10</strong> MIP</td>
<td>88 ± 1</td>
<td>70 ± 1</td>
<td>35 ± 1</td>
<td>0.08:1:12.60</td>
<td>63.8 ± 0.1</td>
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<td></td>
<td>(409)</td>
<td>(32)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>86 ± 2</td>
<td>61 ± 1</td>
<td>32 ± 1</td>
<td>0.13:1:14.15</td>
<td>75.7 ± 0.1</td>
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<td></td>
<td>(392)</td>
<td>(28)</td>
<td>(4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td>86 ± 2</td>
<td>79 ± 2</td>
<td>1:10.89</td>
<td>63.3 ± 0.1</td>
<td>128 ± 1</td>
</tr>
<tr>
<td></td>
<td>(390)</td>
<td>(36)</td>
<td></td>
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</tbody>
</table>

\(^1\text{T:FM:XL= template : functional monomer : cross-linker (mol) ratio in the polymers,}^2\text{Degree of cross-linking compared to the initial ratio - C=C- and -C=O- and in the polymers.}^3\text{Polymers produced using T:FM = 1:4, FM:XL = 1:5 and I:TM of 1:100.}
Table 3.1 (continuation). Imprinting results of polymers synthesized in various template: functional monomer (TM), functional monomer: cross-linker (MX) and initiator:total monomer (IM) ratios.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition T : FM : XL (^1)</th>
<th>Degree of Cross-linking (^2)</th>
<th>Hydrodynamic size, (d_H) (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% incorporation (μmol/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGDMA</td>
<td>MAA</td>
<td>Template</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MX2</td>
<td>MIP&lt;sub&gt;C&lt;/sub&gt;</td>
<td>93 ± 2 (311)</td>
<td>78 ± 2 (130)</td>
<td>10 ± 2 (4)</td>
</tr>
<tr>
<td></td>
<td>MIP&lt;sub&gt;T&lt;/sub&gt;</td>
<td>93 ± 2 (310)</td>
<td>83 ± 2 (138)</td>
<td>31 ± 0 (13)</td>
</tr>
<tr>
<td></td>
<td>NIP</td>
<td>93 ± 2 (366)</td>
<td>84 ± 1 (57)</td>
<td>1:2.21</td>
</tr>
<tr>
<td>IM5</td>
<td>MIP&lt;sub&gt;C&lt;/sub&gt;</td>
<td>98 ± 1 (410)</td>
<td>92 ± 2 (77)</td>
<td>26 ± 1 (12)</td>
</tr>
<tr>
<td></td>
<td>MIP&lt;sub&gt;T&lt;/sub&gt;</td>
<td>98 ± 1 (410)</td>
<td>93 ± 2 (77)</td>
<td>50 ± 1 (10)</td>
</tr>
<tr>
<td></td>
<td>NIP</td>
<td>98 ± 1 (409)</td>
<td>88 ± 2 (73)</td>
<td>1:5.58</td>
</tr>
<tr>
<td>IM10</td>
<td>MIP&lt;sub&gt;C&lt;/sub&gt;</td>
<td>96 ± 1 (401)</td>
<td>84 ± 2 (70)</td>
<td>21 ± 1 (4)</td>
</tr>
<tr>
<td></td>
<td>MIP&lt;sub&gt;T&lt;/sub&gt;</td>
<td>96 ± 0 (398)</td>
<td>82 ± 1 (69)</td>
<td>52 ± 3 (11)</td>
</tr>
<tr>
<td></td>
<td>NIP</td>
<td>97 ± 1 (404)</td>
<td>83 ± 1 (69)</td>
<td>1:5.83</td>
</tr>
<tr>
<td>IM500</td>
<td>MIP&lt;sub&gt;C&lt;/sub&gt;</td>
<td>64 ± 2 (266)</td>
<td>56 ± 0 (47)</td>
<td>16 ± 1 (3)</td>
</tr>
<tr>
<td></td>
<td>MIP&lt;sub&gt;T&lt;/sub&gt;</td>
<td>61 ± 2 (256)</td>
<td>57 ± 2 (48)</td>
<td>22 ± 0 (5)</td>
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<tr>
<td></td>
<td>NIP</td>
<td>88 ± 2 (366)</td>
<td>69 ± 1 (57)</td>
<td>1:6.41</td>
</tr>
<tr>
<td>IM1000</td>
<td>MIP&lt;sub&gt;C&lt;/sub&gt;</td>
<td>40 ± 0 (165)</td>
<td>39 ± 2 (32)</td>
<td>8 ± 1 (2)</td>
</tr>
<tr>
<td></td>
<td>MIP&lt;sub&gt;T&lt;/sub&gt;</td>
<td>43 ± 2 (179)</td>
<td>38 ± 2 (32)</td>
<td>12 ± 1 (3)</td>
</tr>
<tr>
<td></td>
<td>NIP</td>
<td>75 ± 3 (314)</td>
<td>39 ± 2 (32)</td>
<td>1:9.70</td>
</tr>
</tbody>
</table>

\(^1\)T:FM:XL = template : functional monomer : cross-linker (mol) ratio in the polymers; \(^2\)Degree of cross-linking compared to the initial ratio -C=O- and -C=C- and in the polymers. \(^3\)Polymers produced using T:FM = 1:4, FM:XL = 1:5 and I:TM of 1:100.
3.2.1. Variation of template to functional monomer ratios (T:FM)

In this study, MIPs of CAF and THP were synthesized with various template: functional monomer ratios (T:FM = 1:2, 1:6 and 1:8) and compared to the MIP prepared using the accepted T:FM of 1:4. Polymers were thermally synthesized in an oil bath at 60 °C using AIBN as the initiator, MAA as the functional monomer, EGDMA as the cross-linker and ACN as porogen. The formulation was based upon previously reported studies on different systems using CAF and THP as templates: I:TM of 1:100 and volume of porogen/ total monomer of 10mL/mmol. In the case of the FM:XL used for this set of experiments, we opted for the most commonly used and accepted ratio of 1:5.

3.2.1.1. Particle size and morphology

In the SEM images taken and as shown in Figure 3.2, discrete microspheres were synthesized in all systems. The $d_{hl}$ obtained for the NIPs of this system ranges from 89-104 nm with the highest polydispersity index of 1.0 (TM4) and low of 0.2 (TM6-N) as shown in Figure 3.3. The smallest $d_{hl}$ (CAF = 66 ± 1 nm and THP = 70 ± 1 nm) and highest PDIs (1.0) were recorded for the two MIPs when T:FM is 1:2. Generally, the MIP particles got bigger as the concentration of template in the feed is varied except for TM8-THP (particle size comparable to that of TM2).
Figure 3. 2. SEM images of microspheres synthesized at various template: functional monomer (T:FM) ratios. TM2-N (A), TM2-C (B) and TM2-T (C), TM4-N (D), TM4-C (E) and TM4-T (F), TM6-N (G), TM6-C (H) and TM6-T (I), TM8-N (J), TM8-C (K) and TM8-T (L). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS.
3.2.1.2. Polymer composition

In order to quantify the amount of the components (EGDMA, MAA and the template) in the polymer, the unreacted components left in the post polymerisation solution were measured by $^1$H NMR. Comparison between the initial amount in the pre-polymerisation solution with the assumption that the difference between the initial and the unreacted amounts has been incorporated in the polymer. Briefly, the following peaks were monitored for quantitative $^1$H NMR: EGDMA (O-$\text{CH}_2$ signal) at 4.32 ppm, the combination of the signals of -$\text{CH}_2=\text{CH}_2$- for MAA and EGDMA at 5.96 and 6.39 ppm, and -N-$\text{CH}_3$ at 4.23 ppm and 3.84 ppm for CAF and THP (see Section 2.1 for details of the Experimental).

Table 3.1 shows that the amount of EGDMA in the NIPs and the two MIPs (as calculated with respect to the initial amount) is between 83-93%. The 10% difference in the amount of EGDMA in varying the T:FM ratios translates to 0.01 mmol, which is considered a negligible difference. Conversely, the amount of MAA in the polymers ranges from 75-88% for the NIPs.
and 74-87% for the MIPs. The result of the incorporation of the monomers for the MIPs suggest that changing the concentration of template in the feed has minimal effects on the incorporation of the monomers in the resulting polymers. The resulting FM:XL of the polymers (1:5-6), is similar in all cases and close to the starting ratio of 1:5. Polymer yields were in the range of 83-91% for the NIPs, and 84-93 % for the two MIPs.

It is well known in the field that the degree of crosslinking is related to the strength of the cavity produced by the imprinting process. The more conformationally defined the imprinted cavity, the more selective and binding efficient the polymers. In this study, the double bond conversion was correlated to the degree of crosslinking in the polymers; residual double bonds were calculated from the ratios of the peak height of the –C=O (1730 cm\(^{-1}\)) and –C=C (1650 cm\(^{-1}\)) in the polymers with respect to the pure EGDMA. As was expected, the degree of cross-linking of NIPs is comparable as shown in Figure 3.4, with values ranging from 68-71%. Similarly the degree of cross-linking in CAF MIPs appears to be more unaffected in changing the concentration of the template in the feed. Correspondingly, the THP MIPs showed minor changes in the degree of cross-linking as the concentration of template in feed is changed: with values ranging from ~61-64%.
Figure 3. The degree of cross-linking in the polymers synthesized at various T:FM ratios. Residual double bonds in the polymer were quantified using infrared spectroscopy and compared to the double bonds of the EGDMA monomer.

3.2.1.3. Template incorporation

The amount of template incorporated in the polymers in varying T:FM was also calculated according to the procedure discussed in Section 2.3 and the data calculated in the analyses is shown in Figure 3.5. The amount of template incorporation is expressed in terms of percentages or the amount of template in the polymers with respect to the starting concentration of template in the feed. TM2-C and TM8-C polymers incorporated comparable percentages of CAF in the polymers, which is the highest recorded incorporation of CAF in this system (70 and 64 %, respectively), and the lowest was incorporated by TM4-C polymers (18 ± 1%). TM2-C preserved its T:FM feed ratio but lower T:FM in the polymers (between 1:10-1:18) was obtained for the other TM polymers in Table 3.1. The preservation of the T:FM ratio in the polymers of TM2-C MIP suggests that the template acts as nucleation point during the polymerisation, pulling the monomer inwards and producing particles with smaller $d_H$ which was in accordance to the results published by Yoshimatsu. The gradual decrease in the CAF in the polymers from...
TM8 to TM4 experiments follows the expected results based from Le Chatelier’s principle. From TM8 to TM4, the concentration of MAA in the polymerisation feed is decreasing, therefore, the high concentration of MAA in the TM8 experiment pushes the reaction forward, producing more T-FM complexes and incorporating more CAF in the process. However, the comparable CAF incorporation of TM2 with TM8 can be due to the presence of high concentration of template that favours the formation of the T-FM complexes, which is also in accordance with Le Chatelier’s principle. The incorporated templates do not necessarily equate to binding sites and this will be discussed in the succeeding section (Section 3.2.1.4).

The amount of THP incorporated by the polymers followed the same trend as the CAF incorporation: TM2-T and TM8-T contained essentially identical percentages of THP in the polymer. In order to explain the comparable THP incorporation of TM2 and TM8-T polymers, the interactions of MAA and THP were observed in their corresponding complexes. Similarly, it is only the TM2-T polymers that preserved the starting T:FM and the rest incorporated lower than the starting T:FM ratio. It is however noteworthy to mention that THP polymers incorporated higher amount of template in the polymers compared to CAF polymers in all T:FM experiments. This suggests a stronger interaction of THP than CAF with the functional monomer.
3.2.1.4. Template rebinding studies

The maximum saturation period for the two MIP system employed was 18 hours following previously reported study\textsuperscript{145} and gave a 70-90\% template binding from the rebinding solution (100 \(\mu\)M ). The affinity of the NIPs towards CAF is comparable, as expected, with values ranging from 3.6-4.6 \(\mu\)mol/g. In terms of the MIP binding, TM2-C rebound the highest amount of template (6.6 \(\pm\) 0.4 \(\mu\)mol/g) and the lowest was recorded by TM4-C with value of 4.7 \(\pm\) 0.2 \(\mu\)mol/g. In terms of binding site conversion (i.e. the ratio of the amount of template incorporated with respect to the amount of template rebound), TM4-C polymers recorded the highest conversion of imprinted sites to binding sites with the value of 9.5\% which is due to the low incorporation of template and a relatively higher MIP binding while the lowest binding site conversion was recorded by TM2-C with only 1.8 \% conversion despite high template incorporation. The low binding site conversion observed with TM2-C could be attributed to the formation of complexes with weaker interactions with the functional monomer due to the presence of excess CAF in the feed and insufficient concentration of MAA forming incomplete
or partial binding sites. Due to comparable NIP binding towards CAF, the resulting IF is also comparable with lowest of 1.3 to a high of 1.6. The results of the rebinding suggest that the concentration of template in the feed, down to T:FM of 1:8 (11% CAF) does not affect the binding performance of the polymers. The results of the rebinding experiments illustrated that the amount of template incorporated (Section 3.2.1.3) does not necessarily equal or get converted to binding cavities efficiently.

![Graph showing CAF incorporation and NIP binding performance](image)

**Figure 3.** CAF incorporation, MIP and NIP binding performance of polymers synthesized at various T:FM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution with 70-90% binding from the rebinding solution. Post rebinding solutions were analyzed by HPLC. Template incorporation in μmol/g is also shown for comparison purposes.

The affinity of the NIP towards THP is also comparable with values ranging from 3.6-4.3 μmol/g as can be seen in Figure 3.7. In the case of MIP binding, no significant difference was observed, with the highest recorded MIP binding of 6.6 ± 0.7 μmol/g shown by TM8-T polymers and the lowest by TM4-T polymers with 5.4 ± 1.0 μmol/g. Due to high template incorporation but relatively low MIP binding, TM2-T polymers recorded the lowest binding site conversion.
(1.60%), which was also observed in the CAF counterparts. The highest binding site conversion was calculated in TM6-T polymers which is equivalent to 7.45%. Due to close range of MIP and NIP binding in between T:FM ratio, there is also a minor difference in the recorded IF of this system (IF= 1.5). Similar to CAF counterparts, due to equivalent binding and IF of the polymers synthesized in variable concentration of template, it indicates that the binding capacity of the polymers is not dependent on the concentration, down to T:FM of 1:8 (11% THP), of the template in the feed.

Figure 3. THP incorporation, MIP and NIP binding performance of polymers synthesized at various T:FM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution with 70-90% binding from the rebinding solution. Post rebinding solutions were analyzed by HPLC.

Overall, while THP has been demonstrated to provide stronger interaction with the functional monomer MAA than CAF, the incorporated template was not efficiently converted to high affinity binding sites more so with the TM2 polymers for both MIP systems. The incorporated templates in both CAF and THP system in TM2 could be due to incomplete or
partial imprinting that did not give high fidelity binding sites. Moreover, comparable IF values were obtained which suggests that even in the lowest concentration of templates (TM8), there is already enough amount of template to form the equilibrium concentration of T-FM complexes.

**3.2.2. Variation of functional monomer to cross-linker ratio (FM:XL)**

In this study, three sets of precipitation polymers with various functional monomer to cross-linker ratios (FM:XL) using two template system (CAF and THP) were produced: 1:2 (MX2) with 33% functional monomer, 1:5 (17%, MX5) and 1:10 (9%, MX10). The I:TM ratio was kept constant as the previous section (Section 3.2.1) at 1:100 and the volume/total monomer ratio at 10.00mL/mmol. A T:FM of 1:4 was used.

**3.2.2.1. Particle size and morphology**

The DLS measurements showed increasing $d_{H}$ with decreasing concentration of MAA in the feed (from MX2 to MX10) which is also visible in the SEM images taken for this system (Figure 3.8). The particle sizes of the NIPs range from 88-128 nm and more polydispersed (PDI of 0.5-1.0) compared to its MIPs counterparts with PDI of 0.1-0.3. CAF MIPs ranges from 84-120 nm with PDI of 0.1-0.3, which was also observed in the THP MIPs with sizes ranging from 65-115 nm and a PDI of 0.1-0.3. A speculation regarding the differences in the sizes of the MIPs and the NIPs was offered by Long$^{53}$ and emphasized that the presence of the template pulls the components towards each template molecule making the particle smaller. The resulting morphology of the microspheres are shown in Figure 3.9.
Figure 3. Hydrodynamic sizes, $d_H$, of microspheres synthesized in various FM:XL ratios. Measurements were conducted using Dynamic Light Scattering (DLS) and used ACN as the dispersant.
3.2.2.2. Polymer composition

As shown in Table 3.1, the incorporation of EGDMA of the CAF polymers is comparable regardless of the concentration of MAA in the feed with values ranging from 88-93%. The percentage of MAA incorporated in the polymers ranges from 70-80%, also similar to the MAA incorporation in the previous section of this chapter (Section 3.2.1.2). Unlike MX2 and MX5 polymers, MX10-C incorporated lower amount of MAA in the polymers resulting to a slightly lower FM:XL (1:13) than the starting ratio.
A similar trend was observed with the EGDMA incorporation of THP polymers and the values were comparable to the percentage of EGDMA in the CAF polymers in each FM:XL Ratios ranged from 86-93% with the highest displayed by MX2-T polymers. The MAA incorporation, on the other hand, showed a wider range of 61 (for MX10) to 83%. As with the CAF polymers, MX10-T recorded a lower FM:XL in the resulting polymers (1:14) but with a higher concentration of MAA in the feed, the initial FM:XL was preserved (MX5-T = 1:5 and MX2-T = 1:2).

As shown in Figure 3.10, the degree of cross-linking in the MX2 polymers is lower than the other MX polymers, for both MIPs and NIPs but in agreement with the values recorded for the TM polymers (Section 3.2.1.2). NIPs exhibited a degree of cross-linking within the range of 59-71 %, CAF MIPs showed cross-linking between 50-71 % and THP MIPs with 58-76%.

Figure 3.10. Degree of cross-linking in the polymers synthesized at various FM:XL ratios. The residual double bonds in the polymers were quantified using Infrared spectroscopy and compared to the double bonds of the EGDMA monomer.
3.2.2.3. Template incorporation

In the case of MX-C polymers, the incorporation of CAF was observed to be decreasing with increasing concentration of MAA in the feed (Figure 3.11). The highest CAF incorporated in this system was $35 \pm 1\%$ recorded by MX10-C and reduced by 3.5 times to $10 \pm 1\%$ displayed by MX2-C. Lower incorporation of CAF in a higher amount of MAA in the feed could be attributed to the possibility of MAA preferring to dimerize with itself than interact with CAF thus lowering the concentration of template-monomer complex. Due to low incorporation of CAF in the polymers, the resulting T:FM ratio in the polymers (Refer to Table 3.1) is lower than the starting ratio of 1:4: MX2 recorded a T:FM of 1:32, MX5 with 1:18 and MX10 with 1:8.

For the THP incorporation, on the other hand, the highest recorded THP incorporation in this system was exhibited by MX5 polymers and the other investigated ratios, 1:2 and 1:10, had similar amounts of THP incorporated in the polymers (~32 %, 1.5 times lower). The increase in the incorporated THP upon increasing the concentration of MAA in the feed could be due to the formation of a more stable T-FM complex which is based from Le Chatelier’s principle. However, adding more MAA in the feed promotes dimerization of MAA instead of interacting with THP, thus lowering the template incorporation in the microspheres. The difference in the incorporation of THP and CAF could be due to the difference in the MAA units that can form complexes with each template.

For THP, addition of more MAA from MX10 to MX5, enhances the interaction between MAA and THP since THP can form two H-bond interactions with MAA while CAF can only form one H-bond interactions. Thus, in MX10 there is a possibility that all of the MAA has been used for the efficient formation of T-FM complex with CAF and increasing the MAA concentration favours the dimerization of MAA. Similar to the MX CAF MIPs, the resulting
T:FM ratios in the polymers were lower than its starting T:FM: MX2-T gave a T:FM of 1:11, MX5 with 1:6 and MX10 with 1:8. The resulting T:FM of THP MIPs is higher compared to CAF MIPs which indicates a stronger interaction of THP than CAF with MAA based on an additional interaction point (hydrogen attached to the nitrogen) on the THP molecule.

![Figure 3](image)

**Figure 3.** Percentages of CAF and THP incorporated in the polymers in various FM:XL ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

### 3.2.2.4. Template rebinding studies

Figure 3.12 summarizes the incorporation and the binding performance of the MIPs and NIPs towards the CAF template. In the case of NIP, the amount of CAF bound of MX10 and MX5 microspheres were similar (2.4 ± 0.1 μmol/g and 3.6 ± 0.2 μmol/g, respectively) and increased 4 times to 12.0 ± 1.2 μmol/g for MX2 polymers, which could be due to the high concentration of MAA in the polymers that can interact and rebind CAF (T:FM =1:32). In terms of MIP binding, the MX2-C bound the highest amount of CAF equivalent to 14.1 ± 0.3 μmol/g and MX10-C bound the lowest with only 3.8 ± 0.1 μmol/g CAF bound. The high MIP binding of MX2-C polymers is due to the formation of a higher concentration of T-FM complexes because of higher concentration functional monomer incorporated in the polymer. Additionally, MX2-C exhibited
higher conversion of imprinted to binding sites with value of 25.6 % and the conversion drastically reduced to 7.6 % in using MX10-C polymers. The lower binding exhibited by MX10-C polymers is consistent with the published results of Yoshimatsu et al.\textsuperscript{99} which is attributed to the lower concentration of T-FM complexes formed during the imprinting process.\textsuperscript{93, 99, 137, 147-148} MX10-NIP gave a low affinity towards CAF resulting to higher IF (1.6) compared to other FM:XL investigated despite of low MIP binding, consistent with the demonstrated results of Yoshimatsu et al.\textsuperscript{99} They proposed that the non-specific binding capability of NIPs is proportional to the MAA units in the feed. As expected, an increase in the non-specific binding was observed in MX2 NIP \textsuperscript{21, 93, 98} which gave the lowest IF (IF=1.2) for this system. Additionally, a loss of binding capability was observed with MX2 polymers (for both MIP and NIP) which can be due to the lower degree of cross-linking consistent with the results presented by Yoshimatsu et al. \textsuperscript{99} in which they attributed to the loss of rigidity of the cavity of the polymers. \textsuperscript{26, 129, 137}
Figure 3. 12. Comparison of the amount of CAF incorporated, rebound by the MIPs and NIP prepared in various FM:XL ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analyzed by HPLC.

It is shown in Figure 3.13 that MX2-T polymers incorporated the highest THP (177.3 ± 1.3 μmol/g) for this system, and incorporation of THP decreases 3.9 times to 45.9 ± 0.1 μmol/g in MX10-T polymers. The investigation of the affinity of the NIP towards THP, showed that MX2 NIP gave the highest binding with THP equivalent to 12.0 ± 1.2 μmol/g and dropped 3.4 times to 3.6 ± 0.2 μmol/g for MX5-NIP and 5.7x to 2.1 ± 0.4 μmol/g for MX10 NIP. Similarly, MX2-T polymers exhibited the highest MIP binding of 14.7 ±1.1 μmol/g and dropped to 4.0 ± 0.2 μmol/g. This binding behaviour is similar to what was observed in the CAF system. Calculating for the efficiency of the imprinting process, MX2-T and MX10-T polymers displayed comparable binding site conversions (~8.5%). In all cases, a slightly higher MIP binding for MX-THP compared to MX-CAF polymers was observed, providing higher IFs suggesting a greater interaction between THP and MAA.
Summary of the THP incorporation and rebinding performance of the MIPs and NIPs prepared in various FM:XL ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analysed by HPLC.

The high MIP binding displayed by MX2 polymers is largely due to non-specific binding that gave low imprinting factors for both MIP systems. In addition, the binding results suggest that both CAF and THP systems prefer low concentration of MAA and high concentration of EGDMA in the feed (FM:XL = 1:5-10). This is not consistent with the demonstrated evidence in bulk polymers that the concentration of the template or the monomer should be increased in order to produce more binding sites due to the formation of higher concentration of template-monomer complex. Lowering the amount of MAA also arises to an equal increase in the amount of EGDMA in the fee, suggesting that appropriate rigidity of the polymers is also an important aspect to consider. This is consistent with the results published by Rosengren et al. Therefore, there should be a balance between the cross-linker and functional monomer to produce efficient MIPs.
3.2.3. Variation of amount of initiator to total monomer ratio (I: TM)

Polymers were synthesized thermally (60°C) using EGDMA as the cross-linker, MAA as the functional monomer and acetonitrile (ACN) as porogen. Despite of the comparable MIP binding exhibited by MX10 and MX5, we opted to use the more commonly employed FM:XL of 1:5 for all the I:TM experiments. In addition, 1:5 is also the widely used FM:XL ratio in precipitation polymerisation. The T:FM ratio was kept constant at 1:4 as well as the volume/monomer ratio at 10mL/mmol. Five different I:TM ratios: 1:5 (IM5), 1:10 (IM10), 1:100 (IM100), 1:500 (IM500) and 1:1000 (IM1000), were used to produce CAF (e.g. IM5-C) and THP (e.g. IM5-T) MIPs.

3.2.3.1. Particle Size and morphology

Based on published literature, one of the physical properties of the polymers that is evidently affected by the concentration of initiator in the polymerisation feed is the particle size. However, the \( d_h \) of the NIPs measured by DLS ranges from 83-140 and does not follow any trend with respect to the concentration of initiator as the \( d_h \) of IM5 and IM1000 are comparable as shown in Figure 3.14. Also, the PDI values of the NIPs is comparatively higher than its corresponding MIPs in a lower concentration of initiator. The comparable \( d_h \) of IM5 and IM1000 suggests that the particle size of NIP is affected by the rate of polymerisation and number of nucleation sites: in IM5, the more radical species are present forming higher concentration of nuclei incorporating more monomer into the growing polymer chain resulting to bigger particles. In IM1000 on the other hand, even though few nuclei are formed, the growing polymer nuclei can coalesce and form larger particles.

The particle sizes of the MIPs, however, increased with increasing concentration of initiator (Figure 3.14) coinciding with the results shown by several studies. In the case
of the CAF MIPs, a gradual increase from 95-136 nm was observed, similarly with THP MIPs, increasing $d_H$ was observed with increasing concentration of initiator from 90-132 nm. It was also observed that the PDI values of the microspheres produced in a lower concentration of initiator (1:100-1:1000) were lower than the values obtained in higher concentrations of initiator, consistent with the results published by Wang. The effect of the concentration of initiator on the particle size of the MIPs indicate that the presence of the template in the feed could intensify the effects of the concentration of initiator in the particle sizes. It would seem that the particle sizes of the MIPs are more affected by the number of nucleation sites: higher concentration of initiator means faster rate of reaction therefore more smaller nuclei in a short amount of time and aggregates that give particles a larger diameter.

SEM images were taken in similar fashion as the previous sections (Sections 3.2.1.1 and 3.2.2.1) and confirmed the differences in the particle sizes, as the I:TM ratio is varied and is shown in Figure 3.15.
Figure 3. Hydrodynamic sizes, $d_H$, of microspheres synthesized at various I:TM ratios. Measurements were conducted using Dynamic Light scattering (DLS) and using ACN as the dispersant.
Figure 3. SEM images of microspheres synthesized using different initiator : total monomer ratios (I:TM). IM1000-N (A), IM1000-C (B) and IM1000-T (C), IM500-N (D), IM500-C (E) IM500-T (F), IM100-N (G), IM100-C (H) IM100-T (I), IM10-N (J), IM10-C (K) IM10-T (L), and IM5-N (M) IM5- C(N) and IM5-T (O). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS.
3.2.3.2. Polymer composition

Similar method was employed in the determination of the polymer composition of the IM polymers: unreacted components (EGDMA, MAA and templates) left in the post polymerisation solution were quantified by $^1$H NMR spectroscopy using 1,4-dioxane as the reference standard. Table 3.1 shows that the amount of all components in the polymer decreases with decreasing amount of initiator. (regardless if it’s a MIP or a NIP and the nature of the template used). The decrease in the incorporation of the monomers in decreasing concentration of initiator was also reported by Stover et al. They proposed that the efficiency of polymerisation at low concentration of initiator is the result of a low concentration of monomers in the feed in precipitation polymerisation.$^{152}$

There is no considerable difference in the amount of EGDMA uptake of the NIP synthesized in higher amount of initiator (88-98% from I-1:5 to I-1:500) and it dropped to 75 ± 3% when the amount of initiator is decreased to 1:1000 (0.1%). This behavior was also observed in the amount of MAA in the polymers. But the onset of the drop in the amount of MAA is when the I:TM is 1:100. Due to low conversions of monomers in lower concentration of initiator, lower FM:XL (1:6-1:10) was calculated compared to the starting ratio of 1:5, indicating that EGDMA has a higher reactivity compared to MAA even at low concentration of initiator. This resulted in a higher concentration of XL in the polymers (or lower FM:XL ratio in the polymers)

Similarly, for the CAF polymers, the amount of EGDMA calculated in the polymers decreases with decreasing concentration of initiator in the feed: from a high of 98 ± 1% shown by IM5-C to a low of 39 ± 2% shown by IM1000-C. A similar effect in the incorporation of MAA in the CAF polymers was observed: decreasing with decreasing concentration of initiator, from 92 ± 2% shown by IM5-C to 39 ± 2% by IM1000-C. Since both monomers (EGDMA and
MAA) showed a decreasing trend in decreasing concentration of initiator, the resulting FM:XL in the CAF polymers preserved the initial FM:XL of 1:5. The lower incorporation of MAA in the NIP and the preservation of the starting FM:XL in the MIP suggests that the template can act as an efficient nucleation points during polymerisation\textsuperscript{143, 154-155} incorporating higher amount of MAA even in low concentration of initiator. The \textsuperscript{1}H NMR-based yield obtained for CAF imprinted polymers ranges from 24-97 % and is higher than the isolated yield which is only in the range of 37-84 %.

Interestingly, similar trends were observed in the THP polymers and the values recorded is not significantly different from the values obtained from CAF polymers: the highest recorded value for the incorporation of EGDMA is 98 ± 1% (410 \textmu mol/g) showed by IM5-T and the lowest of 40 ± 0% (165 \textmu mol/g) showed by IM-1000-T, and for the incorporation of MAA, the highest value is 93 ± 2% (77 \textmu mol/g) exhibited by IM-5-T and lowest was 38 ± 2% (32\textmu mol/g). These values preserved the initial FM:XL ratio of 1:5 in the polymers similar to CAF polymers and resulted to an NMR based yield of 25-97 %. The isolated yield obtained for THP system is 45-85 %, which is slightly lower than the NMR based yield.

It can be seen in Figure 3.16 that between the NIP and the MIP, regardless of the template used, there is no considerable difference in the amount of cross-linking in the polymers with values ranging from 62-74%, which indicates that EGDMA has not completely reacted even in high concentration of initiator in both MIPs and NIPs. This is in contradiction with the findings reported by reported studies.\textsuperscript{152, 156} in which they observed that the degree of cross-linking of the microspheres were proportional to the concentration of initiator used in the polymerisation process. The observed range between each initiator concentration investigated
was between 48-54% which could be attributed to the different monomer, initiator and the ratio of the solvent to the amount of monomer in the feed.

Figure 3. 16. The degree of cross-linking in the polymers synthesized at various I:TM ratios. Residual double bonds were quantified using Infrared Spectroscopy and compared with the double bonds of EGDMA.

3.2.3.3. Template incorporation

Template incorporation of the polymers (both CAF and THP) was also observed to be decreasing with decreasing concentration of initiator as shown in Figure 3.17. In the case of CAF incorporation, the highest recorded amount of template incorporated by the polymers (IM5-C) is 26 ± % (12 μmol/g) and the lowest is 8 ± 1% (2 μmol/g). The incorporation of THP on the other hand, in all cases is higher than the CAF incorporation: with the highest value of 50 ± 1% (10 μmol/g) and the lowest of 12 ± 1% (3 μmol/g). In theory, the concentration of the initiator in the feed is directly proportional to the rate of the reaction in FRP system. Based on the calculated template incorporated in the polymers, it would seem that the two MIP systems investigated favour the faster polymerisation reaction due to the fast entrapment of the template-
functional monomer complex/cluster (T-FM) or which was referred to as “snap freezing” by Turner et al. Additionally, in all I:TM ratios, the amount of THP incorporation is higher than CAF incorporation, with the highest value of THP incorporation (50 ± 1%) almost twice as much as the highest recorded CAF incorporation (26 ± 1%) in IM5 experiments. This is another evidence suggesting that the strength of interaction and number of binding points of the template and functional monomer favours the formation of the association cluster between the template and functional monomer.

Figure 3. CAF and THP incorporation in the polymers at various I:TM ratios measured by 1H NMR using 1,4-dioxane in DMSO-\textit{d}_6 as the reference standard.

3.2.3.3. Template rebinding studies

The effects of the concentration of initiator in the affinity of the NIPs towards CAF is minimal given the minor differences in the binding performance in each I:TM ratios. The highest NIP binding was 3.59 ± 0.15 \( \mu \)mol/g and the lowest of 2.82 ± 0.12 \( \mu \)mol/g. In terms of MIP binding, the amount of CAF rebound by the polymers was highest in high concentration of
initiator as shown in Figure 3.18: with the highest of 4.91 ± 0.24 µmol/g exhibited by IM5-C and a lowest of 3.57 ± 0.710 µmol/g exhibited by IM1000-C. Due to low incorporation and relatively high MIP binding of IM100-C, this polymer recorded the highest binding site conversion of 9.6%. The lowest was 6.2% showed by IM500-C polymers. Due to minimal changes in the amount of CAF rebound by the NIP, this resulted to an IF of 1.1-1.7, with IM5-C recording the highest IF.

![Figure 3.18](image)

**Figure 3.18.** Comparison of the amount of CAF incorporated, rebound by the MIP and the NIP polymers synthesized at various I:TM ratios. Polymers were incubated for 18 hours in 100µM template rebinding solution and the post rebinding solutions were analyzed by HPLC.

The NIP binding performance towards THP is marginally affected by the concentration of initiator with changes in the amount rebound minor compared to its MIP counterparts. The highest NIP binding observed is equivalent to 3.86 ± 0.28 µmol/g by IM1000-T NIP and the lowest was exhibited by IM5-T NIP with value of 3.07 ± 0.29 µmol/g as shown in Figure 3.19. In comparison, the MIP binding showed that the highest THP rebound by IM-5-T with a value of 5.97 ± 0.36 µmol/g and a low of 4.59 ± 0.21 µmol/g by IM1000-T. Considering the conversion of the imprinted sites to binding sites, the highest conversion (6.3 %) was recorded by IM1000-T.
due to a lower incorporation of template. The lowest was by IM5-T with only 5.0% conversion. The low binding site conversion calculated for IM5-T suggests that the high incorporation of THP might be simply due to partial imprinting, not due to the formation of high fidelity binding sites. Similar to CAF counterparts, the system that recorded the highest IF value is the system with the highest concentration of initiator, IM5-T (2.0). The lowest is shown by IM1000-T (1.2). Both systems recorded an increasing NIP binding from IM5 to IM1000, however, higher MIP binding was observed towards THP molecule, which can be due to more interaction points of the THP molecule than CAF.

Figure 3. 19. Comparison of the amount of THP incorporated in the polymers, rebound by the imprinted and non-imprinted polymers synthesized in various I:TM ratios. Polymers were incubated for 18 hours in 100μM template rebinding solution and the post rebinding solutions were analyzed by HPLC.

The amount of initiator in the feed appears to have an effect on the amount of template incorporated and the binding performance of the polymers. From this study, both MIP systems favour fast polymerisation reactions and this could be due to the immediate trapping of the T-FM complexes formed during the polymerisation process. This could be due to the “snap-freezing” of the T-FM complexes, put forward by Turner et al. to explain the better performance of the
MIPs from microwave induced initiation compared to thermally-synthesized MIPs, allowing the preservation of the three dimensional arrangement leading to the formation of stronger binding cavities in the polymers.\textsuperscript{79} However, the binding site conversion (amount of template rebound/amount of template incorporated) of IM5 microspheres recorded lower binding site conversion than the IM1000 counterparts. This could be due to the disruption of the T-FM complexes by the heat given off during the decomposition of high concentration of initiator, as was proposed by Mijangos et al.\textsuperscript{77} Despite the higher binding site conversion of the IM1000 polymers, this ratio is still not an advisable I:TM ratio to be used since the polymerization is difficult to control due to its sensitivity to the presence of oxygen. Additionally, IM1000 produced the lowest yield among all of the IM polymers synthesized. Therefore, based on the calculated IF values and higher percentage yield, this study showed that a higher concentration of initiator is more preferable to use (I:TM < 1:100) with the optimum being the I:TM ratio of 1:5.

3.2.4. Polymer Surface area

Due to instrument constraints, surface areas of only a small number of representative polymers were determined by the Brunauer–Emmett–Teller (BET) method and tabulated in Table 3.2. The systems chosen were (1) the low and high FM:XL ratios: MX2 (FM:XL = 1:2) and MX10 (FM:XL = 1:10) with constant ratios of I:TM = 1:100 and T:FM = 1:4 and (2) low and high T:FM ratios: TM2 (T:FM = 1:2) and TM10 (T:FM = 1:10) with constant parameters of I:TM = 1:100 and FM:XL = 1:5). The effect of the initiator concentration on MIP performance and its correlation with surface area are further discussed in Chapter 4, thus the I:TM microspheres were not included in the surface area analysis for this chapter.
In varying the FM:XL ratio in the feed, the surface area of the NIP ranges from 70-250 m²/g, with MX2 NIP giving a higher surface area (3.6 times) than its MX 10 counterpart. Thus, the surface area of the NIP is increasing with increasing concentration of FM (decreasing concentration of XL) which was observed in previous experiments by Golker and Rosengren et al. with monolithic polymers. The increasing trend in the surface area of the particles coincides with the decreasing trend observed with the $d_H$ of the microspheres.

The range of surface areas observed for the MIPs, on the other hand is wider than the NIP; CAF MIPs have surface areas ranging from 30-132 m²/g and THP MIPs from 71-672 m²/g. It would appear that further investigation is warranted as the surface areas obtained for MIPs are variable and, due to limited data, could not be correlated to the physical properties and binding performance of the MIPs.

**Table 3.** BET surface area (m²/g) data for selected CAF and THP MIPs and NIPs.

<table>
<thead>
<tr>
<th></th>
<th>MX2¹</th>
<th>MX5²</th>
<th>MX10⁵</th>
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<td></td>
<td>NIP</td>
<td>TM2³</td>
<td>TM10⁴</td>
</tr>
<tr>
<td>NIP</td>
<td>254.0 ± 2.4</td>
<td>121.5 ± 8.2</td>
<td>71.2 ± 2.7</td>
</tr>
<tr>
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<td>29.9 ± 0.23</td>
<td>132.0 ± 1.02</td>
</tr>
<tr>
<td>MIP_T</td>
<td>672.5 ± 9.3</td>
<td>71.2 ± 2.7</td>
<td>148.5 ± 6.25</td>
</tr>
</tbody>
</table>


**3.3. Summary**

In this chapter, the effects of changing the different ratios of the components of the feed (T:FM, FM:XL and I:TM) in the different polymer properties were investigated.
Polymer yields ranged from a low of 25% for those prepared using low concentration of initiator (IM1000 MIPs) to a high of 98% for those prepared at high concentration of crosslinker (MX10 MIPs). The degree of crosslinking remained in a close range between 50% to 70%.

In terms of FM:XL ratio in the polymers, a lower concentration of initiator (IM500 and IM1000, FM:XL=1:5) and a higher concentration of crosslinker (MX10) did not preserve the FM:XL formulation resulting in polymers of higher XL content than the feed.

In the case of template incorporation, it was observed that higher template (CAF and THP) was incorporated in the MIPs containing higher concentration of functional monomer (~80 μmol/g for TM8 and ~100 μmol/g for MX2), template (~350 μmol/g TM2) and initiator (~75 μmol/g IM5) in the feed relative to the others in the same set of experiments. However, the incorporated template has not been translated to high fidelity binding sites and. Except for the MX2, which demonstrated the highest MIP binding in both CAF and THP system, exhibited comparable (about 1.5 μmol/g difference) MIP binding. Conversely, all NIPs showed comparable template binding (except for MX2 which was synthesized with higher concentration of functional monomer, resulting in IF in a close range between 1.2-2.0). In all cases, the incorporation and MIP binding of THP polymers are higher than its CAF counterparts signifying the stronger interaction of THP with the functional monomer than CAF.

The particle sizes of the microspheres measured is in the range 60-150 nm and was demonstrated to be affected by the concentration of initiator (increases with increasing concentration of initiator) and concentration of functional monomer (increases with decreasing concentration of functional monomer). The differences in the surface area of the MIPs and the NIP (30-675 m²/g) revealed that more accurate binding capacity can be obtained if it is
normalized with respect to surface area. However, due to limited surface area results obtained, it is recommended that further investigation should be conducted.

Our results suggest that the T:FM ratio between 1:2 to 1:8 will result in comparable binding capacities regardless of the level of template incorporation and can maintain low NIP binding. Results also showed that there should be a balance between the rigidity of the polymers and the concentration of functional monomer that can form T:FM complexes to produce binding efficient polymers. Therefore FM:XL should be kept in between 1:5-1:10. It has also been demonstrated that a faster reaction time is more favoured thus the optimum I:TM should be maintained above 1:100.

For this study, the best performing CAF and THP MIPs were obtained using T:FM = 1:4, FM:XL = 1:5 and I:TM = 1:5 giving a binding capacity of 4.91 ± 0.24 μmol/g and IF of 1.8 for CAF MIP and 5.97 ± 0.36 μmol/g and IF of 2.0 for THP MIP. Thie precipitation MIPs have been prepared using ACN porogen at 10mL/mmol total monomers.
Chapter 4
Precipitation imprinting of phenolic templates: Effect of the nature of the template and initiator concentration

4.1 Introduction

The success of molecular imprinting is dependent on the formation of a stable complex or association cluster between the functional monomer and the template \(^{16,21,159}\) which is dependent on various factors: the shape of the template and the number of functional groups of the template that can form interaction with the functional monomer. It was illustrated in several reports that the more interaction points between the template and the functional monomer, the more binding efficient the resulting molecularly imprinted polymers will be. \(^{16,21,128,159}\) However, Spivak et al. have shown that, in some cases, the shape of the template has a more prevalent effect over the number of functional groups or interaction points between the template and the functional monomer on the efficiency of the polymers.\(^{146}\) Results presented in Chapter 3 suggested that the nature of the template seems to affect the final properties of the polymers (polymer composition, binding performance). Theophylline was imprinted more efficiently in comparison with caffeine, which was speculated to be due to the stronger interaction of the functional monomer with theophylline.

Microspheres produced by precipitation polymerization have proven to be sensitive to any changes in the composition of the polymerization feed and these changes affect the binding performance of the resulting microspheres.\(^{26,49,77-78,99,160-161}\) Results of Chapter 3 demonstrated...
that the amount of initiator in the feed affects the composition of the polymers and ultimately the binding performance of the polymers: both caffeine and theophylline MIP systems displayed higher imprinting and binding efficiencies in higher amount of initiator or faster reaction.

In order to analyse further the effect of the nature of the template in the imprinting and binding efficiencies, and physical properties of the resulting MIPs, this chapter focuses on three MIP systems for different phenolic templates (Figure 2.1) with varying number of interaction points (hydroxyl group) synthesized by precipitation polymerisation. These templates were also chosen because of their simple and flat structures of comparable size. Among the three experiments conducted in Chapter 3, the effects of varying the amount of initiator to total monomer (I:TM) is more evident in the physical properties and binding efficiencies of the resulting MIPs thus, for this chapter, the three phenolic templates were synthesized in varying I:TM ratios employing one of the accepted FM:XL and T:FM ratios investigated in Chapter 3: FM:XL of 1:5, T:FM of 1:4. In addition, the chosen FM:XL and T:FM ratios were the commonly used ratios in molecular imprinting technology. Furthermore, the effects of temperature were assessed and compared in terms of imprinting and binding efficiencies by producing 3 MIP systems in two different methods of initiation; thermal and photochemical.

4.2. Results and Discussion

In Chapter 3, it was demonstrated that template incorporation and binding performance of MIPs prepared by precipitation polymerisation were affected by the concentration of initiator used for polymerisation, particularly, MIPs for theophylline and caffeine performed better at higher concentration of AIBN (e.g. I:TM = 1:5). In this chapter, we further investigated the effects of the concentration of the initiator on the performance of MIPs prepared by precipitation
polymerisation using phenolic templates ((3,5-dimethylphenol (1OH), 5-methylbenzene-1,3-diol (2OH) and Benzene-1,3,5-triol polymers (3OH), refer to Figure 2.1 for the structures)) with varying number of interaction points with the functional monomer. Moreover, the effects of the different mode of initiation (thermal and photochemical) on the imprinting and rebinding capacities of the microspheres were also compared and assessed.

4.2.1. Thermally-synthesized imprinted microspheres

In this study, MIPs for 1OH, 2OH and 3OH were synthesized by precipitation polymerisation at 60 ºC using AIBN as initiator, acetonitrile as porogen (10 mL/mmol of total monomer), EGDMA as the cross-linker, MAA as the functional monomer. The effect of the amount of initiator was assessed for every MIP system at five different I:TM ratios: 1:100 (1% AIBN), 1:50 (2%), 1:25 (4%), 1:10 (9%) and 1:5 (17%) keeping the T:FM:XL ratio of 1:4:20 constant.

4.2.1.1. 3-5, Dimethylphenol MIPs (T-1OH)

4.2.1.1.1. Polymer Composition

Polymer composition was calculated based on the unpolymerized monomers and residual template in the post polymerisation solution with respect to the pre polymerisation mixture by ¹H NMR spectroscopy using the following peaks: EGDMA (O-CH₂ at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH₂=C- less the O-CH₂(EGDMA) peak), and 6.75 ppm for 1OH. As shown in Table 4.1, the incorporation of EGDMA in the polymers is not significantly different between the MIP and the NIP at all the I:TM ratios. In addition, the incorporation of the functional monomer, MAA, was also observed to be unaffected by the presence of the template in all cases. However, the composition of EGDMA and MAA within the polymers was observed to increase with increasing concentration of initiator, i.e. increase
monomer conversion to polymer from a low of 89% for I:TM ratio 1:100 to a high of 98% for I:TM ratio 1:5, resulting in an FM:XL ratio of 1:5, in all cases, equivalent to the feed ratio. This result is not surprising because higher concentration of initiator will produce higher concentration of free radicals in solution resulting in faster rate of polymerisation. The isolated yield of the polymers is lower (82-89%) than the NMR–based yields, which can be attributed to the formation of short chained oligomers that did not reach the critical mass to precipitate out of the solution or due to the loss during the extraction of template and drying process.
**Table 4.1.** Composition and degree of cross-linking of thermally-synthesized 3,5-dimethylphenol (T-1OH) imprinted and non-imprinted polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM&lt;sup&gt;1&lt;/sup&gt; (%initiator)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition</th>
<th>Reacted Double Bonds&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% incorporation (μmol/g)</td>
<td>T:FM:XL&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EGDMA</td>
<td>MAA</td>
<td>Template</td>
</tr>
<tr>
<td>T-1OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs</td>
<td>99 ± 0 (824)</td>
<td>92 ± 1 (153)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>98 ± 1 (813)</td>
<td>97 ± 1 (162)</td>
</tr>
<tr>
<td>T-1OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs</td>
<td>97 ± 1 (812)</td>
<td>92 ± 1 (154)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>98 ± 1 (816)</td>
<td>93 ± 2 (153)</td>
</tr>
<tr>
<td>T-1OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs</td>
<td>96 ± 1 (797)</td>
<td>90 ± 1 (150)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>96 ± 1 (800)</td>
<td>92 ± 2 (155)</td>
</tr>
<tr>
<td>T-1OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs</td>
<td>92 ± 0 (770)</td>
<td>85 ± 0 (142)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>94 ± 0 (782)</td>
<td>90 ± 1 (150)</td>
</tr>
<tr>
<td>T-1OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs</td>
<td>91 ± 0 (754)</td>
<td>82 ± 1 (137)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>93 ± 0 (773)</td>
<td>85 ± 2 (142)</td>
</tr>
</tbody>
</table>

<sup>1</sup>I:TM = initiator (mmol): total monomer (mmol EGDMA+ mmol MAA), <sup>2</sup>% initiator = amount (mol) of initiator/amount of total monomer (mol), <sup>3</sup>T:FM:XL = template : functional monomer : cross-linker (mol) ratio in the polymers, <sup>4</sup>Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and >C=O with respect to their peak height ratio in pure EGDMA.

While the concentration of initiator was found not to affect the incorporation of the monomers, template incorporation within the polymers was observed to decrease with increasing amount of initiator in the feed. T-1OH-1:100 polymers recorded the highest amount of template incorporation (48 ± 1%, 20.07 μmol) which gradually decreased to 14 ± 1% (5.78 μmol) with T-1OH-1:5 as illustrated in Figure 4.1. These results indicate that the polymerisation at I:TM ratio
of 1:5 could be too fast and does not give enough time for the 1OH molecules to interact with the functional monomers or it could be due to the disruption of the complex formation between the template and the functional monomer attributed to the production of heat from the decomposition of the AIBN according to Mijangos et al.\textsuperscript{77}. This, however, is in contrast with the results obtained for THP and CAF MIPs (Chapter 3) which showed higher template incorporation at higher initiator concentration.

![Graph](image)

**Figure 4.** Percentages of 1OH incorporated in the polymers in various I:TM ratios. Measured by \textsuperscript{1}H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

As summarized in Table 4.1, there is minimal difference (~3\%) in the degree of crosslinking between the MIP and NIP indicating that the presence of the template did not affect the extent of crosslinking. The range of the degree of cross-linking was observed to be marginal (60-75\%), with a difference of 3 \% in between levels of initiator. These observations in the degree of cross-linking is consistent with the results obtained for theophylline and caffeine MIPs (Chapter 3). The least cross-linked polymers were recorded by T-1OH-1:5 MIPs and NIPs (73-
75%) indicating the presence of higher concentration of double bonds in the polymers due to faster termination of the polymerisation reaction attributed to the higher amount of initiator.

4.2.1.1.2. Morphology and Particles Sizes

The SEM images in Figure 4.2 show all T-1OH polymers to be discrete microspheres of average size, measured by DLS, ranging from 80-120 nm with narrow polydispersity indices (PDI) of 0.01-0.06. It was also observed that particle sizes increased with increasing concentration of initiator which is in agreement with published results.\textsuperscript{149, 151, 162-164} The increase in the $d_H$ of the microspheres in increasing concentration of initiator was also observed with CAF and THP system (Section 3.2.3.1) which was attributed to the coagulation of smaller nuclei.\textsuperscript{17 152}

NIP particles were observed to have a bigger $d_H$ then the MIPs and this difference is more noticeable in higher amount of initiator indicated in the insets in Figure 4.2. The inability to control the size of the MIP and the NIP of a given system has been documented in previous research,\textsuperscript{49, 99} which was attributed to the presence of the template in the pre-polymerisation mixture\textsuperscript{165-166} during nucleation stage wherein the presence of the template causes an attractive pull towards the center making the imprinted polymers smaller than the NIP counterparts.\textsuperscript{167}
Figure 4. SEM images of T-1OH microspheres in varying amount of initiator in the polymerization feed. T-1OH-1:100 MIP (A) and NIP (B), T-1OH-1:50 MIP (C) and NIP (D), T-1OH-1:25 MIP (E) and NIP (F), T-1OH-1:10 MIP (G) and NIP (H), T-1OH-1:5 MIP (I) and
4.2.1.1.3. Template Rebinding Studies

The results of the time binding experiments are given in Figure 4.3. T-1OH binding was monitored at 15, 30, 60, 120 and 180 mins and binding saturation was observed after 60 minutes. The saturation period obtained for T-1OH polymers is relatively faster than other MIP systems using various phenolic templates (e.g. 12168, 20169-170 and 24171 hours). Subsequent binding assays were measured after 60 minutes – 50 min incubation period with shaking and 10 min of centrifugation.

Figure 4.3. Time binding experiment of 1OH imprinted polymers (P-1OH-1:50) and non-imprinted counterpart. 10.0 mg polymers were incubated at different time periods using 100 µM DMP solution.

Figure 4.4 shows the comparison of the amount of 1OH (µmol 1OH/ mass of polymers) incorporated in the polymers during the imprinting process and the amount of 1OH rebound by the polymers prepared at various I:TM ratios after 1 hour of incubation of 10.0 mg polymers in 1.00 mL of 100 µM 1OH. Template incorporation of T-1OH polymers markedly decreased with
increasing concentration of initiator, with T-1OH-1:100 exhibiting 4 times the template incorporation of T-1OH-1:5 (124 ± 0.8 µmol/g versus 32.7 ± 1.2 µmol/g). The decrease in the template incorporation could be attributed to the lower branching density in the polymers. It was reported in several papers that the amount of initiator affects the mechanical properties of the polymers, such as the branching density. Branching density is defined as the fraction of repeated units in a polymer which contains a branch point, thus higher branching density means shorter distances between each branch point. It was reported in previous literature that branching density is proportional to concentration of initiator in the feed.172-175 Higher branching density promotes rigidity in the polymers, and it was suggested by several literature that the binding cavities formed should have the balance of rigidity and flexibility that allows the efficient “trapping” of the template during imprinting process and the same time flexible enough to increase the accessibility of the template during rebinding process.99,176-177 MIP binding was also observed to decrease with increasing concentration of initiator. From 2.09 ± 0.01 µmol/g for T-1OH-1:100 MIP, it decreases to 0.88 ± 0.01 µmol/g for T-1OH-1:5 MIP. Nevertheless, the decrease in the MIP binding from T-1OH-1:100 to 1:5 is not as drastic as the change observed in the calculated template incorporated in the polymers. This could be due to the optimum number of T-FM complexes that can be formed by a given amount of template and functional monomer as proposed by Spivak et al.146

The binding site conversion, however, increased 1.6 times with increasing concentration of initiator, from a low of 1.7% (T-1OH-1:100) to a high of 2.7 % (T-1OH-1:5) brought about by the drastic decrease in the template incorporation with increasing concentration of initiator. These results suggest that high template incorporation at lower concentration of initiator (e.g. T-1OH-1:100) didn’t translate to high fidelity imprints.
The effect of the concentration of the initiator was also evident in the binding of the NIP but the changes are not as evident as the changes observed in the MIP. Gradual decrease in the amount of template bound by the NIP with increasing concentration of initiator was observed; with a high of 1.2 ± 0.1 μmol/g for T-1OH-1:100 to a low of 0.6 ± 0.1 μmol/g for T-1OH-1:5 NIP. The low NIP binding observed in T-1OH-1:5 can be due to the fast polymerisation reaction that the inter-monomer interaction between MAA molecules in the pre polymerisation solution are preserved leaving lower concentration of MAA available to interact with 1OH during the rebinding process. This inter-monomer interaction possibly is disrupted at lower initiator concentration (i.e. 1:50 – 1:100) when polymerisation is slower at 60°C resulting to free MAA being able to interact with and bind 1OH. In all cases, MIP binding was higher than NIP binding giving imprinting factors (IF) in the range between 1.4-1.8, with the highest IF exhibited by T-1OH-1:100 polymers.

Figure 4. 1OH incorporation, MIP and NIP binding performance of polymers synthesized in various I:TM ratios. Polymers were incubated for 60 mins in 100μM template rebinding solution with 40-70% binding from the rebinding solution. Post rebinding solutions were analyzed by 1H NMR. Template incorporation in μmol/g is also shown for comparison purposes.
4.2.1.1.4. Characterization of binding sites

The binding sites of the polymers were characterised using T-1OH-1:100 and analysed by the non-linear Langmuir (NLL) and linearized-Langmuir (LL) models. Based on the assumption that the binding sites formed are homogenous. Figure 4.5 shows the NLL and LL binding isotherms while Table 4.2 gives a summary of binding parameters $N$ and $K_a$ obtained from the two models. The NLL plot of T-1OH-1:100 show that the template loading capacity of the MIP is higher than the NIPs with the MIP recording a $N(2.59 \pm 0.24 \text{ µmol/g})$ almost twice as much as that for its NIP ($1.42 \pm 0.11 \text{ µmol/g}$) counterpart. The comparable $K_a$ values for the MIPs ($1.30 \pm 0.09 \times 10^5 \text{ M}^{-1}$) and its NIPs ($1.20 \pm 0.12 \times 10^5 \text{ M}^{-1}$) signifies the similar affinity of 1OH towards the MIP and NIP which could be due to the single interaction point of the template.\textsuperscript{23,126} Despite the comparable $K_a$, $N$ (MIP) being higher (by 1.8 times) than $N$ (NIP) indicates that the imprinting process has a more prevalent effect on the binding capacity of the MIPs consistent with the IF of 1.8 obtained for the system. The LL model gave $N$ and $K_a$ which are both comparable to the NLL values.

Table 4.2. Affinity constants ($K_a$) and number of binding sites ($N$) of T-1OH polymers determined by the non-linear Langmuir (NLL) and linearized Langmuir (LL) models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>MIP</th>
<th>NIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-linear Langmuir model\textsuperscript{1}</td>
<td>$K \times 10^5 \text{ M}^{-1}$</td>
<td>$1.30 \pm 0.09$</td>
<td>$1.16 \pm 0.12$</td>
</tr>
<tr>
<td></td>
<td>$N \text{ (µmol/g)}$</td>
<td>$2.59 \pm 0.24$</td>
<td>$1.42 \pm 0.11$</td>
</tr>
<tr>
<td>Langmuir model</td>
<td>$K \times 10^5 \text{ M}^{-1}$</td>
<td>$1.33 \pm 0.11$</td>
<td>$1.25 \pm 0.05$</td>
</tr>
<tr>
<td></td>
<td>$N \text{ (µmol/g)}$</td>
<td>$2.49 \pm 0.23$</td>
<td>$1.36 \pm 0.06$</td>
</tr>
</tbody>
</table>

\textsuperscript{1} values were determined from Prism GraphPad using the one-site hyperbola model.
Figure 4. Binding isotherms for T-1OH-1:100 polymers fitted to (A) Non-linear Langmuir model (B) Linearised Langmuir isotherm.
4.2.1.2. 5-Methylbenzene-1,3-diol MIPs (T-2OH)

4.2.1.2.1. Polymer Composition

2OH polymers were characterised as with the 1OH system. Polymer compositions were quantified using $^1$H NMR using 1,4-dioxane as the reference standard and by monitoring the peaks for EGDMA (O-CH$_2$ at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH$_2$=C- less the O-CH$_2$(EGDMA) peak), and 6.47 ppm for 2OH. The initial amounts of the components were compared with the amount of the unreacted components remaining in the post polymerisation solution. As summarized in Table 4.3, high conversions for EGDMA (92-99 % conversion) were observed for both MIPs and NIPs of T-2OH and the presence of the template has no apparent effect on EGDMA conversion for all concentrations of initiator tested. In the case of MAA, there is no significant difference in the % MAA between the MIP and its corresponding NIP, which suggests that the template does not affect the MAA incorporation in the polymers. However, similar to incorporation of EGDMA, MAA conversion, for both MIPs and NIPs, were observed to decrease with decreasing concentration of initiator. Despite the decreasing incorporation of both the EGDMA and MAA in the polymers, the resulting FM:XL ratio in the polymers was preserved in 1:5, as with the 1OH system, with a calculated yield between 91-96%. The isolated yield calculated for T-2OH polymers is in the range of 85-92%, with the highest yield displayed by T-2OH-1:5 polymers and the lowest by T-2OH-1:100 polymers. T-2OH polymers preserved the starting FM:XL (1:5) regardless of the concentration of the initiator, which was also observed in the T-1OH system.
Table 4.3. Composition and degree of cross-linking of thermally-synthesized 5-methylbenzene-1,3-diol (2OH) imprinted and non-imprinted polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM(^1) (%initiator)(^2)</th>
<th>% Incorporation (μmol/g)</th>
<th>Polymer Composition T:FM:XL(^3)</th>
<th>Reacted Double Bonds(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EGDMAXMA Template</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-2OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs 99 ± 0 (825)</td>
<td>10 ± 0 (4.20)</td>
<td>0.03:1:5.0 62.22 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 96 ± 1 (801)</td>
<td></td>
<td>1:5.0 64.88 ± 0.01</td>
</tr>
<tr>
<td>T-2OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs 98 ± 0 (818)</td>
<td>8 ± 1 (3.50)</td>
<td>0.02:1:5.4 67.98 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 98 ± 0 (819)</td>
<td></td>
<td>1:5.4 68.39 ± 0.01</td>
</tr>
<tr>
<td>T-2OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs 96 ± 1 (798)</td>
<td>9 ± 1 (3.80)</td>
<td>0.03:1:5.4 64.81 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 96 ± 1 (799)</td>
<td></td>
<td>1:5.5 67.88 ± 0.01</td>
</tr>
<tr>
<td>T-2OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs 95 ± 0 (790)</td>
<td>12 ± 1 (5.00)</td>
<td>0.04:1:6.7 62.52 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 95 ± 0 (794)</td>
<td></td>
<td>1:5.58 63.31 ± 0.02</td>
</tr>
<tr>
<td>T-2OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs 92 ± 1 (764)</td>
<td>9 ± 1 (3.90)</td>
<td>0.03:1:6.7 60.49 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 92 ± 0 (767)</td>
<td></td>
<td>1:5.6 60.65 ± 0.02</td>
</tr>
</tbody>
</table>

\(^1\)I:TM = initiator (mmol): total monomer (mmol EGDMAXmmol MAA), \(^2\)% initiator = amount (mol) of initiator/amount of total monomer (mol), \(^3\)T:FM:XL = template : functional monomer : cross-linker (mol) ratio in the polymers, \(^4\)Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and -C=O with respect to the peak height ratio of pure EGDMAX.

**Figure 4.6.** illustrates that the amount of template incorporation by the polymers is not affected by the concentration of initiator in the feed as there is no considerable difference in the amount of template incorporated at different I:TM ratios with values ranging from 9 ± 1.2 % (23.9 ± 0.3 μmol/g) to 12 ± 1.0 % (29.6 ± 0.3 μmol/g) These results are different from the results...
obtained for the T-1OH system wherein the amount of template incorporated decreased with increasing concentration of initiator. Additionally, the incorporated amount of 2OH in the polymers is 1.3 – 5.2 times lower than its T-1OH counterparts and this may be attributed to a side reaction of 2OH which is discussed in the succeeding section (Section 2.3.1.4) of this chapter since it is expected that higher amount of 2OH will be incorporated in the polymers based from the additional interaction points compared to 1OH.

![Figure 4.6](image)

**Figure 4.6.** Percentages of 2OH incorporated in the polymers in various I:TM ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

There is no considerable difference in the degree of cross-linking between the MIP and the NIP from each I:TM ratio, as well as between the MIPs and NIPs prepared from varying I:TM ratios as summarized in Table 4.3, with values ranging from 60-69 %, suggesting that the template and the concentration of initiator does not affect the degree of cross-linking in the MIPs and the NIPs as with the 1OH system.
**4.2.1.2.2. Morphology and Particle Sizes**

Increasing trend in the particle sizes of both the MIPs and the NIPs with increasing amount of initiator are observed from the SEM images and the DLS results (Figure 4.7) consistent with the findings for the T-1OH system and the caffeine and theophylline systems (Chapter 3) as well as those reported by previous researchers. The NIP particles appear to be bigger (by ~10 nm) than the MIP counterparts. It is also noteworthy that the polydispersity indices of the particles are significantly different. T-2OH-1:100 gave the lowest PDI of 0.02 and T-2OH-1:50 showed the highest of 0.50. The increase in the PDI and particle size of the microspheres with increasing initiator concentration suggest that particle coagulation occurred during polymerisation as claimed by previous studies.
Figure 4. SEM images of T-2OH-imprinted microspheres in varying amount of initiator in the polymerization feed. T-2OH-1:100 MIP (A) and NIP (B), T-2OH-1:50 MIP (C) and NIP (D), T-1OH-1:25 MIP (E) and NIP (F), T-2OH-1:10 MIP (G) and NIP (H), T-2OH-1:5 MIP (I) and NIP (J). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM.
4.2.1.2.3. Template rebinding studies

According to Figure 4.8, the binding sites of T-2OH-imprinted microspheres are saturated after 45 min of incubation period with 2OH rebinding solution, thus succeeding rebinding measurements were performed after 45 minutes.

![Graph showing template rebinding studies](image)

**Figure 4.8.** Time binding tests using 10.0 mg T-2OH microspheres incubated in 1.00 mL of 100 µM 2OH solution. Amount of template bound to the polymers were analyzed using 
$^1$H NMR spectroscopy using the peak signal at 6.47 ppm for 2OH-H2.

Figure 4.9 shows the amount of the template incorporated and rebound by the MIPs and NIPs prepared at various concentrations of initiator. Minimal and gradual decrease in the template bound by the NIP counterparts with increasing amount of initiator was observed, with the highest value of 1.08 ± 0.02 µmol/g by T-2OH-1:100 to lowest of 0.64 ± 0.03 µmol/g by T-2OH-1:5. The decreasing amount of 2OH rebound by the polymers in increasing concentration of initiator might be due to the increasing MAA incorporation in the polymers which has the higher probability of inter-monomer interaction than interacting with 2OH.

89
Unlike T-1OH in which the template incorporation is decreasing with increasing concentration of initiator, the template incorporation of T-2OH slightly changes with increasing concentration of initiator with values ranging from 20-30 µmol/g but does not follow a decreasing trend. Nevertheless, the amount of template rebound by the T-2OH imprinted microspheres slightly decreased with increasing concentration of initiator. The T-2OH-1:100 MIP managed to rebound 1.34 ± 0.01 µmol/g, which is 1.6 times higher than the template rebound by the T-2OH-1:5 MIP with a value of 0.86 ± 0.03 µmol/g. Calculating the binding site conversion for each I:TM ratio, highest binding site conversion (5.6%) was obtained from T-2OH-1:100 and the lowest by T-2OH-1:5 with only 3.6 % conversion. The binding site conversion of T-2OH polymers is within the range of 3.6-5.6 %, with lowest conversion exhibited by T-2OH-1:5 and the highest by T-2OH-1:100. While MIP binding is, in all cases, higher than NIP binding, the difference is minimal resulting to IF values of between 1.1-1.3.

Figure 4. 9. Amount of 2OH incorporation in the polymers and the rebound of 2OH-MIPs and NIPS in different I:TM experiments analysed by $^1$H NMR monitoring the 2OH peak at 6.47 ppm and the peak at 3.57 ppm for the standard 1,4-dioxane in DMSO-$d_6$. 

90
4.2.1.2.4. Characterization of binding sites.

Similar binding isotherm models were used to analyse the binding sites of T-2OH-1:100 polymers which is shown in Figure 4.10 A (NLL) and B (LL). The calculated binding parameters of T-2OH-1:100 were summarized in Table 4.4. As expected, higher \( N \) was calculated using NLL model for the MIP (1.60 ± 0.09 µmol/g) than the corresponding NIP (1.24 ± 0.11 µmol/g). In terms of the \( K_a \), the MIP recorded an affinity constant of 1.24 ± 0.06 \( \times 10^5 \) M\(^{-1}\) which is 1.3 times higher than its NIP (0.92 ± 0.09 \( \times 10^5 \) M\(^{-1}\) ) counterpart. The low \( N \) calculated for T-2OH-1:100 MIP indicates inefficient imprinting process, and combined with low affinity of the polymers towards 2OH, gave rise to a low IF for this system (IF=1.1). The \( N \) and \( K_a \) values calculated using LL model is comparable with the values obtained using NLL model.

Table 4.4. Affinity constants \((K_a)\) and number of binding sites \((N)\) of T-2OH polymers determined by the non-linear Langmuir (NLL) and linearized Langmuir (LL) models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>MIP</th>
<th>NIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-linear Langmuir model(^1)</td>
<td>( K ) (x(10^5 ) M(^{-1}) )</td>
<td>1.24 ± 0.06</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>( N ) (µmol/g)</td>
<td>1.60 ± 0.09</td>
<td>1.24 ± 0.11</td>
</tr>
<tr>
<td>Linearized Langmuir model</td>
<td>( K ) (x(10^5 ) M(^{-1}) )</td>
<td>1.41 ± 0.05</td>
<td>1.18 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>( N ) (µmol/g)</td>
<td>1.54 ± 0.23</td>
<td>1.17 ± 0.09</td>
</tr>
</tbody>
</table>

\(^1\) values were determined from Prism GraphPad using the one-site hyperbola model.
Figure 4. 10 Binding isotherms for T-2OH-1:100 polymers fitted to (A) Non-linear Langmuir model (B) Linearised Langmuir isotherm.
4.2.1.3. Benzene-1,3,5-triol MIPs (T-3OH)

4.2.1.3.1. Polymer Composition

The incorporation of each of the components in the polymer were quantified using $^1$H NMR using 1,4-dioxane as the reference standard and by monitoring the peaks for EGDMA (O-CH2 at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH2=C- less the O-CH2(EGDMA) peak), and 6.11 ppm for 3OH peak. The initial amounts of the components were compared with the amount of the unreacted components remaining in the post polymerisation solution.

Both EGDMA and MAA components within the polymers do not differ significantly between MIPs and NIPs for all of the I:TM ratios, as summarized in Table 4.5, which indicates that the presence of the template does not affect the incorporation of the EGDMA and MAA in the polymers. These results are in agreement with those obtained for T-1OH and T-2OH as well as for the caffeine and theophylline systems discussed in Chapter 3. However, the concentration of initiator affects the incorporation of EGDMA and MAA in the polymers, with ranges between 91-99% for EGDMA and 87-93 % for MAA, which is also consistent with the results obtained in the two other thermally synthesized systems. The resulting FM:XL of 1:5 is preserved since the incorporation of both monomers decreased with decreasing concentration of initiator. The isolated % yield for this system is in the range of 73-83%, which is lower than the calculated yield of 92-98%, as with the other systems, due to loss during purification.
Table 4.5. Composition and degree of cross-linking of thermally-synthesized Benzene-1,3,5-triol (3OH) imprinted and non-imprinted polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM$^1$ (%initiator)$^2$</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition T :FM : XL$^3$</th>
<th>Reacted Double Bonds$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% incorporation (μmol/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-3OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs 99 ± 0 93 ± 1 4 ± 1 (823) (156) (1.75)</td>
<td>0.01:1:5.4</td>
<td>70.6 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 99 ± 0 91 ± 1 (826)</td>
<td>1:5.5</td>
<td>74.4 ± 0.01</td>
</tr>
<tr>
<td>T-3OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs 98 ± 0 91 ± 1 14 ± 1 (816) (151) (5.36)</td>
<td>0.04:1:5.4</td>
<td>74.2 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 98 ± 0 90 ± 1 (816)</td>
<td>1:5.4</td>
<td>73.5 ± 0.01</td>
</tr>
<tr>
<td>T-3OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs 95 ± 1 88 ± 1 19 ± 1 (795) (146) (7.40)</td>
<td>0.05:1:5.5</td>
<td>73.0 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 96 ± 1 91 ± 2 (801)</td>
<td>1:5.3</td>
<td>71.0 ± 0.01</td>
</tr>
<tr>
<td>T-3OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs 94 ± 0 88 ± 1 23 ± 0 (784) (148) (9.00)</td>
<td>0.06:1:5.3</td>
<td>76.0 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 93 ± 0 89 ± 1 (775)</td>
<td>1:5.12</td>
<td>74.3 ± 0.01</td>
</tr>
<tr>
<td>T-3OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs 91 ± 1 87 ± 0 30 ± 1 (761) (145) (12.31)</td>
<td>0.08:1:5.2</td>
<td>73.9 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 90 ± 1 87 ± 2 (749)</td>
<td>1:5.2</td>
<td>75.1 ± 0.01</td>
</tr>
</tbody>
</table>

$^1$I:TM = initiator (mmol): total monomer (mmol EGDMA+ mmol MAA), $^2$% initiator = amount (mol) of initiator/amount of total monomer (mol), $^3$T:FM:XL = template : functional monomer :cross-linker (mol) ratio in the polymers, $^4$Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and -C=O with respect to the peak height ratio of pure EGDMA.

The increase in the incorporation of the template 3OH within the polymers with decreasing amount of initiator is evident in Figure 4.11. From 29 ± 1 % (with respect to the initial amount in the polymerisation feed) template incorporation by T-3OH-1:100 MIP, it decreased to 22 ± 1 % when the amount of initiator in the feed was doubled (T-3OH-1:50, 2%),
and continuously decreased to only $4 \pm 1\%$ in T-3OH-1:5 MIP. These results indicate that the template-monomer interaction does not favour a fast polymerisation reaction, the $I:TM = 1:5$ which is predicted by Mijangos et al. that the formation of heat due to the high concentration of initiator in the feed can disrupt the T-FM complex formation.

![Figure 4](image.png)

**Figure 4.** Percentages of 3OH incorporated in the polymers in various I:TM ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

The degree of cross-linking (Table 4.5) in the polymers, based from the ratio of the integration of the peaks of the $-C=\text{C}-$ and the $-\text{C}=\text{O}$ in the IR spectra, ranges from 70-76% and appears not directly affected by the concentration of initiator. These calculated degrees of cross-linking coincide with the results obtained from T-1OH and T-2OH systems.

**4.2.1.3.2. Morphology and Particle Sizes**

As with T-1OH and T-2OH, the particles sizes of T-3OH polymers (MIP and NIP) increase with increasing concentration of initiator. It is noticeable in the SEM images in **Figure 4.12** that the microspheres produced of this system (both MIP and NIP) are bigger in higher concentration of initiator, and this was confirmed by the measurements obtained from the SEM
images and DLS measurements. Measurements obtained from DLS, the $d_{H}$ sizes of the T-3OH imprinted microspheres range from $92.2 \pm 1.0$ nm (T-3OH-1:50) to $142.3 \pm 0.2$ nm (T-3OH-1:5) while the sizes of the NIPs range from $93.1 \pm 0.1$ nm to $134.4 \pm 0.6$ nm. The increasing trend in the particle sizes with increasing concentration of initiator has been observed in the two other phenolic MIP systems (T-1OH and T-2OH) consistent with the results of the published studies by Wang and Yang. ¹⁴⁹⁻¹⁵⁰, ¹⁸¹
Figure 4. SEM images of T-3OH polymers produced in varying I:TM ratios T-3OH-1:100 MIP (A) NIP (B), T-3OH-1:50 MIP (C) NIP (D), T-3OH-1:25 MIP (E) NIP (F), T-3OH-1:10 MIP (G) NIP (H), T-3OH-1:5 MIP (I) NIP (J). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM.
4.2.1.3.3. Template rebinding studies

Prior to template rebinding analyses, the saturation period or equilibration time between the template and the polymers was performed using 3OH polymers incubated at various times (20, 30, 45, 60, 75, 90, 105, 120 mins) in 100 μM 3OH solution. Figure 4.13 demonstrates that the equilibration time for T-3OH is 75 minutes. No changes in the amount of template rebound by the polymers were observed after the mentioned periods, thus subsequent binding tests were performed after 75 minute incubation period.

![Figure 4.13](image-url)

**Figure 4.13.** Time binding experiment for T-3OH polymer (T-3OH-1:50). Using 10.0 mg polymers and 100 μM 3OH rebinding solution. ¹H NMR results were quantified using the CH=C- signal of 3OH at 6.11 ppm and using 1,4-dioxane as the reference standard in DMSO-d₆.

**Figure 4.14.** shows that template incorporation and binding for T-3OH MIPs follow a decreasing trend with increasing concentration of initiator which was also observed in T-1OH system. An 8 times decrease in the template incorporation was observed with increasing initiator concentration from 75.3 ± 0.95 μmol/g for T-3OH-1:100 to 9.5 ± μmol/g for T-3OH-1:5. In the case of the 3OH rebinding, T-3OH-1:50 MIP exhibited the highest amount of bound template
(2.18 ± 0.01 μmol/g) and gradually decreased to 1.15 ± 0.01 μmol/g by T-3OH-1:5, which is ~2 times lower than the amount obtained from T-3OH-1:50. The decreasing trend (from lowest to highest initiator concentration) in the template incorporation and MIP binding were also observed with the T-1OH system which is attributed to the higher branching density of the T-3OH-1:5 polymers that limits the trapping and accessibility of template during imprinting and rebinding process. The translation of incorporation sites to binding sites (amount of template rebound with respect to the amount of template incorporated by the polymers) was observed to increase 5 times, from a low of 2.4% with T-3OH-1:100 to 12.2% with T-3OH-1:5.

The amount of template bound by the NIP is comparable regardless of the concentration of initiator with the highest value of 1.00 μmol/g and lowest of 0.85 μmol/g, which is similar to the observed binding of NIP of T-1OH system which was attributed to the probability of inter-monomer interaction due to higher incorporation of MAA in the polymers. Comparing the template bound by the NIP with the corresponding MIP, the calculated imprinting factors ranges from 1.4-1.8 with the highest value recorded both by T-3OH-1:50 and T-3OH-1:100. This behaviour was also observed in the T-1OH system; with the polymers produced in highest concentration of initiator exhibited lowest template incorporation and template rebinding capacity but highest binding site conversion.
Figure 4. 14. Amount of template rebound by the T-3OH MIPs and the NIPs at varying I:TM ratios. Unreacted components remaining in the post polymerisation solution was quantified by \(^1\)H NMR using the -CH=C- peak of T-3OH at 6.11 ppm and 1,4-dioxane in DMSO-\(d_6\) at 3.57 ppm as the reference standard.

4.2.1.3.4. Characterisation of binding sites

Similar binding isotherm models were used to characterize the binding sites of T-3OH-1:100 polymers as shown in Figure 4.15 and the binding parameters, \(N\) and \(K_a\) obtained for each models were summarized in Table 4.6. Figure 4.16 A displays the higher template loading of the MIP with a recorded \(N\) of 2.17 ± 0.12 µmol/g, which is 2 times higher than for its NIP (1.26 ± 0.07 µmol/g). The \(K_a\) recorded for the MIP (1.2 ± 0.11 x10\(^5\) M\(^{-1}\)) and the NIP (1.0 ± 0.12 x10\(^5\) M\(^{-1}\)) are comparable. These binding site characteristics (higher \(N\) MIP but comparable \(K_a\)) were also observed in the other systems which indicate the more prevalent influence of the binding site capacity in the imprinting process consistent with the IF of 1.8 recorded in the template rebinding process. Comparable binding parameters to the NLL model were calculated in using LL model.
Figure 4. Binding properties of the T-3OH-1:100 polymers in different binding models, Non-linear Langmuir model (A) and Linearized Langmuir model (B)
Table 4.6. Affinity constants ($K_a$) and number of binding sites ($N$) of T-3OH polymers determined by the non-linear Langmuir (NLL) and linearized Langmuir (LL) models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>MIP</th>
<th>NIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-linear Langmuir model¹</td>
<td>$K$ ($x10^5$ M$^{-1}$)</td>
<td>1.21 ± 0.11</td>
<td>0.97 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>$N$ ($\mu$mol/g)</td>
<td>2.17 ± 0.12</td>
<td>1.26 ± 0.07</td>
</tr>
<tr>
<td>Linearized Langmuir model</td>
<td>$K$ ($x10^5$ M$^{-1}$)</td>
<td>1.36 ± 0.10</td>
<td>1.12 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>$N$ ($\mu$mol/g)</td>
<td>2.07 ± 0.12</td>
<td>1.18 ± 0.09</td>
</tr>
</tbody>
</table>

¹ values were determined from Prism GraphPad using the one-site hyperbola model.

4.2.1.4. Comparison of the thermally-synthesized MIP systems

All microspheres (MIPs and NIPs) preserved the initial FM:XL ratio of 1:5 regardless of the concentration of initiator and the template. The incorporation of the templates, however, has been found to be dependent on the concentration of initiator. In the case of T-1OH and T-3OH, template incorporation increased with decreasing concentration of initiator. This could be due to the disruption of the T-FM complexes during polymerisation process attributed to the release of heat from the decomposition reaction of high initiator concentration. In addition, this could also be due to the high branching density of the polymers that prevents the access of the template in the polymers during imprinting process.

Comparing the three MIP systems, 1OH incorporated twice as much as that of 3OH at all I:TM ratios. This is in contrary to the expectation that the number of interaction points of the template is proportional to the imprinting efficiency of the polymers due to the stronger interaction between the template and the functional monomer. Moreover, comparable
IF values were calculated for the T-1OH and T-3OH systems due to similar MIP and NIP binding. This could be due to analogous affinity of the only type of functional group of 1OH and 3OH. Therefore, on the basis of IF values, the interaction based theory is not applicable in these systems which is in agreement with the study conducted by Spivak et al.\textsuperscript{146} In terms of binding site conversion, lowest binding site conversion was recorded by T-1OH systems. It would seem that, in the case of T-1OH, most of the incorporated template did not result to high fidelity imprints.

Based from the number of interaction points of 2OH, it is expected that template incorporation of T-2OH polymers is higher than the T-1OH counterparts but lower than the T-3OH counterparts, however, T-2OH polymers incorporated lower template than T-1OH polymers which could be explained by the possibility of side reactions. Previous reports have indicated that, in the presence of radicals, even at low temperature (15 °C)\textsuperscript{182}2OH can undergo side reactions including dimerisation with itself according to the reaction shown in Figure 4.216.\textsuperscript{183-186} \textsuperscript{1}H NMR analysis of the post-polymerisation solution supports this hypothesis. This is supported by the \textsuperscript{1}H NMR results showing a reduction of ~ 16% (equivalent to ~ 16% of the template) of the integration of the 6.47 ppm peak (2OH-H4) that otherwise should have been half the integration of the signal at 7.02 ppm (2OH-H2). The probability of the dimerisation reaction of 2OH is higher in higher concentration of initiator which could explain the low template incorporation in this system.
Figure 4. 16. Dimerisation reaction of 2OH in the presence of a radical. 184

Table 4.7 shows that there is no significant difference between the $K_a$ values (considering the errors) for each MIP system prepared using the same concentration of initiator (I:TM=1:100) based on the NLL model. However, marked difference in the number of binding sites or $N$ has been observed. While T-1OH recorded $N$ comparable to the T-3OH system, these $N$ values are ~1.6 times higher than that obtained for the T-2OH system. The low $N$ value for 2OH polymers combined with the possibility of imprinting the 2OH dimers could be the reason for the short saturation period for T-2OH polymers compared to T-3OH (75 mins) which were both measured by $^1$H NMR. The bigger binding cavities formed due to imprinting 2OH dimers can accommodate higher concentration of 2OH during the time rebinding experiment and since there is a low $N$ formed in T-2OH polymers, these binding sites were saturated faster. In the case of their NIP counterparts, comparable $N$ values for the three systems were calculated (in consideration of the errors) In each system, the $N$ value recorded for the NIP is 2 times lower than the MIP counterpart.

Binding isotherms for the best performing 2OH MIP (T-2OH-1:5) and 3OH MIP (T-3OH-1:50), based on IF, were also determined. T-2OH-1:5 gave the highest imprinting factor (1.34) among the T-2OH MIPs despite its MIP displaying the lowest amount of template bound (0.86 ± 0.03 µmol/g), while T-2OH-1:100 recorded the highest MIP binding (1.34 ± 0.01
μmol/g). Comparing the $K_a$ of the MIPs of T-2OH-1:100 and T-2OH-1:5 (Table 4.7), the $K_a$ of T-2OH-1:5 is 2 times that of T-2OH-1:100, but the $N$ value of T-2OH-1:5 is 2 times lower than T-2OH-1:100. The higher value of $K_a$ recorded for T-2OH-1:5 polymers could be attributed to the fast polymerisation of the T-FM complexes preserving higher order complexes forming high affinity binding sites in lower concentration, which was observed by Turner et al. in comparing the faster polymerisation of caffeine imprinted polymers by microwave with thermal initiation. These results indicate that regardless of the high $K_a$ of T-2OH-1:5 system, the loading capacity of the 2OH to the polymers is limited since there are only small number of binding sites with the calculated affinity towards 2OH which was explained in detail in the proposed mechanism of the formation of binding sites in non-covalent imprinting by Rampey et al. In the case of the NIP of the two systems, comparable values of $K_a$ and $N$ were observed suggesting successful imprinting of 2OH.

The best performing T-3OH system based on the IF (1.83) is T-3OH-1:50 with a MIP binding of 2.18 ± 0.01 μmol/g. The recorded $K_a$ and $N$ values of T-3OH-1:50 is comparable with the T-3OH-1:100 as summarized in Table 4.7. It is noteworthy to mention that the $N$ of T-3OH-1:50 is 1.2 times higher than T-3OH-1:100 which is consistent with the difference in the IF values of the two MIP systems.

The association constants of the three templates with the polymers is, as expected, comparable since there is only one type of functional group (hydroxyl, OH) present in the three templates. Therefore, it is more useful to compare the number of binding sites, $N$ of the polymers rather than its $K_a$ which was also suggested by Ansell and Rampey. It was suggested that the reason that there is a wide range of association constants being reported in a single polymer system is due to the dependency of the $K_a$ values to the range of the concentration of the template
being investigated and therefore, the number of binding sites, N, are a more accurate measurement of the binding efficiency of MIPs. From this, we opted to present the ratio of the N of the MIP and its corresponding NIP (\(N_{\text{MIP}}/N_{\text{NIP}}\)) as a measure of the polymer efficiency. It is shown in Table 4.7 that the \(N_{\text{MIP}}/N_{\text{NIP}}\) of the polymers is comparable with the calculated IF of each system suggesting that the ratios of N can be a possible alternative measurement of the binding efficiencies of the polymers.

**Table 4.7.** Binding site properties of the MIPs of the three system.

| Polymers | Non-linear Langmuir model\(^1\) (NLL) |  |
|----------|--------------------------------------|--|---|
| MIP system | I:TM | \(K\) (X10^2 M\(^{-1}\)) | \(N\) (µmol/g) | \(N_{\text{MIP}}/N_{\text{NIP}}\) |
| T-1OH | 1:100 MIP | 1.30±0.09 | 2.59±0.24 | 1.82 |
| | | 1.16 ±0.12 | 1.42±0.11 | |
| T-2OH | 1:100 MIP | 1.24±0.06 | 1.60±0.09 | 1.29 |
| | | 0.92±0.09 | 1.24±0.11 | |
| | 1:5 MIP | 2.50±0.16 | 0.92±0.02 | 1.29 |
| | | 2.20±0.15 | 0.71±0.11 | |
| T-3OH | 1:100 MIP | 1.21±0.11 | 2.17±0.12 | 1.72 |
| | | 0.97±0.12 | 1.26±0.07 | |
| | 1:50 MIP | 0.99±0.16 | 2.56±0.13 | 1.92 |
| | | 0.83±0.13 | 1.33±0.10 | |

\(^1\) values were determined from Prism GraphPad using the one-site hyperbola model
In the case of the particle sizes, similar effects were observed in the three systems, that is, particle sizes increase with increasing concentration of initiator, with sizes ranging from 80-160 nm and MIPs observed to be smaller than its NIP counterparts. This is consistent with the finding noticed by the group of Long et al. where MIPs are generally smaller than the NIP due to the attractive forces towards the template/center that affects the size of particle during nucleation stage. 53

4.2.1.5. Cross-reactivity studies

Cross-reactivity studies, i.e. non-competitive binding, were conducted using the best performing MIP systems (chosen in terms of their exhibited IFs): T-1OH-1:100 for T-1OH, T-2OH-1:5 for T-2OH and T-3OH-1:50 for T-3OH, against the other two analogues. The results are summarised in Figure 4.17 and Table 4.8.

Table 4. 8. Selectivity indices of the thermally synthesized MIPs investigated for cross-reactivity studies calculated from data presented in Figure 4.19A.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Selectivity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1OH</td>
</tr>
<tr>
<td>T-1OH-1:100</td>
<td>1.00</td>
</tr>
<tr>
<td>T-2OH-1:5</td>
<td>1.00</td>
</tr>
<tr>
<td>T-3OH-1:50</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Selectivity index (SI) = IF<sub>analogue</sub>/IF<sub>template</sub>
The binding capacities of the NIP towards its template and the analogues are comparable as shown in Figure 4.17 B: T-1OH-1:100 NIP bound ~1.2 μmol/g of 2OH and 3OH, T-2OH-1:5 rebound ~ 0.7μmol/g of 1OH and 3OH and T-3OH-1:50 bound ~ 0.9 μmol/g of 1OH and 2OH after the corresponding incubation periods. The comparable NIP binding towards the template and the other analogues indicates that the NIPs do not possess the capability to discriminate the structural differences of the three templates as expected with superficial binding.
As shown in Table 4.8, T-1OH-1:100 MIP displayed comparable low selectivity towards 2OH (SI = 0.78) and 3OH (SI = 0.73). This is probably due to the slightly bigger size of 1OH (5.21 x 5.94 Å), forming larger imprints allowing access to the slightly smaller 2OH with size measured in its widest point 5.84 x 5.55 Å and 3OH (5.55 x 5.55 Å) molecules. In the case of T-2OH-1:5 MIP, SI ≥ 1 was recorded for both 1OH and 3OH indicating its non-selectivity. This could be due to the imprinting of the dimerized 2OH (~16% of the concentration of the 2OH incorporated) instead of 2OH, which is bulkier than 2OH alone thus the cavities formed are bigger allowing access to any of the analogues which also explains the higher value of SI (poor selectivity) of 2OH than T-1OH-1:100 polymers investigated. Unlike the other two systems, T-3OH-1:50 polymers displayed moderate discrimination against 1OH (SI = 0.58) and 2OH (SI = 0.67) molecules which could be influenced by size and interaction. The alkyl groups of 1OH make it slightly bigger than the other two phenolic templates investigated, and based from this, less 1OH was rebound than 2OH analogues by T-3OH polymers. Additionally, 2OH has more interaction points than 1OH that could facilitate the rebinding process of 2OH to the T-3OH polymers.

4.2.1.6. Molecular modelling and $^1$H NMR Interaction Studies

In order to further understand the results of the rebinding and the cross-reactivity studies molecular modelling and $^1$H NMR titration was conducted in using the three phenolic templates. The interaction between the phenolic template and functional monomer MAA was investigated by molecular modelling (Spartan Wavefunction ‘14) and $^1$H NMR studies employing the 1:4 T:FM ratio used in all imprinting experiments.
The computer generated simulation of a 1:4 1OH:MAA cluster given in Figure 4.18 A shows H-bonding interaction between the O atom of one molecule of MAA with the H atom of 1OH (2.19 Å) while the three other MAA molecules are interacting with one another via H-bonding between their oxygens and hydrogens. These inter-monomer interactions are expected due to the lack of interaction points with the template and have been documented before. 49, 187-190 The H-bonding between 1OH and MAA is also evident in the 1H NMR experiment summarized in Figure 4.19 A. The hydrogen of 1OH in position 2 (1OH-H2) shows the highest downfield movement of 0.06 ppm which indicates that the interaction of the hydroxyl group of 1OH attached to carbon in position 1 created a partially negative environment to the adjacent carbon (1OH-C2) and the attached 1OH-H2 hydrogen. The H-bonding interaction, which involves the electron acceptance of the hydroxyl group of 1OH, caused a shielding effect causing an upfield movement (0.062 ppm) to the carbon to where it is attached (1OH-C1). The binding energy of this cluster was estimated from semi-empirical mechanic PM3 method (Spartan Wavefunction ‘14) equivalent to $\Delta E_{\text{binding}} = -62.64 \text{ kJ/mol.}$
Figure 4. Electrostatic potential maps, molecular interactions and corresponding distances in 1OH-4MAA cluster between the template 1OH (A), and the analogues 2OH (B) and 3OH (C) measured by Spartan ‘14 v1.1.8. Intermonomer distances observed (data not shown) were also observed with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding interactions.
Figure 4. Chemical shifts of protons (A) of MAA and each of the three template monitored by $^1$H NMR and carbons (B) monitored by $^{13}$C NMR. Molecular structures of the template; 1OH $R_1$ and $R_2 = CH_3$, 2OH $R_1 = OH$ and $R_2 = CH_3$ and 3OH $R_1$ and $R_2 = OH$, and the functional monomer MAA with the atom labelling for the NMR interaction studies.
The interactions of the 2OH and MAA were also analyzed in similar fashion as 1OH. From Figure 4.20 A, the two hydroxyl groups of the 2OH are observed to be interacting with all of the four molecules of MAA and the interactions involve the oxygen and the hydrogen of the hydroxyl moieties of 2OH and the calculated $\Delta E_{binding}$ is -103.24 kJ/mol. It was observed in the molecular modelling image that the hydroxyl group of 2OH (2OH-C1) interacts with MAA-1 and MAA-2 with H-bond distances of 2.10 and 2.17 Å, respectively. The other hydroxyl group of 2OH (2OH-C3) interacts with MAA-3 and MAA-4 with 2.17 and 2.10 Å H-bond distances respectively. These sets of interactions are effective since all four moles of MAA are converging towards the template forming a shape-specific cavity for 2OH. This cavity is favoured in imprinting process which explains the higher binding site conversion for the 2OH MIP system than its 1OH system counterparts. The interactions observed in the simulation of 2OH and 4 molecules of MAA are supported by the movements in the chemical shifts recorded from the $^1$H NMR and $^{13}$C NMR studies performed. The movement of the proton in position 4 (2OH-H4) to the deshielded region is possibly due to the more prevalent H-bond donation of the hydroxyl group attached to 2OH-C3 to two different molecules of MAA. The 2OH-H2 proton recorded the highest movement in chemical shift (0.017 ppm) which can be attributed to the extent of the combination of H-bond interactions of the two hydroxyl groups attached to 2OH-C1 and 2OH-C3. This is supported by the movement of the carbon in position 2 which exhibited a movement of 0.020 ppm. The movements of 2OH-C2 (0.020 ppm) and 2OH-C4 (0.010 ppm) to the upfield region also confirmed the H-bond acceptance interaction of the oxygen atoms of the hydroxyl group of 2OH.
Figure 4. 20. Electrostatic potential maps, molecular interactions and corresponding distances in 2OH-4MAA cluster between the template 2OH (A), and the analogues 1OH (B) and 3OH (C) measured by Spartan '14 v1.1.8. Intermonomer distances observed (data not shown) were also observed with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding interactions.
Figure 4.21A shows the set of interactions of the most stable complex formed between the template 3OH and 4 molecules of MAA from computer generated images. The simulation of the 3OH with MAA, shows that the hydrogens of only two of the three hydroxyl groups are interacting with the carbonyl oxygen of only two molecules of MAAs displaying H-bonding interaction with an average distance of 2.2 Å and a $\Delta E_{\text{binding}}$ -67.60 kJ/mol. The four molecules of MAA formed a network of H-bonding interactions forming a cavity encapsulating the 3OH template, which may be the reason for the creation of high fidelity binding sites manifested by high binding site conversion presented in the previous section. The H-bond interaction between hydroxyl hydrogen (3OH-C3) and the carbonyl oxygen of MAA was confirmed by the movement of the chemical shift (0.0010 ppm) of the proton 3OH-H2 towards the upfield region detected by $^1$H NMR. The carbon to where the hydroxyl groups are attached (3OH-C1) recorded a movement of 0.0120 ppm towards the deshielded region, validating the deshielding effect due to the donation of H-bond to MAAs. This H-bond donation interaction also produced movement to the adjacent carbon nuclei (3OH-C2) of 0.010 ppm towards the deshielded region.
Figure 4. Electrostatic potential maps, molecular interactions and corresponding distances in 3OH-4MAA cluster between the template 3OH (A), and the analogues 1OH (B) and 2OH (C) measured by Spartan ‘14 v1.1.8. Inter-monomer interactions were also observed (data not shown) with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding interactions.
The movements of the proton and carbon nuclei of the functional monomer were also monitored by $^1$H and $^{13}$C NMR, which both showed that all of the MAA signals exhibited movements indicating several interactions which supports the results from simulation studies of MAA dimerisation by H-bonding regardless of the template used. The allylic hydrogen at 1.85 ppm is swamped by the peak signal of acetonitrile at 2.10 ppm, thus for this case is not discernible. The two vinylic hydrogen signals of MAAs displayed movements with values ranging from 0.001-0.010 ppm. As for the movements of the carbon nuclei, the recorded shifts is from 0.10-0.20 ppm for the four carbon atoms of MAA.

Further molecular modelling experiments were conducted in an attempt to explain the results of the cross-reactivity studies. This time, the minimum energy conformation of the monomer cluster formed after template interaction was frozen and the template was replaced with the analogues tested. It is noteworthy to mention that the modelling experiments conducted are based on the most stable complex formed between the template and the functional monomer and did not take into account the heterogeneity of the binding sites in the polymers. Figure 4.18 illustrates that similar interaction were exhibited by 2OH (Figure 4.18 B) and 3OH (Figure 4.18 C) when used as analogues in the cavity imprinted with 1OH. The moderate selectivity of the T-1OH-1:100 polymers towards 2OH and 3OH analogues could be due to the smaller sizes of 2OH and 3OH than the template (1OH) that can easily fit into the cavity imprinted with 1OH. In the case of 2OH imprinted polymers, similar spatial arrangements were observed when the two analogues 1OH (Figure 4.20 B) and 3OH (Figure 4.20 C) replaced 2OH around the monomer cluster of 4 MAAs. However, in the case of 1OH, the H of the lone hydroxyl group of the 1OH displayed interaction with the oxygen atom of MAA-3 (bond distance of 2.19 Å) and the hydroxyl hydrogen of MAA-4 (bond distance of 2.11 Å) of the cluster which could explain the
non-selectivity of T-2OH-1:5 towards the 1OH analogue. With the 3OH analogue, on the other hand (Figure 4.20 A), 2 of the 3 hydroxyl groups showed analogous H-bond interactions and distances (2.1-2.2 Å) as was observed with 2OH with the 4 MAA units in the cluster. In addition, since 3OH is smaller than 2OH, it could easily fit in to the 2OH sphere resulting in non-selectivity of the 2OH MIP. There is also the possibility of imprinting the 2OH dimer specifically for T-2OH-1:5 which was synthesized in high concentration of initiator. The measurement of the dimer in its widest point is 10.57 X 5.89 Å. If this was the case, both 1OH and 3OH could easily fit into the imprinted sites. In addition, the cavity formed by imprinting the 2OH dimer exhibited stronger H-bonding interaction as indicated by the H-bond distances observed in molecular modelling in Figure 4.22: 1.80 Å with MAA-1, 1.84 Å with MAA-3 and 1.90 Å with MAA-4. Based upon the molecular modelling of the cluster and the dimer, the estimated ΔE_{binding} is – 729.55 kJ/mol. This value implies that the formation of the 2OH dimer with MAA cluster is the most stable among the other clusters (1OH, 2OH and 3OH with 4 units of MAA), which is the reason for the high probability of imprinting the 2OH dimer instead of the 2OH. Among the three imprinted polymers investigated for cross-reactivity studies, T-3OH-1:50 polymers exhibited the highest selectivity towards its template. In replacing 3OH with 1OH in the 4 MAA clusters formed during the imprinting process (Figure 4.21 B), a weak H-bonding was observed with a distance of only 2.20 Å, which explains the low selectivity of the polymers towards the 1OH molecule. In investigating the interaction of 3OH in the MAA cluster formed in imprinting 2OH, it revealed that similar H-bond interactions were observed in using 3OH as analogue which can be the reason for the comparable selectivity of the T-2OH-1:5 polymers towards 3OH.
Figure 4. 22. Electrostatic potential maps, molecular interactions and corresponding distances in between the 2OH dimer and 4 MAA measured by Spartan ‘14 v1.1.8. Inter-monomer interactions were also observed (data not shown) with distances ranging from 2.0-2.4 Å which corresponds to weak H-bonding interactions.

4.2.1.7. Polymer surface area and porosity

Due to constraints in instrument access, surface areas were measured only for selected samples given in Table 4.9. In order to determine the effect of the nature of the template on the morphology of the resulting polymers three MIP systems prepared in low concentration of initiator were selected (T-1OH, T-2OH and T-3OH-1:100). In addition, to determine the effects of the concentration of initiator in the morphology of the polymers, T-2OH-1:100 and T-2OH-
1:5 were chosen. Samples were measured by nitrogen absorption and analyzed using BET. The NIPs of the three T-1:100 systems showed comparable surface areas: T-1OH-1:100 NIP recorded a surface area of $90.86 \pm 1.11 \text{ m}^2/\text{g}$, T-2OH-1:100 NIP recorded a surface area of $90.32 \pm 0.87 \text{ m}^2/\text{g}$ and T-3OH-1:100 NIP gave a value of $110.36 \pm 1.54 \text{ m}^2/\text{g}$. Interestingly, their MIPs gave variable surface areas. T-1OH-1:100 MIP showed a surface area equivalent to $84.87 \pm 0.52 \text{ m}^2/\text{g}$ which is comparable to the surface area of its NIP which suggests that the presence of 1OH did not affect the surface area of the polymer. T-2OH-1:100 MIP, on the other hand, recorded a surface area surface area of $132.95 \pm 2.29 \text{ m}^2/\text{g}$ 1.5 times higher than its NIP counterpart, and T-3OH-1:100-MIP recorded a value half of the measured surface area of its NIP counterpart ($51.98 \pm 0.46 \text{ m}^2/\text{g}$). For the 2OH and 3OH polymers, the effect of the presence of the template is more pronounced than 1OH polymers.

In the case of varying the concentration of initiator, it would seem that lower surface area is exhibited by the polymers (both MIP and NIP) synthesized in higher concentration of initiator: T-2OH-100 MIP measured $132.95 \pm 2.29$ and T-2OH-1:5 MIP gave a surface area of $34.68 \pm 0.51$, and T-2OH-1:100 NIP recorded a surface area of $90.33 \pm 0.87$ and T-2OH-1:5 NIP with $33.30 \pm 0.37$. 
Table 4.9. BET surface areas (m²/g), average pore volumes (cm³/g) and average pore sizes (Å) of selected microspheres.

<table>
<thead>
<tr>
<th>MIP system</th>
<th>I:TM</th>
<th>BET surface area (m²/g)</th>
<th>Av Pore Volume (cm³/g)</th>
<th>Av Pore Size (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIP</td>
<td>NIP</td>
<td>MIP</td>
</tr>
<tr>
<td>T-1OH</td>
<td>1:100</td>
<td>84.87 ± 0.52</td>
<td>90.86 ± 1.11</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>25.87 ± 0.13</td>
<td>-</td>
<td>0.029</td>
</tr>
<tr>
<td>T-2OH</td>
<td>1:100</td>
<td>132.95 ± 2.29</td>
<td>90.33 ± 0.87</td>
<td>2.945</td>
</tr>
<tr>
<td></td>
<td>1:5</td>
<td>34.68 ± 0.51</td>
<td>33.30 ± 0.37</td>
<td>0.037</td>
</tr>
<tr>
<td>T-3OH</td>
<td>1:100</td>
<td>51.98 ± 0.46</td>
<td>110.36 ± 1.54</td>
<td>0.027</td>
</tr>
</tbody>
</table>

The average pore volumes measured for the selected MIPs and NIPs range from 0.027-2.945 (cm³/g) and 0.050-0.112 (cm³/g), respectively. Examining the pore volume obtained for T-2OH-1:100 MIP, the magnitude of the value is 100x compared to the other values measured for the MIPs, and could be erroneous, thus, we recommend repeating the measurement. In the case of pore sizes, comparable pore sizes for the MIP and the NIP of 1OH were observed while for the 2OH and 3OH, the difference is more pronounced as the number of hydroxyl increases (T-3OH-1:100 MIP = 10Å). Nevertheless, it is not clear from this results if, aside from the porogen, the pore size is also affected by the nature of the template.

Due to instrument access constraints, pore sizes were only measured for two representative samples in order to compare the effect of the concentration of initiator in the porosity of the polymers. Pore sizes are normally categorized into three sizes: micropores (< 2 nm), mesopores (2-50 nm) and macropores (> 50 nm)\textsuperscript{191} From Figure 4.23, it can be observed that the MIP of T-1OH-1:100 is more porous than the T-1OH-1:5 MIP counterpart with a pore
volume ratio of 3 and is consist of high proportion of macropores with average pore widths of 69-285 Å. T-1OH-1:5 MIP on the other hand, contains only four types of pores with average pore widths of 82-228 Å. The pore sizes observed with the MIPs of the two systems are wide enough to allow easy access of the template 1OH with a size of only 5.21 x 5.94 Å at its widest points. The higher porosity observed in T-1OH-1:100 polymers could be the reason for the higher MIP binding recorded than the T-1OH-1:5 MIP. The results of the porosity measurements demonstrated that the concentration of the initiator affects the porosity (cm$^3$/g) of the resulting MIPs, however, it is still recommended to measure other systems (T-2OH and 3OH with I:TM of 1:100 and 1:5) in order to obtain a more comprehensive result relating the effect of initiator to the porosity of the polymers.

Figure 4. 23. Incremental pore volume versus pore width plots for MIPs of T-1OH-1:100 and 1:5.
4.2.1.7.1. Binding with respect to surface area

The binding capacities of the MIPs are more commonly expressed with respect to mass (μmol/g). This method is acceptable if the sizes of the MIPs and NIPs are comparable to one another. Therefore, this can be applied to monoliths prepared by bulk polymerisation, wherein polymers are ground and sieved into desired size ranges, thus the difference in the particle size of the MIP and NIP is insignificant. As for microspheres, especially prepared by precipitation polymerisation, some published documents reported noticeable differences in the sizes of the MIPs and NIPs, which can affect the normalization of the imprinting efficiency.49, 99 Due to this observable difference in the particle sizes, it is equally important to express the binding performance of the polymers with respect to surface area (μmol/m²).143, 192 193-194

In the case of T-1OH-1:100 polymers, the IF calculated with respect to the mass and surface area is comparable (~1.8-1.9) due to comparable surface area of the MIP and NIP (Figure 4.24), which was also in agreement with the particle size or \(d_H\) observed in DLS. In contrast to T-1OH polymers, T-2OH polymers exhibited a lower IF (0.9) when normalized in terms of surface area. The calculated IF normalized in terms of surface area indicated higher non-specific binding and proves the unsuccessful imprinting of 2OH. Lastly, normalizing the binding performance of T-3OH-1:100 polymers with respect to surface area, it showed that the IF increased 2 times, from IF = 1.8, it increased ~2 x to IF= 3.8 and this is due to the significant difference in the surface area between T-3OH-1:100 MIP and NIP. The measured surface area for the T-3OH-1:100 system revealed that despite of the comparable particle sizes of the MIP and the NIP, the difference is significantly different. Thus, the normalization of the binding performance with
respect to surface area is a more accurate measurement of the binding performance of MIP microspheres.

**Figure 4.** Comparison of the binding capacity of the T-1:100 MIP systems using different phenolic templates normalized with respect to mass (A) and surface area (B).
In order to further understand the imprinting efficiency of T-2OH system, T-2OH-1:5 was also normalized with respect to surface area and as seen in Figure 4.25, the IF of T-2OH-1:5 polymers did not change significantly. In the case of T-2OH-1:100 polymers, even though the template bound by MIP normalized with respect to surface area is lower than its corresponding NIP, in consideration of the errors, the values are comparable.

Figure 4. 25. Comparison of the binding capacity of T-1OH polymers in different concentrations of initiator normalized with respect to mass (A) and surface area (B).
4.2.2. Photochemically synthesized imprinted microspheres

The previous section (Section 4.2.1) discussed the thermally-synthesized MIPs, particularly the effect of the concentration of initiator in the imprinting and binding efficiencies of polymers using templates with one (1OH) and multiple points (2OH and 3OH) of interaction. In this part of the study, the three MIP systems using the three phenolic templates 1OH, 2OH and 3OH were photo-chemically synthesized by precipitation polymerisation. Photo-chemical polymerisations were carried out using an immersion apparatus supplied by the Ace-Hanovia Lamp Division of Ace Glass Incorporated. The lamp used was a 450-watt, medium-pressure, quartz, mercury arc, Type Number 7825-34 while maintaining the temperature of the water bath between 27-35°C. The total energy radiated by the lamp is 40-48% in the UV portion of the spectrum (222.4 -366.0 nm and maximum watts of 25.6) and is enough to initiate the formation of radical species from AIBN, which forms at 360 nm. Similar formulations and components were used as the thermally-synthesized counterparts: EGDMA as the cross-linker, MAA as the functional monomer, acetonitrile as porogen and AIBN as the initiator, with T:FM:XL of 1:4:20 and solvent to monomer ratio of 10 mL/mmol.

4.2.2.1. 3,5-dimethylphenol MIPs (P-1OH)

4.2.2.1.1. Polymer composition

The incorporation of each of the components in the polymer were quantified in similar way as the thermally synthesized polymers: using $^1$H NMR using 1,4-dioxane as the reference standard and by monitoring similar peaks for EGDMA (O-CH2 at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH$_2$=C- less the O-CH$_2$ EGDMA peak), and 6.75
ppm for 1OH peak. The initial amounts of the components were compared with the amount of the unreacted components remaining in the post polymerisation

P-1OH MIPs incorporated similar amounts of EGDMA as with their NIP counterparts for all of the I:TM experiments conducted which range from 68-74\% with respect to the initial amount as tabulated in Table 4.10. Similar behaviour was also observed with the incorporation of MAA, no considerable difference was noted in comparing the imprinted and non-imprinted polymers and between the microspheres synthesized using different amounts of initiator. These translated to a FM:XL ratio of 1:6 with\% yield between 65-76 \%. The MIPs and the NIPs for each I:TM experiment have comparable isolated yields except for the P-1OH-1:5 polymers wherein the NIP has an average of 93.3 ± 0.1 \% and the MIP has only 76.2 ± 14.2 \%. The low isolated yield might be attributed to the loss of polymers during extraction and drying process.
Table 4.10. Composition and degree of cross-linking for photo-chemically-synthesized 3,5-dimethylphenol (P-1OH) polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM&lt;sup&gt;1&lt;/sup&gt; (%initiator)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition T :FM : XL&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Reacted Double Bonds&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% incorporation (μmol/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-1OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs 70 ± 1 (582) 62 ± 1 (104) 13 ± 1 (5.20)</td>
<td>0.05:1:5.6</td>
<td>67.4 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 69 ± 2 (572) 60 ± 1 (101)</td>
<td>1:5.7</td>
<td>66.2 ± 0.01</td>
</tr>
<tr>
<td>P-1OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs 68 ± 1 (565) 60 ± 0 (100) 21 ± 0 (8.80)</td>
<td>0.09:1:5.6</td>
<td>69.5 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 67 ± 1 (566) 59 ± 1 (98)</td>
<td>1:5.8</td>
<td>68.4 ± 0.03</td>
</tr>
<tr>
<td>P-1OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs 70 ± 0 (584) 58 ± 1 (96) 21 ± 0 (8.90)</td>
<td>0.09:1:6.1</td>
<td>65.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 66 ± 1 (556) 64 ± 0 (98)</td>
<td>1:5.7</td>
<td>66.8 ± 0.01</td>
</tr>
<tr>
<td>P-1OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs 74 ± 1 (614) 57 ± 2 (96) 21 ± 1 (9.00)</td>
<td>0.09:1:6.4</td>
<td>61.1 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 73 ± 1 (611) 70 ± 2 (117)</td>
<td>1:5.2</td>
<td>65.3 ± 0.01</td>
</tr>
<tr>
<td>P-1OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs 73 ± 1 (606) 62 ± 1 (103) 21 ± 1 (8.70)</td>
<td>0.08:1:5.9</td>
<td>68.9 ± 0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs 70 ± 2 (587) 68 ± 1 (117)</td>
<td>1:5.2</td>
<td>69.5 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>1</sup>I:TM = initiator (mmol): total monomer (mmol EGDMA+ mmol MAA), <sup>2</sup>% initiator = amount (mol) of initiator/amount of total monomer (mol), <sup>3</sup>T:FM:XL = template : functional monomer :cross-linker (mol) ratio in the polymers, <sup>4</sup>Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and -C=O with respect to the peak height ratio of pure EGDMA.

The incorporation of the template within the P-1OH polymers from 1:100 to 1:10 I:TM ratios is comparable (21%) and dropped to 13 ± 1% when I:TM = 1:5 as summarized in Figure 4.26. This result might be due to the disruption of the T-FM complex due to the high concentration of initiator which was discussed in the published study of Mijangos et al. Unlike
its thermal counterpart, the amount of template incorporated by P-1OH MIP are ~2 times lower than that of T-1OH which is probably due to the possible side reaction of 1OH under UV irradiation. Photodegradation of the three phenolic templates was observed when the templates were exposed under UV radiation. In the case of 1OH, 25% of 1OH degraded after 24 hr exposure to UV radiation as quantified using $^1$H NMR. Further evidence is provided by the observed movement of the chemical shifts of 1OH (from 6.52 to 6.75 ppm to 6.12 and 6.17 ppm) in the post polymerisation solution which suggests that 1OH could have reacted to form a dimer \textsuperscript{195-196}. This side reaction is discussed further in the next section of this chapter.

![Graph showing percentages of 1OH incorporated in the photo-chemically synthesized polymers in various I:TM ratios.](image)

**Figure 4.** 26. Percentages of 1OH incorporated in the photo-chemically synthesized polymers in various I:TM ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

### 4.2.2.1.2. Morphology and Particle Size

The difference in $d_H$ particle sizes of the photochemically synthesized 1OH polymers for every I:TM experiments is more noticeable than their thermally synthesized counterparts for both MIP and NIP. The particle sizes of the microspheres measured by DLS and SEM are gradually
increasing with increasing amount of initiator in the feed, P-1OH-1:100 polymers (~225 nm) is triple the size of the P-1OH-1:5 (~75 nm) polymers. Dry particle size of P-1OH-1:100, estimated from SEM images, were ~100 nm smaller than the DLS-derived sizes with average values of 130.8 ± 6.7 nm for the MIP and 144.6 ± 3.1 nm for the NIP. Despite the differences in the measured sizes between the SEM and DLS, the apparent size difference between the 1:5 and 1:100 polymers are obvious in the SEM images shown in Figure 4.27. It also noticeable that the polymers of P-1OH-1:5 (Figure 4.27 C and D) are more aggregated and interconnected to one another and appears to approach monolithic morphology, which has been documented in previous researches.162, 197-198 The formation of smaller particles at lower reaction temperature has been documented in previous study conducted by Li et al. and attributed to increase in viscosity that led to a decrease in the solubility of the monomers in the solvent forming smaller particles.162, 197 Moreover, the bulk-like morphology of the P-1OH-1:5 polymers might be attributed to the combination of the decrease of the quality of the solvent (e.g. viscosity) and as well as the overall reaction that led to domino effects of complete collapse of the polymer network during nucleation stage followed by the loss of colloidal stability that led to overlapping and cross-linking of the surfaces of individual microspheres.152, 198-199
Figure 4. SEM images of photochemically synthesized polymers. P-1OH-1:100 MIP (A) and NIP (B) and P-1OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM.

The degree of cross-linking in the polymers tabulated in Table 4.10 recorded values ranging from 65-69%. It suggests that the concentration of initiator does not affect the degree of cross-linking present in the polymers which was also observed in the thermal counterparts of 1OH system.
4.2.2.1.3. Template Rebinding Studies

Figure 4.28 illustrates the comparison of the amount of template incorporated and rebound by the polymers prepared at various I:TM ratios. The amount of template rebound by the P-1OH imprinted polymers synthesized is comparable to one another: with the lowest of 0.81 ± 0.01 µmol/g in using P-1OH-1:5 and highest of 1.0 ± 0.01 µmol/g in using P-1OH-1:100. The decreasing amount of template rebound in increasing concentration of initiator was similar to the trend observed in thermally synthesized counterparts, however the changes in P-1OH system in between I:TM ratios are not as pronounced as the thermal counterparts. Since the amount of template incorporated is similar for the polymers synthesized from I:TM = 1:100 to 1:10, comparable binding site conversions were also recorded for the mentioned polymers (1.3-1.4%) and increased to 1.9% when the I:TM = 1:5. In the case of the NIPs, comparable amount of templates were rebound with values ranging from 0.45 to 0.60 µmol/g with decreasing concentration of initiator. In view of the fact that comparable values were calculated for the template rebound by the MIP and the NIP, the calculated IFs are close and ranged from a low of 1.5 (P-1OH-1:50) to a high of 1.8 (P-1OH-1:5).
Figure 4. 28. Comparison of the amount of template incorporated and binding performance by P-1OH polymers produced in different I:TM ratios measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard. 10.0 mg polymers incubated in 100 µM 1OH rebinding solution for 60 mins.

4.2.2.2. 5-Methylbenzene-1,3-diol MIPs (P-2OH)

4.2.2.2.1. Polymer composition

The amount of each component in photochemically-synthesized 5-methylbenzene-1,3-diol (P-2OH) MIPs were calculated as the other previously discussed system: the unreacted components in the post polymerisation solutions were quantified using 1,4-dioxane as the reference standard (3.57 ppm) and by monitoring the peaks for EGDMA (O-CH$_2$ at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH$_2$=C- less the O-CH$_2$(EGDMA) peak), and 6.47 ppm for 2OH peak. It was recorded that the amount of EGDMA incorporated (Table 4.11) in the polymers synthesized in high concentration of initiator does not have considerable differences between the MIP and the NIP, however in lower amount of initiator (1:50 and 1:100), the differences in the EGDMA in between the MIP and the NIP is higher: for
the 1:50 imprinted polymers the amount of EGDMA is 71 ± 1% (659 ± 17 µmol/g) and for the its non-imprinted counterpart is 86 ± 1% (715 ± 6 µmol/g) and for the P-2OH-1:100 MIP it contains only 69 ± 1% (571 ± 6 µmol/g) and 74 ± 1% (620 ± 7 µmol/g) for its NIP counterpart. The amount of EGDMA also decreases with decreasing concentration of initiator in the feed, and the maximum decrease is observed when the amount of initiator is decreased from 1:50 to 1:100. Correspondingly, the amount of MAA in the polymers between the MIP and the NIP is comparable to one another in all of the variation of I:TM ratios conducted, but the drop in the amount of MAA in the polymers is noticeable in the presence of low amount of initiator in the feed. Due to low incorporation of MAA of both the MIP and the NIP, the resulting FM:XL in the polymers is between 1:5-1:7 and this is later manifested to the NMR based % yield of between 67-86 % with the lowest yield by P-2OH-1:100. The calculated isolated yield (65-75%) coincides with the NMR-based yield.
Correspondingly, the template incorporation for the this system is low, similar to its thermal counterpart.. This occurrence is probably due to the side reaction of 2OH that was pointed out in the discussion of thermally synthesized 2OH polymers in section 2.3.1.2. Nevertheless, Figure 4.29 shows that the concentration of initiator does not affect the incorporation of template in photochemically synthesized polymers as can be seen in the minor changes in between I:TM ratios. The highest incorporation is displayed by P-2OH-1:5

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM&lt;sup&gt;1&lt;/sup&gt; (%initiator)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Reacted Double Bonds&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EGDMA</td>
<td>MAA</td>
<td>Template</td>
</tr>
<tr>
<td>P-2OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs</td>
<td>88 ± 0</td>
<td>69 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(730)</td>
<td>(116)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>88 ± 0</td>
<td>63 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(730)</td>
<td>(106)</td>
</tr>
<tr>
<td>P-2OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs</td>
<td>87 ± 1</td>
<td>80 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(724)</td>
<td>(133)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>86 ± 1</td>
<td>72 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(718)</td>
<td>(120)</td>
</tr>
<tr>
<td>P-2OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs</td>
<td>79 ± 1</td>
<td>66 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(661)</td>
<td>(111)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>84 ± 2</td>
<td>73 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(701)</td>
<td>(121)</td>
</tr>
<tr>
<td>P-2OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs</td>
<td>71 ± 1</td>
<td>69 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(659)</td>
<td>(116)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>86 ± 1</td>
<td>76 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(715)</td>
<td>(128)</td>
</tr>
<tr>
<td>P-2OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs</td>
<td>69 ± 1</td>
<td>48 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(571)</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td>74 ± 1</td>
<td>53 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(620)</td>
<td>(88)</td>
</tr>
</tbody>
</table>

<sup>1</sup>I:TM = initiator (mmol): total monomer (mmol EGDMA+ mmol MAA).<sup>2</sup>% initiator = amount (mol) of initiator/amount of total monomer (mol).<sup>3</sup>T:FM:XL = template : functional monomer :cross-linker (mol) ratio in the polymers. <sup>4</sup>Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and -C=O with respect to the peak height ratio of pure EGDMA.
with 17 ± 1% (6.90 ± 0.40 µmol/g) incorporation and the lowest were displayed by P-2OH-1:50 with only 10 ± 1% (4.30 µmol/g) incorporation.

**Figure 4.** Percentages of 2OH incorporated in the photo-chemically synthesized polymers in various I:TM ratios. Measured by $^1$H NMR using 1,4-dioxane in DMSO-$d_6$ as the reference standard.

### 4.2.2.2.2. Morphology and Particle Size

SEM images of selected polymers produced at I:TM = 1:100 and 1:5 are shown in Figure 4.30. Polydisperse microspheres were obtained in P-2OH-1:100 polymers, with imprinted polymers (Figure 4.30 A) appear to be bigger in diameter than its NIP (Figure 4.30 B). Increasing the amount of initiator to 1:5, changes the morphology of the polymers and this seems to approach monolithic polymerisation as was also observed in the P-1OH system which is attributed to several factors (decrease in the quality of solvent and the loss of colloidal stability of individual particles. As for the $d_{hl}$ measured by DLS, a larger value were recorded for the P-2OH-1:5 polymers, which does not correspond with the sizes shown, in the SEM, especially for the P-2OH-1:5, and this is potentially due to the aggregation of the particles.
Figure 4. 30. SEM images of photochemically synthesized polymers. P-2OH-1:100 MIP (A) and NIP (B) and P-2OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM.

As shown in Table 4.11, there are no differences in the amount of cross-linking in the polymers in each I:TM ratios, indicating that the concentration of initiator does not affect the cross-linking degree in the MIP as well as the NIP. This occurrence is in agreement of the results obtained in the thermal counterparts, T-2OH. Additionally, the minor differences in the degree of cross-linking between the MIP and the NIP imply that the polymerisation process is not affected by the presence of the template.
4.2.2.2.3. Template Rebinding Studies

As shown in Figure 4.31, the highest amount of template incorporated is 44.7 ± 0.4 μmol/g exhibited by P-2OH-1:5 and the lowest is 33.0 ± 0.3 μmol/g afforded by P-2OH-1:10. Similar to its thermal counterparts, the dimerisation of the template 2OH is highly probable and this is aggravated by the fact that the three templates are photochemically reactive and known for their capabilities as a radical scavenger.\textsuperscript{182,200}

Ten mg of polymers were incubated for 45 mins (with shaking) with 100 μM rebinding solution. The amount of template bound to the polymers of each I:TM ratio was quantified by monitoring the peak of 2OH at 6.47 ppm corresponding to –CH=C–, and comparing it to the peak of the reference standard, 1,4 dioxane at 3.57 ppm. Results of the template rebinding experiments (Figure 4.31) demonstrated that there is no observable trend with respect to the concentration of the initiator. P-2OH-1:50 rebound the highest amount of 2OH (1.75 ± 0.07 μmol/g) which is almost twice as much as the lowest template rebound exhibited by P-2OH-1:10 (0.94 ± 0.05 μmol/g). In terms of the binding site conversion the highest conversion was displayed by P-2OH-1:50 (5.21%) and the lowest was shown by P-2OH-1:5 (2.32%). The combination of low incorporation of template and high MIP binding gave a higher binding site conversion for P-2OH-1:50. The unpredictable trend in the MIP binding and binding site conversion was also observed in the thermal counterpart of 2OH which is attributed to the side reactions that 2OH can undergo (Section 2.3.1.4) In considering the NIP binding, the lowest is observed in higher concentration of initiator (P-2OH-1:5; 0.73 ± 0.04 μmol/g and P-2OH-1:10; 0.73 ± 0.03 μmol/g) and the highest NIP binding recorded was 1.31 ± 0.02 μmol/g shown by P-2OH-1:50. In calculating the IF, no significant disparity between I:TM ratios which is in the range of 1.2-1.3, which suggest that this system produced low performing polymers.
Figure 4. 31. Comparison of the template incorporation and the binding performance of the MIP and NIP of P-2OH polymers synthesized in various I:TM experiments. Amount of 2OH were quantified by $^1$H NMR using 1,4 dioxane in DMSO-$d_6$ as reference standard and monitoring the –CH=C- peak at 6.47 ppm of 2OH.

4.2.2.3. Benzene-1,3,5-triol polymers MIPs (P-3OH)

4.2.2.3.1. Polymer composition

The amount of each component in photochemically-synthesized Benzene-1,3,5-triol polymers (P-3OH) were calculated the same way as the other previously discussed systems: the unreacted components in the post polymerisation solutions were quantified using 1,4-dioxane as the reference standard (3.57 ppm) and by monitoring the peaks for EGDMA (O-CH2 at 4.32 ppm) and MAA (5.10 and 5.65 ppm for MAA and EGDMA and CH2=C- less the O-CH2(EGDMA) peak), and –CH=C- peak at 6.11 ppm for 3OH. There is no considerable differences in the incorporation of EGDMA in between the MIP and the NIP of each I:TM ratio (Table 4.12) as was observed in the other photochemical systems discussed previously (P1OH and P-2OH). However, the concentration of initiator seems to affect the incorporation of EGDMA in the NIP as the amount decreases with decreasing amount of initiator: from 90 ± 1 (751 µmol) by P-3OH-1:5 it dropped to 67 ± 1% (567 µmol) by P-
Similarly, the amount of MAA incorporated by the NIP decreases with decreasing concentration of initiator keeping a closer range of values, from the high of 72 ± 1% (119 µmol) to a low of 61 ±1% (102 µmol). Despite of the decreasing monomer conversions in the polymers in decreasing concentration of initiator, the resulting FM:XL are comparable ~1:6, which is slightly lower than the starting FM:XL. The higher incorporation of the cross-linker in the polymers indicates the higher reactivity of EGDMA than MAA even at low concentration of initiator. Moreover, the low conversions of monomers resulted to decreasing yields in decreasing concentration of initiator: from a high of 89 ± 1% to a low of 67 ± 1%.

Similarly for its MIP counterparts, decreasing conversion of EGDMA was also observed: the amount of EGDMA incorporated decreases from 88 ± 1% (732 µmol) to a low of 68 ± 2% (570 µmol). In the case of MAA, comparable values were incorporated, from 64-69% (~620 µmol). The resulting FM:XL in the polymers is comparable with the FM:XL recorded for the NIP counterparts: FM:XL of 1:6 which signifies the higher reactivity of EGDMA than MAA even in the presence of a template. The conversion of monomers also resulted to decreasing %yield of the polymers in decreasing concentration of initiator: from a high of 86 ± 1% to a low of 68 ± 2%. The isolated yield measured for the MIP and the NIP is in the range of 67-78 % which is lower than the NMR-based yield.
Table 4.12. Polymer composition and cross-linking density in the photochemically synthesized Benzene-1,3,5-triol (P-3OH) polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>I:TM(^1) (%initiator)(^2)</th>
<th>EGDM A</th>
<th>MAA</th>
<th>Template</th>
<th>Polymer Composition T:FM :XL(^3)</th>
<th>Reacted Double Bonds(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-3OH-1:5</td>
<td>1:5 (17%)</td>
<td>MIPs</td>
<td></td>
<td></td>
<td>88 ± 0 (732) 69 ± 1 (115)</td>
<td>24 ± 1 (10.00)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td></td>
<td></td>
<td>90 ± 1 (751) 72 ± 1 (120)</td>
<td>1:6.3 (115)</td>
</tr>
<tr>
<td>P-3OH-1:10</td>
<td>1:10 (9%)</td>
<td>MIPs</td>
<td></td>
<td></td>
<td>83 ± 1 (689) 69 ± 1 (115)</td>
<td>24 ± 1 (10.20)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td></td>
<td></td>
<td>85 ± 1 (697) 69 ± 1 (114)</td>
<td>1:6.1 (114)</td>
</tr>
<tr>
<td>P-3OH-1:25</td>
<td>1:25 (4%)</td>
<td>MIPs</td>
<td></td>
<td></td>
<td>79 ± 2 (656) 64 ± 2 (108)</td>
<td>24 ± 1 (8.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td></td>
<td></td>
<td>81 ± 1 (678) 69 ± 1 (115)</td>
<td>1:5.9 (115)</td>
</tr>
<tr>
<td>P-3OH-1:50</td>
<td>1:50 (2%)</td>
<td>MIPs</td>
<td></td>
<td></td>
<td>73 ± 1 (605) 68 ± 1 (114)</td>
<td>16 ± 1 (6.80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td></td>
<td></td>
<td>75 ± 2 (628) 67 ± 1 (113)</td>
<td>1:5.6 (113)</td>
</tr>
<tr>
<td>P-3OH-1:100</td>
<td>1:100 (1%)</td>
<td>MIPs</td>
<td></td>
<td></td>
<td>68 ± 2 (570) 64 ± 2 (106)</td>
<td>11 ± 1 (4.70)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NIPs</td>
<td></td>
<td></td>
<td>68 ± 1 (567) 61 ± 1 (102)</td>
<td>1:5.6 (102)</td>
</tr>
</tbody>
</table>

\(^1\)I:TM = initiator (mmol): total monomer (mmol EGDM A + mmol MAA), \(^2\)% initiator = amount (mol) of initiator/amount of total monomer (mol), \(^3\)T:FM:XL = template : functional monomer :cross-linker (mol) ratio in the polymers, \(^4\)Correlated to degree of cross-linking in the polymer, residual double bonds measured from FTIR using the ratio of the peak height of -C=C- and -C=O with respect to the peak height ratio of pure EGDM A.

The differences in the amount of template incorporated by the polymers is noticeable as the concentration of the initiator was varied: with the highest of 24 ± 1% (10.0 μmol/g) by P-3OH-1:5 polymers and lowest of 11 ± 1% (4.70 μmol/g) by P-3OH-1:100 polymers, making the final T:FM ratios between 1:11-1:25. Similar to the 2OH system, 3OH polymers...
incorporated higher amount of template in the polymers in faster polymerisation reaction or higher amount of initiator and decreases with decreasing amount of initiator.

Table 4.12 also summarizes the degree of cross-linking accounted for the polymers synthesized at various I:TM ratios. The degree of cross-linking in the polymers synthesized in lower concentration of initiator (1:10-1:100) have comparable degree of cross-linking: with a range of between 58-65% cross-linking for the NIP and 60-65% for the MIPs.

4.2.2.3.2. Morphology and Particle Size

Both MIPs and NIPs of P-3OH-1:100 appear to be polydispersed particles with a $d_H$ of 105.7 ± 0.6 nm (PDI = 0.10) for MIP and 101.5 ± 0.2 nm (PDI = 0.10) for NIP. These sizes are consistent with the particles size recorded for the P-2OH-1:100 polymers. As was expected, the morphology of P-3OH-1:5 polymers are different, it did not produce discrete particles as exhibited in Figure 4.32 C and D, which was similar to the morphology observed in the other photo-chemically synthesized polymers synthesized in high concentration of initiator. The $d_H$ measured by DLS for P-3OH-1:5 MIP is 123.7 ± 3.4 nm and 166.3 ± 1.8 nm for the NIP, which do not correspond to the size of the particles shown in the SEM images, and this is probably due to aggregation of the smaller particles interlinked to one another. The P-3OH polymers with the lowest degree of cross-linking produced smaller particles interconnected together as was observed with P-1OH and P-2OH polymers produced in the same concentration of initiator.
Figure 4. 32. SEM images of the photochemically synthesized P-3OH polymers. P-3OH-1:100 MIP (A) and NIP (B) and P-3OH-1:5 MIP (C) and NIP (D). Insets are the hydrodynamic size of the microspheres with the corresponding polydispersity indexes (PDI) measured by DLS and SEM.

4.2.2.3.3. Template Rebinding Studies

Final assessment for every system is the template rebinding capacity of the polymers. The binding performance of the photo-chemically synthesized polymers were assessed in similar method as the 3OH thermal counterparts. Each polymer of different I:TM systems (10.0mg) was incubated for 75 mins in 100 µM rebinding solution and the results of the binding performances of the P-3OH polymers were quantified using $^1$H NMR by monitoring the –CH=C- peak of 3OH at 6.11 ppm and are summarized in Figure 4.33.
Template incorporation of the P-3OH polymers is higher in higher concentration of initiator; highest recorded is 69.3 ± 0.93 μmol/g by P-3OH-1:10 MIP and the lowest is 38.2 ± 0.42 μmol/g and showed by P-3OH-1:100 MIP. In the case of the template rebinding performance, the NIP binding are comparable (~1.0-1.4 μmol/g). Similarly, comparable binding was also observed with the MIP counterparts ranging from 1.3-2.0 μmol/g which was also observed in the previous photochemical systems (P-1OH and P-2OH) that the template incorporation calculated is not efficiently converted to binding sites. The comparable MIP binding of the polymers in this system suggest that there exist an optimum equilibrium concentration of T-FM complexes that can be formed, which was also observed in the previously discussed systems. Surprisingly, in calculating for the binding site conversion, P-3OH-1:100 exhibited the highest conversion with a value of 5.0 % due to low incorporation of template and P-3OH-1:10 have the lowest conversion with value of 1.9 % due to high incorporation but low MIP binding. In contrast with the thermal counterpart, lower imprinting efficiency was observed in higher concentration of initiator which may be attributed to the formation of high concentration of radicals that increases the probability of the dimerisation of the phenolic templates which accounts for ~30% loss of templates (with respect to the initial amount). In terms of the NIP binding highest is 1.38 ± 0.01 μmol/g which is shown by P-3OH-1:5 polymers and the lowest of 0.94 ± 0.02 μmol/g showed by P-3OH-1:10. In calculating for the IF of each I:TM ratios, the comparable MIP binding ranges from 1.51 to 1.98 μmol/g which indicates that the concentration of initiator does not affect the binding capacity of the polymers as was observed in the previously discussed photochemical systems.
4.2.2.4. Comparison of the photochemically synthesized polymers

The incorporation of EGDMA and MAA varies from one I:TM experiment to the other, more so in different template system, however, the resulting FM:XL ratio in the polymers remains in a close range of between 1:5-6. While variable effects were observed in the template incorporation with different I:TM ratios, (Figure 4.34 A) it is noticeable that in I:TM of 1:5, comparable amount of templates were incorporated by the three MIPs. It is also the I:TM ratio that incorporated the least amount of templates in the three MIPs, similar to the thermally synthesized systems. This observation was attributed to the disruption of the T-FM complexes due to additional heat released due to higher concentration of initiator and the probability of higher branching density that prevented the efficient imprinting of templates in the polymers. In most I:TM ratios, 2OH was the least incorporated among the three templates and this could be due to the higher probability of dimerization of 2OH compared to 1OH and 3OH.
Figure 4. Comparison of the template incorporation (A), MIP (B) and NIP (C) binding performance of the MIP and NIP of the photochemically synthesized polymers. Amount of each templates were quantified by $^1$H NMR using 1,4 dioxane in DMSO-$d_6$ as reference standard.
As for the amount of cross-linking in the polymers, it appears to be unaffected by the concentration of initiator and the nature of the template as comparable degree of cross-linking were calculated for the three systems keeping a small range between 58-69 %.

In terms of the binding performances, it seems like P-1OH MIPs are not affected in varying the concentration of initiator in the feed since comparable amount of templates were rebound by different I:TM system as shown in Figure 4.34 B. Unlike P-1OH polymers, the amount of templates rebound by the P-2OH and P-3OH systems fluctuated in varying I:TM ratio and did not illustrated any trend. It is noteworthy to mention that in most I:TM ratios, P-3OH bound the highest.

Similarly, P-1OH NIPs comparable amount of 1OH in various concentration of initiator that resulted to a range of IF values between 1.6 to 1.8 according to Figure 4.34 C. Correspondingly to their respective MIPs, fluctuating NIP binding was also observed with the P-2OH and P-3OH NIPs and this recorded a variable IF values ranging from 1.2 to 1.4. In consideration of the IF values calculated for the three MIPs systems, P-1OH recorded the highest IF values, similar to the thermally-synthesized polymers. These results served as an incongruity of the interaction-based theory, which was also in parallel with the study conducted by Spivak et al. 146

The three MIP systems produced in I:T =1:100 produced discrete particles with $d_H$ sizes ranging from 100-150 nm with PDIs of 0.10-0.30. Moving to higher concentration of initiator, the morphology of the polymers changed from particles to monolithic which was explained thoroughly by Downey which was attributed to the solvency conditions and the poor quality of solvent during the polymerisation process. 198
4.2.3. Comparison of Thermal vs Photochemical initiation method.

Photochemically synthesized polymers deviate from the expected results that in precipitation polymerisation, regardless of the concentration of initiator, discrete particles are obtained. However, at high amount of initiator (I:TM= 1:5), the photochemically synthesized polymers appear to be a combination of smaller particles and monolithic gross morphology, which was not observed in all thermally synthesized polymers. This might be due to several reasons; first is due to auto-acceleration effect that is characterize by a decrease in the rate of termination caused by a drastic increase in the rate of polymerisation and a simultaneous increase in the molecular weight of the growing polymer. And it might be due to the temperature of the polymerisation solution that reduced the quality of the solvent (e.g. solvency and viscosity of the porogen) and the reaction, leading to the collapse of the colloidal stability of individual particles.\textsuperscript{198, 201} Moreover, comparable degree of cross-linking was observed between the thermal and photochemically-synthesized polymers.

At lower concentration of initiator (I:TM= 1:25-1:100), template incorporation within the photochemically synthesized polymers are lower than the thermally-synthesized polymers which is attributed to insufficient kinetic energy of molecules at lower temperature to collide with the functional monomer and form interaction. At higher concentration of initiator (I:TM= 1:5-1:10), however, the amount of template incorporated by the thermally-synthesized polymers is lower than the photochemically synthesized polymers which can be attributed to the disruption of the T-FM complexes due to the exothermic reaction of radical formation. In addition, higher concentration of initiator, there is a higher probability of the dimerisation of the templates due to the formation of higher concentration of radical species in the reaction. This reaction is supported by the appearance of peaks in the $^1$H NMR of the post polymerisation solutions of 1OH and 3OH in higher concentration of initiator.
Therefore, the decrease in the concentration of the templates from the initial concentration is a combination of incorporation in the polymers and the dimerisation calculated incorporated template. The possible dimerisation reactions of the 1OH and 3OH are shown according to Figure 4.35. The dimerisation reaction of 1OH given in Figure 4.35 A is supported by the emergence of an additional peak in NMR spectra of P-1OH in the post polymerisation solution due to the aromatic proton (labeled as D1OH-H11) as shown in Figure 4.35 B. Correspondingly, an appearance of another peak was observed in the post polymerisation solution of the 3OH system due to the aromatic proton at 7.1 ppm. (labeled as proton D3OH-H3 in Figure 4.35 D) which is attributed to the formation of a 3OH dimer with the structure shown in Figure 4.35 C. In the case of 2OH, higher incorporation was recorded with the photochemically synthesized polymers which could be due to the fact that the dimerisation of 2OH is a kinetically driven reaction as was presented by Thavasi et al. 182
Figure 4. Proposed dimerisation reaction of 1OH (A) and 3OH (C) under UV irradiation with the partial NMR spectra of initial (bottom spectra) and post polymerisation (top spectra) solutions of P-1OH (B) and P-3OH (D).

Comparable MIP binding was observed between the thermal and the photochemically synthesized polymers which indicates that in a given I:TM and T:FM, there is an optimum concentration of T-FM complexes that can be formed thus changing the temperature will not increase the binding capacity of the MIP. This is in accordance to the study of Spivak showing that there exist a maximum amount of T-FM complexes that can be formed in a given amount of the components in the feed. Higher NIP binding was observed in photochemical polymers resulting to lower IF than thermal polymers which is probably due to the limited solubility of the monomers and lower frequency of collisions at lower temperature in photochemical initiation which decreases the conversion of monomers in the polymers leaving more units of MAA that can interact with the template during rebinding.
process. In both initiation methods, IOH polymers demonstrated higher values of IF than the other two MIPs systems, signifying that these systems are another exception from the interaction-based theory, an accordance with the findings of Spivak et al. that in some cases, the number of interaction points does not necessarily equate to more binding efficient MIPs.146

Another factor that can lower the incorporation of the template is the formation of radical species other than the one formed from the initiator. This is possibly because of the wide range of the emitted radiation of the UV lamp employed for the photo-chemical synthesis (200-400 nm), which is enough to promote the formation of radical species from the components in the polymerisation reaction. This was evident in the experiment conducted in which a solution of MAA and EGDMA (1:5), without the addition of AIBN, was placed in the photochemical cabinet using the same UV lamp employed for the photochemical synthesis of MIP, and a cloudy solution was obtained after 24 hours. The cloudiness is attributed to the formation of short-chained oligomers that did not reach critical mass to precipitate out of the solution.

Overall, the thermally-synthesized polymers are more binding efficient due to higher IFs calculated for this system which is due to the low NIP binding recorded for this system. In addition, the temperature of the solution during the polymerisation reaction reduces the quality of the solvent lowering the solubility of the monomers and templates in the photochemical initiation that lowers the number of collisions in between the components of the reaction that affects the monomer conversion and binding performance of the polymers.
4.3. Summary

The properties and binding efficiency of thermally and photo-chemically synthesized polymers using three phenolic templates were assessed and compared. Additionally, the effect of varying the concentration of initiator was also correlated to the binding performance of microspheres of the three MIP systems.

The particle size was mainly affected by the concentration of initiator; it increased with increasing concentration of initiator. Looking at the polymer composition, the FM:XL ratio in the resulting polymer is unaffected by the presence and the nature of the template. It is also unaffected by the concentration of initiator and reaction temperature that led to the preservation of the starting FM:XL of 1:5. However, photochemical initiation incorporated lower monomers than the thermal counterparts that led to lower %yields. The degree of crosslinking, on the other hand, was found to be unchanged and remained comparable in given system. In the case of template incorporation, it is affected by the 1) nature of the template in which higher incorporation of 1OH was observed in both thermal and photochemical initiation compared to other phenolic templates  2) the concentration of initiator, increasing templates are incorporated in the polymers in decreasing concentration of initiator, and  3) reaction temperature in which template incorporated within thermally-synthesized polymers is decreasing with increasing concentration of initiator and this is opposite to the results calculated for photochemically-synthesized polymers. Nevertheless, the template incorporated does not necessarily form high fidelity imprints. Based from the rebinding results, it seems that there is an optimum equilibrium concentration of T-FM complexes that can be formed in a given T:FM since MIP binding is comparable in spite of the concentration of initiator, nature of template and reaction temperature.

In characterizing the binding sites of the three MIP systems prepared from the same I:M ratios, comparable $K_a$s were measured which suggest that the affinity of the MIP towards
its template is comparable. However, the nature of the template affected the number of binding sites, \( N \), formed in the resulting polymers. Moreover, varying the concentration of initiator increases the \( K_a \) and decreases the \( N \) of the system as was demonstrated by T-2OH-1:100 and T-2OH-1:5 polymers.

Cross-reactivity studies of the best performing MIP for each MIP system demonstrated that T-3OH MIP is the most selective towards its template giving selectivity indices of 0.58 and 0.67 for 1OH and 2OH, respectively.

Template effects were also manifested in the surface area, pore sizes and pore volumes of the MIPs. More pronounced difference in the properties of the MIP and the NIP were observed in 3OH system. Due to the difference in the surface area between the MIP and the NIP of each system, binding capacities were normalized with respect to surface area. Normalization of the binding capacity with respect to surface area led to an increase in MIP binding and ultimately higher IF (3 times higher) which is illustrated by T-3OH-1:100 polymers. We believe that more accurate assessment of the binding performance of precipitation MIPs can be obtained when normalised against surface area considering the variability of the physical properties of the microspheres.

For this study, the most binding efficient 1OH and 3OH imprinted polymers are those prepared in lowest concentration of initiator (T-1OH-1:100 and T-3OH-1:100) by thermal initiation, showing the highest MIP binding, with relatively low NIP binding resulting the high IF in their respective sets.
Chapter 5
Imprinting efficiency of a ‘Stoichiometric’ Pyridine-based functional Monomer in precipitation polymerization

5.1. Introduction

Molecular imprinting using small templates is common and proved to be efficient using commercially available functional monomers and crosslinkers. As the size of the templates increases, such as biomolecules, the imprinting process becomes more challenging. Biomolecules impose difficulty in imprinting due to their chemical instability and the size and complexity of their binding sites. Unlike small molecules, large molecules have the tendency to become trapped into the cross-linker networks of the polymers which is problematic in the template removal process. In addition, biomolecules also have multiple potential binding sites, which can increase the number of non-specific binding sites and ultimately lead to poor selectivity of materials.

The specificity of biological molecules does not only rely on the shape and size conformability, but the multiple binding points of the receptor also needs to be in congruent with those of the substrate. This concept was then applied to molecular imprinting to produce more selective materials by designing functional monomers capable of forming an array of interactions with the template. Researchers aim to mimic the molecular recognition capabilities of the biological molecules that involves a donor-acceptor-donor (DAD) hydrogen bonding interactions by using a acylamido-pyridine and pyrimidine-
based\textsuperscript{207} or a urea-based\textsuperscript{104} custom-designed functional monomers. Compounds with imide functionality are one of the most commonly investigated templates for these custom designed functional monomers because the trans-amide group of these monomers can form the DAD/ADA hydrogen bond array of interaction with the imide group (Figure 2) affording high affinity binding sites.\textsuperscript{208-209}

![Diagram of DAD/ADA hydrogen bonding array](image)

**Figure 5.1.** Illustration of the DAD/ADA hydrogen bonding array of the trans-amide group\textsuperscript{209} of a bis-acylamidopyridine-based compound and an imide functionality.

One of the widely studied custom-designed functional monomer is the 2,6-bis-(acrylamido) pyridine (BAAPy, 1, Figure 2), which has been widely explored in imprinting imide-containing templates, such as fluorouracil\textsuperscript{103}, cyclobarbital\textsuperscript{102} and barbiturates\textsuperscript{112}. Due to the displayed specificity of the BAAPy-synthesized MIPs towards small molecules, researchers moved to using more complex and bulkier templates like riboflavin\textsuperscript{110}, glutamic acid\textsuperscript{104} and uracil derivatives\textsuperscript{108,207}. In a more recent study, the application of BAAPy in imprinting process of a more complex uracil-containing compound, nucleosides, was proven to be efficient in bulk format. In this study, 2′3′5′-tri-O-acyl Urds with different alkyl chain lengths (attached to the ribose ring) were used as dummy templates for the recognition of Urd nucleosides. Among the tested targets, the nucleoside with a single alkyl chain, 2′,3′,5′-tri-O-
acetyluridine (TAU) MIPs, showed higher binding capacity, selectivity and specificity than the other tested templates as determined by frontal chromatography.  

Herein, we present a detailed assessment of the imprinting efficiency and binding performance of TAU MIPs prepared by precipitation polymerisation using BAAPy (see Figure 2 for structure) as functional monomer which, to the best of our knowledge, have not yet been fully investigated. As an added value to this study, we also present the application of quantitative nuclear magnetic resonance spectroscopy (qNMR) for polymer composition and \textit{in situ} binding measurements. We found that the stoichiometric 1:1 T:FM ratio has not been maintained in precipitation polymerisation and an optimal TAU:BAAPy ratio of 1:2.5 was obtained for MIP microspheres prepared in acetonitrile without agitation. This precipitation MIP afforded affinity constant and binding capacity higher than its bulk counterpart. Molecular modelling, NMR studies and selectivity assays indicate that, aside from the DAD/ADA hydrogen bond interaction, BAAPy also interacts with the acetyl groups of TAU. Imprinting efficiency (i.e. template incorporation) and binding capacity of precipitation MIPs have also been shown to be affected by the initiator concentration and method of agitation.

\subsection*{5.2. Results and Discussion}

BAAPy as a functional monomer in bulk imprinting of uracil derivatives with variable acyl group chain lengths has been explored by the group of Krstulja.\textsuperscript{108-109} the investigated uracil-based targets, TAU (2) imprinted MIPs have been shown to exhibit the highest affinity (number of TAU binding sites = 3.42 \textgreek{m}ol/g, $K_a = 1.7 \times 10^4$ L/mol) and selectivity. While BAAPy has been extensively used in bulk imprinting, its utility in precipitation polymerisation has been limited. There has only been one report on BAAPy-based microspheres for solid phase extraction of barbiturates in human urine samples. This current
study evaluates the performance of BAAPy as a functional monomer in precipitation imprinting of TAU, particularly monitoring both imprinting and binding efficiencies.

5.2.1. Synthesis of MIPs

5.2.1.1. Bulk Polymerisation

TAU MIPs were first synthesized by bulk polymerisation using the 1:1:20 TAU:BAAPy:EGDMA formulation of Krstulja, et al. but employing AIBN, instead of azo-bis-dimethylvaleronitrile (ABDV), at 60°C using chloroform (BP-1:1-C) and acetonitrile (BP-1:1-A) as porogens. While Krstulja, et al. have shown chloroform as an efficient porogen, we were also keen to use acetonitrile in order to compare with MIP microspheres also generated in acetonitrile. TAU was reported to exhibit comparable solubility in both solvents (≥ 100 mM). As can be seen in Figure 5.2, the polymers obtained from both acetonitrile (BP-1:1-A) and chloroform (BP-1:1-C) are highly porous. While microspheres seem to be formed at the surface of the MIPs, both MIPs and NIPs generally showed bulk morphology expected from bulk molecular imprinting process in the presence of limited amount of porogen.
The composition of MIPs and NIPs was determined indirectly by calculating the amounts of left-over (unpolymerised) monomers and template in solution post-polymerisation with respect to the pre-polymerization mixture by $^1$H NMR spectroscopy (see Experimental). The results, summarised in Tables 5.1 and 5.2, show high conversions for EGDMA ($\geq 95\%$) and BAAPy ($\geq 92\%$) in the NIPs resulting in FM:XL mol ratios of 1:20 (BP-1:1-A) and 1:21 (BP-1:1-C) approximating the feed formulation of 1:20. The BAAPy conversion in the MIPs, on the other hand, was slightly lower at 64\% and 77\% for chloroform and acetonitrile-porogenated MIPs, respectively, while the EGDMA conversion remains high (94\%) and comparable to that of the NIPs, resulting in FM:XL mol ratios of 1:29 and 1:25,
respectively. Nevertheless, TAU incorporation, i.e. imprinting, within the polymers for both BP-1:1-C and BP-1:1-A while moderate, 143 ± 1 µmol/g (60%) and 158 ± 5 µmol/g (66%), respectively, with respect to the reaction feed (240 µmol/g), afforded T:FM ratios of 0.9:1 and 0.8:1, respectively, approximating the expected 1:1 stoichiometric T:FM relationship also obtained by Krstulja, et al.\textsuperscript{108} We surmised that the lower BAAPy conversion in the MIPs is due to the formation of the TAU:BAAPy complex which is less soluble in the porogen than the uncomplexed TAU and BAAPy. Turbidity tests confirmed our hypothesis. We observed a decrease in transmittance (from 98% to as low as 40% between 400 and 700 nm) upon the addition of TAU to a BAAPy solution in acetonitrile and chloroform indicating formation of insoluble species.
Table 5.1: TAU imprinting results for bulk (BP) and precipitation (PP) polymers.

<table>
<thead>
<tr>
<th>Polymers¹</th>
<th>Feed Polymer Imprinted</th>
<th>Rebound</th>
<th>TAU:BAAPy:EGDMA mole ratios²</th>
<th>Particle Size, nm (PDI)</th>
<th>TAU³, µmol/g⁴</th>
<th>IF⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.78: 1: 25</td>
<td>32-45 µm</td>
<td>158 ± 5</td>
<td>3.57 ± 0.11</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>NIPs 1:20</td>
<td>1:20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP-1:1-C</td>
<td>MIPs 1:1:20</td>
<td>0.86: 1: 29</td>
<td>143 ± 1</td>
<td>4.07 ± 0.2</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>NIPs 1:21</td>
<td>1:21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.4:1:16 (0.774)</td>
<td>337 ± 1</td>
<td>98 ± 2</td>
<td>3.64 ± 0.03</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>NIPs 1:18</td>
<td>1:18</td>
<td>368 ± 2 (0.794)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.84: 1: 22</td>
<td>not measured</td>
<td>173 ± 3</td>
<td>0.29 ± 0.02</td>
<td>1.5</td>
</tr>
<tr>
<td>A-St⁶</td>
<td>NIPs 1:19</td>
<td>1:19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.58: 1: 18</td>
<td>not measured</td>
<td>153 ± 4</td>
<td>1.22 ± 0.10</td>
<td>1.4</td>
</tr>
<tr>
<td>A-Rd⁷</td>
<td>NIPs 1:23</td>
<td>1:23</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.29: 1: 19 (0.374)</td>
<td>387 ± 1</td>
<td>73 ± 2</td>
<td>1.14 ± 0.10</td>
<td>2.1</td>
</tr>
<tr>
<td>A-150⁸</td>
<td>NIPs 1:20</td>
<td>1:20</td>
<td>490 ± 1 (0.507)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>MIPs 1:1:20</td>
<td>0.16:1:20 (0.716)</td>
<td>468 ± 1</td>
<td>38 ± 2</td>
<td>0.56 ± 0.01</td>
<td>1.2</td>
</tr>
<tr>
<td>A-1200⁹</td>
<td>NIPs 1:21</td>
<td>1:21</td>
<td>380 ± 1 (0.417)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹A= acetonitrile, C= chloroform; ²Only FM:XL for NIPs; ³TAU in feed = 240 µmol/g except for PP-2:1-A = 480 µmol/g; ⁴µmol/g=µmol template / g total monomers; ⁵Imprinting factor = Bound MIP/Bound NIP; ⁶St = Stirred; ⁷Rd= Rolled; ⁸Initiator:total monomer (BAAPy + EGDMA) mol ratio = 1:50; ⁹Initiator:total monomer ratio = 1:200; Note: Initiator:total monomer ratio of all other polymers = 1:131.
Table 5.2. Feed and polymer composition for bulk (BP) and precipitation (PP) MIPs and NIPs.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Composition mmol (%)</th>
<th>MIPs</th>
<th>NIPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AIBN TAU BAAPy EGDMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AIGN BAAPy EGDMA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>BP-1:1-A</td>
<td>0.0137 0.1790 0.1790 3.5803</td>
<td>0.1790 3.5803</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.1% (60%) (77%) (94%)</td>
<td>(96%) (95%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>BP-1:1-C</td>
<td>0.0137 0.1790 0.1790 3.5803</td>
<td>0.1790 3.5803</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.1% (60%) (77%) (94%)</td>
<td>(96%) (95%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A</td>
<td>0.0038 0.0238 0.0238 0.4761</td>
<td>0.0238 0.4761</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.0% (32%) (89%) (80%)</td>
<td>(92%) (83%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A-</td>
<td>0.0038 0.0238 0.0238 0.4761</td>
<td>0.0238 0.4761</td>
<td></td>
</tr>
<tr>
<td>St</td>
<td>(0.0% (65%) (80%) (90%)</td>
<td>(98%) (94%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A-</td>
<td>0.0038 0.0238 0.0238 0.4761</td>
<td>0.0238 0.4761</td>
<td></td>
</tr>
<tr>
<td>Rd</td>
<td>(0.0% (38%) (63%) (58%)</td>
<td>(64%) (75%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A-</td>
<td>0.0100 0.0238 0.0238 0.4761</td>
<td>0.0238 0.4761</td>
<td></td>
</tr>
<tr>
<td>I50</td>
<td>(0.0% (28%) (95%) (92%)</td>
<td>(94%) (93%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>Polymer</td>
<td></td>
</tr>
<tr>
<td>PP-1:1-A-</td>
<td>0.0025 0.0238 0.0238 0.4761</td>
<td>0.0238 0.4761</td>
<td></td>
</tr>
<tr>
<td>I200</td>
<td>(0.0% (14%) (84%) (85%)</td>
<td>(72%) (78%)</td>
<td></td>
</tr>
</tbody>
</table>

1A= acetonitrile, C= chloroform; 2Polymer composition estimated by NMR; 3Same amount used in NIPs; 4St = Stirred; 5Rd=Rolled; 6Initiator:total monomer (BAAPy + EGDMA) mol ratio = 1:50; 7Initiator:total monomer ratio = 1:200; Note: Initiator:total monomer ratio of all other polymers = 1:131.
5.2.1.2. Precipitation Polymerisation

TAU MIPs (PP-1:1-A) were subsequently synthesised by precipitation polymerisation following the bulk formulation with chloroform and acetonitrile (10 mL per mmol monomer) as porogens. Polymers prepared in chloroform and even with 50% chloroform/50% acetonitrile by volume resulted in gels so only PP-1:1-A was subjected to further characterisation. As shown in Figure 5.3, PP-1:1-A are spherical particles with average hydrodynamic sizes ($d_H$) of 337 and 368 nm for MIP and NIP, respectively, measured by DLS. Particle aggregation is evident from SEM images which is consistent with their broad PDIs (0.8) obtained from DLS measurements.

![SEM images of precipitation polymers PP-1:1-A MIP (A) and NIP (B).](image)

As with bulk imprinting, the FM:XL ratio in the PP-1:1-A feed was kept at 1:20. However, the conversion of EGDMA was lower (Table 5.2), i.e. ~80%, while that of BAAPy higher (~90%), than what was observed in BP polymers resulting in a higher BAAPy:EGDMA ratio of 1:18 for NIP and 1:16 for MIP. Unlike the bulk process, we did not observe precipitation of the TAU:BAAPy complex (as indicated by a decrease in solution
transmittance) and we presume that the degree of BAAPy and EGDMA conversions is a function of their copolymerisation tendencies. Interestingly, the TAU:BAAPy ratio obtained was 1:2.5 (i.e. 0.4:1) as only 98 ± 1 µmol/g (41%) was incorporated. a deviation from the 1:1 stoichiometric relationship obtained with bulk MIPs and expected from BAAPy-based uracil MIPs, Our results seem to suggest that template-monomer interaction is influenced and can be optimised by solvent dilution. Beijer et al. have extensively studied the interaction of BAAPy with uracil derivatives and have shown that the DAD H-bond induced 1:1 complex only prevail if no other functional group or interaction sites, other than the imide, is present.\textsuperscript{211} TAU, on the other hand, has three ester functionalities surrounding the ribose ring which can be possible points of interaction with BAAPy. Evidence to this effect was obtained from \textsuperscript{1}H NMR titration and molecular modelling template-monomer interaction studies.

### 5.2.2. TAU-BAAPy Interaction Studies

The interaction between BAAPy and uracil derivatives by the ADA/DAD H-bond mechanism (Figure 1) has been well documented\textsuperscript{211-213} displaying high association constants (2 to 1.7X10\textsuperscript{4} M\textsuperscript{-1})\textsuperscript{108,211}. In the case of TAU, Krstulja\textsuperscript{108} has alluded to an interaction study by \textsuperscript{1}H NMR spectroscopy monitoring the amido proton of BAAPy. We have extended this study to other \textsuperscript{1}H and \textsuperscript{13}C nuclei and have also conducted supplementary semi-empirical molecular modelling.

The computer generated 1:1 TAU:BAAPy complex (Spartan ‘14 v1.1.8) given in Figure 5.4. A shows the ADA/DAD H-bonding interactions to be the predominant with distances between the interacting atoms of 1.8 Å. Nevertheless, while these H-bonding arrays are still evident when the TAU:BAAPy ratio is decreased to 1:3, mimicking the PP-1:1-A system, the amido protons of the other two BAAPy units have also been observed to
interact with the carbonyl oxygen 6’ and 10’ of TAU. The distances between the ADA/DAD H-bond interacting atoms have also been shown to slightly increase to 2.1-2.2 Å, suggesting weaker interactions than with the 1:1 ratio, but this have been compensated by the formation of two additional H-bond interactions with two other BAAPy units.

**Figure 5.4.** The predominant hydrogen bonding interaction points (distances $2.2 \geq 2.5 \, \text{Å}$) between BAAPy and TAU measured by Spartan '14 v1.1.8 in a 1:1 (A) and 1:3 (B) TAU:BAAPy clusters.

To verify the interactions observed from molecular modelling, $^1$H and $^{13}$C NMR titration experiments were carried out at 60ºC, the reaction temperature, monitoring movements in chemical shifts ($\geq 0.2$ ppm) of protons and carbons, respectively, brought about by interactions between BAAPy and TAU. Figure 5.5 and 5.6 give selected $^1$H and $^{13}$C
NMR spectra, respectively, showing peak shifts of interacting nuclei. These peak movements are also illustrated in Figure 5.6.

The ADA/DAD hydrogen bond array interactions between the imide group of TAU and the amide group of BAAPy are evident from the chemical shift movements of the amido protons (Figure 5.5 A). The H-bond donating amido proton of TAU (TAU-3, see Figure 2.1 for proton/carbon assignments) showed a marked upfield peak movement presumably upon interaction with the H-bond acceptor nitrogen (BAAPy-1) of BAAPy. Conversely, the amido protons of BAAPy (BAAPy-7a,b) experienced a downfield chemical shift movement in the presence of TAU attributed to enhanced deshielding by amido oxygens TAU-2 and TAU-4 of TAU. Consequently, carbons TAU-2 and TAU-4 would have been more shielded and thus underwent a shift upfield (Figure 5.6, B).

The chemical shift movements of other carbon nuclei indicate additional interactions between TAU and BAAPy aside from the DAD/ADA H-bonding array. In particular, TAU acyl carbons 6’ and 10’ (TAU-6’ and TAU-10’) as well as their adjacent methyl groups TAU-7’ and TAU-11’, respectively, exhibited upfield shifts which could be attributed to additional shielding brought about by the interaction of the acyl oxygens with the amido proton of BAAPy. These interactions are evident in the computer image generated for the 1:3 TAU:BAAPy complex. The carbons BAAPy-8a,b of BAAPy also showed movements indicating interactions of the amido oxygens with, most possibly, the amino hydrogen of TAU or its own. We have certainly observed from molecular modelling that, at ≥1:4 TAU:BAAPy ratios, intra-BAAPy interactions predominate consistent with the ¹H NMR titration results which show negligible peak movement of the TAU amido nitrogen at 1:5 TAU:BAAPy ratio. It would seem that BAAPy carbons BAAPy-2,6 also experienced the deshielding of the adjacent amido hydrogens by the TAU-2 and TAU-4 amido oxygens causing an downfield peak movement at 1:1 TAU:BAAPy stoichiometry. However, at lower
TAU:BAAPy ratios (i.e. ≥1:2) the peaks reversed to upfield shifts indicating a change in electron density in their proximity. This suggests that at 1:1 stoichiometric ratio, the ADA/DAD H-bonding array is the predominant interaction between TAU and BAAPy and that BAAPy participates in other interactions at lower TAU:BAAPy ratios.
**Figure 5.** Partial $^1$H NMR spectra of a pure BAAPy (A), pure TAU (B) 1:1 mole ratio of TAU: BAAPY (C) and 1:3 mole ratio of TAU:BAAPY (D), measured at 60ºC in $d$-DMSO, showing marked chemical shift movements of protons involved in TAU-BAAPy interactions.
Figure 5. 6. Partial $^{13}$C NMR spectra of a pure BAAPy (A), pure TAU (B) 1:1 mole ratio of TAU: BAAPY (C) and 1:3 mole ratio of TAU:BAAPY (D), measured at 60°C in $d$-DMSO, showing marked chemical shift movements of protons involved in TAU-BAAPy interactions.
Figure 5.7. Chemical shifts of selected protons (A) carbons (B) of TAU and BAAPY measured by measured by $^1$H and $^{13}$C NMR respectively. Note that $\Delta$ chemical shift = chemical shift of the mixture - chemical shift of the pure solution of TAU or BAAPy.
5.2.3. Template rebinding studies

Krstulja et al.\textsuperscript{108} reported the binding performance of TAU bulk MIPs using frontal chromatography and recorded high imprinting factors (IF = 48) based on the difference of retention factors between MIP and NIP. For this study, we opted to use batch binding assays and developed an \textit{in-situ} quantitative solution \textsuperscript{1}H NMR protocol to measure the unbound TAU left in solution, as with HPLC, without the need to separate the polymeric particles. The range of the concentration used is in accordance to the range of concentration of TAU used in Krstulja’s study.\textsuperscript{108} Employing 1,4-dioxane as a reference standard, this \textit{in-situ} method was applied to rebinding tests at analyte concentration of ≥ 10 µM giving results that are comparable to the conventional method that involves separation of polymer particles prior to measurements. TAU rebinding tests were first conducted to determine the optimum TAU rebinding time using PP-1:1-A. Maximum binding capacity was achieved after 60 min for (Figure 5.8), subsequent binding assays were measured after 60 min of incubation and an additional 15 min of test sample preparation.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.8.png}
\caption{In-situ time-binding tests using 10.0 mg of PP-1:1-A microspheres incubated in 0.5 mL of 100 µM TAU solution.}
\end{figure}
5.2.3.1. TAU rebinding efficiency

Figure 5.9 gives the rebinding results for bulk (BP-1:1-A and BP-1:1-A) and precipitation (PP-1:1-A) polymers (10.0 mg in 0.500 mL of 100 mM TAU) after 1 hour of incubation. As earlier presented in Table 5.1, 64% and 77% of the TAU added in the feed formulation of chloroform and acetonitrile-porogenated MIPs, respectively, was incorporated the monolithic MIPs resulting in a 1:1 stoichiometric T:FM ratio, but only rebound 2.8% (4.1 ± 0.2 µmol/g) and 2.3% (3.6 ± 0.1 µmol/g) of it, respectively. These results suggest that most of the incorporated template was not converted to high fidelity imprints in bulk imprinting, with some possibly destroyed during grinding of the monoliths. Conversely, their respective NIPs also recorded comparable TAU binding of 2.4 ± 0.1 µmol/g (BP-1:1-C) and 1.8 ± 0.2 µmol/g (BP-1:1-A) giving imprinting factors of 1.7 and 2.0, respectively.

![Figure 5.9. TAU incorporation and binding efficiency of PP-1:1-A microspheres and bulk polymers BP-1:1-A and BP-1:1-C. 10.0 mg of polymers were incubated in 0.500 mL of 100 µM TAU solution for 1 hour prior to quantitative ¹H NMR analysis.](image-url)
In contrast to the BP polymers, PP-1:1-A only incorporated 41% (98 µmol/g) of the TAU feed resulting in a 1:3 TAU:BAAPy ratio in the polymer, rather than 1:1. As presented in the previous section, both molecular modelling and NMR titration experiments support formation of 1:3 TAU:BAAPy complexes due to the presence of the acyl groups in TAU, in addition to its imide functionality, capable of interacting with the amido proton of BAAPy. Nevertheless, PP-1:1-A MIP managed to rebind 3.7% (3.64 ± 0.03 µmol/g) of the imprinted TAU, 1.5 times higher than that of BP-1:1-A (2.4%). These results suggest that imprinting is more efficient by precipitation polymerisation than by bulk. In the case of the non-imprinted polymers, PP-1:1-A NIP gave a TAU binding (1.23 ± 0.02 µmol/g) 1.5 times lower than that of BP-1:1-A NIP (1.81 ± 0.18 µmol/g) resulting in an imprinting factor of 3.0, higher than that of BP-1:1-A (i.e. 2.0).

5.2.3.2. Characterisation of Binding Sites: Binding Isotherms

Binding isotherms for PP-1:1-A and its bulk counterpart BP-1:1-A are presented in Figure 5.10 as non-linear (NL) and linearized Langmuir (LL) curves. These approximations assume homogeneous binding but have also been conveniently used for heterogeneous MIP systems. Binding parameters $K$ (binding affinity constant) and $N$ (total number of binding sites) derived from both models summarised in Table 5.3 are in close agreement.
Figure 5.10. Binding isotherms of BP-1:1-A and PP-1:1-A polymers fitted to non-linear (A) and linearized (B) Langmuir models. Isotherms obtained using 10 mg polymer incubated for 1 hr in 0.500 mL of 1 to 100 µM TAU solution. Free TAU was measured by in-situ quantitative $^1$H NMR spectroscopy.
Table 5.3. Binding affinity constants ($K$) and number of binding sites ($N$) for BP-1:1-A and PP-1:1-A estimated from non-linear (NL) and linearised (LL) Langmuir curves given in Figure 5.10.

<table>
<thead>
<tr>
<th>Model</th>
<th>Parameter</th>
<th>BP-1:1-A</th>
<th>PP-1:1-A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIP</td>
<td>NIP</td>
</tr>
<tr>
<td>Non-linear Langmuir</td>
<td>$K$ (M$^{-1}$)</td>
<td>3.4 ± 0.3 × 10^4</td>
<td>0.9 ± 0.3 × 10^4</td>
</tr>
<tr>
<td></td>
<td>$N$ (µmol/g)</td>
<td>4.54 ± 0.22</td>
<td>3.34 ± 0.81</td>
</tr>
<tr>
<td>Linearised Langmuir</td>
<td>$K$ (M$^{-1}$)</td>
<td>3.0 ± 0.4 × 10^4</td>
<td>0.6 ± 0.1 × 10^4</td>
</tr>
<tr>
<td></td>
<td>$N$ (µmol/g)</td>
<td>4.59 ± 0.38</td>
<td>4.24 ± 0.53</td>
</tr>
</tbody>
</table>

1 values were determined from Prism GraphPad using the one-site hyperbola model. Errors are at 95% confidence level.

As an expected consequence of molecular imprinting, both BP and PP MIPs showed higher TAU binding and total binding sites ($N$) than their non-imprinted counterparts. Likewise, $K$ for MIPs are also higher than that for NIPs indicating that higher affinity binding sites for TAU were created during molecular imprinting.

Krstulja et al.\textsuperscript{108} have reported $K$ values of $1.2 \times 10^3$ M$^{-1}$ and $0.3 \times 10^3$ M$^{-1}$ for MIP and NIP, respectively, an order of magnitude lower than the values we obtained ($3.4 \times 10^4$ M$^{-1}$ and $0.9 \times 10^4$ M$^{-1}$, respectively), for a TAU/BAAPy system equivalent to our BP polymers also analysed using the NL model and comparable concentration range ($\leq 100$ µM). Nevertheless, both calculations recorded a $K_{MIP}$ 4 times higher than the corresponding $K_{NIP}$ confirming efficient imprinting of TAU in both cases. In the case of $N$, Krstulja et al. obtained values twice as high as ours, 10.65 µmol/g vs 4.54 µmol/g for MIP and 4.88 µmol/g vs 3.34 µmol/g for NIP, and a higher $N_{MIP}/N_{NIP}$ ratio of 2.2 compared to only 1.4 in this study. It is noteworthy that Krstulja et al. generated their polymers at a temperature of 40°C (vs 60°C in this present study) and have employed frontal chromatography for binding calculations which could account for the difference in $K$ and $N$ values obtained by the two studies.
Our results also showed the $K$ for PP MIP ($7.5 \pm 0.8 \times 10^4 \text{ M}^{-1}$) to be 2 times higher than that for BP MIP ($3.4 \pm 0.3 \times 10^4 \text{ M}^{-1}$) and 10 times higher than its corresponding NIP. Conversely, $N$ for PP MIP ($5.60 \pm 0.39 \mu\text{mol/g}$) is slightly higher than that for its bulk counterpart ($4.54 \pm 0.22 \mu\text{mol/g}$) and twice as much as the $N$ of its corresponding BP-NIP. Both PP and BP NIPs afforded comparable $K$’s and $N$’s. These results indicate that precipitation polymerisation was able to generate higher affinity binding sites for TAU which could be attributed to a stronger T:FM interaction provided by a maximal interaction by virtue of the 1:3 TAU:BAAPy ratio. Previous studies\textsuperscript{123, 217} have also demonstrated that precipitation polymerization yields more homogenous and higher affinity constants imprinted polymers compared to bulk polymerisation.

5.2.4. Selectivity Studies

PP-1:1-A have been shown to possess higher affinity binding sites than its bulk counterpart while showing a non-stoichiometric TAU:BAAPy ratio of 1:3. Molecular modelling and NMR studies conducted on this system suggests favourable interactions, other than the ADA/DAD H-bond array, involving the acetyl groups in the ribose ring of TAU. Thus, selectivity studies for PP-1:1-A were conducted against analogues 2,3,5-tri-O-acetyl cytidine, TAC and Urd, (Figure 2.1). Unlike TAU, Urd does not have the three acetyl groups in the ribose ring while TAC does possess the three acetyl groups in the ribose ring but not the imide group.

Results of the non-competitive cross-binding assays on PP-1:1-A MIP are given in Figure 5.11. While the template TAU was rebound at $3.64 \pm 0.03 \mu\text{mol/g}$, only $2.50 \pm 0.01 \mu\text{mol/g}$ of Urd was bound decreasing to $1.56 \pm 0.03 \mu\text{mol/g}$ with TAC. Similarly, in competitive binding assays, while the total bound quantities of TAU+Urd and TAU+TAC
mixtures are comparable, less TAC was bound than Urd (0.91 ± 0.07 µmol/g vs 1.33 ± 0.09 µmol/g). These results indicate that analyte binding is predominantly governed by the DAD/ADA hydrogen bond as shown by the significant amount of bound Urd compared to the non-imide containing TAC. Nevertheless, binding of Urd under non-competitive condition is 30% lower than TAU suggesting that the interaction of BAAPy with the acetyl groups in the ribose ring of TAU (not found in Urd) also enhances TAU binding. Further, even with the disruption of the DAD/ADA hydrogen bonding array, TAC still registered a moderate binding of 43% (non-competitive) and 23% (competitive) with respect to TAU suggesting the importance of the acetyl groups in the ribose ring. It is noteworthy that the amino group of TAC could also interact with BAAPy and could also be responsible for its binding.

Figure 5.11. Binding capacities of PP-1:1-A MIP in non-competitive cross-binding and competitive assays against Urd (Ur) and 2',3',5'-tri-O-acetyl-cytidine (TAC). 10.0 mg of polymers were incubated for 1 hour prior to 1H NMR analysis using 0.500 mL of 100 µM of analyte for non-competitive rebinding and equimolar concentration (50 μM) of TAU and analogue for competitive rebinding.

To verify the interactions between BAAPy and the analogues which we alluded to as responsible for the competitive and non-competitive binding results, we subjected the two analogues to molecular modelling calculations, using the previously generated 1:3
TAU:BAAPy cluster presented in Figure 5.4, B, by ‘freezing’ the 3 BAAPy units in place and replacing TAU with either Urd or TAC. As can be seen in Figure 16A, Urd interacts with one BAAPy unit via the DAD/ADA hydrogen bonding array and no interaction was observed with the other two BAAPy units which, with TAU, showed interactions with the acetyl groups in the ribose ring. With TAC (Figure 5.12, B), interaction was evident between BAAPy units 2 and 3 and the acetyl groups of TAC, similar to what was observed with TAU. BAAPy unit 1 also interacted with the amino group of TAC but the DAD/ADA hydrogen bond array of interaction was not maintained. These molecular modelling results are consistent with the cross- and competitive binding analyses.

Figure 5.12. Urd:BAAPy (A) and TAC:BAAPy (B) 1:3 clusters showing interaction points and their corresponding distances measured by Spartan ‘14 v1.1.8. The BAAPy cluster was generated using TAU as template (see Figure 5.4, B) and frozen in place.
5.2.5. Other Factors

5.2.5.1. Effect of Initiator Concentration

Mijangos et al. 77, 160 have compared the effects of the amount of the initiator [1,1’-azobis(cyclohexane-1-carbonitrile)] in the bulk imprinting of (+)-ephedrine at 80°C and found that, apart from its effect on polymer rigidity, imprinted polymers produced in lower amount of initiator (1 %, initiator:total monomer (I:TM) ratio = 1:13) performed better than the MIPs produced in higher amount of initiator (5%, I:TM ratio = 1:65). They hypothesised that the heat of reaction brought about by high amount of the initiator in the feed disrupts the complex formation between the template and the functional monomer, reducing the affinity and selectivity of the MIPs.

In this study, PP-1:1-A, prepared with I:TM ratio of 1:131 following published formulation108 was compared with two other TAU precipitation MIP systems prepared with I:TM ratios of 1:50 (PP-I-1:50) and 1:200 (PP-I-1:200) using the same formulation and porogen as PP-1:1-A. While Yang et al. observed that higher concentration of initiator resulted in bigger and polydisperse particles149, 151, this trend was not observed in our systems as the particles appear to be aggregated, polydisperse and of comparable size.
Figure 5.13. SEM images of PP-1:1 microspheres produced by varying the initiator:total monomer (I:TM) ratios in acetonitrile at 60°C: 1:50 (PP-I-1:50) MIP (A) and NIP (B); 1:131 (PP-1:1-A) MIP (C) and NIP (D); 1:200 (PP-I-1:200) MIP (E) and NIP (F). Refer to Table 5.1 and 5.2 for additional details of their synthesis.

While the FM:XL ratios of the polymers were not markedly affected by the concentration of initiator in the feed, the T:FM ratio was significantly affected. From 1: 2.5
T:FM ratio obtained from PP-1:1-A, it decreased to 1:6.25 (14% template incorporated) when the I:TM ratio was reduced to 1:200 but increased to 1:3.44 (28% template incorporated) when the I:TM ratio was increased to 1:50. Analyses of the binding capacities of the polymers (Table 5.1, Figure 5.14) showed PP-1:1-A (IF = 3.0) to be better performing than both PP-I-1:50 (IF = 2.1) and PP-I-1:200 ((IF = 1.2). The drastic reduction in the imprinting and binding efficiencies of PP-I-1:200 compared to PP-1:1-A suggests that slow polymerisation reaction at 60 °C does not favour the formation of imprints which could be because slow reaction provides an opportunity for the template-functional monomer complex to be disrupted. While template incorporation and binding were markedly higher with PP-I-1:50 than with PP-I-1:200, they were still observed to be lower than those for PP-1:1-A. We speculate that for this reaction, the polymerisation rate was faster, as evidenced by the early onset of precipitation, and we speculate that the equilibrium concentration of the TAU-BAAPy complexes has not yet been reached that resulted to low specific binding capacity.

![Figure 5.14. Comparison of the template (TAU) incorporation (i.e. imprinted) and binding capacities of PP-1:1 microspheres produced by varying initiator (AIBN):total monomer (I:TM) ratios in acetonitrile at 60°C: 1:50 (PP-I-1:50), 1:131 (PP-1:1-A) and 1:200 (PP-I-1:200). Refer to Table 5.1 and 5.2 for additional details of their synthesis.](image)
5.2.5.2. Effect of Agitation

Molecular imprinting produced by precipitation polymerisation has been carried out with and without agitation. 49,6 In previous studies have illustrated that most systems favour gentle rocking or no form of agitation at all because it assists in the formation of more binding efficient polymers 151, 218 and more mono-dispersed particles. 149.

In the case of the precipitation system under study, the effect of agitation, by vigorous stirring (at ~130 rpm) and gentle rolling (at ~9.5 rpm), were investigated under the same conditions as the non-agitated PP-1:1-A. Both polymerisation mixtures subjected to agitation produced highly aggregated particles with a “cauliflower” morphology (Figure 5.15), consistent with those observed by Yang et al. 151 for their particles from stirred precipitation polymerisation mixture. As shown in Table 5.1 and 5.2, the conversions and FM:XL ratios (based on EGDMA, measured by qNMR) obtained for these systems varied slightly and, more notably, their T:FM ratios. While PP-1:1-A gave a 1:2.5 stoichiometry, PP-1:1-A-St and PP-1:1-A-Rd afforded 1:1 and 1:2 ratios, respectively. It would seem that agitation has affected the formation of the T:FM association cluster, quite possibly by disrupting the weaker associations but maintaining the strong DAD/ADA hydrogen bond array resulting in a 1:1 (or close to) stoichiometric T:FM ratios. However, while TAU incorporated in PP-1:1-A-St (173 ± 3 μmol/g) is twice higher than that of PP-1:1-A (98 ± 3 μmol/g), PP-1:1-A recorded a binding capacity ten times higher (3.64 ± 0.03 μmol/g) than the stirred equivalent (0.29 ± 0.01 μmol/g) (Table 5.1, Figure 5.16). The higher uptake of the template by PP-1:1-A-St is probably due to “superficial” incorporation of the template which does not necessarily form high fidelity cavities in the polymers. These results suggest that the interaction between the functional monomer and the template is disrupted or reduced when the polymerisation
reaction is agitated. On the other hand, the amount of TAU incorporation (152 ±2 μmol/g) in PP-1:1-A-Rd, which was subjected to a more gentle form of agitation than stirring (rolled at 9.5 rpm), is less than that for PP-1:1-A-St but higher than for PP-1:1-A consistent with the above hypothesis. Conversely, its binding capacity is less than that of PP-1:1-A but higher than that of PP-1:1-A-St.

Figure 5.15. SEM images of microspheres of PP-1:1-A-Rd MIP (A) and NIP (B), PP-1:1-A-St MIP (C) and NIP (D). See Figure 5.3 for SEM images of PP-1:1-A.
Figure 5.16. Comparison of the template uptake, template bound by the MIP and the NIP of the polymers obtained from PP-1:1-A (no agitation), PP-1:1-A-Rd (rolled at ~9.5 rpm) and PP-1:1-A-St (stirred at ~130 rpm).

5.3. Summary

The efficiency of the stoichiometric non-covalent imprinting of BAAPy with TAU due to their strong DAD/ADA hydrogen bond array interaction has been observed in bulk polymerisation process. This study is the first to investigate and assess the imprinting and template rebinding efficiencies of the TAU/BAAPy MIP system prepared by precipitation polymerisation. Template incorporation and batch rebinding as well as polymer composition was measured by quantitative NMR spectroscopy.

We found that the stoichiometric 1:1 T:FM ratio exhibited by the TAU/BAAPy bulk MIP has not been maintained in precipitation polymerisation and an optimal TAU:BAAPy ratio of 1:2.5 was obtained for MIP microspheres prepared in acetonitrile (PP-1:1-A) without agitation from a 1:1 TOUAU:BAAPy feed. The PP-1:1-A microspheres afforded a $K$ of $1.7 \times 10^4$ M$^{-1}$ and a binding capacity of 3.69 µmol/g (41% of the measured incorporated TAU) higher than its bulk counterpart BP-1:1-A ($K = 3.4 \pm 0.3 \times 10^4$ M$^{-1}$, $B_{MIP} = 4.54 \pm 0.22$).
µmol/g) despite incorporating 1.5 times more TAU. Molecular modelling and NMR studies indicate that, aside from the DAD/ADA hydrogen bond interaction, BAAPy also interacts with the acetyl groups of the ribose ring of TAU supporting the formation of the 1:3 TAU:BAAPy complex. Non-competitive cross-rebinding and competitive assays using PP-1:1-A against analogue 2,3,5-tri-\textit{O}-acetyl cytidine (TAC), which possess three acetyl groups in the ribose ring but not the imide group, showed significant TAC binding which suggests that BAAPy also interact with the acetyl groups. Nevertheless, cross- and competitive binding assays against Urd resulted in Urd binding higher than that against TAC which indicates that the DAD/ADA hydrogen bond array is the predominant interaction between TAU and BAAPy.

Imprinting efficiency and binding capacity of precipitation MIPs have also been shown to be affected by the initiator concentration and the method of initiation. We found that, for the MIP system under study (PP-1:1-A), both template incorporation and rebinding are favoured by a moderate initiator concentration, i.e. I:TM ratio of 1:131, also employed by other groups.\textsuperscript{103-104, 108-109, 207} Lowering the initiator concentration to achieve an I:TM ratio of 1:200 lowered template incorporation by a factor of 2.6 and binding capacity by a factor of 6.5. On the other hand, increasing the I:TM ratio to 1:50 also reduced template incorporation by 1.3 and binding capacity to 3.2. We attributed this to imprints that are not properly formed due to disruption of the template-monomer complex because of a slow reaction or due to the formation of partial imprints that causes formation of loosely attached templates. It is also interesting to note that while vigorous agitation by stirring showed high template incorporation, it gave very low template rebinding which we found could be improved by mild agitation (gentle rolling at \(~9 \text{ rpm}\)). However, highest translation of template incorporation to rebinding was obtained with the MIP prepared without agitation which we
speculated to be due to the undisrupted and optimal formation of T:FM complexes producing more high fidelity imprints within the polymers.

Interestingly, while the imprinting efficiencies (i.e. template incorporation with respect to the initial concentration in the polymerisation feed) measured for the better performing TAU MIPs generated in this study were moderate, 41% for PP-1:1-A and 60% for BP-1:1-C, their rebinding capacities were only between 3-4% of the incorporated template.
Chapter 6
Ionic Liquids as porogen in precipitation imprinting of propanolol

6.1. Introduction

Solvents have important roles in the molecular imprinting process: They bringing all the components into a homogeneous phase and make the polymers porous, hence, they are also know as porogens. Additionally, previous research suggest that the porogens also facilitate and enhance the template-monomer interaction during the imprinting process. Porogens can also affect the morphological properties of the polymers, such as porosity and surface area. These properties ensure the accessibility of the binding sites for the removal and rebinding of the template. Low solubility porogens have been linked to MIPs with larger pore sizes (higher surface area) because of the early formation of polymers during synthesis. Conversely, high solubility porogens produces MIPs and NIPs with smaller sized pores or materials with higher surface areas. Most conventional porogens used in molecular imprinting are volatile organic solvents as they fulfil the solvency requirements of the most of the involved components in the process. Organic solvents such as methanol, tetrahydrofuran (THF), dichloromethane, chloroform and acetonitrile were assessed to enhance binding efficiencies of the resulting polymers. Finding a greener alternative to VOCs is favourably advantageous due to the toxic effects brought about by these types of solvents. Potential alternative are the room temperature ionic liquids (RTILs). RTILs or simply ILs (ionic liquid) were originally used as a solvent in polymerisation process but were observed to increase reaction rates and molecular weight of the polymers. They also improve the reaction conversion, allowing the
potential to prepare high molecular weight polymers using a low concentration of catalyst in reverse ATRP of MMA. 229-230

ILs are organic salts that are in liquid form at room temperature. They have low melting points, are thermally stable, have high viscosity and have negligible vapour pressure. 221, 225, 227, 232-234 ILs are typically composed of a poorly coordinated large organic cation and a small organic or inorganic anion so they are highly polar (but considered as a non-coordinating solvent). 234-235 Due to their properties, ILs have gained attention as the “green” alternative solvent for organic synthesis. 222, 228, 231 There are a wide variety of ILs that are available and easily synthesized, however but the most commonly used in polymerisation reactions are the imidazolium-based ILs (Figure 7.2)

The use of imidazolium based ILs as porogens in molecular imprinting by a non-covalent approach was first been reported by Booker et al. in 2006. 70 It was illustrated that the microspheres prepared by precipitation polymerisation using the two imidazolium –based IL: bmImBF4 and bmImPF6, showed an accelerated polymerisation rate. The increase in the polymerisation rate in using ILs as porogen was attributed to the combination of its polarity and viscosity. It was proposed that Harrisson et al. 236 that ILs has the ability to lower the activation energy of the propagation stage of the polymerisation reaction due to its polarity. Aside from lowering the activation energy of the polymerisation reaction, it was also found to decrease the termination rate due to its high viscosity. 236-238 Among the different porogens, it was the bmImBF4-synthesized MIPs which exhibited the greatest selectivity and binding performance 2 times higher than the MIPs synthesized under traditional precipitation polymerisation. It was also demonstrated that the previously mentioned advantages of ILs in terms of the polymerisation reaction was still observable even at low temperatures (5 °C). 70 Recently, different imidazolium-based ILs were employed to synthesize propranolol imprinted monoliths. Among the imidazolium-based ILs employed, 1-butyl-3-
methylimidazolium hexafluorophosphate (bminPF₆) afforded the highest template binding and gave an imprinting factor of IF= 2.3. Several other studies followed suit which demonstrated the successful use of ILs in imprinting different types of templates. Most involved the application of IL-MIPs as a sorbent material in SPE in trace analyses of different compounds. Sun et al. illustrated that IL-MIPs displayed a good selective recognition of its template against other analogues and showed higher recoveries of quinolones (78-94%) in animal tissue samples.239 In another study, 1-allyl-3-methylimidazolium bromide was used as a porogen in the molecular imprinting of α-chloro-dichlorodiphenyldichloroethane and showed high recovery of the templates in celery samples.240

Figure 6. 1. Structure of an imidazolium ion

This study is an extension of a previous investigation on the use of 1-butyl-3-methylimidazolium hexafluorophosphate (PF₆) as porogen in the synthesis of MIPs. Previously, it was demonstrated that trans-aconitic acid-imprinted microspheres produced using ILs as porogen demonstrated comparable efficiency and selectivity compared to VOC-synthesized MIPs. In another study, monoliths produced using PF₆ as porogen showed lower binding performance when using the template propranolol (PNL) compared to chloroform, CHCl₃ prepared MIP. This behaviour was attributed to the lower surface area and number of pores. However, fine-tuning the rebinding conditions enhanced the MIP binding and imprinting factors of PF₆ polymers.23 These contradicting results led our group to the investigation of the microspheres prepared by precipitation polymerisation using the PNL as
a template. We followed the optimized formulation of Yoshimatsu et al.\textsuperscript{99} to validate the effects of the ILs in the imprinting process.

6.2. Results and discussion

Series of publications were reported by Booker et al. regarding the applications of ILs as porogen in MIPs synthesis. In the first report, the applications of ILs as porogens in molecular imprinting of cocaine and trans-aconitic acid led to an accelerated polymerisation rate. This was also observed at lower temperature without sacrificing selectivity of the resulting polymers.\textsuperscript{70} Results showed that ILs-synthesized microspheres produced by following the formulation of the precipitation polymerization showed product formation in less time than the VOCs-synthesized polymers at 60 °C (8 hours vs 18 hours for ILs as porogens and VOCs as porogens). Additionally, it was demonstrated that the selectivity of the IL synthesized MIPs systems (using trans-aconitic acid as template) synthesized at 60 °C was at par with or better than the VOCs synthesized MIP systems (IF= 1.0 for ILs-synthesized polymers vs IF= 0.98 for VOCs-synthesized polymers). This improvement in the properties of the MIPs produced by ILs led to the investigation of different ILs as porogen in another MIP system, where propranolol was used as a template.

In a separate study,\textsuperscript{73} the selectivity and template rebinding capacity of a propranolol (PNL) MIP monolithic system synthesized using VOC (CHCl\textsubscript{3}) were compared with the MIP systems synthesized in an IL, PF6. The recorded imprinting factors for MIP synthesized in bmImPF\textsubscript{6} (IF= 1.98) is ~3 times lower than the IF observed for MIP synthesized in CHCl\textsubscript{3} (IF = 4.64). The binding isotherms of MIP-PF\textsubscript{6} and MIP-CHCl\textsubscript{3} expressed as Scatchard plots yielded higher values of $K_a$ and number of binding cavities for MIP-CHCl\textsubscript{3} ($K_a = 2.99 \times 10^4$.
M\(^{-1}\) and \(N = 0.49 \text{ μmol/g, 1.37 X10}^5 \text{ mole}\) than MIP-PF\(_6\) (\(K_a = 6.25 \text{ X10}^{-4} \text{ M}^{-1}\) and \(N = 9.77 \text{ X10}^{-6} \text{ mole}\)). Scanning electron micrographs of the two MIP systems also showed different morphologies. Analysis of the physicochemical properties of the two systems revealed the differences of the two MIP systems. BET surface area analyses of the two systems illustrated that MIP-CHCl\(_3\) (306 m\(^2\)/g) has 40% higher surface area than MIP-bmImPF\(_6\) (185 m\(^2\)/g). PALs pore sizes and relative number of pores were also assessed and showed that there are two distinct pore sizes present in the two MIP systems: Type A (small) and B (large). Analysis of the template size, it showed that the type of pore accommodating the template at its widest diameter is the Type B pores of the polymers with sizes ranging from 7.06 nm (MIP) and 11.15 nm (NIP). The MIP-CHCl\(_3\) had significantly smaller Type B pore size (2.19 nm), and a larger relative number of 8.5% vs MIP-PF\(_6\) of 7.06 nm pore size with a relative number of only 3.0%. From the pore size and its relative number analyses, it was assumed that the higher template binding capacity displayed by the MIP-CHCl\(_3\) was attributed to the greater number of Type B pores, despite of its smaller size.

The differences in the binding performance of the two formats synthesized in PF\(_6\) indicated that the trans-aconitic acid imprinted microsphere format\(^{70}\) is more efficient than the propranolol imprinted monoliths.\(^{73}\) In order to clarify and gain better understanding of the role of ILs as a porogen in precipitation polymerisation and expand more information on the initial results in the applicability of the ILs in molecular imprinting by precipitation polymerisation, a similar template system (propranolol, PNL) was analysed. The highest MIP binding of 700 μg/mL recorded in imprinting PNL uses the formulation of Yoshimatsu et al., where TRIM was used as a cross-linker, MAA as the functional monomer, ACN as porogen and a T:FM:XL of 1:2.5:4:0. This formulation was followed for comparative purposes\(^{99}\)
6.2.1. Synthesis of Polymers

Thermal polymerisation (60°C) of propranolol (PNL) imprinted polymers using the two porogens: ACN (ACN-PP) and PF6 (IL-PP) were conducted following the formulation reported by Yoshimatsu et al. (T:FM:XL =1: 2.8: 3.5)\textsuperscript{49}

It was noted that the rate of polymerisation in IL-PP was faster compared to the ACN-PP system. White particulates started forming after 60 mins, for IL-PP and after 180 mins, for ACN-PP showing thermal polymerisation. The increase in the polymerisation rate in using ILs as porogen was also reported by Booker et al. \textsuperscript{70,72,226}

6.2.2. Polymer composition

Table 6.1 shows the polymer composition of the two MIP systems produced in different porogens. In terms of the amount of components in the polymers, ACN-PP MIP incorporated higher amount of MAA (82 ± 2 %) but lower amount of TRIM (78 ±2) compared to its NIP (62 ± 1% MAA and 94 ± 1 % TRIM). This may be attributed to the presence of the template in the system. We speculated that during the imprinting process, the interaction of the template with the functional monomer could have promoted the incorporation of more functional monomer into the polymer. The calculated FM:XL ratio in the MIP is 1:1.4 and 1:2.2 for the NIP which is higher than the starting FM:XL of 1:1.5.
Table 6.1. Polymer composition of An-PP and IL-PP MIP and NIP.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Incorporated components in the polymers</th>
<th>Polymer Composition T:FM:XL</th>
<th>Degree of Cross-linking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% incorporation (μmol/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRIM</td>
<td>MAA</td>
<td>PNL</td>
</tr>
<tr>
<td>ACN-PP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIPs</td>
<td>78 ±2</td>
<td>82 ±2</td>
<td>62 ±1</td>
</tr>
<tr>
<td>(392)</td>
<td>(275)</td>
<td>(82)</td>
<td></td>
</tr>
<tr>
<td>NIPs</td>
<td>94 ±1</td>
<td>62 ±1</td>
<td></td>
</tr>
<tr>
<td>(472)</td>
<td>(207)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-PP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIPs</td>
<td>88 ±3</td>
<td>64 ±1</td>
<td></td>
</tr>
<tr>
<td>(440)</td>
<td>(85)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NIPs</td>
<td>83 ±3</td>
<td></td>
<td>1:1.3¹</td>
</tr>
<tr>
<td>(417)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹template : functional monomer : cross-linker (mol) ratio in the polymers were compared with the initial ratio of 1(133 μmol) : 2.5 (333 μmol) : 3.75 (500 μmol)
²Amount of cross-linking compared to the initial ratio -C=C- and -C=O- and in the polymers.
³Calculated with the assumption that all MAA (333 μmol) is incorporated in the polymers.

In the case of the IL-PPs, the amount of TRIM in the IL-PP MIP (88 ± 3%, 816 μmol) is almost equal to amount in its NIP counterpart (83 ± 3%, 816 μmol). This indicates that the presence of the template does not have an effect on the TRIM incorporation in the polymers.

In measuring the amounts of unpolymerized TRIM, the -O-CH₂- peak of TRIM was used because no double bond peak (due to MAA nor TRIM) was visible in the ¹H NMR spectra of both the MIP and the NIP. The absence of the double bond peaks and the presence of the -O-CH₂- peak of TRIM imply two things: complete conversion of MAA to polymer or an occurrence of a secondary reaction between TRIM and MAA in which all double bonds have reacted/cross-linked but soluble in PF6. This secondary reaction is possible considering that imidazolium-based ILs have been known to increase the acceleration rate of the polymerisation process due to the combination of two factors: high polarity and viscosity of ILs.

To confirm the occurrence of a secondary reaction, the post-polymerisation solutions were dialysed against water after collecting the IL-PP polymers. Interestingly, after removal
of IL, water-insoluble polymer fractions were obtained for both MIP and NIP. It is also worth mentioning that IL-PP MIPs had lower amount of second fraction compared to its NIP counterpart. It was insufficient for further characterization and this second fraction (IL-PP-2 NIP) will be discussed in a separate section (Section 6.2.5).

The amount of template incorporated (with respect to the starting concentration of PNL) within the ACN-PP MIP (62 ±1 %, 82 µmol) is similar to the amount of template incorporated within the IL-PP MIP (64 ± 1%, 84 µmol) as shown in Table 6.1. This suggests that the IL porogen does not seem to affect the template incorporation in the polymers. It has been established that polar porogens interfere in the complex formation of the template and the functional monomer \(16, 46, 62, 219\) which was not observed given the wide difference in dielectric constants between the two porogens. \(\varepsilon_{\text{ACN}} = 3.45^{243}\) and \(\varepsilon_{\text{BF}_6} = 11.4\).\(^{244}\) Quite possibly, the high viscosity of the ILs counteracted the polarity effect with poor solvation of the template in the viscous ILs. This could have led to a high concentration of template and monomer in the surrounding active site of nucleation\(^{236}\) while promoting a fast polymerisation reaction.

The amount of cross-linking in the polymers is correlated with the ratios of the peak integrations of the -C=O and -C=C- of the crude cross-linker and in the polymers. Table 6.1 shows that the degree of cross-linking between the polymer systems was significantly different from each other. The degree of cross-linking has been deduced to be 67.3 ± 0.02% and 68.0 ± 0.02% for the MIP and NIP, respectively, of IL-PP polymers, and 54.7 ± 0.01% and 59.7 ± 0.01% for the MIP and NIP, respectively, of ACN-PP polymers. The calculated degree of cross-linking for the IL-PP polymers revealed that 67-68% of the starting monomers were cross-linked. The 10% difference in the degree of crosslinking between the IL and ACN polymers could be due to the proven effect of ILs in increasing the rate of polymerisation (observed in the study by Booker \textit{et al.}\(^{70}\)). The high viscosity of ILs\(^{236, 241}\)
limits the monomer movements of the monomers in the solution and therefore increase the probability of polymerisation of the growing chain.

Acetonitrile produced discrete microspheres (Figure 6.3 A and B) with $d_H$ values of 185.3 ± 0.1 nm for the imprinted polymer and $d_H = 160.8 ± 0.1$ nm for the non-imprinted polymers. On the other hand, IL-PP produced a “spongy” morphology observed for both the imprinted polymer and for its non-imprinted counterpart. This morphology is consists of small particles linked together in a network which is similar to a sponge (Figure 6.3 C and D). Two particle sizes were detected for IL-PP polymers as shown in the particle size distribution graph in Figure 6.4 obtained by DLS. For the IL-PP MIP (Figure 6.4 C) the particle sizes recorded were 217 ± 0.1 nm and 877.1 ± 0.2 nm with a PDI of 0.3 For its NIP counterpart, the particle sizes detected are 281.6 ± 0.7 and 957.9 ± 0.9 nm with a PDI of 0.5 (Figure 6.4 D) obtained with DLS. Based from the PDI values of the IL-PPs, it indicates that this system is consists of particles with various sizes, which is noticeable in the SEM images in Figure 6.3 C and D. Booker et al. reported a smaller particle size range with sizes ranging only from 300-500 nm (for both MIP and NIP). This can be attributed to a different cross-linker used (divinylbenzene). 71
Figure 6. SEM micrographs of ACN-PP MIP (A) and NIP (B), and IL-PP MIP (C) and NIP (D). Insets are the particle sizes ($d_H$) measured by dynamic light scattering in nm.
Specific Surface Area (BET) & total pore volume using nitrogen gas was conducted on the four different polymers (ACN and PF6 MIP and NIPs) by N₂ gas absorption. The collected data is given in Table 6.2.

As shown in Table 6.2, comparable pore sizes were observed with the IL-PP MIP and NIP indicating that the presence of the template does not affect the pore sizes of the polymers. This is consistent with the results presented by Booker et al.\textsuperscript{73} In terms of the pore volume, IL-PP NIP (0.449 cm$^3$/g) recorded two times the pore volume recorded for its MIP (0.230 cm$^3$/g).
cm$^3$/g) counterparts. Similar to the pore volume, a significant difference in the surface area of the IL-PP NIP (327 m$^2$/g) and MIP (168 m$^2$/g) was also recorded. These significant differences in the pore volume and BET surface area between the MIP and the NIP were not consistent with the published results of Booker et al.$^{73}$ This could be due to the difference in the cross-linker and format (Booker used DVB as crosslinker and monoliths as MIP format) employed$^{73}$

In the case of ACN-PP polymers, the average pore volume and pore sizes of the MIP and the NIP are comparable but different BET surface areas were measured (Table 6.2). The surface area of ACN-PP-NIP is 1.25 times higher than its corresponding MIP and an indication that the presence of the template in the polymerisation solution has an effect in the morphology of the polymers. Despite of the differences in the VOC system presented by Booker (CHCl$_3$ as porogen, DVB as cross-linker and monoliths as MIP format), the similarities in the morphology of the MIP and the NIP were consistent.

In comparison of the two systems, it was illustrated that PF6 made a more porous NIP. This was based from the higher pore volume and surface area compared to its MIP counterparts, which is not observed with the MIP and NIP of ACN-PP. Additionally, the pore sizes and pore volumes of the IL-PP polymers (both MIP and NIP) were higher compared to ACN-PP polymers. Given the size difference between ACN (with size of < 0.5 nm) and PF6 (with the size of imidazolium moiety of 0.967 x 0.371 nm and the PF6 ion with the size of 0.328 x 0.328 nm), PF6 can produce larger pore sizes compared to ACN. Moreover, the structured arrangement of PF6 has illustrated the possibility of forming a micellar structure in the other layer, that can create larger pore sizes in the polymers.$^{73, 245}$
Table 6.2. BET surface area data, pore volume and sizes of ACN-PP polymers and IL-PP polymers.

<table>
<thead>
<tr>
<th>Polymer systems</th>
<th>Ave Pore Volume (cm³/g)</th>
<th>Ave Pore Size (Å)</th>
<th>BET surface Area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN-PP NIP</td>
<td>0.231</td>
<td>26</td>
<td>351</td>
</tr>
<tr>
<td>MIP</td>
<td>0.200</td>
<td>28</td>
<td>281</td>
</tr>
<tr>
<td>IL-PP NIP</td>
<td>0.449</td>
<td>55</td>
<td>327</td>
</tr>
<tr>
<td>MIP</td>
<td>0.230</td>
<td>55</td>
<td>168</td>
</tr>
</tbody>
</table>

6.2.3. Template Rebinding Studies

Figure 10 shows the comparison of the amount of template incorporated and bound by each of the two MIP systems. Comparable percentages of PNL incorporation was observed in the two polymer systems (IL-PP = 64 ± 1%, 476 ± 11 µmol/g and ACN-PP = 62 ± 1%, 525 ± 10 µmol/g). In the case of MIP rebinding performance, ACN-PP MIP bound 38.45 ± 0.3 µmol/g (77% binding) of PNL and a 1.1 times increase in the MIP binding was observed in IL-PP MIPs (41.3 ± 1.0 µmol/g, 83% binding). The PNL binding by the ACN-synthesized polymers obtained in this study was higher than the results of Booker Ref, wherein only 48% was rebound in using CHCl₃ as porogen in the monolith synthesis. This difference in template rebinding between the VOC MIPs could be due to three synthetic inconsistencies: (1) the difference in the dielectric constants of the two porogens ($\varepsilon_{ACN} = 3.45$ and $\varepsilon_{CHCL3} = 4.81$), (2) the difference in the imprinting method, (3) the crosslinker. Solvent polarity affects the formation of the T-FM complex: polar solvents disrupt the formation of this complex. Thus, in using CHCl₃ as porogen, lower template rebound was observed. Booker employed bulk polymerisation which could lower the binding capacity of the polymers due to the damages caused by grinding and sieving of monoliths. Moreover, Booker also employed divinyl benzene, a cross-linker with two polymerisable double bonds and a vinyl benzene
ring that adds rigidity to the polymers. These can also affect the rigidity of the polymers and hence, the binding performance. Similar binding site conversions were calculated for the two systems: IL-PP MIPs displayed 8.7% and CAN-PP MIPs showed 7.3%.

In the case of NIP binding performance, IL-PP NIP (33.0 ± 0.3 µmol/g) bound two times greater than the ACN-PP NIP (19.4 ± 0.3 µmol/g), and gave a lower imprinting factor (IF= 1.25). This could be due to the difference in the solvent used for rebinding studies from the porogen used for the synthesis. Another possible reason for this is that IL-PP NIPs (55 Å) have larger pore sizes compared to ACN-PP NIPs (26 Å) that can accommodate greater concentrations of PNL.
The binding performance of the polymers with respect to surface area was also calculated and shown according to Figure 6.4 B. With respect to surface area, IL-PP MIP and NIP gave PNL binding of 0.25 µmol/m² and 0.10 µmol/m², respectively, increasing the resulting IF to 2.44 from 1.3. The large difference in surface area between the IL-PP NIP and the MIP (NIP ~1.9 times higher) resulted in an increase in the different amount of PNL bound by the MIP and the NIP, and higher IF. Normalized binding performance in terms of surface area of IL-PP polymers is a more reliable measurement of the efficiency of the MIP.
Due to the differences in the sizes and the surface area of the microspheres produced by precipitation polymerisation. In the case of ACN-PP polymers, the amount of PNL bound by ACN-PP MIP and NIP translated to 0.14 µmol/m² and 0.06 µmol/m², respectively, resulting in a higher IF of 2.46. Thus, expressing the binding performance with respect to surface area, the binding efficiency of IL-PP polymers (measured by IF) is at par with ACN-PP polymers.

6.2.4. Other Physical Characterisation

6.2.4.1. X-ray diffraction

The X-ray diffractions of ACN-PP polymers revealed that the microspheres are porous and do not have an ordered structure. Additionally, comparable X-ray diffraction patterns of the MIPs and its corresponding NIPs were observed. This suggests that the presence of the template does not have an effect in the crystallinity or the ordered structure of the polymers. Similarly, the IL-PP MIP and NIP afforded similar curves indicating that the nature of the porogen does not change the amorphous nature of the polymers.
6.2.4.2. Differential Scanning Calorimetry

The thermal stability of the polymers was analysed initially by differential thermal analysis (DTA) to obtain degradation temperature and control the subsequent differential scanning calorimetry (DSC) experiments. The recorded temperature at which the polymer starts to degrade was at ~250 °C. This was used as the limit for the determination of glass transition temperature of the polymers.

As shown in Figure 6.6 and Table 6.3, the $T_g$ of ACN-PP MIP (~110 °C) is lower than the $T_g$ of ACN-PP NIP (120 °C) as can be seen in Figure 6.6. This difference might be attributed to the imprinting effect of the template in the MIP. It leaves empty cavities upon the removal of the template, thus, requiring less energy to disrupt the polymeric network.\(^{246-248}\)

Similarly, for the IL-PP system, IL-PP MIP exhibited lower $T_g$ than the corresponding NIPs, which is consistent with the results obtained by previous studies.\(^{249-251}\) The slightly higher $T_g$s of the ACN-PP polymers (MIP and NIP) suggests that ACN promotes more rigid
polymers than PF6. The average pore size and pore volume (Table 6.2) of IL-PP are about twice those of ACN-PP resulting in lower $T_g$.

**Table 6.3.** Glass transition temperatures $T_g$, of the ACN-PP and IL-PP polymers observed using DSC.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>T$_g$ (°C)</th>
<th>Average T$_g$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACN-PP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>105</td>
<td>110</td>
</tr>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>115</td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 1</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Trial 2</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>IL-PP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIP</td>
<td>110</td>
<td>105</td>
</tr>
<tr>
<td>Trial 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial 2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NIP</td>
<td>115</td>
<td>110</td>
</tr>
<tr>
<td>Trial 2</td>
<td>105</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6.6.** DSC curves of ACN-PP MIP and NIP, IL-PP MIP and NIP and the second fraction of IL-PP (IL-PP-2) NIP using DSC with a maximum temperature of 250 °C and a heating rate of 30°C/ min.
6.2.5. Characterization of the ionic liquid dispersible fraction from IL-PP polymer (IL-PP-2)

The IL-PP-2 fraction was obtained after exhaustive purification via dialysis and washing with methanol overnight with stirring. In order to determine the composition of IL-PP-2, it was subjected to NMR analyses. The $^1$H NMR spectrum of the IL-PP-2 NIP shown in Figure 6.8, confirms the presence of PF6. The signals observed at 1.78 and 4.15 ppm are the hydrogens of the butyl group in position 2 in the PF6 structure shown in Figure 6.8, and the hydrogen of the carbon of the butyl chain adjacent to the N (position 1, Figure 6.8), respectively. The broad peaks observed from ~1-2 ppm were the –CH$_2$- and the –CH$_3$ of the polymerized monomers. The O-CH$_2$ peak of TRIM at 4.47 ppm was not prominent as it probably overlaps with the IL peaks but an enlarged version would show broad peaks at ~5.6 and ~6.1 ppm which could be due to the unpolymerised double bonds of TRIM. IL-PP-2 is most likely short chained branched (but not crosslinked) oligomers of MAA and TRIM attached to the imidazolium ring of the PF6 IL. This is because of this fraction is soluble in a chloroform and water: methanol 1:5 mixture. It is very likely that crosslinked particles were also formed but were likely filtered off.
In order to further analyse the composition and properties of IL-PP-2, the IR and the $T_g$ of the polymer were obtained. The IR spectrum of IL-PP-2 NIP shows the presence of peaks attributable to the monomers. It showed similar features to the IL-PP fraction. However, unlike the $^1$H NMR spectrum, the PF6 moiety was not visible.

**Figure 6. 7.** $^1$H NMR spectrum of the second fraction (IL-PP-2) in CDCl$_3$ after second dialysis against water and washed with methanol.

**Figure 6. 8.** Overlaid IR (UATR) spectra of IL-PP- NIP and IL-PP-2 NIP.
The DSC test of IL-PP-2 (Figure 6.6) was also completed using the same parameters employed for the IL-PP polymers. It can be seen in Figure 9 that the DSC curve only showed one baseline, and no $T_g$ in comparison with the IL-PP polymers. This indicates that IL-PP-2 is oligomeric and non-crosslinked.

Based upon the tests conducted the isolation of the second fraction IL-PP-2 could be due to the possibility of PF6 as a source of radical species which has been observed previously by Kano et al.\textsuperscript{252} To confirm, TRIM, MAA and PF6, following the IL-PP formulation, but without the presence of an initiator, and a pure PF6 as a control, were placed in a water bath at 60 °C for 24 hours. The reaction tube with MAA and TRIM became cloudy indicating the formation of insoluble polymers suggesting that PF6 has the ability to initiate polymerisation, possibly as a radical initiator in the cationic radical form \textsuperscript{226}. A similar behaviour of ILs was reported by Biedron et al. where they proposed that imidazolium-based ILs can undergo chain transfer reactions with methyl methacrylate.\textsuperscript{226} The radical forming capability of ILs could most likely be responsible for the formation of the oligomers (IL-PP-2) suspended in the post polymerisation solution of IL-PP, which was not observed in ACN-PP solutions.

6.3. Summary

The properties and binding efficiency of the polymers synthesized in two porogens: acetonitrile and IL, bmImPF$_6$ (PF6) were assessed. Similar to other polymers synthesized in Chapters 3,4 and 5, microspheres were produced in using ACN as porogen with sizes ranging from 160-185 nm. In the case of IL-PP polymers, a “spongy” morphology (due to the porous morphology that resembles a sponge) was observed. This morphology could be due to the phase separation between the growing polymeric chain and the porogen that occurs during
the polymerisation reaction.\textsuperscript{253-254} Moreover, two populations of particles were detected by DLS with the larger sizes ranging from 875-958 nm and the smaller sizes ranging from 215-285 nm, with MIPs were smaller than their corresponding NIPs. Template and TRIM incorporation of the two polymer systems was comparable with one another.

Based upon the measured surface area, pore volume and pore sizes of the IL-PP and ACN-PP polymers, it can be justified that the effect of the presence of the template in the polymerisation is more noticeable when using ILs as a porogen in MIP synthesis. In comparing the effects of the two porogens, it seems that PF6 makes larger pore sizes and higher pore volumes compare to ACN as porogen. This can be attributed to the higher viscosity of PF6.

In the case of binding performance normalized in terms of mass of the polymers, ACN-PP polymers recorded higher IF than the IL-PP polymers. This signifies the efficiency of ACN-PP polymers compared to IL-PP polymers. Additionally, different morphology was obtained from the polymers, specifically the MIP, produced in using IL as porogen. The morphology had a lower surface area compared to ACN synthesized polymers. The binding capacity of polymers with respect to surface area is a more reliable measurement of the binding capacities of the polymers.\textsuperscript{134,255} This was demonstrated by the increased in the MIP binding and IF calculated in both IL-PP and ACN-PP polymers.

Thermal stability of the polymers was also assessed and it showed that the $T_g$ of the MIP is lower than that of the NIP, with a difference of 5°C and 10°C observed for IL-PP and ACN-PP polymers, respectively. The lower $T_g$ of IL-PP could be related to its larger average pore size and higher average pore volume compared to ACN-PP polymers. Both IL and ACN polymers showed similar microstructure as assessed by X-ray crystallography.
The isolated second fraction, IL-PP-2 illustrated that PF6 has the capability to form radicals and initiate polymerisation which was also observed in previous studies.
Chapter 7
Summary and Recommendations

7.1. Summary

This study aims to further understand the mechanistic aspects of the non-covalent approach of molecular imprinting produced by precipitation polymerisation, with emphasis on the effect of the nature of template, temperature, the formulation and nature of porogen on the properties and binding performance of the microspheres. Additionally, we have successfully utilised NMR as the main tool for quantitative measurements, i.e. determining polymer compositions (by analysing post-polymerisation solutions) and template rebinding. To the best of our knowledge, this is the first time that NMR has been applied to the full extent for MIP characterisation.

Chapter 3 compared the physical properties and binding performance of CAF and THP imprinted polymers prepared using EGDMA as crosslinker, MAA as functional monomer, ACN as porogen by precipitation polymerisation with porogen to total monomer volume ratio of 10 mL/mmol. In all cases, THP is more efficiently incorporated within the polymer and rebound indicating a stronger interaction between THP and the functional monomer MAA than with CAF. Template incorporation was affected by the T:FM ratio with the highest incorporation recorded by TM2 (polymers produced with T:FM =1:2, FM:XL 1:5 and I:TM= 1:100) and TM8 (polymers produced with T:FM = 1:8, FM:XL 1:5 and I:TM = 1:100). However, regardless of the efficiency of template incorporation, MIP binding capacities are comparable. In the case of NIPs, all except that of TM2, gave comparable binding resulting in a close range of IF=1.3-1.6. The high NIP binding of TM2 could be attributed to the presence of higher concentrations of FM. It would seem that the T:FM ratios of between 1:2-1:8, which coincides with the recommended T:FM ratio in the literature 21, 98,
for precipitation and bulk imprinting of THP and CAF would still yield comparable binding performance. In evaluating the effect of the FM:XL ratio in the feed, we found that there should be a balance between the concentration of cross-linker, which provides the rigidity of the polymers, and the concentration of the functional monomer, that can form the optimum concentration of T:FM complexes to produce the binding efficient polymers. Higher binding efficiencies were obtained between FM:XL ratios of 1:5-1:10 (9-17 % FM or 83-91 % XL with respect to the amount of total monomer) which is in agreement with literature finding. The particle sizes (90-135 nm) appear to be larger in higher concentration of initiator (AIBN in all cases). Additionally, higher concentrations of initiator increases the amount of template incorporation in the MIPs and gave higher MIP binding. The binding efficiency of the MIP and the yield started to deteriorate at I:TM of 1:500, thus it is advisable to use I:TM ratios of $\geq 1:100$.

In Chapter 4, the effects of the concentration of initiator, reaction temperature and strength of T:FM interaction on the resulting MIPs was assessed. Three different phenolic templates with varying number of hydroxyl groups capable of forming H-bond interaction with the functional monomer were employed. For this system, MIPs were prepared using MAA as the functional monomer, EGDMA as the cross-linker, AIBN as the initiator and following the formulation 1:4:20 for the T:FM:XL ratio and 10mL/mmol for the total volume to mmol of monomers. Microspheres were produced by precipitation polymerisation using ACN as porogen either thermally (60°C) or photochemically (27-35 °C). Based on the results of the CAF and THP system (Chapter 3), the range of the concentration of initiator used for the experiments in Chapter 4 was kept between 1:5-1:100. The effects of the initiator and strength of interaction in the thermally-synthesized 2OH MIPs (T-2OH) were difficult to assess. The results were quite random most likely due to the complications brought about by the dimerisation of 2OH particularly during photochemical initiation. Nevertheless, both T-
1OH and T-3OH MIPs followed similar trend in imprinting efficiency (template incorporation) - higher template incorporation with lower concentration of initiator. However, higher incorporation of 1OH compared to 3OH was observed. The observed binding performance, of T-1OH and T-3OH, with respect to mass, increases with decreasing concentration of initiator. As with the THP and CAF MIPs, (Chapter 3) incorporated template was not translated efficiently to binding sites, with rebinding efficiencies only 2-12% of the incorporated and only 1-5% for the photochemically-synthesized counterparts. Non-competitive selectivity studies also demonstrated that T-3OH is the most selective towards its template. This is an indication of the formation of high fidelity imprints due stronger interaction with the functional monomer due to higher number of interaction points.

Template effects were revealed in the surface area, average pore volume and average pore size of the polymers. Due to instrument constraints, only selected set of polymers that represents the three template MIP systems and set of polymers prepared in different concentration of initiator were selected. The BET surface area measured ranged from 25-135 m²/g. MIPs prepared in low concentration of initiator (e.g. I:TM =1:100) recorded lower surface areas than the corresponding NIPs, with more prominent effects observed for 2OH and 3OH polymers. Lower BET surface area was measured for polymers prepared in higher concentration of initiator (e.g. 1:5) compared to those of prepared in lower concentration of initiator (I:TM=1:100). The average pore volumes measured for the selected MIPs and NIPs ranged from 0.027- 2.945 (cm³/g) and 0.050-0.112 (cm³/g), respectively. Comparable pore sizes for the MIP and the NIP of 1OH were observed while for the 2OH and 3OH, the difference is more pronounced as the number of hydroxyl increases (T-3OH-1:100 MIP = 10Å). Nevertheless, it was not clear from these results if, aside from the porogen, the pore size was also affected by the nature of the template.
The binding capacity of the polymers was dependent upon the particle size or active surface area of the polymers. From the significant difference in the surface area of the polymers (especially the T-3OH system), normalizing the binding capacity of the polymers with respect to surface area is a more reliable assessment of the polymer binding performance. This resulted to an increase in the binding capacities of the polymers and as well as the difference in the MIP and NIP binding. This was especially seen with T-3OH system where the surface area of the MIP and NIP was significantly different.

Unlike the thermal counterparts, the imprinting efficiency and binding performance of photochemically synthesized polymers are more unpredictable. This is attributed to the fact that these phenolic templates can form radical species upon exposure to UV radiation and presence of another radical species which often leads to dimerisation of the templates with itself.

In Chapters 3 and 4, polymers were prepared by non-covalent, non-stoichiometric approach using the most commonly used functional monomer, MAA. In order to further investigate the mechanism of the stoichiometric non-covalent imprinting, in Chapter 5, polymers were synthesized using a custom-designed functional monomer, 2,6-bis-(acrylamido) pyridine (BAAPy). This monomer was capable of forming an array of H-bond interactions (donor-acceptor-donor/acceptor-donor-acceptor, DAD/ADA) with an imide-containing template, 2′,3′,5′-tri-O-acetyl uridine (TAU). This study is the first to investigate the imprinting and template rebinding efficiencies of the TAU/BAAPy molecularly imprinted polymeric (MIP) system prepared by precipitation polymerisation using ACN as porogen. Compared to the monolithic counterpart prepared using CHCl₃ as porogen, the stoichiometric 1:1 T:FM ratio has not been maintained in precipitation polymerisation. Also, an optimal TAU:BAAPy ratio of 1:2.5 was obtained in acetonitrile, without agitation, affording an affinity constant (1.7 x 10⁴ M⁻¹) and a binding capacity (3.69 µmol/g) higher than its bulk
 counterpart. Molecular modelling, NMR studies and selectivity assays against the analogues uridine (Urd) and 2,3,5-tri-\(O\)-acetyl cytidine (TAC) indicate that, aside from the DAD/ADA hydrogen bond interaction, BAAPy also interacts with the acetyl groups of TAU. Template incorporation and rebinding in precipitation MIPs are favoured by a moderate initiator concentration, i.e. initiator:total monomer (I:TM) ratio of 1:131 and without any form of stirring or agitation. While the imprinting efficiencies for the best performing bulk and precipitation TAU MIPs generated in this study were moderate (41% and 60%, respectively) their rebinding capacities were only between 3-4% of the incorporated template.

In Chapter 6, the application of ILs as porogen in imprinting PNL by precipitation polymerisation was explored as a continuation of the research made by Booker et al. Using TRIM as cross-linker, MAA as functional monomer, and the T:FM:XL ratio of 1:1.5:4, the binding capacities of the polymers produced in PF6 were compared with the polymers synthesized in the most commonly used porogen in precipitation MIPs, ACN (ACN-PP). Comparable percentages of PNL were incorporated by the microspheres prepared using ACN (62 ± 1%, ACN-PP) and PF6 (64 ± 1%, IL-PP). Different morphologies were observed in the two system: ACN produced discrete microspheres while PF6 produced a spongy porous material with two population sizes ranging from 200-900 nm, which are 5 times larger than the microspheres produced in ACN. This demonstrated that the binding capacity of the IL-PP polymers the recorded IF of IL-PP polymers were lower than that of the ACN-PP polymers. The BET surface area of the polymers was within the range of 160-350 m\(^2\)/g with lower BET surface area for MIPs in both systems (ACN and IL-PP). However, the surface area of the IL-PP MIP is two times lower than its NIP counterpart. Higher average pore sizes were obtained for IL-PP polymers (55 Å) than ACN-PP polymers (26-28 Å). Comparable average pore volume were observed for CAN-PP MIP and NIP (~0.200 cm\(^3\)/g) while significantly higher average pore volume was measured for IL-PP NIP (0.449 cm\(^3\)/g) than IL-PP MIP (0.230
cm$^3$/g). Due to the difference in the morphology and particle sizes, the binding capacities were expressed with respect to surface area and showed that the MIP binding and IF values of IL-PP MIP was at par with the ACN-PP MIP. The $T_g$ of the MIP is lower than that of the NIP, with a difference of 5°C and 10°C observed for IL-PP and the ACN-PP polymers, respectively. The lower $T_g$ of IL-PP could be related to its larger average pore size and higher average pore volume compared to ACN-PP polymers. Both IL and ACN polymers showed similar microstructure as assessed by X-ray crystallography. It was also observed that IL can facilitate the formation of short-chained oligomers, even without the presence of any initiator.

Table 7.1 is a summary of the binding parameters and imprinting efficiencies of the systems included in this study. Comparing the three non-stoichiometric non-covalent imprinted systems (Chapter 3, 4 and 6), the highest recorded template incorporated in the CAF and THP polymers (75%) is 1.5 times higher than that of thermally-synthesized phenolic imprinted polymers (50%, 1OH). This, in turn, is 1.3 times times lower than the PNL incorporation (60-65%) incorporation which can be attributed to the difference in the crosslinker employed (EGDMA vs TRIM). These values (non-stoichiometric, non-covalent imprinting) coincides with the values obtained in using stoichiometric, non-covalent (BAAPy) system by precipitation polymerisation in which the polymers incorporated between 14-35%. However, looking at the bulk polymerisation of the BAAPy system, the monoliths incorporated 95-96% of the starting feed concentration of the template,. This is considerably higher than the incorporated template obtained from the previous systems. It is also interesting to note that the effect of the concentration of initiator on template incorporation observed between the xanthine derivatives (CAF and the THP) and the phenolic templates followed an opposing trend system. The xanthine MIP systems incorporated a higher concentration of template (CAF and THP with 26-50% vs phenolic templates with 4-14%) at higher concentration of (1OH, 3OH) templates (I:TM =1:5). Also,
lower initiator concentration was favoured by the phenolic template system. This suggests that the time required to achieve the optimum T-FM complexes varies according to the nature of the templates and the starting T:FM ratio in the feed. Nevertheless, MIP binding capacity is not directly related to the template incorporation. The polymer yield, however, is significantly affected, with the MIPs prepared at very low I:M ratio showed poor conversion as an effect of the slow rate of polymerisation. It would seem from our results that it is better to keep the I:TM ratios $\geq 1:100$ for precipitation imprinting.

CAF and THP are one of the most commonly studied templates in molecular imprinting with the corresponding $K_a$s and $N$ of each of the template system have been published. The association constants for caffeine was determined to be within the range of $1.62-5.43 \times 10^3 \, \text{M}^{-1}$ and $N$ of $16.98 \, \mu\text{mol/g}$ which was calculated by the Scatchard model.\textsuperscript{141} In the case of theophylline, the association constants range from $1.53 \times 10^4$ to $1.0 \times 10^8$\textsuperscript{100,142} and $N$ of $0.016-1.3 \, \mu\text{mol/g}$. These values were determined from MIP systems of polymer feed composition and concentration of rebinding solution different from those employed in this study which can affect the calculation of $K_a$s of the system according to Rampey et al.\textsuperscript{126} Thus, those published results cannot be used for direct comparison to our systems. The $K_a$ and $N$ obtained for the phenolic systems obtained from our study, were $0.99-2.50 \times 10^5 \, \text{M}^{-1}$ and $0.71-2.59 \, \mu\text{mol/g}$, respectively, both of which were calculated by a non-linear Langmuir model. Surprisingly, in comparing these values to that of the BAAPy system, the $K_a$’s of the phenolic systems are 3.5 times greater as expected due to their heterogeneity brought about by using non-stoichiometric functional monomer. However, in the case of $N$, the phenolic systems ($0.71-2.59 \, \mu\text{mol/g}$) recorded 2.2 times lower than the BAAPy system ($5.60 \pm 0.39 \, \mu\text{mol/g}$). Based from these results, it suggests that $N$ could be a more effective assessment of the imprinting efficiency of the MIPs. This was explained by Rampey et al. as being the
results of the dependency of the $K_a$ values to the analytical concentration range of the rebinding solution being tested rather than the actual MIPs itself. $^{126}$
Table 7.1. Comparison of imprinting efficiencies and binding parameters of the MIP systems included in this study.

<table>
<thead>
<tr>
<th>Template</th>
<th>Functional monomer</th>
<th>Crosslinker</th>
<th>Porogen (Volume per mmol Monomer)</th>
<th>Reaction Temp (˚C)</th>
<th>Imprinting efficiency (Template incorporation)</th>
<th>MIP binding (μmol/g), Binding site conversion(%)</th>
<th>Imprinting Factors</th>
<th>Ka (M⁻¹)</th>
<th>N (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF</td>
<td>MAA</td>
<td>EGDMA</td>
<td>ACN (10mL/mmol)</td>
<td>60</td>
<td>20-75 %</td>
<td>3.6-14.7 μmol/g, 5-10%</td>
<td>1.1-1.7</td>
<td>a1.53-1.00x10⁸</td>
<td>a 16.98</td>
</tr>
<tr>
<td>THP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2-2.0</td>
<td>b1.62-5.43x10⁶</td>
<td>b0.016-22.8</td>
</tr>
<tr>
<td>1OH</td>
<td></td>
<td></td>
<td></td>
<td>27-35</td>
<td>10-25 %</td>
<td>0.81-1.75 μmol/g, 1-5 %</td>
<td>1.5-1.8</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>2OH</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td>1.2-1.3</td>
<td>Not determined</td>
<td>Not determined</td>
</tr>
<tr>
<td>3OH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2-1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAU</td>
<td>BAAPy</td>
<td></td>
<td>ACN (10mL/mmol)</td>
<td>60</td>
<td>14-37%</td>
<td>0.56-4.07 μmol/g, 3-4%</td>
<td>1.2-3.0</td>
<td>7.5x10⁴</td>
<td>5.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ACN (3mL/mmol)</td>
<td></td>
<td></td>
<td></td>
<td>1.7-2.0</td>
<td>3.4x10⁴</td>
<td>4.54</td>
</tr>
<tr>
<td>PNL</td>
<td>MAA</td>
<td>TRIM</td>
<td>ACN (12 mL/mmol)</td>
<td>60</td>
<td>60-65%</td>
<td>38.45-41.25 μmol/g, 37-43%</td>
<td>2.0 (2.5)*</td>
<td>2.99x10⁴</td>
<td>0.137</td>
</tr>
<tr>
<td>PNL</td>
<td></td>
<td>PF6</td>
<td>ACN (12 mL/mmol)</td>
<td></td>
<td></td>
<td></td>
<td>1.3 (2.4)*</td>
<td>6.25x10⁴</td>
<td>9.77</td>
</tr>
</tbody>
</table>

*Imprinting factors with respect to surface area, Values from previous studies a⁷⁹, 14¹, b¹⁴² 10⁰, c using DVB as a cross-linker, CHCl₃ as porogen and monolith format and d System using DVB as cross-linker and monolith format⁰⁷¹.
7.2. Recommendations for Future Work

Due to the complications that can occur in photochemical initiation (e.g. dimerisation of templates and polymerisation of EGDMA and MAA without initiator), further studies regarding the effects of temperature could involve the use of an azo initiator that can produce radicals at lower temperature (e.g. Azo-bis-dimethyl valeronitrile). Our study was only limited to free radical polymerisation, thus, this investigation could be extended to the investigation of the imprinting and binding efficiency of polymers using controlled radical polymerisation (e.g. ATRP or RAFT).

Additionally, the reactivity of the phenolic templates used in Chapter 4 (both thermally and photochemically) made the assessment difficult. Thus, further investigation could involve the use of a model system using different sets of templates with lower reactivity at higher temperatures. Moreover, this set of templates could contain varying number of interaction points to better understand the effects of the nature of template/strength of interaction between the template and the functional monomer in the recognition mechanism of MIPs. It was only the effect of concentration of initiator that was investigated in Chapter 4. Therefore, the model system could also involve the assessment of the effects of varying the formulations (e.g. FM:XL or T:FM), as was conducted in Chapter 3 (CAF and THP system) or volume/monomer ratio in the feed.

Binding performance of the MIPs and NIPs is commonly normalized in terms of mass and this is only applicable when working with monoliths, since the MIPs and NIPs were ground and sieved to the desired sizes, thus there is no significant differences in the sizes of the particles. However, in systems with microspheres where the size of the MIPs is different from its NIP counterparts, the binding performance should be normalized in terms of surface area. Therefore, it is more beneficial and useful to express the binding performance of the
polymers normalized in terms of surface area. In the absence of BET surface area and particle size measurements (e.g. by SEM or DLS), selectivity of the polymers towards its target molecule, evaluated by selectivity indices could be used to assess the efficiency of a MIP system.

Among the systems investigated, one of the efficient systems we proposed based upon the MIP binding and IF is the TM2 polymers. However, the sizes difference of the MIPs and the NIP of this system is significant, thus normalization with respect to surface area is recommended. This is in addition to our recommendation that surface areas of the MIP systems discussed in Chapter 3 be further investigated.
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