2015•2016
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Advanced MIPs for pesticide detection

Promotor :
Prof. dr. Thomas JUNKERS

Geoffrey Stijfs

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Foreword and Acknowledgements

I never expected that I would end up doing a master thesis in the scientific environment of *Organic* and *Bio-Polymer* Chemistry (*OBPC*) when I started my study of Biomedical Sciences. But as the years passed I wanted to see other sides of research, different from cell studies and clinical laboratory work. Throughout my senior training it became clear that it was a good decision. The experience I gained, whether it be in the field of laboratory work, scientific thinking, experimental conduct or analysis, is priceless. I feel I have grown as a scientist and as a person throughout these past few years.

I have a couple of people to thank for that, starting with prof. dr. Thomas Junkers. He did a great job of keeping us interested while at the same time training us to become excellent scientific researchers and speakers. Secondly, the people of OBPC are so nice. They made my time as a senior intern very enjoyable. Gijs, Stephan, Joachim, Jorne, Ruben, Dries, Roald and Wouter all come to mind. But even more so I want to express my gratitude to Erika for having patience with me and my somewhat chaotic way of conduct and showing me how to conduct correct research. Lastly, I have to mention Jeroen Vrijsen, my colleague senior intern and friend since starting the master study. His undeniable ability to relativize while still maintaining his perfectionistic nature is outrageously enviable.

None of this would have been possible though if it was not for the support of my friends, parents and beautiful girlfriend. Mom, dad, I love you. Baby brother, I love you. Robin old pal, you know it. Jos, my neighborhood buddy, you know it too. But most of all Julie, who made me believe in myself and give it my all, showed me that I can be whatever I want to be with focus and dedication. I love you.

And with that I think everybody will be smothered in love for the rest of this thesis. However, there is someone I may have forgotten to thank. That would be you, the person reading this document right now. Thank you for taking the time to read my thesis. I hope you enjoy it and that it is to your satisfaction.

List of abbreviations

Synthetic Organic Compounds:	SOCs
Neonicotinoids:	neonics
Vlaamse Milieu Maatschappij:	VMM
Predicted No Effect Concentration:	PNEC
wastewater treatment plants:	WWTP
Molecularly Imprinted Polymers:	MIPs
Free Radical Polymerization:	FRP
Dichloromethane:	DCM
Methacrylic acid	MAA
Ethylene glycol dimethacrylate:	EGDMA
Non-Imprinted Polymer:	NIP
N,O-bis(methacryloyl)ethanolamine:	NOBE
Azobisisobutyronitrile:	AIBN
N,O-bis(methacryloyl)threonine:	threonine-NOBE
Batch Rebinding:	BR
Phosphate Buffered Saline:	PBS
Diethylether:	Et_2O
Saturated:	Sat.
Porcine Pancreas Lipase:	PPL
Methanol:	MeOH
Parts per million:	ppm
Parts per billion:	ppb
Sodium chloride:	NaCl
Ammoniumchloride:	NH_4Cl
Sodium hydrogen carbonate:	$NaHCO_3$
Disodium hydrogen phosphate:	Na_2HPO_4
Chloroform:	$CHCl_3$
Thionylchloride:	$SOCl_2$
4-methoxyphenol:	MEHQ
Glacial acetic acid:	CH_3COOH

Abstract

In 60% of the tested water sites in Flanders imidacloprid, the most used neonicotinoid insecticide, exceeds the predicted no effect concentration. Imidacloprid is harmful to the environment but no efficient purification procedure exists to date. Molecularly Imprinted Polymers (MIPs), highly sensitive materials with cavities formed by non-covalent interactions that are complementary to the target, are ideal for these purification purposes. These cavities are specific due to the hydrogen bonds between target and matrix and due to the fact that they are an exact match to the target. Unfortunately, exact imprints can only be formed if enough functionalities are present in the MIP matrix. These functionalities are mostly provided by monomers, but crosslinkers are needed to ensure matrix stability. We hypothesized that MIPs synthesized with functionalized crosslinkers would improve binding efficiency versus conventional systems through incorporation of more functionalities in MIPs.

To achieve this goal, N,O-bis(methacryloyl)ethanolamine (NOBE) and threonine-NOBE, a NOBE analogue with an extra acidic functionality, were synthesized through adjusted literature procedures. MIPs were synthesized with these compounds and their binding efficiencies compared with ethylene glycol dimethacrylate and methacrylic acid MIPs, more conventional MIP building blocks. The impact of using functional crosslinkers on specific binding efficiency was evaluated by static absorbance experiments (figure 1).

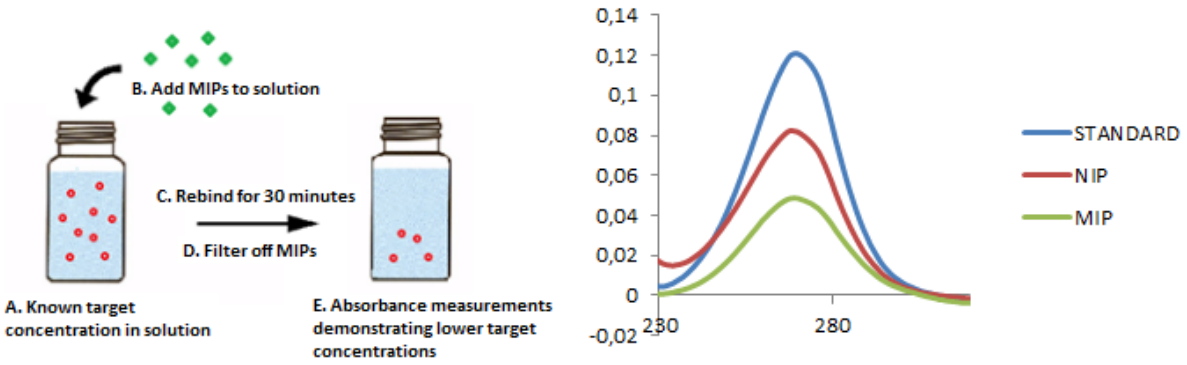


Figure 1: Schematic representation of an absorbance experiment.

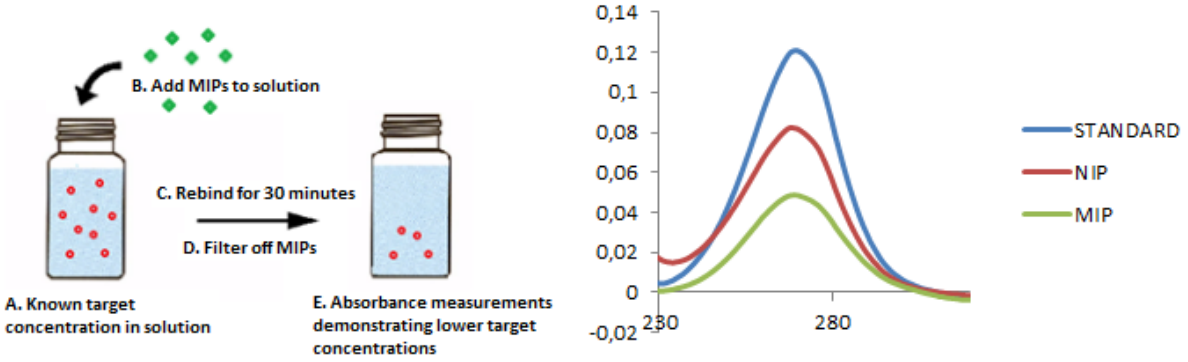
During this thesis, NOBE was successfully synthesized and isolated with a yield of 30%. Threonine-NOBE was synthesized in a two-step synthesis route with low overall conversion of around 10%, but could not be isolated yet.

NOBE MIPs were successfully synthesized and their binding efficiencies evaluated with respect to the conventional EGDMA/MAAMIPs. In conventional systems, no significant difference in binding was observed between MIP and NIP in acetonitrile or buffered solution. NOBE-MIPs do show specificity towards imidacloprid in acetonitrile, but not in buffered solution. It would seem that the use of functional crosslinkers can increase the specific binding efficiency of MIPs towards imidacloprid. However, to ascertain the validity of this statement and ensure the possibility of future widespread employment of functional crosslinkers in MIPs, it is imperative to do the same studies with other template molecules. Regardless, functional crosslinkers have the potential to become great assets in the road to increasingly better sensing devices.

Samenvatting

In 60% van de geteste watersites in Vlaanderen wordt imidacloprid, het meest gebruikte neonicotinoïde insecticide, teruggevonden in concentraties die hoger zijn dan de voorspelde concentratie zonder effect. Imidacloprid is schadelijk voor het milieu maar tot op heden is er geen efficiënte zuiveringsprocedure beschikbaar. Molecularly Imprinted Polymers (MIPs) zijn zeer specifieke materialen die beschikken over target-specifieke bindingssites als gevolg van een polymerisatie van monomeer en crosslinker rond een target dat gefixeerd is door non-covalente interacties. De gevormde caviteiten zijn zo specifiek omdat ze exact overeenkomen met het target. Helaas kunnen exact overeenkomende caviteiten enkel gevormd worden als er voldoende functionaliteiten aanwezig zijn in de MIP matrix. Deze functionaliteiten zijn afkomstig van monomeren, maar zij zijn weinig vertegenwoordigd in MIPs omdat er veel crosslinker ingebouwd moet worden om de stabiliteit van de matrix te verzekeren. Er werd dus een hypothese gesteld dat MIPs bestaande uit functionele crosslinkers een betere bindingsefficiëntie zouden vertonen ten opzichte van conventionele systemen.

Teneinde deze stelling te bewijzen werden N,O-bis(methacryloyl)ethanolamine (NOBE) en threonine-NOBE, een NOBE analoog met een extra zuurgroep, gesynthetiseerd volgens aangepaste literatuur en gebruikt in MIP synthese. Hun bindingsefficiëntie werd beoordeeld en vergeleken met [(ethyleen glycol dimethacrylaat)-co-(methacrylzuur)] MIPs. Bindingsefficiëntie werd beoordeeld door middel van statische absorbantie metingen (figuur 1).



Figuur 1: Schematische voorstelling van een absorbantie experiment.

Tijdens deze thesis werd NOBE succesvol gesynthetiseerd en geïsoleerd met een rendement van 30%. Threonine-NOBE werd gesynthetiseerd in een tweestapsreactie met een lage conversie rond 10%, maar kon nog niet gezuiverd worden.

NOBE MIPs werden gesynthetiseerd en hun bindingsefficiënties geëvalueerd op basis van de conventionele EGDMA/MAA MIPs. De conventionele systemen vertoonden geen specifieke binding in acetonitrile en buffer. NOBE systemen deden dat wel in acetonitrile, maar niet in buffer. Het lijkt er dus op dat het gebruik van functionele crosslinkers de specifieke binding van MIP voor imidacloprid kan verhogen. Echter, om deze stelling te valideren en de mogelijkheid tot algemeen gebruik van functionele crosslinkers in MIPs te verzekeren dienen dezelfde studies uitgevoerd te worden met andere target molecules. Ongeacht bezitten functionele crosslinkers de potentie om onmisbaar te worden op de weg naar steeds betere sensor applicaties.

Table of contents

Chapter I – Introduction

1 *Pesticides in surface water*

The past few decades, the quality of surface and ground water has improved. This is one of the rare successes in the field of environmental management. The restriction of the use of several synthetic organic compounds (SOCs) has contributed greatly to this observed improvement. However, the concentration of several upcoming contaminants, like third generation pesticides, is steadily rising (figure 2). This implies that current water purification is not extensive enough and needs to be addressed, especially when considering their risks. Several pesticides and their degradation products are already being monitored to determine the degree of contamination and water quality[1].

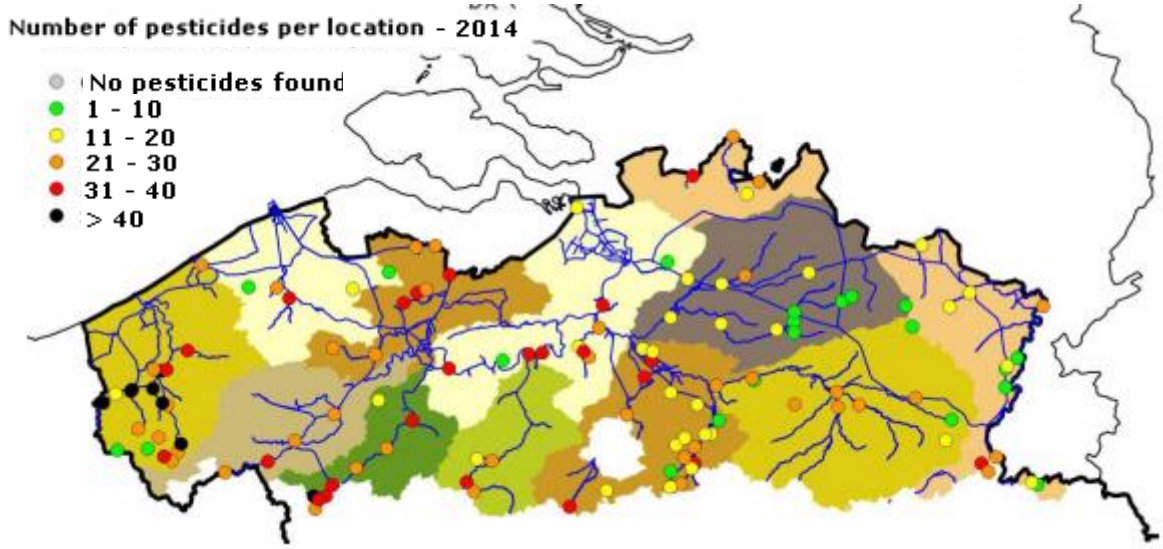


Figure 2: Pesticide contamination survey in Flanders, Belgium[1].

One specific group of third generation pesticides, the neonicotinoids or neonics, has been a controversial subject since 2006. Neonics were introduced for commercial use in the late 90's and have a chemical structure similar to nicotine. Neonics interact with certain receptors on the synapses of neurons much like nicotine. Overstimulation of the central nervous system by neonics is often fatal for small insects. A big advantage of neonics is their 'water solubility', allowing for the introduction of these products into irrigation water, which results in a widespread absorption into food crops, but also accumulation in ground water. Additionally, neonics persist in the environment. Their hazardous nature led to

extensive environmental monitoring by the VMM in 2014 to better assess neonic occurrence in Flanders.

When neonics were first introduced, they were considered a valuable third generation pesticide due to their low-toxicity towards bees and other beneficial insects. However, researchers have recently discovered that neonics can be harmful to bees through low level contaminations in pollen and nectar. Moreover, an environmental study by VMM[2] concluded that neonic contamination is more persistent than expected. One particular neonic, imidacloprid, is found in 80% of all tested measuring sites. In 70% of the cases the predicted no effect concentration (PNEC) was exceeded (figure 3).

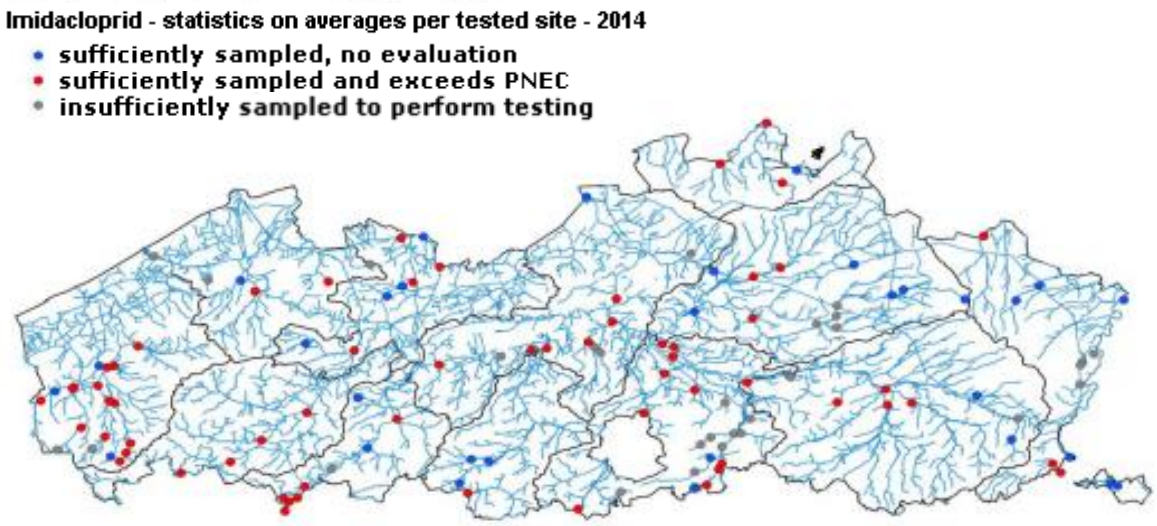


Figure 3: Evaluation of imidacloprid concentration in Flanders, Belgium[2].

Recently, VMM has also published a comparative study between influent- and effluent concentrations in wastewater treatment plants (WWTP), concluding that most pesticide products that were tested in this study were inadequately removed in the effluent from WWTP. This 'purified' water holds a significant share of pesticide pollution of surface waters in Flanders[1]. Hence, it is necessary to develop more efficient waste water purification for the pesticides that are not removed sufficiently, especially when taking into account that waste water from WWTPs eventually is released back into the water circulation, resulting in a steady increase of harmful pesticide compounds in waste- and drinking water alike.

2 *Molecularly Imprinted Polymers for wastewater treatment*

2.1 *Current water treatment*

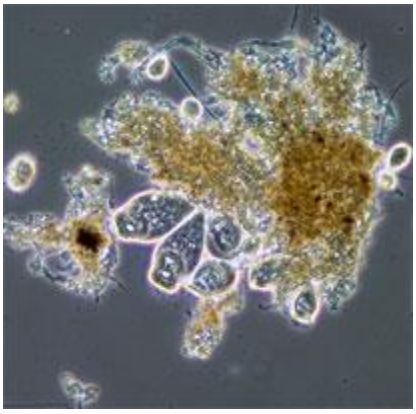


Figure 4: Activated sludge.

Today, waste water is purified biologically by employing a mass of microorganisms - better known as activated sludge - to degrade organic matter to carbon dioxide, water and other organic compounds (figure 4). Drinking water is purified with relatively cost efficient methods in the form of activated carbon [3], UV-desinfection, sedimentation and oxygenation. However, waste water is released into surface water after purification. This means that any contamination that is still present in waste water after purification will still end up in surface water.

Research by VMM revealed that effluent from WWTP still contains several SOCs in extremely low concentrations, usually around the parts per billion (ppb). Even though this seems negligible, international norms state that pesticides that remain in water with an average concentration above the PNEC have the potential to inflict chronic effects on the environment. This is the case for imidacloprid and in order to avoid it, different approaches need to be taken to further improve water quality. Carbon black cannot be used for the complete removal of persistent pesticide contaminants as it is not specific enough to detect them in these concentrations[4]. It is thus necessary to develop more personalized, specific materials for these compounds. The required properties for such a material can be found in Molecularly Imprinted Polymers (MIPs).

2.2 *Molecularly imprinted polymers*

MIPs are 3-Dimensional, crosslinked, polymeric materials that are formed around target molecules, like a lock around a key (figure 5). The target molecule interacts with the monomer and crosslinker through hydrogen bonding, after which bulk polymerization of crosslinker and monomer solidifies and fixes the structure in place. After grinding of the polymerized material, the target molecules are washed out, resulting in a MIP with cavities that are complementary to the target molecule[5].

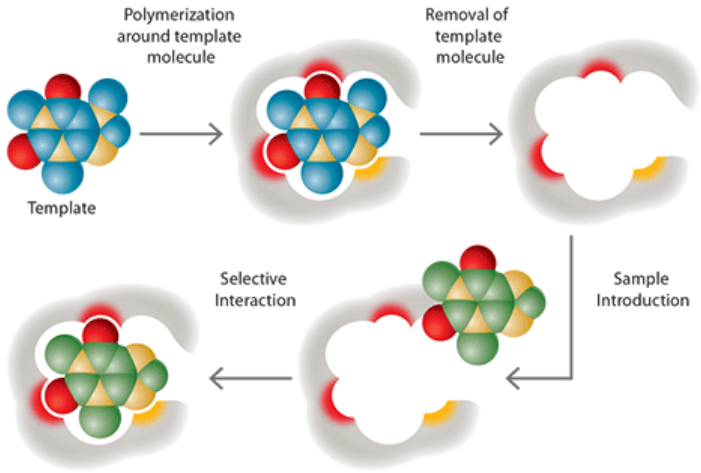


Figure 5: the principle of MIPs.

MIPs are most commonly produced with Free Radical Polymerization (FRP) because it is fast and compatible with a large range of components to be used in synthesis, in contrast with other methods. There are multiple other approaches that can be utilized for the synthesis of MIPs, all having an influence on the eventual properties of MIPs[7].

MIPs are highly versatile smart materials that have already shown their value in purifying- and detection purposes. Recent advancements in the area have demonstrated the use of MIPs in the recognition of organophosphorus- and triazine pesticides using MIP nanospheres and solid phase extraction MIPs respectively[8,9].

2.2.1 *Porogen*

The solvent used in MIP polymerization also influences their properties. Porogenic solvents are preferred in MIP synthesis as they will increase material porosity. It has also been experimentally determined that MIPs generally show the highest binding efficiency towards their target in the porogen that was used during polymerization. Even though the synthesized MIPs are destined for use in water based samples, their synthesis in water is not possible as it will lead to competition between water and the target molecule due to the strong tendency of water to form hydrogen bonds. Therefore, MIPs will be synthesized in porogens such as chloroform, dichloromethane (DCM) or acetonitrile. This will lead to an efficient formation of specific cavities for the target.

2.2.2 *Monomer*

In MIPs, monomers provide functionalities that undergo hydrogen bonding with target molecules, thus fixing the target for efficient formation of specific cavities during polymerization. Consequently, more monomer should equal more specific cavities in MIPs due to the presence of more functionalities.

The most common monomers employed in MIP synthesis are 4-vinyl pyridine (4-VP) and methacrylic acid (MAA; figure 6). The popularity of MAA comes from its potential to polymerize at the carbon-carbon double bond, thus maintaining its acidic functionality and hydrogen bridge forming capacity, an essential property of this monomer that ensures a good fixation of the target molecule in MIP synthesis.

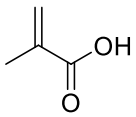


Figure 6: methacrylic acid.

2.2.3 *Crosslinker*

The benefit of using crosslinkers in MIPs is the fact that they ensure the formation of a rigid structure. Since MIPs deal with specific cavities, it is imperative that these cavities remain specific and will not lose their shape or orientation over time.

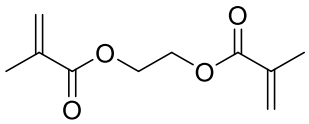


Figure 7: Ethylene glycol dimethacrylate.

[(ethylene glycol dimethacrylate)-co-(methacrylic acid); EGDMA-co-MAA] Is a well-known and studied MIP system, with EGDMA (figure 7) serving as crosslinker. Several studies with this system have shown high affinity towards the imprinted molecule as opposed to their non-imprinted polymer (NIP) counterparts[10].

Considering that the chance of forming a hydrogen bond between target and MIP component is dependent on the amount of functionalities in the polymerization mixture and the potential to form perfect imprints, more functionalities should equal more specific cavities. MIP systems studied today are mainly composed of crosslinkers that do not provide many functionalities[11]. However, it is necessary to incorporate this much crosslinker into the system, as it will ensure the stability of the specific cavities created. If a high percentage of crosslinker is used in the synthesis of MIPs, there will be less functional monomers available to form specific cavities. Conversely, if a low percentage of crosslinker is incorporated, a lot functionalities will be available in the system but the cavities will not be stable enough. It would thus seem that it is not possible to increase the amount of monomer in MIPs without decreasing the rigidity of the system.

2.2.4 *Functional crosslinker*

Functional crosslinkers are molecules that combine crosslinker and monomer. The benefit of this is the potential to create systems that are highly crosslinked, while maintaining a high degree of functionalization. These properties make functional crosslinkers ideal for use in MIP synthesis.

N,O-bis(methacryloyl)ethanolamine (NOBE) (figure 8) is such a functional crosslinker. Aside from the benefits functional crosslinkers provide, NOBE also has a hydrogen bridge donor in the form of the amide functionality, as opposed to two hydrogen bridge acceptors in the form of ester bonds in EGDMA. This could lead to more defined imprints of the target molecule. Lastly, the amide bond also serves to increase the stability of the matrix.

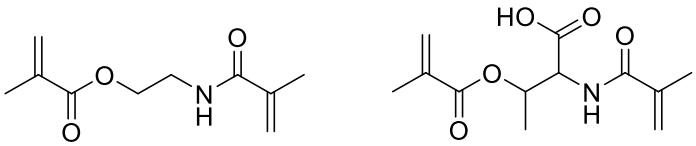


Figure 8: Structure of NOBE (left) and threonine-NOBE (right).

Literature has already shown that NOBE MIPs exhibit increased binding capacities towards several templates[12]. In order to potentially increase the selectivity and stability of the MIP system even more, a derivate of NOBE can be synthesized starting from the amino acid L-Threonine. The introduction of an extra functionality to NOBE through the synthesis of threonine-NOBE will result in more stable MIPs while maintaining or increasing the specificity of the matrix towards the target molecule.

Even though it seems promising that functional crosslinkers can be used to stabilize a MIP system while maintaining or even increasing the specific binding of it, very little is known on the properties and behaviour of the functional crosslinkers in MIP synthesis. For this thesis, it was hypothesized that threonine-NOBE MIPs would give the best target-MIP interactions and thus provide the optimal system for imidacloprid detection and extraction. To prove their better target-MIP interactions, their binding efficiencies were compared to conventional EGDMA-co-MAA MIPs.

Chapter II - Materials & Methods

1 *Materials*

Triethylamine (99%, 121-44-8), Methacryloylchloride (99%, inhibited with 200 ppm MEHQ, AcroSeal®, 920-46-7), $NaHCO_3$ (99.5%, 144-55-8), NaCl (7647-14-5), NH_4Cl (12125-02-9), Ethylene Glycol Dimethacrylate (EGDMA, 98%, 97-90-5) and Thionylchloride ($SOCl_2$, 99.7%, 7719-09-7) were bought from Acros Organics. Ethanolamine (141-43-5) was obtained from Tokyo Chemistry Industry CO., LTD. Dichloromethane (DCM, 75-09-2), Ethylacetate (141-78-6), Hexane (110-54-3), Hydrochloric Acid (HCl, ~37%, 7647-01-0), Acetonitrile (75-05-8), Chloroform ($CHCl_3$, 67-66-3), Glacial Acetic Acid (anal., 64-19-7) and L-Threonine (72-19-5) were acquired from Fisher Scientific UK. F-96 Greiner Bottom well plates, Diethylether (Et_2O, 60-29-7) and Methanol (MeOH, 67-56-1) were purchased from VWR Chemicals. Na_2HPO_4 (7558-79-4) Was obtained from Merck. Acetone (67-64-1), Methacrylic Acid (MAA, 99%, inhibited with 250 ppm MEHQ, 79-41-4), Azobisisobutyronitrile (AIBN, 98%, 78-67-1) and Aluminium Oxide (Al_2O_3, activated, basic, 1344-28-1) were procured from Sigma-Aldrich. Lastly, dry silica (LC 60A 70 – 200 micron chromatographic silica media, 14808-60-7) and Confidor systemic insecticide were purchased from Davisil® and Bayer: CropScience respectively.

All synthesized and extracted compounds were qualified with meltpoint determination using an Electrochemical IA900 Series Digital Meltpoint Apparatus and a Varian Inova spectrometer at 400 MHz for ^{1}H-NMR using a 5 mm OneNMR PFGprobe (Agilent Technologies Inc, Santa Clara, CA, USA). The synthesized MIP systems were pulverized *via* ball-milling (Fritsch Premium Line Pulverisette 7). Purification of the crushed MIPs was achieved with an Accelerated Solvent Extraction Device (Dionex ASE 350) or Soxhlet extraction. Batch Rebinding (BR) experiments were measured in a UV-VIS spectrophotometer (Agilent Technologies) at a range from 800 to 200nm. When the BR experiment was performed in buffer, the samples were measured in a microplate reader (FluoStar) with a sample volume of 150µL per well.

2 Methods

2.1 Imidacloprid extraction

Imidacloprid was precipitated from Confidor® Systemic Insecticide and recrystallized in acetone until pure. ^{1}H-NMR (δ/$CDCl_3$): 3.50 (t, 2H), 3.78 (t, 2H), 4.51 (s, 1H), 7.31 (d, 1H), 7.67 (dd, 1H), 8.17 (broad s, 1H), 8.28 (d, 1H). ^{13}C-NMR (δ/CDCl3): 161.81, 152.13, 149.85, 139.65, 130.34, 125.33, 45.88, 45.73, 42.06. t_{melt} = 143.8° C.

2.2 NOBE synthesis

To a 3-necked round bottom flask containing 83.35 mmol (5 mL) ethanolamine and 215.43 mmol (30 mL) triethylamine, a solution of 200 mmol (20 mL) methacryloylchloride in 150 mL dry DCM was added dropwise under inert atmosphere in an ice bath. After 2 hours, the precipitate was filtered off and the formed reaction products were extracted out of the remaining solution using water, $NaHCO_3$ (sat.), NH_4Cl (sat.) and brine, respectively. Afterwards, the product was isolated through column chromatography (Et_2O and 90:10 hexane:ethylacetate). ^{1}H-NMR (δ/$CDCl_3$): 6.09 (1H, s), 5.66 (1H, s), 5.57 (1H, s), 5.31 (1H, s), 4.28 (2H, t), 3.61 (2H, quad), 1.93 (3H, s), 1.91 (3H, s).

2.3 Threonine-NOBE synthesis

2.3.1 L-threonine α-methyl ester hydrochloric acid synthesis

219 mmol (20 g) of L-threonine was reacted with 550 mmol (40 mL) thionylchloride in MeOH for 75 minutes at room temperature, according to adjusted literature procedure[13]. Afterwards, L-threonine α-methyl ester hydrochloric acid was precipitated in cold diethylether. ^{1}H-NMR (δ/$CDCl_3$): 1.46 (3H, d), 3.84 (3H, s), 4.16 (1H, m), 4.33 (1H, q), 8.34 (3H, s).

2.3.2 N,O-bis(methacryloyl)threonine α-methyl ester synthesis

65.2 mmol (9.978 g) L-threonine α-methyl ester hydrochloride was dissolved in 100 mL DCM together with 176,62 mmol triethylamine. In a separate flask, 117.18 mmol MAA was dissolved in 50 mL DCM, together with 119.204 mmol (24.595 g) DCC. Both flasks were cooled to 0° C prior to adding one flask to the other in one fraction. Afterwards, 11.9 mmol DMAP was added and the reaction was allowed to run at 0° C for 30 minutes prior to letting it run at room temperature for 5 days. Lastly, the formed precipitate during the reaction was filtered off and threonine-NOBE α-methyl ester was purified by column chromatography using 75:25

hexane:ethylacetate as eluent. ^{1}H-NMR (δ/$CDCl_3$): 1.30 (3H, d), 1.88 (3H, s), 1.97 (3H, s), 3.70 (3H, s), 4.85 (1H, dd), 5.39 (1H, s), 5.43 (1H, m), 5.54 (1H, s), 5.57 (1H, s), 6.03 (1H, s) 6.41 (1H, s).

2.3.3 *N,O-bis(methacryloyl)threonine synthesis*

26.20 mmol (7.052 g) threonine-NOBE α-methyl ester underwent an oxidation reaction with 4 g Porcine Pancreas Lipase (PPL, 300 U/mg) in 150 mL 1x Phosphate Buffered Saline (PBS). The mixture was shaken for 6 days before filtration of PPL. After evaporation of the solvent, the product was redispersed in hexane to form a cloudy solution. After separation and evaporation, ^{1}H-NMR revealed the formation of threonine-NOBE, although isolation was not yet possible. ^{1}H-NMR (δ/$CDCl_3$): 1.30 (3H, d), 1.88 (3H, s), 1.97 (3H, s), 4.85 (1H, dd), 5.42 (1H, s), 5.44 (1H, m), 5.55 (1H, s), 5.76 (1H, s), 6.03 (1H, s) 6.46 (1H, s).

2.4 *Imidacloprid MIP/NIP synthesis*

2.4.1 *EGDMA-co-MAA MIPs*

0.5 mmol (255.2 mg) imidacloprid, 2 mmol (170 μL) MAA and 10 mmol (1.9 mL) EGDMA were dissolved in 3.5 mL acetonitrile. Upon dissolution, 0.06 mmol (10 mg) AIBN was added to the solution and the mixture was purged with nitrogen for 5 minutes before allowing it to polymerize at 68° C for 24 hours. The corresponding NIP systems were synthesized exactly the same, but in the absence of imidacloprid. Afterwards, the MIP systems were ground at 500 rpm for 150 seconds and purified in a Dionex ASE 350 at 50° C under elevated pressure. The rinsing volume per cycle was set to 20 mL and each system underwent 4 cycles with 5% acetic acid in MeOH, 8 cycles with 5% acetic acid in acetonitrile and 8 cycles with MeOH, ensuring complete purification.

2.4.2 *NOBE-co-MAA MIPs*

NOBE-co-MAA systems were synthesized following the same general procedure as EGDMA-co-MAA MIPs, with 0.25 mmol (127.6 mg) imidacloprid, 1 mmol (85 μL) MAA, 5 mmol (965 mg) NOBE and 0.03 mmol (5mg) AIBN. NOBE MIPs were ball-milled at 300 rpm for 150 seconds and purified using soxhlet extraction with 50:50 CH_3COOH:MeOH for 5 days followed by MeOH for 3 days.

2.5 *BR experiments*

2.5.1 *Experiments in organic solvents*

5 mg of polymer was introduced in a 5 mL solution with a known concentration of target molecule. After 30 minutes, the polymer was filtered off and the target concentration of the residual solution was determined with UV-VIS spectrophotometry.

2.5.2 *Experiments in buffer*

1 mg polymer was added to 1mL of phosphate buffer (1mmol, pH 7) with a known target concentration. After 30 minutes, the MIPs were filtered off and the supernatans was measured in the FluoStar microplate reader.

Chapter III – Results & Discussion

1 *NOBE synthesis*

According to literature, MIP systems to date are not composed of enough functionalities to ensure a high enough homogeneity of specific cavities throughout MIP matrices solely through non-covalent interactions. It would thus be necessary to covalently attach templates to functional monomers. However, this is not possible for most templates. Additional functionalities can potentially solve this imprinting problem[13]. Therefore, functional crosslinkers were introduced in MIP synthesis[14]. The functional crosslinker used in this study was NOBE, a relatively known crosslinker that has already been tested in literature. However, it has never been tested in a pesticide detection system. Moreover, the synthesis of NOBE poses a lot of problems in terms of exothermicity and self-polymerization. Also the differing reactivities of both functionalities of the starting product (ethanolamine) can lead to side products and thus lower yields.

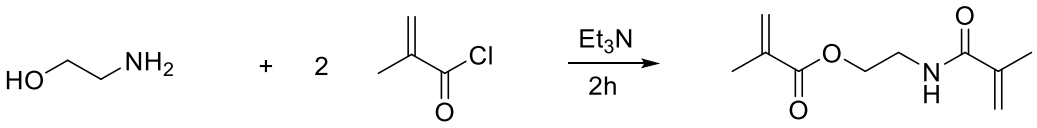


Figure 9: Schotten-Baumann reaction for NOBE synthesis.

1 eq. of ethanolamine reacted with 2 eq. of methacryloylchloride in the synthesis of NOBE (figure 9). Et_3N was added to the mixture to form $Et_3NH^+Cl^-$ salt in order to avoid the Micheal addition of HCl to the methacrylate.

Different reaction conditions were tested in an attempt to optimize NOBE synthesis (table 1). Shortening the reaction time from 48 hours to 2 hours resulted in equal NOBE yields (± 30%) after isolation. This is possibly due to the high reactivity of methacryloylchloride, causing the reaction to end in several hours. It is probably also possible to continue with the work up of the reaction immediately after all reagents have been added to the mixture, but the decision to wait at least 2 hours was taken because it ensures complete reaction of ethanolamine with methacryloylchloride and its impurities.

Dilution and subsequent dropwise addition of methacryloylchloride to the reaction mixture was attempted in order to counteract the exothermicity of the reaction and in that way avoid side product formation, but did not increase yields. The low yields are in line with literature findings[15] (± 30%) and can be ascribed to the abundance of side products that are being formed during the synthesis. These side products partially come from methacryloylchloride. This product was only 75% pure when used. The impurities in the bottle were methacryloylchloride derivates (determined *via* ^{1}H-NMR, figure 10). All derivates still possess an acid chlorine functionality and will thus also react with ethanolamine, NOBE synthesis will result in a mixture of around 10 products. The high amount of impurities in the bottle of methacryloylchloride is due to exposure to water in the air over time.

Table 1: Tested reaction conditions in the optimization of NOBE synthesis.

Reaction time (h)	Temperature (°C)	Isolated Yield (%)
0	RT	27.60
0	40	28.00
2	RT	27.92
2	40	26.80
24	RT	28.20
24	40	27.43
48	RT	25.00
48	40	25.26

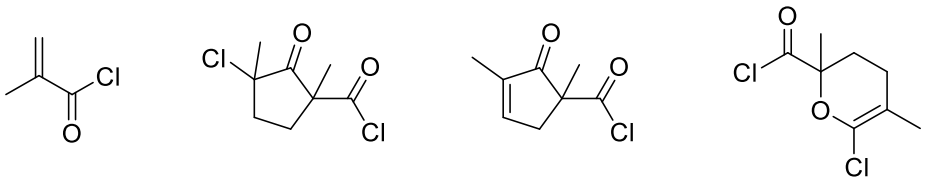


Figure 10: different compounds formed by methacryloylchloride in bottle.

2 *Threonine-NOBE synthesis*

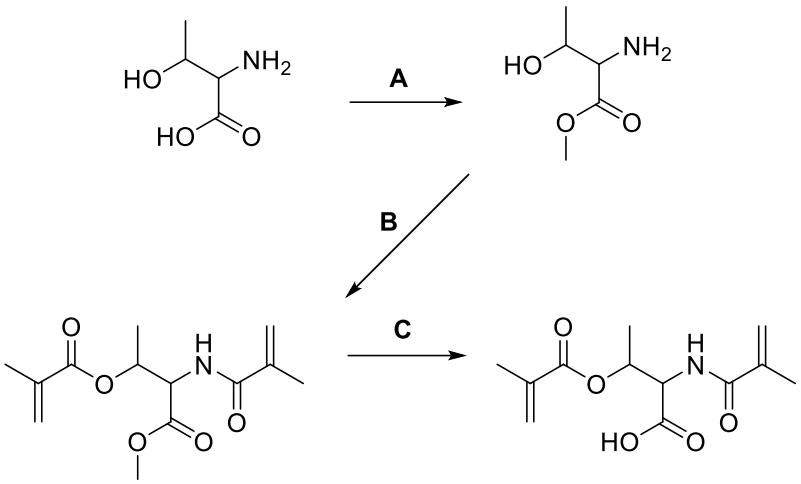


Figure 11: Threonine-NOBE synthesis. A. $SOCl_2$ in MeOH, 75 minutes reaction time; B. Reaction with 2 eq. MAA and Et_3N in DCM, in DCC/DMAP mechanism, 5 days reaction time; C. enzymatic oxidation with PPL in 1x PBS, 6 days reaction time.

In order to create even more functionalities for good imprinting on the crosslinking molecules, threonine-NOBE was synthesized (figure 11). This has never been reported in literature, but a general synthesis pathway to threonine-NOBE, starting from L-threonine, is DCC/DMAP coupling. The mild nature of DCC/DMAP coupling reactions, combined with the uptake of any released water by DCC and the ability to perform the reaction at room temperature makes it an ideal approach to synthesize molecules that self-polymerize easily[16]. However, if DCC/DMAP were to be performed on L-threonine, the α-carboxylic acid of L-threonine would react with the alcohol functionality on another L-threonine molecule, which results in polymerization. To prevent this, an esterification of the α-carboxylic acid was first performed.

The synthesis of L-threonine α-methyl ester was first attempted with an Amberlyst-15 catalyst according to literature procedure[17], as it was described as a mild method for the esterification of amino acids with minimal work up. However, post workup, no esterification could be observed in ^{1}H-NMR. In a second reaction, L-threonine was dispersed in MeOH and $SOCl_2$ was added dropwise to the mixture due to its harmful nature (figure 11; **A**). $SOCl_2$ will form an acid chlorine functionality on L-threonine, making it very reactive. This functionality will immediately react with MeOH, forming HCl (g) and L-threonine α-methyl ester in the process. After stirring for 75 minutes, L-threonine α-methyl ester hydrochloric acid was obtained in 70% isolated yield.

Subsequently, DCC/DMAP coupling with MAA and Et_3N in DCM was performed (figure 11; **B**). MAA was used in this reaction instead of methacryloylchloride due to the higher purity of MAA versus the acid chlorine. Regardless, the DCC/DMAP coupling for the synthesis of threonine-NOBE α-methyl ester was successfully achieved with 46% isolated yield.

Afterwards, the α-carboxylic ester group of threonine-NOBE α-methyl ester was removed through enzymatic oxidation with Porcine Pancreas Lipase (PPL; figure 11; **C**). PPL was used because it was shown to be the most selective towards the α-carboxylic ester group instead of the ester bond originating from the alcohol functionality of L-threonine[15]. ^{1}H-NMR spectra show around 30% conversion to threonine-NOBE. A complete scheme of threonine-NOBE synthesis can be found in figure 11.

Even though threonine-NOBE α-methyl ester was successfully converted to threonine-NOBE, it was not possible to isolate it as it had become insoluble in any conventional solvent after work up. The insolubility can be explained by autopolymerization which results, in the case of threonine-NOBE, in a crosslinked structure.

The overall conversion of L-threonine to threonine-NOBE comes down to 9.66%. This is mainly due to the fact that the synthesis pathway consists of a protection step, coupling- and deprotection step. Three work up and isolation procedures result in the loss of a large amount of product. The enzymatic oxidation to threonine-NOBE is also yield-inefficient, perhaps due to loss of activity of the enzyme through prolonged exposure to suboptimal conditions during oxidation.

It clear that multi-step synthesis of threonine-NOBE is not efficient enough and needs to be optimized further in an attempt to increase overall conversions. Besides optimization, different approaches towards threonine-NOBE synthesis, such as single-step synthesis, can be explored. A single-step synthesis pathway for threonine-NOBE eliminates intermediary work-up procedures and should result in higher yields. Increasing the number of enzymatic oxidation cycles to maximize conversion could also serve to increase yields.

3 *Imidacloprid MIP/NIPs*

To investigate the potential benefit of NOBE in MIPs with imidacloprid as target molecule, binding efficiencies of NOBE MIPs were evaluated in comparison to conventional EGDMA-co-MAA systems. Not only does EGDMA closely resemble NOBE, it is also a well-studied crosslinker in MIP synthesis and evaluation. Hence, it can serve as a guideline for the evaluation of NOBE in MIPs.

To achieve a good comparison of conventional versus novel systems, first, the optimal rebinding parameters for EGDMA/MAA based MIPs for imidacloprid were tested through BR experiments. Once these parameters were known, they could be extrapolated to NOBE systems to evaluate their performance under the ideal conditions for EGDMA/MAA systems.

To date, several MIP systems have been evaluated in their potential to extract pesticides from contaminated samples. EGDMA/MAA systems usually show good specific binding towards the target pesticide[18,19]. However, little research has actually been performed on imidacloprid as a target molecule in MIPs[19]. Also, the binding efficiency of studied systems was usually determined with MIP solid phase extraction cartridges, with an additional washing step prior to quantification of binding efficiencies. It is possible that this washing step can remove loosely bound targets, which can result in higher specific binding. This, however, has not been experimentally confirmed yet.

In this thesis, binding efficiencies were evaluated through static absorption experiments, better known as batch rebinding experiments (BRs). A BR (figure 12) entails the introduction of the polymer matrix into a sample with known target concentration. The polymer will bind part of the target and after filtration of the polymer, the residual solution should show a reduced absorbance in spectrometry. Ideally, absorbance of samples should decrease more for MIPs than for NIPs, as MIPs have specific cavities and should thus bind the target more efficiently. The degree with which absorbance decreases after a BR gives information on how much target was actually taken up by the polymers. The point of doing the quantification of binding efficiencies in this way is that there is no additional washing step involved, thus maintaining most aspecific bonding that occurs between matrix and target molecule as well. This quantification technique will be more trustworthy in future "insert and measure" applications.

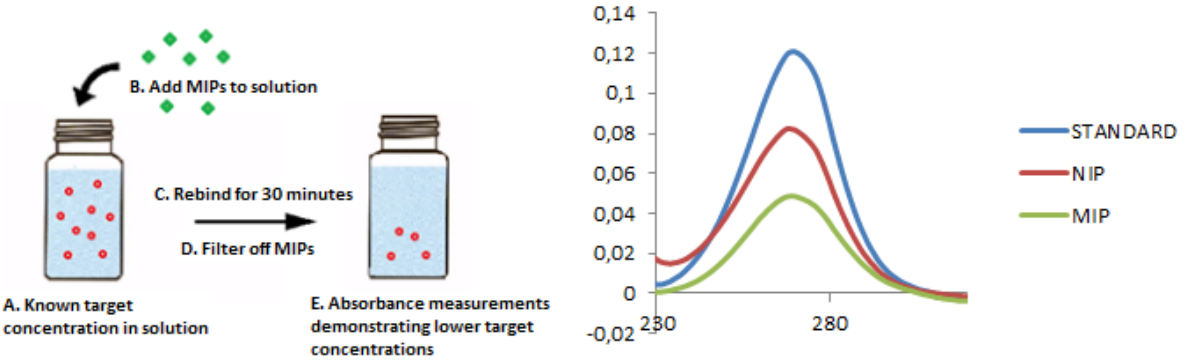


Figure 12: Schematic representation of an absorbance experiment.

Lastly, even though NOBE and threonine-NOBE are a central topic in this thesis, the efficiency of NOBE MIPs was compared to EGDMA/MAA MIPs. Therefore, EGDMA/MAA MIPs were evaluated first with regards to their optimal rebinding times and binding efficiencies in various solvents.

3.1 *EGDMA-co-MAA MIPs*

In order to obtain more information on the ideal rebinding times for EGDMA/MAA MIPs with imidacloprid as a target, a BR was performed for different samples with equal target concentrations and rebinding times varying from 30 minutes to 8 hours. The results (figure 13) demonstrated a significant difference in binding efficiencies between MIP and NIP towards imidacloprid in rebinding times of 30 minutes and 1 hour, but no difference in binding efficiencies at longer rebinding times. It was believed that the instalment of an equilibrium between bound and unbound states over time was responsible for the observed trend. The decision was thus made to perform a repeat experiment *in triplo* of short rebinding times (figure 14) to validate this theory.

Surprisingly, the repeat experiment revealed that there was no better binding efficiency for EGDMA/MAA MIPs than NIPs for short rebinding times. Hence, it would seem that the instalment of an equilibrium is not responsible for the observed data. Another possibility is that most of the interactions between MIP and target molecule occur with the matrix instead of the specific cavities and these interactions are the cause for the similar binding efficiencies between MIP and NIP.

Another possibility is that there simply are no specific cavities in this system. These possibilities all need to be investigated further before drawing any conclusions though, as they are based on too little data. Regardless, since these materials are

destined for sensing applications, short rebinding times of 30 minutes were chosen for further tests.

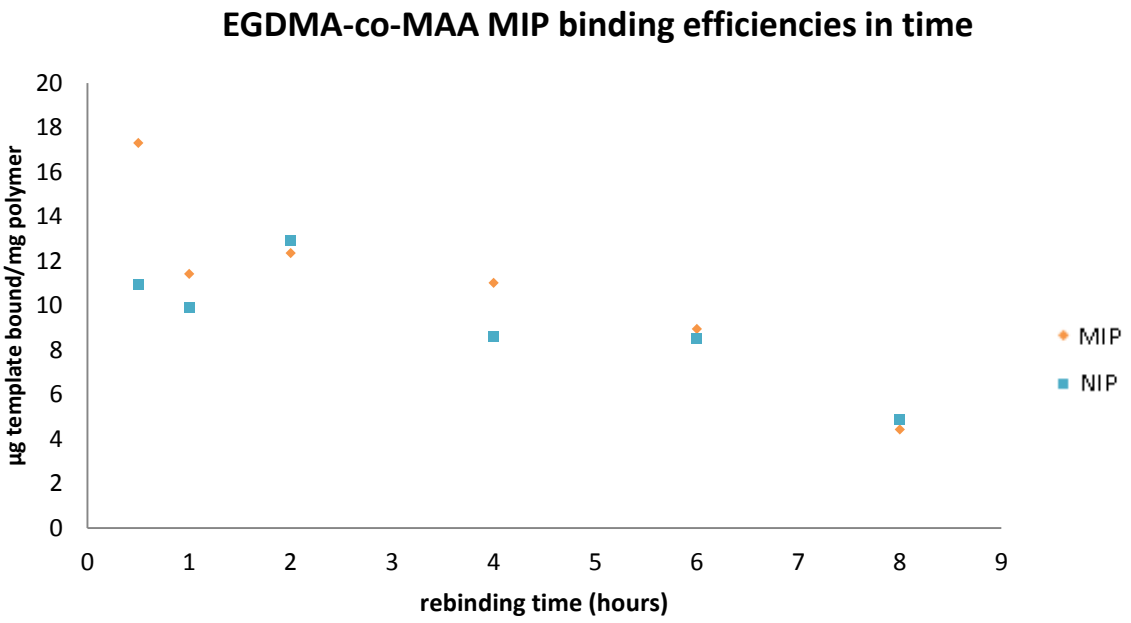


Figure 13: EGDMA-co-MAAMIP/NIP binding efficiencies in time.

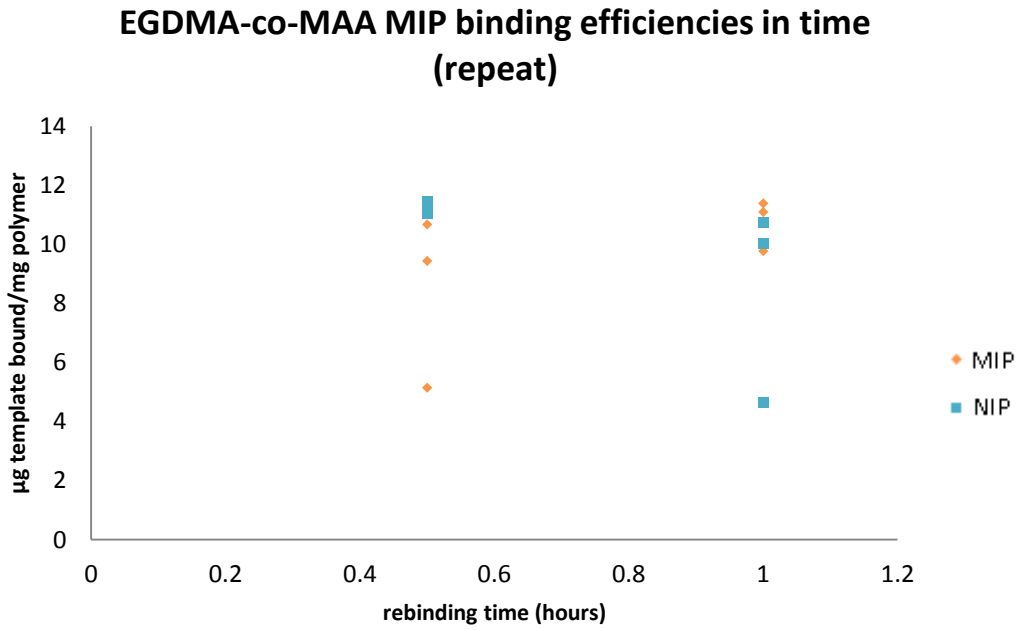


Figure 14: *in triplo* repeated BR experiment for binding efficiency in time.

To determine the detection- and saturation limits of EGDMA-co-MAA MIPs, BRs for differing target concentrations were performed in acetonitrile (figure 15) and buffer (figure 16). No significant difference was observed in binding efficiencies between MIP and NIP in acetonitrile nor buffer. However, the data points for EGDMA/MAA binding efficiencies in buffer (300 – 500 ppm) may indicate MIP binding efficiency is slightly better than NIP. However, this cannot be confirmed on one experiment and this test will have to be repeated to exclude the possibility of a scatter.

It is also possible that 300 ppm is the concentration threshold at which the specific cavities of the MIP are saturated and the target molecule starts binding on the MIP matrix. This, combined with the possibility that the NIP matrix starts saturating at this target concentration, would explain the increase of target uptake by the MIPs above a concentration threshold of 300 ppm. However, NIPs do not show signs of saturation in the data, with steadily increasing binding efficiencies with increasing target concentration. Then again, it is possible that the scatter of the data is too high to observe the saturation of NIPs. to gain more security on these conclusions, repeat experiments should be performed to reveal trends in the data that cannot be deduced from one data set.

A potential explanation for the fact that specific binding also does not occur in buffer based samples is the fact that imidacloprid does not favour aqueous environments. This implies that imidacloprid will bind to MIP and NIP, regardless of specific cavities, as it will seek out the most favourable environment. It is not the polar solvent that removes specific binding; it is the apolar nature of the target molecule in a polar solvent that obscures the specific binding, as it will associate with any matrix that is less polar than the solvent. For example, NIPs bind target aspecifically while MIPs bind target specifically. At a certain concentration, both NIP and the specific cavities of the MIP are saturated, but MIP can still bind target on its matrix, while NIP cannot anymore. MIP binding efficiencies will thus continue to rise with increasing target concentrations. In this way specific binding is obscured but it still occurs. However, this theory has to be thoroughly investigated before accepting it.

It has become clear that EGDMA-co-MAA MIPs are not specific enough at low target concentrations to be useful in detection- and extraction devices for imidacloprid.

However, comparative data has been obtained and the step towards MIP systems composed of the functional crosslinker NOBE can be made.

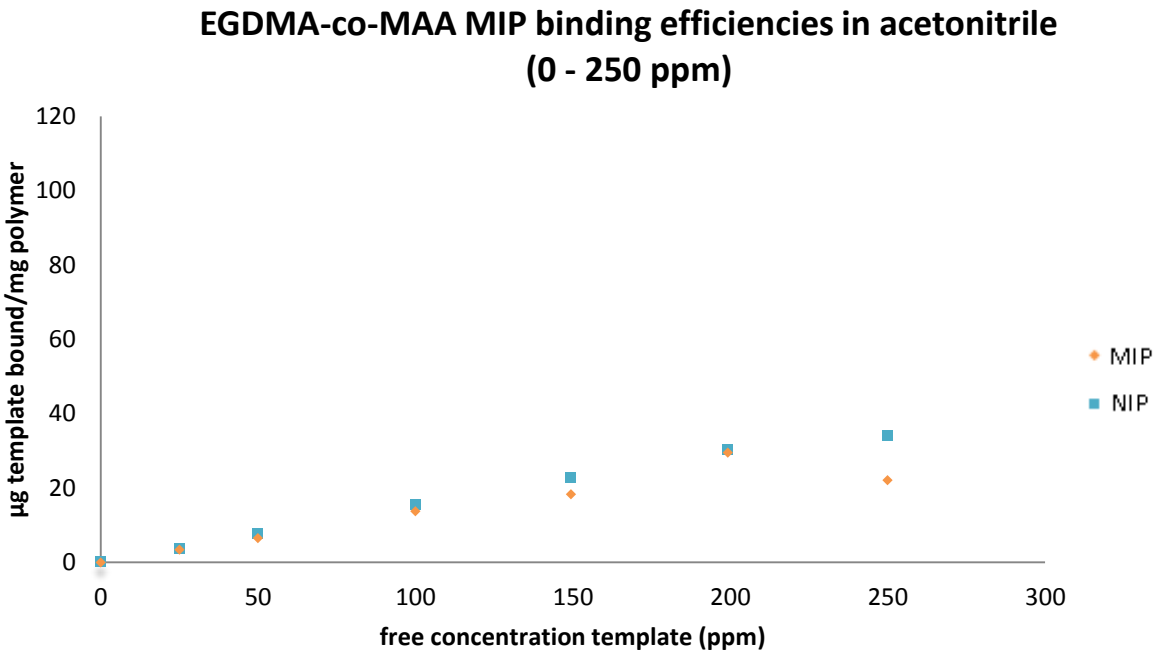


Figure 15: EGDMA-co-MAA binding efficiencies in acetonitrile.

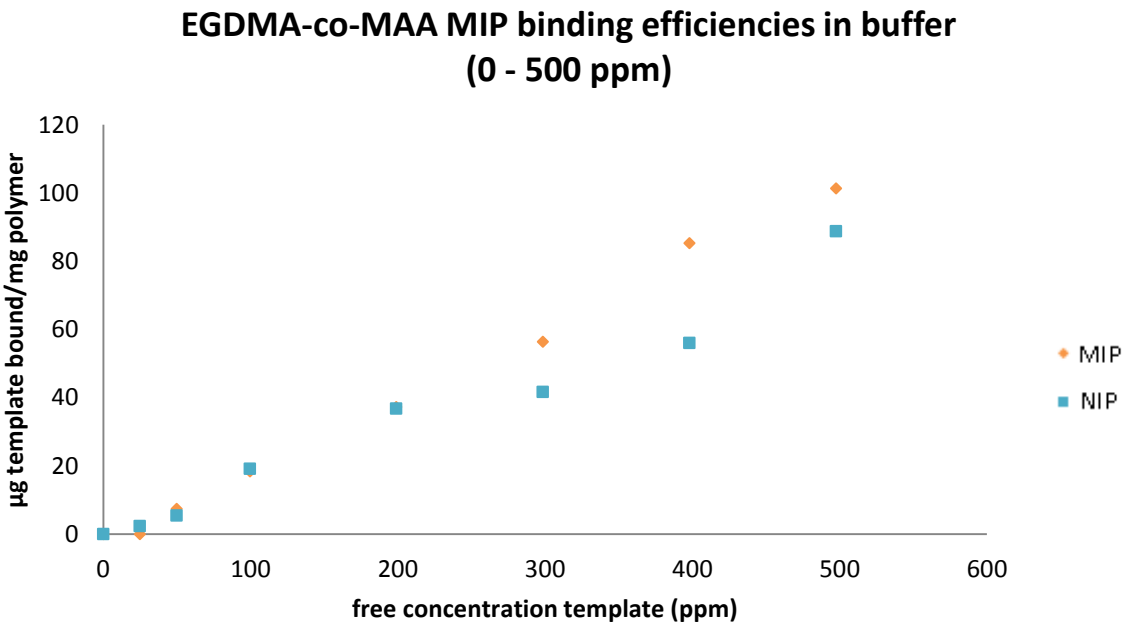


Figure 16: EGDMA-co-MAA binding efficiencies in buffer.

3.2 *NOBE MIPs*

BRs of NOBE systems were performed at 30 minute rebinding times. This time was chosen with respect to BRs performed for EGDMA/MAA and the fact that BRs of both systems are intended for comparison. Therefore, as much conditions as possible were kept equal. NOBE MIPs were tested for their binding efficiency towards imidacloprid in acetonitrile (figure 17) and buffer (figure 18), in varying concentration ranges.

For BRs in acetonitrile, it was observed that NOBE MIPs bind imidacloprid equally well as EGDMA-co-MAA MIPs with binding efficiencies up to 50 µg/mg polymer at a free target concentration of 750 ppm. However, a big discrepancy can be observed between NOBE MIP and NIP binding efficiencies as opposed to conventional systems. This indicates that specific binding took place in NOBE MIPs, which did not in EGDMA-co-MAA MIPs. It is this specific binding that makes this material more suited for implementation into detection and extraction devices, as it can already specifically bind imidacloprid at a concentration of 50 ppm. It is also this observation that confirms the hypothesis that the introduction of more functionalities into the MIP matrix can lead to more target-specific cavities and will thus increase the specific binding of the MIP system.

It should be noted that this result is merely a proof of principle and in order to gain more certainty on this statement, multiple repeat experiments should be performed under various conditions. Although, with a consistent imprint factor (= ratio between specific and aspecific bonding of MIPs versus NIPs) of around 2, functional crosslinkers already prove to be potentially essential compounds in future MIP systems.

Lastly, two NOBE MIP BRs were performed in buffer, with the focus being placed on lower target concentrations (0 ppm to 150 ppm) to assess the potential of NOBE MIPs to work in low concentration ranges. No specific binding was observed for NOBE MIPs in buffer. At higher target concentrations (> 25 ppm), the spread between data points becomes increasingly bigger, with binding efficiencies for MIP and NIP in the same range. The big spread is potentially also caused by the apolar nature of imidacloprid, causing it to stick to any apolar compound (MIP and NIP alike).

It should be noted that BRs for NOBE MIPs in buffer were performed up to 150 ppm target concentration, whereas BRs in buffer for EGDMA/MAA MIPs ranged up to 500 ppm, with a potential increased binding occurring at around 300 ppm. Thus, it is possible that the same theory regarding specific binding in EGDMA systems holds true, but it will need to be investigated first.

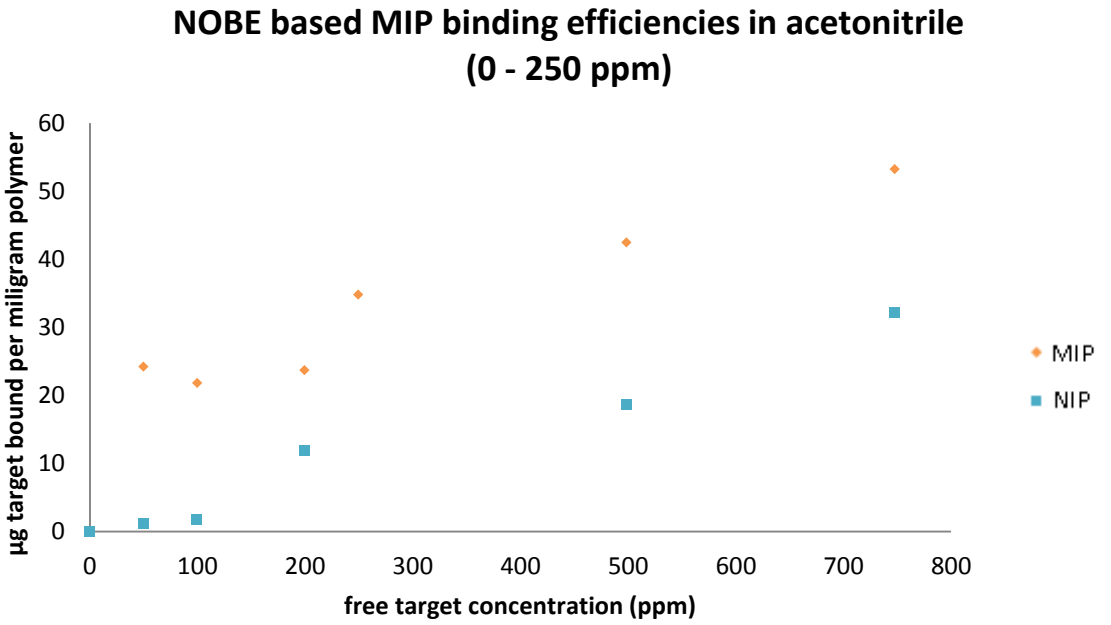


Figure 17: NOBE MIP binding efficiencies in acetonitrile (0 – 250 ppm).

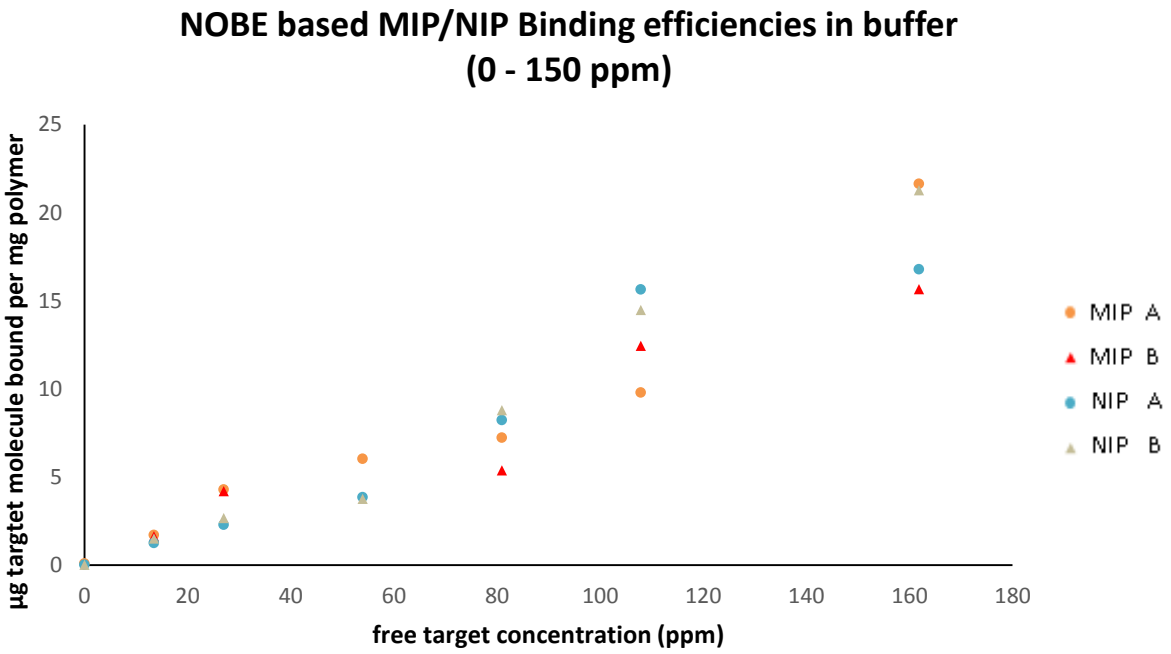


Figure 18: NOBE MIP binding efficiencies in buffer (0 – 150 ppm):

So while NOBE increases the binding specificity of MIPs when compared to conventional systems in acetonitrile, it does not in buffer. The presence of specific binding in acetonitrile is an interesting observation, as it confirms the hypothesis that more functionalities in fact increase the specific binding of MIPs. However, several repeat experiments need to be performed in order to be certain about the consistency of this conclusion.

The absence of specific binding in buffer is probably related to the polarity of MIP matrix, target molecule and solvent. Additionally, it can also be related to other factors, aside from the presence of specific cavities, which independently influence specific binding of NOBE MIPs to their target. If these factors can be determined, efforts can be made to bypass them and ultimately increase the specificity of NOBE MIPs in samples containing solvents with properties that negatively impact specific binding.

Chapter IV – Conclusion & Outlook

The results presented in this thesis show that the use of functional crosslinkers like NOBE in the development of MIP systems for imidacloprid detection and extraction could lead to the development of novel filtration devices, provided that the specific binding efficiencies of these MIP systems are optimalized for aqueous environments. Even though NOBE MIPs show an increased specific binding efficiency in organic solvents, their binding capacity versus the EGDMA-co-MAA system is lower. Furthermore, NOBE MIPs show no specific binding in aqueous samples.

The materials studied here are intended for purification purposes of water samples. Therefore, the specific binding efficiency in water needs to be increased. This can be achieved with threonine-NOBE under the hypothesis that a further increase in functional groups in MIPs will lead to even more specific bindingsites. The effective synthesis of threonine-NOBE was already a big step towards this future research. However, it is a time-consuming and yield-inefficient synthesis pathway and requires optimization and consistency before threonine-NOBE can effectively be employed in MIP synthesis.

One optimization route could be the synthesis of threonine-NOBE in milli flow reactors, as literature has already described that these reactors can carry out batch reactions faster and more efficiently with often times better yields[20]. With flow chemistry it would be possible to continuously and autonomously produce threonine-NOBE while avoiding side reactions, ultimately resulting in higher yields.

Another interesting future topic in the use of these functional crosslinkers would be a screening study with various targets and their structure analogues in order to see if it is possible to generalize our hypothesis.

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