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Design and development of chiral and achiral molecularly imprinted stationary phases

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DESIGN AND DEVELOPMENT OF CHIRAL AND ACHIRAL MOLECULARLY IMPRINTED STATIONARY PHASES

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DEDICATION

To my parents and my wife

My parents have always been the support behind all of my career endeavors. I would not be in my current position had it not been for their strong support of education in the household. Also, my wife has been there by my side through most of my graduate career and I thank her so much for all she has done.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

α	Separation factor
AcOH	Acetic Acid
AIBN	2,2'-Azoisobutyonitrile
AMPSA	2-acrylamido-2-methyl-1-propanesulfonic acid
Binol	(R)-(+)-1, 1′-Bi-2-naphthol
BOC	*t*-Butyl carbamate
Boc-tyr	BOC-L-tyrosine
CBZ-trp	CBZ-L-tryptophan
CBZ-ser	CBZ-L-serine
$CHCl_3$	Chloroform
$CDCl_3$	Deuterated Chloroform
CH_2Cl_2	Methylyne Chloride
CH_3CN	Acetonitrile
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dicholoromethane
DCU	N, N'-Dicyclohexylurea
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DVB	divinylbenzene
EDAM	N,N'-ethylenedimethacrylamide
EDMA	ethylene glycoldimethacrylate
EI	electrospray ionization
EGDMA	Ethylene glycol dimethacrylate
FAB	fast atom bombardment
FM	functional monomer
FT-IR	Fourier transform infrared
HEMA	hydroxyethylmethacrylate
HCl	Hydrochloric acid
HOAc	Acetic acid
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
IR	Infrared
k'	Capacity factor
MAA	Methacrylic acid
$MgSO_4$	Magnesium Sulfate
MeCN	Acetonitrile
MeOH	Methanol
MIPs	Molecularly imprinted polymers
NMR	Nuclear magnetic resonance
$NaHCO_3$	Sodium bicarbonate
NOBE	N, O-bismethacryolethanolamine
OMNiMIPs	One monomer molecularly imprinted polymers

PETEA	pentaerythritol tetraacrylate
PETRA	pentaerythritol triacrylate
PPC	Pre- Polymer Complex
SPE	Solid Phase Extraction
THF	Tetrahydrofuran
TRIM	Trimethacrylate
tR	Retention time
UV	Ultraviolet

ABSTRACT

Although the cross-linker can comprise over 80% of the polymer composition, improving the nature of the cross-linker in molecularly imprinted polymers has not been studied extensively. The goal of this research is to develop novel cross-linking monomers to either use in the **O**ne **M**o**N**omer **M**olecularly **I**mprinted **P**olymer system (OMNiMIP) or use in conjunction with other commercially available cross-linkers and functional monomers. Chapter 2 contains research into the understanding of the performance of a new cross-linking monomer (N, O - bismethacryloyl ethanolamine, NOBE) discovered in the Spivak Research Group. The ability of this monomer to outperform traditional two monomer systems in a multiple template imprinting method was tested in Chapter 3.

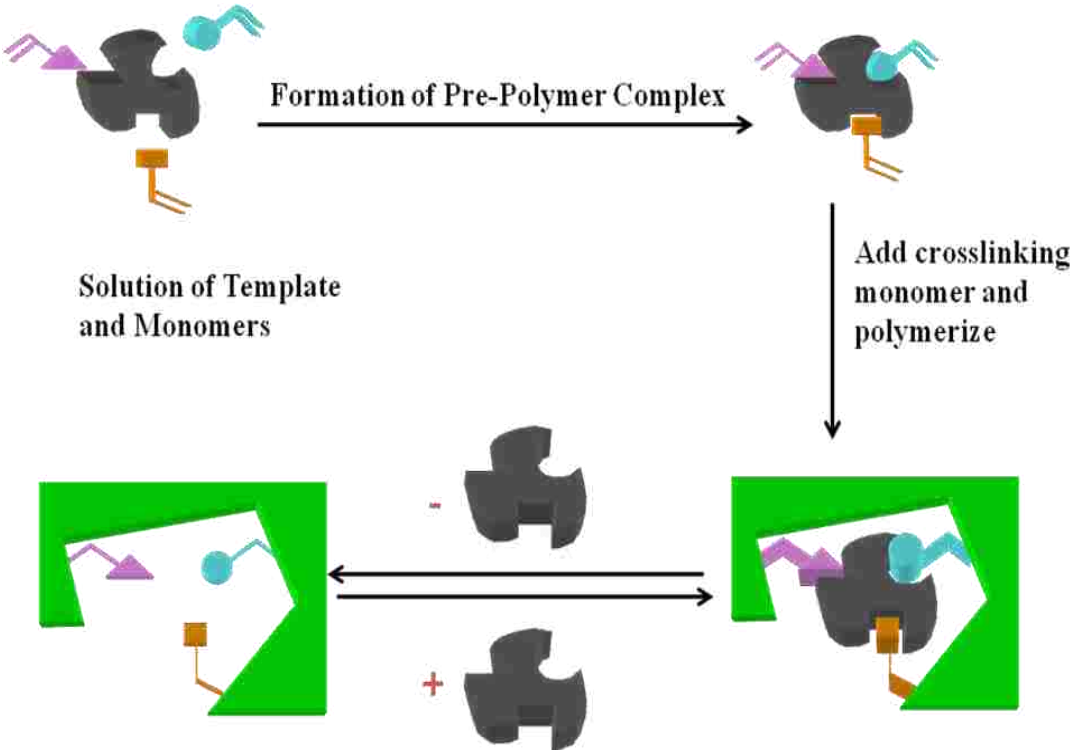
Chapters 4 and 5 discuss research that is related to the design and analyses of chiral cross-linking monomers. Several chiral monomers based on amino acid precursors were first developed to determine if increasing steric bulk would affect the ability of the polymer material to create a molecularly imprinted polymer. With this study, however, a surprising result was discovered when analyzing templates with the same and opposite stereochemistry. Due to the unique ability of the chiral monomers, an imprinted polymer containing a racemic mixture of a single template was prepared. The results did not show separation and further studies are under current study using monomers that contain more ionic and hydrogen bonding sites. Chapter 5 details the development and synthesis of several chiral multi-hydrogen/ionic bonding monomers.

Chapter 6 describes research performed in collaboration with the Ye research group at Lund University. This research reports the use of NOBE to selectively imprint a neurological peptide fragment. Chapter 6 also briefly details future work needed in the development of novel cross-linkers.

CHAPTER 1: INTRODUCTION TO MOLECULARLY IMPRINTED POLYMERS

1.1 Molecularly Imprinted Polymers

Molecular imprinting is a useful technique for making durable and inexpensive materials for applications such as analytical detection, separations, and biological assays.[1-4] An imprinted polymer is created when a template molecule interacts with functional monomers through covalent/non-covalent interactions in a solution to form a pre-polymer complex (PPC), which is then polymerized together with a cross-linker (**Scheme 1.1**). Following the removal of template the resulting polymer has site specific cavities for the template molecule. Thus, molecular imprinting creates selective recognition sites inside polymer matrices.



Scheme 1.1. Imprinting process showing interaction of template, functional monomers, and cross-linker.

The concept of imprinting was first conceived by Polyakov in the 1930s, when he used silica matrices to study the adsorption and desorption of molecules into a silica matrix.[5] During the time of Polyakov's reported findings, several scientists were debating the selectivity of antibodies in nature. Among this group was Linus Pauling, who adhered to the belief that antibody formation only took place in the presence of the antigen and therefore would explain the high affinity for the antigen.[6] Dickey then applied this theory to the inorganic silica system described by Polyakov. In 1949 Pauling reported on a study in which he showed selective rebinding of a dye in the silica matrices.[7] Subsequently, there have been several other studies performed on the silica matrices to show specific uptake of only the imprinted molecule. However, the dawn of the current method of imprinting in organic matrices was first developed by Guenter Wulff, and is the primary method used in imprinting today.[8] Wulff developed the polymers for use as an enzymatic mimic; that is, he tried to make a polymer that had the same binding capacity as those found in natural enzymes. Despite Wulff's contributions it was the research of Klaus Mosbach that really propelled the imprinting world.[8] The Mosbach group was able to study and optimize the current standard in organic molecular imprinting.

1.2 Methods for Producing Molecularly Imprinted Polymers

Bulk monolithic polymerization is the most common method of producing MIPs. This method requires the need for grinding and sieving of the polymer before using in any application (i.e. HPLC, SPE, etc.). While being the simplest method of production, this method leads to irregular shaped particles often over a broad size range. Irregular shapes and sizes lead to reduced separation performance and greater column pressures in chromatography. Therefore, the

key to achieving the optimal separation is uniform particle size and shape. The uniform particle size allows for the highest packing density of imprinted polymer material and lower column pressures. A separate concern with bulk polymerization is that only a small fraction of the polymer (~20%) is available for the chromatographic analysis, the remaining 80% of the polymer is lost in the grinding process in the form of fine particles ("fines") that fall through the sizing sieves. Although several methods have been shown to give uniform particle size and nearly 100% recovery of polymer material, only precipitation and suspension polymerization have shown promissing applicability towards commercialization.[5]

1.2.1 Precipitation Polymerization in Imprinted Polymers

Precipitation polymerization was first used in the field of imprinting by the Mosbach group in 1999. The Mosbach group polymerized theophylline and estradiol together with methacrylic acid (MAA), ethylene glycoldimethacrylate (EDMA), and trimethylolpropane trimethacrylate (TRIM) as the functional monomer and cross-linkers, respectively. The average particle sizes for the precipitation polymerization ranged from 0.2 to 0.3 μm. The particles provided high affinity along with high selectivity and allowed for imprinted polymers to be used in capillary electrochromatography, solid-phase microextraction, and chemical sensing.[9]

Since the inception of precipitation polymerization for producing molecularly imprinted polymers, several monomer/template combinations have been used. Nearly all of the combinations used have produced particle sizes of sub-micron size that have both high affinity and high selectively.[10-12] The Spivak research group in collaboration with the Ye research group has successfully developed microparticles via precipitation polymerization using a single bi-functional monomer (discussed further in Chapter 6).[13]

1.3 Covalently Linked Molecularly Imprinted Polymers

The specific interactions of the template with the monomer/polymer system can be obtained through two main bonding systems, covalent and non-covalent bonding.[9-14] Covalent imprinting gives only template-monomer covalent connections that can rebind either covalently or non-covalently, whereas, non-covalent imprinting can give a multitude of exchanges between templates and monomers (**Figure 1.1**). Despite the seemingly great potential of covalent imprinting to greatly minimize non-specific bonding; the procedures required to remove the templates from covalently imprinted polymers can also damage the polymer thus reducing performance.[1] Also, covalent imprinting is only useful for a select group of compounds (i.e. alcohols (diols), aldehydes, ketones, amines and carboxylic acids), leaving out a vast majority of analytes.[15-20]

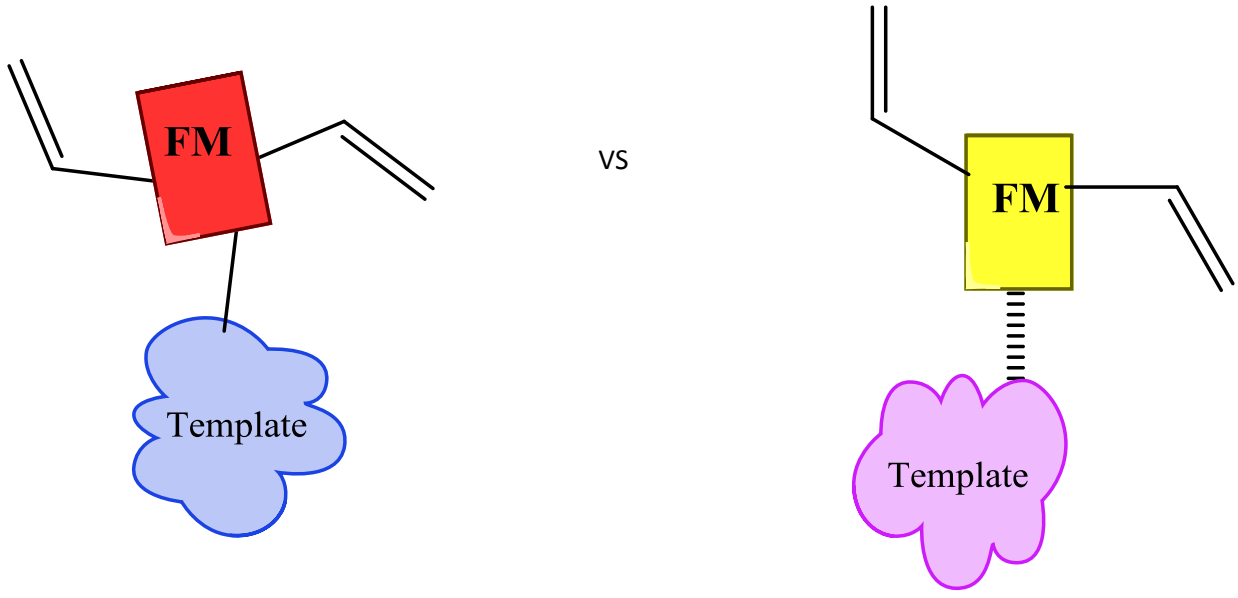


Figure 1.1 Covalent versus Non-covalent imprinting methods showing the functional monomer (FM) attached to the template through covalent interactions and the functional monomer bonding to the template through non-covalent forces.

1.4 Non-covalent Molecularly Imprinted Polymers

The non-covalent approach to imprinting allows for a greater range of analytes available for imprinting, and is the closest matching system to the many systems found in nature.[26] Non-covalent imprinting is based on molecular interactions such as hydrogen-bonding (H-bonding), ionic bonding, and dipole-dipole interactions. This approach, which is both very simple and robust, was first introduced by the Mosbach group.[4] The non-covalent method of imprinting has been dominated using methacrylic acid (**1.1**) as the primary functional monomer in the imprinting field. However, there has been a steady stream of different monomers used for specific imprinting applications.[27, 28] The other commercially available functional monomers used in molecularly imprinted polymers include acid, base, and neutral compounds. Acid and base containing functional monomers interact with the template through acid-base interactions along with a smaller extent of hydrogen bonding. The acid containing functional monomers (**Figure 1.2**) include methacrylic acid, 4-vinylbenzoic acid (**1.2**), acrylic acid (**1.3**), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA) (**1.4**), (2-trifluoromethyl)acrylic acid (**1.5**), itaconic acid (**1.6**), and 2-(methacryloyloxy)ethyl phosphate (**1.7**).[29-37]

The basic functional monomers (**Figure 1.3**) are N-vinylimidazole (**1.8**), 4-vinylpyridine (**1.9**), 2-vinylpyridine (**1.10**), N,N-diethylaminoethyl methacrylate (**1.11**), and aminostyrene (**1.12**).[38-46] Neutral monomers (**Figure 1.4**) afford bonding with the template only through hydrogen bonding or hydrophobic interactions. The common neutral functional monomers are hydroxyethylmethacrylate (**1.13**), methacrylamide (**1.14**), acrylamide (**1.15**), and vinyl pyrrolidone (**1.16**).[47-52]

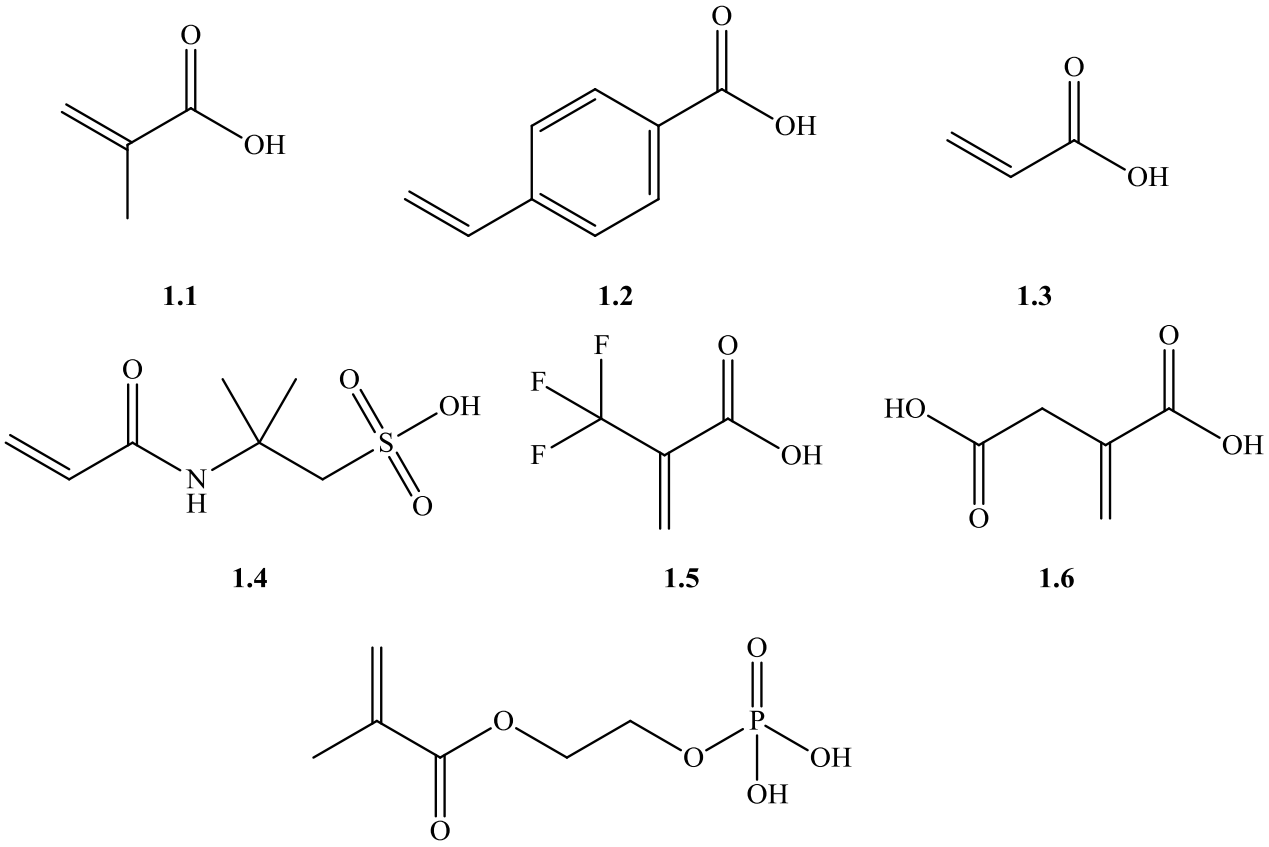


Figure 1.2 Figure depicting the acidic functional monomers used in imprinting.

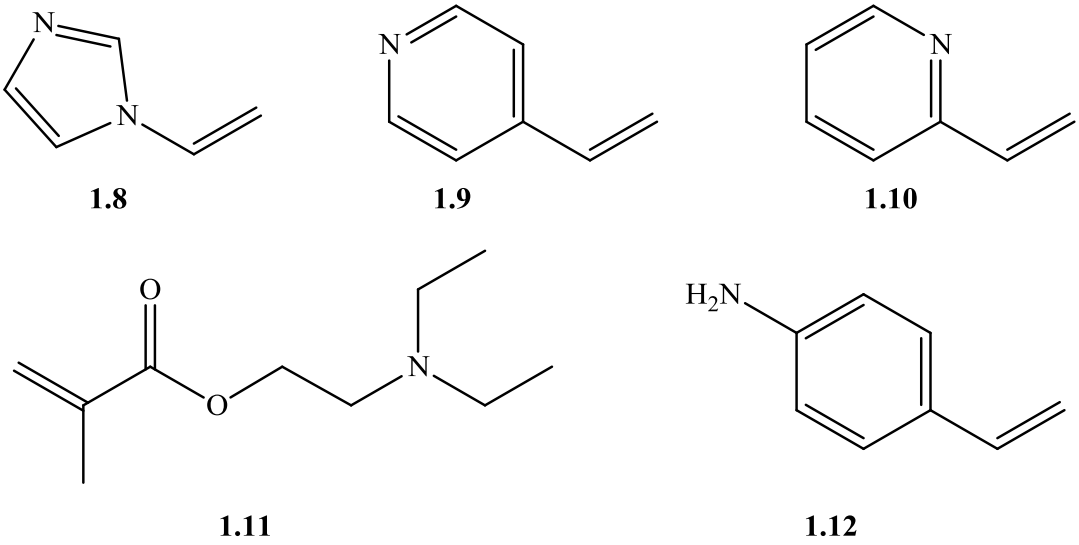
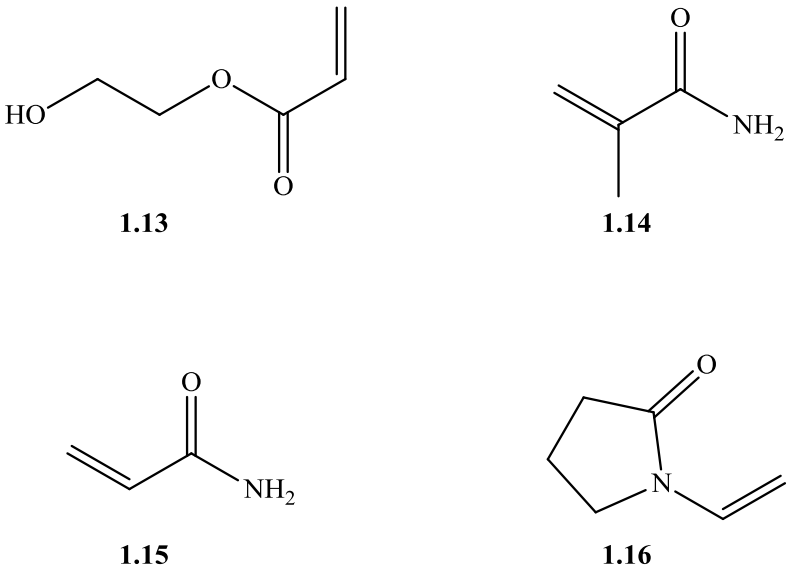


Figure 1.3 Figure depicting the basic functional monomers used in imprinting.



1.13 **1.14**

1.15 **1.16**

Figure 1.4 Figure depicting the neutral functional monomers used in imprinting.

1.5 Development of Cross-linkers in Imprinting Technology

The development of cross-linkers**,** despite having a large impact on the formation of the polymer matrix, has lagged behind the development and expansion of functional monomers. The cross-linker imparts the rigid framework (polymer matrix) necessary for the formation and retention of specific cavities for the chosen templates. The polymer matrix is generally considered an inert component that does not influence the template interaction with the functional monomer. The first comparison of cross-linking monomers came from the Wulff research group.[53, 54] The Wulff group compared the cross-linkers ethyleneglycol dimethylacrylate (EGDMA, **1.17**) and divinylbenzene (DVB, **1.18**) (**Figure 1.5**) for their performance in terms of separation factor (α). In nearly every case studied, the Wulff group found that EGDMA outperformed DVB. The Wulff study lead to primarily all imprinted polymers using EGDMA as the cross-linker and any cross-linking derivatives used since have originated from the design of

EGDMA. However, in select applications, multifunctional acrylate cross-linkers have shown improved performance over EGDMA. The multifunctional cross-linkers (**Figure 1.5**) include

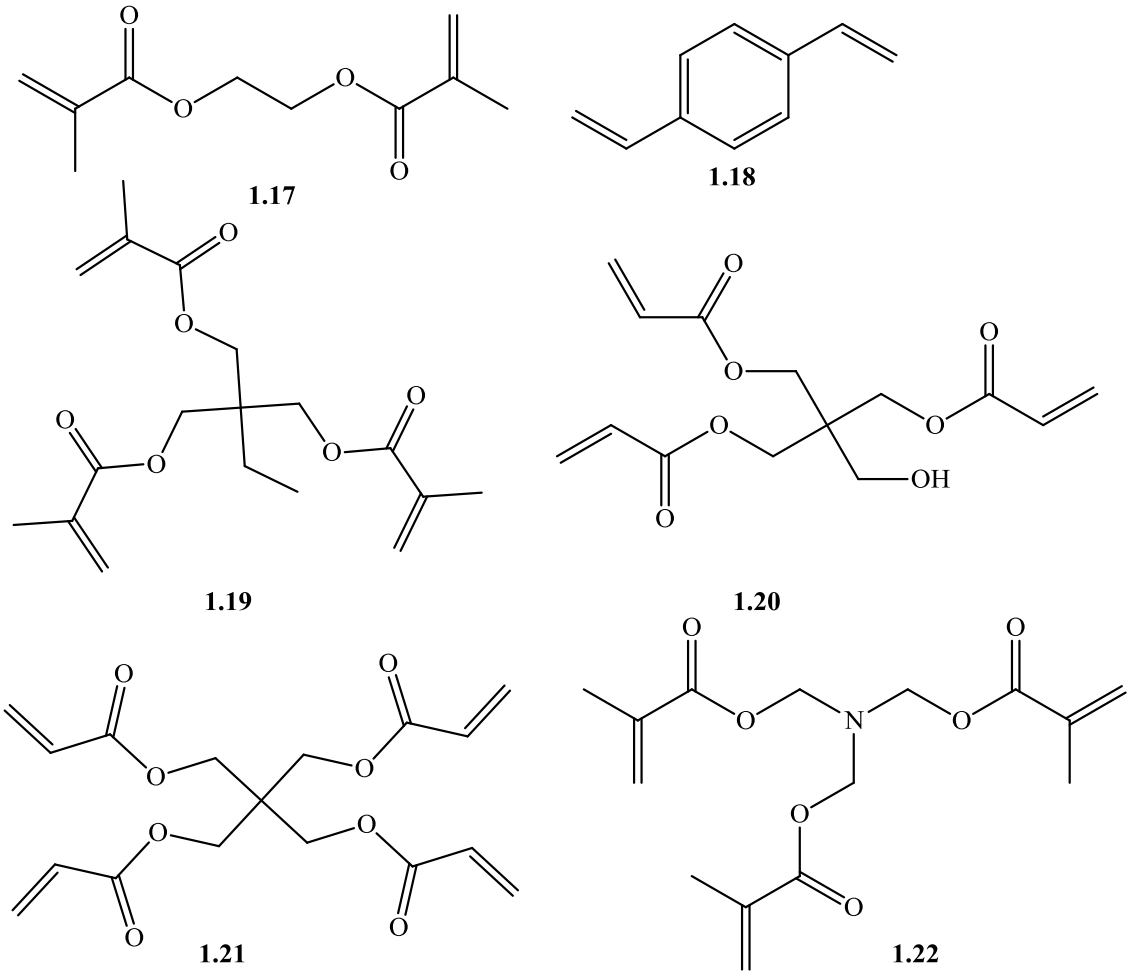


Figure 1.5 Figure depicting the cross-linking monomers used in imprinting.

trimethylolpropane trimethacrylate (TRIM) (**1.19**), pentaerythritol triacrylate (PETRA) (**1.20**), pentaerythritol tetraacrylate (PETEA) (**1.21**), and triethanolamine trimethacrylate (**1.22**).[55-60] As seen in the structures of the multifunctional cross-linkers they

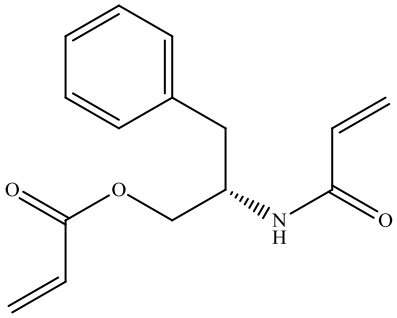


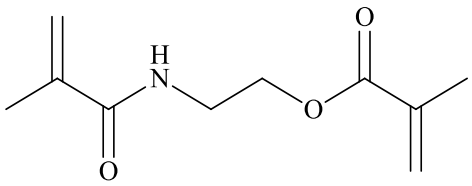
Figure 1.6. Structure of compound 1.23

still, for the most part, retain their inert status in the polymer matrix. The Mosbach group introduced the first cross-linker containing other functionality in the carbon backbone which is derived from an amino acid (N,O-bisacryloyl-L-phenylalaninol, **1.23**).[29] This cross-linker did not show increased performance when polymerized collectively with acrylic acid as the functional monomer. The Wulff research group produced several other amino acid cross-linking derivatives used to make reversible covalent bonds to templates using Schiff's base chemistry.[61] In spite of this, the limitations of covalent imprinting, as stated above, hinder the use of Wulff's Schiff's base connections.

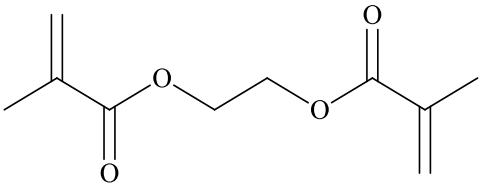
1.6 Development of the Hybrid Cross-linker in the Spivak Research Group

The Spivak research group developed a novel cross-linking monomer for use in molecular imprinting called N, O-bismethacryolethanolamine (NOBE) **(1.24)**. The inspiration behind the design of NOBE was to improve the performance of ethylene glycol dimethacrylate (EGDMA) (**1.17)** by adding sites where hydrogen bonding can occur. As stated above, EGDMA is a common crosslinking monomer used with methacrylic acid (MAA) as the functional monomer for making molecularly imprinted polymers; but EGDMA has very little hydrogen bonding capacity. The lack of bonding ability causes the EGDMA to be an inert component in the final polymer product. Another cross-linking monomer that has extensive hydrogen bonding capability is N,N'-ethylenedimethacrylamide (EDAM)(**1.26**); however, Shea and coworkers have shown that this molecule exhibits little solubility in the organic solvents needed to make an imprinted polymer.[22] Organic solvents are required to generate the highest performance values in non-covalent imprinted polymers that use hydrogen bonding as the main interactive force. Organic solvents also help promote ionic interactions when using polar aprotic solvents and other non-covalent forces used in the complexing of template and monomer.

Ethylene glycol dimethacrylate (EGDMA), on the other hand, is soluble in most organic solvents. A combination of the solubility properties of EGDMA and the hydrogen bonding



1.24



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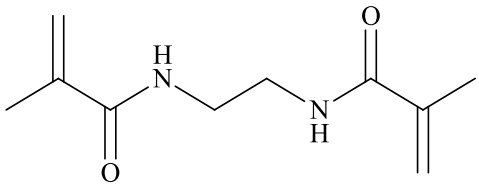

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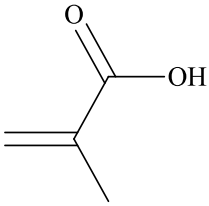
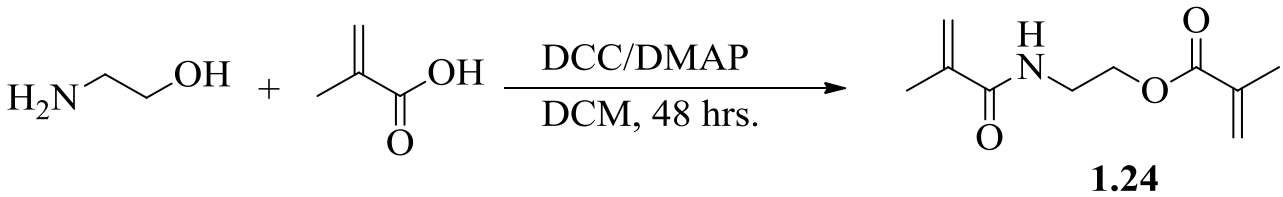


1.1

Figure 1.7: Structures of functional monomer and cross-linkers used to create NOBE (**1.24**).

properties of EDAM would be the best possible solution for a monomer with the properties of organic solubility and hydrogen bonding capacity. NOBE was synthesized to fit these criteria. NOBE has the solubility properties of EGDMA and the hydrogen bonding properties of EDAM, which proved to be a better monomer than EGDMA. The synthesis of NOBE is shown in **Figure 1.7**.



1.24

Figure 1.8: Synthetic pathway for NOBE.

NOBE was originally developed only for use as a new crosslinking monomer. The initial results showed NOBE/MAA polymers outperformed the corresponding EDGMA/MAA polymers (**Table 1.1**). However, further studies performed by Sibrian-Vazquez and Spivak showed that NOBE alone can provide higher performance than when used with a functional monomer (**Table 1.2**).[62] This is evaluated using the separation factor alpha (α), calculated using equation 1**.** The value of alpha (α) can give a measurement of the binding of one specific template to the polymer. In equation 1, the enantioselectivity is given as the ratio of the capacity factor of the imprinted enantiomer over the capacity factor of the non-imprinted enantiomer.

Equation 1:

$$\text{Capacity Factor} = k = \frac{V(t) - V(o)}{V(o)}$$

V(t) = retention volume
V(o) = dead volume

$$\text{Enantioselectivity} = \boldsymbol{\alpha} = k'_L/k'_D$$

The discovery of NOBE's ability to outperform the two monomer system led to a new beginning for imprinting. The need for adding a crosslinker and functional monomer separately has become obsolete, since NOBE (1) can perform as both. This has lead to the era of One MoNomer Molecularly Imprinted Polymers (OMNiMIPs). **Figure 1.8** shows the scheme of imprinting when NOBE is used as the lone monomer, which eliminates the need for calculating the amount of functional monomer and crosslinker to use. Also, there is no longer any wondering about solubility issues with the functional monomer, crosslinker, and template.

1.7 Contributions to Molecular Imprinting

The goal of this research is to better understand the performance of NOBE and analogues derived from NOBE to gain insight into the synthesis of novel MIPs. During the course of this study extensive investigation on the performance of NOBE under many different conditions was

carried out as shown in Chapters 2 and 3. Also, chirality was introduced into the cross-linker backbone, which has opened a new field of MIP research. The results of the initial studies are shown in Chapter 4. The promising results from the initial chiral monomer study lead to several other chiral monomers synthesized and analyzed as shown in Chapter 5. The main theme of Chapters 4 and 5 is the development of chiral monomers and both their performance in normal imprinting techniques, and their performance in racemic or scalemic imprinting. Chapter 6 shows the other applications of imprinted material developed in the Spivak Research Group and provides suggestions for the future work in the development of novel materials and methods for the synthesis of molecularly imprinted polymers.

Figure 1.9: Scheme of non-covalent imprinting using NOBE.

Table 1.1: Comparison of the separation factor (α) NOBE/MAA and EGDMA/MAA polymers.

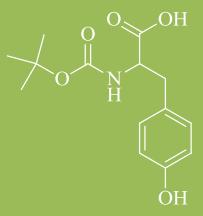
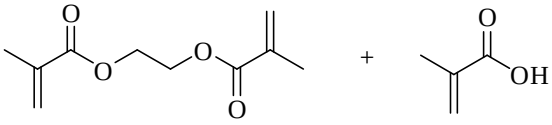
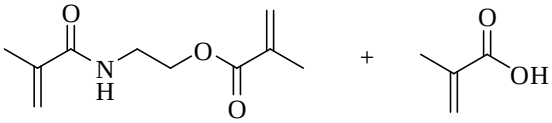
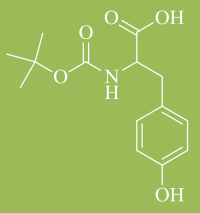
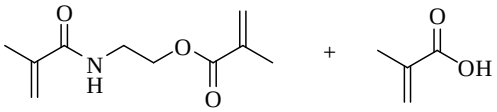
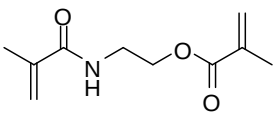
Monomer Combination	Template 
	**α** **1.65**
	**2.32**

Table 1.2: Comparison of the separation factors (α) NOBE/MAA and NOBE polymers.

Monomers	Template 
	**α** **2.32**
	**3.71**

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CHAPTER 2: DEVELOPMENT AND CHARACTERIZATIONS OF OMNIMIPS

Part 1. Design, Development and Characterization of NOBE

2.1. Introduction and Background

The current strategy for forming organic molecularly imprinted polymers was first developed by Wulff, and is primarily used in the area of separations.[1] Wulff developed imprinted polymers for use as an enzymatic mimic; the polymer he made had the same binding capacity as those found in natural enzymes. The polymers specifically rebound optically active templates that were mixed with the monomer prior to polymerization. The post polymerization materials have been dubbed "antibody mimics" because they have interactions with the templates that are near the level of the affinity of antibodies.[2]

Molecularly imprinted polymers are typically composed of a functional monomer and a cross-linking monomer. The template interacts with the functional monomer, and the crosslinker forms the network that has the specific cavity for the template. The principle of imprinting depends upon the intermolecular (covalent/non-covalent) interactions of the template with the specific binding site in the polymer. Non-covalent interactions include ionic bonding, hydrogen bonding, and Van der Waals forces. The non-covalent approach is the most prevalent method due to the ease in removing the template and it closely matches how enzymes and antibodies bind in nature.[3-4] The template can simply be extracted using the non-covalent method; whereas the covalent method requires chemical reactions to remove the template.

The method of molecular imprinting begins with a solution of functional monomers and template which form a prepolymer complex (PPC) and which is then polymerized in the presence of a cross-linking monomer (**Figure 2.1**). The resulting polymer forms specific cavities that show specific recognition properties for the template molecule. The recognition properties

are due to the specific interactions of the template with the shape and functionality of the polymer.[5, 6]

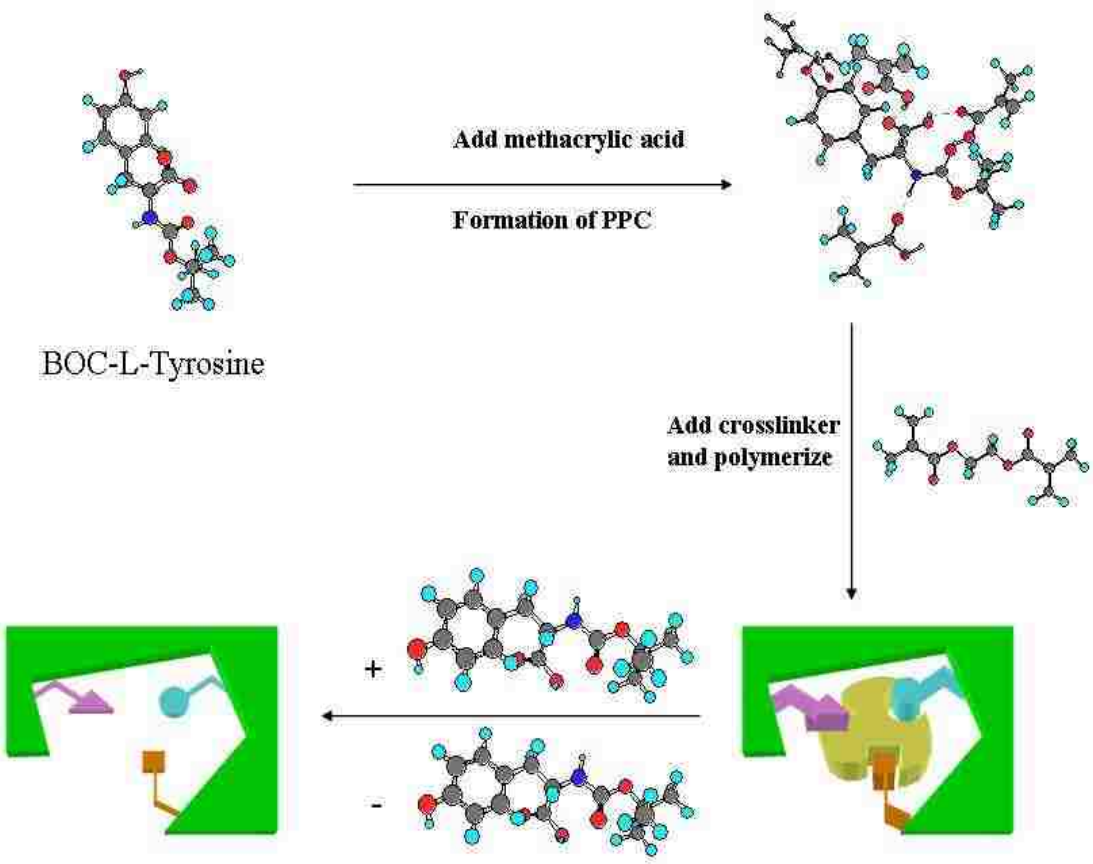


Figure 2.1: Scheme of imprinting using non-covalent interactions.

The advent of NOBE and OMNIMIPS that were discussed in Chapter 1 allows for a new era in the field of imprinting. The superior performance of NOBE over the two monomer system was previously described by Sibrian-Vasquez.[7,8] However, she did not test the limits of NOBE over varying experimental situations. To fully characterize and analyze NOBE, several experiments were done varying polymerization solvents, analyzing the effect water has on the separation performance, inter and intra molecular infrared (IR) studies of NOBE/NOBE and NOBE/template interactions, and varying the amount of initiator used in the polymerization process. Each of the studies listed afforded a clearer understanding of the nature of the improved

performance of NOBE over the old two monomer system. Furthermore, the results gathered in these experiments will give useful insight into further uses of NOBE (Chapters 3 & 6).

2.2 Project Goals

The goals of this project were:

- Fully characterize the performance capabilities of NOBE in a traditional imprinting role.
- Determine the full extent of the inter and intra molecular bonding occurring in between NOBE/NOBE interactions and NOBE/Template interactions.

2.3. Experimental

2.3.1. Synthesis of N, O-bismethacrylethanolamine (NOBE) 1.24

Synthesis of NOBE (**1.24**) was modified from a previously published report.[7] To a 500 mL round bottom flask equipped with a magnetic stir bar 250 mL dichloromethane (DCM) was added. 1 equivalent of ethanolamine (4 g) (65.49 mmol) is added to the DCM. The mixture is then cooled to 0 ºC. After cooling the solution, 0.2 equivalents 4-dimethylaminopyridine (DMAP) (1.6 g) (13.1 mmol) was added. Methacrylic acid (2.8 equivalents) (183.4 mmol) is then added and the solution is allowed to cool to 0 ºC. Next, 2 equivalents of N,N'-dicyclohexylcarbodiimide (DCC) (27.0 g) (131 mmol) is added slowly (2 g per minute), the mixture is then covered with a nitrogen balloon and allowed to stir at room temperature for 48 hours. The resulting solution was filtered to remove the N, N'-Dicyclohexylurea (DCU) and extracted (4 x 15 mL HCl (aq) & 8 x 15 mL sat. $NaHCO_3$ solution). The organic layer is then

filtered and dried (anhy. $MgSO_4$). The resulting solution is concentrated by half and columned (50/50 hexane/ethyl acetate). The pure product gave 10 g (77%).

2.3.2 Polymer Preparation

The following procedure was used for imprinted polymers employing the new cross-linking monomer. In a 13 x 100 mm test tube, (0.21g, 0.76 mmol) of boc-L-tyrosine was dissolved in 3.0 mL of acetonitrile. To this solution, NOBE (2.5g 12.7 mmol) was added, and (0.025g, 0.152 mmol) of AIBN. The solution was purged by bubbling nitrogen gas into the mixture for 5 min, then capped and sealed with teflon tape and parafilm. The samples were inserted into a photochemical reactor, which was immersed in a constant temperature bath. A standard laboratory UV light source (medium pressure 450 W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed at the center of the polymer mixtures. The polymerization was initiated photochemically at 20°C and the temperature maintained by both the cooling jacket surrounding the lamp and the constant temperature bath holding the entire apparatus. The polymerization was allowed to proceed for 8 h and then used for chromatographic experiments.

2.3.3. Chromatographic Experiments

Removal of the template was achieved by Soxhlet extraction with methanol for 48 h. Then the polymers were ground using a mortar and pestle, the particles were sized using U.S.A. Standard Testing Sieves, and the fraction between 25-37 μm was collected. The particles were slurry packed, using a solvent delivery module, into stainless steel columns (length, 75 mm; i.d., 2.1 mm) to full volume for chromatographic experiments. The polymers were then washed on line for 12 h using acetonitrile/acetic acid: 99/1, at a flow rate of 0.1 mL/min to remove any

residual template. HPLC analyses were performed isocratically at room temperature (21°C). The flow rate in all cases was set at 0.1 mL/min using a mobile phases consisting of acetonitrile/acetic acid: 99/1 or acetonitrile, a substrate concentration of 0.1 mM boc-L-tryosine and 0.1 mM boc-D-tyrosine in acetonitrile, and a wavelength detection of 260 nm. The void volume was determined using acetone as an inert substrate. The separation factors (α) were measured as the ratio of capacity factors k'_L/ k'_D. The capacity factors were determined by the relation $k' = (V_t - Vo)/ V_o$, where V_t is the retention volume of the substrate, and *Vo* is the void volume.

2.4. Results and Discussion

2.4.1. Solvent Effects

The underlying mechanism responsible for molecular recognition in imprinted polymers is believed to arise from the complex of the templates with the monomer, and the shape selective polymer cavity formed around the template. Sellergren had previously studied the effect on the hydrogen bonding of solvents and how this relates to an imprinted polymer.[9] The results show the polymers made in solvents with less hydrogen bonding gave a higher separation factor (α). Our goal was to determine how NOBE fits into the previously reported conclusions about solvent effects on the polymers. Several NOBE polymers were made using different solvents. The solvents chosen range from non-polar to polar and non-hydrogen bonding to hydrogen bonding. The solvents chosen were: acetonitrile, chloroform, toluene, methanol, and N, N-dimethylformamide (DMF). The solvents were chosen both for their polarity and hydrogen bonding characteristics, but also for their ability to solubilize NOBE.

NOBE was polymerized in each of the solvents, then binding investigated using high performance liquid chromatrography (HPLC) with acetonitrile and chloroform as the mobile

phase. NOBE that was imprinted in the less polar solvents was expected to have increased performance. The less polar solvents have no hydrogen bonding capacity and will lead to stronger monomer to template interaction, thus improving the selectivity of the polymer. However, somewhat surprising results were observed when α was calculated. **Table 2.1** shows the results when acetonitrile was used as the mobile phase in the chromatographic analyses.

Table 2.1: Separation factors (α) of NOBE in different solvents.

Polymerization Solvent	k'_D	k'_L	α
Acetonitrile	2.76	10.75	3.90±0.02
Chloroform	4.17	11.25	2.70±0.04
Toluene	2.23	4.69	2.10±0.03
Methanol	3.28	3.61	1.10±0.01
N,N-dimethylforMamide	2.39	2.61	1.09±0.01

[a]0.1mL/min flow rate; .01 mM injection concentration, 260 nm detection. [b]values are approximate.

Although, chloroform or toluene are much less polar than acetonitrile, and should give a higher α than the other solvents, the values were significantly lower. This can be explained by a very interesting trend noticed in EGDMA and MAA imprinted polymers, which shows that the polymers perform the best when analyzed using the solvent they were polymerized in.[10] This effect is caused by an increase in non-selective binding although an exact explanation for this effect is not yet clear. It is believed that the sites formed in the polymers are also influenced by the solvent used as well as the template. Therefore it is believed that the shapes will only exactly fit the template when analyzed in the solvent used for polymerization. It was then decided to change the mobile phase and perform a crossover study, in which the mobile phase is changed in the HPLC from acetonitrile to chloroform. Toluene, methanol, and DMF were not chosen. This

is because toluene gives too high of an absorbance reading by Ultra Violet (UV) detection method, and no binding was expected for methanol and DMF due to the protic nature of these two solvents.

The template used in the original imprinted polymers was Boc-L-tyrosine, but BOC-L-tyrosine is not soluble in chloroform. This problem actually gave good indications that NOBE interacted extremely well with the template when chloroform was used as the solvent, since the template was soluble in the monomer solution prior to polymerization. Despite many attempts the template would not dissolve, even at very dilute concentrations in chloroform. This problem led to performing a crossover study with a different template, 1, 1'-Bi-2-naphthol. The new template was analyzed in the same manner as was the tyrosine template. The results are shown in **Table 2.2**.

Table 2.2: Results of the crossover study with NOBE and $CHCl_3$ and CH_3CN using 1, 1'-Bi-2-naphthol as the template.

Mobile Phase[a]	Alpha (α)
100 % $CHCl_3$	15[b]
99/1 % $CHCl_3$/AcOH	6.24±0.06
99/1 % CH_3CN/AcOH	5.82±0.12
100% CH_3CN	12[b]

[a]0.1mL/min flow rate; .01 mM injection concentration, 260 nm detection. [b]values are approximate.

The value for the 100 % chloroform run was difficult to determine, since the imprinted template's signal was broad and weak. The 100 % CH_3CN run gave similar results as the 100 % chloroform. The results in the table indicate the primary mechanism in the improved performance of NOBE is the extensive hydrogen bonding in the matrix of the polymer. Aprotic

solvents such as chloroform and acetonitrile enhance the hydrogen bonding network, and lead to improved performance in the polymers.

2.4.2 Effect of Water on the Chromatographic Performance of NOBE

A comparative study was performed to determine the performance of NOBE when polymerized in the presence of water against the performance of EGDMA/MAA polymers under identical conditions to determine if NOBE has a higher tolerance to the hydrogen bond breaking capabilities of water. The breaking up of the hydrogen bonding network formed in the pre-polymer complex (PPC), lowers the selectivity in the imprinted polymer. The change in selectivity can be seen in the lower α values. **Table 2.3** gives the alpha (α) values for NOBE and EGDMA/MAA polymers formulated with different percentages of water in the solvent/porogen.

Table 2.3. Alpha (α) values of NOBE and EGDMA/MAA.

% WATER	EGDMA/MAA	NOBE
0 %	1.78±0.08	3.9±0.01
1 %	1.67±0.11	3.0±0.07
10 %	1.34±0.13	1.2±0.08

[a]0.1mL/min flow rate; .01 mM injection concentration, 260 nm detection. [b]Values are approximate.

The results show NOBE performed better at low water concentrations, compared to EGDMA/MAA, essentially because NOBE starts at a much higher enantioselectivity factor (α) and maintains superiority over low water regimes. The EDGMA/MAA polymer performs better at the high concentrations of water because the amount of functional monomer/template is isolated compared to the NOBE/template interaction. It appears that NOBE is both a functional monomer and crosslinker, and is more tolerant to water at low concentrations, but the same

reason can also explain the poor performance at high water concentrations. The water negatively interacts with the hydrogen bonding in the pre-polymer complex and disrupts the interaction with the template. While the chance of a low percentage of water disrupting the bonding in a polymer that is 100 % functional monomer (NOBE) is small; the higher percentage can displace more of the bonding sites in NOBE than in EGDMA/MAA. This can be explained because the EGDMA/MAA polymers have a more ionic bonding character that may tolorate binding under aqueous conditions better than that of NOBE.

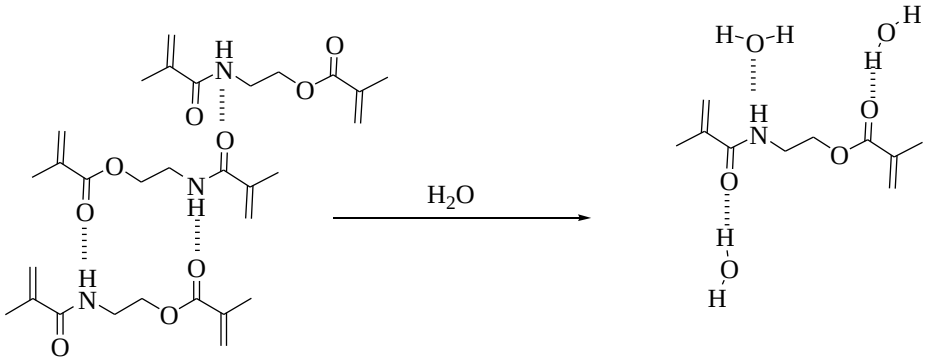


Figure 2.2: Effect water has on the polymer matrix.

The whole hydrogen bonding matrix of NOBE can become disrupted at higher concentrations of water as shown in **Figure 2.2**. The dramatic decrease in performance in the NOBE polymers at high water concentrations is caused by the decrease in hydrogen bonding throughout the polymer matrix. The hydrogen bonding in the polymer matrix is believed to help remove non-selective interactions from occurring in the pre-polymer complex and during polymerization, allowing for a polymer matrix with lower accessible sites for non-selective bonding. The matrix of EGDMA/MAA polymers is not composed of a hydrogen bonding network and therefore is more tolerant to water at higher concentrations.

2.4.3. IR Studies on the NOBE Polymers

Several infrared spectra (IR) were taken on the NOBE polymers. The purpose of this study was to determine if the hydrogen bonding in the NOBE polymer occurred in the dimer formation or the matrix formations under varying conditions (**Figure 2.3**). Hydrogen bonding will stretch and weaken the covalent bond (X-H), where the hydrogen is connected to. This stretching and weakening will cause the vibrational frequency to become lowered. In our case the bending vibrational mode of the N-H bond (amide bone II) in amides will be studied.[11] Two series of IR studies were performed: 1. increasing concentrations of BOC-L-tyrosine with NOBE and 2. decreasing concentrations of pure NOBE. The two series will tell the extent of hydrogen bonding throughout the NOBE matrix. **Figure 2.4** shows the spectra for increasing concentration of BOC-L-tyrosine and **Figure 2.5** shows the dilute NOBE spectra.

As **Figure 2.4** shows the increase in the tyrosine causes more hydrogen bonding to be in place and thus lowers the frequency of the N-H bond. **Figure 2.5** shows that in diluting NOBE the extent of hydrogen bonding throughout the NOBE matrix is decreased causing a decrease in the frequency. The results show that NOBE exists as a matrix supported through extensive hydrogen bonding networks. Also, the gradual change indicates that there are networks, not dimmers as the primary species in the pre-polymer complex.

2.4.4. Effects of Initiator Concentration on the Performance of NOBE Based MIPS

With NOBE entering imprinting into the OMNiMIP era, the formulation to make the polymer has become extremely simple. The formulation now requires simply weighing the template out, adding solvent and monomer, and adding in the initiator. With the template comprising 5 % of the solution the chance of creating an error in the polymer is reduced,

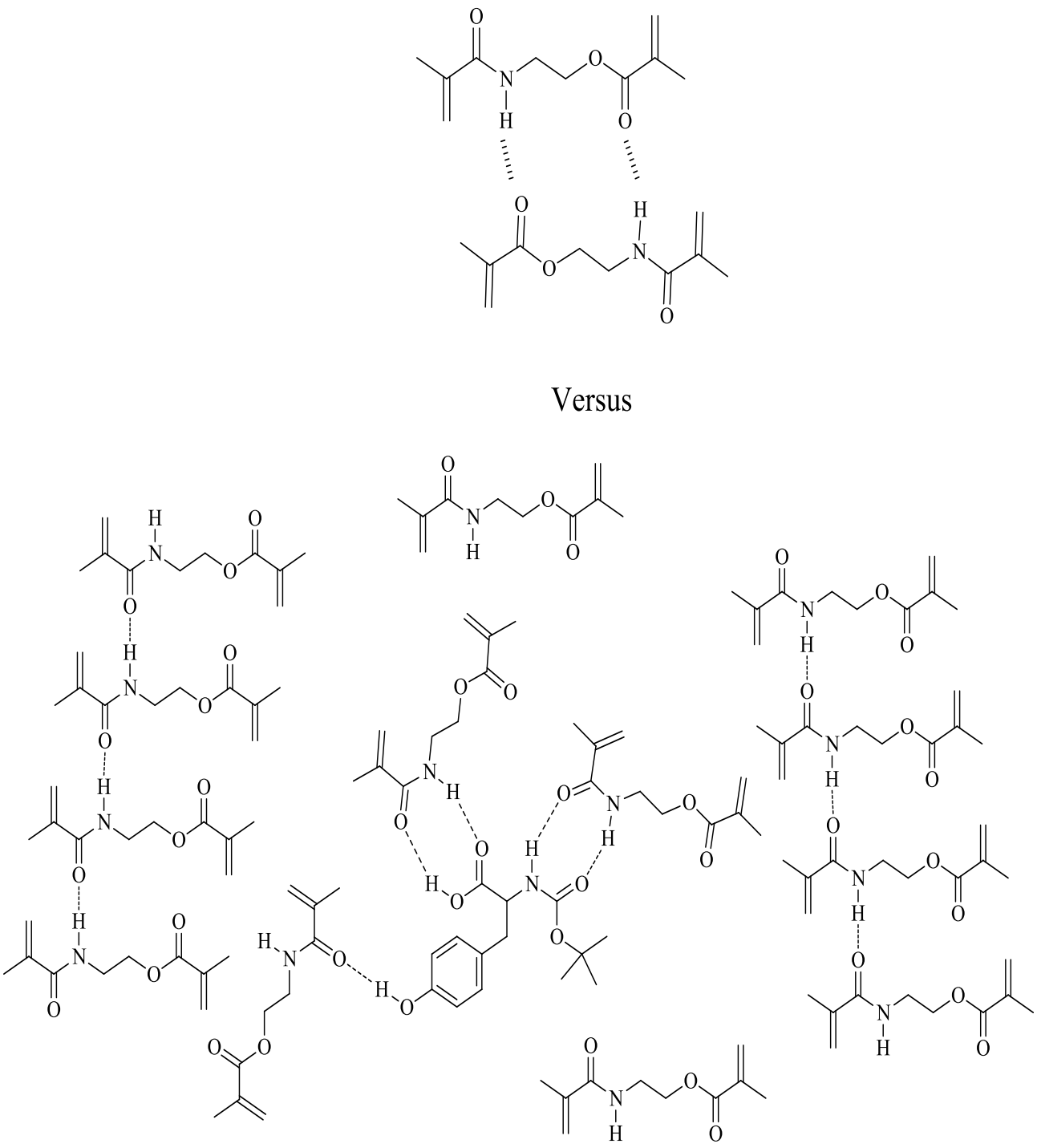


Figure 2.3: Dimer versus matrix formation in a solution of NOBE and template.

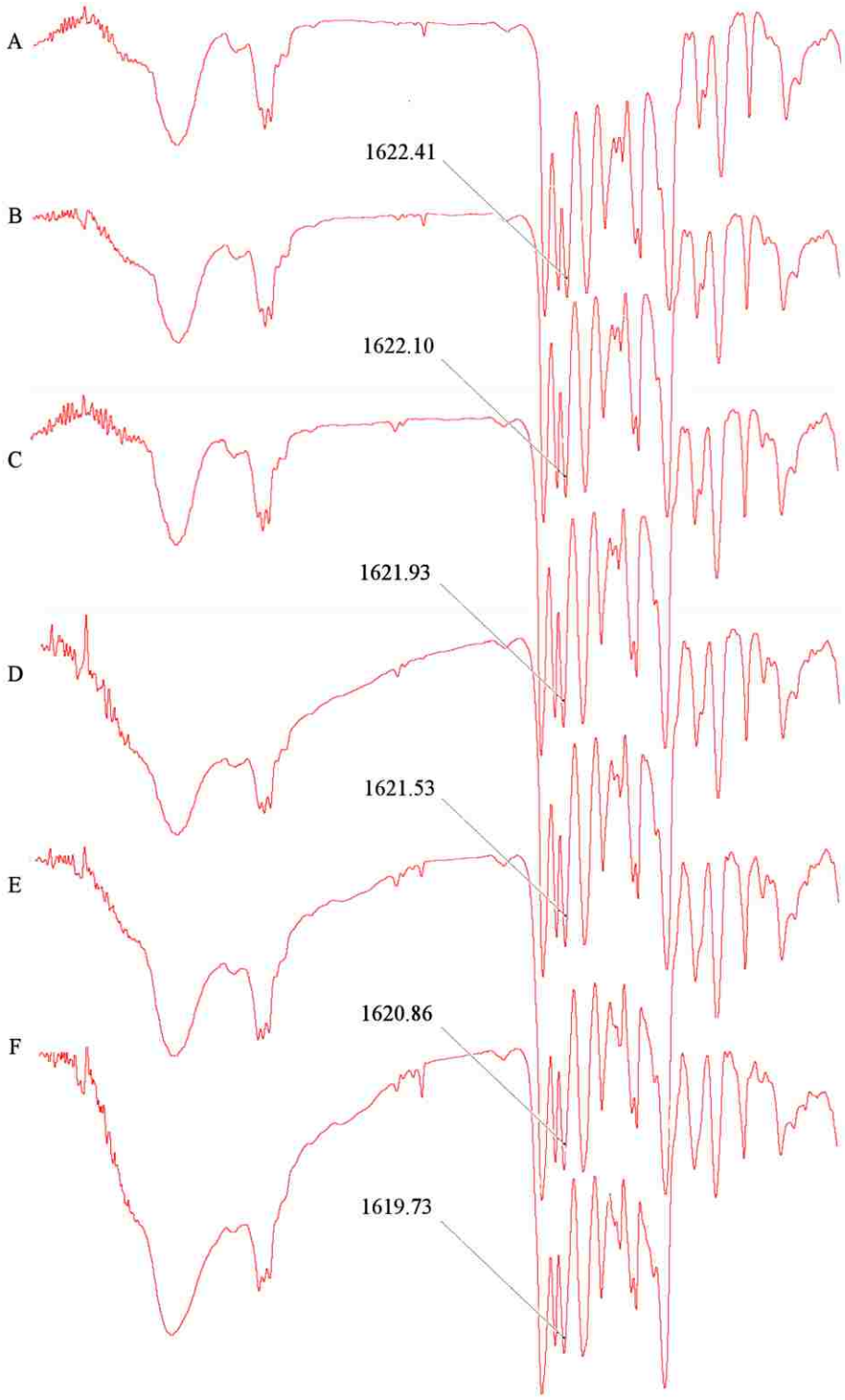


Figure 2.4: IR Spectra of A) pure NOBE B) NOBE with 2 mol% t-BOC-L-tyrosine, C) NOBE with 5 mol% t-BOC-L-tyrosine, D) NOBE with 10 mol% t-BOC-L-tyrosine, E) NOBE with 20 mol% t-BOC-L-tyrosine, and F) NOBE with 30 mol% t-BOC-L-tyrosine.

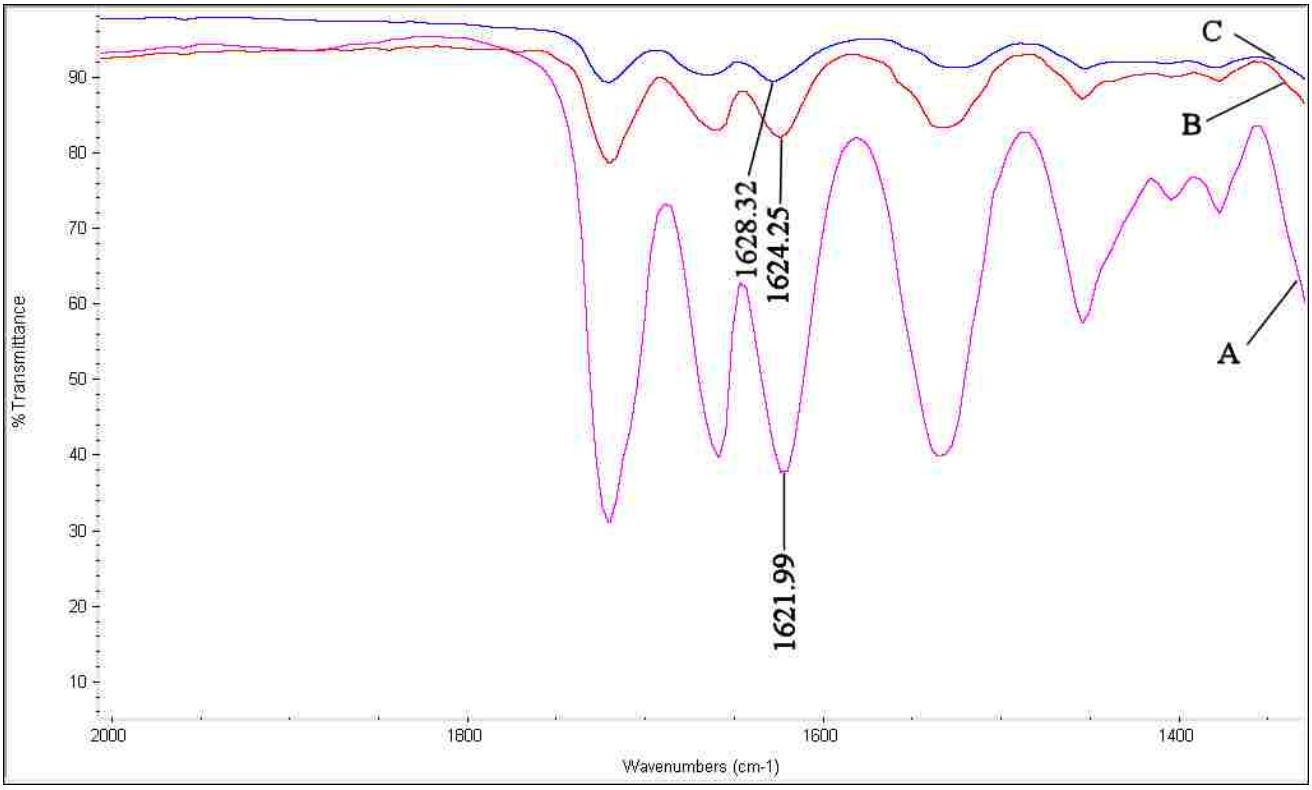


Figure 2.5: IR Spectra of increasing NOBE concentration in Fluorolube A) 4.84mol/kg, B) 2.28mol/kg, and C) 1.08mol/kg.

however, is only at a 1 % concentration in the solution. This is a relatively small amount of initiator (azobisisobutyronitrile (AIBN)) and can lead to a large experimental error because the percentage is low, the amount needed to cause an error goes up. Initiator concentrations of 0.5 %, 1.0 %, 2.0 %, and 2.5 % were added to the polymerization mixtures and the resulting polymers were analyzed using HPLC. The alpha values for the polymers are shown in **Figure 2.6**.

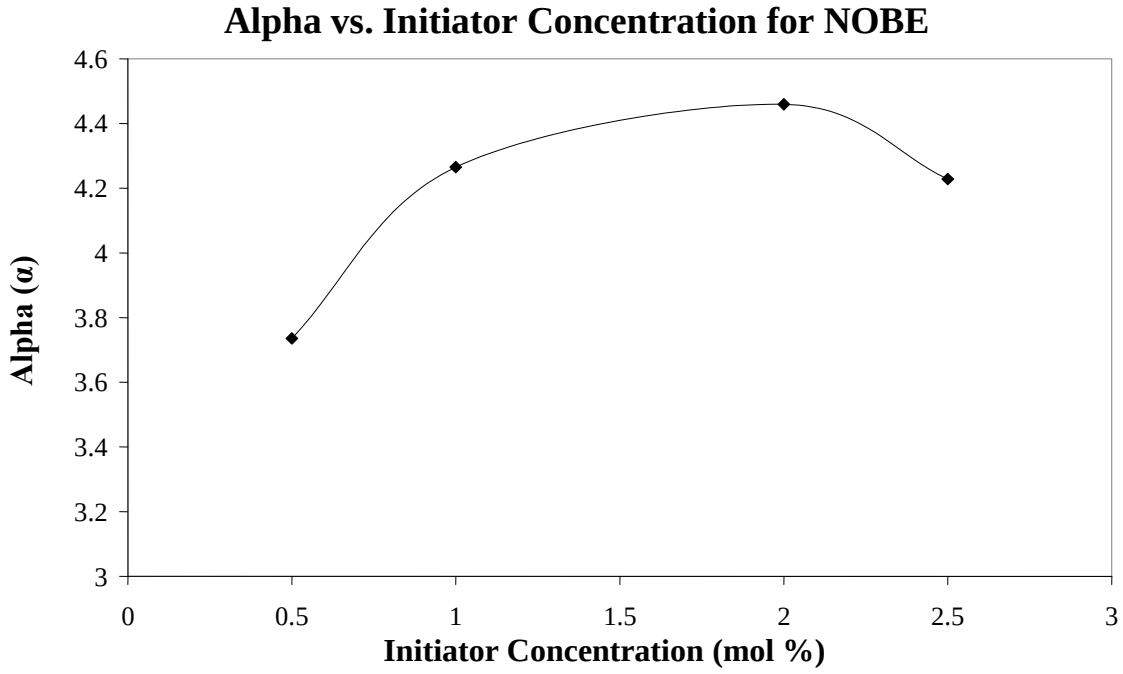


Figure 2.6. Alpha (α) versus concentration for NOBE polymers.

Significant changes in alpha (α) are noticeable when the concentration of the initiator changes. Possible explanations for the lower values at the two extremes, 0.5 and 2.5 %, are that the polymerization proceeded too rapidly or too slowly and the physical characteristics of the polymers can be altered depending on the amount of initiator included in the matrix. Since the pre-polymer complex is in equilibrium from the complexed form to the non-complexed form, the slow and fast polymerizations could polymerize the monomer in the non-complexed state thus giving lower alpha (α) values. Piletsky and co-workers performed several studies on the influence of polymerization conditions on imprinted polymers. Their results state that one of the critical factors determining the performance of the polymer is how much cross-linking occurs before the polymer becomes insoluble in solution. This is a critical factor because if the polymer

falls out of solution when not complexed to the template, the performance of the polymer will go down.[12,13]

2.5. Conclusions

The discovery of N, O-bismethacrylethanolamine (NOBE) has lead to a new discovery in making molecularly imprinted polymers, namely improved performance using one functional cross-linker as monomer. Through a series of experiments the tolerance of NOBE to different conditions (solvent effects, water tolerance, and effect of initiator concentration) was analyzed. Also IR studies were performed to determine the extent of hydrogen bonding throughout the NOBE polymer matrix. The results show that molecular recognition in MIPs using NOBE is primarily due to strong hydrogen bonding in the pre-polymer complex, NOBE can perform well under low water conditions but not high water conditions. NOBE gives the highest alpha vales at an initiator concentration of 2 %.

Part 2. Studies on the Length of the Carbon Backbone of NOBE

2.6 Introduction

NOBE was compared to two similar achiral monomers, shown in **Figure 2.7**, to determine the optimal spatial arrangement in the carbon backbone. The two compounds shown in **Figure 2.7** contain either one less carbon atom (**2.3**) or one more carbon atom (**2.4**) when compared to NOBE in the carbon backbone. The intended design of monomer **2.4** was to prove that the increased degree of freedom in molecular motion of the flexing cross-linker would allow for the monomer to create greater non-selective sites in the resulting polymer. In addition, the increased length allows for more polymer motion (swelling or shrinking) when exposed to varying solvent conditions. This motion was hypothesized to decrease the binding capability of

monomer **2.4,** because the increased polymer chain motion can permanently erase the imprinting effect by random motion. By the same argument monomer **2.3** was expected to show improved performance by keeping the pre-organized binding site rigid in its template selective form.

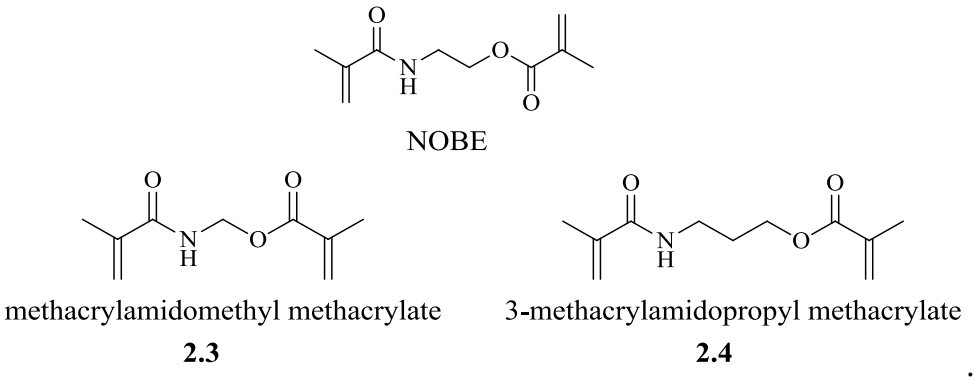


Figure 2.7. NOBE and analogs used to compare MIP performance versus cross-linker length.

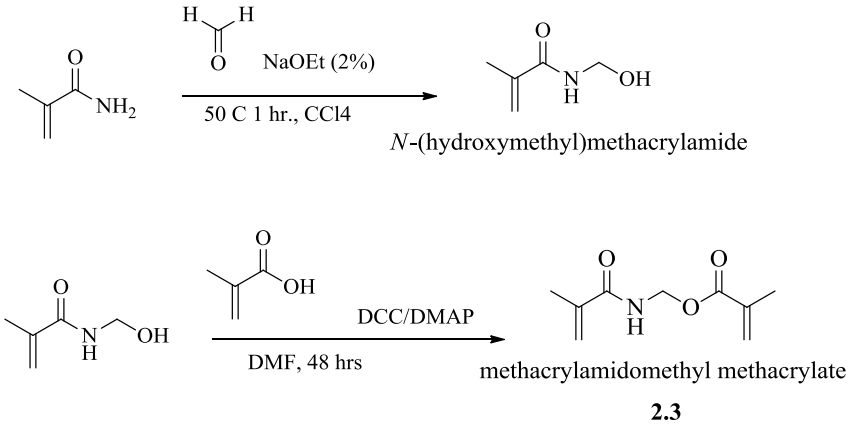
2.7 Project Goals

The goal of this project was:

- To determine the optimal size of the cross-linking monomer in relation to NOBE.

2.8. Experimental

2.8.1 Synthesis of Monomer 2.3 (methacrylamidomethyl methacrylate).

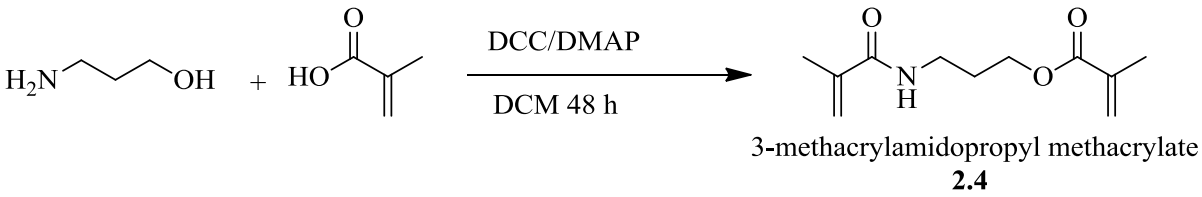


Scheme 2.1. Synthetic pathway for monomer **2.3**.

N-(hydroxymethyl)methacrylamide Methacrylamide (2 grams, 23.50 mmol) was added to 100 mL of carbon tetrachloride (CCl_4) and allowed to stir for 5 minutes. To the flask was added para-formaldehyde (0.5 grams) and a 2% by weight of sodium ethoxide (NaOEt) in ethanol. The resulting solution was then heated to 50ºC and allowed to stir for 1 hour. The mixture was then vacuum distilled (59-65ºC, 0.8 Torr) to yield the product as a clear oil in 50 % yield (1.35 grams). ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.10 (1H, s), 5.79 (1H, s), 5.70 (1H, s), 5.21 (2H, s), 3.65 (1H, s), 1.98 (3H, s). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.22, 141.36, 118.15, 68.8, 19.72.

methacrylamidomethyl methacrylate N-(hydroxymethyl)methacrylamide (1 gram, 8.69 mmol) was dissolved in 25 mL of dimethyl formamide (DMF) in a flask equipped with a magnetic stirrer. Methacrylic acid (MAA) (0.75 grams, 8.70 mmol) was added and the solution was allowed to cool to 0ºC while stirring. After 20 minutes of stirring at 0ºC DCC (1.81 grams, 8.80 mmol) and DMAP (0.11 grams, 0.087 mmol) was added to the solution. The resulting mixture was covered with a nitrogen balloon and allowed to stir at room temperature for 48 hours. The resulting DCU was then filtered and the crude product was isolated via rotary evaporation. The crude product was then dissolved in EtOAc and extracted with 1N HCl (3 x 15 mL) and a saturated solution of $NaHCO_3$ (4 x 15 mL). The organic phase was then dried over $MgSO_4$ and the product was isolated using rotary evaporation. The product was further purified by flash chromatography using a 70/30 mixture of EtOAC/Hexane. The final product while under the vacuum of the rotary evaporator would undergo spontaneous autopolymerization leaving only a 20% yield. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.05 (1H, s), 6.48 (1H, s), 6.40 (1H, s), 5.82 (2H, s), 5.78 (1H, s), 5.71 (1H, s), 2.01 (3H, s), 1.97 (3H, s). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 169.32, 167.24, 141.38, 137.84, 123.74, 118.16, 19.5, 17.9.

2.8.2 Synthesis of Monomer 2.4 (3-methacrylamidopropyl methacrylate)

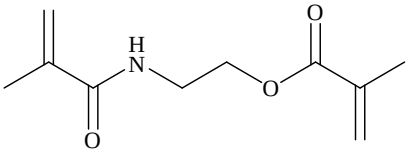
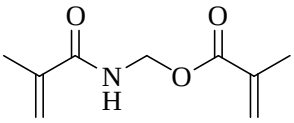
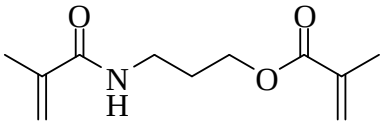


Scheme 2.2. Synthetic pathway for monomer **2.4**.

3-methacrylamidopropyl methacrylate 3-Amino-1-propanol (2 grams, 26.63 mmol) was dissolved in 200 mL of DCM in a flask equipped with a magnetic stirrer. Methacrylic acid (MAA) (5.92 grams, 66.57 mmol) was then added to the flask and the MAA/3-amino-1-propanol solution was mixed and allowed to cool to 0ºC. DCC (12.07 grams, 58.58 mmol) and DMAP (0.034 grams, 0.27 mmol) was added to the solution. The mixture was then covered with a nitrogen balloon and allowed to stir at room temperature for 48 hours. The resulting DCU was then filtered and the crude product was isolated via rotary evaporation. The crude product was then dissolved in DCM and extracted with 1N HCl (3 x 15 mL) and a saturated solution of $NaHCO_3$ (4 x 15 mL). The organic phase was then dried over $MgSO_4$ and the product was isolated using rotary evaporation. The product was further purified by flash chromatography using a 50/50 mixture of EtOAC/Hexane. Upon solvent evaporation the product was a light yellow oil in 80% yield. ^{1}H NMR ($CDCl_3$, 250 MHz): δ 8.07 (1H, s), 6.51 (1H, s), 6.45 (1H, s), 5.72 (1H, s), 5.68 (1H, s), 4.21 (2H, t), 3.18 (2H, t), 2.01 (3H, s), 1.98 (3H, s), 1.84 (2H, m). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 168.97, 167.52, 141.56, 136.02, 125.64, 118.95. 62.78, 36.82, 28.79, 19.64, 17.77.

The monomers were polymerized and characterized using HPLC. The template (boc-L-tyrosine) used is the same as which was used for the NOBE studies. The results comparing the new monomers to NOBE are shown in **Table 2.4**.

Table 2.4: Comparison of NOBE and monomers **2.3** and **2.4**.

Monomer	α
	**3.7**±0.02
	**1.56**±0.08
	**1.27**±0.12

[a]0.1mL/min flow rate; .01 mM injection concentration, 260 nm detection. [b]values are approximate.

The results for the three carbon monomer **2.4** were expected. The alpha (α) value decreased because of the increased flexibility versus NOBE in the polymer matrix. The increased flexibility in the monomer moves the hydrogen bonding functionality around, increasing entropy and losing fidelity of the original imprinted site. The results for the one carbon monomer **2.3** were not as expected. The results were expected to improve when compared to NOBE because of the reduction in random motion; however, the α values were dramatically lower. The one carbon monomer **2.3** was a softer polymer when compared to NOBE and the three carbon monomer **2.4**, which leads one to believe the monomer was not fully polymerized. The lack of complete polymerization is a possible explanation of the poor performance.

2.9. Conclusion

The natural progression of research included studies varying the linear structure on NOBE to form two separate compounds (**2.3** & **2.4**). The two new compounds contained either one less carbon (**2.3**) in the carbon backbone when compared to NOBE and one more carbon (**2.4**) than NOBE. The results for monomer **2.3** where decreased because of the reactive nature of this monomer. As noted in the experimental section, Monomer **2.3** was prone to autopolymerization during purification and lead to a material that contained partially polymerized soluble compounds and therefore was not able to fully produce a good binding site. The performance of monomer **2.4** was reduced because of the increase range of motion arising from the extra carbon in the backbone increasing entropy and losing conformity of a good imprinted site. The extra carbon gave a material that would swell and shrink more than NOBE. This physical change in the polymer morphology can cause the poor performance for monomer **2.4**. This small study helped to determine that NOBE was the right size of cross-linker and any derivatives should be derived from NOBE.

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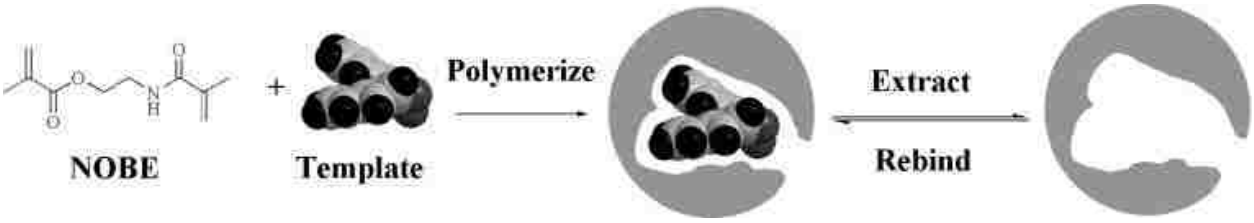
CHAPTER 3: IMPRINTING MULTIPLE TEMPLATES USING OMNiMIPS

Part 1. Multi-analyte Imprinting Capability of OMNiMIPs Versus Traditional Molecularly Imprinted Polymers*

3.1. Introduction

Molecular imprinting is an evolving technique that provides materials capable of molecular recognition which can be applied to analytical devices, detectors, assays, and separation formats.[1-4] The method of molecular imprinting is carried out by polymerization of one or more monomers in the presence of a template molecule, followed by removal of the template to leave a binding cavity with selectivity toward the template molecule (**Scheme 3.1**). In most of the accounts of molecular imprinting, a single template molecule is used to create the



Scheme 3.1. Outline of the molecular imprinting strategy using the crosslinker NOBE.

specific binding site of the MIP. However, the imprinting process does not have to be limited to a single template, and several compounds can be imprinted simultaneously (**Scheme 3.2**). Advantages of multiple-template imprinting are that several different classes of compounds can be extracted, separated, assayed, detected, or otherwise analyzed at one time.[5-12] The simultaneous separation of several compounds on one stationary phase would be of use, for example, in the analysis of pharmaceutical formulations. Alternatively, a detector incorporating

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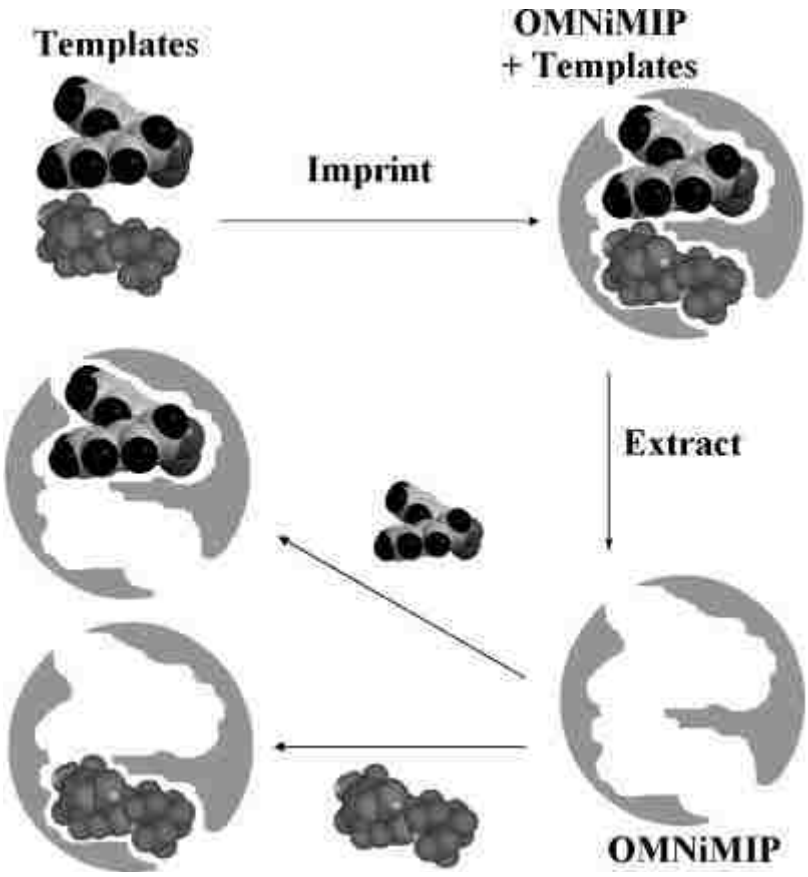


Scheme 3.2. OMNiMIP formation in the presence of multiple templates for multi-analyte binding.

an MIP to multiple templates would be capable of detecting one (or more) of a family of possible contaminants in biological or environmental systems. While this could also be achieved by mixing the individually imprinted polymer particles, this method requires the synthesis and processing of several polymers, and may provide materials with decreased binding and selectivity (vide infra). Moreover, some applications of imprinted polymers require that the material remains intact, such as membranes[13, 14] or monolithic columns[15, 16].

A few examples have been reported on different MIP polymers, or polymer mixtures, that have been imprinted with more than one template. The first example was reported by Sreenivasan and Sivakumar, who imprinted both salicylic acid and hydrocortisone in the same

MIP formulated with hydroxyethylmethacrylate (HEMA) as the functional monomer and ethylene glycol dimethacrylate (EGDMA) as the crosslinker.[6] The MIP made with both templates showed selective uptake of both salicylic acid and hydrocortisone, however, the selectivity was reduced relative to MIPs made with only one template or the other. The reduced selectivity was presumably due to the dilution of the number of binding sites per gram of the polymer mixture for each of the templates. Soon after, this group used acrylic acid (AA)/EGDMA formulated polymers to show analogous effects for MIPs imprinted with three templates: cholesterol, testosterone, and hydrocortisone.[8] Similarly, Dickert et al. introduced the idea of ‘‘double molecular imprinting’’ using two templates that were simultaneously imprinted in a crosslinked polyurethane thin film.[7] The double imprinted MIP was able to recognize both the templates, while polymers imprinted with only one of the templates primarily exhibited a preference for that template only. Schweitz et al. used capillary electrochromatography to analyze an MIP made with metoprolol and atenolol simultaneously, versus MIPs imprinting each of these templates singly.[10] In this report, the MIP made with the mixture of templates showed better resolution than the singly imprinted polymers; template–template interactions were speculated to have been the underlying reason for this. The same research group also found that resolution of R and S propranolol appeared better for the simultaneously imprinted material versus the mixed particles. Furthermore, the authors demonstrated that resolution of enantiomers of simultaneously imprinted templates could be systematically controlled and improved by changing the ratio of templates in the pre-polymer mixture. In another report, Suedee et al. imprinted tetracycline and its degradation products in a traditional bulk polymer formulation using EGDMA as the crosslinker and methacrylic acid (MAA) as the functional monomer.[12] The imprinting factors for this mixed MIP were either equal, or in some cases less than the imprinting

factors found for an identically formulated MIP to only the template tetracycline. Examples of physically mixing particles of MIPs made separately using two different templates were reported as early as 1998; for example, Bowman et al. imprinted separately the templates propranolol, atenolol, and timolol using traditionally formulated EGDMA/MAA MIPs.[17] Under HPLC conditions, the mixed bed particle column showed reduced capacity factors versus columns packed with the singly imprinted polymers, as noted for earlier examples. The authors noted that mixing the individual MIPs appeared to ‘‘blend’’ the molecular recognition properties of the different template materials, allowing differential binding of a library of related molecules that were not actually imprinted. Sabourin et al. found that an MIP mixture of three singly imprinted polymers was able to simultaneously separate mixtures of racemates or diastereomers of the three different compounds.[5]

Recently, we have discovered a simpler approach to MIP formation that utilizes a single crosslinking monomer, N, O-bismethacryloyl ethanolamine (NOBE), in addition to the template, solvent and initiator (**Scheme 3.1**).[18] We have coined the term ‘‘OMNiMIPs’’ (One MoNomer Molecularly Imprinted Polymers) to describe this approach, which eliminates variables such as choice of functional monomer and crosslinker, the ratio of functional monomer/crosslinker, and the ratio of functional monomer/template which normally complicate the MIP design.[19] In addition to developing an easier method for the formation of new MIP materials, there are fundamental differences in OMNiMIPs versus traditional imprinted materials. For example, we have found that higher binding capacities can be obtained for OMNiMIPs as the template loading is increased up to 20–25% template.[20] On the other hand, MIPs formed using the commonly used formulation (EGDMA and MAA) often lose binding and selective properties at 10% or less template loading.[21, 22] Due to the higher template loading that is possible with OMNiMIPs, and

the corresponding increase in binding capacity, it was anticipated that OMNiMIPs would have a greater capability to imprint a number of different analytes simultaneously compared to traditionally formulated MIPs. This assumption was tested for a binary template system, (R)-1,1'-bi-2-naphthol and BOC-L-tyrosine, for NOBE based OMNiMIPs versus traditionally formulated EGDMA/MAA molecularly imprinted polymers.

3.2. Project Goals

The goals of this project were:

- To test NOBE's ability to imprint more than one template simultaneously.
- Compare NOBE ONMIMIPs versus EGDMA/MAA polymers in multi-analyte imprinting.

3.3. Materials and Methods

3.3.1. Materials

EGDMA and MAA were purchased from Sigma-Aldrich and distilled in vacuo to remove inhibitors prior to polymerization. NOBE was synthesized by a previously published method.[18] Sodium bicarbonate, MgSO4, BOC-L-tyrosine, BOC-D-tyrosine, and 2, 2'-azo-bis-isobutyronitrile (AIBN) were all purchased from Aldrich Chemicals and used without further purification. Flash chromatography was carried out with silica gel, 32–63mm from Science Adsorbents Inc. HPLC grade solvents were obtained from commercial suppliers and used without further purification.

3.3.2. OMNiMIP Polymer Formulation using NOBE

For the OMNiMIPs incorporating 0.05 molar equivalents of template: BOC-L-tyrosine (0.214 g, 0.761 mmol, 0.05 eq.), (R)-(+)-1,1'-bi-2-naphthol (0.218 g, 0.761 mmol, 0.05 eq.), or a mixture of both were dissolved in dry acetonitrile (3.0 ml) with subsequent addition of NOBE (3.00 g, 15.2 mmol, 1 eq.) and azo-bisisobutyronitrile (AIBN; 0.0500 g, 0.304 mmol, 0.02 eq.) to the solution. The solutions were transferred via pipette into 13mm X 100mm screw top test tubes, purged with nitrogen gas for 5 min, capped and sealed with Teflon tape and Parafilm, and then inserted into a photochemical reactor with a constant temperature bath maintained at 20ºC. A standard laboratory ultraviolet light source (medium pressure 450Wmercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed into the photoreactor. The solutions were then photopolymerized for 8 h at 20ºC. Control polymers were synthesized under the same conditions in the absence of either template.

3.3.3. EGDMA/MAA Polymer Formulation

Similar to the procedure for the NOBE imprinted polymers, EGDMA/MAA imprinted materials were formulated with BOC-L-tyrosine (0.244 g, 0.867 mmol, 0.05 eq.), (R)-(þ)-1, 10-bi- 2-naphthol (0.248 g, 0.867 mmol, 0.05 eq.), or a mixture of both dissolved in dry acetonitrile (3.0 ml). To the template solutions were added EGDMA (2.75 g, 13.9 mmol, 0.8 eq.) and MAA (0.299 g, 3.47 mmol, 0.2 eq.), along with AIBN (0.0569 g, 0.347 mmol, 0.02 eq.). The solutions were transferred into 13mm X 100mm screw cap test tubes, purged with nitrogen for 5 min, and sealed with Teflon tape and Parafilm. Photopolymerization conditions were the same as that of the NOBE polymers. Control polymers were synthesized under the same conditions in the absence of either template.

3.3.4. Chromatographic Evaluations

The template was removed from the imprinted polymers by Soxhlet extraction with methanol for 48 h. The polymers were ground with a mortar and pestle, using USA Standard Testing Sieves to collect particles with diameters between 25 and 37 mm. The particles were slurry packed using a Beckman 112 Solvent Delivery Module into steel columns (length 100 mm; inner diameter 4.1 mm) to full volume for chromatographic experiments. After packing, HPLC analyses were performed using a Hitachi L-7400 UV Detector and L-7100 pump. The columns were equilibrated online for 12 h using a mobile phase consisting of acetonitrile/acetic acid (99:1, v/v) at a flow rate of 0.10 ml/min to remove any remaining template. The actual HPLC analyses were performed isocratically at room temperature (21ºC), at a flow rate of 1.0 ml/min using acetonitrile/acetic acid (99:1, v/v) as the mobile phase. Analytes (BOC-L tyrosine, BOC-D-tyrosine, (R)-(þ)-1, 1'-bi-2-naphthol, and S-(-)-1,1'-bi-2-naphthol, and the racemates of both tyrosine and 1,1'-bi-2-naphthol) were dissolved in HPLC grade acetonitrile and detected at a wavelength of 260 nm; the substrate injection concentration was 1.0 mM. For the mixed polymer packed column beds, the particles were physically mixed in a scintillation vial and agitated for 10 min in 20 ml acetone prior to packing into a chromatography column.

For all imprinted polymers and polymer mixtures, the separation factor, α, was measured as a ratio of capacity factors k'enantiomer 1/k'enantiomer 2, with k' determined by the following relation: $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the analyte and t_0 is the retention time of the void volume measured using acetone. The percentage loss in selectivity between a higher separation factor found for the singly imprinted polymer (α_2) and the lower separation factor found for multiple-template imprinted or mixed bed columns (α_1) was calculated using the

equation $[(\alpha_2-\alpha_1)/(\alpha_2-1)]$ X (100)%; where the minimum value for α_1 is 1, which is subtracted from the denominator as a normalization factor.

3.4. Results

Molecular recognition by the imprinted polymers in this study was measured by comparison of separation factors for enantiomers of the analytes. Enantioselectivity is the best measure of the molecular imprinting effect because molecular recognition depends solely on geometrical differences of the enantiomeric analytes, and eliminates any partitioning differences that would arise from molecules with different physical properties.[2] The two templates investigated for multiple-template imprinting were chosen from those that had been previously studied for molecular imprinting utilizing both the NOBE OMNiMIP system and traditional EGDMA/MAA molecularly imprinted polymers.[19] Although selectivity was not found for BOC-L-tyrosine in this study by the EGDMA/MAA imprinted polymer, this is likely due to the fact that the earlier publication reported HPLC data for each enantiomer separately[19]; whereas, the separation factor values reported here are from HPLC of racemic mixtures. Separation factors for racemic mixtures of 1,1'-bi-2-naphthol or BOC-tyrosine were determined for the following three types of OMNiMIP formulations for chromatographic columns:

1. OMNiMIPs imprinted with 5 mol% (R)-1,1'-bi-2-naphthol or BOC-L-tyrosine (entries 1 and

2. OMNiMIPs imprinted with 5 mol% (R)-1,1'-bi-2-naphthol and 5 mol% BOC-L-tyrosine (10 mol% total for both templates, entry 3).

3. OMNiMIPs imprinted with 2.5 mol% (R)-1,1'-bi-2-naphthol and 2.5 mol% BOC-L-tyrosine (5 mol% total for both templates, entry 4).

4. Physically mixed particles of entries 1 with 2 in equal amounts (entry 5).

5. Physically mixed particles of entry 2 with non-imprinted polymer (entry 6).

Table 3.1. Comparison of separation factors and losses in selectivity for OMNiMIPs in different formats versus traditionally formulated EGDMA/MAA (80/20) imprinted polymers.

Entry	Imprinted polymer	Selectivity for (R)-(þ)-1, 10-bi-2-naphthol (α)	Loss in selectivity for R-1,10-bi-2-naphthol (%)[c]	Selectivity for BOC-L-tyrosine (α)	Loss in selectivity for BOC-L-tyrosine (%)[c]
1	NOBE OMNiMIP (5% (R)-(+)-1,1'-bi-2-naphthol)[a]	8.9	—[d]	n/d[e]	—[d]
2	NOBE OMNiMIP (5% BOC-L-tyrosine)[a]	n/d[e]	—[d]	2.9	—[d]
3	NOBE OMNiMIP (5% (R)-(+)-1,1'-bi-2-naphthol + 5% BOC-L-tyrosine)[a]	8.4	6.3	2.5	21.1
4	NOBE OMNiMIP (2.5% (R)-(+)-1,1'-bi-2-naphthol + 2.5% BOC-L-tyrosine)[b]	4.4	57.0	1.0	100
5	Mixed bed: i. NOBE OMNiMIP (5% (R)-(+)-1,1'-bi-2-naphthol)[a] physically mixed with: ii. NOBE OMNiMIP (5% BOC-L-tyrosine)[a]	2.6	79.7	1.0	100
6	Mixed bed: i. NOBE OMNiMIP (5% (R)-(+)-1,1'-bi-2-naphthol+ 5% BOC-L-tyrosine)[a] physically mixed with: ii. NOBE (NON-IMPRINTED)	1.0	100	1.0	100
7	EGDMA/MAA (5% (R)-(+)-1,1'-bi-2-naphthol)[a]	3.2	—[d]	n/d[e]	—[d]

Table 3.1. Continued.

Entry	Imprinted polymer	Selectivity for (R)-(þ)-1, 10-bi-2-naphthol (α)	Loss in selectivity for R-1,10-bi-2-naphthol (%)[c]	Selectivity for BOC-L-tyrosine (α)	Loss in selectivity for BOC-L-tyrosine (%)[c]
8	EGDMA/MAA (5% BOC-L-tyrosine)[a]	n/d[e]	—[d]	1.0	—[d]
9	EGDMA/MAA (5% (R)-(+)-1,1'-bi-2-naphthol + 5% BOC-L-tyrosine)[a]	1.9	59.1	1.0	n/a[f]
10	EGDMA/MAA (2.5% (R)-(+)-1,1'-bi-2-naphthol þ 2.5% BOC-L-tyrosine)[b]	1.0	100	1.0	n/a[f]
11	Mixed bed: i. EGDMA/MAA (5% (R)-(+)-1,1'-bi-2-naphthol)[a] physically mixed with: ii. EGDMA/MAA (5% BOC-L-tyrosine)[a]	1.0	100	1.0	n/a[f]
12	Mixed bed: i. EGDMA/MAA (5% (R)-(+)-1,1'-bi-2-naphthol + 5% BOC-L-tyrosine)[a] physically mixed with: ii. EGDMA/MAA (NON-IMPRINTED)	1.0	100	1.0	n/a[f]

[a]Imprinted polymer formulated with 0.05 molar equivalents of specified templates.
[b]Imprinted polymer formulated with 0.025 molar equivalents of specified templates.
[c]Calculated from the equation in the Chromatographic evaluation subsection under the Materials and Methods section.
[d]Not applicable.
[e]Not determined.
[f]No selectivity available to detect losses.

Looking at **Table 3.1**, entry 1 shows a separation factor of 8.9 for enantiomers of 1,1'-bi-2-naphthol on the OMNiMIP imprinted with only (R)-(+)-1,1'-bi-2-naphthol. Focusing on the resolution of binapthol enantiomers, the high α value affords good separation as shown in chromatogram ''a'' of the cascade plot in Figure 1. Similarly, entry 2 shows a separation factor of 2.9 for the OMNiMIP imprinted with only the BOC-L-tyrosine template. The initial test for the ability of an OMNiMIP to imprint more than one template simultaneously is shown in entry 3. For this polymer, both (R)-(+)-1,1'-bi-2-naphthol and BOC-L-tyrosine were used as templates, each in the same mole per cent as that used in entries 1 and 2 (i.e., 5 mol% each). The separation factor for a racemic mixture of 1,1'-bi-2-naphthol was evaluated first, giving a value of 8.4 as shown in column 3 of entry 3. This value is very close to that of the (R)-(+)-1,1'-bi-2-naphthol single imprinted polymer, displaying only 6.3% loss in selectivity as shown in the fourth column of **Table 3.1**. Correspondingly, chromatogram ''b'' shows a similar resolution to that of chromatogram ''a'' in **Figure 3.1**, showing minimal interference on (R)-(+)-1,1'-bi-2-naphthol imprinting by additional templates. Next, the separation factor for a racemic mixture of BOC-L-tyrosine was evaluated, again giving an α value only moderately lower (21.1% loss in selectivity) than that of the singly imprinted OMNiMIP in entry 2.

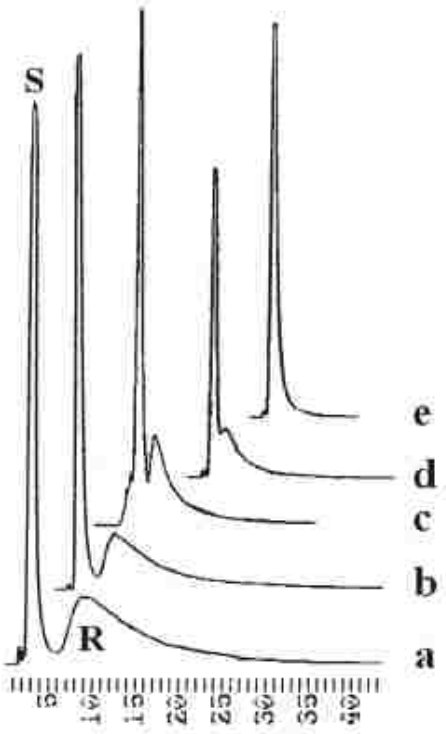


Figure 3.1. Elution profiles of a racemic mixture of 1,1'-bi-2-naphthol on different HPLC column formats incorporating the NOBE based OMNiMIP.
Chromatograms a–e correspond to entries 1, 3, 4, 5, and 6 in Table 1respectively.

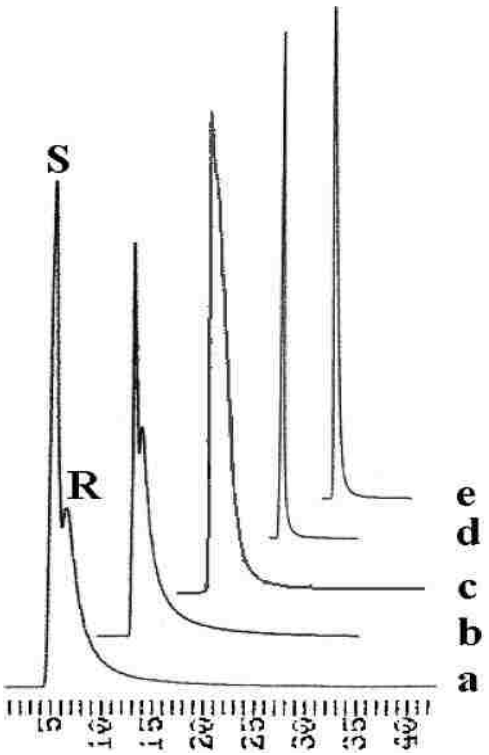


Figure 3.2. Elution profiles of a racemic mixture of 1,1'-bi-2-naphthol on different HPLC column formats incorporating polymers imprinted EGDMA/MAA. Chromatograms a–e correspond to entries 7, 9, 10, 11, and 12 in Table 1 respectively.

These results were then compared to the performance of the ‘‘mixed bed’’ column packed with equal amounts of imprinted polymer from entries 1 and 2. Entry 5 shows that for this case, the measured selectivity for (R)-(+)-1,1'-bi-2-naphthol from a racemic mixture dropped 79.7%. Furthermore, there was complete loss of enantioselectivity toward BOC-L tyrosine when the racemic mixture was eluted on the mixed bed column. It was hypothesized that the lower enantioselectivity was due in part to the reduced amount of template, and thus the number of binding sites in the polymer for each template, effectively imprinted per gram of the mixed polymer material. In other words, 2.5 mol% of the OMNiMIP in the mixed bed column was effectively imprinted with (R)-(+)-1,1'-bi-2-naphthol, and the same for BOC-L-tyrosine; and this was not a fair comparison with the OMNiMIPs imprinting 5.0 mol% of each template (entries 1 and 2). Therefore, a more equitable comparison was made by simultaneously imprinting 2.5 mol% of each template, and the results in entry 4 show 57.0% loss of selectivity for (R)-(þ)-1,10-bi-2-naphthol and 68.4% for BOC-L-tyrosine. These results do indicate a more fair comparison to the mixed bed OMNiMIP in entry 5, since the reduction in the α values for entries 4 and 5 are similar in magnitude with respect to the singly imprinted OMNiMIPs. However, the 2.5 mol% multiple-template OMNiMIP in entry 4 does have significantly better separation factors than the

mixed bed column in entry 5, which is clearly reflected in the better resolution of peaks in chromatogram ‘‘c’’ versus chromatogram ‘‘d’’ in the cascade plot of **Figure 3.1** which correspond to entries 4 and 5 respectively. A third comparison was made with a mixed bed column formulated with equal mixtures of the multiple-imprinted polymer and non-imprinted polymer (entry 6), which would also provide a mixed bed column with 2.5 mol% of each template effectively imprinted. In this case, entry 6 shows complete loss of selectivity, indicating that mixing imprinted polymers with non-imprinted polymers has an even greater detrimental effect on binding and selectivity of imprinted materials than any other combination (e.g., entries 3–5). The loss in separation factor is verified by chromatogram ‘‘e’’ in **Figure 3.1**, which shows a single peak for both enantiomers of 1,1’-bi-2-naphthol.

All the OMNiMIPs above were compared to traditionally formulated EGDMA/MAA (80/20) imprinted polymers that were equivalently prepared. Entry 7 in **Table 3.1** reports a separation factor of 3.2 for 1,1’-bi-2-naphthol on the (R)-(+)-1, 1’-bi-2-naphthol MIP, which does not provide adequate resolution of enantiomers as shown in chromatogram ‘‘a’’ in the cascade plot of **Figure 3.2**. In fact, the 2.5 mol% multiple-imprinted OMNiMIP (chromatogram ‘‘c’’ in **Figure 3.1**) appears to provide better resolution than the singly imprinted EGDMA/MAA polymer for binapthol enantiomers. Moreover, no separation (α=1) was seen for tyrosine on its imprinted polymer (entry 8); thus, no further changes in tyrosine resolution were expected, which was validated by entries 9–12. The results for the simultaneously imprinted EGDMA/MAA MIPs in entries 9 and 10 reveal a 59.1 and 100% loss in selectivity respectively for (R)-(+)-1,1’-bi-2-naphthol, and complete loss of BOC-tyrosine selectivity in both cases. The mixed bed columns (entries 11 and 12) showed complete loss of selectivity for 1,1’-bi-2-

naphthol, and continued lack of selectivity for BOC-tyrosine as expected. For chromatographic comparison of EGDMA/MAA polymers to OMNiMIPs, the remaining chromatograms ‘‘b’’, ‘‘c’’, and ‘‘d’’, corresponding to entries 9–12 are shown in the cascade plot in **Figure 3.2**.

An unanticipated finding from this study was that mixing the multiple-template imprinted polymer with non-imprinted polymer resulted in complete loss of selectivity. An especially clear example of this is seen for enantioselectivity of 1,1’-bi-2-naphthol derivatives eluted on the different OMNiMIP column formats. While the mixed bed OMNiMIP combining the two singly imprinted polymers showed a large loss in selectivity versus the (R)-(+)-1,1’-bi-2-naphthol imprinted polymer (entry 5), the mixed bed column incorporating the multiple-template imprinted OMNiMIP along with non-imprinted polymer suffered complete loss of selectivity (entry 6). Both of these mixed bed columns should have the same number of binding sites for (R)-(+)-1,1’-bi-2-naphthol, corresponding to 2.5 mol% of the polymer. Therefore, it can be postulated that the inclusion of non-imprinted polymer in a mixed bed column format is the cause of the enormous loss of selectivity seen. To see if this phenomenon is general, the singly imprinted (R)-(+)-1,1’-bi-2-naphthol OMNiMIP was combined with non-imprinted polymer in a mixed bed format. The resulting separation factor of 1.5 (entry 1 of **Table 3.2**) of this mixed bed column indicates loss of nearly all selectivity of the original imprinted OMNiMIP (entry 1 of Table 1) that existed prior to mixing with non-imprinted polymer. Furthermore, there was complete loss of selectivity of the BOC-L-tyrosine OMNiMIP upon mixing with non-imprinted polymer (entry 2 of Table 2); the complete loss may be due to the significantly lower separation factor relative to the 1,1’-bi-2-naphthol imprinted polymer. For the EGDMA/MAA imprinted polymer, a similar phenomenon was observed where complete loss of selectivity is found for the (R)-(+)-1, 1’-bi-2-naphthol imprinted EGDMA/MAA polymer (entry 3 of **Table 3.2**).

Meanwhile, the BOC-L-tyrosine imprinted polymer continued to exhibit lack of selectivity both prior to, and after mixing (entry 4 of **Table 3.2**). Collectively, the examples of mixed bed columns that incorporate non-imprinted polymer consistently show total loss, or at least a severe loss, in selectivity that was originally present in the imprinted polymer component.

3.5. Discussion

The first important observation from this study is the finding that OMNiMIPs are more effective for multi-analyte molecular imprinting versus traditionally formulated MIPs, supported by data presented in **Table 3.1** and the corresponding chromatograms in the cascade plot in **Figure 3.1**. The origins of this improvement may be due to the greater amount of functional monomer available in the OMNiMIP for interacting with the templates. For OMNiMIPs, the NOBE crosslinker incorporates the amide group for hydrogen bonding to the templates, providing essentially 100 mol% of interactive functional groups. For traditional MIPs that use a mixture of monomers, such as EGDMA and MAA, there is always a limit on the amount of interactive functional monomer that can be used. This is a consequence of the minimum level of crosslinking needed in MIPs to maintain the structural features of the template-binding site.

Previous studies on EGDMA/MAA imprinted polymers have empirically determined that approximately 80 mol% crosslinker (EGDMA) generally provides the crosslinking needed for optimum molecular recognition in MIPs.[2] Therefore, this concentration of crosslinker was chosen for this study, leaving 20 mol% of the functional monomer MAA for interaction with the templates. Further research on template to monomer ratio in EGDMA/MAA imprinted polymers has shown that increase in template to monomer ratio initially increases the selectivity to a point, after which selectivity decreases.[22] The initial increase is postulated to arise from the formation

of more binding sites in the MIP, which should increase the performance of the material. However, at some critical template to monomer ratio, the selectivity of the imprinting material decreases. The reduced selectivity is a result of the reduced percentage of functional monomer available for interacting with the template, relative to OMNiMIPs. Therefore, OMNiMIPs may be able to better maintain the needed increase in functional monomer for interaction with greater template concentration ranges versus EGDMA/MAA imprinted polymers.

Table 3.2. Separation factors for single-template imprinted polymers mixed with non-imprinted polymer

Entry	Imprinted polymer	**Selectivity for (R)-(+)-1,1'-bi-2-naphthol (α)**	**Selectivity for BOC-L-tyrosine (α)**
1	Mixed bed: i. NOBE OMNiMIP (5% (R)-(+)-1,1'-bi-2-naphthol)[a] physically mixed with: ii. NOBE (NON-IMPRINTED)	1.5	n/d[b]
2	Mixed bed: i. NOBE OMNiMIP (5% BOC-L-tyrosine)[a] physically mixed with: ii. NOBE (NON-IMPRINTED)	n/d[b]	1.0
3	Mixed bed: i. EGDMA/MAA (5% (R)-+)-1,1'-bi-2-naphthol)[a] physically mixed with: ii. EGDMA/MAA (NON-IMPRINTED)	1.0	n/d[b]
4	Mixed bed: i. EGDMA/MAA (5% BOC-L-tyrosine)[a] physically mixed with: ii. EGDMA/MAA (NON-IMPRINTED)	n/d[b]	1.0

[a]Imprinted polymer formulated with 0.05 molar equivalents of the specified templates.
[b]Not determined.

In this study, when two analytes were imprinted, the combined concentration of templates requiring functional monomer increased. For the OMNiMIPs, the increase in concentration on adding both templates does not appear to overtax the available interactive functional monomer. As a result, the performance of the multiple-template OMNiMIP would be anticipated to be similar to that of imprinting one or the other template, which is verified by comparing entries 1 and 2 with entry 3. On the other hand, the limited amount of functional monomer in the EGDMA/MAA imprinted polymer appeared to be adequate for 5 mol% (R)-(+)-1,1'-bi-2-naphthol; however, upon further addition of 5 mol% BOC-L-tyrosine in the polymer formulation, the selectivity of the imprinted polymer was significantly reduced. The loss in selectivity may be due to the overall decrease in the functional monomer/template ratio required by the (R)-(+)-1, 1'-bi-2-naphthol template for forming high affinity binding sites. Thus, the higher analyte capacity of OMNiMIPs facilitates multiple template imprinting.

A second important observation is that chromatographic resolution, and ultimately enantioselectivity, of multiple-template imprinted OMNiMIPs surpassed that of columns comprising a mixture of differently imprinted particles. For (R)-(+)-1,1+-bi-2-naphthol, the multiple-template OMNiMIP in entry 4 of **Table 3.1** gave an a value 4.4, whereas the mixed bed column in entry 5 yielded a significantly lower a value of 2.6. These two entries were chosen for comparison because both column materials incorporate 2.5 mol% of templated sites for (R)-(+)-1,1'-bi-2-naphthol and BOC-L-tyrosine. Corresponding entries in **Table 3.1** for BOC-L-tyrosine showed an a value of 1.6 for the simultaneously imprinted OMNiMIP versus 1.0 for the mixed bed column. The trend may be explained by the topology of the binding sites. In the mixed bed column, each individual particle is imprinted with either R-binapthol or BOC-L-tyrosine. Focusing on (R)-(+)-1,1'-bi-2-naphthol, this analyte will undergo separation in the R-binapthol

imprinted particles. However, it will undergo remixing in the BOC-L-tyrosine particles which only interact non-specifically with (R)-(þ)-1,1'-bi-2-naphthol, essentially having the same effect as ''dead volume'' in chromatography. For the simultaneously imprinted OMNiMIP, each particle has evenly distributed binding sites and non-selective sites. Furthermore, the topology of the (R)-(+)-1,1'-bi-2-naphthol binding sites are in close enough proximity to maintain a steady separation at each theoretical plate within the column, without any dead volume remixing. The same trends in selectivity are seen for BOC-L-tyrosine. For the EGDMA/MAA imprinted polymers, an equivalent comparison between multiple-template imprinted materials (entry 10 in **Table 3.1**) and the mixed bed column (entry 11 in **Table 3.1**) is not possible because both examples do not exhibit any enantioselectivity for either template.

A third observation is that the examples of mixed bed columns that incorporate non-imprinted polymers consistently show total loss, or at least a severe loss, in selectivity that was originally present in the imprinted polymer component. The underlying cause may again be due to remixing of enantiomers in the non-imprinted polymer particles which act as ''dead volume'' for chromatography. The remixing may be more severe when non-imprinted material is used, versus material imprinted with a different template, because the functional monomers tend to complex with each other eliminating to a large degree the binding interactions with template. For a material imprinted with a molecule different than the analyte, functional groups are still available for interaction with the analyte, albeit non-selectively. While this enhanced binding is non-selective, it may inhibit the remixing process by slowing the analyte transport and maintaining separation to a greater extent versus non-imprinted material.

3.6. Conclusions

Multiple-template imprinting in OMNiMIPs was found to have better performance versus traditionally formulated EGDMA/MAA imprinted polymers. The imprinting of two templates simultaneously provided nearly the same enantioselectivity for each template as the singly imprinted OMNiMIP for each template. EGDMA/MAA multiply imprinted polymers suffered greater losses in selectivity, although molecular recognition for each template was observed. It should be noted that EGDMA/MAA MIPs are generally best for imprinting amine-bearing molecules via ionic interactions, whereas in the examples presented here, only hydrogen bonding interactions were available for the templates. In contrast, NOBE based OMNiMIPs do not hydrogen-bond strongly with amine-based compounds, making the two different polymer formulations complementary in their application. The imprinting in the OMNiMIP appears to be unaffected by a mixture of templates, as long as the capacity of the functional monomer is not overtaxed and the templates do not interfere with each other. Furthermore, imprinting two analytes in a mixture simultaneously was found to provide better performance versus physically mixing the particles from two templates imprinted separately. Mixed particle systems may only be an advantage in cases where templates interact with each other, and cannot be imprinted simultaneously. An even greater improvement is anticipated for the multiple-template imprinting of three or more templates, versus mixing particles from three or more imprinted polymers which should cause a greater decrease in selectivity due to increased analyte remixing effects during chromatography. In addition, this is the first report of mixing imprinted polymers with non-imprinted polymers; and while relevant to this study, this procedure appears to be particularly detrimental toward loss of selectivity and not viable in practice. It can be concluded from the

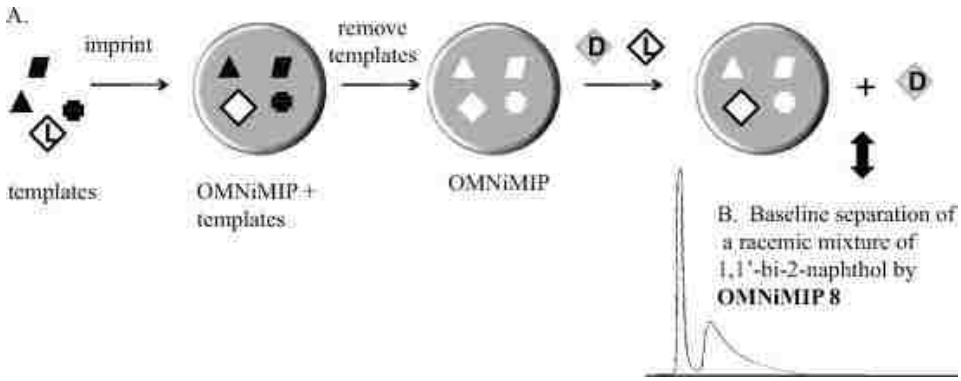
results that imprinting mixtures of templates simultaneously is the best method for producing multi-analyte molecular recognition in imprinted polymers.

Part 2: Analyte Separation by OMNiMIPs Imprinted with Multiple Templates*

3.7 Introduction

The formation of polymer materials in the presence of a template is a method for creating polymers that have a bias toward rebinding of the template molecule versus other compounds. Most often referred to as molecularly imprinted polymers (MIPs), these materials are useful in the fields of separations, sensors, assays, and catalysis.[2, 3, 23] Usually a single template in pure form is imprinted for molecular recognition of that template alone, striving for low cross-selectivity with other molecules. However, in some cases cross-selectivity may be useful for particular applications. For example, chromatographic protocols (solid phase extraction, HPLC, etc.) by MIPs may target one of a family of compounds.[6-8, 24-26] Furthermore, cross-selectivity by a MIP is useful for application to non-imprinted molecules with similar features as the template, extending its utility beyond molecular recognition of the template.[27] An interesting third example utilizes MIPs for development of drug targets related to the template.[28-31]

However, for some applications it would be of use to create a material that can bind to a variety of molecular targets with unrelated structures. Researchers in molecular imprinting have achieved this by imprinting more than one template simultaneously (**Scheme 3.3**). In most examples of imprinting multiple templates, the binding affinity and selectivity for each analyte is significantly reduced in comparison to the corresponding single template imprinted polymer; although in some cases the binding properties are comparable.[12] There has also been an interesting report of improved selectivity by a multi-templated imprinted polymer versus the singly imprinted MIP.[10]



Scheme 3.3. Illustration of enantioselective binding by an OMNiMIP imprinted with multiple templates. *(Reproduced with permission from Biosensors and Bioelectronics 25 (2009) 604–608)

Recently we have reported a study on multi-analyte imprinted polymers that compared the performance of traditionally formulated ethyleneglycol dimethacrylate-co-methacrylic acid (EDGMA-co-MAA) with MIPs formed from a single crosslinking monomer.[32] Imprinted polymers formulated with a single crosslinker have the acronym OMNiMIPs, which stands for *O*ne *MoN*omer *M*olecularly *I*mprinted *P*olymers, and have been shown to have enhanced binding and selectivity versus traditional (EDGMA-co-MAA) MIPs. To date, the best crosslinking monomer found for OMNiMIPs is N,O-bismethacryloyl ethanolamine often referred to as NOBE.[33] OMNiMIPs made with NOBE that have been imprinted with a single enantiomer of a wide scope of compounds showed significantly enhanced enantioselectivity versus EDGMA-co-MAA MIPs in all cases, except for templates functionalized with amine groups. This improved performance was also seen for OMNiMIPs imprinted with two templates. A further interesting aspect of simultaneously imprinting the two templates was that the performance of this multi-analyte imprinted polymer showed significantly better selectivity than physically mixing the particles of the singly imprinted polymers (maintaining equal amounts of template-imprinted

sites). This highlights the importance of multi-analyte imprinting for materials capable of recognizing multiple target molecules.

From previous studies, it was found that the binding capacity of OMNiMIPs increases with increasing amounts of template until approximately 20–25 mol% template loading with respect to the monomer.[20] Template loadings greater than 25 mol% resulted in gradual loss of binding capacity. Thus, the binding capacity is maximized at 25 mol% template loading, where the ratio of monomer to template is 3:1; i.e. there are three NOBE monomers available to interact with each template molecule. This suggests that the highest affinity sites require approximately three monomers surrounding the template molecule; indicating that as the number of monomers surrounding the template decreases from three, the molecular recognition correspondingly decreases. This is significant because, previous studies on multi-analyte imprinting in OMNiMIPs were carried out below the maximum template loading. Thus, simultaneous imprinting using 10% of each of two templates performed nearly the same as singly imprinted polymers imprinted with 10 mol% of either template. The next step was to test the effects on imprinting if template loading is increased higher than 25 mol% for a multi-analyte imprinted polymer. To carry out this study, four different templates were imprinted simultaneously, each with a template loading of 10 mol%. With a total template loading of 40 mol%, it was anticipated that the binding performance would decrease because the capacity for rebinding sites has a maximum quantity in the range of 20–25%.

3.8 Project Goals

The goals of this project were:

- Determine the ability of NOBE to imprint up to four templates simultaneously.

- To push the template loading limit of NOBE up to 40 mol%.

3.9. Materials and Methods

3.9.1. Materials

All templates and their enantiomers were purchased from Sigma-Aldrich and used without further purification. HPLC grade solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed using silica gel, 32–63 μm from Science Adsorbents Inc. *N*,*O*-bis(methacryloyl)ethanolamine (NOBE), was synthesized according to literature procedure.

3.9.2. Polymer Preparation

Polymers were made using the One Monomer Molecular Imprinted Polymer (OMNiMIP) method.[19]The polymers made from NOBE were either imprinted individually with BOC-L-tyrosine (BOC-tyr), (R)-(+)-1, 1′-Bi-2-naphthol (Binol), CBZ-L-tryptophan (CBZ-trp), CBZ-L-serine (CBZ-ser); or various mixtures of all four. The NOBE polymers imprinted with each individual template were synthesized as follows: BOC-L-tyrosine (0.287 g, 0.00102 mol, 0.1 eq.) or (R)-(+)-1, 1′-Bi-2-naphthol (0.292 g, 0.00102 mol, 0.1 eq.) or CBZ-L-tryptophan (0.345 g, 0.00102 mol, 0.1 eq.) or CBZ-L-serine (0.244 g, 0.00102 mol, 0.1 eq) was dissolved in dry acetonitrile (3.0 mL). To the dissolved template was added NOBE (2.00 g, 0.0102 mol, 1 eq.), then azobis-isobutyronitrile (AIBN; 0.033 g, 0.000204 mol, 0.02 eq.). The multi-analyte imprinted polymers were prepared by first combining 0.00102 mol of each template, dissolved in 3.0 mL total of dry acetonitrile. As before, NOBE (2.00 g, 0.0102 mol, 1 eq.) was added, followed by AIBN (0.033 g, 0.000204 mol, 0.02 eq.). The pre-polymerization solution for each formulation was transferred via pipette into 13 × 100 mm screw top test tubes, purged with

nitrogen gas for 5 min, capped and sealed with Teflon tape and parafilm. The test tubes were inserted into a photochemical reactor maintained at a constant temperature of 20 °C. A standard laboratory ultraviolet light source (medium pressure 450 W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed into the photoreactor, and the solutions were then photopolymerized for 8 h at 20 °C. The template was removed from the imprinted polymers by Soxhlet extraction with methanol for 48 h. The polymers were ground with a mortar and pestle, using U.S.A. Standard Testing Sieves to collect particles with diameters between 25 and 37 μm.

3.9.3. Chromatographic Evaluations

The OMNiMIP particles were slurry packed using a HPLC solvent delivery module into stainless steel columns (length 100 mm; inner diameter 2.1 mm) to full volume for chromatographic experiments. After packing, the columns were equilibrated on line for 12 h using acetonitrile—acetic acid (99:1, v/v) at a flow rate of 0.100 mL/min to remove any remaining template. HPLC analyses were performed isocratically at room temperature (21 °C) using a Hitachi L-7400 UV Detector and L-7100 pump. The flow rate was set at 0.1 mL/min using acetonitrile/acetic acid (99:1, v/v) as a mobile phase. The racemic substrates (0.5 mM each enantiomer) were dissolved in HPLC grade acetonitrile for injection onto the column, and detected at a wavelength of 260 nm to determine separation factors. Various mixtures of some or all of the templates were also injected on the HPLC to determine resolution of the different analytes. For all imprinted polymers and mixtures, the separation factor, α, was measured as a ratio of capacity factors k'_L/k'_D of the two enantiomers, with k' being determined by the following relation: $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the imprinted/non-imprinted substrate

and t_0 is the retention time of the void volume. The void volume was determined using acetone as an inert substrate.

3.10. Results and Discussion

3.10.1. Evaluation of Enantioselective Performance for Multi-analyte Imprinted Polymers

The best measure of the imprinting effect is enantioselectivity because the properties of both enantiomers are the same, except for their three-dimensional orientation in space. MIPs can create complementary three-dimensional binding sites that bind only one enantiomer through the formation of shape selective cavities and pre-organization of interactive groups within the binding cavity. The majority of MIPs are to single templates, with the objective of separating the template (as the analyte) from all other analytes. If a different template is imprinted in another polymer, rebinding of that template will be specific versus other analytes. However, imprinting the two templates simultaneously does not guarantee a MIP capable of separating both analytes. This is not due to a lack of imprinting of the templates; the MIP merely creates sites that preferentially bind each of the templates. On the other hand, the magnitude of rebinding depends on many factors that are not affected by the imprinting process; e.g. the strength of the functional monomer-template complex, contributions of non-specific effects to overall binding, the binding site heterogeneity, etc.[26] What can be expected from a polymer imprinted with two templates is that both analytes can be removed simultaneously from a more complex mixture. It should also be noted that as the number of templates simultaneously imprinted increases, the chances for similar chromatographic retention increases. Thus, enantioselectivity is a better assessment of the imprinting effect than evaluation of the MIPs ability to separate the different templated compounds.

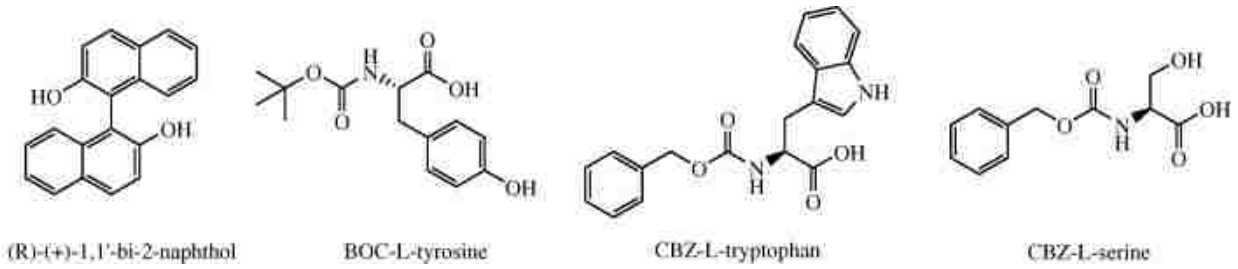


Figure. 3.3. Templates used for formation of multi-analyte OMNiMIPs.

For this study, four different templates (**Figure 3.3**) were chosen from earlier studies showed the best binding and selectivity properties in OMNiMIPs. Each template was imprinted singly and compared to multi-analyte imprinted polymers formed with two, three, and all four of the templates simultaneously. All the multi-analyte imprinted polymers incorporated Binol, the template exhibiting the best selectivity in a singly imprinted polymer. Multi-analyte MIPs with two templates included tyrosine for one, and tryptophan for the other; both have shown comparable selectivity that is slightly less than that of Binol. One triply imprinted polymer was formed with Binol, BOC-tyr, and CBZ-trp; and the last MIP formed from all four templates where CBZ-ser had previously shown the lowest (but good) selectivity in a singly imprinted polymer. The imprinting effect for all the imprinted polymers was first evaluated by comparison of enantioselectivity of each of the templates as analytes on the different polymers, rather than separation of each of the templates from another. The enantioselective separation values (α) determined by chromatographic retention studies are shown in **Table 3.3**. Binding for each analyte on its own imprinted polymer showed comparable results to similar studies previously reported.[19] However, changes emerge for the multiple imprinted polymers. Focusing on Binol, when this template is imprinted with another template such as BOC-tyr or CBZ-trp there is a negligible difference in the imprinting effect of either template, reflected in the alpha values.

This also is comparable to earlier studies, where the total amount of template imprinted was below 25 mol%.

However, when 30 mol% of templates are imprinted (i.e. 10 mol% of three different templates), there is a significant drop in enantioselectivity (28%). Furthermore, when 40 mol% of templates are imprinted (i.e. 10 mol% of four different templates), there is a larger decrease in the imprinting effect (37%). This indicates that once the total template concentration is greater than the optimal imprinting capacity of 25%, there is a steady decrease in effective imprinting.

Table 3.3. Comparison of separation factors for OMNiMIPs that imprint different sets of templates.

OMNiMIP	Templates imprinted in OMNiMIP	(R)-(+)-1,1'-bi-2-naphthol	BOC-L-tyrosine	CBZ-L-tryptophan	CBZ-L-serine
1	(R)-(+)-1,1'-bi-2-naphthol (10mol%)	8.5	–[a]	–[a]	–[a]
2	BOC-L-tyrosine (10mol%)	–[a]	4.1	–[a]	–[a]
3	CBZ-L-tryptophan (10 mol%)	–[a]	–[a]	3.9	
4	CBZ-L-serine (10 mol%)	–[a]	–[a]	–[a]	2.3
5	1. (R)-(+)-1,1'-bi-2-naphthol (10mol%) 2. BOC-l-tyrosine (10mol%)	8.2	3.5	–[a]	–[a]
6	1. (R)-(+)-1,1'-bi-2-naphthol (10mol%) 2. CBZ-l-tryptophan (10mol%)	8.3	–[a]	2.8	–[a]
7	1. (R)-(+)-1,1_-bi-2-naphthol (10mol%) 2. BOC-l-tyrosine (10mol%) 3. CBZ-l-tryptophan (10mol%)	6.0	3.1	2.3	–[a]

Table 3.3 Continued

8	1. (R)-(+)-1,1_-bi-2-naphthol (10mol%) 2. BOC-l-tyrosine (10mol%) 3. CBZ-l-tryptophan (10mol%) 4. CBZ-l-serine (10mol%)	3.7	2.9	2.3	2.3
[a] Enantioselectivity was not determined for these compounds.					

Similar results are seen for BOC-tyr and CBZ-trp, where enantioselectivity steadily decreases as greater amounts of template are added. However, the impact in the imprinting effect for CBZ-ser was not severe, even in the MIP formed with 40 mol% of combined templates. Before conducting this study, two opposing effects were hypothesized to occur when overloading the imprinting capacity of OMNiMIPs using multiple templates. The first possibility entertained the idea of one of the templates dominating interactions with the NOBE monomer, maintaining the imprint efficiency for that template, while the other templates suffer reduced imprint selectivity. The second possibility was that all templates experience loss of imprinting efficiency due to a reduced number of monomer molecules available to imprint each template molecule. **Table 3.3** reveals that loss of selectivity occurs for nearly all analytes as the imprinted polymer is imprinted beyond optimal capacity with multiple templates, instead of a single template maintaining its imprinting efficiency at the expense of the other templates. It is also interesting to note that while Binol provided the best singly imprinted polymer, it also showed the greatest loss in imprint efficiency as more templates were added beyond the optimal capacity of the polymer (**Figure 3.4**); the opposite appears true for serine.

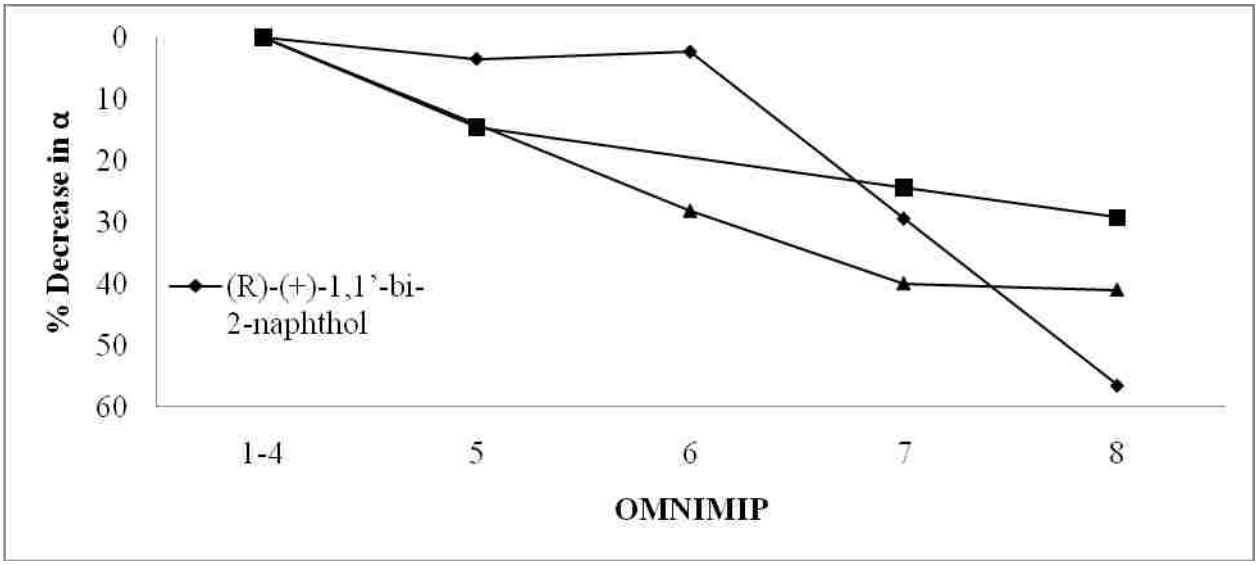


Figure 3.4. Loss of enantioselectivity for each analyte as the amount and number of templates is increased.

3.11. Evaluation of Overall Binding by Multi-analyte Imprinted Polymers

The overall performance of multi-analyte imprinting is best represented by **Figure 3.5**, which shows the results for chromatographic analysis of all eight enantiomers of the four compounds surveyed in this study. The first peak in **Figure 3.5** represents the "D" enantiomers of all four compounds, while the second peak represents the "L" enantiomers of all four compounds. It is not surprising that the group of "L" enantiomers elute at similar times, because the imprinting method generally does not directly control the relative retention of the imprinted templates. Instead, molecular imprinting provides retention of the imprinted species versus non-imprinted species; thus, resolution can only be expected between imprinted and non-imprinted species. It would not be anticipated that imprinted templates would have significant separation unless the templates themselves bound the monomers with substantially different binding energies. This effect is not under the control of the imprinting process, just a coincidence of template selection.

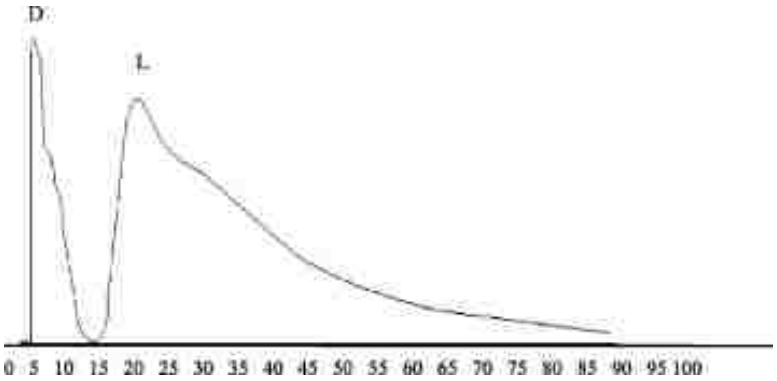


Figure 3.5. Elution profile of all eight enantiomers of the compounds shown in Figure 3.3.

However, it is clear that the multiple template OMNiMIP 8 is in fact capable of separating one of the imprinted family of compounds from a mixture of those and other compounds, *including the enantiomers of the imprinted templates* (**Figure 3.3**).

3.12. Effect of Multi-analyte Imprinting on Porosity and Surface Area

It was anticipated that increasing the percent template would affect the morphology of the OMNiMIP materials; for example, surface area and porosity could change as the template concentration increased. Looking at **Table 3.4**, there is an increasing trend in the total pore volume as the concentration of template increases from 10% for OMNiMIPs 1–4, 20% for OMNiMIPs 5–6, 30% for OMNiMIP 7, and 40% for OMNiMIP 8. The entries for OMNiMIPs 1–4 and 5–6 appear to depend only on template concentration, indicating that changes are independent of the molecular structure of the template, or that the templates are very similar in polarity and functional groups. Part of the porosity of the imprinted materials originates from the cavity left by the template after removal; and as the template is increased, more cavities lead to an increase of pores as well as increase in surface area. There is also a rise in the average pore diameter as the template concentration increases which could be due to template aggregation giving larger cavities, or changes in the polarity of pre-polymer solution which can affect the

phase separation kinetics leading to changes in porosity. Overall, the imprinted polymers show gradual trends in porosity and surface area that reflect the concentration of the templates and the formation of imprinted binding site cavities.

Table 3.4. Effects of different template loadings on surface area and porosity for multi-analyte OMNiMIPs.

	Total pore volume [a] (mL/g)	Surface area[b] (m^2/g)	Average pore size[c] (Å)
OMNiMIP 1–4[d]	0.187	50	170
OMNiMIP 5	0.199	54	225
OMNiMIP 6	0.200	54	226
OMNiMIP 7	0.211	57	232
OMNiMIP 8	0.226	60	251

[a] BJH cumulative adsorption pore volume.
[b] Determined using the BET model.
[c] BJH average pore diameter.
[d] Average results of OMNiMIPs 1–4.

3.13. Conclusions

OMNiMIPs have been shown to have higher binding capacities than traditionally formulated MIPs. The increase in binding capacity (i.e. the number of binding sites) comes from the increase in template loading that is possible now that the functional monomer is also the crosslinker. The four templates used in this analysis displayed the best imprinting performance, determined in an earlier study[19] the following order of highest imprinting effect to lowest: 1. (R)-(+)-1,1′-bi-2-naphthol; 2. BOC-L-tyrosine; 3. CBZ-L-tryptophan; 4. CBZ-L-serine (shown in **Figure 3.3**). Initial results previously published showed only minimal to modest differences in the imprinting effect between singly imprinted and multiple imprinted polymers when the cumulative template loading of multiple templates in the polymer remains below 20–25 mol%. The current study has replicated these results (OMNiMIPs 5 and 6), and further shown that increased loading of multiple templates past this range results in significant lowering of the imprinting effect. **Figure 3.4** shows the greatest losses in enantioselectivity for (R)-(+)-1,1′-bi-2-

naphthol by OMNiMIPs 7 and 8; while the decreases in imprinting effect for BOC-L-tyrosine and CBZ-L-tryptophan are less severe. Overall, it appears that compounds which show better enantioselectivity in singly imprinted polymers will continue to show better performance in multi-analyte imprinted polymers, as indicated in **Table 3.3**. However, once the combined template concentration surpasses a critical template loading, what was once the best binding template ((R)-(+)-1,1′-bi-2-naphthol) suffers the greatest losses in imprinting effect.

3.14 References

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CHAPTER 4: CHIRAL EFFECTS OF ALKYL-SUBSTITUTED DERIVATIVES OF *N,O*-BISMETHACRYLOYL ETHANOLAMINE ON THE PERFORMANCE OF ONE MONOMER MOLECULARLY IMPRINTED POLYMERS (OMNiMIPs)*

4.1 Introduction

Molecularly imprinted polymers (MIPs) are analytical materials that have widespread use for applications in separations and sensors.[1-3] Improvements in MIP methodology are continuing to progress, including efforts toward new formats[4], new applications[5], and new materials[6,7]. Our group has long been involved with the development of novel materials for molecular imprinting, in particular in the development of novel crosslinking monomers.[8-10] One of the early findings in our group was that molecular recognition in MIPs is enhanced when the functional groups interacting with the template molecule are part of the crosslinking monomer.[11] During these investigations, we discovered a much simpler approach to MIP formation which utilizes a single crosslinking monomer, *N,O*-bismethacryloyl ethanolamine (NOBE, **1**), in addition to template, solvent, and initiator (**Scheme 4.1**).[12,13] We refer to these materials as **o**ne **m**o**n**omer **m**olecularly **i**mprinted **p**olymers (OMNiMIPs). This approach eliminates several complications that typically occur from the use of multiple functional monomers and crosslinkers, such as:

– what type of functional monomers to use

– how many functional monomers to use

– how much of each functional monomer to use

– what type of crosslinker to use

– the ratio of functional monomer/crosslinker

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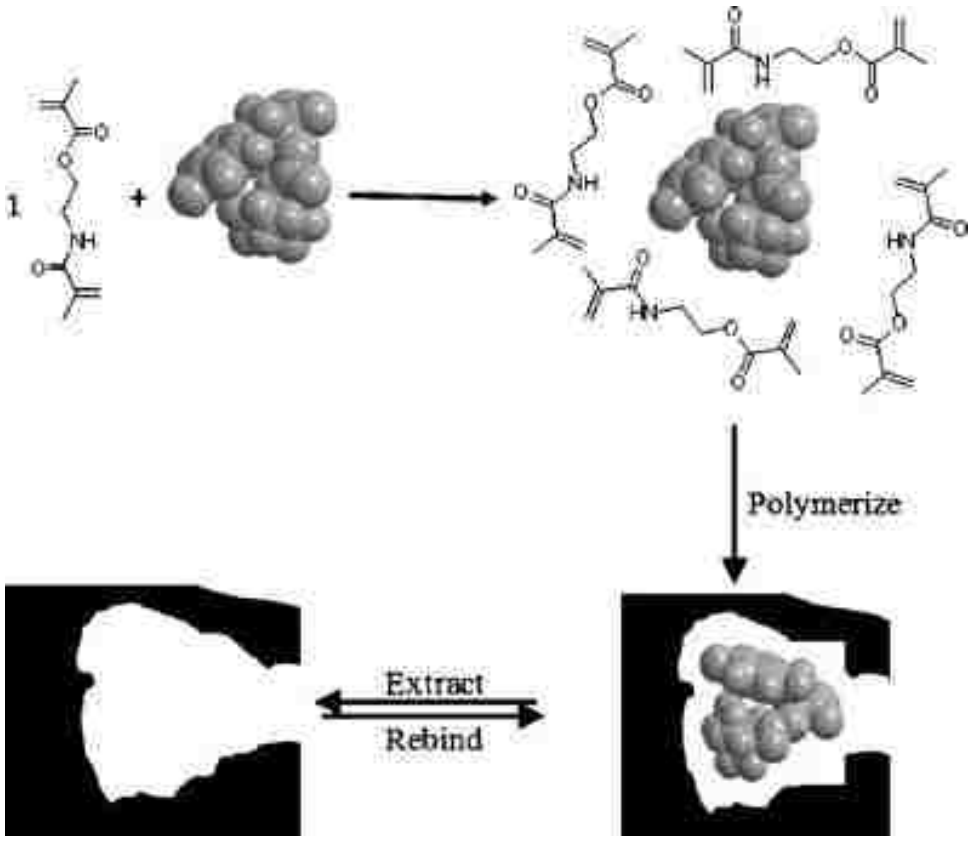


Scheme 4.1. Outline of the simple OMNiMIP imprinting strategy using BOC-L-tyrosine as template

In addition to the elimination of the above variables that make molecular imprinting difficult, the general performance of the OMNiMIP materials using NOBE was found to be superior over the traditionally employed methacrylic acid/ethyleneglycol dimethacrylate MIPs. It is anticipated that the simple OMNiMIP methodology may become the standard for MIP fabrication, providing a reliable and easy method for important bioanalytical applications. While NOBE has been identified as a useful lead compound, the performance of the OMNiMIP strategy may be further optimized by the development of new and better crosslinkers. Therefore, initial studies toward further functionalization of NOBE, and the impact on MIP performance, are reported here.

4.2 Project Goals

The goals of this project were:

- To synthesize and analyze chiral derivatives of NOBE containing varying steric side chains.
- To analyze to possibility of racemic imprinting using the novel chiral monomers.

4.3 Experimental

4.3.1 General

Unless otherwise indicated, chemicals were purchased from Aldrich and used without further purification. Solvents were obtained from commercial suppliers and used as received. Reactions under anhydrous conditions were performed in dry glassware under N_2 atmosphere. Reactions were monitored by thin-layer chromatography using 0.25 mm Macherey–Nagel silicagel glass plates (60F-254) with fractions being visualized by UV light. Column chromatography was carried out with flash silica gel, 32–63 μm from Science Adsorbents Inc.

^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker DPX-250 spectrometer for compounds dissolved in $CDCl_3$ unless otherwise. Chemical shifts (δ) are given in ppm relative to $CDCl_3$ (7.24 ppm, ^{1}H; 77.00 ppm, ^{13}C) unless otherwise indicated. IR spectra were obtained as neat samples on a Nicolet AVATAR 320 FT-IR unless otherwise indicated. High-resolution mass spectra (HRMS) were obtained on a Finnigan MAT900 double sector instrument, under fast atom bombardment (FAB, liquid sims) ionization or electrospray ionization (EI). Imprinted polymerization was performed in a photochemical turntable reactor (ACE Glass Inc.), which was immersed in a constant-temperature bath. A standard laboratory UV light source (a Canrad–Hanovia medium pressure 450-W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed at the center of the turntable. HPLC columns were packed using a

Beckman 1108 solvent delivery module, into stainless steel columns (length 100 mm, i.d. 2.1 mm) to full volume for chromatographic experiments. HPLC analyses were performed isocratically at room temperature (21 °C) using an Hitachi L-7100 pump with an Hitachi L-7400 detector. Pore size measurements were obtained in a Quantachrome AUTOSORB-1 AS-1.

4.3.2 Monomer Synthesis

General All chemicals were purchased from Sigma–Aldrich and used without further purification, except for the amino acids which were purchased from Lancaster Synthesis. All solvents used were dried using a pur-solve system (a system that pushes the solvent through alumina canisters). The amino alcohols were each synthesized using previously described methods.[14-16] Two grams of each amino alcohol was then mixed with methacrylic acid (MAA) (2.5 eq) and 4-dimethylaminopyridine (DMAP) (0.2 eq) at 0 °C in 250 mL of dichloromethane (DCM) in a 500-mL round bottom flask, equipped with a magnetic stir bar for 15 min. Dicyclohexylcarbodiimide (DCC) was then added slowly, keeping the temperature below 5 °C. The temperature was then slowly increased to room temperature and the reaction was left to run under a nitrogen balloon for 48 h. The solution was then filtered and extracted (2×15 mL 0.5 N HCl and 4×15 mL 0.5 M $NaHCO_3$). The organic extract was then evaporated in a rotary evaporator and taken up in 40 mL of ethyl acetate. The product was further purified by flash chromatography (EtOAc/hexanes mixture).

2-Methacrylamidopropyl methacrylate (5) L-Alaninol (or D-alaninol) (2.073 mL) was added to DCM (300 mL) and cooled to 0 °C. To this solution MAA (11.3 mL) and DMAP (0.65 g) were added. After 10 mins, DCC (11 g) was added and the mixture was allowed to stir for 2 days. The DCU was filtered and the organic phase was extracted with 0.5 M HCl (4×200 mL) and a saturated solution of $NaHCO_3$ (8×200 mL), dried over $MgSO_4$, and the solvent evaporated

under vacuum to give a light yellow oil. The product was isolated by flash chromatography using EtOAc/hexanes (50:50) in a 75% yield: ^{1}H NMR ($CDCl_3$, 250 MHz): δ1.2 (3 H, t); 2.0 (6 H, d); 4.0 (1 H, m); 4.1 (2 H, d); 5.6 (1 H, s); 5.2–6.2 (4 H, s); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 17.2, 17.9, 19.5, 43.0, 71.2, 119, 125.5, 136, 142, 168, 169; FT-IR (cm^{-1}): 3,406.45 (broad), 2,970.5, 1,711.06, 1,664.15, 1,626.23, 1,363.27, 909.30, 733.92; HRMS (FAB) ($M+H^+$) calcd. 211.12, found 211.1187.

2-Methacrylamido-3-methylbutyl methacrylate (6) L-Valinol (2.16 mL) was added to DCM (300 mL) and cooled to 0 °C. To this solution MAA (8.77 mL) and DMAP (0.47 g) were added. After 10 min, DCC (8.0 g) was added and the mixture was allowed to stir for 2 days. The DCU was filtered and the organic phase was extracted with 0.5 M HCl (4 × 200 mL) and a saturated solution of $NaHCO_3$ (8 × 200 mL), dried over $MgSO_4$, and the solvent evaporated under vacuum to give a light yellow/orange oil. The product was isolated by flash chromatography using EtOAc/hexanes (50:50) in a 74% yield: ^{1}H NMR:δ 1.0 (6 H, d); 1.8 (1 H, m); 2.0 (6 H, t); 4.1 (1 H, m); 4.3 (2 H, m); 6.5 (1 H, s); 5.5–6.3 (4 H, s); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 18.1, 18.5, 19.1, 29.6, 53.4, 64.5, 119.2, 125.9, 135.8, 140.1, 167.4, 168.4; FT-IR (cm^{-1}): 3,350.81 (broad), 2,963.28, 1,717.95, 1,656.50, 1,621.56, 1,533.79, 1,454.1297.98, 1,168.39, 939.99; HRMS (FAB) ($M+H^+$) calcd. 239.15, found 239.1482.

2-Methacrylamido-4-methylpentyl methacrylate (7) L-Leucinol (2.16 mL) was added to DCM (300 mL) and cooled to 0 °C.To this solution MAA (8.77 mL) and DMAP (0.47 g) were added. After 10 min, DCC (8.0 g) was added and the mixture was allowed to stir for 2 days. The DCU was filtered and the organic phase was extracted with 0.5 M HCl (4 × 200 mL) and a saturated solution of $NaHCO_3$ (8 × 200 mL), dried over $MgSO_4$, and the solvent evaporated under vacuum to give a yellow oil. The product was isolated by flash chromatography using EtOAc/hexanes

(50:50) in a 74% yield: ^{1}H NMR: δ 1.2 (6 H, d); 1.7 (1 H, t); 2.1 (2 H, t); 2.2 (6 H, s); 4.2 (1 H, d); 4.4 (1 H, m); 5.49–6.15 (4 H, s); 6.1 (1 H, s); ^{13}C NMR ($CDCl_3$, 62.5 MHz): δ 17.8, 18.2, 18.5, 22.2, 22.8, 24.8, 40.7, 46.7, 66.3, 119.2, 125.9, 128.9, 135.8, 140.1, 167.3, 168.0; FT-IR (cm^{-1}): 3,320.64 (broad), 2,957.06, 1,784.28, 1,720.69, 1,656.43, 1,620.06, 1,532.73, 1,296.83, 1,168.98, 1,052.18, 939.78; HRMS (FAB) ($M+H^+$) calcd. 253.17, found 253.1698.

4.3.3 Polymer Preparation

The following procedure was used for imprinted polymers employing the new crosslinking monomers. In a 13 × 100-mm test tube, BOC-L-tyrosine or BOC-D-tyrosine (5 mol %) was dissolved in 3.0 mL of MeCN. To this solution was added 2 g of monomer, and AIBN (1 mol%). The solution was purged by bubbling nitrogen gas into the mixture for 5 min, then capped and sealed with Teflon tape and Parafilm. The samples were inserted into a photochemical reactor, which was immersed in a constant-temperature bath. A standard laboratory UV light source (medium pressure 450-W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed at the center of the turntable. The polymerization was initiated photochemically at 20 °C and allowed to proceed for 8 h, while the temperature was maintained by both the cooling jacket surrounding the lamp and the constant-temperature bath holding the entire apparatus.

4.3.4 Quantification of Extracted Template

A 20-mL aliquot of each Soxhlet extraction solution from OMNiMIP5–7 (total extraction volume 300 mL) was removed and evaporated to dryness. The resulting solid material was then weighed and 5 mg (approximately 1% of the total weight of solids) of the material was dissolved in $CDCl_3$. To the resulting solution was added 0.05 mL of CH_2Cl_2. ^{1}H NMR was used to calculate the relative areas of the signal corresponding to BOC (9H) with the signal for CH_2Cl_2

(2H) as the basis for calculating the total moles of BOC-L-tyrosine in the NMR sample. This value was multiplied by 1,500 for the total moles of BOC-L-tyrosine in the original 300-mL extract.

4.3.5 Chromatographic Evaluations

Removal of the template was achieved by Soxhlet extraction with MeOH for 48 h. The polymers were then ground using a mortar and pestle, the particles were sized using USA Standard Testing Sieves, and the fraction between 25 and 37 μm was collected. The particles were slurry packed, using a solvent delivery module, into stainless steel columns (length 100 mm, i.d. 2.1 mm) to full volume for chromatographic experiments. The polymers were then equilibrated on-line for 12 h using MeCN/acetic acid (99:1) at a flow rate of 0.1 mL min^{-1} to remove any residual template. HPLC analyses were performed isocratically at room temperature (21 °C). The flow rate was set at 0.1 mL min^{-1} using MeCN/acetic acid (99:1) as mobile phase. The substrate concentration was 0.1 mM *t*-BOC-L-tyrosine and 0.1 mM *t*-BOC-D-tyrosine dissolved in MeCN, and detected at a wavelength of 260 nm. The void volume was determined using acetone as an inert substrate. The separation factors (α) were measured as the ratio of capacity factors $k' = k'_L / k'_D$. The capacity factors were determined by the relationship $k' = (V_t - V_0) / V_0$, where V_t is the retention volume of the substrate, and V_0 is the void volume.

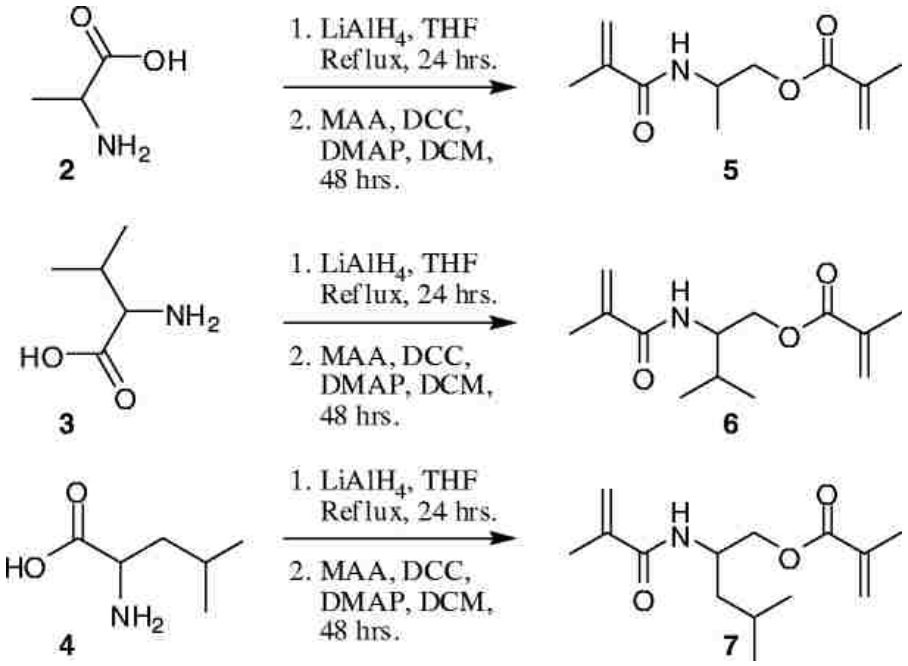
4.3.6 Porosity Measurements

A sample of polymer (350–500 mg) was degassed at 150 °C/3 h under vacuum. The adsorption and desorption isotherms were obtained using a 20-min equilibration time. Surface areas were determined according to the BET model, pore volumes and size distributions according to the BJH model.

4.4 Results and Discussion

4.4.1 Preparation of Monomers and Polymers

The series of NOBE derivatives initially investigated for improved MIP performance are shown in **Scheme 4.2**. These derivatives are easily obtained from the readily available amino acid starting materials, and provide changes to the imprinting matrix without any additional hydrogen-bonding interactions that would change the nature of the template binding. The smallest change is the addition of a single methyl group (compound 2), and compounds 3 and 4 systematically introduce larger substituents at the same position, for investigation of trends in the effects of sterics (and possibly hydrophobicity) on the performance of polymers formed by the OMNiMIP method. For the synthesis of the monomers, the overall conversion of the amino acids to the crosslinking monomers is shown in **Scheme 4.2**. In the first step, each of the amino acids 2–4 was reduced to the corresponding amino-alcohol; which was subsequently coupled to two equivalents of methacrylic acid (MAA) to give the final crosslinkers 5–7.



Scheme 4.2. Scheme for the synthesis of the new monomers **5**–**7**

As described in the experimental section, the newly synthesized crosslinkers were utilized for imprinting BOC-L-tyrosine, which has been shown to create a highly enantioselective OMNiMIP.[12]

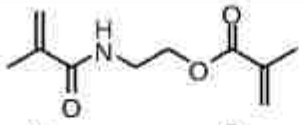
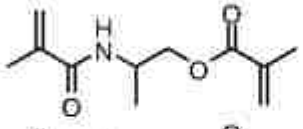
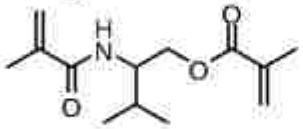
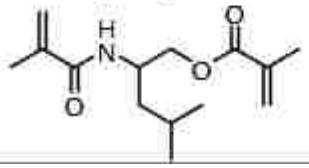
4.4.2 Chromatographic Binding Evaluation

Using BOC-L-tyrosine as the template, MIPs were fabricated using the new monomers according to the experimental protocols, and molecular recognition performance evaluated using HPLC. In addition to capacity factors, **Table 4.1** reports the separation factor for enantioselectivity of L- versus D-BOC-tyrosine on each of the imprinted polymers. Enantioselectivity (α) is used as the primary figure of merit in evaluating MIP materials, because there are no differences in partitioning effects between the enantiomers, and all selective recognition is the result of the imprinting effect. Therefore, enantioselectivity is the most direct measure of the imprinting effect. With NOBE as the first entry in **Table 4.1**, the subsequent monomer entries are arranged in order of increasing size of the substituent appended to central ethylene group.

Looking at the enantioselectivity values in **Table 4.1**, in comparison to NOBE only the alanine-derived monomer **5** performs at the same level of enantioselectivity. Chromatograms showing complete separation of enantiomers by OMNiMIP1 and OMNiMIP5 are shown in **Figure 4.1**. Thus, the addition of the methyl group in the case of the alanine-based monomer does not appear to have a deleterious effect on the performance of OMNiMIPs. On the other hand, OMNiMIPs made using the valine **6** and leucine **7** derived monomers showed little or no imprinting effect. The poor enantioselective performance seen for OMNiMIP3 and OMNiMIP4 (made using the valine **6** and leucine **7** monomers respectively) is likely due to the steric

blocking of necessary hydrogen bonding by the amide group of the monomers (and the corresponding imprinted polymers) to the template molecule. Without complexation of the imprinting monomers with the template molecule, imprinting cannot take place. This seems to be

Table 4.1. Binding and enantioselectivity comparison for MIPs imprinted with BOC-L-tyrosine using monomers **5**–**7** compared to NOBE (**1**), determined by HPLC[a]

Entry	Monomer	k'_L	k'_D	α
OMNiMIP1		9.17	2.48	3.7
OMNiMIP5-L[b]		5.40	1.42	3.8
OMNiMIP6-L[b]		2.31	2.31	1.0
OMNiMIP7-L[b]		1.09	0.91	1.2

[a]HPLC conditions: particle size 25–37 μm; column size 100 × 2.1 mm; mobile phase MeCN/acetic acid (99:1); analytes (1 mM Boc-L-tyrosine, 1 mM Boc-D-tyrosine, acetone (used to determine void volume)) were all detected at 270 nm; flow rate 0.1 mL min^{-1}; sample volume injected 5 μL[b] For this study, the L enantiomer of this monomer was used.

particularly the case for OMNiMIP7 which had very low capacity factors (shown in **Table 4.1**), indicating the polymer had difficulty binding the templates at all. Furthermore, monomer **6** was difficult to polymerize, and only approximately half of the monomer converted to the needed highly crosslinked polymer that was subsequently tested for chromatographic selectivity. This poor polymer conversion may also be responsible for the poor selectivity seen for OMNiMIP6 as a result of inadequate formation of binding sites.

While the enantioseparation of OMNiMIP5 and OMNiMIP1 are comparable, the additional methyl group on alanine-based crosslinker of OMNiMIP5 also provides chirality to

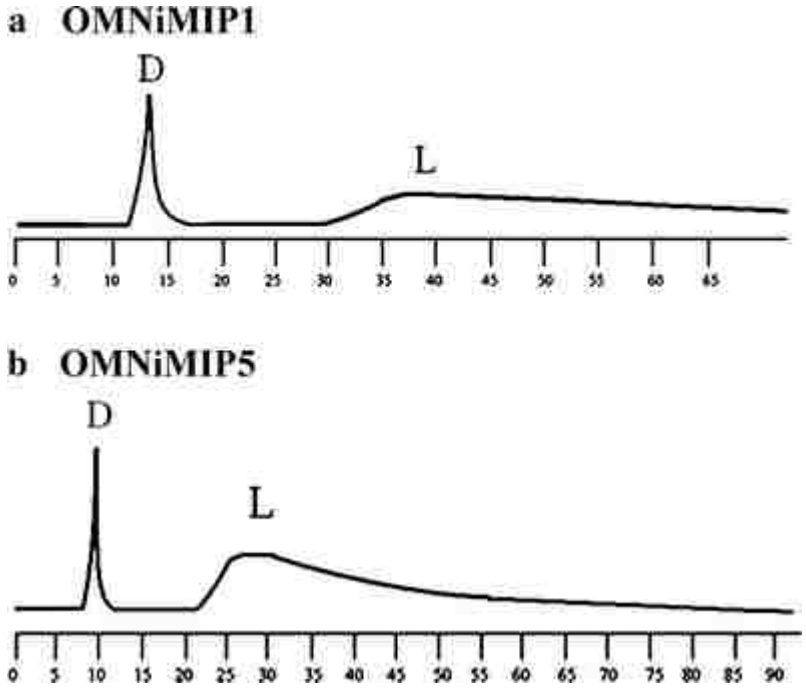


Figure 4.1 HPLC chromatograms for resolution of mixtures of D- and L-BOC-tyrosine on a column packed with OMNiMIP1 (**a**) compared to a column packed with OMNiMIP5 (**b**)

the imprinted polymer system. The influence of this chiral center toward the imprinting effect was investigated. Thus, monomer **5** was synthesized in both L and D forms, and each of these crosslinkers was imprinted in one case with BOC-L-tyrosine, and in the other case with BOC-D-tyrosine. Subsequently, each of these OMNiMIPs was evaluated chromatographically and the results shown in **Table 4.2**. The first observation of interest is that the diastereomeric pairs of crosslinking monomer with BOC-tyrosine do not form equivalent OMNiMIPs; instead, the enantioseparation performance is very different. For example, if the L enantiomer of monomer **5** is used to imprint BOC-L-tyrosine, the separation factor is significantly larger than if this monomer is used to imprint BOC-D-tyrosine. Thus, monomer–template combinations that are diastereomeric in the solution phase give rise to OMNiMIPs with significantly different performance. The control polymer in entry 5 shows that if no template is used, there is a negligible degree of enantioselectivity which indicates that the chiral backbone of the polymer

itself does not provide any enantioselectivity without the imprinting process. While a similar effect has been seen for molecularly imprinted polymers incorporating a chiral functional monomer[11,17], this is the first demonstration of this effect in OMNiMIPs and evaluation of all four diastereomeric pairs. One of the most surprising aspects of this study is that the relatively non-interactive methyl group of monomer **5** could induce such drastic effects.

Table 4.2 Capacity and separation factors for racemic mixtures of BOC-tyrosine on OMNiMIPs made using different stereoisomer combinations of crosslinker **5** with BOC-tyrosine as template[a]

Entry	OMNiMIP	Template	k'_L	k'_D	Separation factor (α)
1	OMNiMIP5-L	BOC-L-tyrosine	5.4	1.8	3.8
2	OMNiMIP5-L	BOC-D-tyrosine	2.1	4.6	2.3
3	OMNiMIP5-D	BOC-L-tyrosine	4.4	1.9	2.4
4	OMNiMIP5-D	BOC-D-tyrosine	2.0	7.3	3.6
5	OMNiMIP5-L	No Template	2.0	1.95	1.03

[a]HPLC conditions: particle size 25–37 μm; column size 100 × 2.1 mm; mobile phase MeCN/acetic acid (99:1); analytes (1 mM Boc-L-tyrosine, 1 mM Boc-D-tyrosine, acetone (used to determine void volume)) were all detected at 270 nm; flow rate 0.1 mL min^{-1}; sample volume injected 5 μL

The different binding behavior of OMNiMIPs originating from the different complexes may be may be due to differences in the concentration of the pre-polymer complex. An increase in pre-polymer complex for one diastereomer over the other would lead to a proportional increase in the number of enantioselective binding sites in the subsequently formed OMNiMIP, which would be expected to cause an increase in the apparent separation factor. If this were the case, then the difference in performance would be determined by solution-phase considerations. In addition, differences in enantioselectivity may arise from geometrical aspects of the chiral binding site that is formed during the polymerization. A second observation from **Table 4.2** is that enantiomeric pairs of crosslinking monomer with BOC-tyrosine appear to form equivalent OMNiMIPs. For example, if the L enantiomer of monomer **5** is used to imprint BOC-L-tyrosine, the separation factor is approximately the same as the D enantiomer of monomer **5** used to

imprint BOC-D-tyrosine. Thus, the overall effects of diastereomeric or enantiomeric pairs of monomers with template appear to be reversible.

Next, the performance of imprinting a racemic template mixture using the L enantiomer of crosslinker **5** was investigated. Without a chiral monomer, imprinting a racemic mixture is not anticipated to create differential binding sites for enantiomeric templates/analytes; thus, enantioseparation would not be possible. However, with the L-chiral crosslinker **5**, the separation factors in **Table 4.3** show that the resulting OMNiMIP was partially successful. The capacity factors of each of the pure enantiomers eluted one at a time, on the OMNiMIP prepared with racemic template, showed significant differences. This is illustrated in **Table 4.3** with an "effective separation factor" (α′) calculated from the ratio of the capacity factors for the pure enantiomers eluted one at a time. However, if a racemic mixture of the BOC-tyrosine is eluted on this same column, enantiomeric resolution is not obtained, and a single broad elution peak is obtained. In comparison, the peaks for the pure enantiomer analytes were considerably narrower, as shown in the Supplementary Material. The lack of resolution of a racemic mixture points to a small energetic difference between the diastereomeric complexes formed in the OMNiMIP as a result of low differentiation in the binding geometries in the polymer binding site; however, no inference can be made from this data on different numbers of binding sites.

Table 4.3 Capacity factor, apparent separation factor (α), and effective separation factor (α′) for enantiomers of BOC-tyrosine on OMNiMIP5-L imprinted with a racemic mixture of BOC-tyrosine[a]

Entry	Analyte	k'_L	k'_D	Separation factor
1	BOC-L-tyrosine	4.8		α′ = 2.4
2	BOC-D-tyrosine (racemic)		2.0	
3	BOC-tyrosine	12.0	12.0	α = 1.0

[a]HPLC conditions: particle size 25–37 μm; column size 100 × 2.1 mm; mobile phase,MeCN/acetic acid (99:1); analytes (1 mM Boc-L-tyrosine, 1 mM Boc-D-tyrosine, acetone (used to determine void volume)) were all detected at 270 nm; flow rate 0.1 mL min^{-1}; sample volume injected 5 μL.

Physical characterization of the OMNiMIPs made from compounds **1** (OMNiMIP1) and **5–7** (OMNiMIP5–OMNiMIP7) are shown in **Table 4.4**. Soxhlet extraction provided nearly quantitative removal of the template for each the polymers. Surface area and pore measurements for OMNiMIP5 are provided in **Table 4.4** for comparison with the corresponding data reported in the literature for OMNiMIP1. The surface area and average pore size appear to be similar for these two polymers; thus the additional methyl group on the monomer structure for OMNiMIP5 does not create any changes in the morphology of the polymer matrix. Because of the poor chromatographic performance of OMNiMIP6 and OMNiMIP7, surface data are not provided at this time.

Table 4.4 Physical characterization of final processed OMNiMIPs

	Amount of template extracted[b] (% of original template loading)	**Surface area[c] (m^2 g^{-1})**	**Average pore size[d] (Å)**	**Total pore volume[e] (mL g^{-1})**
OMNiMIP1[a]	85	45.8	166	0.191
OMNiMIP5	87	51.0	208	0.126
OMNiMIP6	94	----------	----------	----------
OMNiMIP7	92	----------	----------	----------

[a]Surface area and pore data acquired from ref. 12
[b]Quantitation by method described in Experimental
[c]Determined using the BET model on a seven-point linear plot
[d]BJH cumulative adsorption pore volume
[e]BJH average pore diameter

4.5 Conclusions

Much of the literature on the development of MIP materials has previously focused on the development of functional monomers for molecular imprinting. Recent work by our group has shown that functional monomers are more effective when incorporated into a crosslinking format.[11] This has led to a revolution in the development of crosslinking monomers for molecular imprinting, culminating in the OMNiMIP process. OMNiMIP methodology is sensitive to crosslinker design, which must act as the matrix and functional group interaction

with the templates simultaneously. Fortuitous discovery of the ability of compound 1 provided the first example of OMNiMIP methodology; however, crosslinker structure had not been optimized for the best binding and selective performance. The three new OMNiMIP crosslinkers reported herein comprise the first study on the effects of derivatization of compound 1. The alkyl groups in compounds 5–7 were chosen to correlate simple changes in the structure of 1 with the binding and selectivity performance of the imprinted polymers. It appears from this study that a small steric change, such as the addition of a methyl group to the central ethylene spacer group, does not inhibit the formation of selective imprinting sites. However, crosslinker derivatives with rather large substitutions create OMNiMIPs with poor binding and selectivity properties. This is probably a result of large groups near the adjacent amide, blocking necessary binding interactions with the amide group.

The addition of a chiral center afforded by OMNiMIP5 offers opportunities for enantioselectivity not available to the achiral OMNiMIP1 monomer. Enantiopure monomers were synthesized for imprinted polymers designated OMNiMIP5-L or OMNiMIP5-D, polymerized from the L or D form of monomer 5 respectively. Imprinting chiral templates using these monomers leads to diastereomeric complexes at the pre-polymer stage and in the final polymer. The data in **Table 4.2** appear to show that selectivity by OMNiMIPs in entries 1 and 2, using the L enantiomer of crosslinker 5 for imprinting L and D BOC-tyrosine respectively, arises from diastereomeric complexes, which result in different enantioselectivity values. On the other hand, entries 1 and 4 give roughly the same enantioselectivity values and appear to arise from enantiomeric complexes. Next, a racemic template mixture was imprinted in OMNiMIP5-L, and found to exhibit enantioselective factors if the analytes are eluted singularly; however, resolution is lost for elution of the racemic mixture. Last, morphological features of OMNiMIP5 and

OMNiMIP1 are similar, thus any differences in racemic imprinting do not arise from differences in macroscopic properties. Future work will investigate derivatives capable of improved enantioselectivity using crosslinker 5 as the new lead compound.

4.6 References

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CHAPTER 5. DESIGN AND SYNTHESIS OF CHIRAL NOBE ANALOGS WITH ADDITIONAL HYDROGEN BONDING INTERACTIONS

5.1 Introduction

One of the main limitations to full commercial use of molecularly imprinted polymers (MIPs) is the necessity of an enantiopure template. In traditional MIPs, several milligrams of the pure template must be used to create the enantioselective binding sites formed in imprinted materials.[1] Following the partial success of the chiral monomer studies shown in Chapter 4, a closer study the chiral center in the backbone of the cross-linking monomer was warranted. Also, the influence of whether chiral molecular recognition in MIPs can be improved by addition of hydrogen bonding functionality in the chiral cross-linker will be analyzed. The improved hydrogen bonding capabilities may allow for the development of a material that does not require a chiral pure template. An example, put forth by Mosbach and Lindner, of a chiral monomer (also referred to as a chiral selector) showed selective preference of one enantiomer when polymerized with a mixture of enantiomers.[2] However, this was only achieved with certain templates that are known to have high selectivity for binding to only one form of the enantiomer. Other earlier examples required strong reversible covalent type interactions (i.e. boron ester formation) to achieve separation from a mixture.[3] The development of novel chiral monomers with greater template affinities will reduce the limitations and drawbacks of current imprinted polymers and chiral selectors and allow a broader commercial use for MIPs.[4]

Several monomers (**Figure 5.2**) with varying hydrogen bonding abilities were developed and used in racemic (50/50 mixture of enantiomers) imprinting techniques. Hydrogen bonding in the cross-linker backbone has been shown to have a powerful influence on molecular recognition

by MIPs. This was described previously in literature by the unexpected reduction of non-selective binding interactions by a monomer (N,O-bismethacryloyl serine, NOS, **5.1**) containing

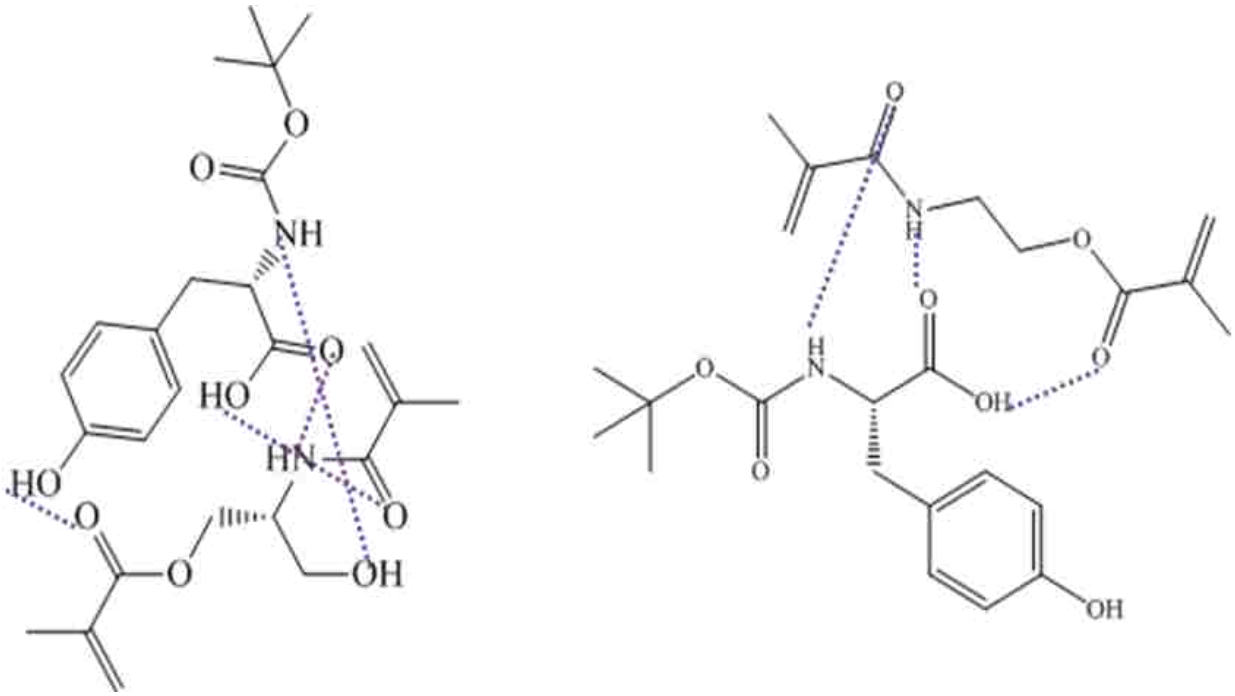
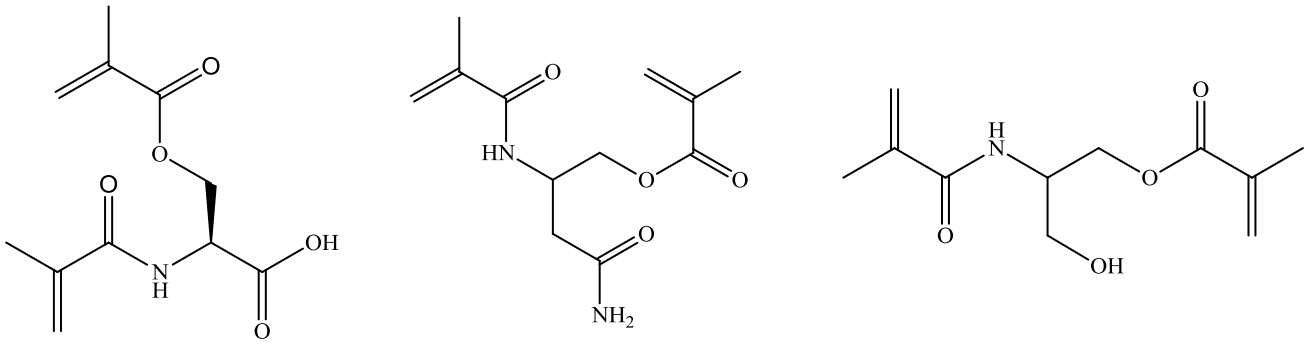


Figure 5.1. Illustration depicting the possible difference between NOBE and a cross-linker with additional hydrogen bonding functionalities when interacting with Boc-L-tyrosine.

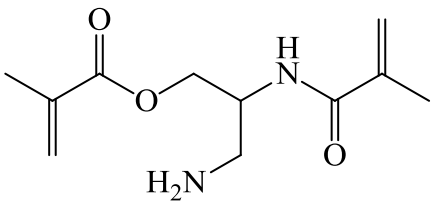
carboxylic acid functionality as part of the backbone.[5] Although the reasons for this are not fully understood, one possibility for this is that hydrogen-bonding interactions may provide fewer non-selective interactions (or less influential non-specific interactions) versus ionic monomers used for imprinting. Another possibility, depicted in **Figure 5.1**, is that the additional bonding sites can allow for stronger complexation in the pre-polymer complex, allowing for greater selective bonding of one enantiomer over the other.

The new designs for OMNiMIPs with additional hydrogen bonding, shown in **Figure 5.2**, were based on serine as seen in NOS as well as asparagine and glutamine. The synthetic steps

for transforming asparagines into the corresponding monomer, **5.2**, are shown in **Scheme 5.2**. The synthetic route



5.1 **5.2** **5.3**



5.4

Figure 5.2. New chiral functional cross-linking monomers containing additional hydrogen bonding functionality.

is similar to that for the chiral monomers shown in Chapter 4, although a milder reduction was employed for the transformation of the carboxylic acid to the corresponding alcohol in the first step.[6]

The next monomer design incorporates the hydroxyl group as the additional hydrogen bonding factor (monomer **5.3** in **Scheme 5.3**). The synthesis of this monomer thus far has given a low yield because of the lack of solubility of the starting materials.

The final monomer design attempted was similar to that of monomer **5.3**. Monomer (**5.4**),

but incorporated amine functionality in place of the hydroxyl group shown in monomer **5.3**. The addition of the amine functionality was expected to act as a complementary monomer to NOS. NOS has an acid functionality whereas monomer **5.4** contains a basic functionality. The synthesis of monomer **5.4** is shown in **Scheme 5.4**. NOS and monomer 5.4 can imprint opposite templates.

Overall, the additional interaction of the monomer and template due to the hydrogen bonding substituent (present in the new monomers) arising from the chiral center is believed to be the cause of stronger diastereomeric complexes in the pre-polymer complex. As was described in Chapter 4 and shown in Figure 5.1 the additional hydrogen bonding functionality will have the same effect as a chiral selector, but will not be limited to the select templates that will only match to certain chiral selectors. Thus, the ability of the corresponding polymer to selectively bind preferentially one enantiomer of many different chiral compounds will be enhanced. This capability will be a revolution in the field of imprinting.

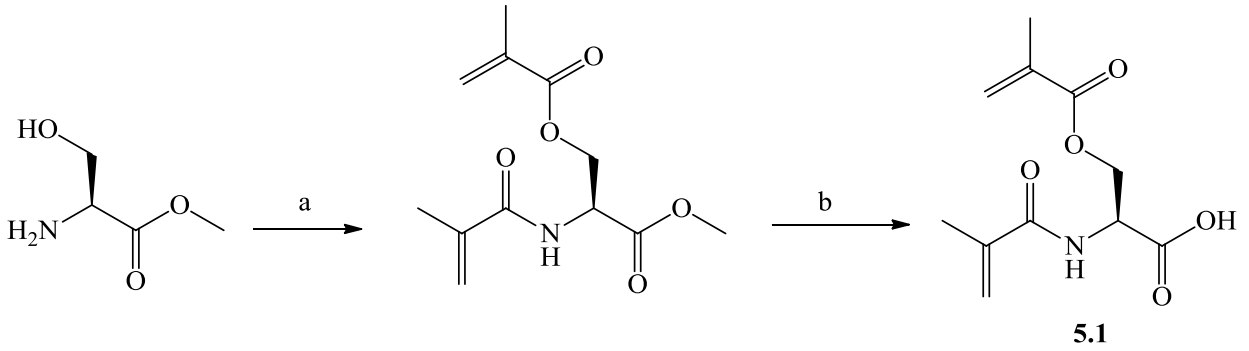
5.2 Project Goals

The goals of this project were:

- To synthesis chiral cross-linking monomers containing additional bonding capacity (H-bonding, ionic).
- To analyze the new monomers for the ability to achieve racemic imprinting.

5.3 Experimental

5.3.1 Synthesis of *N,O*-Bismethacryloyl, L-Serine (5.1)[5]



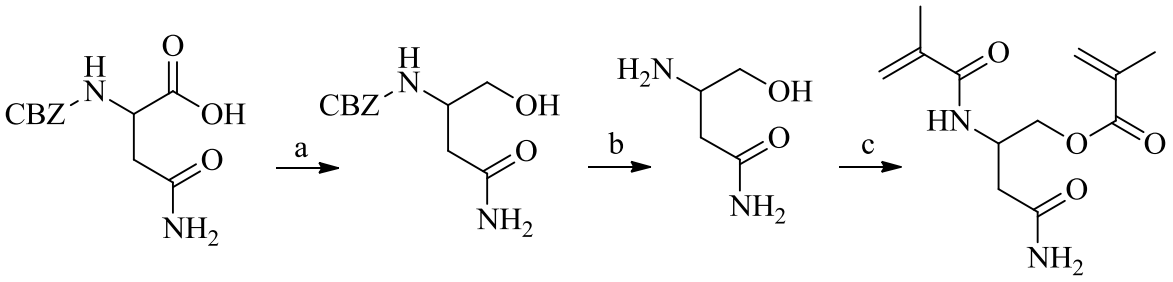
5.1

Scheme 5.1. Synthetic scheme for momomer 5.1. (a) MAA/Et_3N/DMAP/DCC/CH_2Cl_2, rt/5 d; PPL pH = 7.5 (PBS), rt/72 h.

N,O-Bismethacryloyl, L-Serine-Methyl Ester. L-Serine R-methyl ester hydrochloride (0.467 g, 3 mmol) was dissolved in dichloromethane (DCM) (15 mL) and cooled to 0 °C, followed by dropwise addition of Et_3N (0.607 g, 6 mmol). In another flask methacrylic acid (0.517 g, 6 mmol) and 4-dimethylaminopyridine (DMAP) (0.0733 g, 0.6 mmol) were dissolved in DCM (30 mL), and the resulting solution was cooled at 0 °C. To this flask was added the hydrochloride solution in one portion. After 5 min, N,N'-dicyclohexylcarbodiimide (DCC) (1.238 g, 6 mmol) was added to the cooled solution at 0 °C and stirred additional 30 min. After this period, the temperature was allowed to rise to room temperature and the reaction mixture was stirred 5 days. The DCU was filtered, the organic phase was extracted with 0.5 M $NaHCO_3$ (2 x 15 mL), 0.5 M sodium citrate (2 x 15 mL), dried over $MgSO_4$, and the solvent was evaporated under vacuum giving an orange oil. The product was isolated as a yellow oil by flash chromatography using EtOAc/hexanes 50/50 in 71% yield. 1H NMR (CDCl3, 250MHz): δ 6.67-6.70 (1H, d), 5.99 (1H, 4.80-4.87 (1H, m), 4.41-4.43 (2H, dd), 3.69 (3H, s), 1.88 (3H, s), 1.82 (3H, s).

N,O-Bismethacryloyl, L-Serine. In a 100 mL amber bottle with cap was dissolved *N,O*-bismethacryloyl, L-serine R-methyl ester, (0.334 g, 1.3 mmol), in acetone (5 mL) followed by the addition of 40 mL of 0.1 M phosphate buffer of pH 7.5. To this mixture porcine pancreatic lipase, EC 3.1.1.3 (100 mg), was added. The mixture was sonicated for 1 min and then shaken for 72 h at room temperature. The reaction mixture was acidified to pH 3.0 with 1.0 M HCl. The aqueous phase was extracted with EtOAc (3 x 20 mL), and the combined organic extracts were washed with water (2 x 20 mL). The organic phase was dried over $MgSO_4$ and the solvent evaporated under vacuum to give a yellow oil. The product was isolated by flash chromatography using only EtOAc to give an isolated yield of 61.8%. 1H NMR (CDCl3, 250 MHz): δ 10.68 (1H, s), 6.87-6.90 (1H, d), 6.04-6.05 (1H, d), 5.74-5.75 (1H, d), 5.54-5.55 (1H, d), 5.37-5.38 (1H, d), 4.85-4.91 (1H, m), 4.51-4.52 (2H, dd), 1.91 (3H, s), 1.85 (3H, s).

5.3.2 Synthesis of *N,O*-Bismethacryloyl L-asparagine (5.2)



5.2

Scheme 5.2. Synthetic steps for monomer **5.2**: (a) i. NMM ii. i-$BuCO_2Cl$. iii. $NaBH_4$/MeOH; THF, -10°C, N_2. (b) Pd/C, H_2, MeOH (c) $H_2C=C(CH_3)COCl/Et_3N/CH_2Cl_2$, 40ºC/24h.

N-Carbobenzoxy-L-asparaginol. To a stirred solution of the *N*-protected amino acid **5.2** (1.00g, 3.76 mmol) in dry THF (15 mL) at –10 ºC, *N*-methylmorpholine (0.334g, 3.3 mmol) was added followed by isobutyl chloroformate (0.451g, 3.3 mmol). After 10 min. $NaBH_4$ (0.34g, 9 mmol)

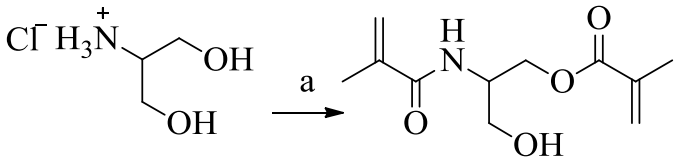
was added in one portion. Then dry MeOH (30 mL) was added dropwise to the mixture over a period of 10 min at 0°C. The solution was stirred for additional 10 min and then neutralized with 1N HCl (6 mL). The organic solvents were evaporated under reduced pressure and the product was extracted with EtOAc (3 x 21 mL). The organic phase was washed with 1N HCl (12 mL), H_2O (30 mL), 5% $NaHCO_3$ (15 mL), and H_2O (2 x 30 mL), dried over $MgSO_4$, and the solvent evaporated under reduced pressure. A light yellow oil was obtained, this was dissolved in EtOAc (15 mL) and then hexane (200 mL) was added. The mixture was allowed to stand at 0°C overnight to allow crystallization. Light crystal needles were formed and washed with hexane. 1H NMR ($CDCl_3$, 400 MHz): δ 8.03 (1H, s), 7.33-7.48 (5H, m), 7.16 (2H, s), 5.09 (1H, s), 3.90 (1H m,), 3.65 (1H s,), 3.50 (1H, d), 3.25 (1H, d), 2.52 (1H, d), 2.27 (1H d,). 13C NMR ($CDCl_3$, 100 MHz): δ 173.60, 155.61, 136.11, 128.90, 128.90, 127.66, 127.15, 127.15, 66.08, 65.21, 51.82, 33.18.

L-asparaginol. The amino alcohol **2.14** (1.26g, 4 mmol) was treated with 40 mL of 2M HCl in ethyl ether. The temperature was kept at 0 °C/6 h and then it was increased to room temperature and the reaction mixture was stirred for additional 18 hours. The excess of HCl and ether was evaporated first under a stream of N2 and then under vacuum. The residue, a white solid was filtered out, washed with ethyl ether (3 x 20 mL), and dried at room temperature. Yield 94%. 1H NMR ($CDCl_3$, 400 MHz): δ 7.16 (1H, s), 5.11 (2H, s), 3.65 (1 H , s) 3.50 (1H, d), 3.25 (1H, d), 2.52 (1H, d), 2.27 (1H, d). 13C NMR ($CDCl_3$, 100 MHz): δ 173.62, 67.85, 49.51, 35.92.

N,O-Bismethacryloyl L-asparaginol. L-asparaginol (0.5 g, 1.9 mmol) was dissolved in DCM (15 mL) and cooled to 0 °C, followed by drop wise addition of Et3N (0.607 g, 6 mmol). In another flask methacrylic acid (0.344 g, 4 mmol) and DMAP (0.0733 g, 0.6 mmol) were dissolved in

DCM (30 mL), and the resulting solution was cooled at 0 °C. To this flask the hydrochloride solution was added in one portion. After 5 min, DCC (0.824 g, 4 mmol) was added to the cooled solution at 0 °C and stirred for an additional 30 min. After this period, the temperature was allowed to rise to room temperature and the reaction mixture was stirred for 5 days. The DCU was filtered, the organic phase was extracted with 0.5 M $NaHCO_3$ (2 x15 mL), 0.5 M sodium citrate (2 x 15 mL), dried over $MgSO_4$, and the solvent was evaporated under vacuum giving an orange oil. The product was isolated as yellow oil by flash chromatography using EtOAc/hexanes 50/50 in 71% yield. 1H NMR ($CDCl_3$, 400 MHz): δ 8.03 (1H, s), 7.16 (2H, s), 6.48 (1H, s), 6.40 (1H, s), 5.79 (1H, s), 5.70 (1H, s), 4.50 (1H, d), 4.41 (1H, m), 4.25 (1H, d), 2.52 (1H, d), 2.28 (1H, d), 2.01 (3H, m), 1.98 (3H, m). 13C NMR ($CDCl_3$, 100 MHz): δ 173.62, 168.65, 167.25, 141.38, 136.00, 125.24, 118.17, 67.28, 47.45, 33.43, 19.62, 17.89.

5.3.3 Synthesis of *N,O*-Bismethacryloyl Serinol (5.3)



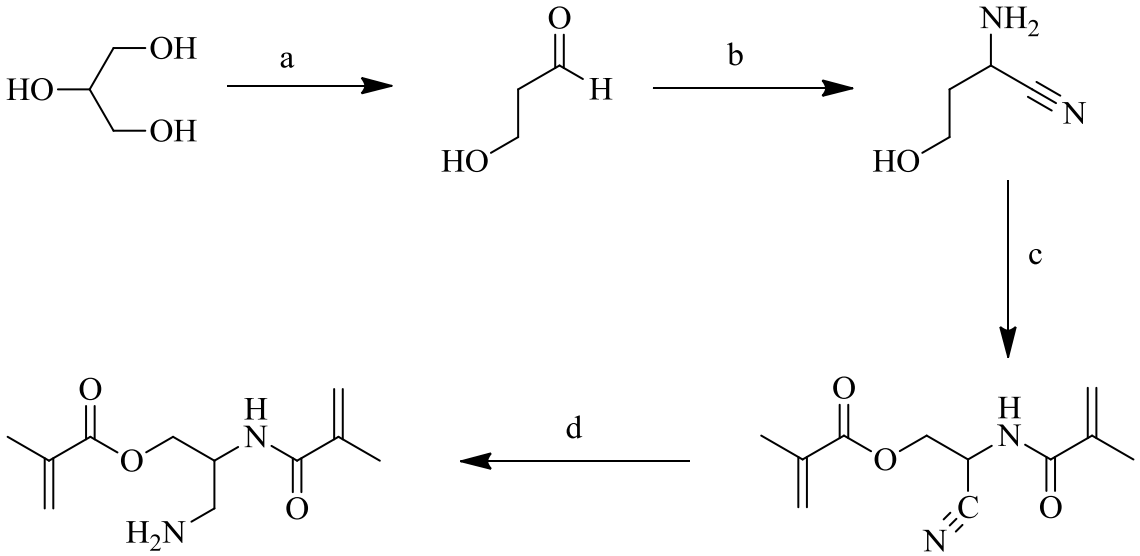
5.3

Scheme 5.3. Synthesis for monomer **5.5**: (a)$H_2C=C(CH_3)COCl/Et_3N$, THF/DMF, 50ºC/16h.

N,O-Bismethacryloyl serinol. Serinol (0.5 g, 5.4 mmol) was dissolved in THF/DCM (50/50) (15 mL) and cooled to 0 °C, followed by drop wise addition of Et_3N (0.607 g, 6 mmol). In another flask methacryloyl chloride (1.11 g, 10.8 mmol) was dissolved in THF/DCM (50/50) (30 mL), and the resulting solution was cooled at 0 °C. To this flask was added the serinol solution in one portion. The temperature was allowed to rise to 50ºC and the reaction mixture was stirred for 16

hours. The organic phase was extracted with 0.5 M $NaHCO_3$ (2 x15 mL), 0.5 M sodium citrate (2 x 15 mL), dried over $MgSO_4$, and the solvent was evaporated under vacuum giving a light yellow oil. The product was purified as yellow oil by flash chromatography using EtOAc 100% in 55% yield. 1H NMR ($CDCl_3$, 400 MHz): δ 8.05 (1H, s), 6.48 (1H, s), 6.40 (1H, s), 5.79 (1H, s), 5.70 (1H, s), 4.50 (1H, d), 4.27 (1H, m), 4.25 (1H, d), 3.65 (1H,s), 3.50 (1H, d), 3.25 (1H, d), 2.01 (3H, s), 1.98 (3H, s). 13C NMR ($CDCl_3$, 100 MHz): δ 168.64, 167.22, 125.27, 118.16, 64.23, 60.33, 53.88, 19.46, 17.91.

5.3.4 Synthesis of 3-amino-2-methacrylamidopropyl Methacrylate (5.4)



5.4

Scheme 5.4. Synthesis for Monomer 5.4: (a) Lactobacillus reuteri, 30ºC, 45 min[7]; (b) NH_3/NaCN, r.t., 1h[8]; (c) $H_2C=C(CH_3)COCl/Et_3N$, THF/DMF, 50ºC/16h; (d) $NiCl_2$: $NaBH_2$, dry EtOH, r.t, 15 min.[9]

3-hydroxypropionaldehyde. Glycerol (2.00 g, 21.73 mmol) was dissolved in 5 mL of deionized water. To this solution was added 200 mg of Lactobacillus reuteri. The solution was then stirred for 8 hours. The solution was then filtered to remove Lactobacillus reuteri and vacuum distilled

to purify the resulting 3-hydroxypropionaldehyde. Yield 90% 1H NMR ($CDCl_3$, 400 MHz): δ 9.72 (1H, s), 3.86 (2H, t), 3.65 (1H, s), 2.59 (1H, s). 13C NMR ($CDCl_3$, 100 MHz): δ 202.20, 54.82, 45.47.

2-amino-4-hydroxybutanenitrile A filtered solution of 0.723 g (13.51 mmol) of ammonium chloride in 50 mL of water is placed in a 500 mL round-bottomed flask. The flask was placed in an ice bath and cooled to 5–10°C. A solution of 1 g (13.51 mmol) of 3-hydroxypropionaldehyde in 50 mL of ether is added while stirring. Then a solution of 0.637 g (13 mmol) of sodium cyanide in 3.5 mL of water is added, with stirring, at such a rate that the temperature never exceeds 10°C. The reaction mixture is stirred for one hour after all the sodium cyanide has been added and allowed to stand overnight. The ether layer is separated and the aqueous liquor is extracted with six 30 mL portions of ether. The ether extracts are combined and the ether is distilled. The residue is diluted with 80 mL of methyl alcohol. The solution is cooled and saturated with ammonia gas. The reaction mixture is allowed to stand for two or three days, and the excess ammonia is removed over vacuum. The methyl alcohol is removed by distillation as completely as possible. The product resulted as viscous oil. Yield 70%. 1H NMR ($CDCl_3$, 400 MHz): δ 5.11 (1H, s), 3.80 (2H, t), 3.65 (1H, s), 3.62 (1H, t), 2.07 (2H, q). 13C NMR ($CDCl_3$, 100 MHz): δ 116.21, 56.68, 38.95, 37.22.

2-cyano-2-methacrylamidoethyl methacrylate 2-amino-4-hydroxybutanenitrile (0.5g, 5 mmol) was dissolved in THF/DMF (50/50) and allowed to cool to 0ºC while stirring. The solution was added with Et_3N (1.02 g, 10 mmol) drop wise and the solution was allowed to cool to 0ºC while stirring. Methacryloyl chloride (1.04 g, 10 mmol) was slowly added over 10 min. The mixture was then allowed to stir at 50ºC for 16 hours. The organic phase was extracted with 0.5 M

$NaHCO_3$ (2 x15 mL), 0.5 M sodium citrate (2 x 15 mL), dried over $MgSO_4$, and the solvent was evaporated under vacuum giving a light orange oil. The product was purified by flash chromatography using EtOAc 100% in 55% yield. 1H NMR ($CDCl_3$, 400 MHz): δ 8.12 (1H, s), 6.57 (1H, s), 6.44 (1H, s), 5.66 (1H, s), 5.73 (1H, s), 5.17 (1H, t), 4.79 (1H, d), 4.58 (1H, d). 13C NMR (CDCl3, 100 MHz): δ 170.21, 168.32, 142.54, 136.81, 124.12, 117.49, 68.51, 43.57, 19.75, 17.69.

3-amino-2-methacrylamidopropyl methacrylate 2-cyano-2-methacrylamidoethyl methacrylate (1.00 g, 4.42 mmols) was dissolved in 10 mL dry EtOH while stirring. $NiCl_2$ (0.57 g, 4.42 mmols) was added and the solution was allowed to stir under N_2 gas for 10 min. $NaBH_4$ was slowly added and the reaction was allowed to stir at room temperature for 15 min. The reaction was quenched by the addition of 1N HCl. The product was purified by extraction using EtOAc, saturated $NaHCO_3$(aq) and isolated via flash chromatography (100% EtOAc) to give a viscous clear oil. Yield 80%. 1H NMR ($CDCl_3$, 400 MHz): δ 8.12 (1H, s), 6.57 (1H, s), 6.44 (1H, s), 5.66 (1H, s), 5.73 (1H, s), 5.11 (2H, s), 4.50 (1H, d), 4.25 (1H, d), 3.00 (1H, d), 2.75 (1H, d). 13C NMR (CDCl3, 100 MHz): δ 170.21, 168.32, 142.54, 136.81, 124.12, 117.49, 65.31, 56.12, 40.73, 19.75, 17.69.

5.3.5. Polymer Preparation

The following procedure was used for imprinted polymers employing the new cross-linking monomers. In a 13 × 100-mm test tube, BOC-L-tyrosine, BOC-D-tyrosine, (R)-(+)-1,1’-bi-2-naphthol, (1S, 2S) - (-)-1,2-Diphenylethylene, (1R, 2R) - (+) -1,2-Diphenylethylene, (S)-(-)-1,1'-Bi(2-naphthylamine), or (R)-(+)-1,1'-Bi(2-naphthylamine) (5 mol %) (**Figure 5.3**) was dissolved

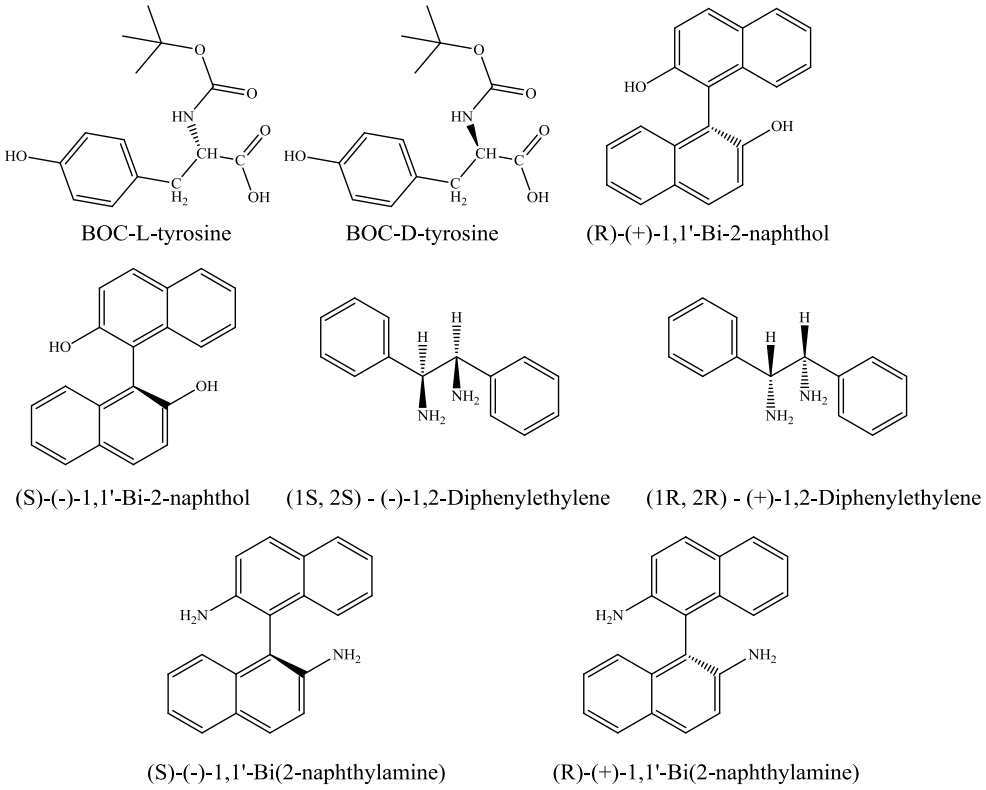


Figure 5.3. Templates used in the chiral imprinting studies.

in 3.0 mL of MeCN. To this solution 2 grams of monomer was added, and AIBN (1 mol%). The solution was purged by bubbling nitrogen gas into the mixture for 5 minutes, then capped and sealed with Teflon tape and Parafilm. The samples were inserted into a photochemical reactor, which was immersed in a constant-temperature bath. A standard laboratory UV light source (medium pressure 450-W mercury arc lamp) jacketed in a borosilicate double-walled immersion well was placed at the center of the turntable. The polymerization was initiated photochemically at 20 °C and allowed to proceed for 8 hours, while the temperature was maintained by both the cooling jacket surrounding the lamp and the constant-temperature bath holding the entire apparatus.

5.3.6 Determination of Solubility in Acetonitrile (ACN)

Monomers 5.1-5.4 were added drop-wise to 5 mL of acetonitrile and allowed to stir for 5 minutes. Following stirring the solutions were left to stand for 30 minutes. The solutions were then visually analyzed to determine the solubility of the monomers in acetonitrile.

5.3.7. Chromatographic Evaluations

Removal of the template was achieved by Soxhlet extraction with MeOH for 48 h. The polymers were then ground using a mortar and pestle, the particles were sized using USA Standard Testing Sieves, and the fraction between 25 and 37 μm was collected. The particles were slurry packed, using a solvent delivery module, into stainless steel columns (length 100 mm, i.d. 2.1 mm) to full volume for chromatographic experiments. The polymers were then equilibrated on-line for 12 h using MeCN/acetic acid (99:1) at a flow rate of 0.1 mL min^{-1} to remove any residual template. HPLC analyses were performed isocratically at room temperature (21 °C). The flow rate was set at 0.1 mL min^{-1} using MeCN/acetic acid (99:1) and MeCN as mobile phases. The substrate concentration was 0.1 mM of the templates shown in **Figure 5.2** dissolved in MeCN, and detected at a wavelength of 260 nm. The void volume was determined using acetone as an inert substrate. The separation factors (α) were measured as the ratio of capacity factors k_L/k_D. The capacity factors were determined by the relationship $k = (V_t - V_0)/V_0$, where V_t is the retention volume of the substrate, and V_0 is the void volume.

5.4. Results/Discussion

As described in the Experimental section, the newly synthesized cross-linkers were utilized for imprinting BOC-L-tyrosine, BOC-D-tyrosine, (S)-(-)-1,1'-bi-2-naphthol, (R)-(+)-

1,1'-bi-2-naphthol, (1S, 2S) - (-)-1,2-Diphenylethylene, (1R, 2R) - (+) -1,2-Diphenylethylene, (S)-(-)-1,1'-Bi(2-naphthylamine), or (R)-(+)-1,1'-Bi(2-naphthylamine) to create OMNiMIPs imprinted with scalemic/racemic combinations of templates. Monomer **5.1** (NOS) has undergone the most extensive investigation due to the high solubility NOS displays in organic solvents. Monomers **5.2-5.6** have not shown the same solubility (**Table 5.1**) as that of NOS and have not been fully analyzed and will not be discussed in this chapter.

Table 5.1. Solubility of new cross-linking monomers in acetonitrile (ACN).

Cross-linker	Solubility
Monomer 5.1	Fully miscible
Monomer 5.2	Partially miscible
Monomer 5.3	Immiscible
Monomer 5.4	Immiscible

Similar to past studies on BOC-L/D-tyrosine which has shown enhanced imprinting performance using NOBE, a series of NOS/EGDMA imprinted polymers were synthesized and evaluated using chromatographic methods.[5] Previous studies performed by Sibrian-Vasquez and Spivak showed an increase in separation factor (α) followed by a decline in separation factor when imprinting nicotine in NOS at varying cross-linker concentrations when with EGDMA.[11] At 25 mol% NOS the separation factor began to decrease. As a result, a study varying the

amounts of NOS and EGDMA from 0% NOS to an OMNIMIP of NOS (100% NOS) was performed to determine the optimum ratio of NOS/EGDMA using boc-L-tyrosine. The results located in **Table 5.2** and **Figure 5.4** demonstrates the ability of NOS to perform as an OMNIMIP.

Table 5.2. Capacity factors (k') and enantioselectivity (α) for NOS/EGDMA polymers using Boc-L-tyrosine as the template.

% NOS: %EGDMA	k_D'	k_L'	(α)
0:100	1	1	1.0±0.2
25:75	0.62	0.43	1.4±0.05
50:50	0.96	0.61	1.6±0.09
75:25	0.55	0.32	1.7±0.07
100:0	0.64	1.30	2.0±0.12

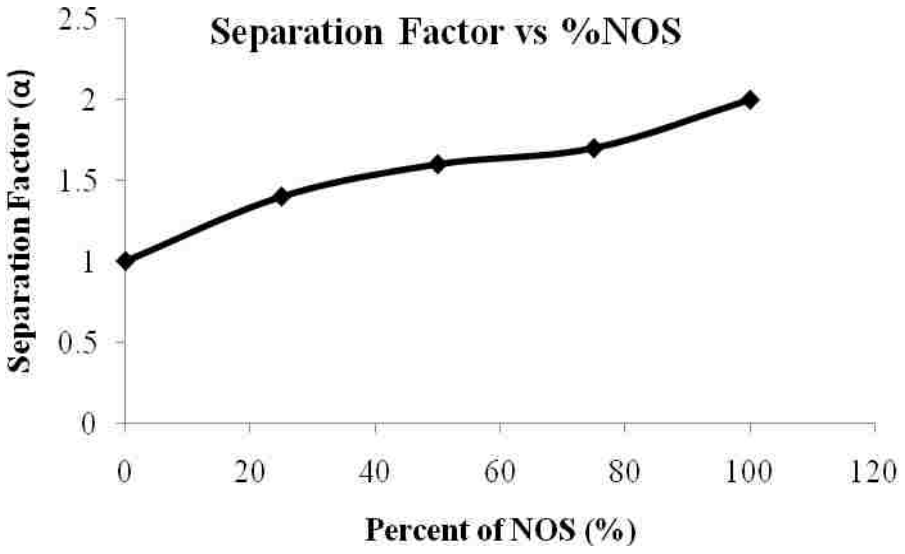
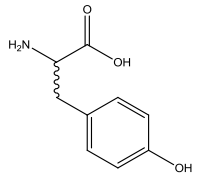
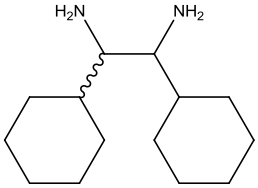


Figure 5.4. Chart demonstrating the linear trend in separation factor (α) in NOS/EGDMA polymers imprinted with Boc-L-tyrosine.

Although NOS has the highest performance as an OMNIMIP, NOBE still can outperform when imprinting a single enantiomer of Boc-tyrosine (α = 3.8 (NOBE) vs. 2.0 (NOS)). This result is likely due to the increased in non-selective binding in NOS (**Table 5.2**) when compared to the NOBE polymers (**Table 2.1**, Chapter 2) as shown by the capacity factors. The results also suggest this limitation can still be overcome with the strong hydrogen bonding polymer matrix present in OMNIMIPs.

NOS does not perform at the same level of NOBE when using templates that can only hydrogen bond. However, the unique ability of NOS to form ionic interactions allows for stronger interactions with amine containing compounds. Therefore, the ultimate step in the analyses of NOS and the other monomer listed in **Figure 5.3** is the ability to imprint a mixture of enantiomers from a compound and achieve enantioselective separation. Two OMNIMPs using NOS were prepared using BOC-L/D-tyrosine and R/S-1,2-dicyclohexylethane-1,2-diamine as templates. The OMNIMIP using NOS that imprinted BOC-L/D-tyrosine did not show selective binding for either template. The more surprising result was the separation factor for the diamine compounds ((1S, 2S) - (-)-1,2-Diphenylethylene, (1R, 2R) - (+) -1,2-Diphenylethylene) (**Table 5.3**). The ionic interactions present in the NOS monomer/template interactions favored (1S, 2S) - (-)-1,2-Diphenylethylene over (1R, 2R) - (+) -1,2-Diphenylethylene leading to an enantioselectivity factor or α = 6.6. When repeated with other non-amine containing compound similar results were seen as in the analyses of BOC-L-tyrosine. Preliminary results with (S)-(-)-1,1'-Bi(2-naphthylamine) and (R)-(+)-1,1'-Bi(2-naphthylamine) imprinted NOS polymers show enhanced performance for one enantiomer over the other when analyzed separately. More studies are needed to fully understand the nature of the ionic/chiral interactions present and exploit this ability to add to the field of imprinting.

Table 5.3. Results on racemic imprinting using NOS.

Template in 100% of NOS	k_D'	k_L'	Separation Value (α)
BOC-Tyrosine: 	**0.280**	**0.300**	**1.1**
1,2-dicyclohexylethane-1,2-diamine 	**0.002**	**0.700**	**6.6**

5.5. Conclusions/Future Work

The continued positive results using NOS will allow for a comprehensive determination of the possibilities of this monomer for chiral separation. Preliminary results suggest NOS is only able to achieve high selectivity with amine containing compounds. This ability will be further investigated as part of future research projects. Furthermore, the other monomers show little organic solubility and will be part of future projects to determine applicability in imprinting.

5.6. References

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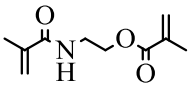
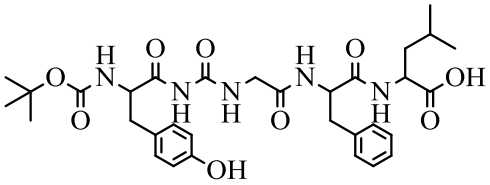
CHAPTER 6: FUTURE WORK AND OTHER APPLICATIONS FOR OMNIMIPS

6.1 A Direct Strategy for Peptide Analysis Using Molecularly Imprinted Polymers under Non-aqueous Conditions*

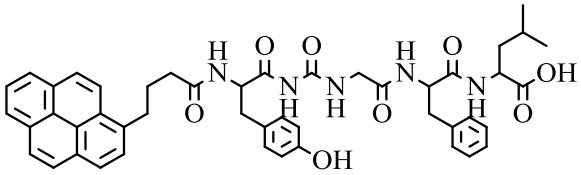
Throughout this dissertation, several new monomers for use in imprinting were discussed. The most studied monomer is NOBE (**6.1**, **Figure 6.1**), and has shown many abilities besides those describe in Chapters 2 and 3. A separate ability of NOBE is to form micro-sized and nano-sized particles that can be used in biological and pharmaceutical applications. This ability was utilized in collaboration with the Le research group at Lund University, Lund, Sweden.[1] NOBE micro and nano particles were used to imprint peptide fragments to achieve separation from a mixture of peptides and proteins. The ability to separate useful peptide fragments will help to develop a new understanding of how individual peptides function and aid in the understanding of certain neurological and disease processes. Furthermore, the high cost of current commercial available separation and isolation media prohibits the wide-scale research that is required to unlock valuable scientific and medical discoveries.[2]



6.1



6.2



6.3

Figure 6.1. Structures of compounds used in micro particle peptide imprinting study.

*Reprinted with permission from: Yoshimatsu, K.; LeJeune, J.; Spivak, D. A.; Ye, Peptide-imprinted polymer microspheres prepared by precipitation polymerization using a single bi-functional monomer *Analyst*, **2009**, (4),719-724.

For this study, a neuropeptide, Leuenkephalin (**6.2, Figure 6.1**) was used as a model to study the feasibility of the proposed approach (**Scheme 6.1**). The *N*-terminal protected sequence, Boc-Leu-enkephalin (**6.3, Figure 6.1**) was used as a template, and the recently developed *N*,*O*-bismethacryloyl ethanolamine (NOBE) as a bi-functional monomer (having both binding and polymerizable moieties) to prepare peptide imprinted microspheres using a precipitation polymerization protocol.[3] After polymerization, polymer particles were collected by ultracentrifugation, and washed repeatedly with methanol:acetic acid (90:10, v:v) to remove the template. A non-imprinted polymer was synthesized under the same conditions except for omission of the template, and used as a control for comparison. Both the imprinted and the non-imprinted polymers were obtained as spherical beads (diameter 1-5 μm) with an apparently broad size distribution (**Figure 6.2**), which may be narrowed through further optimization of the reaction conditions.

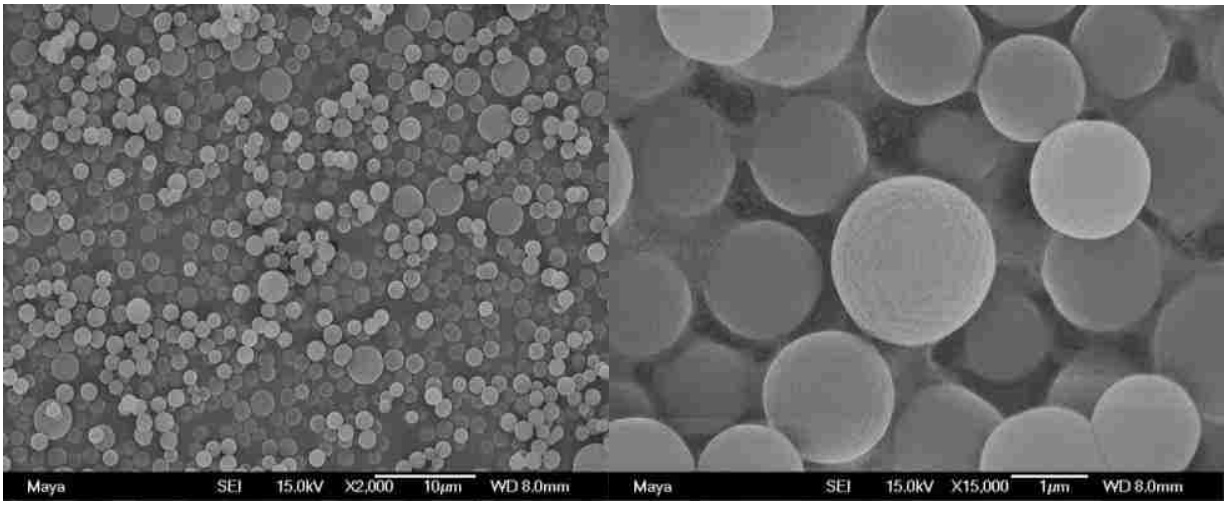
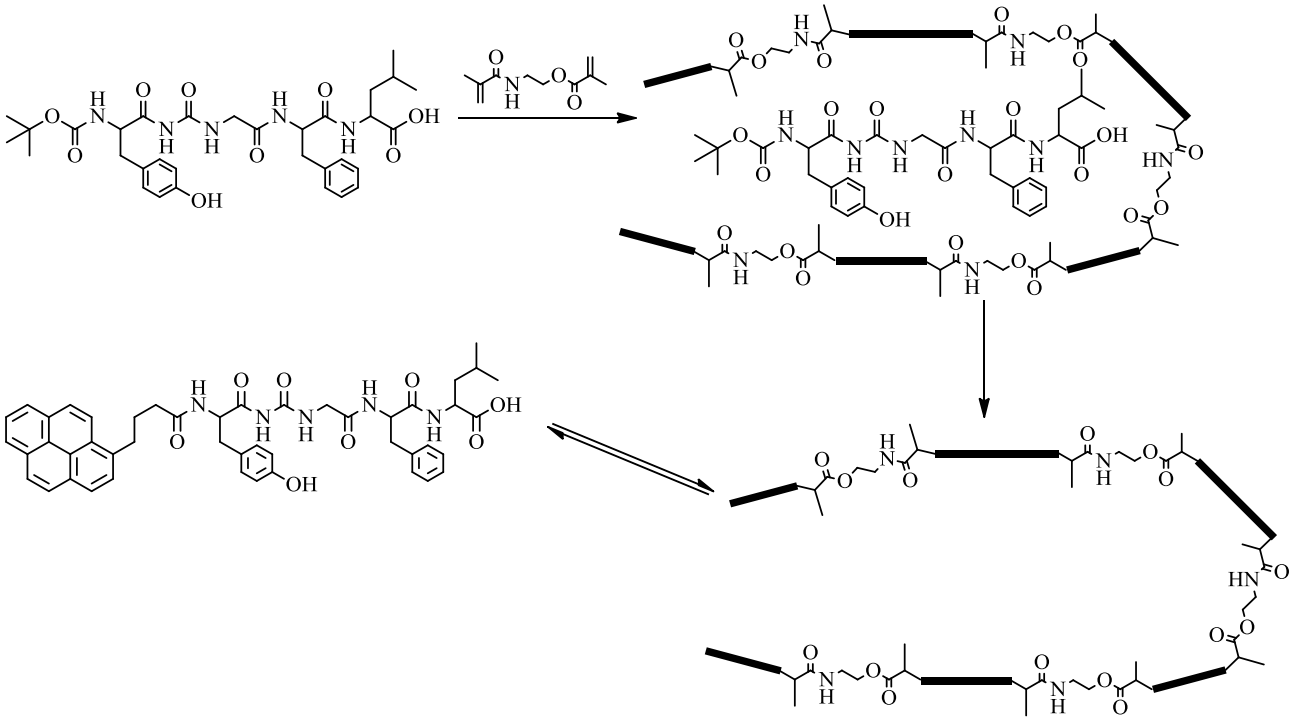


Figure 6.2. SEM images of molecularly imprinted microspheres (a) and non-imprinted microspheres (b).



Scheme 6.1. Preparation of peptide-imprinted polymer (top right), and application of the MIP for analysis of fluorescently tagged target peptide (bottom left).

The imprinted polymer beads were first tested for their specific binding for the original template. Boc-Leu-enkephalin was incubated with different amount of polymers in acetonitrile. After incubation and centrifugation, the concentration of free peptide remaining in supernatant was quantified by HPLC-MS, from which the percentage of Boc- Leu-enkephalin bound to the polymers was calculated. As shown in **Figure 6.3**, the imprinted polymer bound much more the template than the non-imprinted polymer, indicating that the former has apparently much higher affinity for the peptide because of the imprinted binding sites. At a polymer concentration of 5 mg mL^{-1}, the uptake of template by the imprinted polymer (46%) was almost 6 times of that by the non-imprinted polymer. Of potentially greater interest, nonspecific peptide binding, as judged from the template uptake contributed by hydrogen bond interactions with the amide moiety of NOBE.[4] The imprinted sites showed very interesting cross-recognition for a fluorescent analogue

of the template, Pyr-Leu-enkephalin (**6.3**), for which the Boc protection group on the *N*-terminal was replaced by a bulkier pyrene derivative (**Figure 6.3**). Because the two enkephalin

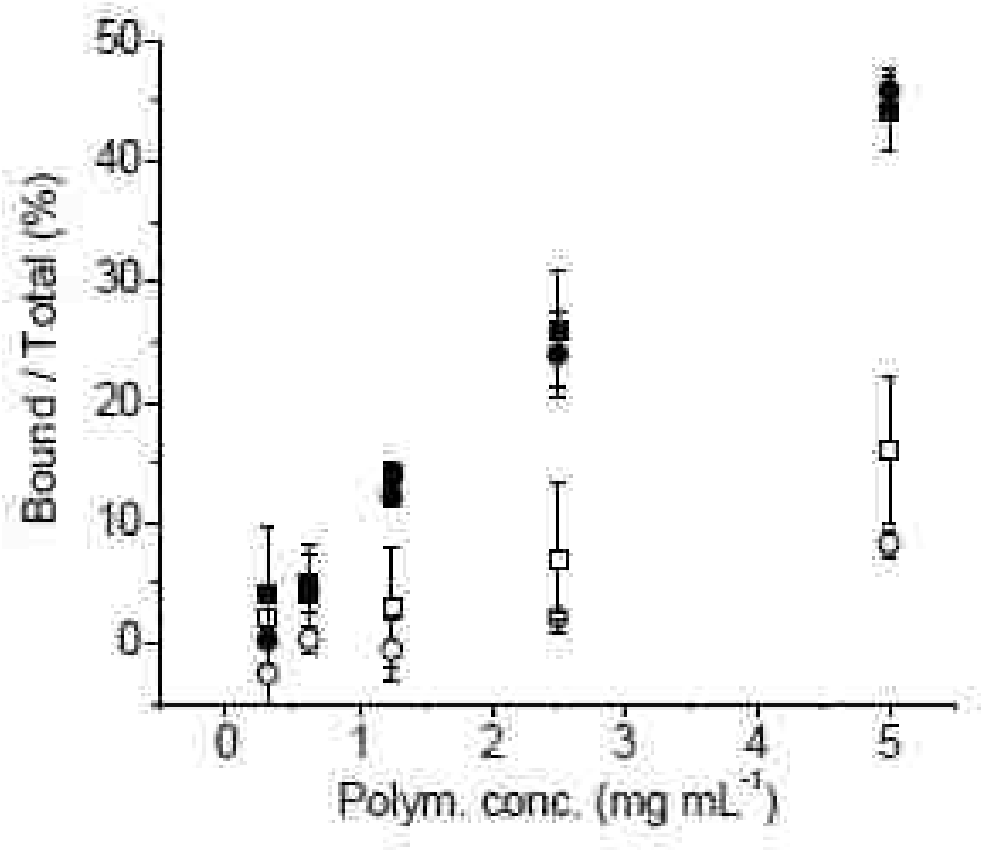


Figure 6.3. Uptake of Boc-Leu-enkephalin (circle) and Pyr-Leu-enkephalin(square) by the imprinted polymer (filled) and the non-imprinted polymer (open). The initial concentration of the peptide derivatives was 15 μM.

derivatives displayed almost identical binding profiles with the imprinted and the control polymers, we conclude that the specific binding of the peptides takes place mainly through hydrogen bond interaction between the NOBE units and the free carboxyl group of the peptides (**Scheme 6.1**).

Selectivity of the imprinted sites was studied by challenging the polymers with several related compounds, and measuring their percentage of uptake by the imprinted and the control

polymers (**Table 6.1**). While Boc-Leu-enkephalin and Pyr-Leu-enkephalin showed similarly high specific binding (judged as the difference between the imprinted and the control polymers), the test compounds lacking the Leuenkephalin sequence had no specific binding (entries 3-5). Since 1-pyrenebutyric acid showed very low binding, it can be postulated that the pyrene moiety itself in the peptide derivatives did not contribute to any specific recognition. The test compounds containing free amino group (entries 4 and 5) showed relatively high non-specific adsorption, which has been observed in previous studies using NOBE.[4]

Table 6.1. Uptake of different test compounds (%) by the imprinted and the control polymers in acetonitrile.[a]

Entry	Test compounds	Uptake by the polymers (%)	
		Imprinted	Control
1	**Boc-Leu-enkephalin**	45.9 ± 1.5	8.2 ± 3.8
2	**Pyr-Leu-enkephalin**	43.5 ± 1.9	15.7 ± 1.2
3	**1-Pyrenenbutyric acid**	4.7 ± 1.0	1.2 ± 2.4
4	**Leu-enkephalin-Pyr**	25.8 ± 4.8	27.0 ± 2.5
5	**1-Pyrenemethylamine**	52.9 ± 0.5	55.0 ± 2.3

[a]Polymer conc. 5 mg mL-1. Total conc. of test compounds 15 μM. Pyrene-containing compounds were quantified by fluorescent spectrometer, the others by HPLC-MS. Data are mean value ± standard deviation (n = 3).

To study the feasibility of combining chemical tagging and MIP-based peptide analysis, Leu-enkephalin (5 μM) was treated in PBS buffer (pH 7.4) with 10 equivalents of 1-pyrenebutyric acid *N*-hydroxysuccinimide ester to introduce a hydrophobic moiety at the *N*-terminal of the peptide. The reaction mixture was dried and re-dissolved in acetonitrile, thereafter taken up with 5 mg of polymer microspheres. The polymers were washed with acetonitirle two times, before the fluorescent peptide was eluted with acetonitrile:water

(50:50,v:v) and quantified by fluorescent intensity measurements. **Figure 6.4** shows the fluorescent emission spectra of the eluted samples collected from the imprinted and the control polymers. The fluorescence intensity of the solution eluted from the imprinted polymer [(1.36 ± 0.10) × 105 CPS] was more than 2 times of that obtained from the control polymer [(6.07 ± 0.65) × 104 CPS]. This showed clearly the potential of using MIPs for selective extraction and simultaneous assay of small peptides in complex biological samples.

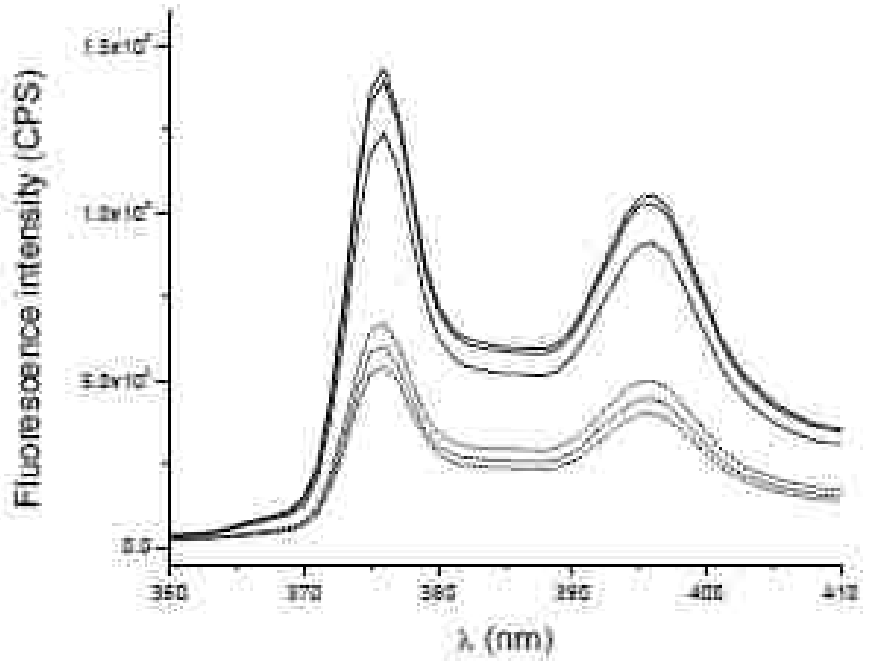


Figure 6.4. Fluorescent spectra of tagged peptide eluted from the imprinted polymer (solid line) and the control polymer (dashed line).

In this study a promising new approach for peptide analysis using molecularly imprinted polymers was displayed. The key of this new strategy is using an inert protection group for peptide modification, so that the tagged target peptide can be partitioned into organic solvents to be selectively enriched and clarified with MIPs before analytical quantification. This strategy should be equally useful for protein analysis in combination with enzymatic digestion. The

shortened peptide sequences, after *in situ* chemical modification, should be easily recognized by MIPs with specially designed target binding sites.

6.2 Future Work

The continued development of novel imprinted materials will lead to a new era in imprinting. The new materials will have the ability to imprint multiple compounds and multiple enantiomers at the same time. Several new monomers will be produced in the Spivak Research Lab that will pursue the ability of chiral imprinting as well as other applications (i.e. sensing, catalysis, and bulk environmental separations) of the novel materials developed.

6.3 References

1. Yoshimatsu, K.; LeJeune, J.; Spivak, D. A.; Ye, Peptide-imprinted polymer microspheres prepared by precipitation polymerization using a single bi-functional monomer. *Analyst*, **2009**, (4),719-724.
2. Boonen, K.; Landuyt, B.; Baggerman, G.; S Husson, S. J.; Huybrechts, J.; Schoofs, L. Peptidomics: The integrated approach of MS, hyphenated techniques and bioinformatics forneuropeptide analysis. *Journal of Separation Science*, **2008**, 31 (3), 427-445.
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4. Sibrian-Vazquez, M.; Spivak, D. A. Molecular Imprinting Made Easy. *Journal of the American Chemical Society* **2004**, 126, 7827-7833.

APPENDIX A: NMR SPECTRA FOR CHAPTER 2

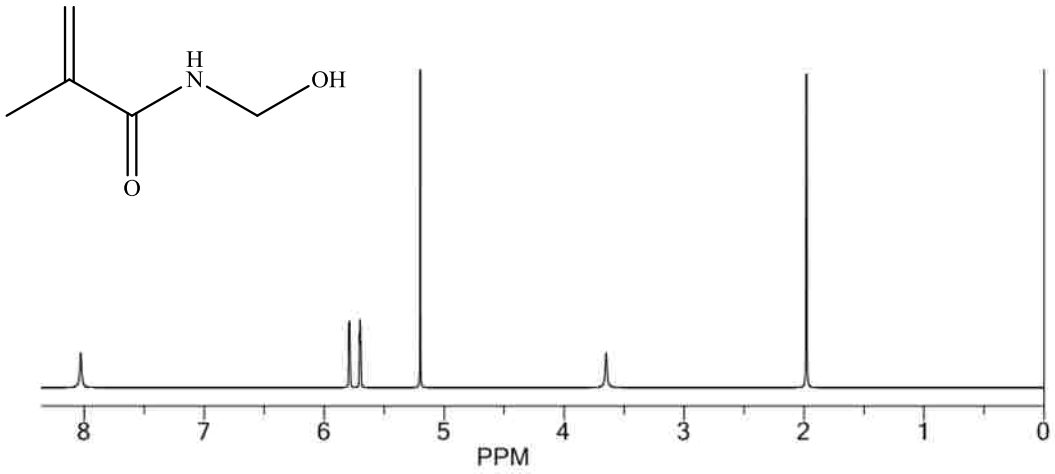


Figure A. 1. ^{1}H NMR for compound 2.13.

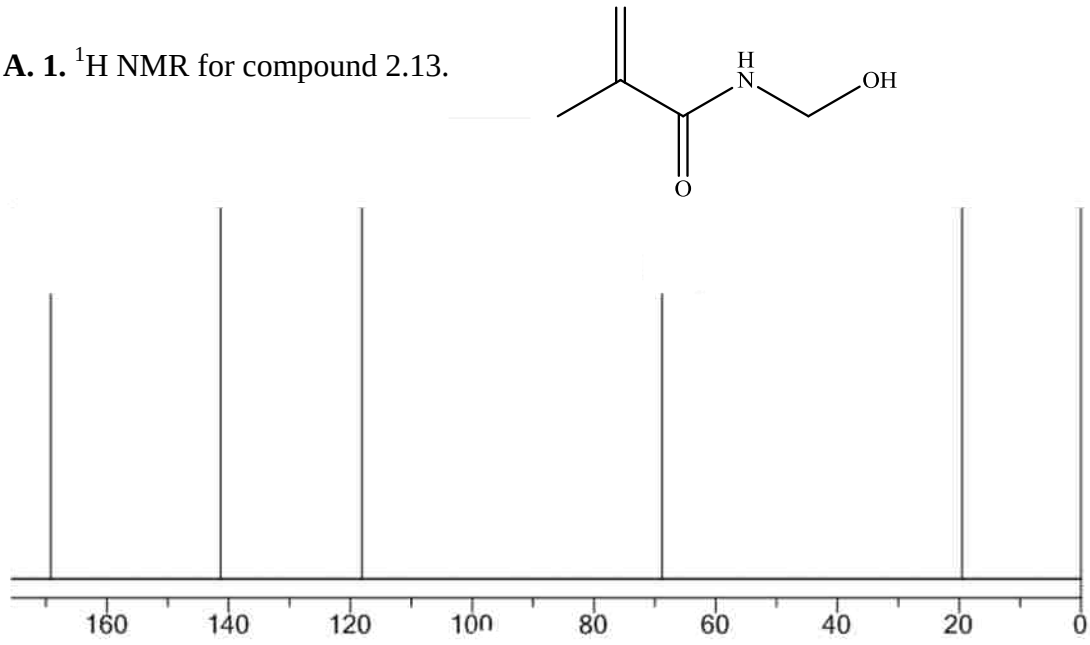


Figure A. 2. ^{13}NMR for compound 2.13.

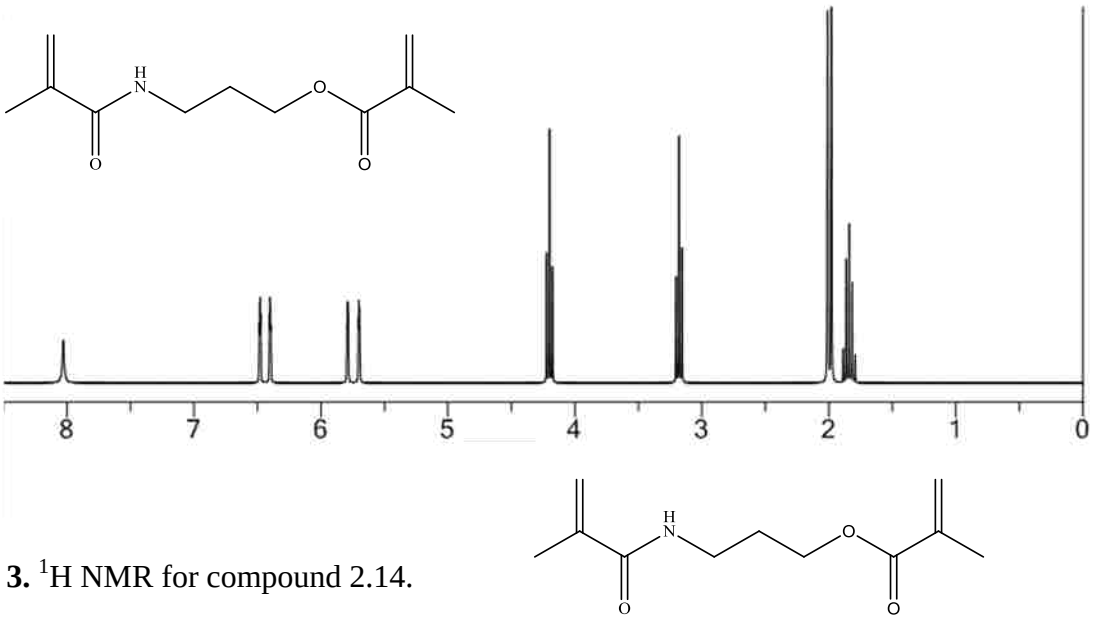


Figure A. 3. ^{1}H NMR for compound 2.14.

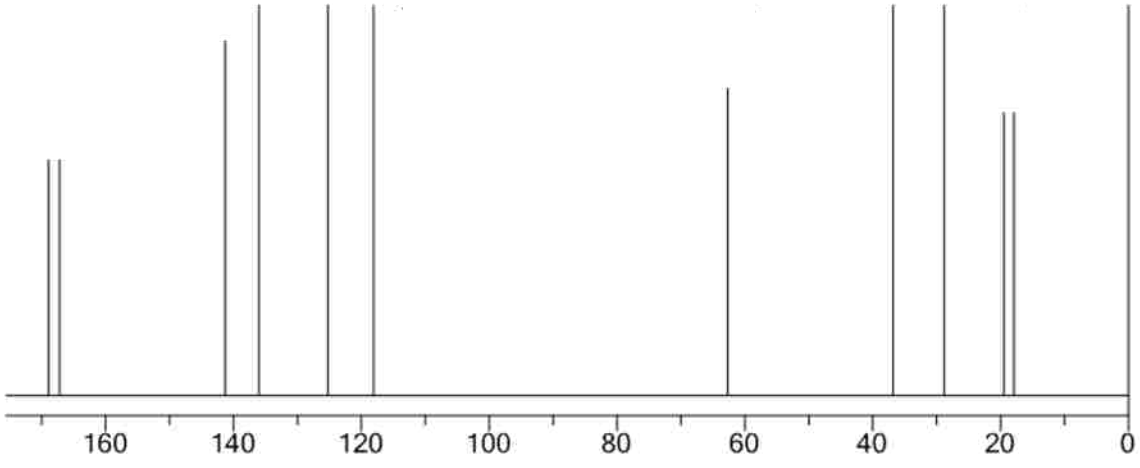


Figure A. 4. ^{13}C NMR for compound 2.14.

APPENDEIX B: NMR SPECTRA FOR CHAPTER 4

Figure B. 1 ^{1}H NMR for compound 5.

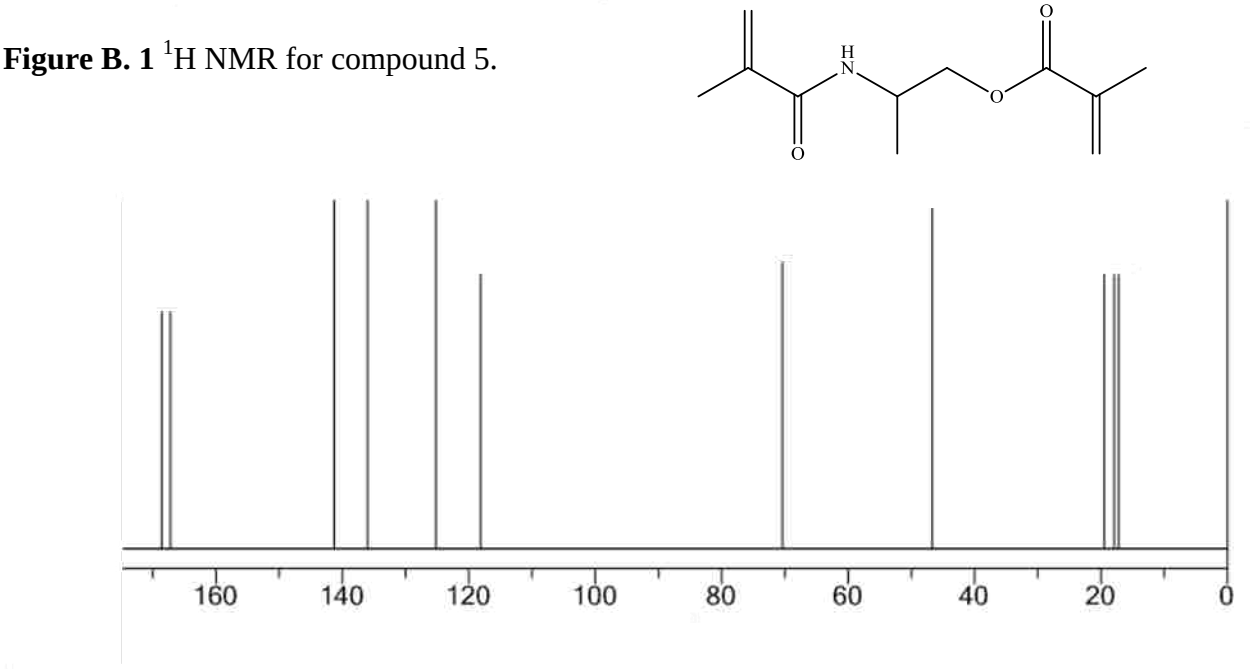


Figure B. 2. ^{13}C NMR for compound 5.

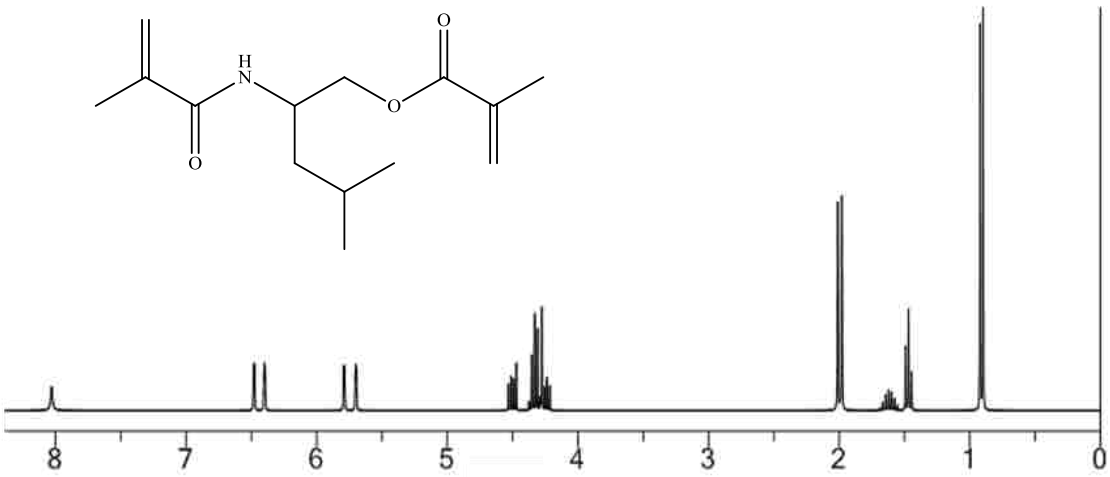


Figure B. 3 ^{1}H NMR for compound 6.

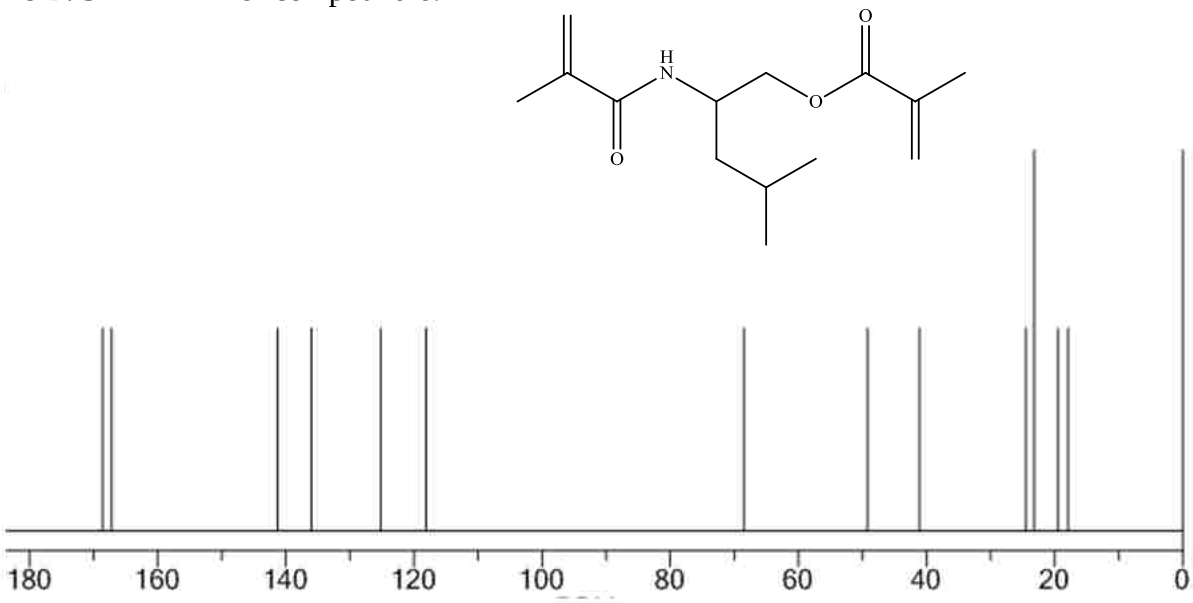


Figure B. 4 ^{13}C NMR for compound 6.

Figure B. 5 ^{1}H NMR for compound 7.

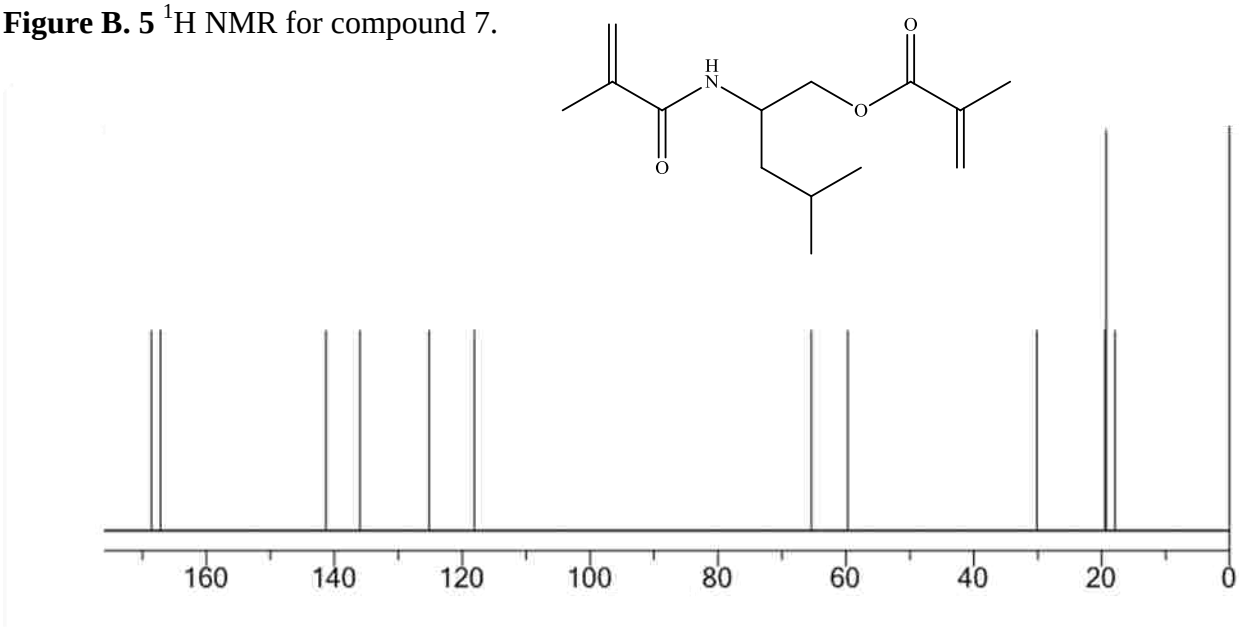
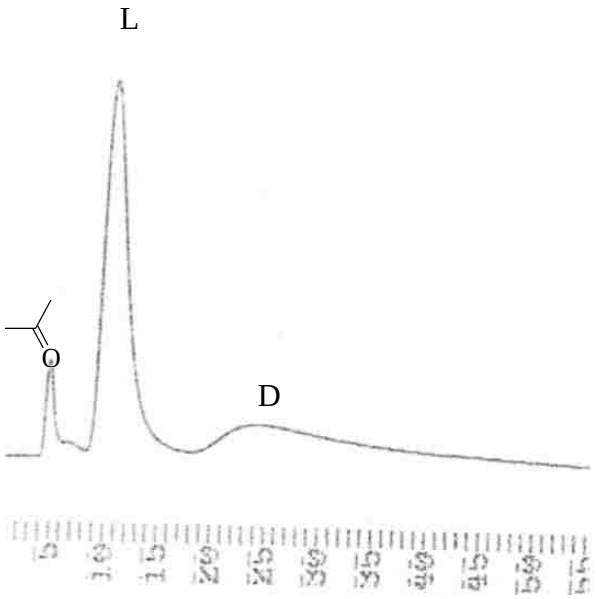


Figure B. 6 ^{13}C NMR for compound 7.

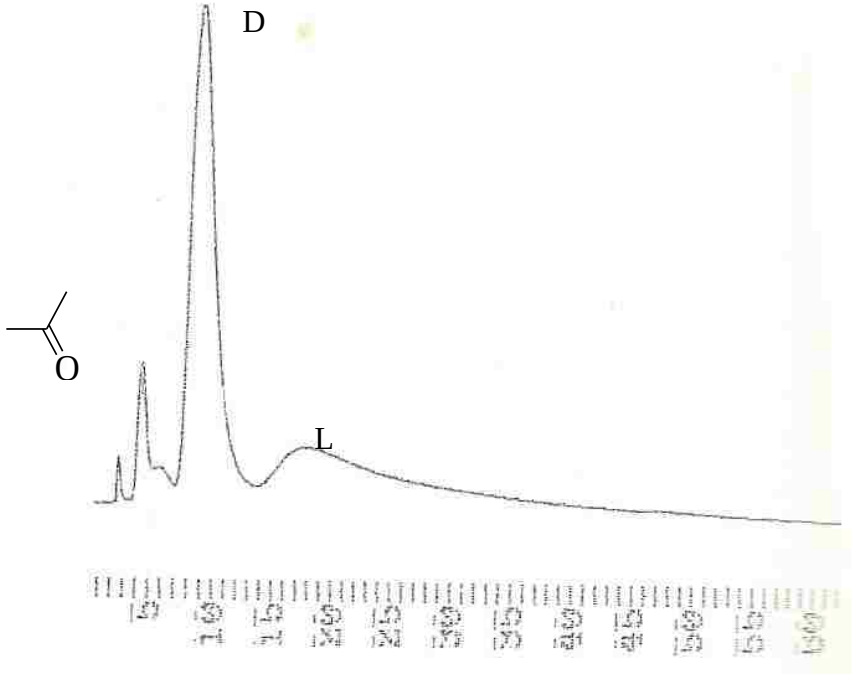
APPENDIX C. SUPPLEMENTARY MATERIAL FOR CHAPTER 4.

Figure A. Chromatograms from data in table 2.

1. Chromatogram made from OMNIMiP5-D imprinted with BOC-D-tyrosine, injected with racemic mixture of BOC-tyrosine.



2. Chromatogram made from OMNIMiP5-D imprinted with BOC-L-tyrosine, injected with racemic mixture of BOC-tyrosine.



3. Chromatogram made from OMNIMiP5-L imprinted with BOC-D-tyrosine, injected with racemic mixture of BOC-tyrosine.

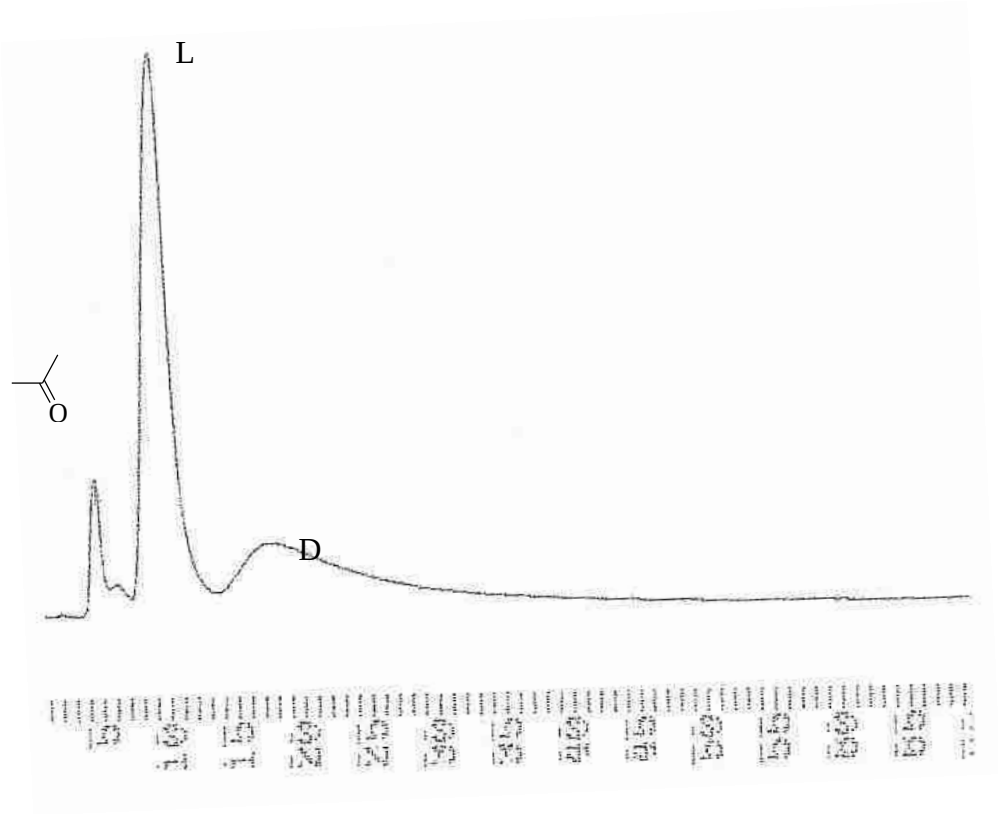
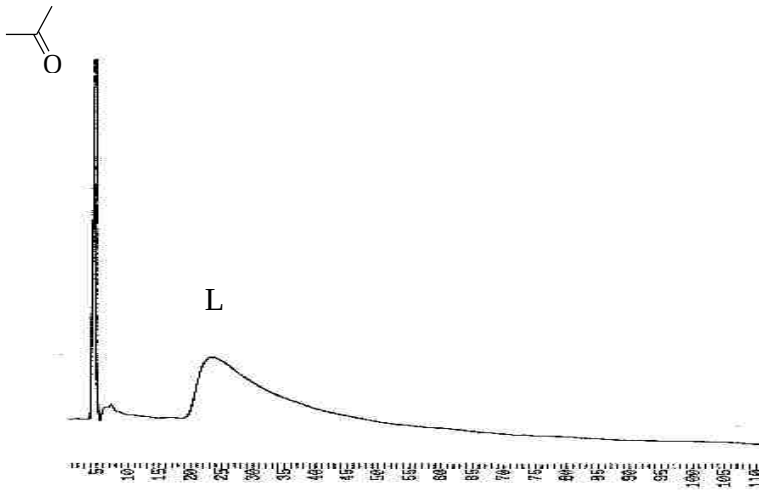
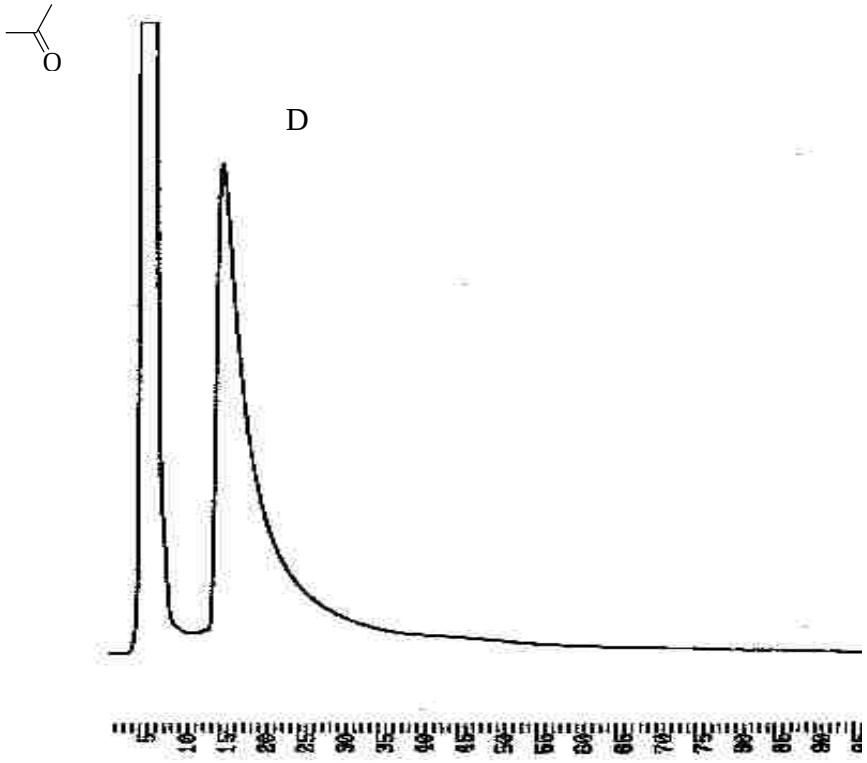


Figure B. Chromatograms from data in table 3.

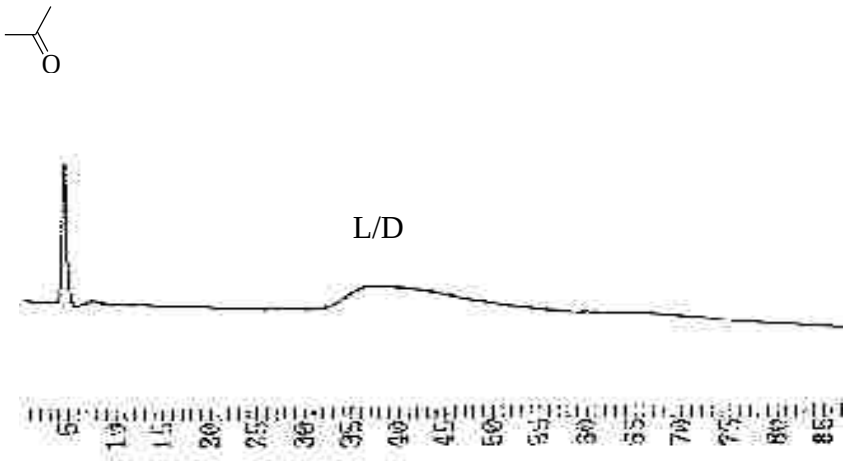
1. OMNIMiP5-L imprinted with racemic BOC-tyrosine, injected with BOC-L-tyrosine



2. OMNIMiP5-L imprinted racemic BOC-tyrosine, injected with BOC-D-tyrosine



3. OMNIMiP5-L imprinted with racemic BOC-tyrosine, injected with racemic BOC-tyrosine



APPENDIX D. NMR SPECTRA FOR CHAPTER 5

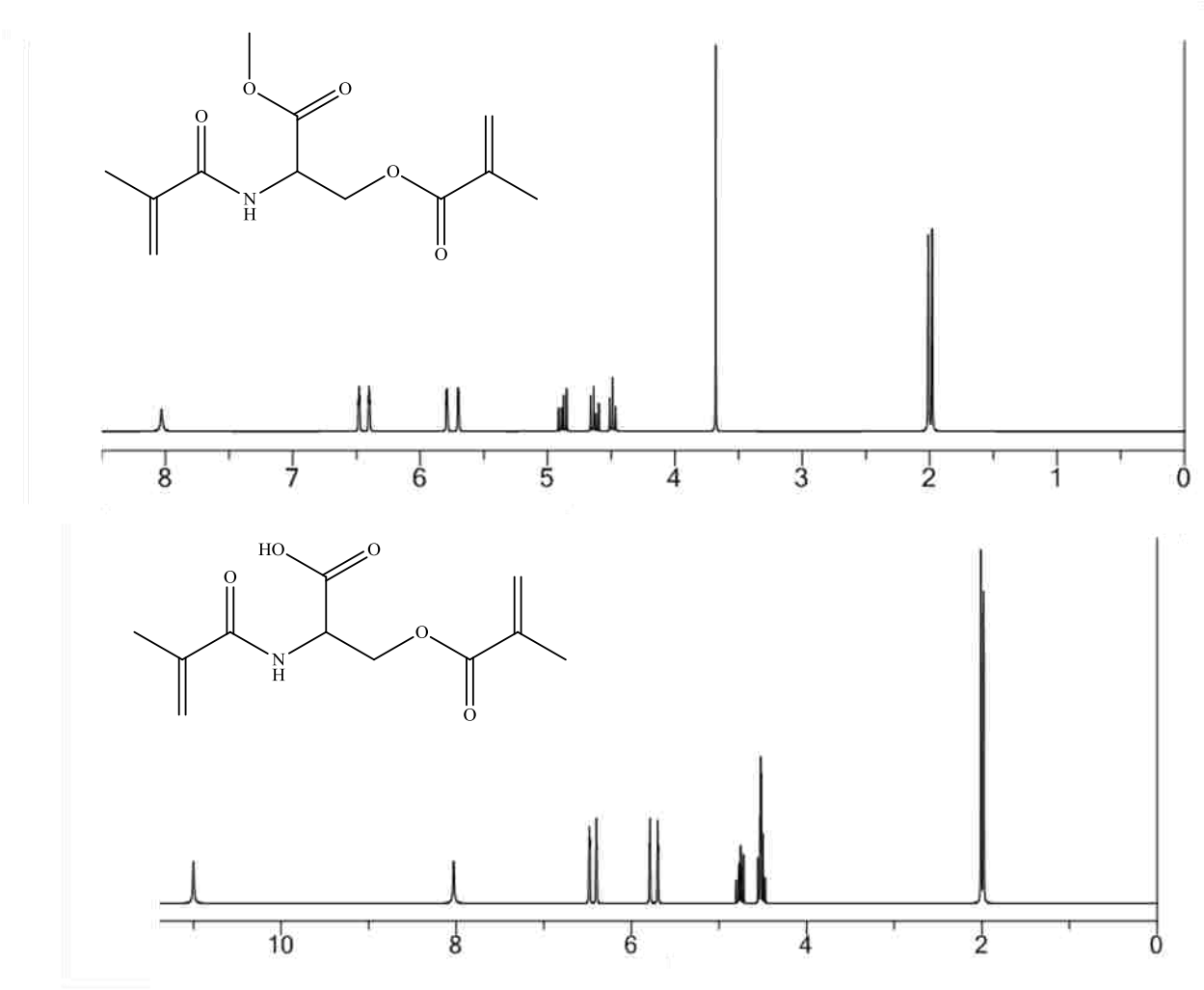


Figure D. 1. ^{1}H NMR for compound 5.1

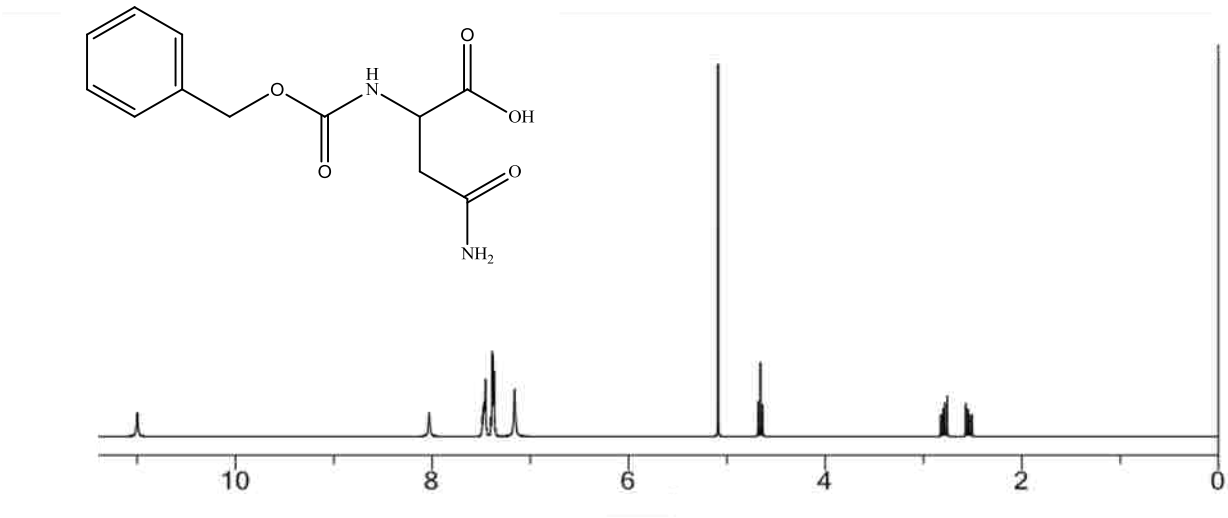


Figure D. 2 ^{1}H NMR for precursor of compound 5.2.

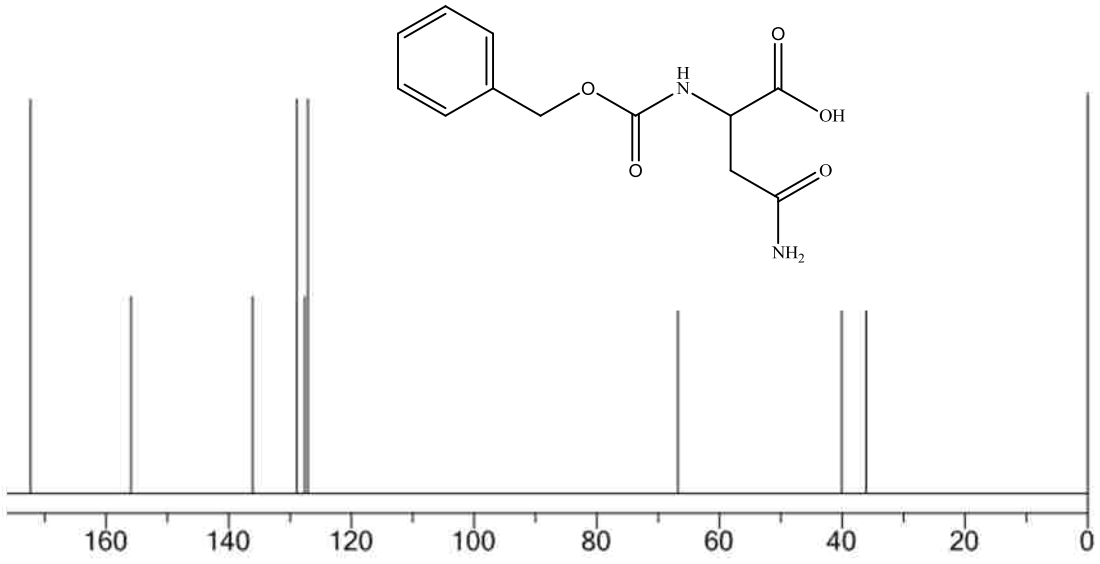


Figure D. 3 ^{13}C NMR for precursor of compound 5.2.

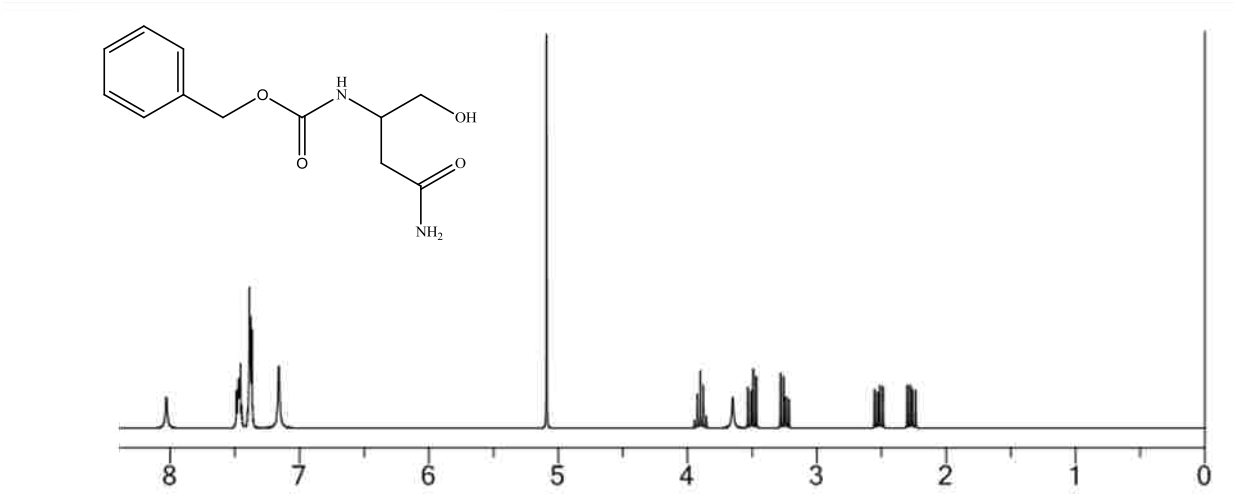


Figure D. 4 [1]H NMR for precursor of compound 5.2.

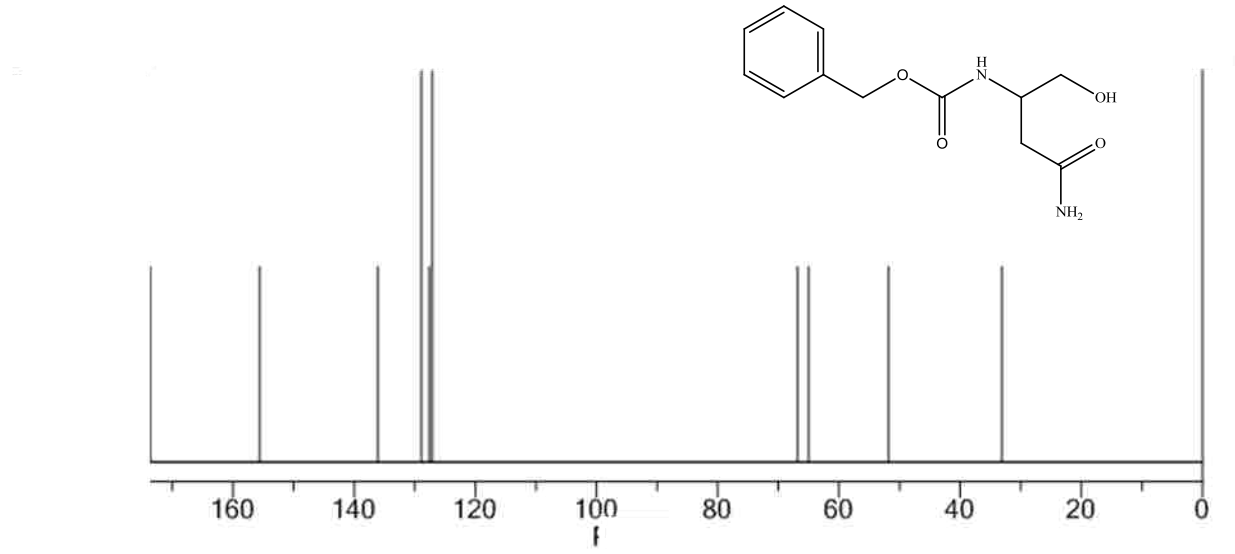


Figure D. 5 [13]C NMR for precursor of compound 5.2.

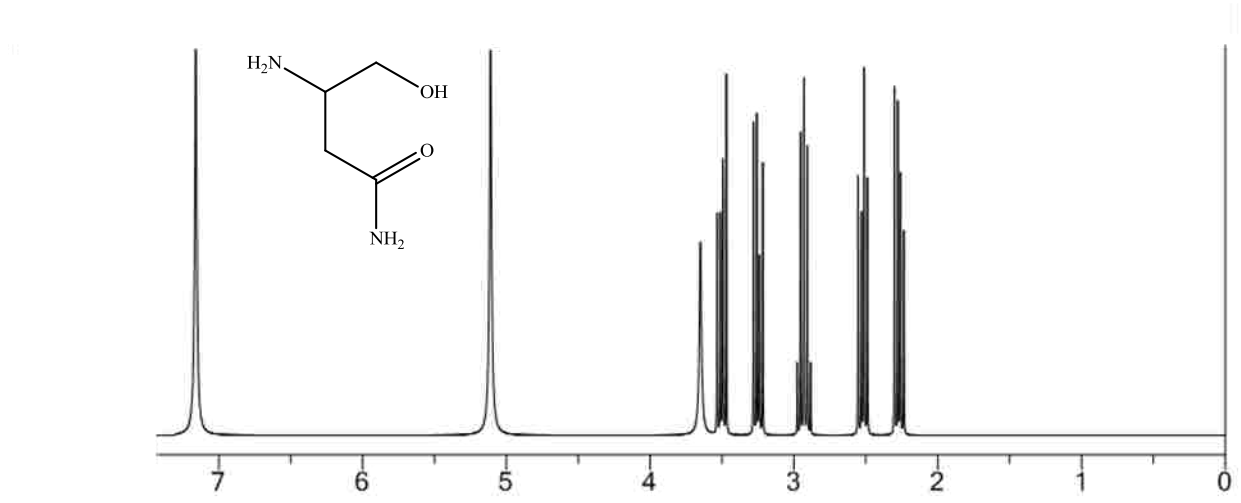


Figure D. 6 ^{1}H NMR for precursor of compound 5.2.

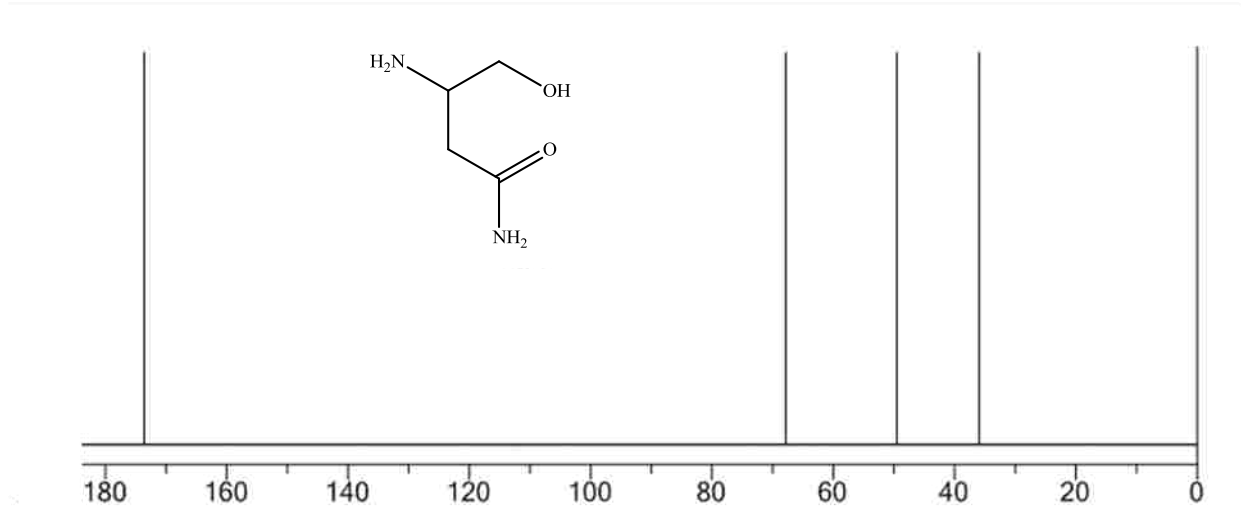


Figure D. 7 ^{13}C NMR for precursor of compound 5.2.

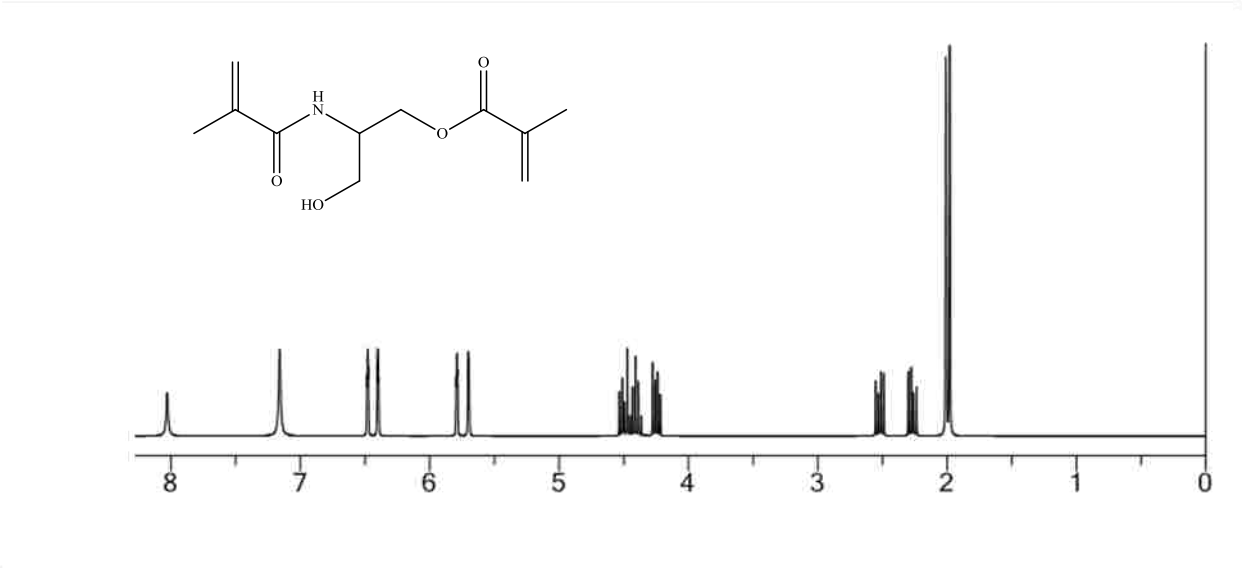


Figure D. 7 ^{1}H NMR for precursor of compound 5.2.

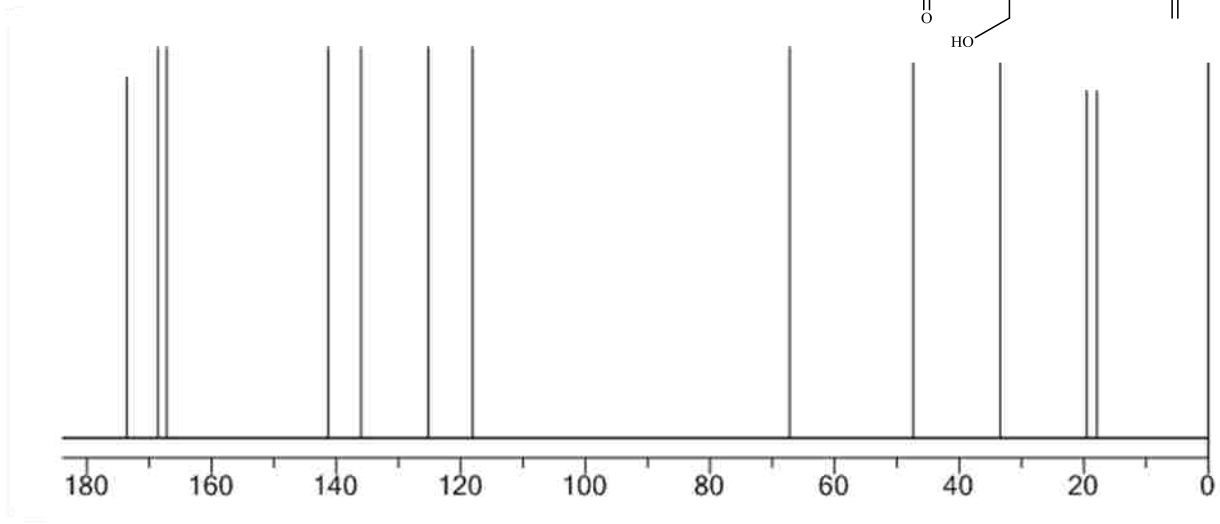


Figure D. 8 ^{13}C NMR for precursor of compound 5.2.

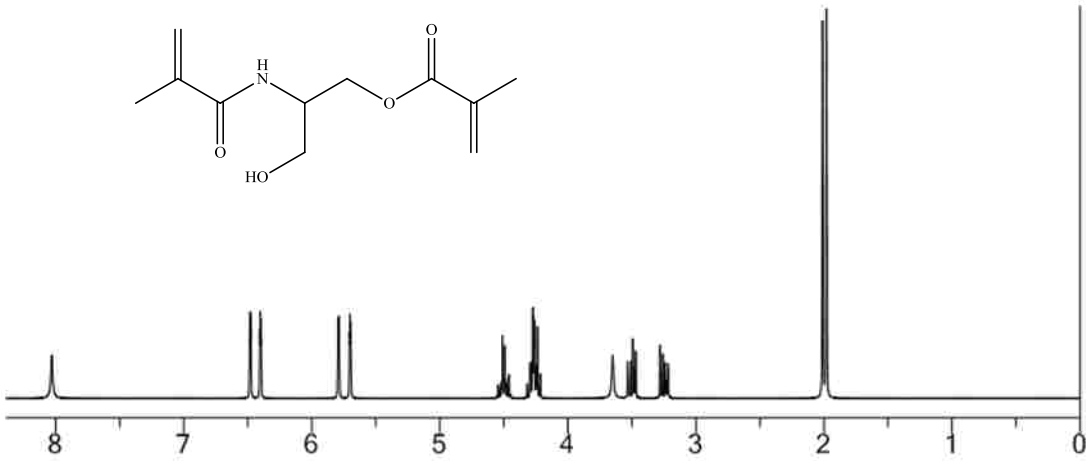
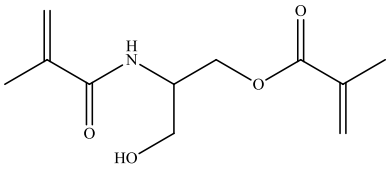


Figure D. 9 ^{1}H NMR for precursor of compound 5.3.



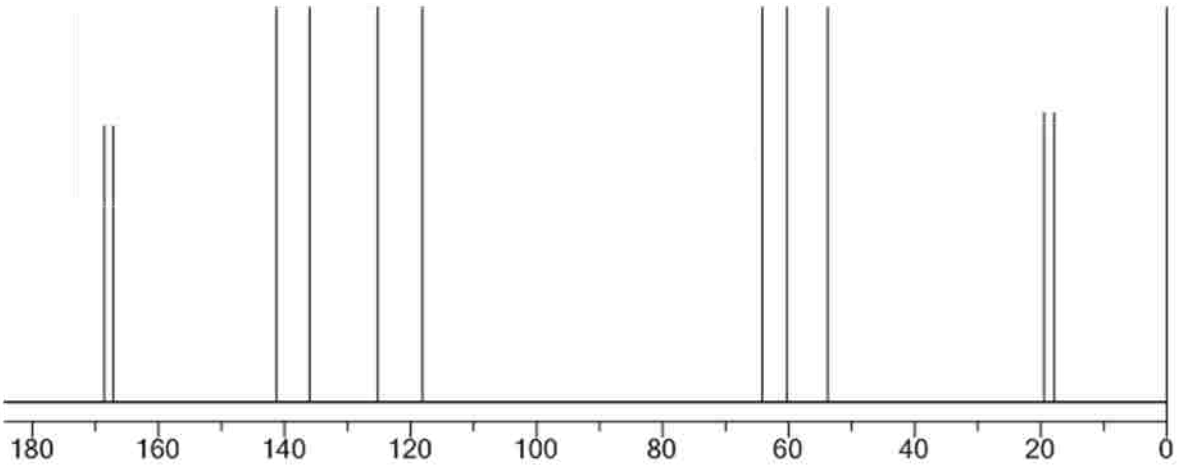


Figure D. 10 ^{13}C NMR for precursor of compound 5.3.

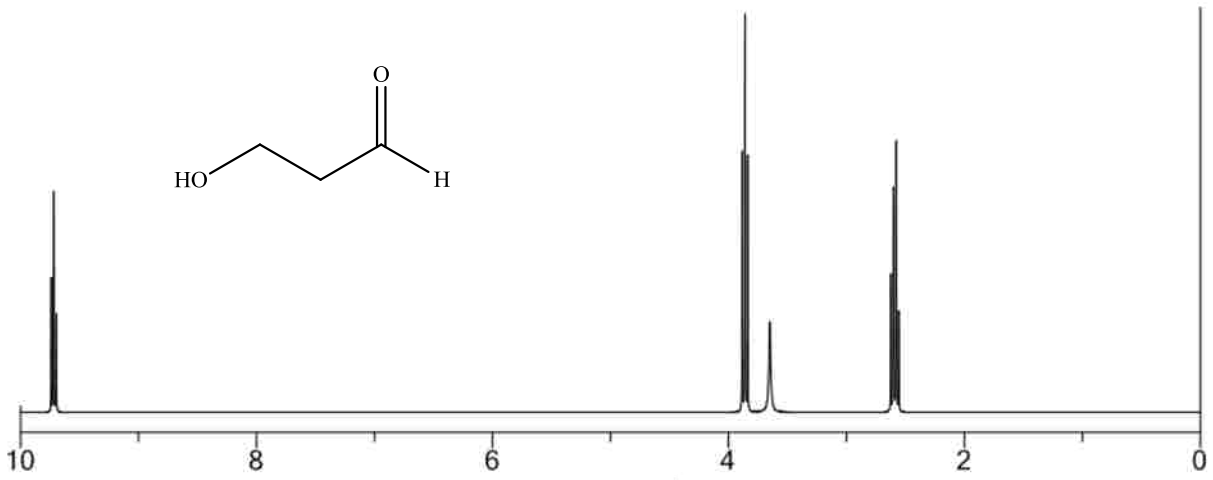


Figure D. 11 ^{1}H NMR for precursor of compound 5.4.

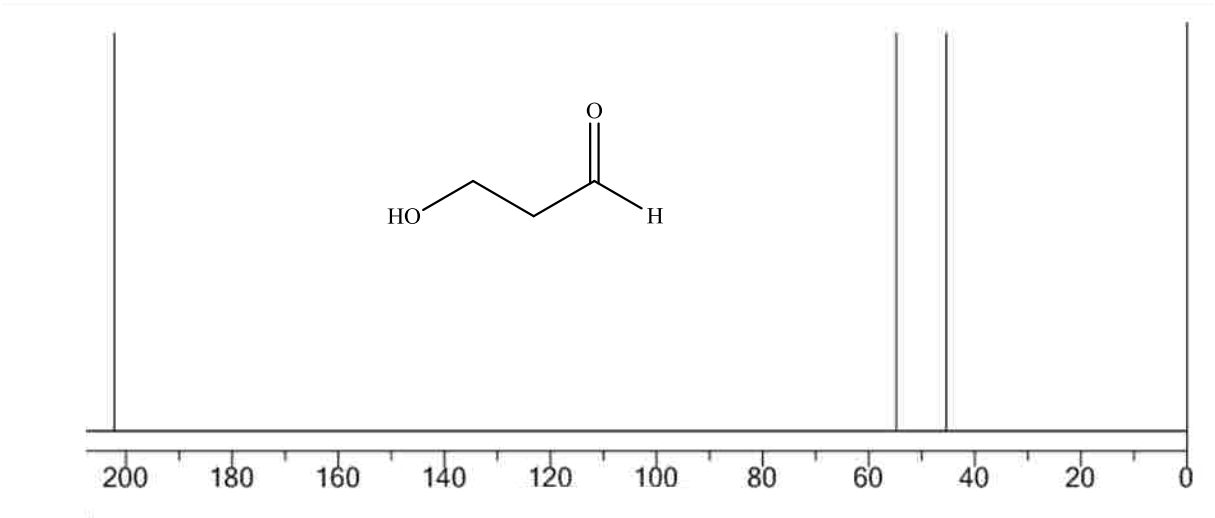


Figure D. 12 ^{13}C NMR for precursor of compound 5.3.

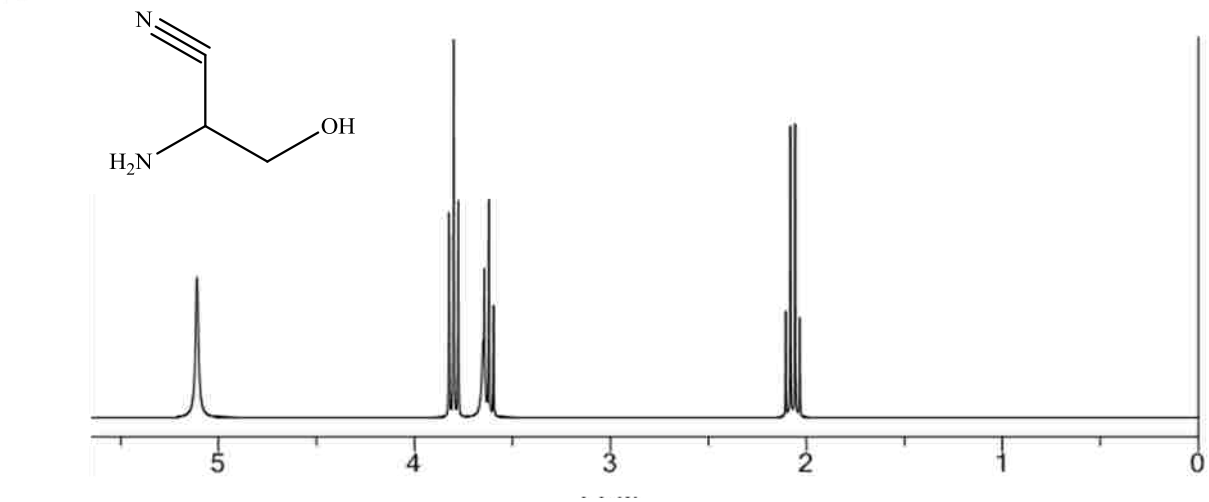


Figure D. 13 ^{1}H NMR for precursor of compound 5.4

Figure D. 14 ^{13}C NMR for precursor of compound 5.3.

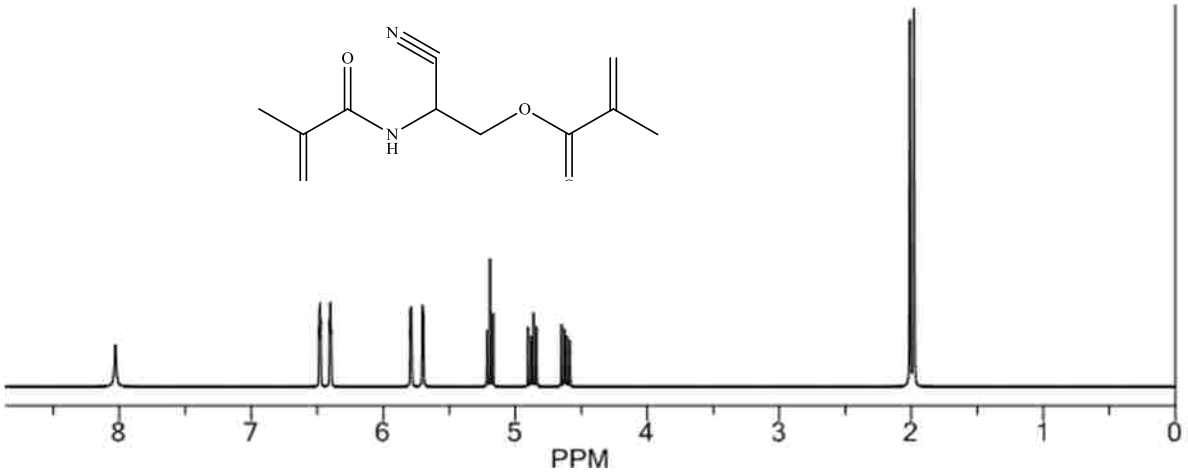


Figure D. 15 ^{1}H NMR for precursor of compound 5.4

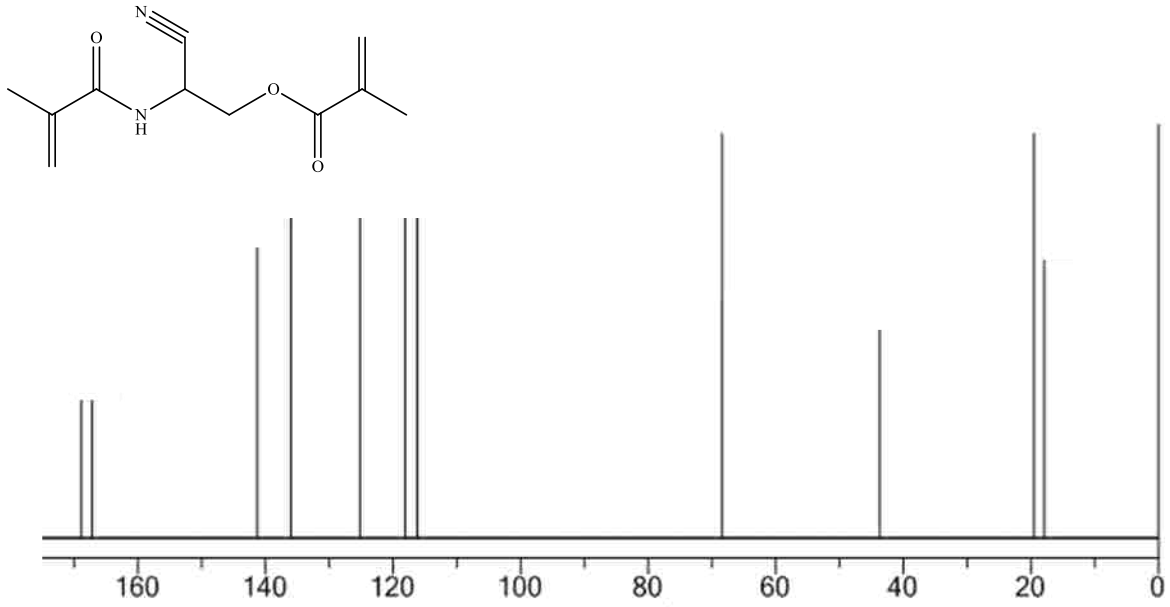


Figure D. 16 ^{13}C NMR for precursor of compound 5.4

Figure D. 17 ^{1}H NMR for compound 5.4

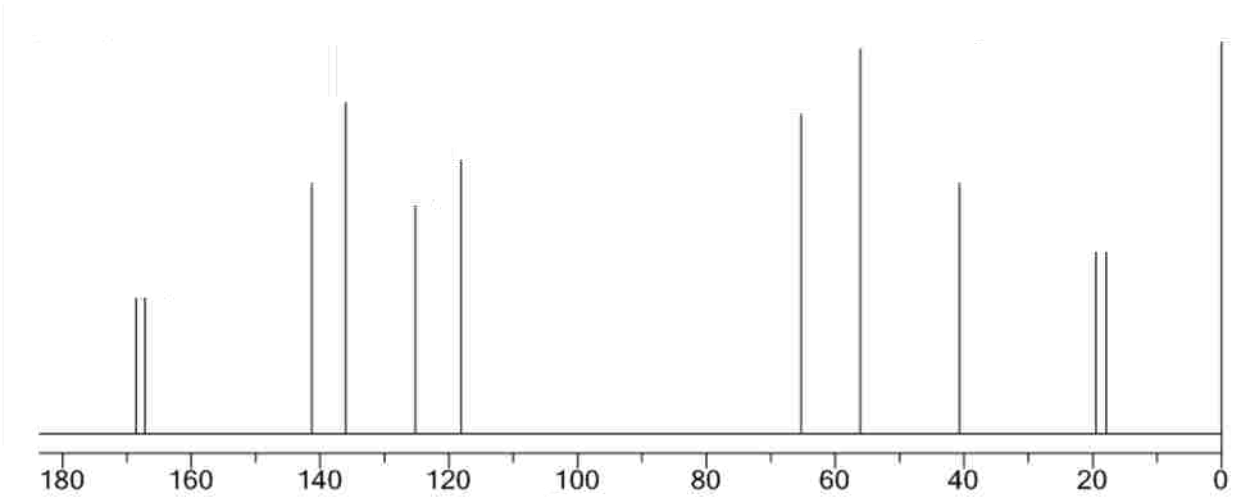


Figure D. 16 ^{13}C NMR for compound 5.4

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Jason Paul LeJeune was born in Welsh, Louisiana. He earned his Bachelor Degree, majoring in chemistry at McNeese State University in 2003. Jason went on to pursue a doctoral degree in chemistry in 2004 at Louisiana State University, working under the mentorship of Dr. David Spivak in developing novel molecularly imprinted polymers. Jason has participated in numerous regional, national and international meetings presenting his research results and garnered awards. He was the recipient of Hoffmann- LaRoche Younger Investigator Award during the Affinity 2007 in New York.

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