Novel molecularly imprinted polymers – membranes, microspheres, photoswitchable particles

Ph.D. thesis

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1. Introduction

Molecular imprinting is a technology which can create selective adsorption binding sites in a polymer matrix. This interesting technique is based on the fact that the target molecule (template) is present during the polymer synthesis and chemically interacts with so-called “functional” monomers. Molecularly imprinted polymers (MIPs) are able to rebind the target molecule selectively after the template removal. The efficiency of the imprinting process is tested in parallel on a so-called nonimprinted polymer (NIP) which does not contain the template molecule during the synthesis.

I carried out most of my research work at the Department of Inorganic and Analytical Chemistry, at the Budapest University of Technology and Economics. During more than a decade our group gained expertise in molecular imprinting within diverse fields. It contributed to the pioneering development of molecularly imprinted solid phase extraction (MISPE) in the framework of a European project. Furthermore, significant research was done in pursuit of the better theoretical understanding of MIPs. Lately, one of the greatest remaining challenges in molecular imprinting is in the group’s central interest; that is protein imprinting, which was successfully solved by the design of novel nanostructures.

Originally, molecularly imprinted polymers were synthesized exclusively by the bulk polymerization method resulting in a hard polymer monolith, which needed tedious processing. The monolith had to be crushed, ground and sieved, resulting in irregular particles, which were not optimal for most applications. This process could even destroy the formed binding sites. Having recognized these undesired consequences of bulk polymerization new approaches were put forward to create narrow disperse regular microspheres or membranes which could fit much better to the intended application.

My doctoral work mainly focuses on the development of new molecularly imprinted polymer formats following some earlier results of the group. By a previously introduced modified precipitation polymerization spherical microparticles were obtained even at high monomer concentration, with the use of special solvents, for example paraffin oil. This contradicts to the observations in traditional precipitation polymerization where the monomer loading is typically less than 5 v/v%, otherwise macrogelation occurs. I have studied this unexplained phenomenon in terms of how the particles evolve and what types of polymerization condition are responsible for the monodisperse particle formation. The method has several advantages over the traditional precipitation polymerization. These are the reduced solvent need, close to 100% yield, enhanced template-functional monomer complexation and wide variety of applicable apolar solvent, which are all favourable in molecular imprinting.

I have also investigated the MIP-membrane format since membranes, in general, can provide a convenient format for specific applications, such as filtration and solid phase extraction. Our research group has earlier established a new method for the rapid optimization of MIPs by modifying commercially available multiwell membrane filterplates with the selective polymer. I have prepared such composite membrane filterplates by incorporating propranolol selective MIPs into the glass fiber membranes. Using the 24-well filterplates I have elaborated and optimized a solid phase extraction protocol for the fast, high-throughput quantitation of β-blockers from urine and blood samples. Through this work I have shown that the MIP composite membrane filterplates are especially well-suited for the solid phase extraction of biological samples where small sample volumes are typical and can be competitors of the MISPE cartridge format. They are prepared in a more straightforward way

1 In MIP terminology “bulk polymerization” actually refers to solvent polymerization with high monomer loading, whereas in classical polymer chemistry in bulk polymerization no solvent is used at all.
in one step by carrying out the polymer synthesis directly on the support membrane as opposed to MISPE cartridges where preformed MIP beads have to be packed into the syringe barrels. The multiwell filterplate format allows high-throughput sample pretreatment and is amenable to automation.

A major concern with molecularly imprinted membranes (MIMs) is their low adsorption capacity due to the limited amount of selective polymer that can be built into them. The integration of MIP nanoparticles with the membrane format might solve this problem due to their well-defined morphology and increased surface area. So far there are only a few reports on MIP micro/nanoparticle–modified membranes,\textsuperscript{13} where the presynthesized polymer beads are incorporated into the membrane support in a consecutive step. I have devised a novel approach to create polymer nanoparticles \textit{in situ} inside the membrane pores in a one-step synthetic procedure. This was made possible by the application of the modified precipitation polymerization in the multiwell filterplate membranes.

During my studies I had the opportunity to carry out part of my research work at the University of Geneva supported by the SCIEX Fellowship of the Swiss Confederation. Here, I designed novel photoswitchable MIP particles with the use of a photochromic compound, spiropyran combining my expertise with MIPs with that of the Swiss group with spiropyrans. Nowadays exciting, new functional materials are built utilizing this class of compounds that exhibit photocontrollable properties for example photocontrolled wettability, shrinking, and swelling. Yet, there had been only one example in an earlier study which used a spiropyran-based functional monomer to build a photoswitchable MIM.\textsuperscript{14} Other photoswitchable MIPs use azobenzene derivatives as functional monomers being responsible for both the selective recognition and the photocontrolling of the template binding. We offer a novel approach for the synthesis of photoactivatable MIPs. Here the photochromic monomer is solely responsible for the actuation of the polymer backbone and thereby of the binding sites, which are formed from a separate functional monomer. This gives a generic route to endow well-established MIPs with photoswitchable feature by the incorporation of a photochromic monomer. The concept has been verified by preparing photoswitchable polymer particles for the template terbutylazine.

In \textit{Chapter 2 - Background} I overview the polymerization techniques currently in practice for MIP synthesis along with some general concepts and application fields.

In \textit{Chapter 3 – Materials and methods} I list the chemicals and consumables that I used in my work. The methods were separated into two sections; in \textit{General methods} the applied analytical and characterization methods are given that were used in many parts of my research work. \textit{Specific methods} are overviewed subsequently which inform the reader about the different polymerization methods, instrument set-ups, and experimental conditions specifically related to the different research topics.

\textit{Chapter 4} describes the synthesis, optimization, characterization and the use of spiropyran-based MIP particles in photocontrolled analyte binding-release experiments.

The most important findings of the precipitation polymerization at high monomer loadings are summarized in \textit{Chapter 5}. I have extended the group’s earlier findings with methacrylic acid–ethylene glycol dimethacrylate based polymers to a wider range of functional monomers, crosslinkers and solvent systems, as well.

\textit{Chapter 6} gives an insight into the synthesis and application of the MIP-membrane filterplate as a MISPE device for biological sample preparation.

\textit{Chapter 7} presents the results of the MIP particle–membrane synthesis approach.

In the \textit{Summary} I give a final conclusion of my doctoral work and the most important scientific results are summarized in the thesis points.
2. Background

2.1. What are molecularly imprinted polymers?

Molecular imprinting is a technology which can create predetermined selectivity toward a selected analyte (template) in a polymer matrix. Molecularly imprinted polymers (MIPs) can be synthesized when the target molecule, acting as a template, is present in the prepolymerization solution and orientates suitable functional monomers around itself by self-assembly. The position of the functional monomers is stabilized by strongly crosslinking the forming polymer network. After the polymerization had taken place, the template is removed revealing binding sites complementary in size and shape to the template molecule which are able to rebind the target molecule selectively (Figure 2.1). These sorbents bearing a predetermined selectivity towards a template offer many application opportunities; they can be used for instance as chromatographic stationary phase, separation media in sample pretreatment, recognition element in sensors, and catalyst, to name a few.

![Figure 2.1 Scheme of molecular imprinting](image)

2.2. Covalent and non-covalent approaches in imprinting technology

The non-covalent approach

In this technique non-covalent interactions, for example H-bonding, electrostatic and hydrophobic interactions drive the self-assembly phenomenon via template-functional monomer complexation. Functional monomers have to be rationally selected to provide preferably multiple interaction points, thus enhancing binding site fidelity. The main benefit of the non-covalent approach is that the template removal is relatively facile, the template can be extracted from the polymer matrix by disrupting the secondary forces. The rebinding is also achieved by non-covalent interactions. It is important to promote selective interactions with the careful selection of the polymerization conditions. Typically, non-polar solvents are used in order to preserve for instance H-bonding, which, in many cases, is responsible for the successful imprinting. The functional monomer is frequently applied in excess in order to shift the complex formation equilibrium. However, this monomer surplus can give rise to non-specific binding sites where the rebinding is not due to the preformed shape-complementarity but is driven merely by one-point interactions between the template and the functional groups of the polymer backbone. The so-called stoichiometric imprinting...
overcomes this drawback. In this case functional monomers with very high affinity toward the template are employed. The equilibrium is completely shifted toward the complex formation, therefore these monomers can be applied in stoichiometric ratio with the template. However, they are commercially not available and organic synthesis is necessary for their preparation.¹⁸

**The covalent approach**

The pioneering work in covalent imprinting was carried out by Wulff et al.¹⁹ This type of imprinting method forms covalent bonds between the template and the functional monomer prior to polymerization. This bond remains intact while the polymer matrix is formed. After the synthesis, the template-monomer bond is cleaved and the template rebinding takes place similarly by a chemical reaction. The advantage of the method is that there is an inherently strong interaction between the template and the monomer albeit covalent bonds which are easily cleavable and can be reformed are relatively scarce. Carbohydrates and boric acid derivative functional monomers were used to demonstrate the applicability of the approach.

**The semi-covalent approach**

In this method the synthesis of the polymer is carried out according to the covalent approach but the template rebinding takes place with non-covalent interactions. Whitcombe et al. introduced the technique using a sacrificial spacer on the template 4-vinylphenyl carbonate ester cholesterol derivative.²⁰ The template was removed by cleaving a certain C-C bond and the binding site was able to interact with the OH-group of cholesterol via non-covalent interactions. This technique also requires organic synthetic steps for the modification of the template, thus its application has been limited in the imprinter community.

In practice the abovementioned non-covalent approach is far the most wide-spread of all because of its simplicity and wide variety towards many types of compounds and also probably due to the limited number of easily cleavable covalent bonds that can be utilized in covalent imprinting.

**2.3. Preparation of molecularly imprinted polymers based on the non-covalent approach**

In molecular imprinting the use of monomers which are able to interact with the template of interest is a prerequisite. The functional monomer must contain a polymerizable vinyl or (meth)acrylate group, and functional group(s) for instance carboxyl group, amino group or aromatic ring to form the desired interaction with the template. A selection of typically used commercially available functional monomers is presented in Figure 2.2. Generally, the first step to design a MIP is to rationally select the potential functional monomers for the target molecule. For this purpose spectroscopic techniques, such as ultraviolet-visible (UV-Vis), fluorescence, nuclear magnetic resonance (NMR) methods can be applied with which the monomer-template interaction can be investigated in solution before embarking on polymer synthesis.¹⁸,²¹-²³ In molecular imprinting generally high crosslinking ratio is used in order to conserve the formed binding sites. An optimization is needed to find out the conditions where the highest selective binding capacity is achieved. The high-crosslinking ratio has additional benefits in MIPs intended for chromatographic use where the mechanical stability and robustness is one of the basic prerequisites for application.
Crosslinking monomers contain minimum two polymerizable bonds like ethyleneglycol dimethacrylate (EGDMA), divinylbenzene (DVB) but tri- and tetrafunctional monomers were also successfully exploited in the MIP field. Some crosslinkers are shown in Figure 2.3. It has been shown that trimethylolpropane trimethacrylate (TRIM), a trifunctional monomer is superior compared to EGDMA in case of lower crosslinking level. It can provide a higher load capacity and the amount of functional monomer can safely exceed the amount of crosslinker without loss of performance. 

'OMNiMIPs' (one monomer molecularly imprinted polymers) was an interesting approach presented by the Spivak-group in which a special monomer containing two polymerizable bonds (N,O-bismethacryloyl-ethanolamine, NOBE) was developed which could act simultaneously as a functional monomer and a crosslinker. They investigated the H-bonding ability of this monomer with different classes of templates for example carboxylic acids, alcohols and amines. Significant improvement could be achieved for the first two classes in their enantioseparation compared to a traditional methacrylic acid (MAA)-EGDMA polymer. The amido group of NOBE provided strong H-bonding interaction between template and monomer.

In the preparation of MIPs a solvent is also applied in the prepolymerization mixture serving multiple functions. First of all, it has to be able to solubilize all the compounds. Secondly, it often acts as a pore-forming agent. Adequate pore structure can be advantageous (but not necessary) for the transport of the template to and from the binding sites. In the non-covalent approach the selection of a proper solvent which does not disrupt the secondary interactions.
between the template and the functional monomer is very important. Non-polar solvents, dichloromethane, toluene, chloroform and acetonitrile are typically used. In cases where selective hydrophobic interactions are responsible for the specific interactions, a polar medium, for instance water-methanol mixture is used.\textsuperscript{26} The initiator is another essential additive in the prepolymerization solution. AIBN (2,2’-azobis-isobutyronitrile), ABDV (2,2’- azobis-dimethylvaleronitrile) are typical initiators for thermal and UV polymerization, but benzoin ethyl ether (BEE) can be also utilized in UV polymerization. Bubbling inert gas through the solution is an important step to avoid the inhibitory effect of oxygen.

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/2.3.png}
\caption{Crosslinking monomers typically used in molecular imprinting (EGDMA: ethylene glycol dimethacrylate; TRIM: trimethylolpropane trimethacrylate)}
\end{figure}
\end{center}

\subsection{2.4. Synthesis methods}

For a long time, MIPs were produced exclusively by bulk polymerization, which requires labour-intensive processing of the obtained hard monolith by crushing, grinding and sieving. In order to obtain regularly sized polymer beads other polymerization techniques found their way into the MIP field for instance precipitation, emulsion, suspension and multi-step swelling polymerization. These synthetic techniques are overviewed in Figure 2.4 and will be discussed in detail below along with the synthesis of MIP-membranes.
Figure 2.4 Overview of polymerization methods suitable to prepare MIPs (A) bulk polymerization, (B) polymer monolith in situ prepared in a column, (C) precipitation polymerization, (D) polymerization in sacrificial silica beads, (E) thin MIP films in silica pores using iniferters (F) multi-step swelling and polymerization, (G) suspension polymerization
2. Background

2.4.1. Bulk polymerization

Imprinted polymers are often prepared by bulk polymerization, which is typically triggered by thermal or photo-initiation. This method has been the most popular; and can be a good starting point for the researchers who are new to the field of imprinting. The technique does not need exceptional skills or expensive instruments, and extensive literature is available. The template, functional monomer, crosslinking monomer and initiator are dissolved in a suitable solvent typically in a concentrated solution, for instance, a 4:3 solvent:total monomer ratio. After bubbling an inert gas through the mixture polymerization can be initiated thermally, chemically, by UV light or γ-ray irradiation. After the hard polymer monolith is formed, a tedious procedure follows the easy synthesis. The monolith has to be crushed and ground in a mortar, wet-sieved and a certain size fraction (typically 25-50 µm) of particles has to be collected. The template removal can be carried out for example by Soxhlet-extraction or by batch mode consecutive washing steps with an appropriate solvent. H-bonding organic solvents like methanol/acetic acid mixtures are typical. Generally, the template extraction is conducted until no template can be detected in the washing solution. Here, it is important to note that it is very difficult, if not impossible to remove all the template from the polymer. Even after extensive washing a constant, slow leaching of the template out of the polymer can be observed, which is called ‘bleeding’ in MIP terminology. All these procedures cause a substantial loss of the polymer sometimes amounting to 75% of the initially obtained bulk material. At the end of the preparation process irregular particles in a wide size range are obtained which are far from being ideal for many applications.

2.4.2. Polymerization techniques of particulate polymers

2.4.2.1. Precipitation polymerization

This polymerization method is a wide-spread technique to create spherical beads. Essentially, the same recipes are used as in bulk polymerization with the main difference that the monomer concentration is typically between 2-5 v/v% (dilute conditions). An advantageous feature is that no surfactants or stabilizers are needed unlike in suspension polymerization which can adversely affect the template-functional monomer interaction. The monomers are completely solubilized in the polymerization solvent but the forming polymer is not therefore it precipitates out from the solution during polymerization. High crosslinking ratio has to be employed in order to obtain regular monodisperse microspheres. This requirement coincides with the one in molecular imprinting to obtain stable imprinted sites. Precipitation polymerization is often carried out either in acetonitrile or in a mixture of acetonitrile and toluene. The latter one provides high porosity to the beads.

The pioneering work of MIP synthesis by precipitation polymerization was carried out by Wang et al.27 They were able to synthesize imprinted particles for theophylline with a diameter of ~5 µm which is suitable for HPLC-column packing. The template affected the particle size and the imprinted polymer beads were smaller than the nonimprinted control. However, this phenomenon cannot be considered as a general rule of thumb because the opposite trend can also be observed.28 An extensive study performed by Yoshimatsu et al. revealed that a fine tuning of particle size is possible by using two crosslinkers, TRIM and DVB and varying their ratio. The size of the synthesised particles increased from 130 nm to 2.4 µm depending on the extent of the TRIM feed.29
It is worth mentioning that in conventional precipitation polymerization the dilute conditions do not favour the monomer-template complexation and through this the formation of selective binding sites. A modified precipitation polymerization method developed earlier in our group addresses this drawback. In this technique paraffin oil in combination with toluene was used as solvent and the monomer concentration was close to that applied in bulk polymerization. Spherical polymer particles with approximately 2 μm diameter were prepared. The particles were imprinted for the template terbutylazine and tested as HPLC stationary phase.\textsuperscript{11} This technique was further investigated in my research work, and the results can be found in Chapter 5.

Recently, core-shell structured stationary phases have been introduced in liquid chromatography, which significantly increased the separation efficiency. This trend also calls for new synthetic strategies in the preparation of MIPs. In a two-step precipitation polymerization method the imprinted polymer shell was created on the surface of DVB seeds according to the scheme shown in Figure 2.5. With the core-shell MIPs enhanced chromatographic performance was achieved and an in-line sample preparation technique for the determination of thiabendazole from fruit juice samples has been developed.\textsuperscript{30,31}

![Figure 2.5 Scheme of the preparation of core-shell MIP microspheres by precipitation polymerization\textsuperscript{31}](image)

\subsection*{2.4.2.2. Suspension polymerization}

This polymerization technique involves two phases. An organic-solvent based phase containing the monomers and the initiator is dispersed with continuous agitation in an immiscible medium for instance water and the polymerization takes place in the organic phase. The final particle size depends on the size of the dispersed droplets and it is in the range of tens of micrometers. An additional surfactant or stabilizer is needed for the stability of the dispersion but it can interfere with the template-functional monomer interactions and its
removal after polymerization is often difficult. The use of an aqueous medium can be deleterious on the template-functional monomer complexation in case the selective interaction is based on hydrogen-bond formation. However, when electrostatic or hydrophobic interactions are responsible for the binding this method can be a facile, straightforward way for MIP bead synthesis. A MIP obtained with suspension polymerization was successfully applied in the HPLC separation and determination of 4-aminopyridine and 2-aminopyridine using an aqueous buffer/methanol mixture as the mobile phase. By careful selection of the eluent pH, 4-aminopyridine and 2-aminopyridine could be resolved on the MIP column but not on a commercial C18 column.

A modified method to circumvent the deteriorating effect of water was proposed by Mayes and Mosbach who used a liquid perfluorocarbon phase for the dispersion. The authors could achieve baseline separation of Boc-phenylalanine enantiomers. The bead size was controlled between 5 and 50 µm by the adjustment of the stabilizing agent content. However, the measurements revealed a high-degree of non-specific binding. This fact was explained by the covalent grafting of a layer of fluorinated surfactant which could not be removed and caused non-specific binding due to its high hydrophobicity.

Another modified suspension polymerization technique was proposed by Kempe and Kempe using mineral oil as the continuous phase. Their propranolol-imprinted polymers were used in radioligand binding assays.

Recently, an interesting paper presented the synthesis of chiral MIP stationary phases without the use of stabilizer in an aqueous suspension system. The authors used chloroform as the organic phase, and the practically non-porous polymer beads were used for enantioseparation in HPLC.

2.4.2.3. Emulsion polymerization

This technique is similar to suspension polymerization in terms of the use of two immiscible phases. Typically, the monomer (organic phase) is dispersed into an aqueous phase and the monomer droplets are stabilized with surfactants. Generally, this technique gives polymer beads ranging from tens of nanometers to some hundreds. The concerns, one can raise, are partly the same in this method as in suspension polymerization, that is the aqueous continuous phase can disrupt the template-monomer interactions. Additionally, the surfactant can adversely affect the imprinting process. Another drawback is the cumbersome and sometimes inadequate removal of the amphiphilic molecules from the synthesized polymer.

Emulsion polymerization was first introduced into the MIP field in 1992. Copper ion selective polymers were synthesized by the use of a fatty acid type functional monomer which served simultaneously as a surfactant during the polymerization. The binding sites for the metal ions were located on the surface of the nanometer sized particles since the metal ions did not dissolve in the organic phase and the imprinting was confined to the oil/water interface.

Miniemulsion polymerization was successfully utilized for the creation of Boc-L-phenylalanine-anilide imprinted beads. In this technique a powerful ultrasonication forms the monomer droplets and determines the actual size of the particles. Improved binding capacity was achieved for the targeted enantiomer compared to its counterpart and also to the nonimprinted polymer.

In an interesting work of Priego-Capote et al. polymerizable, surfactant-like functional monomers were utilized for the imprinting of propranolol in miniemulsion polymerization. Due to its amphiphilic characteristics the monomer resided on the interface of the two phases and the imprinted sites could be created on the surface of the polymer nanoparticles. The
crosslinker content had to be optimized to achieve a stable emulsion. The MIP nanobeads were used as pseudostationary phase in capillary electrochromatography (CEC) for enantiomer separation.

2.4.2.4. Polymerization in preformed beads

Silica supported MIPs

This technique is a straightforward way to create microspherical beads since the MIP preparation is carried out in the pores of spherical silica particles commonly used in liquid chromatography. The pores of the silica support are filled up with the imprinting polymerization solution by gentle agitation or sonication while special care is devoted to avoid any monomer mixture remainings on the outer surface of the silica particles. The pores of the silica can be considered as microreactors where bulk polymerization takes place. The obtained free-flowing particles have the same size as the silica support and can be packed into HPLC columns. Alternatively, the porous silica particles may be used as a sacrificial support, i.e. in a post-treatment step the silica skeleton can be removed by etching with a concentrated hydrogen fluoride (HF) solution. Two variants of the sacrificial method have been realized. In the first case the prepolymerization mixture contains a suitable porogen. Therefore, the polymer structure that remains after the etching of silica will be porous with imprinted cavities and it can be considered as the negative image of the silica support.

In the second case, which was first demonstrated by Yilmaz et al., the template is covalently anchored to the silica through spacer arms. No porogen is used in this case and accordingly the pores in the particle originate exclusively from the etching of silica. This so-called hierarchical imprinting technique provides an easy way to prepare spherical beads with well-defined porosity.

Another interesting approach was the grafting of thin MIP films on silica beads by the adsorption of the initiator on the silica support. Since the initiator was immobilized onto the silica surface prior to polymerization formation of the polymer was restricted to the pore surfaces. The authors were able to improve the kinetic characteristics of the polymer by tuning the thickness of the imprinted film on the surface of the silica. The L-phenylalanine anilide imprinted polymer stationary phase was able to reach baseline resolution between enantiomers within 5 minutes.

The use of a conventional azo-initiator in this technique can be problematic because it might lead to polymerization in the solution phase, too. One option to circumvent this problem is the use of the so-called iniferers. These compounds decompose into two active radicals, one able to initiate polymerization and fixed on the surface, the other able to terminate the growing macromolecule. Another option is to use the reversible addition-fragmentation chain transfer (RAFT) polymerization which is a controlled radical polymerization technique and it is able to generate polymers with low polydispersity and a desired molecular weight, with the use of RAFT agents, such as 2-phenylprop-2yldithiobenzoate.

A nanometer-thin layer of MIP film was polymerized onto the surface of silica particles. L-phenylalanine anilide imprinted polymers were made with and without the RAFT agent and were packed into HPLC columns. In elution chromatography higher retention and improved enantioselectivity was achieved with the polymer that was synthesized with the RAFT agent.

Multi-step swelling and polymerization

Most of the contributions to MIP-multi-step swelling techniques were carried out by Haginaka’s group. This method is another suitable technique to create microspherical MIP particles although it needs several steps to attain the desired polymer format. Typically, in the
first step one needs to disperse polystyrene seed particles (~1 µm) in water with the admixing of a microemulsion containing the initiator, an activating solvent (dibutyl phthalate) and a surfactant (sodium dodecyl sulphate). Gentle stirring at room temperature is provided until the microemulsion droplets are absorbed into the seed particles. In the second step an aqueous emulsion of the monomers (both functional and cross-linking), initiator, porogen, a stabilizer (polyvinyl alcohol) and the template are added to the swollen seed particle dispersion and stirred further for several hours. After making sure that the droplets are absorbed into the seed particles the dispersion is deoxygenated with inert gas and the polymerization is initiated. As it can be seen the synthesis method is a bit complicated, which probably hinders its wider application. Moreover, here again the aqueous medium can adversely affect the template-monomer complex if it relies on H-bonding.

2.4.2.5. Nanogels

This type of polymer format is relatively new in the MIP-field and such particles are mainly used as enzyme-mimics or plastic antibodies similar in size to natural antibodies.\textsuperscript{46,47} This method does not involve the compartmentalization or the precipitation of the monomers. The very high dilution (<1 v/v% monomer concentration) of the system allows to create very small particles of some tens of nanometer without the need for surfactants. The polymerization is conducted in a good solvent where polymer precipitation does not occur. The formed polymer nanospheres are swollen with the solvent. A careful investigation is needed to establish an optimum concentration below which nanogels can be obtained. High crosslinking ratio is disadvantageous in this technique because it decreases the solubility of the polymer chains and leads to precipitation. The first paper dealing with imprinted nanogels was published in 2001.\textsuperscript{48} The authors prepared \( \alpha \)-D-mannopyranoside imprinted nanogels by the covalent approach. The template was preferentially bound to the imprinted polymer compared to the L-enantiomer. Due to their flexibility and facile dispersibility into colloidal solutions nanogels are favoured formats for catalytic applications which has been recently reviewed.\textsuperscript{46}

2.4.3. Polymer monoliths

A single piece of polymer rod prepared in situ in an HPLC column is called a monolith. Polymer monoliths as imprinted stationary phases were developed as early as 1993 by the group of Matsui.\textsuperscript{49} The prepolymerization solution is filled into an empty chromatographic column and the column is kept at elevated temperature to initiate the polymerization. The obtained polymer can be directly used to perform chromatographic experiments. The inherent benefit is that there is no need to process the synthesized bulk material and the column is ready-to-use. The difficulty is to produce a pore structure which allows rapid eluent flow and also provides a high surface area for adsorption, therefore the pore-forming solvent has to be carefully selected. Toluene-isooctane mixtures have proven to be good porogens for the production of super-porous imprinted polymer monoliths.\textsuperscript{50,51} The proportion of isooctane should be optimized, bearing in mind that by raising its concentration more macropores are created but at the same time the fragility of the monolith increases. Controlled living polymerization has also been utilized for the preparation of MIP monoliths. A RAFT agent, dibenzyl trithiocarbonate was used for enrofloxacin imprinting. This agent has a substantial influence on polymer morphology and provides more adjustable conditions to tune the macropore size and surface area.\textsuperscript{52}
A recent review gives an in-depth summary of imprinted monolithic stationary phases both for HPLC and CEC applications.  

2.4.4. Membranes  

2.4.4.1. Self-supported membranes  

Self-supported molecularly imprinted membranes have been synthesized using different approaches i.e. by i) dry or wet polymer solution phase inversion; ii) in situ crosslinking polymerization and by the iii) sol-gel process. In self-supported MIMs selective binding sites are formed simultaneously with the porous structure of the membrane from the same building blocks, therefore it is difficult to achieve a high number of accessible binding sites and efficient membrane separation at the same time.  

2.4.4.2. Composite membranes  

The preparation of MIP-composite membranes allows the use of a support material with an optimized pore structure which is sequentially modified with a different polymer providing the selective recognition function. MIP-composite membranes were first prepared from commonly used methacrylate polymers by filling the pores of a glass filter or polymer microfiltration membrane (pore-filling membranes). Thin film composite membranes were first synthesized by Hong et al by photopolymerization of an ultrathin MIP film across the surface of a microporous alumina membrane. These approaches result in mainly microporous membrane materials where the diffusional transport of the template is enhanced compared to other molecules due to its interaction with the imprinted sites, or the binding of the template changes the pore structure and thereby the membrane permeability/conductivity. In thin-layer composite MIMs the base membrane exhibiting an appropriate pore structure and surface area is coated with the molecularly imprinted polymer so that the membrane permeability is not affected drastically. In these cases high performance affinity membrane adsorbers are obtained where the template is retarded by the imprinted sites. The first realization of this concept used a special photoreactive polymer that was grafted from appropriate monomers in the presence of the template. Later the supporting macroporous membranes were modified with a photoinitiator from which photo-grafting of the selective polymer was started. Further, simplified procedures wet the support membrane with the pre-polymerization mixture and initiate the polymerization. MIP particle composite membranes use preformed micro/nanoparticles for embedding into a macroporous membrane structure resulting in affinity adsorber membranes. This approach was first realized by entrapping molecularly imprinted nanoparticles between polyamide membrane discs which served as a support. The significance of the integration of MIP nanoparticles and membrane technology lies in the fact that the well-defined morphology and high surface area of the particles may result in increased specific binding capacity of the membranes, today being a major concern with state-of-the-art MIM adsorbers.
2. Background

2.5. Characterization techniques

2.5.1. Physical methods

Morphological information about the polymers can be acquired by scanning electron microscopy (SEM) measurements. The size and shape of the particles can be studied and particle size distribution analysis can be performed on the obtained SEM micrographs. N\textsubscript{2} sorption porosimetry allows a detailed surface characterization of the imprinted polymers. Sorbed gases and solvents are removed from the polymer in vacuum then the adsorbed amount of nitrogen is measured at different relative pressures. Also the desorption isotherms can be recorded. Several parameters such as specific surface area, average pore size, pore volume and pore size distribution can be calculated based on different adsorption models. Swelling tests can also reveal information about the polymers. The dry polymers with a known volume are equilibrated with a solvent and then the volume change is registered. The volume swelling ratio is the volume of the solvent swollen polymer divided by the volume of the dry polymer.

An interesting study combining all three abovementioned characterization methods studied zearalenone selective polymers in terms of the effect of different functional monomers and porogens.\textsuperscript{69}

2.5.2. Chemical methods

Most of the chemical measurements aim to reveal the selectivity, and the binding capacity of the developed polymer.

2.5.2.1. Equilibrium batch rebinding measurement

The equilibrium batch rebinding measurement is probably the most straightforward way to characterize MIPs. We can obtain either entire binding isotherms or just measure at one concentration point for example in order to rapidly screen a polymer library for evaluation and selection of an optimal polymer composition. The binding isotherm plots the adsorbed amount on the polymer as a function of the equilibrium concentration. The dry polymer is weighted into a glass vial or a plastic sample container, and a certain amount of the template solution in a given concentration is pipetted onto the particles. After homogenization the suspension is left to reach equilibrium with constant agitation. Following centrifugation the concentration of the unbound analyte in the supernatant is quantified by HPLC. In Section 3.3.2.1. it can be seen how the results can be calculated from raw data. Polymers have to be washed carefully before the experiment because the residual template inside the polymer can cause false results by changing the equilibrium between the liquid and the solid phase. If the template relatively weakly adsorbs onto the polymer, very small phase ratio (solvent volume/polymer mass) is required otherwise the concentration change in the supernatant will be very low and can be quantified only with a large experimental error. Nevertheless, this method allows one to thoroughly investigate the binding properties (adsorption isotherms) and the selectivity of MIPs in different solvents, at different temperatures even from solutions containing multiple analytes.
2. Background

2.5.2.2. Characterization as liquid chromatographic stationary phase

Frontal chromatography is a well-established method for the characterization of MIPs. In this case the imprinted and nonimprinted polymer is packed into an empty HPLC column. The mobile phase contains the template in varying concentrations and breakthrough curves are recorded. One can calculate the breakthrough volume from the first derivative of the chromatogram and from this the bound amount of template can be readily obtained. The method is suitable for the precise measurement of the adsorption isotherm. The fitting of the binding isotherm can provide further characteristics of the imprinted polymer, for instance binding affinity constant, binding site heterogeneity and binding site concentration. A pioneering work in MIP frontal chromatography was done by Sajonz et al.\textsuperscript{70}

Elution chromatography is also a frequently used technique for the characterization of MIPs. Here, the template is injected onto a column filled with the MIP or the nonimprinted polymer (NIP) and the retention time serves as the primary data from which the retention factor is calculated. The imprinting factor (IF), which is the ratio of the retention factors \(k\) on the MIP and the NIP, is widely used to give information about the imprinting effect albeit it is not suitable for accurate comparison of the results (see next section). The chromatographic testing of MIP sorbents can also focus on a better understanding of their adsorption behavior.\textsuperscript{71-74} It is important to mention that the data obtained with this technique cannot be easily used for instance in sensor applications since in chromatography the characterization is carried out under non-equilibrium conditions, whereas in sensors static equilibrium is achieved.

2.5.2.3. Interpretation of the results

Extensive efforts are made to produce imprinted polymers that mainly possess specific binding sites by minimizing the nonspecific binding. Hence, it is always a requirement to characterize the nonimprinted polymers to justify the imprinting efficiency on the MIP. Unfortunately, we can still find papers in the literature which do not investigate the template binding on NIPs and do not confirm the imprinting effect which significantly reduces the value of the work. It is also an important aspect to investigate the binding of structurally non-related compounds on the polymer. Here again, it is necessary to carry out the tests also on the NIP. If we see disparate binding of the non-related compound on the imprinted and nonimprinted polymer, it can signal a difference in the specific surface areas of the polymers, which, in turn, means a different number of non-specific binding sites, as well. Hence, an increased template binding on the MIP compared to the NIP is not necessarily the consequence of the imprinting.

Previously, in our group Tóth et al. pointed out that \(k\) values, selectivity \((\alpha)\) or imprinting factors obtained from elution chromatography for the comparison of different MIPs are not suitable.\textsuperscript{6} These depend on such experimental variables as column length and diameter, injection volume, flow rate and template concentration. The dependency of \(k\) and IF on the injected sample concentration can be seen in Figure 2.6. Nevertheless, if the experimental variables are kept constant different polymeric stationary phases can be characterized and compared using the IF. This behavior is the consequence of a general feature of MIPs, namely they exhibit nonlinear adsorption isotherm. This feature renders the characterization of MIPs more difficult.
2. Background

Figure 2.6 Concentration dependence of the retention factor and imprinting factor in elution chromatography.\textsuperscript{6}

Considering the weaknesses of the imprinting factor, the distribution ratio (or partition coefficient) was suggested as a possible interpretation tool for the evaluation of MIPs.\textsuperscript{7} This value can be simply calculated from batch rebinding data:

\[
D = \frac{q}{c}
\]

Eq. 1

where \(D\) is the distribution ratio [L kg\(^{-1}\)], \(q\) is the concentration on the solid phase [mol kg\(^{-1}\)], and \(c\) is the equilibrium concentration in the liquid phase [mol L\(^{-1}\)].

Practically, to get the \(D\) value one needs to measure one point on the adsorption isotherm. Two typical adsorption isotherms are shown in Figure 2.7. The best way to characterize MIPs is to measure their adsorption isotherm together with that of the NIP and compare the two. This, however, requires a lot of accurate measurements and is rarely done by the researchers working in molecular imprinting. It would also be of great value to obtain isotherms with analogs or non-related compound to gain information about the selectivity of the polymer, but I have found only one such example in the literature.\textsuperscript{75} The usefulness of adsorption isotherms was also emphasized by Castell et al.\textsuperscript{76}

Figure 2.7 Adsorption isotherms, A: linear, B: nonlinear

Adsorption isotherms can be subjected to quantitative analysis by different adsorption isotherm models. The number of binding sites and the association constant(s) can be calculated from fitting parameters and can serve as possible alternative for comparison of MIPs and NIPs. Langmuir, biLangmuir, Freundlich, Freundlich-Langmuir, and Tóth isotherm models were applied for MIPs in most cases establishing a certain mathematical correlation between the bound and free amount of template (analyte). Two main categories exist among the binding models; one treats MIPs as having one or several classes of binding sites, and the
other operates with continuous binding site distribution model. The two most frequently used models are the following:

**Langmuir model**

In this case the MIP has only one type of binding site which binds the template with 1:1 stoichiometry and equal affinity.

\[ B = \frac{NKF}{1 + KF} \]  

Eq. 2

where \( B \) is the bound concentration [mol g\(^{-1}\)], \( N \) is the number of binding sites [mol g\(^{-1}\)], \( K \) is the association constant [M\(^{-1}\)], \( F \) is the free equilibrium concentration [mol L\(^{-1}\)].

**Freundlich model**

This continuous distribution model has been widely used for MIPs since it can give a better approximation of the broad unimodal distribution of the binding sites compared to discrete binding models and the binding site heterogeneity can be quantified.\(^7\) This model is a power function of the equilibrium concentration, and the bound amount of analyte in the polymer phase can be calculated with

\[ B = aF^m \]  

Eq. 3

where \( B \) is the concentration of analyte in the polymer phase in units of [mol g\(^{-1}\)], \( F \) is the equilibrium concentration in the solution phase in [M], \( a \) is the preexponential factor [mol g\(^{-1}\) (M\(^m\))] and \( m \) is the heterogeneity index (unitless). From the fitting parameters, \( a \) and \( m \), one can calculate physical characteristics. The heterogeneity index, \( m \) can have a value between 0 and 1 where 1 corresponds to an entirely homogeneous binding. The method proposed by Rampey et al. can be used to calculate the affinity distribution, number of binding sites and average weighted affinity from the binding isotherms.\(^7\)

The number of binding sites with a given affinity is:

\[ N(K) = 2.303am(1-m^2)K^{-m} \]  

Eq. 4

where \( K \) is the affinity constant, calculated as the reciprocal concentration, \( a \) is the preexponential factor, \( m \) is the heterogeneity index, and \( N(K) \) is the number of binding sites with a given affinity.

The number of binding sites per gram polymer is:

\[ N_{K_{min}-K_{max}} = a(1-m^2)(K_{min}^{-m} - K_{max}^{-m}) \]  

Eq. 5

The weighted average affinity constant is:

\[ K_{K_{min}-K_{max}} = \left( \frac{m}{m-1} \right) \frac{K_{min}^{-m} - K_{max}^{-m}}{K_{min}^{-m} - K_{max}^{-m}} \]  

Eq. 6
where $K_{\text{min}} = \frac{1}{F_{\text{max}}}$ and $K_{\text{max}} = \frac{1}{F_{\text{min}}}$. These calculations are limited and valid only in the experimentally determined concentration range.

2.6. Application fields

Molecularly imprinted polymers attracted attention in diverse fields of analytical chemistry and beyond. Their utilization in separation science is probably the most exploited area. These selective sorbents can be used as a stationary phase in liquid chromatography, in capillary electrophoresis, and capillary electrophoresis.87-88 Abundant literature is available concerning solid phase extraction on MIPs.82-87 There is an increasing potential for the use of MIPs as recognition element is sensors.88-91 We can even find applications for organic synthetic purposes as MIPs can be applied in catalytic reactions.46

In the present section I would like to give a small overview of the application fields without covering all aspects.

2.6.1. Separation science

MIPs have many advantages compared to other stationary phases which can put them in the interest of a chromatographic expert. The high crosslinking degree which is a major requirement for the fixation of the binding sites makes them mechanically stable; they can resist even high pressure without a collapse. They are applicable in a wide pH range and they can withstand extreme chemical conditions. They can be stored without loss of performance as a dry powder for several years, considerably longer than natural antibody-based affinity media.

The porosity of the imprinted material is an important property to be controlled. Generally, the micropores (<2 nm) are undesired in a polymer matrix because of diffusion limitations. However, in many cases the polymer has several hundred m$^2$·g$^{-1}$ specific surface which derives mainly from the micropores.

2.6.1.1. Chromatographic stationary phase

Molecularly imprinted chromatographic stationary phases were first developed to separate enantiomers92 and many papers even today deal with this problem. Several reviews also give further insight into the chromatographic application and characterization of MIPs.78-80,93,94 Indeed, one of the potential areas of MIPs in chromatography is enantioseparation if other chiral stationary phases are expensive or not available. Imprinted polymers can be synthesized in the laboratory, easily handled, and a chromatographic method for the targeted separation purpose can be developed. Many examples of MIP enantioseparations can be found in the literature concerning amino acids, drugs, carboxylic acids, peptides etc.95-99 The desired affinity towards the enantiomers is achieved via imprinting with the selected (S)- or (R)-isomer creating shape-complementary binding sites in the polymer matrix. After the extraction of the template and packing the obtained polymer into HPLC-columns the racemate mixture can be resolved. The chromatogram exhibits two peaks with a predictable elution order. Generally, the first peak is relatively sharp and represents the less retained nonimprinted enantiomer, while the second peak, corresponding to the imprinted form, shows peak broadening and tailing. This phenomenon is attributed to the combined result of several factors:

- particles which are too large or irregular
- inadequate column packing
- slow mass transfer due to the existence of micropores
- binding site heterogeneity
- nonlinear adsorption behaviour of the imprinted binding sites

The most wide-spread application of MIP chromatography is probably to test the imprinting efficiency in dynamic mode even when the desired application will not use the MIP in column format. Typically, binding properties derived from HPLC-characterization are utilized in the development of MISPE (molecularly imprinted solid phase extraction) methods. The required pH, eluent strength, eluent modifiers etc. can be selected for the MISPE application on the basis of the chromatographic characterization.

In the last few years many reviews dealt partially or wholly with the application of MIPs in capillary electrochromatography presenting different approaches for their synthesis and evaluating their performance.\(^{100-102}\) By the combination of capillary electrochromatography and molecular imprinting, efficient and highly selective separations have been achieved. Many applications have appeared recently in the literature for the enantioseparation of chiral compounds\(^ {38,50,51,105}\) or the separation of structural analogs in such systems,\(^ {104,105}\) sometimes even from real samples.\(^ {106,107}\) One should mention, however, that although the resolution and efficiency increase, the peak shapes usually do not improve in MIP based CEC systems, as they are an intrinsic property of MIPs.

### 2.6.1.2. Solid phase extraction sorbent

Molecularly imprinted polymers have been extensively studied as solid phase extraction sorbents since 1994.\(^ {108}\) This application field has proven to be the most promising one because we can find many MISPE sorbents that are now commercially available. The selective sorbents are used in the sample clean-up of different matrices for instance of environmental, food or biological origin. Two important goals can be achieved when using a MISPE method: a) selective removal of interferences from a complex sample and b) preconcentration of the target analyte to reach the detectable concentration of the given analytical method. A major concern in low-level analyte determination with MISPE is the bleeding of the template from the polymer matrix. This problem has been overcome by the use of a 'dummy template'.\(^ {109}\) In this case instead of the analyte to be measured a closely related structural analog is used for the synthesis of the imprinted polymer. Using chromatography or mass spectrometry the bleeding will not cause any false results in the analysis. When loading an aqueous sample onto the MISPE cartridge all the less polar components are retained due to hydrophobic, reversed-phase interactions. Thus, after complete drying of the polymer a selective washing step has to be applied with an apolar solvent to wash down the interfering compounds meanwhile selectively retain the target. It is worth mentioning that most of the MIPs perform selectively in apolar solvents where secondary interactions are facilitated. The elution is generally performed with a polar H-bonding solvent which can disrupt the specific bonds between the polymer and the analyte molecules.

In order to develop water-compatible polymers which can selectively extract the analyte from aqueous samples hydrophilic functional monomers and crosslinkers, polar polymerization solvents or stoichiometric imprinting were employed.\(^ {18,110,111}\) Sample preparation can be also carried out online in a chromatographic system with a MIP column used in combination with a commercial HPLC column. This performs as an online molecularly imprinted solid phase extraction systems. A short column, usually not longer than 1-2 cm, and with 2-5 mm internal diameter, is filled with the MIP polymer and placed into the
injector loop of an HPLC instrument prior to the analytical column.\textsuperscript{112} The MISPE column is responsible for the selective retention of the target analytes while the undesired matrix components are washed away. In a consecutive step the purified and preconcentrated sample is eluted from the MISPE column onto the analytical column where the separation of the targets is carried out.

Solid phase extraction can utilize different polymer formats and these also appeared in MIP applications. Nowadays beside the traditional off-line cartridge mode we can find examples for on-line\textsuperscript{112} protocols. Improved batch methods could be achieved by the incorporation of magnetic particles into the polymer.\textsuperscript{113} Molecularly imprinted stir-bar sorptive extraction, solid phase microextraction and combined liquid membrane-MIP techniques also perform well in the MIP arena.\textsuperscript{114-116}

Since MISPE is the most exploited application area of MIPs novel techniques addressing present drawbacks are expected to emerge in the forthcoming years.

\subsection*{2.6.1.3. Selective removal of impurities}

Beside the analytical applications presented above the targeted removal of impurities or undesired compounds from different samples can be of practical importance.

Hoshino et al. developed molecularly imprinted nanoparticles which were able to selectively capture the peptide melittin (bee venom) in the blood stream of mice.\textsuperscript{47} After intravenously administering the toxic compound and subsequently the molecularly imprinted nanoparticles, the imprinted polymer significantly increased the survival rate of the animals and also reduced peritoneal inflammation and weight loss. The authors investigated the behavior of the particles in the blood stream and their accumulation in different organs by fluorescence imaging. Experiments proved that the selective cavities created by molecular imprinting were responsible for the clearance of melittin since nonimprinted particles did not achieve considerable neutralization.

Another group prepared imprinted polymers for the selective removal of the spoilage agent riboflavin from beer.\textsuperscript{117} The originally high non-specific binding on the polymer was suppressed by a basic post-synthesis treatment where the non-reacted methacrylate groups were hydrolyzed rendering the polymer more hydrophilic. Two types of alkaline treatment were evaluated, a batch-mode and an on-line mode. The latter one provided milder conditions since the alkaline solutions could be injected in a controlled way onto the polymer filled chromatographic column. In this case the imprinted binding sites were not degraded but the hydrophobic non-specific binding could be diminished.

An interesting application field of MIPs in industry is the selective removal of genotoxic impurities from pharmaceutical products. In a study carried out by Székely et al. 1,3-diisopropylurea was selected as the target template and methacrylic acid, a common functional monomer was used for imprinting.\textsuperscript{118} It has been observed that the imprinted polymers performed better when a base was present during the synthesis and thus, the ionic form of the functional monomer could interact through Coulomb forces with the template. The removal of the impurity was tested using a hundred-fold excess of the active pharmaceutical components. Even under this experimental condition 80\% of the model impurity could be removed while less than 20\% of the active compound was retained on the polymer sorbent. In a further study the authors could improve the purification method by choosing a suitable solvent to achieve a reduced level of active pharmaceutical ingredient loss.\textsuperscript{119}
2.6.2. Binding assays

Molecularly imprinted polymers are sometimes referred to as plastic antibodies because they can show similar affinity constants and selectivity as natural antibodies under certain conditions. Their main benefits over natural antibodies are the easy and cheap preparation, their long-term stability and resistance to harsh conditions. They can perform well in organic solvents, and assays can be developed for small molecules without the need of conjugating them to a large carrier molecule which is a necessary step in antibody production. A significant difference between MIPs and monoclonal antibodies is that the binding sites are heterogeneous in the MIPs. High affinity sites can be utilized when the polymer is used well below its binding capacity i.e. in very dilute solutions. Generally, MIPs can be used in competitive MIAs (molecularly imprinted sorbent assay) because the relatively large size of a MIP particle hinders the sandwich assay format. A pioneering work was published in Nature in 1993 in which the authors described a MIA targeting theophylline and diazepam. The MIP presented there exhibited very similar cross-selectivity compared to natural antibodies and the paper also highlighted the stability of these artificial antibodies. Among competitive MIAs there are two subgroups: homologous and non-homologous assays. In homologous assays the competitor is a radiolabeled variety of the analyte whereas in non-homologous assays it is a structurally different one, such as a fluorescent analog of the analyte. Radioactive labeling raises environmental concerns therefore fluorescent labeled or enzyme-linked non-homologous assays are preferred nowadays.

2.6.3. Sensors

In sensor devices the MIP serves as the recognition element. In case of a binding event a chemical or physical signal is generated and transduced to a detector. In immunosensor-type devices the immobilized MIP concentrates the analyte on the surface of the detector and some kind of labeling is needed. They often apply fluorescence-based signal generation: for instance the analyte itself is fluorescent and its binding can be detected directly (this is scarcely possible), or the fluorescent labeled competitor is added and the measurement is similar to a displacement assay. It is also possible to label the polymer matrix with a fluorophore which changes its spectroscopic properties upon analyte/template binding. Receptor-type sensors detect the physico-chemical changes upon the template binding event such as mass, reflectivity, conductivity, permeability or surface potential. These methods provide a distinct advantage over immunosensor-type approaches that is no labeling is required. The development of imprinted nanostructures for sensor applications is a rapidly extending field nowadays. The modification of quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) chips with electropolymerizable surface imprinted MIP thin-films offer exciting new approaches for the detection of large biomacromolecules. In our laboratory Bognár et al. developed a technique based on nanosphere lithography where protein conjugated beads were immobilized on a gold coated QCM chip. After their deposition, a conducting polymer was grafted from the surface of the chip in a controlled manner around the beads. Dissolving the spherical beads protein-imprinted sites were liberated in the polymer and the sensor exhibited superior affinity toward the template avidin compared to different analogs. Lautner et al. introduced a novel photolitographic method where protein surface imprinted polymer microbands were prepared directly on SPR chips using sacrificial polycarbonate
The polymer exhibited one order of magnitude higher binding capacity than the nonimprinted polymer and was able to differentiate between analogous proteins.

2.6.4. MIPs in organic synthesis

MIPs can be prepared as selective protecting agents for the enantioselective synthesis of amino acids. For instance a polymerizable levodopa derivative was used as template in covalent imprinting and after hydrolysis a reactive aldehyde and a specific cavity for the L-amino acid was created. The alkylation reaction of glycine in the presence of this polymer cavity was enantioselective due to the imprinted site which made possible for the alkylation agent to attack from a predetermined direction. Molecularly imprinted polymers can be used as enzyme-mimics, too. For this kind of application one needs to imprint with the transition state analog of the substrate and this can be done either by covalent or non-covalent approaches.

An early example of MIP catalysis was presented by Mosbach et al. in the hydrolysis of p-nitrophenyl acetate. The selected transition state analog was p-nitrophenyl methyl phosphonate. The imprinted polymer exhibited higher catalytic activity compared to the NIP. In enzyme mimics highly crosslinked bulk polymers proved to be unfavourable due to the slow diffusion of the substrate to the active sites, instead nanogels were introduced. These crosslinked colloidal matrices are soluble both in aqueous and organic solvents, and their benefits are the high surface-to-volume ratio and consequently the easy accessibility of the active sites. An interesting work exemplified how catalytic nanogels could mimic aldolase type I enzymes. The polymerizable functional monomer was able to imitate the enamine-based activity of the enzyme. A reversible covalent bond could be formed between the monomer and the transition state analog. The imprinted polymer was synthesized in the form of nanogels which were easy-to-handle, soluble in aqueous medium and provided similar dimensions as natural enzymes. The turnover rate proved to be very high compared to other imprinted catalysts and comparison with the nonimprinted counterpart confirmed the effect of imprinting.

2.6.5. Special MIPs with controlled properties

Beside the predetermined selectivity, molecularly imprinted polymers have been endowed with other stimuli-responsive properties. Thermoresponsive MIPs were prepared by the use of poly(N-isopropylacrylamide). A dopamine-imprinted polymer was prepared for the selective extraction of androgenic drugs from urine. At elevated temperature the binding affinity and binding capacity was increased and temperature modulation could increase the clean-up efficiency of the solid phase extraction. Hua et al. targeted bovine serum albumine to create thermoresponsive MIP hydrogels using the functional monomer N-[3-(dimethylamino)propyl]-methacrylamide. The conformational memory of the polymer could be modulated by changing the temperature and the ionic strength.

Magnetic properties facilitate the handling of the imprinted sorbent, for example in sorbent assays where typically small, micro- or nanometer-sized particles are used, and the separation by centrifugation is tedious. Different strategies were tested for the successful incorporation of magnetic nanoparticles into an imprinted polymer matrix, and the particles were used as sensor for the analyte pyrene. In our laboratory, protein-imprinted magnetic microrods were prepared by Ceolin et al. A conductive polymer containing superparamagnetic nanoparticles was electrochemically grown into the pores of a sacrificial polycarbonate membrane onto which previously the protein template, avidin was physisorbed.
This was followed by the dissolution of the polycarbonate support along with the removal of protein stamps from the surface of the rods which possessed surface-imprinted binding sites.

pH-responsive molecularly imprinted polymers were prepared for bisphenol-A with acryloylamylose functional monomer containing carboxylic groups. Reversible binding and release of the template could be observed when switching the pH of the solution between 4.5 and 8.5, respectively. A major class of stimuli-responsive MIPs is light-controllable smart materials. Since these polymers were of interest in my research work, I overview the state-of-the-art of their development in detail.

Until now, relatively few papers have been published in the literature on molecularly imprinted polymers with photoswitching properties. With one exception these approaches use azobenzene type monomer units as the photocontrollable element in the imprinted material that undergo cis-trans isomerization upon photomodulation. In these approaches the azobenzene unit concurrently serves as the functional monomer. As azobenzene itself does not contain appropriate functional groups for the interaction with the template, many research groups have developed new functionalized azobenzenes for this purpose using aniline, carboxyl, bisurea, diaminopyridine, sulfonic acid and pyridine moieties. Zhang’s group have introduced precipitation polymerization for the straightforward synthesis of photoswitchable azobenzene-based MIPs to create directly spherical microparticles. Beside azobenzenes, spiropyans are commonly used to create photoresponsive intelligent materials. Spiropyans can adopt two different structures, a closed, rather non-polar spiropyran (SP) form and an open, highly polar merocyanine (MC) form. Upon photoinduced modulation the molecule can switch between its closed and open form. UV-irradiation transforms the molecule to MC cleaving the C-O bond while the ring-reclosure can be achieved by visible light or thermal stimuli. MC exists in two forms: a charge-separated, zwitterionic form appears in polar solvents, while in non-polar solvents the quinoidal form of the molecule is preferred (Figure 2.8). Despite their extensive research only one publication from 1994 refers to molecular imprinting with a spiropyran acrylate monomer but there has been no follow-up of this work. Here, a tryptophan-imprinted nonporous membrane was used in flow dialysis experiments. The permeation of the template through the imprinted membrane could be efficiently controlled by switching the MC form on and off by irradiation with UV and visible light, respectively. This system, however, lacked mechanical strength, as the fragile imprinted membrane cracked upon photoisomerization in the dialysis chamber, and therefore it was unusable in practical applications.

![Figure 2.8 Photoisomerization of spiropyran](image-url)
Nowadays, we can even find combined, stimuli-responsive MIPs which can be modulated by multiple impacts. Photoresponsive and magnetic properties were integrated into imprinted microspheres and were successfully used in the sample clean-up of caffeine from tap water, cola and tea samples.\textsuperscript{140} In another work, polymer microspheres prepared by atom transfer radical precipitation polymerization have been endowed with photo- and thermoresponsive features.\textsuperscript{136} The grafting of thermoresponsive poly(N-isopropylacrylamide) brushes increased surface hydrophilicity, and the particles could selectively recognize the template 2,4-dichlorophenoxyacetic acid in pure aqueous medium.
3. Materials and methods

3.1. Materials

In the table below the chemicals and reagents used throughout the experiments are shown.

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>methacrylic acid (MAA)</td>
<td>Aldrich</td>
<td>99%</td>
</tr>
<tr>
<td>ethylene glycol dimethacrylate (EGDMA)</td>
<td>Sigma</td>
<td>97%</td>
</tr>
<tr>
<td>trimethylolpropane trimethacrylate (TRIM)</td>
<td>Aldrich</td>
<td>techn. grade</td>
</tr>
<tr>
<td>4-vinylpyridine (4-VPy)</td>
<td>Aldrich</td>
<td>95%</td>
</tr>
<tr>
<td>methacrylamide (MAAm)</td>
<td>Sigma</td>
<td>98%</td>
</tr>
<tr>
<td>2-hydroxy ethyl methacrylate (HEMA)</td>
<td>Aldrich</td>
<td>97%</td>
</tr>
<tr>
<td>methyl methacrylate (MMA)</td>
<td>Sigma</td>
<td>99%</td>
</tr>
<tr>
<td>divinylbenzene (DVB)</td>
<td>Sigma</td>
<td>80%, techn. grade</td>
</tr>
<tr>
<td>azobisisobutyronitrile (AIBN)</td>
<td>Fluka</td>
<td>98%</td>
</tr>
<tr>
<td>benzoin ethyl ether (BEE)</td>
<td>Aldrich</td>
<td>97%</td>
</tr>
<tr>
<td>rac-propranolol HCl</td>
<td>Fluka</td>
<td>98%</td>
</tr>
<tr>
<td>rac-oxprenolol HCl</td>
<td>Fluka</td>
<td>98%</td>
</tr>
<tr>
<td>terbutylazine</td>
<td>Fluka</td>
<td>anal.std.</td>
</tr>
<tr>
<td>ametryn</td>
<td>Fluka</td>
<td>anal.std.</td>
</tr>
<tr>
<td>prometryn</td>
<td>Fluka</td>
<td>anal.std.</td>
</tr>
<tr>
<td>atrazine</td>
<td>Fluka</td>
<td>anal.std.</td>
</tr>
<tr>
<td>phenytoin</td>
<td>Sigma</td>
<td>99%</td>
</tr>
<tr>
<td>diclofenac Na salt</td>
<td>Sigma</td>
<td>anal.std.</td>
</tr>
<tr>
<td>ibuprofen Na salt</td>
<td>Fluka</td>
<td>98%</td>
</tr>
<tr>
<td>ketoprofen</td>
<td>Sigma</td>
<td>98%</td>
</tr>
<tr>
<td>naproxen</td>
<td>Fluka</td>
<td>anal.std.</td>
</tr>
<tr>
<td>adiponitrile</td>
<td>Fluka</td>
<td>98%</td>
</tr>
<tr>
<td>toluene (TOL)</td>
<td>Prolabo</td>
<td>100%</td>
</tr>
<tr>
<td>acetonitrile (MeCN)</td>
<td>Merck</td>
<td>grad. grade</td>
</tr>
<tr>
<td>paraffin oil (PO)</td>
<td>local pharmacy</td>
<td>Ph. Eur.</td>
</tr>
<tr>
<td>caprylonitrile (heptyl cyanide, CAP)</td>
<td>Aldrich</td>
<td>97%</td>
</tr>
<tr>
<td>ammonium acetate</td>
<td>Aldrich</td>
<td>98%</td>
</tr>
<tr>
<td>trifluoroacetic acid (TFA)</td>
<td>Sigma</td>
<td>98%</td>
</tr>
<tr>
<td>acetic acid (AcOH)</td>
<td>Merck</td>
<td>96%</td>
</tr>
<tr>
<td>phosphoric acid</td>
<td>Aldrich</td>
<td>85%</td>
</tr>
<tr>
<td>disodium hydrogen phosphate 12-hydrate</td>
<td>Reanal</td>
<td>99%</td>
</tr>
<tr>
<td>sodium dihydrogen phosphate monohydrate</td>
<td>Fluka</td>
<td>99%</td>
</tr>
<tr>
<td>methanol (MeOH)</td>
<td>Merck; Prolabo</td>
<td>grad. grade, techn.grade</td>
</tr>
<tr>
<td>dichloromethane</td>
<td>Merck</td>
<td>99.9%</td>
</tr>
<tr>
<td>chloroform</td>
<td>Panreac</td>
<td>99.8%</td>
</tr>
</tbody>
</table>
3. Materials and methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>Supplier</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>methyl tert-butyl ether</td>
<td>Merck</td>
<td>99.8%</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium tetrafluoroborate (BMIM’BF₄⁻)</td>
<td>Sigma</td>
<td>97%</td>
</tr>
<tr>
<td>trihexyl(tetradecyl)phosphonium tris(pentafluoroethyl) trifluorophosphate (PH3 T FAP)</td>
<td>Merck</td>
<td>98%</td>
</tr>
<tr>
<td>tetrahydrofuran (THF)</td>
<td>Panreac</td>
<td>99.9%</td>
</tr>
<tr>
<td>1-octanol</td>
<td>Fluka</td>
<td>99.5%</td>
</tr>
<tr>
<td>carbon tetrachloride</td>
<td>Reanal</td>
<td>99%</td>
</tr>
<tr>
<td>1,1,1-trichloroethane</td>
<td>Fluka</td>
<td>90-95%</td>
</tr>
<tr>
<td>n-hexane</td>
<td>Prolabo</td>
<td>95%</td>
</tr>
<tr>
<td>n-heptane</td>
<td>Merck</td>
<td>99%</td>
</tr>
<tr>
<td>n-decane</td>
<td>Sigma</td>
<td>99%</td>
</tr>
<tr>
<td>n-tetradecane</td>
<td>Aldrich</td>
<td>99%</td>
</tr>
</tbody>
</table>

1’-(2-methacryloyloxyethyl)-3’,3’-dimethyl-6-nitrospiro(2H-1benzopyran-2,2’-indoline) (spiropyran methacrylate, SPMA) monomer was synthesized following previously reported procedures. Commercially available methacrylate monomers were purified before use by using a hydroquinone inhibitor remover column (Sigma-Aldrich). 4-vinylpyridine was distilled in vacuo prior to use. DVB was purified by percolation through an alumina sorbent bed.

Water was purified with a Milli Q Direct 8 system (Millipore, France).

Salt forms of template molecules were converted to the free base or free acid form prior to imprinting. HCl salts of propranolol and oxprenolol were dissolved in excess sodium hydroxide solution and were extracted in methyl tert-butyl ether before use. The sodium salt of diclofenac was dissolved in excess hydrochloric acid solution and was extracted in methyl tert-butyl ether before use.

β-blocker-free urine sample was obtained from a healthy male volunteer. Blank blood plasma sample with citrate phosphate dextrose anticoagulant was purchased form the National Blood Service (Budapest, Hungary).

24-well 10 mL UNIFILTER microplates with GF/C glass microfiber membranes (pore diameter 1.2 μm, membrane thickness 260 μm, membrane diameter 12 mm) were from Whatman (Maidstone, UK). Spin-X centrifuge tube with filter inset (0.45 μm cellulose acetate filter, 2 mL) was from Corning Inc. (Corning, NY, USA).

3.2. General methods

In this section methods that have been used for the characterization of the polymers throughout the work are given.

3.2.1. HPLC methods

The measurement of template (or other analyte) binding on the polymers was carried out by equilibrium batch rebinding experiments, solid phase extraction and filtration tests. In all these cases the analyte concentration of the samples was determined by HPLC.

In Table 3.1 all the applied HPLC methods for the different target compounds are summarized (the relevant chapter number to which the method is connected is indicated in parentheses).
### Table 3.1 Liquid chromatographic methods

<table>
<thead>
<tr>
<th>Analyte</th>
<th>System</th>
<th>Column</th>
<th>Mobile phase composition (v/v%)</th>
<th>F (mL min(^{-1}))</th>
<th>V(_{\text{inj}}) (µL)</th>
<th>Detec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>terbutylazine</td>
<td>JASCO HPLC System</td>
<td>Phenomenex Luna C-18, 4.6×125 mm</td>
<td>40 % water and 60% acetonitrile, isocratic</td>
<td>1</td>
<td>20</td>
<td>UV: 230 nm</td>
</tr>
<tr>
<td>prometryn ametry</td>
<td>JASCO HPLC System</td>
<td>Phenomenex Luna C-18, 4.6×125 mm</td>
<td>50 % water and 50% acetonitrile, isocratic</td>
<td>1</td>
<td>20</td>
<td>UV: 230 nm</td>
</tr>
<tr>
<td>atrazine (Chapter 4)</td>
<td>JASCO HPLC System</td>
<td>Phenomenex Luna C-18, 4.6×125 mm</td>
<td>40 % water and 60% acetonitrile modified with 0.1% AcOH, isocratic</td>
<td>1</td>
<td>20</td>
<td>UV: 260 nm</td>
</tr>
<tr>
<td>ketoprofen (Chapter 4)</td>
<td>JASCO HPLC System</td>
<td>Phenomenex Luna C-18, 4.6×125 mm</td>
<td>40 % water and 60% acetonitrile modified with 0.1% AcOH, isocratic</td>
<td>1</td>
<td>20</td>
<td>UV: 230 nm</td>
</tr>
<tr>
<td>mixture of triazines and ketoprofen (Chapter 4)</td>
<td>Perkin Elmer Series 200 HPLC</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>68% phosphate buffer (pH 3.0, c=10 mM) and 32% acetonitrile, isocratic</td>
<td>0.6</td>
<td>10</td>
<td>UV: 230 nm</td>
</tr>
<tr>
<td>naproxen, diclofenac, ibuprofen (Chapter 5)</td>
<td>Perkin Elmer Series 200 HPLC</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>50% phosphate buffer (pH 3.0, c=10 mM) and 50% acetonitrile, isocratic</td>
<td>0.6</td>
<td>10</td>
<td>UV: 233 nm</td>
</tr>
<tr>
<td>propranolol (Chapter 6)</td>
<td>Perkin Elmer Series 200 HPLC</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>72% phosphate buffer (pH 3.0, c=10 mM) and 28% acetonitrile, isocratic</td>
<td>0.6</td>
<td>10</td>
<td>UV: 215 nm</td>
</tr>
<tr>
<td>propranolol (Chapter 6)</td>
<td>Perkin Elmer Series 200 HPLC coupled to Perkin elmer Sciex API 365 triple quadrupole mass spectrometer</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>methanol and ammonium acetate buffer (pH 4.0, 10 mM), gradient</td>
<td>0.4</td>
<td>10</td>
<td>MRM: m/z 260.3 → m/z 116.0 transition</td>
</tr>
<tr>
<td>terbutylazine (Chapter 7)</td>
<td>Perkin Elmer Series 200 HPLC</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>30 % water and 70% acetonitrile, isocratic</td>
<td>0.6</td>
<td>10</td>
<td>UV: 233 nm</td>
</tr>
<tr>
<td>mixture of triazines and phenytoin (Chapter 7)</td>
<td>Perkin Elmer Series 200 HPLC</td>
<td>Merck LiChroCart Purospher Star RP-18e, 125×3.0 mm</td>
<td>50 % water and 50% acetonitrile, isocratic</td>
<td>0.6</td>
<td>10</td>
<td>UV: 233 nm</td>
</tr>
</tbody>
</table>
3.2.2. N$_2$-sorption measurements

Nitrogen adsorption/desorption isotherms were measured at 77 K, using a Quantachrome NOVA2000 computer controlled apparatus. Samples were outgassed at ambient temperature for overnight. The apparent surface area $S_{BET}$ was derived according to the BET model. The Dubinin-Radushkevich (DR) model was used to evaluate the micropore range of the isotherm. The micropore volume $V_{micro}$ and the average diameter of the micropores $d_{micro}$ were calculated. The equivalent surface area of the micropores $S_{micro}$ was estimated with the assumption that all the molecules defined by $V_{micro}$ contribute to a close-packed monolayer within the micropores. The total pore volume ($V_{tot}$) was calculated from the amount of nitrogen vapor adsorbed at relative pressures of 0.99 (Chapter 5) and 0.90 (Chapter 7), on the assumption that the pores are then filled with liquid nitrogen. An average pore diameter, $d_{ave}=4V_{tot}/S_{BET}$ was derived from the total pore volume and $S_{BET}$, assuming cylindrical geometry. Pore size analysis in the mesopore range was performed from the adsorption branch by the Quantachrome software using the Barrett, Joyner and Halenda (BJH) method.

3.2.3. Scanning electron microscopy

The morphological characterization of the polymer microparticles and composite membranes was carried out by scanning electron microscopy (SEM) using a JEOL JSM-5500LV instrument (or JEOL JSM-6510LV at the University of Geneva). Samples were sputter coated with Au/Pd prior to analysis. Cross-sectional view of the membranes was obtained after freeze fracturing them in liquid nitrogen. Particle size analysis was accomplished visually with the ImageJ software (National Institute of Health) selecting 200 individual particles from the SEM images of each sample. Number-average diameter ($D_n$), weight-average diameter ($D_w$), and uniformity (U, polydispersity index) were calculated by the following formulas:

$$D_n = \frac{\sum_{i=1}^{k} n_i D_i}{\sum_{i=1}^{k} n_i}; \quad D_w = \frac{\sum_{i=1}^{k} n_i D_i^3}{\sum_{i=1}^{k} n_i}; \quad U = D_w / D_n; \quad \text{Eqs. 7-9}$$

where $D_i$ denotes the individual diameter of a particle obtained from the SEM image and $n_i$ is the number of particles with a specific diameter.

3.2.4. Equilibrium batch rebinding measurements

MIPs and nonimprinted polymers were weighted into polypropylene microtubes. The solution of the template (or analyte of interest) was pipetted on the particles or directly on the composite filterplate membranes in toluene or in acetonitrile. The phase ratio was set at 60, i.e. 60 µL solvent/mg polymer was applied in the experiments. The samples were shaken on a Fisher Vortex Genie 2 or on a Grant-Bio PTR-35 Multi-rotator until equilibrium was reached. The samples were centrifuged on a Hermle Z 100 M or on an Eppendorf Minispin microcentrifuge to separate the particles. Filterplate samples were percolated into a collector plate on a VacMaster-96 vacuum manifold applying gentle vacuum. The supernatant or percolated liquid was evaporated under a gentle air stream in case of toluene and reconstituted in eluent or diluted to eluent composition when using acetonitrile.

The concentration of the unbound analyte ($c_e$) was determined with the appropriate HPLC method according to Section 3.2.1.
3. Materials and methods

From the equilibrium concentration the bound concentration of analyte can be calculated according to the following equation:

\[
q_e = \frac{(c_o - c_e)V}{m},
\]

Eq. 10

where \(c_o\) and \(c_e\) are the initial and equilibrium concentration [mol L\(^{-1}\)] of the analyte, respectively. \(V\) is the volume of solution [L], \(m\) is the mass of the dry polymer [kg], and \(q_e\) is the adsorbed concentration expressed in [mol kg\(^{-1}\)]. From \(q_e\) one can calculate the distribution coefficient, \(D\) [L kg\(^{-1}\)]:

\[
D = \frac{q_e}{c_e}
\]

Eq. 11

3.3. Specific methods

3.3.1. Preparation of the polymers

3.3.1.1. MIP microspheres bearing photoswitchable spiropyran unit (Chapter 4)

The template terbutylazine, the functional monomer MAA, spiropyran methacrylate (SPMA), the crosslinker TRIM or EGDMA and AIBN initiator were weighted into a screw cap glass vial. The components were dissolved in toluene (2 w/v% monomer concentration). After deoxygenating the solution with nitrogen, the polymerization vessel was placed into a waterbath thermostated at 60\(^\circ\)C for 48 hours in the dark. The precipitated polymer particles were collected by centrifugation and extensive batch-mode washing was performed by changing the washing solvent, methanol-acetic acid (9:1) several times until no template was detected in it by HPLC. Finally, the polymers were washed with methanol, and left overnight for complete drying in a ventilated hood. Simultaneously, a nonimprinted polymer (NIP) was prepared in the same manner as the MIP except that the template was omitted from the prepolymerization mixture. The detailed composition of the studied polymers (PS1-PS6) is summarized in Table 3.2.

<table>
<thead>
<tr>
<th></th>
<th>template (mmol)</th>
<th>MAA (mmol)</th>
<th>SPMA (mmol)</th>
<th>EGDMA (mmol)</th>
<th>TRIM (mmol)</th>
<th>AIBN (mg)</th>
<th>toluene (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS1 MIP</td>
<td>0.08</td>
<td>0.32 [16%]</td>
<td>-</td>
<td>1.59 [84%]</td>
<td>-</td>
<td>3.4</td>
<td>16.8</td>
</tr>
<tr>
<td>PS1 NIP</td>
<td>0.08</td>
<td>-</td>
<td>0.10 [5%]</td>
<td>1.58 [79%]</td>
<td>-</td>
<td>3.8</td>
<td>19.2</td>
</tr>
<tr>
<td>PS2 MIP</td>
<td>0.08</td>
<td>0.32 [16%]</td>
<td>0.10 [5%]</td>
<td>1.38 [69%]</td>
<td>-</td>
<td>4.3</td>
<td>21.3</td>
</tr>
<tr>
<td>PS2 NIP</td>
<td>0.08</td>
<td>-</td>
<td>0.30 [15%]</td>
<td>1.60 [80%]</td>
<td>-</td>
<td>4.5</td>
<td>22.5</td>
</tr>
<tr>
<td>PS4 MIP</td>
<td>0.02</td>
<td>0.10 [5%]</td>
<td>0.30 [15%]</td>
<td>0.49 [39%]</td>
<td>-</td>
<td>4.5</td>
<td>22.5</td>
</tr>
<tr>
<td>PS4 NIP</td>
<td>0.02</td>
<td>-</td>
<td>0.32 [16%]</td>
<td>0.49 [39%]</td>
<td>-</td>
<td>3.1</td>
<td>15.7</td>
</tr>
<tr>
<td>PS5 MIP</td>
<td>0.04</td>
<td>0.16 [16%]</td>
<td>0.45 [45%]</td>
<td>-</td>
<td>0.39 [39%]</td>
<td>3.4</td>
<td>16.8</td>
</tr>
<tr>
<td>PS5 NIP</td>
<td>0.08</td>
<td>-</td>
<td>0.45 [45%]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.2 Chemical composition of the studied MIPs and NIPs

(the mole\% of monomers is indicated in the brackets)
3.3.1.2. Polymer microspheres by the modified precipitation polymerization (Chapter 5)

The polymerization mixture containing the functional monomer, and/or the crosslinker, the initiator and the polymerization solvent mixture was prepared in a glass vial prior to polymerization. The mixture was purged with argon for 1.5 minutes, tightly sealed with a PTFE septum cap and was placed into a thermostat at 60°C for 24 hours. The formed polymers were thoroughly washed with 3×3 mL toluene followed by 3×3 mL methanol and were dried overnight under vacuum.

A typical polymer recipe was as follows:
Functional monomer, (0.147 mmol), crosslinking monomer, (0.733 mmol) and initiator, AIBN (0.011 mmol, 1.8 mg, 1.3 mol% of the monomers) were dissolved in 750 µL of co-solvent/PO mixture (50/50 v/v%). The solvent/monomer volume ratio was 3:1. The molar ratio of crosslinker to functional monomer was 5:1. For the detailed composition of the polymers prepared see Table 3.3

In case of the imprinted polymers a similar recipe was applied, but also the template, diclofenac or naproxen was added to the polymerization mixture in a molar ratio of 1:4 relative to the functional monomer.

Table 3.3 Polymer compositions and morphological properties of the microparticles prepared by the modified precipitation polymerization

<table>
<thead>
<tr>
<th>func. monomer (mole%)</th>
<th>cross-linker (mole%)</th>
<th>Monomer (v/v%)</th>
<th>solvent composition (volume %)</th>
<th>initiator (mole%)</th>
<th>Dₐ (µm)</th>
<th>CV %</th>
<th>D₉₀ (µm)</th>
<th>U°</th>
<th>Polymer morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1 17%</td>
<td>TRIM 83%</td>
<td>33 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>hard monolith</td>
</tr>
<tr>
<td>P2 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>1.84</td>
<td>9.0</td>
<td>1.87</td>
<td>1.020</td>
<td>microspheres</td>
</tr>
<tr>
<td>P3 17%</td>
<td>TRIM 83%</td>
<td>17 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>1.74</td>
<td>8.6</td>
<td>1.79</td>
<td>1.025</td>
<td>microspheres</td>
</tr>
<tr>
<td>P4 17%</td>
<td>TRIM 83%</td>
<td>9 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>1.62</td>
<td>8.7</td>
<td>1.66</td>
<td>1.022</td>
<td>microspheres</td>
</tr>
<tr>
<td>P5 17%</td>
<td>TRIM 83%</td>
<td>5 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>1.36</td>
<td>8.9</td>
<td>1.39</td>
<td>1.023</td>
<td>microspheres</td>
</tr>
<tr>
<td>P6 17%</td>
<td>TRIM 83%</td>
<td>2 %</td>
<td>CHCl₃:PO =50:50</td>
<td>1.3 %</td>
<td>1.30</td>
<td>6.3</td>
<td>1.31</td>
<td>1.011</td>
<td>microspheres</td>
</tr>
<tr>
<td>P7 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl₃:PO =25:75</td>
<td>1.3 %</td>
<td>1.80</td>
<td>14.7</td>
<td>1.93</td>
<td>1.073</td>
<td>segmented particles</td>
</tr>
<tr>
<td>P8 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl₃:PO =75:25</td>
<td>1.3 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>hard monolith</td>
</tr>
<tr>
<td>P9 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>toluene:PO =50:50</td>
<td>1.3 %</td>
<td>2.84</td>
<td>17.8</td>
<td>3.14</td>
<td>1.103</td>
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</tr>
<tr>
<td>P10 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CCl₄:PO =50:50</td>
<td>1.3 %</td>
<td>3.34</td>
<td>18.1</td>
<td>3.66</td>
<td>1.095</td>
<td>segmented particles</td>
</tr>
<tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>C₂Cl₂H₅:PO =50:50</td>
<td>1.3 %</td>
<td>1.51</td>
<td>10.3</td>
<td>1.56</td>
<td>1.032</td>
<td>microspheres</td>
</tr>
<tr>
<td>P12 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>capronitri tle:PO =50:50</td>
<td>1.3 %</td>
<td>2.52</td>
<td>29.2</td>
<td>3.15</td>
<td>1.251</td>
<td>segmented particles</td>
</tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>THF:PO =50:50</td>
<td>1.3 %</td>
<td>1.55</td>
<td>8.9</td>
<td>1.59</td>
<td>1.023</td>
<td>microspheres</td>
</tr>
<tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>octanol:PO =50:50</td>
<td>1.3 %</td>
<td>1.48</td>
<td>8.7</td>
<td>1.51</td>
<td>1.021</td>
<td>microspheres</td>
</tr>
<tr>
<td>func. monomer (mole%)</td>
<td>cross-linker (mole%)</td>
<td>Monomer (mol%)</td>
<td>solvent composition (volume %)</td>
<td>initiator (mole%)</td>
<td>Dn,µm</td>
<td>CV %</td>
<td>Dh,µm</td>
<td>Uν</td>
<td>Polymer morphology</td>
</tr>
<tr>
<td>----------------------</td>
<td>---------------------</td>
<td>--------------</td>
<td>-----------------------------</td>
<td>-----------------</td>
<td>--------</td>
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<td>--------</td>
<td>-----</td>
<td>---------------------</td>
</tr>
<tr>
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<td>TRIM 83%</td>
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<td>cyclohexane:PO=50:50</td>
<td>1.3 %</td>
<td>3.08</td>
<td>21.9</td>
<td>3.53</td>
<td>1.147</td>
<td>segmented particles</td>
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<td>25 %</td>
<td>CHCl3:PO =50:50</td>
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<td>1.03</td>
<td>10.9</td>
<td>1.07</td>
<td>1.039</td>
<td>microspheres</td>
</tr>
<tr>
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<td>TRIM 83%</td>
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<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>3.36</td>
<td>19.0</td>
<td>3.7</td>
<td>1.100</td>
<td>microspheres</td>
</tr>
<tr>
<td>P18 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>1.68</td>
<td>6.9</td>
<td>1.70</td>
<td>1.015</td>
<td>microspheres</td>
</tr>
<tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>3.50</td>
<td>13.6</td>
<td>3.68</td>
<td>1.054</td>
<td>microspheres</td>
</tr>
<tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>3.11</td>
<td>11.5</td>
<td>3.24</td>
<td>1.041</td>
<td>microspheres</td>
</tr>
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<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>2.20</td>
<td>9.3</td>
<td>2.25</td>
<td>1.024</td>
<td>microspheres</td>
</tr>
<tr>
<td>P22 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>2.15</td>
<td>8.4</td>
<td>2.19</td>
<td>1.021</td>
<td>microspheres</td>
</tr>
<tr>
<td>P23 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>very small, irregular particles</td>
</tr>
<tr>
<td>P24 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>linear polymer</td>
</tr>
<tr>
<td>P25 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>irregular particles</td>
</tr>
<tr>
<td>P26 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>2.97</td>
<td>22.5</td>
<td>3.41</td>
<td>1.148</td>
<td>microspheres</td>
</tr>
<tr>
<td>P27 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>2.26</td>
<td>9.9</td>
<td>2.33</td>
<td>1.030</td>
<td>microspheres</td>
</tr>
<tr>
<td>P28 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>1.61</td>
<td>7.1</td>
<td>1.63</td>
<td>1.014</td>
<td>microspheres</td>
</tr>
<tr>
<td>P29 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>1.88</td>
<td>7.7</td>
<td>1.92</td>
<td>1.020</td>
<td>microspheres</td>
</tr>
<tr>
<td>P30 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>1.3 %</td>
<td>3.34</td>
<td>11.0</td>
<td>3.46</td>
<td>1.035</td>
<td>microspheres</td>
</tr>
<tr>
<td>P31 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>0.3 %</td>
<td>2.30</td>
<td>8.8</td>
<td>2.35</td>
<td>1.023</td>
<td>microspheres</td>
</tr>
<tr>
<td>P32 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>0.7 %</td>
<td>1.95</td>
<td>7.2</td>
<td>1.98</td>
<td>1.015</td>
<td>microspheres</td>
</tr>
<tr>
<td>P33 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>2.8 %</td>
<td>1.68</td>
<td>10.2</td>
<td>1.74</td>
<td>1.036</td>
<td>microspheres</td>
</tr>
<tr>
<td>P34 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>5.7 %</td>
<td>1.55</td>
<td>7.2</td>
<td>1.58</td>
<td>1.016</td>
<td>microspheres</td>
</tr>
<tr>
<td>P35 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>8.2 %</td>
<td>1.49</td>
<td>7.3</td>
<td>1.51</td>
<td>1.016</td>
<td>microspheres</td>
</tr>
<tr>
<td>P36 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>CHCl3:PO =50:50</td>
<td>11 %</td>
<td>1.34</td>
<td>12.6</td>
<td>1.40</td>
<td>1.051</td>
<td>irregular particles</td>
</tr>
<tr>
<td>P37 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>Toluene:PO =75:25</td>
<td>1.3 %</td>
<td>2.26</td>
<td>11.1</td>
<td>2.34</td>
<td>1.037</td>
<td>microspheres</td>
</tr>
<tr>
<td>P38 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>Toluene:PO =50:50</td>
<td>1.3 %</td>
<td>2.04</td>
<td>15.3</td>
<td>2.17</td>
<td>1.063</td>
<td>microspheres</td>
</tr>
<tr>
<td>P39 17%</td>
<td>TRIM 83%</td>
<td>25 %</td>
<td>Toluene:PO =25:75</td>
<td>1.3 %</td>
<td>2.82</td>
<td>17.9</td>
<td>2.82</td>
<td>1.096</td>
<td>microspheres</td>
</tr>
</tbody>
</table>

aDn is the number-average diameter of the particles
bDw is the weight-average diameter of the particles
U is the polydispersity index of the particles
UV polymerization
3.3.1.3. UV polymerization of molecularly imprinted composite membranes on multiwell filterplates (Chapter 6, 7)

The membranes of the 24-well filterplate were washed with 5 mL MeOH and dried before use. The polymerization mixture containing the template (0.1 mmol, propranolol or oxprenolol in Chapter 6, and terbutylazine in Chapter 7), the functional monomer (MAA 0.4 mmol), the cross-linker (EGDMA 2 mmol), the UV initiator (BEE 0.03 mmol) and the porogen was prepared in a glass vial prior to polymerization. For propranolol and oxprenolol imprinted polymers 585 µL adiponitrile porogen was used. For the terbutylazine imprinted polymers 1290 µL solvent or solvent mixture was applied, whose composition can be seen in Table 3.4. The polymerization mixture was purged with argon for 2 mins, then 30 µL (in case of propranolol and oxprenolol MIPs) or 50 µL (in case of terbutylazine MIPs) was transferred onto the filterplate membranes under oxygen-free argon atmosphere. The plate was covered with a UV transparent cling film and was placed under two UVC germicidal lamp (35 W, 254 nm, LightTech Ltd., Dunakeszi, Hungary) at a distance of 15 cm as it is shown in Figure 3.1. The irradiation time was 3 hours. Argon flow was maintained during the whole procedure. Nonimprinted polymer (NIP) modified filterplates omitting the template were also prepared for comparison. The template and the other nonpolymerized components were removed by washing with toluene (in case of PO containing solvents) and methanol.

The amount of molecularly imprinted polymer formed in one well was calculated by measuring the weight difference of the filterplate before and after polymerization and dividing it by the number of wells used. 13.0±0.5 mg of polymer was formed in each membrane, which corresponds to approx. 100% conversion of the monomers.

Table 3.4 Polymerization solvents and solvent mixtures used to prepare the terbutylazine imprinted particle composite membranes

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Polymerization solvent</th>
</tr>
</thead>
</table>
| CAP/PO MIP/NIP | 33 % (v/v) caprylonitrile  
67 % (v/v) paraffin oil |
| TOL/PO MIP/NIP | 25 % (v/v) toluene  
75 % (v/v) paraffin oil |
| PH3 T FAP MIP/NIP | trihexyl(tetradecyl)phosphonium tris(pentafluoroethyl) trifluorophosphate |
| BMIM BF4 MIP/NIP | 1-butyl-3-methylimidazolium tetrafluoroborate |

Figure 3.1 UV polymerization set-up on the filterplate
3.3.2. Binding studies

3.3.2.1. Photocontrolled binding and release study of spiropyran-based photoswitchable particles (Chapter 4)

Samples were incubated with the template solution either in acetonitrile or in toluene until equilibrium was reached using the experimental conditions described in Section 3.2.4. Sampling of the supernatant was first carried out without UV light manipulation. After that, the samples were UV irradiated with a Herolab NU-4 UV Hand lamp (4 W, 365 nm) for 10 and 15 min, in toluene and acetonitrile, respectively and were analyzed.

For repetitive photomodulated binding cycles a series of replicate samples containing photoswitchable particles were incubated in equilibrium batch rebinding experiments (see Section 3.2.4.) and one of them was analyzed after each of the following steps by sampling the supernatant. When equilibrium was reached the supernatant was analyzed. After that, the samples were irradiated with UV light using a Herolab NU-4 UV Hand lamp (4 W, 365 nm). 10-minute irradiation time was applied when toluene was used as solvent, while samples in acetonitrile were irradiated for 15 minutes. Thereafter the samples were reincubated under visible white light until equilibrium was reached. A second UV irradiation was applied in the same manner as before. A third cycle with visible light was performed converting the spiropyran units to their closed state.

3.3.2.2. Molecularly imprinted solid phase extraction with the MIP composite membrane filterplates

β-blocker MIP modified membranes (Chapter 6):

Membrane filterplates were assembled onto a VacMaster-96 vacuum manifold (Biotage AB, Uppsala, Sweden) connected to a VacMaster VCU vacuum control unit and a vacuum pump (Ilmvac LKC 131Z, Saskia GmbH, Ilmenau, Germany). The following optimized solid phase extraction (SPE) protocol was used with the filterplate membranes for the extraction of biological samples. First, molecularly imprinted membranes were conditioned by flowing through 1 mL methanol and 1 mL buffer solution of pH 10.0. Then 100 μL sample adjusted to pH 10.0 was loaded onto the composite membranes and kept there for 5 minutes by plugging the outlet of each filterplate well. Afterwards, a washing step with 500 μL water at a flow rate of approximately 0.5 mL min⁻¹ was employed. The polymers were dried by passing air through the wells for 10 minutes, then 100 μL MeCN was percolated through the modified membranes by gravity. Elution was carried out with 4x250 μL MeOH modified with 2% trifluoroacetic acid. For each elution aliquot 5 min residence time and shaking on a Heidolph REAX 2000 orbital shaker (Heidolph Elektro GmbH, Keilheim, Germany) were allowed. The eluate obtained was evaporated under a gentle N₂ stream at 40 °C with a TurboVap LV evaporator (Zymark, Hopkinton, MA) and was reconstituted in 100 μL HPLC eluent for final analysis by high performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS) according to Section 3.2.1.

terbutylazine MIP modified membranes (Chapter 7):

1 mL aqueous sample containing 10⁻⁶ M terbutylazine, atrazine, ametryn, prometryn and 10⁻⁵ M phenytoin was applied onto the composite MIM. The membrane was washed two
times with 250 μL MeCN:water=30:70 mixture and eluted with 2 mL methanol. All fractions were collected and analyzed by HPLC according to Section 3.2.1.

3.3.2.3. Characterization of the terbutylazine imprinted membranes in filtration experiments (Chapter 7)

Water permeability measurements were carried out by percolating water through the membranes using vacuum as a driving force with a VacMaster VCU vacuum control unit and a vacuum pump (IlmVac LKC 131Z, Saskia GmbH, Ilmenau, Germany).

To obtain breakthrough curves 200 μL aliquots of 10⁻⁶ M terbutylazine in MeCN were filtered through the membranes by gravity. The permeates were diluted to eluent composition and were analyzed by HPLC according to Section 3.2.1.

3.3.3. Spectroscopic methods for the characterization of the photoswitchable particles (Chapter 4)

3.3.3.1. Fluorescence microscope imaging

Spiropyran-based MIP polymer particles were incorporated into a plasticized poly(vinyl chloride) thin film drop casted on a glass slide. Imaging was carried out on a Nikon Eclipse Ti inverted microscope with a 20x Plan Fluar lens. For UV illumination the light of a xenon arc lamp (Lambda DG-4, Sutter Instrument Company) was filtered through a 365/20 nm bandpass filter (F49-365 ZET Laser Clean UP, Chroma) and reflected onto the sample using a 593 nm dichroic mirror. The fluorescence images were recorded using a Neo sCMOS camera (Andor). For imaging the ring-reclosure process, a combination of a 413 nm longpass filter (SR-FF02-409/LP-25, Semrock) and a 550 nm shortpass filter (FELS0550, Thorlabs) was used for excitation. This combination allowed sufficient light intensity to trigger the ring closure reaction while still enabling the fluorescence imaging of the open form. A compromise had to be made with the settings since the fluorescent excitation wavelength of the merocyanine form is in the visible wavelength range. The exposure times of the camera were adjusted to give the same maximum signal in both illumination modes.

3.3.3.2. UV-Vis spectroscopy study and photoswitching of the SPMA monomer in solution and the spiropyran-based polymer microparticles in suspension

The spiropyran methacrylate monomer solution and the SPMA-based polymer particle suspension were both prepared in toluene. The UV-Vis absorbance spectra were recorded with a CCD-array detector (CCS200, Thorlabs) using a fast wavelength switchable xenon arc lamp as light source (Lambda DG-4, Sutter Instrument Company), both controlled with the LabView interface. The DG-4 wavelength switching device allowed the illumination with UV-light for triggering photoreactions and with white light for the absorbance measurement. The short time required for spectrum acquisition (< 0.1 s) in every 10 s avoids undesired photoeffects due to the illumination during measurement. Repeated photoswitching cycles with consecutive UV and Vis irradiation were carried out and the absorbance of the MC form was recorded in the monomer solution and in the particle suspension. For UV illumination a 365/20 nm band pass filter (F49-365 ZET Laser Clean UP, Chroma) was used for 160 s and for the subsequent visible light manipulation a 500 nm cut on long pass filter (FEL0550, Thorlabs) was applied for 300 s. Consecutive UV and Vis switching cycles were performed.
The suspension of the spiropyran containing MIP microparticles in toluene was studied under constant agitation with a magnetic stirrer to prevent sedimentation using a 1.0 cm optical path length quartz cuvette. Sample interrogation was carried out at room temperature in a dark box to avoid ambient light.

First-order kinetic rate constants were calculated for the visible light induced back isomerization using the equation

$$\ln\left[\frac{A_t}{A_0}\right] = -kt,$$

where $A_t$ is the absorbance value at time $t$, $A_0$ is the maximum value reached after UV irradiation and $k$ is the rate constant.

### 3.3.4. Characterization of the polymer-solvent interactions in the modified precipitation polymerization (Chapter 5)

To correlate the observed morphology of the polymer particles to their solubility in the polymerization solvent or solvent mixture the Hansen solubility parameter “distance” between the solvent and the polymer system has been estimated. The following equation gives the solubility parameter distance, $R_a$, between two materials based on their respective partial solubility parameter components.

$$2 \left( \delta_D^2 + \delta_P^2 + \delta_H^2 \right) = 4 \left( \delta_D^2 - \delta_P^2 - \delta_H^2 \right) + \left( \delta_D^2 - \delta_P^2 - \delta_H^2 \right) + \left( \delta_D^2 - \delta_P^2 - \delta_H^2 \right),$$

where $\delta_D$, $\delta_P$ and $\delta_H$ represent the dispersion, polar and H-bonding components of the Hansen solubility parameter (HSP), respectively, while subscripts 1 and 2 refer to the solvent and the polymer. This equation was developed from plots of experimental data where the introduction of the constant “4” represented the solubility data as a sphere encompassing the good solvents. When the scale for the dispersion parameter is doubled in comparison with the other two parameters essentially spherical, rather than spheroidal, regions of solubility are found.\(^{142}\)

HSPs of solvents and monomers were taken from literature data\(^{142}\) unless otherwise noted (see Table 3.5). Solubility parameters of compounds without existing literature data have been estimated by the group contribution method of van Krevelen.\(^{143}\) Hansen solubility parameters of solvent mixtures or monomer mixtures were obtained as the weighted average of the components’ respective HSP values using their volume fraction as the weighting factor. $\delta$ of the polymers can only be determined indirectly and depends on the crosslinking density. In our calculations the three dimensional HSP of the polymers has been estimated by that of the constituting monomer mixtures as they are often found to be close in practice.\(^{144}\)
### Table 3.5 Solubility parameters of the solvents and monomers used during the study

<table>
<thead>
<tr>
<th>Solvent/Monomer</th>
<th>$\delta_d$ (MPa$^{0.5}$)</th>
<th>$\delta_p$ (MPa$^{0.5}$)</th>
<th>$\delta_h$ (MPa$^{0.5}$)</th>
<th>$\delta$ (MPa$^{0.5}$)</th>
</tr>
</thead>
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<td>0.0</td>
<td>0.0</td>
<td>14.9</td>
</tr>
<tr>
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<td>15.3</td>
<td>0.0</td>
<td>0.0</td>
<td>15.3</td>
</tr>
<tr>
<td>decane</td>
<td>15.7</td>
<td>0.0</td>
<td>0.0</td>
<td>15.7</td>
</tr>
<tr>
<td>tetradecane</td>
<td>16.2</td>
<td>0.0</td>
<td>0.0</td>
<td>16.2</td>
</tr>
<tr>
<td>paraffin oil$^{(a,c)}$ (mixture of C$<em>{22}$-C$</em>{36}$ branched alkanes)</td>
<td>15.9</td>
<td>0.0</td>
<td>0.0</td>
<td>15.9</td>
</tr>
<tr>
<td>toluene</td>
<td>18.0</td>
<td>1.4</td>
<td>2.0</td>
<td>18.2</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>16.8</td>
<td>0.0</td>
<td>0.2</td>
<td>16.8</td>
</tr>
<tr>
<td>caprylonitrile$^b$</td>
<td>16.0</td>
<td>7.4</td>
<td>3.3</td>
<td>17.9</td>
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$^a$composition identified by gas chromatography-mass spectrometry analysis

$^b$estimated by using a computer software "Hansen Solubility Parameters in Practice (HSPiP)" version 3.145

$^c$HSP values estimated using the van Krevelen method

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### 3.3.5. Determination of the co-solvent content in the polymer phase and the solution phase in the modified precipitation polymerization (Chapter 5)

Polymerization was thermally initiated in three replicate samples using either a polymerization mixture of 4-VPy, TRIM and AIBN in chloroform/PO solvent or MAA, EGDMA and AIBN in toluene/PO solvent. The components were applied in the same proportions as in the typical polymer recipe (see Section 3.3.1.2.). Polymerization was stopped after 3 hours by cooling the samples to room temperature. Microgel particles formed up to this point were separated from the solvent phase in a 2 mL Costar Spin-X centrifuge tube filter by centrifugation at 15,000 rpm at 4°C in an Eppendorf 5430R centrifuge (Eppendorf AG, Hamburg, Germany). Both the filtrate and the separated polymer were analyzed for the co-solvent content. In case of chloroform, the highly volatile solvent was evaporated from the samples and was determined by the weight loss. Toluene concentration was determined by UV absorbance measurements at 260 nm on a Jasco V550 UV-Vis spectrophotometer (Jasco Inc., Easton, MD, USA). Hexane was used to dilute the collected solution phase and to wash out the polymerization solution from the polymer particles prior to photometric analysis.
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

4.1. Introduction

Spiropyrans are probably the most studied class of compounds showing photochromic properties. Their incorporation into a polymer using the concept of molecular imprinting can lead to photoresponsive recognition elements in chemical sensors, light-controlled selective adsorbents or membrane separators. There exists only one early example in the literature on the use of SP in molecularly imprinted polymers with no follow-up. All the few other works use azobenzene type photoisomerizable monomers to create photoswitchable MIPs. In these approaches the photocontrollable monomer has a dual role; it serves both as a photoswitch and a functional monomer, as well. For this purpose, a specific site has to be incorporated into the azobenzene monomer to provide an interaction point with the template. This, of course, needs a lengthy organic synthetic work. Moreover the photoswitchable template binding and release contradicts to the strong and selective template binding. If the azobenzene functional monomer binds the target too tightly it cannot release it upon photoisomerization and vice versa.

In cooperation with the University of Geneva we introduced a versatile approach for the fabrication of photoswitchable MIPs that avoid the above weakness. Our approach separates the two different functions of photoswitching and recognition. Methacrylic acid, a well-established, common functional monomer is used to selectively interact with the template, while a spiropyran methacrylate monomer is responsible for the photocontrolled template binding and release. We speculated that photoisomerization induces structural changes in SPMA and drastic changes in the conformation of the surrounding polymer network, thereby also changing the spatial arrangement of the binding sites which leads to template release. The proposed concept is illustrated in Figure 4.1.

![Figure 4.1 Scheme of the photomodulation of spiropyran-containing MIPs](image)

Precipitation polymerization as a popular route for the preparation of the microspheres was chosen to prepare photoswitchable MIPs due to its simplicity and compatibility with molecular imprinting. We have chosen terbutylazine, a triazine type herbicide as the model template since triazine imprinted polymers exhibit high imprinting efficiency and selectivity.
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

toward their template and its analogs due to the multiple interactions between the template and the functional monomer, methacrylic acid.\textsuperscript{2,3,146}

The obtained polymer exhibited a photoregulated binding behaviour due to the SP-MC structural changes, it was able to uptake and release the analyte upon illumination with visible or UV light. The inherent photoswitching properties of the particles were characterized by fluorescence microscopy and UV-Vis spectroscopy. The selectivity between the imprinted and nonimprinted polymer and the compound specificity were studied by equilibrium batch rebinding measurements.

4.2. Results and discussion

4.2.1. Preparation of photoswitchable terbutylazine imprinted microparticles

Precipitation polymerization was chosen as a straightforward and beneficial route for the synthesis of spherical MIP particles as it was described in Section 2.4.2.1. In this study, neat toluene was chosen as polymerization medium because triazine imprinted polymers have already been prepared with high imprinting efficiency in toluene by bulk\textsuperscript{2,3,146} and precipitation polymerization.\textsuperscript{11} Moreover, spiropyrans are known to exhibit faster photoswitching kinetics in apolar solvents.\textsuperscript{147} The polymer synthesis was carried out thermally because UV initiation could have induced the ring opening and also the photobleaching of the monomer. At elevated temperatures in polar aqueous solvents the SP molecule may open up because of the H-bonded stabilization of the merocyanine form.\textsuperscript{148} Also for this reason the use of the apolar toluene is advantageous in a thermal polymerization approach.

In addition, methacrylic acid was chosen as an established functional co-monomer responsible for the creation of selective recognition sites by interaction with the triazine molecule through multipoint H-bonding.\textsuperscript{149} The required relative amounts of spiropyran and MAA as well as the quantity and type of crosslinker were explored by preparing polymers with different compositions and testing their template binding ability in equilibrium batch-rebinding assays (see Section 3.2.4.). The measurements were carried out in toluene, i.e., in the polymerization solvent where MIPs are expected to exhibit the highest specific binding.\textsuperscript{150} In all these studies, the template concentration was 100 µM. Distribution coefficients (D) were calculated as a useful interpretation tool of the results derived from the batch-binding experiments.\textsuperscript{7}

Figure 4.2 presents the calculated distribution coefficients of the different polymers (PS1-PS6) both for the imprinted and their nonimprinted counterparts. A detailed description of the different polymer compositions is given in Table 3.2. In parallel, all the polymers were tested for their photochromic properties to find whether illumination with UV light brings about a measurable release of the template. A polymer without spiropyran was prepared for comparison using 16 mol% MAA and 84 mol% EGDMA crosslinker (PS1). Subsequently, 5 mol% SPMA and 16 mol% MAA were incorporated into the polymers at the expense of EGDMA (PS2). The original polymer composition gives high imprinting efficiency considering the much higher D value for the MIP (660±75 L kg\textsuperscript{-1}) than that of the NIP (32±8.2 L kg\textsuperscript{-1}). When SPMA was introduced into the polymer matrix (PS2), a slight decrease in the distribution coefficient could be observed but the binding behavior was still quite similar to the initial composition. However, there was no measurable template release upon photoswitching. A further increase of the SPMA content to 15 mol% (PS3) at the expense of the crosslinker (69 mol%) still could not trigger UV initiated template release. Meantime, the distribution coefficient decreased drastically due to the inadequate crosslinking. In another attempt the crosslinking level was kept high at 80 mol% and SPMA was applied in excess
compared to the functional monomer MAA (PS4). However, the concentration of the spiropyran units in the polymer matrix still proved to be insufficient for the photoactivated template release. Furthermore, an additional decrease in the distribution coefficients and imprinting efficiency was observed due to the inadequate amount of the co-monomer MAA.

These findings suggested that a drastically increased SPMA content is required for light modulated template release, along with a high MAA content in order to retain sufficient binding capacity and selectivity. Accordingly, 45 mol% spiropyran monomer was used with 39 mol% EGDMA crosslinker and 16 mol% functional monomer (PS5). Photoresponsive release and binding behavior was now observed but no selectivity between the MIP and the NIP was achieved. This was attributed to the highly reduced level of crosslinking, which is required to conserve the imprinted binding cavities during polymerization.

It has been reported that the trifunctional crosslinking monomer TRIM is superior to the commonly used bifunctional EGDMA when used in lower crosslinking ratios. It can provide a higher load capacity and the amount of functional monomer can safely exceed the amount of crosslinker without loss of performance. Therefore, a polymer with the above mentioned molar composition was synthesized substituting EGDMA with TRIM (PS6). From Figure 4.2, it is clear that this MIP provided a distinct imprinting effect. It also exhibited UV-induced template binding behavior (for experimental results see Section 4.2.5). We can conclude that high amount of spiropyran monomer at the expense of the crosslinking monomer and therefore an efficient, multifunctional crosslinker (in lower molar ratio) are needed to achieve photoswitchable imprinted polymers. In the subsequent experiments this polymer composition was characterized with different techniques.

![Figure 4.2 Distribution coefficients of imprinted (grey) and nonimprinted polymers (white) with and without SP units (asterisk indicates polymers with photoresponsive binding behavior)](image)

4.2.2. Morphological characterization

The morphology of the polymer microparticles with the optimal composition (PS6) was investigated by scanning electron microscopy (SEM) (see Figure 4.3). The imprinted particles exhibited regular spherical shape with a narrow size distribution (mean diameter 1.70±0.2 µm) while the nonimprinted ones were irregular and smaller with a broad size distribution. This trend was observed also with MAA/EGDMA particles without spiropyran functionality which clearly indicates that the template influences the polymer formation. This suggests that the template–monomer complex changes the solubility of the growing polymer chains, thereby altering the polymer morphology. Typically, acetonitrile is used as solvent for
the precipitation polymerization of methacrylate based MIPs resulting in regular, spherical microparticles. Acetonitrile with a solubility parameter (\(\delta\)) of 24.6 MPa\(^{0.5}\) is more polar than EGDMA or TRIM (\(\delta=18.2\) MPa\(^{0.5}\))\(^\text{11}\) and acts as a poor solvent of the forming methacrylate oligomers. This results in an early phase separation during polymerization when the formed polymer nuclei precipitate. Further on, they grow to larger polymer particles by capturing soluble oligomers and monomers from the polymerization solution. Toluene (\(\delta=18.6\) MPa\(^{0.5}\))\(^\text{27}\) is a good solvent of the methacrylate oligomers thus the network remains fully solvated up to high conversion of the monomers and phase separation is delayed. When phase separation finally occurs, the microgel particles are small and discrete. The separate porogen phase contains relatively low amount of unreacted monomer and crosslinker, which fuse the microgel particles together resulting in small, irregular, aggregated particles when the template is absent. In the presence of the template the template-functional monomer complexes incorporated into the methacrylate oligomers increase the polarity of the chains thereby increasing the difference between the solubility parameter of toluene and the forming polymer network. This again leads to early phase separation, the precipitation of the growing polymer chains and the formation of spherical microparticles.

Figure 4.3 SEM images of molecularly imprinted (A) and nonimprinted (B) polymer microparticles containing spiropyran units

4.2.3. Photoisomerization properties of the MIP microspheres

4.2.3.1. Fluorescence microscopy

The photoactivatable properties of the MIP microspheres were characterized and visualized by fluorescence microscopy. The polymer particles were immobilized into plasticized poly(vinyl chloride) on a glass slide (see Section 3.3.3.1.). Figure 4.4 presents screenshots of a movie where the particles can be observed in their different photoswitched states. The first image in Figure 4.4 shows the fluorescence intensity of the incorporated open, merocyanine units in the particles after UV light irradiation. Subsequently, the fluorescence is continuously decreasing when exposed to visible light as observed in Figure 4.4/[2-4]. The reversibility of the photoswitching was confirmed with several alternating UV and Vis cycles and illustrated in Figure 4.4/[5] and [6], respectively.
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

Figure 4.4 Fluorescence microscopy screenshots from a movie demonstrating the reversible photoswitching property of the MIP microparticles (Image [1],[5]: 10 s UV ($\lambda_{\text{irr}}=365/20$ nm, power= 0.4 W cm$^{-2}$); Image [2], [3], [4], [6]: 2, 7, 55, 45 s visible light illumination ($\lambda_{\text{irr}}=413-550$ nm, power= 8 W cm$^{-2}$), respectively

For the same experiment, the fluorescence intensity change with different light manipulation was analyzed by line profiles across two MIP particles in order to follow the activation and deactivation of single particles. The evolution of fluorescence after UV illumination exhibits faster kinetics compared to the reversed process. Fluorescence intensity data of the alternating activation-deactivation cycles as a function of time give information about the repeatability of the photoswitching on these particles. After ten consecutive cycles approximately ~34% decrease in the intensity could be observed which can be attributed to photobleaching triggered by the light source of the fluorescence microscope (Figure 4.5).

Figure 4.5 Photoswitching cycles of spiropyran containing MIP microspheres by fluorescence microscopy (UV $\lambda_{\text{irr}}=365/20$ nm, time=10 s, power=0.4 W cm$^{-2}$; Vis $\lambda_{\text{irr}}=413-550$ nm, time=50 s, power= 8 W cm$^{-2}$)
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

4.2.3.1. Absorbance measurements

The photoswitching properties of the synthesized microparticles and the SPMA monomer were investigated by UV-Vis spectroscopy in cuvette experiments (see Section 3.3.3.2.). Upon UV irradiation of 50 µM spiropyran methacrylate in toluene the absorbance peak of the open, merocyanine form appears with a peak maximum at 610 nm due to the conjugation between the two aromatic rings (Figure 4.6A) and the solution turns blue. Visible light illumination gradually decreases the absorbance peak of merocyanine (Figure 4.6B) and the solution returns to colorless.

The same experiment was carried out with the spiropyran containing MIP microparticles (PS6) in toluene suspension at 250 µg mL⁻¹ concentration. The background of the MIP microparticle suspension is elevated compared to the solution spectrum due to light scattering of the particles, yet processable spectral data could be acquired. A similar, but somewhat broader absorbance peak was observed upon UV irradiation as in the monomer solution (Figure 4.7A). The absorbance peak decreased when visible light was used to switch the MC form back to SP (Figure 4.7B). This confirms the successful incorporation of the spiropyran monomer into the polymer matrix. Spiropyrans immobilized to polymer matrices tend to exhibit a hypsochromic (blue) shift in their UV-Vis spectrum compared to that in solution, indicating a more polar micro-environment. We could not observe such a shift in case of the MIP microspheres which suggests that the methacrylate based polymer is rather apolar.

The back isomerization of the merocyanine form into spiropyran follows first order kinetics. Comparison of the rate constants in solution and in the polymer can indicate how the molecular photoswitching is affected by anchoring the spiropyran moiety to the solid phase. In the solution phase the rate constant was calculated as $1.5 \pm 0.3 \times 10^{-2}$ s⁻¹, while a decreased value of $4.9 \pm 0.2 \times 10^{-3}$ s⁻¹ was obtained for the polymer particles. The decrease is attributed to the hindered molecular motion of the merocyanine in the polymer matrix during photoswitching.

Figure 4.6 UV-Vis spectral changes with 10 s time increments of 50 µM SPMA in toluene irradiating with $\lambda_{irr} = 365/20$ nm UV light for 90 s (A) and with visible light ($\lambda_{irr} > 500$ nm) for 250 s after UV activation (B)
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

Figure 4.7 UV-Vis spectral changes with 10 s time increments of 250 µg mL$^{-1}$ PS6 MIP suspension in toluene irradiating with $\lambda_{\text{irr}}= 365/20$ nm UV light for 90 s (A) and with visible light ($\lambda_{\text{irr}}= >500$ nm) for 250 s after UV activation (B)

It is well-known that undesired photobleaching can occur when spiropyran is exposed to UV light for longer time periods. Therefore, repeated photoswitching cycles were applied to both systems to gain information about the stability of SPMA. SP–MC transformation was repeatedly triggered by six UV and subsequent Vis light irradiation cycles in 50 µM monomer solution and in the polymer particles in toluene. Relative absorbance values were calculated by dividing the measured absorbance with $A_{\text{max}}$. In Figure 4.8, where the relative absorbance is plotted as a function of time of interrogation, relative absorbance values of the monomer solution (red line) are only reduced to a small extent at the end of the consecutive cycles, which suggests a suitable photostability of the monomer in solution.

In case of the particle suspension (black line) the relative absorbance values were calculated after subtracting the absorbance value of 250 µg mL$^{-1}$ PS1 (not photoswitchable) suspension, thus eliminating the contribution of particle scattering. It has to be noted that in the polymer matrix the open merocyanine form is already present to a measurable extent before UV activation indicated by an initial relative absorbance of 0.28 and the particles are pale purple in their dry state. Polar ester groups, carboxyl and hydroxyl groups on the polymer can interact with the merocyanine moiety. Hence, there is a slight shift in the SP$\rightarrow$MC equilibrium due to the interactions between the open merocyanine form and the carboxyl and ester groups on the polymer backbone.

At the end of the sixth cycles the relative absorbance of merocyanine decreased to approximately 63% of its original value. In contrast to the literature where the covalent attachment of 1% w/w spiropyran to a polymer matrix (PMMA) surface increased its photostability, the opposite effect was observed here. This might be related to the high concentration of spiropyran monomer in the porous polymer matrix that may have resulted in the aggregation of merocyanine molecules and the loss of their photoswitching characteristics. Such an aggregation phenomenon was observed with polymer beads that were surface-modified with spiropyran units. Furthermore, when we interrogated a SPMA solution with subsequent photoswitching cycles at elevated concentration (1 mM), the photobleaching of the spiropyran became conspicuous due to the abovementioned aggregation. We have to note here, that in these cuvette experiments the constant motion of the particles in the stirred solution in front of the small illuminated area causes uncertainty in the repeated photoswitchability.
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

4.2.4. Binding properties of the photoswitchable MIP microspheres

The binding kinetics of the spiropyran containing microparticles in 100 µM of terbutylazine was evaluated without UV light manipulation. The measured equilibration time of ~90 mins suggests fast template binding kinetics.

Equilibrium batch rebinding measurements were carried out in the range of 10-3000 µM initial concentration of the template and the template binding was modulated by UV illumination meantime (see Section 3.2.4. and 3.3.2.1.).

Batch rebinding tests were evaluated in the form of adsorption isotherms, as it allows for a reliable characterization of MIPs. The Freundlich model was applied to fit the adsorption isotherms before and after photomodulation and to gain information about the binding sites. The binding isotherms fitted with the Freundlich model for the imprinted and nonimprinted polymers are shown in Figure 4.9.

**Figure 4.8** Photoswitching cycles of 250 µg mL\(^{-1}\) spiropyran containing MIP microsphere suspension (black line) and 50 µM SPMA (red line) in toluene

**Figure 4.9** Binding isotherms of terbutylazine imprinted (black square) and nonimprinted polymers (red circle) in toluene, inset: initial part of the isotherms before UV (solid symbol) and after UV irradiation (open symbol, \(\lambda_{irr} = 365\) nm, time=10 min, power=4 W)
The calculated binding parameters can be found in Table 4.1. The additional binding capacity created by the template results in a difference of 17.2 µmol g\(^{-1}\) between MIP and NIP comparing the respective values at the highest measured equilibrium concentration level of MIP (2109 µM).

The irradiation time was optimized to achieve maximum release of the previously bound analyte. For this purpose MIP microspheres were equilibrated with 10 µM terbutylazine in toluene or acetonitrile and subsequently illuminated with UV light. Samples were taken from the supernatant at different time intervals and their triazine concentration was determined. The percentage of unbound analyte was calculated for each point and plotted against the UV exposure time. The results are shown in Figure 4.10.

Since the ring-opening kinetics is faster in more apolar solvents, less illumination time was necessary to achieve the new equilibrium in toluene. In toluene, the amount of the unbound template increased by 26% within 10 mins compared to the initial value (18%). In acetonitrile complete release of the template could be observed within 15 mins. We have to point out, however, that the template binding is much weaker in acetonitrile compared to toluene which is attributable to the polar nature of this solvent.\(^3,146\)

As it can be seen in Table 4.1, both the number of binding sites (\(N_{K_{\text{min}}-K_{\text{max}}}\)) and the average affinity constants (\(K_{K_{\text{min}}-K_{\text{max}}}\)) are higher in the imprinted polymer compared to the nonimprinted one. The UV irradiation reduces the amount of binding sites (\(N_{K_{\text{min}}-K_{\text{max}}}\)) both on the MIP and the NIP. However, this effect is more pronounced on the imprinted polymer therefore the MIP can be considered more responsive to light illumination. Upon UV light impact also the binding affinity was reduced on both polymers and it was again more significantly affected in the imprinted polymer. One can conclude that photoswitching induces important changes in the binding property of the studied polymer system.
Table 4.1 Freundlich fitting parameters and calculated values of terbutylazine imprinted and nonimprinted polymers with and without UV light manipulation

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<th>MIP after UV</th>
<th>NIP before UV</th>
<th>NIP after UV</th>
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<td>0.42-190</td>
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The cross-selectivity of the photoswitchable molecularly imprinted polymer was assessed with template analogs and a non-related compound both in individual and in mixed solutions of the analytes. Batch rebinding experiments were carried out with three similar triazine derivatives, atrazine, ametryn and prometryn and a structurally different molecule, ketoprofen which has similar hydrophobicity (log \(P=2.81\)) as the template (log \(P=2.98\)).

First, 50 \(\mu\text{M}\) toluene solutions of the individual analytes were applied in the batch rebinding test in a phase ratio of 60 (see Section 3.2.4.). The calculated distribution coefficients are presented in Figure 4.11A. The highest binding on the MIP takes place with terbutylazine. The structurally similar analog, atrazine shows very similar binding to the MIP. Ametryn and prometryn possess less structural resemblance to the template (the chlorine atom is changed to a thiomethyl group) and their distribution coefficient is significantly smaller. This arises from steric hindrance and a reduced access to the specific binding sites. The non-related compound ketoprofen does not show any selective binding since its distribution coefficients on the MIP and the NIP are the same. The triazine analogs also show very similar binding on the NIP. Ketoprofen binds to both polymers with non-specific interactions, as well as the triazine analogs to the NIP. As the number of non-specific binding sites is proportional to the surface area of the polymers we can presume that the imprinted and the nonimprinted polymers have very similar specific surface area. This confirms that the difference in binding of triazine analogs to the MIP and the NIP can be attributed to the selective sites and not to a difference in the surface area of these polymers.

Additionally, the selectivity study was carried out with a mixture of all analytes (50 \(\mu\text{M}\) each component). As it can be seen in Figure 4.11B, the selectivity order shows the same trend toward the template and its analogs on the MIP as observed in the individual solution tests albeit the binding is reduced due to the competitive adsorption of the molecules. The binding of prometryn (the most hydrophobic analyte in the study) is significantly suppressed because in toluene the retention mechanism is driven by normal-phase behavior, i.e. the more polar molecules are preferentially bound to the polymer. The adsorption of ketoprofen is slightly enhanced in the competitive conditions which might be attributed to synergistic effects where the analytes assist the binding of the other compounds for instance by forming H-bonding between each other.
4. Spiropyran-based MIP microparticles containing photoswitchable binding sites

Figure 4.11 Cross-selectivity study of spiropyran-containing MIPs (grey) and NIPs (white) in individual solution of the analytes (A) and in mixed solution (B)

4.2.5. Photoregulated template uptake and release studies

The template and its analog, prometryn were subjected to repeated photocontrolled binding-release cycles in toluene solutions at a concentration of 10 µM (see Section 3.3.2.1.). The reversible operation of the photocontrolled binding sites was tested with alternating UV and visible light illumination. Figure 4.12 presents the photoresponsive binding behavior of the imprinted and nonimprinted polymers.

In the first step before UV light irradiation, the binding sites exist as they were formed during the polymerization, showing a high fidelity toward the template. When the UV light is turned on the spiropyran to merocyanine transformation results in a steric rearrangement of the polymer chains and the conformational change of the binding cavity induces a partial release of the bound molecules. The most significant release is achieved with the template molecule, corresponding to 30% reduction in the binding capacity. The efficiency of release probably correlates with the number of occupied binding sites in the vicinity of the spiropyran molecules. When visible light irradiation is used, rebinding takes place and the initially bound amount of template is regained. A subsequent UV manipulation resulted in the expulsion of a smaller amount of template. This signals a partial reduction of the photoresponsive binding sites, i.e. a fraction of the previously photoadjustable binding sites is not able to release the analyte again. This may be due to a diminution of the switchable spiropyran units, which was also observed in the photocyclic spectroscopic study and was attributed to the photobleaching of the molecules, possible merocyanine aggregations or possible merocyanine-polymer subgroup interactions. In case of prometryn the photoswitching ability is also detected but to a much smaller extent.

The presence of selective binding sites in the imprinted polymer is apparent since the binding capacity in every light manipulated step is higher compared to the nonimprinted counterpart. In the control polymer the presence of photoswitchable binding sites is also detectable since in this polymer the photoswitchable spiropyran unit regulates the non-specific binding.
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Figure 4.12 Binding behavior of the template terbutylazine (MIP: black square, NIP: red circle) and prometryn (MIP: blue diamond, NIP: green triangle) on imprinted and nonimprinted polymers performing repeated photoswitching cycles in 10 μM toluene solutions of the analyte (UV: $\lambda_{irr} = 365$ nm, time=10 min, power=4 W)

4.3. Conclusions

We have demonstrated the preparation and characterization of photoswitchable molecularly imprinted polymer microspheres based on a novel strategy. Instead of using only one photochromic functional monomer, the well-established and widely used methacrylic acid took its role to form the selective binding sites and a spiropyran-based monomer was responsible for the photomodulation of the imprinted cavities.

The separation of the two different functions offers a generic method to synthesize photocontrollable MIPs with a wide selection of targets. This approach eliminates the need to decorate photochromic monomers with functional groups that are capable of binding with the template. Instead, well-established MIP recipes with common functional monomers can be used, adding a polymerizable photoswitchable molecule in a proper amount.

This work revealed the significance of the optimal amount of the spiropyran monomer to achieve photoactivatable molecularly imprinted binding sites. Fluorescence microscopic imaging and spectroscopic characterization supported the photoswitching characteristics owing to the anchored spiropyran units. A detailed characterization of the binding isotherm with the aid of the Freundlich model corroborated both the successful imprinting and the photomodulation of the binding sites. Cross-selectivity tests on analogous compounds and a structurally different one further confirmed molecular imprinting. With the selection of optimal binding conditions complete release of the template could be achieved upon UV irradiation, albeit with a lower binding efficiency. Repeated photoswitching cycles currently suggest that further improvements are necessary in order to increase the long-term stability. We expect that the novel photocontrollable MIPs developed on the basis of this attractive approach may find applications in diverse areas as light assisted solid phase extraction, ligand binding assays and photoresponsive renewable sensor elements.
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

5.1. Introduction

Crosslinked polymer microspheres have attracted significant interest among polymer chemists and materials engineers both in academia and industry owing to their diverse applicability. Spherical particles are used for example in chromatography and biotechnology, in diagnostic tests and assays or in medical treatment and diagnostic imaging.

Precipitation polymerization is one of the frequently used methods for direct microsphere synthesis. The technique was developed by the Stover group in 1993. This technique has become wide-spread among MIP-researchers in the past decade since it is able to create polymer microspheres with narrow size distribution in the range of several hundred nanometers to a few microns without the need of different additives that could interfere with the molecular imprinting process. Its disadvantages stem from the fact that it is conducted in very dilute solutions (typically around 2-5 v/v % monomer concentration). This implies a high solvent need making this technique economically unfavorable. Moreover, the polymerization does not reach completion and typically results in relatively low yield. The low monomer concentration raises further concerns in molecular imprinting. The dilute medium can adversely affect the template-monomer complexation shifting its equilibrium towards the uncomplexed forms hence the number of imprinted binding sites is significantly reduced. Our group has addressed these concerns by using specific solvents or solvent mixtures. As a result molecularly imprinted spherical micro/nanoparticles could be directly prepared in free radical crosslinking copolymerization of MAA and EGDMA in highly concentrated (up to 40 v/v%) monomer solutions.

In my work I have expanded the scope of this new approach to other polymer systems and studied the effect of various polymerization conditions and monomer compositions on the formation, size and morphology of the obtained particles. The poly(4-VPy-co-TRIM) polymer system prepared in paraffin oil/chloroform solvent mixture was studied in more detail (polymer P2 in Table 3.3) and the type and ratio of the functional monomer/crosslinker, the effect of the initiator concentration, the amount, type and composition of the polymerization solvent mixture and the polymerization kinetics were assessed. Morphological differences were explained by examining the solvency conditions and studying the solvent composition in the solution phase and polymer phase during polymer synthesis. Finally, molecularly imprinted polymer microspheres were successfully prepared at high monomer concentrations with this approach for two acidic compounds, diclofenac and naproxen and were characterized by N2 porosimetry and equilibrium batch rebinding experiments.

5.2. Results and discussion

5.2.1 Polymerization kinetics

Throughout the experiments two distinct characteristic particle morphologies were observed; smooth monodisperse microspheres and segmented cauliflower-like microparticles. Accordingly, we have studied the formation of the microparticles in two different polymerization systems giving the above-mentioned typical morphologies. Copolymerization
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

of 4-VPy with TRIM in 1:1 mixture of chloroform/PO (polymer P2 in Table 3.3) resulted in highly monodisperse smooth microspheres, while copolymerization of MAA with EGDMA in 1:1 mixture of toluene/PO (polymer P38 in Table 3.3) afforded segmented microparticles. In order to obtain samples at different phases of the polymerization reaction we have started to polymerize several batches at the same time and stopped them at different time intervals. The polymer particles were washed, weighed and visualized with SEM. In the 4-VPy/TRIM system as early as 10 minutes after the onset of the polymerization the clear solution turned opaque and polymer particles of about 430 nm diameter were obtained (Figure 5.1A).

Figure 5.1 Particles obtained after different polymerization times in the 4-VPy/TRIM/paraffin oil:chloroform system (polymer P2) A) 10 minutes; B) 1 hour; C) 24 hours and the MAA/EGDMA/toluene:paraffin oil system (polymer P38) D) 1 hour, E) 3 hours, F) 24 hours, visualized by SEM measurements. The bars in A–C, in D–E and in F denote 1, 5 and 2 µm, respectively.

The particles grew continuously for 6 hours and after that their size remained constant reaching a final size of 1.84 µm (see Figure 5.1B, C and Figure 5.2). The yield was also rapidly increasing until the 6th hour after that there was only a moderate increase showing the termination of polymerization (Figure 5.2).

Figure 5.2 Change of the particle diameter and yield with polymerization time in the 4-VPy/TRIM/paraffin oil:chloroform system (polymer P2 in Table 3.3).
It has to be pointed out that in contrast to regular precipitation polymerization from dilute monomer solutions the monomer conversion was practically 100% within 10 hours owing to the concentrated polymerization solution.

The poly(4-VPy-co-TRIM) particles were rather monodisperse throughout the polymerization with a polydispersity index between 1.011 and 1.036. Plotting the cubic number-average particle diameter related to the volume or mass of the particles versus the yield gave a linear relationship with a correlation coefficient of r=0.991. From this we can conclude that there is no secondary nucleation or coagulation after the nucleation during the polymerization process. The particles grow consistently after the primary nuclei were formed like in the regular precipitation polymerization.

The formation of poly(MAA-co-EGDMA) particles in 1:1 mixture of toluene/PO has followed a rather different pattern. Samples taken right after the cloud point at 1 hour and later after 3 hours and 24 hours are shown in Figure 5.1D; E and F, respectively. In Figure 1D we can observe that aggregated microgel particles with approx. 100 nm diameter precipitate out from the solution at the onset of the phase separation. In the followings, micropores between the microgel particles are gradually filled up with monomers and oligomers or soluble polymers depositing from the solution phase as can be perceived from Figure 5.1E and F.

We assume that the growth of smooth poly(4-VPy-co-TRIM) microspheres in the presence of chloroform/PO proceeds with the same entropic precipitation mechanism as described by Downey et al. The core of the particles is highly crosslinked but their surface at any instant is a solvent swollen lightly crosslinked gel layer with unreacted double bonds. The inner side of the gel layer continually deswells as further crosslinking takes place while the outer part continuously extends by capturing highly solvated oligomers from the solution.

On the other hand the growth of the cauliflower-like poly(MAA-co-EGDMA) microparticles probably proceeds with enthalpic precipitation mechanism typically observed in low solvency medium. Here, phase separation of microgel particles takes place at an early phase of the polymerization due to their insolubility in the solvent. At this point the solution phase still contains significant levels of monomer and crosslinker. These upon copolymerization fuse the microgel particles together, and also cause significant in-filling of small pores by precipitating onto existing particles due to their limited solubility.

In order to correlate the difference in the observed particle morphologies to the properties of the polymerization medium, the solvation capability of the chloroform/PO and the toluene/PO solvent mixtures was assessed using Hansen solubility parameter (HSP) values (see Section 3.3.4). They are supposedly give a better prediction of solubility than the Hildebrand solubility parameter since they take into account also polar and H-bonding interactions in addition to dispersion forces. In a three dimensional plot of the HSP components (δD dispersion, δP polar and δH H-bonding) where the scale for δD is doubled one can calculate the solubility parameter distance (Ra) of the solvent from the polymer system (see Section 3.3.4). In this space good solvents are “close” to the polymer i.e. their solubility parameter distance is smaller, while poor solvents are “far” from it.

We have calculated the solubility parameter distances of both the individual solvents and the solvent mixtures from the respective polymers (see Table 3.3). From Table 5.1 one can see that paraffin oil is a nonsolvent for the polymers exhibiting high Ra values. This can also be inferred from the fact the paraffin oil cannot solubilize the monomers by itself. Chloroform and toluene have lower Ra values from the respective polymers. Indeed, toluene is considered a good solvent for poly(MAA-co-EGDMA) as it allows the formation of a microporous polymer network with high specific surface area when used alone as solvent (see Table 5.4). Chloroform is an even better solvent for poly(4-VPy-co-TRIM) since bulk polymers that we prepared in chloroform collapsed upon removal of the solvent and were
essentially nonporous in the dry state (see Table 5.4). This is an indication that the solvent remains in the gel during polymerization and does not separate from the polymer phase. Also, from the solubility parameters distance values it can be anticipated that chloroform/PO is a better solvent for poly(4-VPy-co-TRIM) than the toluene/PO mixture for poly(MAA-co-EGDMA).

**Table 5.1** Estimates of the Hansen solubility parameter distances of chloroform, toluene and paraffin oil and their 1:1 mixtures from the poly(4-VPy-co-TRIM) and the poly(MAA-co-EGDMA) polymers

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$R_a$ from poly(4-VPy-co-TRIM)</th>
<th>$R_a$ from poly(MAA-co-EGDMA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>13.9</td>
<td>-</td>
</tr>
<tr>
<td>Toluene</td>
<td>-</td>
<td>68.9</td>
</tr>
<tr>
<td>Paraffin oil</td>
<td>89.0</td>
<td>102.4</td>
</tr>
<tr>
<td>Toluene/PO</td>
<td>-</td>
<td>79.8</td>
</tr>
<tr>
<td>Chloroform/PO</td>
<td>37.3</td>
<td>-</td>
</tr>
</tbody>
</table>

We were curious to see whether this has an implication on how the solvent components behave during polymerization, whether the growing particles have any preference for one of the components or not. In the first instance the microspheres would be swollen with the preferred component and the solution phase would be depleted from it. To shed light on this problem we designed an experiment to separately measure the co-solvent content in the polymer phase and in the solution phase (see Section 3.3.5) in the above two polymerization systems. The polymerization was stopped after 3 hours when already a substantial amount of polymer was formed, which could be conveniently separated from the solvent phase. The chloroform or toluene content of the separated polymer phase and that of the solution phase were determined. These were compared to a theoretical co-solvent concentration that would prevail if no preference of the growing polymer particles to any of the solvent components existed i.e. if the solvent composition were homogeneous throughout the system. The theoretical toluene and chloroform concentration in the 3-hour samples was estimated from their initial value in the polymerization mixture and the amount of polymer formed up to this point considering that the solvent components become more concentrated as polymer precipitates out of the solution. The results for the two polymerization systems can be seen in Table 5.2.

**Table 5.2** Theoretical and measured co-solvent concentrations in the separated polymer and solution phase of the MAA/EGDMA and the 4-VPy/TRIM polymer systems 3 hours after the onset of the polymerization (n=3).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Co-solvent</th>
<th>$Co$-solvent concentration (m/m %)</th>
<th>$Co$-solvent concentration in the solution phase (m/m %)</th>
<th>$Co$-solvent concentration in the polymer phase (m/m%)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-VPy/TRIM</td>
<td>Chloroform</td>
<td>58.9±0.8</td>
<td>54.0±0.3</td>
<td>77.8±2.2</td>
<td>65.9±4.5</td>
</tr>
<tr>
<td>MAA/EGDMA</td>
<td>Toluene</td>
<td>41.2±0.3</td>
<td>42.0±1.8</td>
<td>47.4±2.9</td>
<td>38.0±2.2</td>
</tr>
</tbody>
</table>
From the above data we can draw some interesting conclusions. First of all, it can be seen that 4-VPy/TRIM particles are indeed preferably solvated by chloroform. The chloroform concentration of the solvent phase is about 5 m/m\% smaller than what would be expected if a homogeneous chloroform/PO mixture existed throughout the polymer and the solvent phase. On contrary, the polymer phase contains almost 20 m/m \% more chloroform indicating that it is very much enriched in chloroform. This number might be even higher in reality because centrifugation cannot fully draw out the solution from the interstitial voids between the particles so we measure an average concentration of the polymer and the interstitial solvent.

This effect is hardly observable with the MAA/EGDMA polymer, which was prepared in the mixture of toluene and paraffin oil. The solvent phase after separation of the growing polymer particles contains practically the theoretical amount of toluene while the toluene concentration of the polymer particles is only about 6 m/m\% higher than the calculated theoretical value.

These experiments illuminate why the particle morphology is different when a very good co-solvent (chloroform) or a one with lower solvating capability (toluene) is used in conjunction with a nonsolvent (paraffin oil). In both cases microgel particles become phase separated due to their low solubility in the paraffin oil/co-solvent mixture. In the first case, however, the stability and individuality of the phase separated microgel particles is preserved even at high monomer concentrations because they are highly swollen by the chloroform that they extract from the solvent mixture. With toluene as the co-solvent the precipitated microgel particles become fused early in the polymerization and grow to segmented microparticles.

Nevertheless, the above findings do not explain why we obtained separate particles at high monomer concentration instead of a monolith. To find the reason for this unusual behavior we have synthesized four polymers from 4-VPy and TRIM with the typical polymer composition (see Section 3.3.1.2) that differed only in one component of the polymerization solvent mixture. Paraffin oil, and three lower hydrocarbons, hexane, decane and tetradecane were used in a 1:1 mixture with chloroform. Using paraffin oil, uniform spherical particles were obtained while the other hydrocarbons yielded a hard monolith. It has to be noted that with increasing chain length the hardness of the monolith decreased as it could be somewhat easier crushed. Using tetradecane we could observe that some segments of the monolith were built up of particles (see Figure 5.3). If we look at the solubility parameters of paraffin oil, tetradecane, decane and hexane (see Table 3.5) we can conclude that there are only minor differences between them. Furthermore, all polymerization solvents contained 50\% chloroform, which diminishes the differences between the solubility parameters of the solvent mixtures. However, the estimated molar volume of paraffin oil (463 cm$^3$ mol$^{-1}$) is much larger than that of hexane (132 cm$^3$ mol$^{-1}$), decane (195 cm$^3$ mol$^{-1}$) and tetradecane (262 cm$^3$ mol$^{-1}$). It is well known that solvents with larger molar volume are thermodynamically worse solvents than smaller ones with identical solubility parameters.$^{142}$ This can imply that paraffin oil is thermodynamically even less compatible with poly(4-VPy-co-TRIM) than the lower alkanes studied, although the solubility parameter of the latter ones is somewhat further away from $\delta_{\text{polymer}}$ than that of paraffin oil. It is supposed that being a ‘very bad’ solvent (nonsolvent) paraffin oil separates completely out of the polymer network during polymerization and secludes the growing particles. We have earlier observed similar particle formation with high monomer loadings when we used solvents with large molar volumes like glyceryl trioleate, polyethylene glycols, polypropylene glycols or very incompatible solvents like ionic liquids.

In the followings the different conditions that affect particle morphology in precipitation polymerization using concentrated monomer solutions are studied.
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

5.2. Monomer concentration

Generally, in precipitation polymerization the monomer concentration needs to be set very low, otherwise macrogelation occurs. In the 4-VPy/TRIM system the monomer concentration in the pre-polymerization mixture has been varied between 2-33 v/v% using 1:1 mixture of paraffin oil and chloroform as the solvent (polymers P1-P6 in Table 3.3). The use of the highest, 33 v/v% monomer concentration resulted in a hard monolith but with 25 v/v% and smaller monomer concentrations regular microspheres were formed. The number-average diameter ($D_n$) was 1.3 µm at 2 v/v% monomer concentration and this steadily increased to 1.84 µm at 25 v/v% (Figure 5.4).

Figure 5.3 SEM images of poly(4-VPy-co-TRIM) polymers prepared with alkanes of different chain lengths using chloroform as co-solvent A) hexane B) decane, C) tetradecane, D) paraffin oil. The bars in the pictures denote 10 µm.

Figure 5.4 Variation of the particle diameter with increasing monomer concentration in the 4-VPy/TRIM system (polymers P2-P6, see Table 3.3).

The obtained regular spherical particles were uniform with a relatively smooth surface. The polydispersity index for the different monomer concentrations varied between 1.010 and
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

1.025 (see Table 3.3). In highly concentrated monomer solutions we have observed yield close to 100%, while at the lowest monomer concentration (2 v/v%) the conversion was about 80%.

Our earlier results have shown that in the MAA/EGDMA system the monomer concentration could be increased up to 40 v/v% without encountering macrogelation using paraffin oil/toluene solvent mixture. The average particle size increased with increasing monomer concentration in that system, too. Similar trend have been observed by several authors in the precipitation polymerization of DVB or EGDMA. The increase in size can be explained by the fact, that the solvency of the polymerization medium increases with higher monomer concentration. This allows for oligomeric species of higher molecular weight to grow without precipitation. Consequently, when they are precipitated, less primary particles are formed resulting in larger particles in the end.

5.2.3. Initiator concentration

4-VPy/TRIM polymers have been prepared by varying the initiator concentration between 0.3 to 11 mol% relative to the total monomer concentration (polymers P2 and P31-P36). Initiator concentrations from 0.3 to 8.2 mol% yielded narrow disperse spherical particles but the polymerization with 11 mol% initiator gave somewhat aggregated irregular spheres.

Figure 5.5 Variation of the particle size of poly(4-VPy-co-TRIM) polymer microspheres with increasing initiator concentration.

In Figure 5.5 it can be seen that increasing the initiator concentration the particle diameter monotonously decreases. Meanwhile, the uniformity of the microspheres does not change significantly. Interestingly, other authors have found the opposite effect in precipitation polymerization i.e. the particle size increased with higher initiator concentration. In conventional precipitation polymerization a substantial portion of the initiator radicals may self-terminate and become inefficient due to the low monomer concentration. Therefore particle growth stops upon exhaustion of the initiator, well before all the monomers are converted. The number of particles is not affected by the initiator concentration but with increasing initiator concentrations the particles can grow further and both the particle diameter and the monomer conversion increase. In our case, however, the polymerization yield is close to 100%, irrespectively of the initiator concentration due to the high monomer concentration. The initiator molecules can work much more efficiently, starting new polymer chains instead of self-termination. Therefore, by increasing the AIBN concentration more free radicals and consequently more primary particles are formed and grow during polymerization, resulting in smaller particles since the amount of monomer is the same in each case.
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

These findings are supported by theoretical considerations, as well. In free radical polymerization one can write the reaction rate for initiation, propagation and termination:

\[ v_i = 2k_if[I] \]  
Eq. 14

\[ v_p = k_p[M•][M] \]  
Eq. 15

\[ v_t = k_t[M•]^2 \]  
Eq. 16

where \( v_i, v_p \) and \( v_t \) are the rate of initiation, propagation and termination, \( k_d, k_p \) and \( k_t \) are the rate constants for the initiator decomposition, the chain propagation and termination, \( f \) is the efficiency of the initiator, and \([I], [M•] \) and \([M] \) are the initiator, macroradical and monomer concentration.

In free radical polymerization the rates of initiation and termination are equal in steady-state conditions, considering equation 14 and 16, and this gives as follows:

\[
[M•] = \left( \frac{2k_d f}{k_t} \right)^{\frac{1}{3}} [I]^{\frac{1}{2}} \]  
Eq. 17

This means that the number of the macroradicals (i.e. the number of polymer particles) is proportional to the square root of the initiator concentration.

If we presume 100% conversion and identical monomer concentration in all measurements, the volume of one particle is inversely proportional to \([I]^{1/2}\), therefore the particle diameter, \( D_n \sim [I]^{-1/6} \). This is in good agreement with our results. Fitting of the experimental data (see Figure 5.5) with the equation \( D_n = a[I]^{x} \) gave an exponent \( x = -0.139 \).

5.2.4. The type of functional monomer

Polymers using the typical polymer recipe (see Section 3.3.1.2.) have been prepared by co-polymerizing TRIM with different monofunctional monomers commonly used in molecular imprinting like 4-vinylpiridine, methacrylic acid, methacrylamide, methyl methacrylate and hydroxyethyl methacrylate (polymers P2, P17-P20 in Table 3.3). Interestingly, microspheres prepared from the different functional monomers could be classified into two groups upon their appearance. 4-VPy and MAAm gave smaller spherical particles with narrow size distribution while MAA, MMA and HEMA gave large particles with wide size distribution (Figure 5.6).

**Figure 5.6** SEM images of polymer particles with different functional monomers: A) 4-VPy (P2); B) MAAm (P18); C) MAA (P17); D) HEMA (P19) and E) MMA (P20) The bars in the pictures denote 1 µm.
Particles with MAA, MMA and HEMA functional monomer were composed of a few smaller fused particles. It appears that in an earlier stage of particle growth surface stabilization was not so efficient and the microspheres could aggregate forming multiplets. We have not found any significant difference in the solubility parameters of the monomer mixtures with different functional monomers that could explain the observed variation in particle morphology. However, we speculate that there might be a difference in the relative incorporation rate of the monomers and the crosslinker, therefore the particle surface can contain lower or higher amount of crosslinker. As can be seen in Section 5.2.6 this can have a pronounced effect on particle morphology.

5.2.5. The type of crosslinker

Three different crosslinkers TRIM, EGDMA and DVB-80 have been co-polymerized with 4-VPy in chloroform/PO using otherwise the typical polymerization conditions (polymers P2; P22; P23 in Table 3.3). SEM pictures of the polymer particles with the different crosslinkers are shown in Figure 5.7.

Figure 5.7 SEM images of the polymer particles prepared with A) TRIM (P2); B) EGDMA (P22) and C) DVB-80 (P23) crosslinkers. The bars in the pictures denote 1 µm.

In all three cases a fine powder was obtained but there was a striking difference between the microscopic morphology of the DVB-based and the two other, methacrylate-based polymers. EGDMA and TRIM afforded regular spherical particles although EGDMA-based ones were slightly bigger. Contrarily, with DVB-80 smaller, irregular aggregated polymer particles were obtained. We attribute this behavior to the higher solubility of divinylbenzene in paraffin oil as opposed to the methacrylate crosslinkers which is supported by the observation that DVB is soluble in paraffin oil while the other two monomers are not. This implies that the solubility difference of the forming polymer in the two components of the solvent mixture will be less. We speculate that “extraction” of chloroform into the DVB-containing polymer phase will be less pronounced and the particle surface stabilization will be less effective leading to smaller, aggregated particles.

5.2.6. Ratio of the functional monomer to the crosslinker

As it is well known from the literature a high degree of crosslinking is a prerequisite for the formation of microspheres with low polydispersity in precipitation polymerization. To test whether this observation is valid also at high monomer loadings 4-VPy/TRIM polymers were prepared changing the relative amount of the crosslinker from 0 to 100 mol% in the total monomer content (polymers P2 and P24-P30 in Table 3.3). SEM images of the particles can be seen in Figure 5.8.
With no crosslinker the linear polymer of 4-VPy appeared at the bottom of the vessel as a red coagulum. Below a certain degree of crosslinking no microspheres were obtained only broadly disperse aggregated polymer particles. Between 20 and 40 mol% crosslinker the particles appeared as fused multiplets of smaller particles. This is probably caused by the inadequate stabilization of the particle surface due to the low crosslinking ratio. Narrow disperse spherical particles with smooth surface were obtained between 50 to 84 mol% crosslinker content. An insignificant increase in particle size could be observed changing from 50 % crosslinker content to 60 % and it did not change any more up to 84 % TRIM. Although in the literature we can find different, sometimes even opposite effects of the crosslinking degree on the particle size in different polymerization systems, Goh and Stöver have found that at lower solvency of the medium the microspheres show less of a size dependence on crosslinker level. This is also valid in our system in which the 50% paraffin oil content in the polymerization solvent mixture renders the solvency of the medium rather low.

Interestingly, without any functional monomer (100% TRIM) the particles grow much larger by the coalescence of multiple microspheres. If neat TRIM, a trifunctional crosslinker is polymerized there are a vast number of reactive pendant vinyl groups on the particle surface during polymerization. This might have an adverse effect on particle stabilization, since high concentration of the surface vinyl groups increase the chance that two particles become fused when encounter each other, which has a high probability in the concentrated polymerization system.

![Figure 5.8 SEM images of polymer particles with different crosslinker content relative to the total amount of monomers A) 20 mol% (P25); B) 30 mol% (P26); C) 40 mol% (P27); D) 50 mol% (P28); E) 60 mol% (P29); F) 84 mol% (P2); G) 100 mol% (P30). The bars in the pictures denote 1 µm.](image-url)
5.2.7. The nonsolvent/co-solvent ratio

The composition of the polymerization solvent was studied in two different systems, namely in the poly(4-VPy-co-TRIM) and the poly(MAA-co-EGDMA) systems. In the polymerization of 4-VPy/TRIM chloroform was used as co-solvent, whereas toluene was applied in the copolymerization of EGDMA and MAA. The percentage of the co-solvent in PO has been changed systematically from 75% to 25% (polymers P8, P2 and P7 with 4-VPy/TRIM and P37-P39 with MAA/EGDMA in Table 3.3.). 100% paraffin oil could not solubilize the monomers by itself. With 100% and 75% chloroform and with 100% toluene hard monoliths were obtained. At lower co-solvent ratios particles were formed, however their surface morphology was rather distinct depending on the amount of co-solvent used. As a general rule, the surface roughness of the particles increased with decreasing co-solvent content, i.e. with higher PO content. With high co-solvent content spherical particles with a rather smooth surface were obtained while decreasing the co-solvent content the particles were aggregated and cauliflower-like (Figure 5.9).

To interpret this behavior one has to take into account that by varying the co-solvent/PO ratio the solvency of the medium is changing. Toluene and chloroform are much better solvents of the forming polymer network than paraffin oil and among the two co-solvents chloroform has a better solvency. When high concentration of chloroform or toluene is applied the forming nuclei can grow much larger before they precipitate out due to their better solvation. After phase separation the microgel particles grow by capturing monomers and oligomers from the solution. On contrary, when the concentration of paraffin oil is high the primary nuclei homocoagulate to a substantial extent and form irregularly shaped microparticles with a rough surface.

Goh and Stöver have come to similar conclusions studying the precipitation polymerization of poly(MAA-co-PEGMM-co-EGDMA) in different mixtures of a good solvent and a poor solvent. They have found that discrete spherical particles were obtained
in a certain solvent composition window and with decreasing solvency the surface of the particles changed from smooth to bumpy. These results confirm that higher levels of the good solvent contribute to the colloidal stability of the primary particles formed, while at lower solvency the homo-coagulation period of primary particles was prolonged, resulting in final microspheres with irregular shapes and surfaces.

### 5.2.8. Different co-solvents

Various co-solvents were mixed with PO in a 1:1 ratio and were applied in the co-polymerization of 4-VPy and TRIM (polymers P2, P9-P16 in Table 3.3). The typical polymerization recipe was used as described in Section 3.3.1.2 except in the case of dichloromethane where UV initiation was carried out instead of thermal polymerization due to the high volatility of this solvent. Uniform particles with rather smooth surface were obtained using 1-octanol, THF, dichloromethane, chloroform and 1,1,1-trichloroethane. Segmented particles with a wider size distribution range and a cauliflower-like surface were observed in carbon tetrachloride, caprylonitrile, cyclohexane and toluene (Figure 5.10).

**Figure 5.10** SEM images of poly(4-VPy-co-TRIM) polymer particles prepared with different co-solvents: A) chloroform (P2); B) trichloroethane (P11); C) dichloromethane (P16); D) 1-octanol (P14); E) THF (P13); F) cyclohexane (P15); G) carbon tetrachloride (P10); H) toluene (P9) and I) caprylonitrile (P12) The bars in the pictures denote 1 µm.

In order to correlate the observed particle morphology to the solvation capability of the different co-solvent/PO mixtures, their Hansen solubility parameter distances (see Section 3.3.4) have been calculated from the poly(4-VPy-co-TRIM) polymer. These can be seen in Table 5.3, together with the observed morphology.
Table 5.3 Solubility parameter distances of different co-solvent/PO mixtures from the 4-VPy/TRIM system and the morphology of the obtained particles.

<table>
<thead>
<tr>
<th>Polymerization solvent mixtures (50:50 v/v%)</th>
<th>$R_a$</th>
<th>Particle morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-octanol/PO</td>
<td>14.1</td>
<td>smooth microsphere</td>
</tr>
<tr>
<td>THF/PO</td>
<td>20.1</td>
<td>smooth microsphere</td>
</tr>
<tr>
<td>dichloromethane/PO</td>
<td>28.1</td>
<td>smooth microsphere</td>
</tr>
<tr>
<td>chloroform/PO</td>
<td>37.3</td>
<td>smooth microsphere</td>
</tr>
<tr>
<td>caprylonitrile/PO</td>
<td>43.4</td>
<td>segmented microparticle</td>
</tr>
<tr>
<td>1,1,1-trichloroethane/PO</td>
<td>56.3</td>
<td>smooth microsphere</td>
</tr>
<tr>
<td>toluene/PO</td>
<td>66.3</td>
<td>segmented microparticle</td>
</tr>
<tr>
<td>carbon tetrachloride/PO</td>
<td>82.5</td>
<td>segmented microparticle</td>
</tr>
<tr>
<td>cyclohexane/PO</td>
<td>85.6</td>
<td>segmented microparticle</td>
</tr>
</tbody>
</table>

Paraffin oil–co-solvent mixtures with low solubility parameter distance (below $\approx 60$) can be considered good solvents for the 4-VPy/TRIM system. In these polymerization solvents uniform particles with smooth surface are formed, while in solvents mixtures with higher $R_a$ values segmented and more aggregated particles form. The only exception is the caprylonitrile/PO mixture which has a relatively low solubility parameter distance but it still affords segmented particles. This can be attributed to the fact that it lies in the boundary region of solvency where deviations can occur, especially in case of solvent molecules with high molar volume like caprylonitrile.

5.2.9. Molecular imprinting using precipitation polymerization at high monomer concentrations

MAA/EGDMA based polymer particles prepared in toluene/PO mixtures at high monomer concentrations have been previously imprinted with terbutylazine in our group.\textsuperscript{11} We aimed to extend the method’s applicability toward basic functional monomers targeting acidic templates. Thus, naproxen and diclofenac imprinted polymers were synthesized using 4-VPy as functional monomer. Methacrylate crosslinkers, EGDMA and TRIM were used because DVB did not afford monodisperse microspheres as discussed above. Two different solvent systems have been utilized; chloroform/PO and toluene/PO, both in a 1:1 ratio.

Low temperature N\textsubscript{2} adsorption/desorption isotherm measurements were performed to obtain porosity data about the polymers. For comparison, bulk polymers from 4-VPy and TRIM were also prepared in neat chloroform and toluene with the same monomer composition and the same 3:1 solvent/monomer ratio. Data derived from the isotherms are shown in Table 5.4. An early study of MIPs highlighted that toluene is a good pore forming agent in MAA/EGDMA based polymers while chloroform is not.\textsuperscript{144} This is in concordance with our findings that the bulk polymer prepared in toluene had a very high specific surface area (435 m\textsuperscript{2} g\textsuperscript{-1}) as opposed to the one synthesized in chloroform (17 m\textsuperscript{2} g\textsuperscript{-1}). All the nonimprinted polymer particles, however, exhibited low BET surfaces similar to that of the bulk polymer synthesized in chloroform, even when toluene was used as co-solvent. This is due to the fact that the microgel particles formed during phase separation preserved their individuality during polymerization. They did not become interconnected or aggregated into a space filling macrogel as in bulk polymerization. The latter would have led to the formation of micro-, meso- or macropores and to a higher surface area.
If we compare the polymers prepared with different crosslinkers (TRIM or EGDMA), we can observe that TRIM crosslinked MIPs exhibit increased specific surface area in both solvent systems compared to their nonimprinted counterpart whereas polymers prepared with EGDMA do not show this difference. In case of EGDMA crosslinker and chloroform/PO solvent even a somewhat reduced specific surface area was found for the MIP. Also the total pore volume and the volume of micropores are increased in TRIM crosslinked MIPs compared to the NIP but their micropore diameter is reduced in the presence of the template. Interestingly, this implies that, depending on the type of crosslinker the presence of the template might or might not have an effect on the morphology of the polymer.

**Table 5.4 Porosity data of studied polymer compositions**

<table>
<thead>
<tr>
<th>Functional monomer</th>
<th>Crosslinker</th>
<th>Solvent</th>
<th>MIP/NIP (template)</th>
<th>(S_{\text{BET}}) (\text{m}^2\text{g}^{-1})</th>
<th>(V_{\text{tot}}) (\text{cm}^3\text{g}^{-1})</th>
<th>(V_{\text{micro}}) (\text{cm}^3\text{g}^{-1})</th>
<th>(d_{\text{micro}}) nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-VPy</td>
<td>TRIM</td>
<td>CHCl(_3):PO</td>
<td>MIP (diclofenac)</td>
<td>105</td>
<td>0.098</td>
<td>0.046</td>
<td>2.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIP (diclofenac)</td>
<td>38</td>
<td>0.064</td>
<td>0.014</td>
<td>2.63</td>
</tr>
<tr>
<td>4-VPy</td>
<td>TRIM</td>
<td>tolune:PO</td>
<td>MIP (diclofenac)</td>
<td>68</td>
<td>0.068</td>
<td>0.027</td>
<td>2.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIP (diclofenac)</td>
<td>17</td>
<td>0.040</td>
<td>0.005</td>
<td>2.70</td>
</tr>
<tr>
<td>4-VPy</td>
<td>EGDMA</td>
<td>CHCl(_3):PO</td>
<td>MIP (diclofenac)</td>
<td>17</td>
<td>0.029</td>
<td>0.004</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIP (diclofenac)</td>
<td>28</td>
<td>0.030</td>
<td>0.005</td>
<td>2.60</td>
</tr>
<tr>
<td>4-VPy</td>
<td>EGDMA</td>
<td>tolune:PO</td>
<td>MIP (diclofenac)</td>
<td>25</td>
<td>0.038</td>
<td>0.009</td>
<td>2.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIP (diclofenac)</td>
<td>24</td>
<td>0.038</td>
<td>0.009</td>
<td>2.09</td>
</tr>
<tr>
<td>4-VPy</td>
<td>EGDMA</td>
<td>tolune:PO</td>
<td>MIP (naproxen)</td>
<td>24</td>
<td>0.040</td>
<td>0.009</td>
<td>2.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NIP (naproxen)</td>
<td>24</td>
<td>0.038</td>
<td>0.009</td>
<td>2.09</td>
</tr>
<tr>
<td>4-VPy</td>
<td>TRIM</td>
<td>tolune (bulk)</td>
<td>NIP</td>
<td>435</td>
<td>0.760</td>
<td>0.166</td>
<td>2.08</td>
</tr>
<tr>
<td>4-VPy</td>
<td>TRIM</td>
<td>CHCl(_3) (bulk)</td>
<td>NIP</td>
<td>17</td>
<td>0.039</td>
<td>0.002</td>
<td>3.37</td>
</tr>
</tbody>
</table>

Template binding properties of the different polymers have been assessed in equilibrium batch rebinding measurements in 100 µM toluene solution of diclofenac or naproxen and characterized by calculating their distribution coefficients (see Section 3.2.4). Figure 5.11 presents the obtained results for the MIPs in comparison with their nonimprinted counterpart. Distribution coefficient ratios for MIP and NIP were calculated and shown in the figure to characterize imprinting efficiency. Except for the diclofenac templated polymer synthesized in toluene/PO considerable differences between MIP and NIP could be observed in each case. For naproxen the toluene/PO, while for diclofenac the chloroform/PO solvent medium provided better imprinting efficiency. EGDMA crosslinker was proven to be superior over TRIM in both cases. We must note here though, that the molar ratio of the functional monomer and the crosslinker has not been optimized and was set to the same 1:5 value in both cases. However, TRIM being a trifunctional crosslinker, can be efficient in much lower concentrations, therefore it is reasonable to expect that in a lower molar ratio it could have led to improved imprinting efficiency.
5. MIP microspheres prepared by precipitation polymerization at high monomer loading

Figure 5.11 Distribution coefficients on naproxen (A) and diclofenac (B) imprinted polymers in 100 µM toluene solution of the template using a phase ratio of 60. The ratio of the distribution coefficients on MIP and its respective NIP is indicated above the column pairs.

The selectivity of the diclofenac MIP was further studied in comparison with other structurally similar acidic non-steroidal anti-inflammatory drugs, ketoprofen, naproxen and ibuprofen and a non-related basic β-blocker drug, propranolol in equilibrium batch rebinding experiments. These were performed both in toluene and in MeCN. The distribution coefficients are plotted in Figure 5.12 together with the structure and logP values of the studied compounds. In the apolar toluene (Figure 5.12A) more hydrophilic compounds with lower logP values like ketoprofen and naproxen exhibit high distribution coefficients both on the MIP and the NIP, that is, the polymer behaves like a normal stationary phase. Ibuprofen being more hydrophobic shows much lower binding in toluene. Acid-base interactions also prevail between the 4-VPy-based polymers and the acidic drugs since propranolol, a basic compound, with similar hydrophilicity as ketoprofen and naproxen exhibits only very weak binding on the sorbent. Diclofenac, being the most hydrophobic of all, has almost as high distribution coefficient on the MIP as ketoprofen but low binding on the NIP, indicating successful imprinting. In the polar MeCN (Figure 5.12B) only the template, diclofenac shows high binding on the MIP. This interaction is predominantly due to the imprinted selective sites and not only to nonspecific hydrophobic binding since the NIP binds much less diclofenac. As the specific surface area of the MIP is even somewhat smaller than that of the NIP (see Table 5.4), we can be assured that the enhanced binding on the MIP cannot be attributed to an increased specific surface area.

Figure 5.12 Selectivity of diclofenac imprinted 4-VPy/EGDMA polymer prepared in chloroform/PO. Batch rebinding measurements were carried out in 100µM toluene (A) and acetonitrile (B) solution of the respective compound with a phase ratio of 60. The ratio of the distribution coefficients on MIP and its respective NIP is indicated above the column pairs.
5.3. Conclusion

We have thoroughly investigated a free radical crosslinking copolymerization of different functional monomers and crosslinkers in concentrated monomer solutions (≥25 v/v%) which has led to the formation of micron sized particles. Thermodynamically incompatible solvents with high molar volume alone or in combination with small molecule solvents were used to hinder the formation of bulk polymers and produced microparticles. Narrow disperse smooth microspheres were formed using good solvents as co-solvent, while poorer co-solvents yielded segmented microparticles. It has been experimentally proven that during polymerization after phase separation the ratio of the co-solvent and paraffin oil is changing both in the polymer phase and the solvent phase. The solution is depleted of the co-solvent while polymer particles are enriched in it. The more compatible the co-solvent is with the polymer, the more it is extracted into the precipitated particles.

We have clarified the role of the different polymerization conditions, for example the type of functional monomer, crosslinker and co-solvent and the ratio of the co-solvent/nonsolvent on the final morphology of the resulting particles. Upon these results it is feasible to design new polymerization systems suited for molecular imprinting. This approach substantially extends the choice of the solvents currently used in precipitation polymerization. More apolar solvents, which suit better to the imprinting of H-bonding templates than MeCN become usable in combination with a nonsolvating diluent. Moreover, compared to precipitation polymerization conducted in dilute monomer solutions this approach significantly reduces the solvent need and makes the process more economical with conversion close to 100%. The formation of the template-functional monomer complexes is promoted in the highly concentrated medium leading to higher imprinting efficiency. We can conclude that the method provides additional benefits to conventional precipitation polymerization and facilitates the adaptation of bulk MIP recipes to direct microsphere synthesis.
6. Solid phase extraction of propranolol on multiwell membrane filterplates modified with molecularly imprinted polymer

6.1. Introduction

Previously, our group has integrated MIPs into a high-throughput technology by preparing MIMs in multiwell filterplates. This facilitated the synthesis and screening of substance specific polymer libraries during the optimization process. Based on the abovementioned results, we have envisioned that the MIP modified filterplates can be used in analytical sample preparation for high-throughput solid phase extraction. The multiwell format offers high throughput analysis of samples as opposed to traditional SPE cartridges. It can be operated manually in a similar way as the syringe barrel format using a vacuum manifold but it has the advantage that samples can be handled in parallel, rather than sequentially, using multichannel pipettors. The eluates collected in a microtiter plate can be directly injected into the HPLC by an autosampler eliminating the need for transferring the samples to autosampler vials. To demonstrate the usability of the filterplate membranes in SPE, we have chosen propranolol as the target analyte not only because it has been already imprinted and characterized by many groups but also for its significance in clinical analysis and doping control. Propranolol and other β-blockers are widely prescribed for patients suffering from angina pectoris, hypertension and arrhythmia but they are also misused among sportsmen and therefore they are on the World Anti-Doping Agency (WADA) Prohibited List and are banned in particular sports.

In this work the general steps of the solid phase extraction procedure on the β-blocker imprinted 24-well filterplate membranes have been optimized. We have to emphasize that using the MIM format in SPE mode is not trivial at all, and might need considering experimental parameters that are usually omitted with the syringe barrel format. Traditional SPE sorbents are packed in a high aspect ratio bed (column format) which behaves as a low efficiency chromatographic column offering a relatively long contact time for the analyte with several adsorption and desorption steps. On contrary, the membrane format have a low aspect ratio (thin layer) resulting in very short contact time and virtually one adsorption step. Therefore MIPs that do not have very fast binding kinetics can pose problems such as inadequate analyte binding or release that has to be considered during method development.

Using the optimized protocol the performance of MISPE filterplate membranes in the sample preparation of propranolol containing biological samples have been tested and successfully pre-validated demonstrating the applicability of MIM adsorbers for solid phase extraction of real samples. The high-throughput format enabled the fast and parallel handling of many samples at the same time.

6.2. Results and discussion

6.2.1. Synthesis of MIPs via the modification of filterplate membranes and their characterization

In this work we have followed an earlier reported polymerization recipe to imprint β-blockers and used methacrylic acid as functional monomer and ethyleneglycol dimethacrylate as crosslinker changing the acetonitrile porogen to adiponitrile, a much less volatile but very similar solvent. Volatile solvents cannot be used in this system because the filterplate is kept under a gentle flow of argon during the polymer synthesis.
In the first set of experiments propranolol, the target analyte, was the used template to prepare molecularly imprinted filterplate membranes. These filterplates were used to explore the optimal conditions of a solid phase extraction protocol. To demonstrate the applicability of the membrane filterplate system with urine and blood plasma samples low propranolol concentrations have to be measured. At these low levels, however, even the slight bleeding of the template could lead to false results. For this reason, we applied the structural analog approach\textsuperscript{109}, which has become a common tool in MIP-design and has already been applied to imprint MIMs\textsuperscript{177}. Oxprenolol was selected as a target analog and imprinted filterplate membranes were also produced using this dummy template.

Preliminary equilibrium batch-rebinding experiments to characterize MIP and NIP modified filterplate membranes with the target propranolol were unsuccessful since the results showed a very high level of inaccuracy. This was due to the extremely high distribution coefficients both of MIP and NIP which is well-known from the literature\textsuperscript{7,174}. This manifests in the fact that a very high portion of the template is adsorbed by the polymer during the rebinding step. A slight variation of the adsorbed amount therefore causes a large relative error of the small amount that remained in the liquid phase. This problem can only be overcome if the phase ratio is set very high and indeed in the literature we can find 1:10.000, 1:20.000 phase ratios for the equilibrium batch rebinding measurement of β-blocker imprinted polymers\textsuperscript{7,150,174}. In our system, however, the maximum phase ratio that can be achieved is approximately 1:500 due to the limited volume of the well.

Therefore, we chose a different approach to test the MIP/NIP selectivity of the oxprenolol imprinted filterplate membranes. We compared MIP and NIP membranes using the optimized solid phase extraction protocol with urine samples changing only the type and amount of washing solvent (100 μL MeCN; 500 μL MeCN; 500 μL MeCN+0.5% AcOH; 100 μL MeCN+1% 10mM NaCl). The permeated amount of propranolol was monitored in the washing fractions. We have found that in the washing step at least two times less analyte was eluted from the MIP than from the NIP using all the different washing solvents proving that the imprinted polymer selectively bound propranolol (see Figure 6.1). The highest MIP/NIP selectivity has been achieved using 100 μL MeCN in the washing step where approx. seven times more analyte has been eluted from the nonimprinted than from the imprinted polymer.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{Figure6.1.png}
\caption{Permeated amount on MIP and NIP membranes in different washing solvents after the loading of 100 μL 1500 ng ml\textsuperscript{-1} propranolol (matrix: 1:1 diluted urine, pH 10.0)}
\end{figure}
The surface and the cross-section of the MIP filterplate membranes (Figures 6.2B and D) have been characterized by scanning electron microscopy along with that of the non-modified glass fiber filterplate membrane (Figure 6.2A and C). The surface and cross-sectional structure of the composite polymer are strikingly different.

![Image of SEM images of filterplate membranes.](image)

The molecularly imprinted polymer on the surface of the modified membranes forms a veil-like web between adjacent fibers (Figure 6.2B). However, inside the membrane the polymer is partly deposited in clusters on the glass fibers or in bigger chunks between denser webs of small diameter glass fibers. The nonimprinted and imprinted polymer modified membranes look very similar. The difference between the surface and internal morphology of the composite membrane is probably due to a non-homogeneous distribution of the monomer mixture in the microfiltration membrane i.e. the surface absorbs less polymerization mixture than the bulk.

The permeability of the non-modified glass fiber filterplate membrane and the composite MIM was measured by percolating methanol through them applying a small vacuum. Permeability of the glass fiber membrane was $534\pm73 \text{ mL cm}^{-2} \text{ min}^{-1} \text{ bar}^{-1}$ and that of the MIM was $107\pm46 \text{ mL cm}^{-2} \text{ min}^{-1} \text{ bar}^{-1}$. This decreased permeability still allows for fast filtration on the composite membrane.

### 6.2.2. Optimization of the MISPE conditions

The filterplate MIPs were tested in solid phase extraction experiments. The effect of different parameters that are usually assessed in solid phase extraction (sample solvent,
solid phase extraction of propranolol on multiwell membrane filterplates modified with molecularly imprinted polymer

sample pH, wash/elution solvents and volume) has been investigated. Moreover, residence time of the sample on the membrane and the effect of sample volume have also been studied and turned out to be key factors, as shown below.

6.2.2.1. Sample loading

To promote the best conditions for propranolol binding from aqueous environment the effect of pH was tested. $10^5$ M propranolol solutions were prepared and their pH was set at 3.0, 6.0, 8.0, 10.0 and 11.0. 1 mL of the samples was loaded onto the propranolol imprinted filterplate membranes for 5 minutes, collected and analyzed. Propranolol bound to the membrane after percolating the sample through the MISPE filterplate is shown in Figure 6.3.

Figure 6.3 Propranolol bound to the filterplate membrane after loading 1 mL $10^5$ M solution prepared in aqueous buffers of different pH values (residence time 5 mins)

As can be seen from Figure 6.3, pH 10.0 gave the highest binding from the loaded sample therefore in the optimized protocol the sample pH was set at 10.0. Similar results have been obtained by Andersson measuring the pH dependence of propranolol binding on a similar polymer up to pH 9.0.\textsuperscript{150} Our result closely matches the observations of Sellergren et al. also who studied the effect of mobile phase pH on MIP stationary phases exhibiting an ion-exchange retention mode.\textsuperscript{178} It was found that the highest retention is observed when the mobile phase pH corresponds to the pK\textsubscript{a} of the template. The pK\textsubscript{a} value of propranolol is 9.1\textsuperscript{179} and being a Brønsted base it shows ion-exchange retention mechanism on the imprinted polymer in aqueous media.

Another conclusion of the above experiments is that even at the optimal pH, the binding of the analyte is less than 50%. Based on previous experiences with traditional MISPE cartridges we expected close to 100% binding from aqueous solvent because in this medium the polymer behaves as a reversed phase sorbent and binds the analyte due to its hydrophobicity. To increase the binding efficiency the residence time of the sample in the membrane filterplate wells was extended by plugging the outlets during sample loading. After approximately 40 hours complete binding took place and the whole amount of the drug was bound to the polymer. This means that the binding capacity of the polymer is adequate for the 1 mL sample and either the diffusion from the well to the membrane or the binding kinetics is slow. In order to test these assumptions we have percolated 1 mL $10^6$ M propranolol (pH 10) through the membrane, 1) immediately after sample application, 2) after 5 mins and 3) after 5 mins stirring the applied sample meantime. When the sample flowed through the membrane
Solid phase extraction of propranolol on multiwell membrane filterplates modified with molecularly imprinted polymer

without residing on it 63±4.7% of the analyte was bound to the membrane indicating that the sorption of the analyte is not instantaneous. If the residence time of the sample was increased to 5 mins without any stirring, the bound fraction increased to 75±5.9%. Stirring of the sample during the 5 min period further enhanced the retention to 87±4.4% inferring that diffusion from the well to the surface of the membrane is also a limiting factor. Therefore the sample volume was decreased to 100 µL because this amount is almost fully absorbed into the pores of the membrane, while when we use higher volumes, a significant part of the sample stays above the membrane in the well. It was seen, that when only 100 µL sample was loaded complete binding of the analyte occurred within 5 minutes. The test has been repeated with a more concentrated sample. 100 µL 10⁻⁵ M propranolol was applied to the MIM and the bound fraction was measured after different residence times. In Figure 6.4 it can be seen that binding of the template is quite fast and 5 minutes is enough for the complete adsorption of propranolol from the more concentrated sample, too.

<table>
<thead>
<tr>
<th>TIME / MIN</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOUND / %</td>
<td>90</td>
<td>92</td>
<td>94</td>
<td>96</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 6.4** Variation of the bound amount with residence time after the application of 100 µL 10⁻⁵M propranolol of pH=10.0

It is remarkable, that in case a 1 mL sample is loaded in 10x100 µL aliquots, close to 100% retention can also be achieved, which justifies that the low sample volume is favorable for the sample loading.

A general conclusion can be drawn from the above experiments. Even when the adsorption of the analyte on a MIP is not instantaneous the molecularly imprinted filterplate membranes can still be efficiently used in solid phase extraction by applying only the amount of sample that can be imbibed by the membrane filter. This amount is in an intimate contact with the sorbent, and complete binding can be achieved in the loading step. It is clear that environmental samples that are usually of large volume can pose a problem in this respect but this can also be overcome by allowing very slow filtration. The use of small sample amounts is characteristic of biological matrices, in fact there is a tendency to minimize sample volumes due to ethical considerations or to the limited availability of body liquids in certain animals. In these applications molecularly imprinted membrane filterplates can provide an efficient solution for sample cleanup even if the analyte adsorption is not prompt.

Considering that biological samples often require a protein precipitation step with organic solvent before SPE the effect of sample solvent was also studied. Propranolol samples in buffered aqueous media, in MeCN and in their mixture were prepared and applied to the filterplate membrane. Almost complete binding took place in the pure solvents i.e. MeCN and
aqueous buffer (pH=10.0). In the 2:1 mixture of MeCN and aqueous buffer, which corresponds to an approximate composition of a precipitated plasma sample, almost half of the analyte is lost. This observation corresponds to the results of Kempe et al. who also measured ca. 50% propranolol binding using this MeCN:H$_2$O ratio on the propranolol MIP. The phenomenon is explained by the fact that propranolol binds to the polymer phase through strong electrostatic interactions which are weakened by the presence of water but not fully ceased. The excellent binding on the composite polymer in pure acetonitrile can be explained by the fact that it is very similar to the porogen adiponitrile. In the final protocol aqueous biological samples were directly loaded onto the filterplate membranes after pH adjustment without protein precipitation.

6.2.2.2. Washing steps

In the MISPE protocol washing steps are essential to eliminate interferences and to enhance the selective retention of the analyte. In our system water did not cause any loss of the analyte, and was introduced into the protocol as the first washing step percolating a volume of 500 µL through the filterplate membrane. The purpose of this step is to remove hydrophilic components from the sample matrix.

To remove nonspecific hydrophobic interferences MeCN, MeCN modified with 0.5% acetic acid or 1% 10 mM NaCl, dichloromethane and toluene have been tested. Prior to the washing step a 10-minute drying step was introduced into the protocol to avoid the loss of analyte since residual water in the organic solvent can dramatically decrease the retention of the target. The recoveries obtained in the different washing solvents are depicted in Figure 6.1. Dichloromethane and toluene eluted very small fraction of the applied propranolol both from the MIP and the NIP imparting no selectivity to this step. MeCN by itself and modified with AcOH or NaCl eluted higher amounts of propranolol from the NIP than from the MIP. In the final protocol we have chosen 100 µL MeCN as the organic wash solvent because it eluted only a tiny fraction of the analyte from the MIP and approx. seven times more from the NIP, therefore a high analyte recovery in the SPE procedure could be expected, with efficient removal of interfering substances. This has been verified later with biological samples.

6.2.2.3. Elution

Experiments were carried out to optimize the composition of the elution solvent and the method of elution to recover as much analyte as possible.

Methanol and methanol modified with 1% acetic acid, 1% NaOH and 2% trifluoroacetic acid were tested in these experiments. Preliminary results showed that the elution is more efficient if the solvent is applied in smaller aliquots with a 5-minute residence time and shaking, instead of letting the whole volume to flow through at once. Therefore two consecutive aliquots of 250 µL solvent were applied and analyzed to acquire information about the recovery. MeOH alone eluted 48%, MeOH+1% NaOH eluted 53%, MeOH+1% AcOH eluted 69% and 2% TFA in MeOH eluted 80% of the applied analyte. Therefore, methanol containing 2% trifluoroacetic acid was found to be the most efficient for elution. In the final protocol four 250 µL aliquots of 2% TFA in methanol were applied for 5 minutes for the complete elution of the analyte.
6.2.3. Application to biological samples

In order to demonstrate the applicability of the elaborated MISPE method it has been applied for the sample cleanup of biological samples containing propranolol. In urine samples the minimum required performance limit (MRPL) established by the WADA for this compound is 500 ng mL\(^{-1}\). In plasma samples the therapeutic range of propranolol is 10 to 340 ng mL\(^{-1}\). At such low concentrations bleeding of the propranolol from the polymer can interfere with the measurement, therefore another template, oxprenolol has been chosen for the synthesis of the MIP modified membranes. The filterplates prepared with oxprenolol have been tested with aqueous samples using the optimized SPE method described in Section 3.3.2.2. Checking the permeated amount in the loading, washing and elution steps applying a relatively high concentration propranolol sample (\(2 \times 10^{-5}\) M corresponding to 5180 ng mL\(^{-1}\)) it was verified that this polymer has the same binding properties as the ones prepared with propranolol template. There was no measurable loss of propranolol during sample application and washing while the elution took place with close to 100% efficiency.

In the course of the pre-validation calibration samples were prepared in urine and plasma without using an internal standard and the samples were pretreated on the MIP modified filterplates applying the elaborated MISPE method (see Section 3.3.2.2). Eluates were analyzed by HPLC-MS/MS (see Table 3.1). Linearity of the MISPE-HPLC-MS/MS method was studied by analyzing spiked samples at six different concentration levels in the relevant concentration ranges (urine: 50-1500 ng mL\(^{-1}\); plasma: 10-300 ng mL\(^{-1}\)). Calibration lines were fitted using the least squares method with 1/x weighing. Parameters of the fitted lines together with accuracy values of each calibration point can be found in Table 6.1.

Table 6.1 Parameters of the fitted calibration lines and accuracy values of the calibration points in urine and plasma matrices

<table>
<thead>
<tr>
<th>Concentration (ng mL(^{-1}))</th>
<th>Accuracy*</th>
<th>Concentration (ng mL(^{-1}))</th>
<th>Accuracy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>102 %</td>
<td>10</td>
<td>102 %</td>
</tr>
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<td>125</td>
<td>107 %</td>
<td>25</td>
<td>98.3 %</td>
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<td>89.2 %</td>
<td>50</td>
<td>85.2 %</td>
</tr>
<tr>
<td>500</td>
<td>102 %</td>
<td>100</td>
<td>114 %</td>
</tr>
<tr>
<td>1000</td>
<td>99.4 %</td>
<td>250</td>
<td>105 %</td>
</tr>
<tr>
<td>1500</td>
<td>101 %</td>
<td>500</td>
<td>96.4 %</td>
</tr>
</tbody>
</table>

| Slope                         | 132       | 182                           |
| Intercept                     | 2674      | 2628                          |
| Regression coefficient        | 0.9992    | 0.9971                        |

* Accuracy values are the back calculated concentrations of the calibration points relative to their nominal concentration expressed in percentage

All the calibration points are within the 85-115% acceptance range established for biological samples by the bioanalytical method validation guidance of the U.S. Food and Drug Administration.\(^{[81]}\) Repeatability tests were carried out by analyzing spiked plasma and urine samples in quadruplicate. The recovery was calculated by comparing the peak area of pretreated samples of 250 ng mL\(^{-1}\) concentration to that of directly injected standard solutions.
Recovery values and repeatability data expressed as relative standard deviation are shown in Table 6.2.

**Table 6.2** Recoveries and repeatability data of propranolol from urine and plasma samples (n=4)

<table>
<thead>
<tr>
<th></th>
<th>Urine</th>
<th></th>
<th>Plasma</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovery (%)</td>
<td>R.S.D. (%)</td>
<td>Recovery (%)</td>
<td>R.S.D. (%)</td>
</tr>
<tr>
<td>Propranolol</td>
<td>102</td>
<td>4.05</td>
<td>97.2</td>
<td>6.41</td>
</tr>
</tbody>
</table>

Blank urine and plasma samples were also analyzed and no interfering substances could be detected.

The efficiency of matrix interference removal in the MISPE sample cleanup has been compared with two other methods, namely precipitation of the biological samples with acetonitrile and direct injection of 1:1 diluted blood and urine samples. In HPLC-MS/MS the chromatograms are usually very clean due to the high selectivity of the tandem mass spectrometric detector. However, unremoved matrix components may still interfere with the measurement by causing ion suppression in the ion source of the MS detector. Ion suppression is usually manifested in decreased analyte peaks. Therefore, we have studied the ion-suppression effect in the differently pretreated matrices. Blank plasma and urine samples were pretreated and afterwards were spiked with the analyte and injected. The peak area of these samples was compared to that of aqueous standards of the same concentration. The relative signal of the pretreated post-spiked samples and the standard solutions for the different sample preparation methods is given in Figure 6.5.

**Figure 6.5** Peak area of propranolol in pretreated and post-spiked matrix samples relative to that of the standard solution using different sample preparation techniques: white: 100 ng mL$^{-1}$ urine samples; grey: 100 ng mL$^{-1}$ plasma samples.

From the results it is clear that the cleanest samples showing no matrix effect at all were obtained by the MISPE procedure using the filterplate membranes. Precipitation of the biological samples with organic solvent removed also most of the interferences while direct injection of diluted urine and plasma shows obviously the highest ion suppression effect. These pre-validation data suggest that the new approach using MIP modified microfiltration membranes in filterplates can be a valid substitute of traditional MISPE cartridges.
6.3. Conclusions

A novel, high-throughput molecularly imprinted solid phase extraction method utilizing a composite membrane format is presented. The membrane supported MIPs are situated in multiwell filterplates allowing the rapid synthesis, fast template removal and characterization of the polymer. Propranolol has been chosen as a model analyte and the filterplate membranes were modified with propranolol selective MIPs. Afterwards, a thorough optimization of the sample preparation steps has been carried out. These studies revealed that sample volumes equal to or below 100 μL are suited the best to achieve complete binding of the analyte within a short time. Although this excludes environmental samples from the possible application area of this system, this is a clear advantage in the sample pretreatment of biological samples. In order to test the applicability of the new composite MIP membrane format with real samples we have developed a MISPE method for the clean-up of propranolol from urine samples around the MRPL and from plasma samples in the clinically relevant concentration range. Calibration, specificity and repeatability tests together with matrix effect studies confirmed that the elaborated method suits the requirements of biological sample analysis.

MIMs embedded in the multiwell filterplate setup enable parallel handling of many samples providing high-throughput sample preparation and are amenable to automation, as well. Their preparation is more straightforward and cost-effective than that of the traditional MISPE cartridges which require separate large scale synthesis of spherical particles using substantial amount of solvent. We think that the MIP modified multiwell membrane filterplate can be a competitive format beside the traditional MISPE cartridges and can find its way to routine bioanalytical applications.
7. In situ synthesis of molecularly imprinted nanoparticles in porous support membranes using nonsolvating polymerization solvents

7.1. Introduction

The integration of MIP nanoparticles and membrane technology can eliminate the major concern with MIM adsorbers, namely their limited binding capacity owing to the increased surface area of the nanoparticles. Current procedures for the preparation of MIP particle composite membranes use pre-synthesized nano- or microparticles which are filled between two commercially available membrane supports or built into macroporous membrane phases by phase inversion polymerization. Here, we describe a new approach where the MIP micro/nanoparticles are formed in situ in the pores of a microfiltration membrane therefore they do not need any manipulation after the polymerization reaction.

In this project we combined our expertise with the modified precipitation polymerization (Chapter 5) and the synthesis of MIP composite filterplate membranes (Chapter 6). A macroporous support membrane is wetted with the MIP pre-polymerization mixture which is a highly concentrated monomer solution. Nonsolvating diluents are used in combination with co-solvents or alone. MIP micro- or nanoparticles are formed in the pores of the support membrane and are firmly attached to it. The obtained composite membrane bears a high resemblance to the Empore™ membrane-solid phase extraction discs where adsorber particles are embedded in a polymer filter.

7.2. Results and discussion

7.2.1. Preparation of the MIP particle composite membranes

MIP particle composite membranes have been prepared for the selective binding of the chlorotriazine herbicide, terbutylazine. The target has been imprinted using MAA as the functional monomer and EGDMA as the crosslinker in a 1:4:20 molar ratio. BEE has been used as an initiator and the polymerization was initiated by UV light (254 nm). The porogen/total monomer volume ratio has been kept at 3:1 in all the experiments, since in an earlier work this ratio endowed MIP particles with the highest binding capacity.

To speed up the preparation and characterization of the new composite MIMs we have used glass fiber membrane filterplates like in Chapter 6, where twenty-three support membranes could be modified simultaneously with the MIPs (see Section 3.3.1.3.), washed in a flow-through mode to remove the template and characterized by equilibrium batch rebinding measurements or filtration experiments. For comparison, free particles without using the membrane support have also been prepared.

We have chosen chemically different polymerization solvents (see Figure 7.1) or solvent mixtures (Table 3.4) to explore the scope of this method and establish its potential for later applications. One of the solvents was paraffin oil, which cannot completely solubilize the monomer mixture by itself. Toluene or caprylonitrile was added to it in the smallest possible proportion where homogeneous polymerization mixtures could still be obtained. Toluene is considered as an apolar solvent, while caprylonitrile has similar chemical structure to acetonitrile used as a polar solvent in MIP preparation. Two other solvents have been chosen from the family of room temperature ionic liquids (RTILs). RTILs have already been successfully used as porogens to prepare molecularly imprinted polymers. One of the
In situ synthesis of molecularly imprinted nanoparticles in porous support membranes using nonsolvating polymerization solvents

RTILs that we have chosen was [BMIM][BF₄] which is considered relatively hydrophilic. The other one (triethyl(tetradecyl) phosphonium tris(pentafluoroethyl) trifluorophosphate, PH₃ T FAP) is among the fluoroalkylphosphates which are the most hydrophobic ionic liquids (Table 3.4).

![Figure 7.1 Structure of the compounds used as polymerization solvents: A) caprylonitrile, B) 1-butyl-3-methylimidazolium tetrafluoroborate, C) trihexyl(tetradecyl) phosphonium tris(pentafluoroethyl)trifluorophosphate, paraffin oil is a mixture of C₂₂-C₃₆ alkanes (not illustrated)](image)

All the attempts to incorporate MIP particles into the glass fiber membrane support using the different porogens ended up successfully with conversion close to 100%. During this process approximately 11 mg/cm² polymer could be incorporated into the support membrane. The particles were firmly attached to the fibers of the base membrane and they could not be removed by washing. Water permeability of the base membrane and that of the composite membranes can be seen in Table 7.1 (see Section 3.3.2.3.). Although the permeability of the glass fiber support membrane is decreased due to the modification, still very high water fluxes were obtained on the different composite membranes. Their permeability decreased in the order of CAP/PO MIP > PH₃ T FAP MIP > BMIM BF₄ MIP > TOL/PO MIP. From a practical viewpoint the water flux value on the CAP/PO MIP membrane means that using 11 Hginch vacuum (a routine vacuum setting in SPE experiments) 12 mL water could be percolated through the filterplate membrane within 1 minute, while this value for the least permeable TOL/PO MIP membrane was 0.6 mL.

In comparison with the method where pre-synthesized nanoparticles are sandwiched between two support membranes our procedure provides almost four times more molecularly imprinted polymer per membrane surface area and 1 to 2 magnitudes higher water flux through the membrane.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Water flux (Lm⁻²h⁻¹bar⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmodified glass fiber membrane</td>
<td>146870±19583</td>
</tr>
<tr>
<td>CAP/PO MIP</td>
<td>17448±2826</td>
</tr>
<tr>
<td>PH₃ T FAP MIP</td>
<td>10387±503</td>
</tr>
<tr>
<td>BMIM BF₄ MIP</td>
<td>8648±515</td>
</tr>
<tr>
<td>TOL/PO MIP</td>
<td>844±152</td>
</tr>
</tbody>
</table>

Table 7.1 Water permeability of the unmodified glass fiber support membrane and the MIP particle composite membranes
7. In situ synthesis of molecularly imprinted nanoparticles in porous support membranes using nonsolvating polymerization solvents

7.2.2. Morphology of the MIMs prepared with different solvents

The polymer particles and the composite membranes have been characterized by scanning electron microscopy. Figure 7.2 shows the SEM micrographs of the composite MIMs prepared using different solvents or solvent mixtures. Polymer particles prepared with toluene/paraffin oil or caprylonitrile/paraffin oil are around 1 μm in diameter, while polymers made in the two RTILs are well below 1 micron: PH3 T FAP MIP particles are around 350 nm and BMIM BF4 MIP particles are approximately 500 nm.

![Figure 7.2](image)

**Figure 7.2** SEM micrographs of MIP composite membranes prepared with different polymerization solvents: A) CAP/PO MIM, B) TOL/PO MIM, C) PH3 T FAP MIM D) BMIM BF4 MIP, E) cross-sectional view of a PH3 T FAP MIM F) unmodified glass microfiber membrane. The bars denote 5 μm in Figure A, B, C, D, F and 10 μm in Figure E.

This size difference might be due to the difference in the solvating capability of the polymerization solvents used. The size distribution of the particles is relatively homogeneous. We can observe aggregation of the particles in each membrane.

To obtain information on the porosity of the particles low temperature nitrogen adsorption/desorption isotherms were measured on TOL/PO MIP, CAP/PO MIP, PH3 T FAP MIP and BMIM BF4 MIP particles that were not incorporated into support membrane. Figure 7.3 compares the isotherms of the polymer particles.
7. In situ synthesis of molecularly imprinted nanoparticles in porous support membranes using nonsolvating polymerization solvents

Figure 7.3 Low temperature (77 K) nitrogen adsorption/desorption isotherms of TOL/PO MIP, CAP/PO MIP, PH3 T FAP MIP and BMIM BF4 MIP particles

TOL/PO MIP, CAP/PO MIP and PH3 T FAP MIP exhibit isotherms of Type IIB with a hysteresis loop of H3, which is typical for aggregates with slit-shaped pores. The aggregation is visible in the SEM photographs, too.

BMIM BF4 MIP exhibits an enhanced porosity compared to the other samples in the whole relative pressure range. The shape of the isotherm is not different but the transient H2-H3 type hysteresis is the sign of a complex, interconnected pore network. Porosity data derived from the isotherms is given in Table 7.2.

Table 7.2 Porosity data derived from nitrogen adsorption (77 K)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$S_{\text{BET}}$, m$^2$ g$^{-1}$</th>
<th>$V_{0.90}^*$, cm$^3$ g$^{-1}$</th>
<th>$V_{\text{micro}}$, cm$^3$ g$^{-1}$</th>
<th>$S_{\text{micro}}$, m$^2$ g$^{-1}$</th>
<th>$d_{\text{macro}}$, nm</th>
<th>$d_{\text{ave}}$, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOL/PO MIP</td>
<td>49</td>
<td>0.08</td>
<td>0.014</td>
<td>40</td>
<td>3.1</td>
<td>6.5</td>
</tr>
<tr>
<td>CAP/PO MIP</td>
<td>30</td>
<td>0.045</td>
<td>0.010</td>
<td>27</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>PH3 T FAP MIP</td>
<td>40</td>
<td>0.046</td>
<td>0.014</td>
<td>39</td>
<td>3.2</td>
<td>4.6</td>
</tr>
<tr>
<td>BMIM BF4 MIP</td>
<td>92</td>
<td>0.097</td>
<td>0.036</td>
<td>100</td>
<td>3.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Bulk MIP</td>
<td>455</td>
<td>0.744</td>
<td>0.174</td>
<td>n.d.</td>
<td>1.2</td>
<td>6.5</td>
</tr>
</tbody>
</table>

*volume of the pores not wider than 20 nm

In all the particles the volume of the pores ($V_{0.90}$) is very low. For comparison, we included the data of a bulk MIP having the same composition as the particles. It has been, however, synthesized in toluene, a good pore forming solvent and exhibits a much higher pore volume.
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S\textsubscript{BET} shows a good agreement with the \textit{S}_{\text{micro}} in all cases indicating that the polymer particles are mainly microporous.

### 7.2.3. Equilibrium batch rebinding experiments

The selective binding capacity of the membranes modified with MIP and NIP micro/nanoparticles were evaluated in batch rebinding experiments (see Section 3.2.4.). \(10^{-5}\) M solution of terbutylazine in MeCN was equilibrated with the composite membranes in the filterplate for 24 hours. The phase ratio was set at 60, i.e. the volume of the supernatant was 60-fold the mass of the incorporated particles. Similar experiments were carried out with free MIP and NIP particles that were prepared separately to see if the incorporation into the support membrane has any influence on the binding properties of the polymers. The bound amount of terbutylazine has been calculated and plotted in Figure 7.4.

**Figure 7.4** Bound fraction of the analyte in equilibrium batch rebinding experiments on different MIP and NIP particle composite membranes and free particles. \(10^{-5}\) M terbutylazine in MeCN was equilibrated with the polymers for 24 hours using a phase ratio of 60. (n=3)

We have observed high selective adsorption of the template from acetonitrile on the MIP particles either incorporated into the membrane or free. There was practically no measurable non-specific adsorption on the nonimprinted composite membranes and the non-specific adsorption on the free NIP particles was also very low. We did not found any considerable difference in the selective template uptake of the free particles and the MIP modified membranes. The adsorbed amounts varied from 25% to 44% or 48% in both cases, indicating that the support membrane does not change the binding properties of the MIP particles. Since the template uptake is quite similar for all the MIPs prepared with the different polymerization solvents it can be concluded that none of the solvents had a deteriorative effect on the formation of the recognition sites. All the solvents or solvent mixtures used in this experiment have proven to be appropriate for the MIM preparation. For further experiments the PH3 T FAP polymer membrane has been utilized where the hydrophobic ionic liquid was used as polymerization solvent.

### 7.2.4. Filtration experiments

Since MIM adsorbers are designed to achieve selective separation in flow through mode, we have investigated the kinetics of selective binding during filtration, the breakthrough curves of MIP and NIP membranes and the MIP/NIP selectivity and substance
selectivity in flow through SPE experiments. These tests were carried out on a PH3 T FAP polymer modified membrane.

As a first step, we have investigated the kinetics of template adsorption on the MIP membrane, a factor often overlooked in MIM SPE developments. If the sample is driven too fast through the membrane the analyte cannot equilibrate with the MIP binding sites, therefore the adsorbed amount will depend also on the flow rate.

1 mL $10^6$ M terbutylazine in MeCN was percolated through the composite membrane using different sample throughput times (i.e. different flow rates). The bound amount expressed in percentage can be seen in Figure 7.5.

![Figure 7.5](image)

**Figure 7.5** Variation in the bound fraction with sample application time. 1 mL $10^6$ M terbutylazine in MeCN is applied to the PH3 T FAP MIM.

The sorbed amount on the membrane did not change any more if the sample throughput time was 6 minutes or longer. This shows that 6 minutes is sufficient for the equilibrium saturation of the MIP adsorber. It should be noted that using sample application time of one minute, only one third of the saturation value was achieved. The 6 minute sample throughput time corresponds to approximately 170 µL min$^{-1}$ flow rate i.e. almost 10 bed volumes of sample can be percolated in one minute. In further filtration experiments the sample application time was set to assure equilibrium binding on the membrane.

In a next experiment where the same 1 mL $10^6$ M terbutylazine sample was applied to the membrane in small fractions (200 µL aliquots) the breakthrough curve was obtained (see Section 3.3.2.3.). In each percolated fraction the concentration of the template was quantified and plotted as percentage of the initial concentration against the cumulative volume passed (Figure 7.6).
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Figure 7.6 Breakthrough curve of a PH3 T FAP MIM applying 200 µL aliquots of $10^{-6}$ M terbutylazine in MeCN

From the figure one can see that the imprinted composite membrane completely adsorbs terbutylazine from the first 200 µL percolated volume. The breakthrough volume obtained either from the breakthrough curve by the equal area method or by measuring the retained amount of terbutylazine (see Figure 7.6) was approximately 430 µL (c.a. 25 bed volumes). This means that in equilibrium saturation the MIM binds 4.3 $10^{-10}$ mole terbutylazine when $10^{-6}$ M solution is applied.

In a similar experiment where $10^{-6}$ M terbutylazine in MeCN was percolated through a nonimprinted PH3 T FAP membrane no solute retention was observed at all. This is again a proof of the strong selective recognition capability of the imprinted membrane.

In SPE filtration experiments the PH3 T FAP MIM was tested against its nonimprinted counterpart and its selectivity was assessed towards other structurally related triazine herbicides (ametryn, prometryn, atrazine) and a nonrelated compound, phenytoin. Aqueous samples were prepared containing a mixture of the triazines in $10^{-6}$ M concentration and phenytoin in $10^{-5}$ M concentration. 1 mL sample was loaded on both the imprinted and nonimprinted polymer membranes. This was followed by two washing steps with 250-250 µL MeCN:water=30:70 and an elution step with 2 mL MeOH. This simple protocol was not an optimized procedure, however, earlier studies have shown that the terbutylazine MIP exhibits high selectivity in highly aqueous MeCN:water mixtures and terbutylazine can be eluted with MeOH. During sample application all the analytes were fully retained on the MIP membranes. This is due to the rather hydrophobic nature of MAA-EGDMA polymers. While in the two washing steps only 12% terbutylazine has been eluted from the membrane, already 82% of the nonrelated compound phenytoin has been removed, although it was applied in a tenfold excess. Prometryn was retained in the washing steps almost as well as terbutylazine but 44% ametryn and 52% atrazine has been removed. This shows that the imprinted particles exhibit high selectivity against dissimilar compounds, but recognize structural analogs of the template. The selectivity order is similar to earlier reported ones obtained in elution chromatography on TOL/PO MIP particles.

The MIP/NIP selectivity of the PH3 T FAP polymer membrane has also been assessed in the SPE experiments. Figure 7.7 shows the recovered fraction of terbutylazine on the imprinted and nonimprinted membrane in each step.
In situ synthesis of molecularly imprinted nanoparticles in porous support membranes using nonsolvating polymerization solvents

Figure 7.7 Recoveries in the different steps of the SPE procedure on PH3 T FAP MIP and NIP membranes after the application of $10^{-6}$ M aqueous terbutylazine. (wash 1 and wash 2: 250 μL MeCN:water=30:70 mixture, elution: 2 mL MeOH)

Both polymers bound the template completely from the aqueous sample due to hydrophobic interactions. However, in the two aqueous MeCN washing steps 44% of the analyte has been removed from the NIP and only 12% from the MIP. This confirms that the imprinted membrane selectively binds its template even though the SPE conditions have not been optimized.

The above experiments also suggest that the novel microparticle- or nanoparticle-modified MIMs can be used in analytical sample preparation to selectively extract the analyte from the sample - either in organic or in aqueous media. Examples for the first option can be found in food or environmental analysis where solid samples like soil, grain, and baby food are first extracted with an organic solvent and further purification is needed, whereas aqueous samples are often encountered in environmental and bioanalysis.
7.3. Conclusions

We presented a new technique for the preparation of MIP micro/nanoparticle composite membranes. The synthesis of the MIP particles is carried out inside a commercially available macroporous support membrane. With the aid of nonsolvating polymerization solvents polymer micro- or nanoparticles are formed even at high monomer concentrations (3 to 1 solvent: monomer ratio) in yield close to 100%. These are fixed to the support membrane structure and provide a selective functionalization. As opposed to former methods where MIP particle membranes are prepared in two sequential steps (i.e. formation of micro/nanoparticles mainly by precipitation polymerization, followed by their incorporation into support membranes) this is a simple one-step procedure. The use of the modified precipitation polymerization method is cost-effective compared to the conventional precipitation polymerization where large solvent volumes are needed and the yield is usually less than 100%. With this technique much higher amounts of imprinted polymer can be incorporated into the support membrane than by the thin-film or thin-layer composite methods, thereby the capacity of the new MIM is much higher. The permeability of the membranes allows high flow rates which can reduce internal mass transport limitations.

As a proof-of-concept MIP particle composite membranes using different solvents were prepared for the template terbutylazine and were fully characterized by scanning electron microscopy and N$_2$ porosimetry. The MIP particles incorporated into the membrane show quite low polydispersity and their diameter ranges from 350 to 1000 nm depending on the solvent used. Their template binding selectivity has been proven in equilibrium batch rebinding experiments and in flow through mode. They showed excellent MIP/NIP selectivity in acetonitrile and good substance selectivity against a non-related compound.
8. Summary, thesis points

In my doctoral work I have been involved in the development of molecularly imprinted polymers. My objectives were mainly directed toward the creation of new polymer formats that can be viable alternatives to the traditional bulk polymerization. The newly synthesized polymers have been tested in practice, either as molecularly imprinted solid phase extraction devices for the pretreatment of biological samples or nano/microparticle composite membranes for selective filtration purposes. I also investigated and broadened the scope of a novel precipitation polymerization method yielding monodisperse spherical microspheres in highly concentrated monomer solutions. Furthermore, photoswitchable MIPs were synthesized and characterized in a joint-project in Switzerland by a new, generic approach.

During my PhD studies I could spend a semester at the University of Geneva, where I designed novel, photoactivatable MIPs. In our versatile approach the selective recognition and the photoresponsive function of the MIP are ensured by two different monomers. As a proof of concept, MIP microspheres were synthesized by precipitation polymerization toward terbutylazine, a triazine-type herbicide. Formation of the selective binding sites was based upon H-bonding interactions between the template and the functional monomer methacrylic acid, while a polymerizable spiropyran unit was incorporated into the polymer matrix to provide light-controllable characteristics. A trifunctional monomer, trimethylolpropane trimethacrylate, was used as a crosslinker. The imprinted particles exhibited considerable morphological differences compared to their nonimprinted counterpart as observed by scanning electron microscopy. The imprinting effect was further confirmed by equilibrium rebinding studies. The photoresponsive feature of the polymer particles was visualized by fluorescence microscopy and further characterized by UV-Vis spectroscopy. The template binding behavior could be regulated by alternating UV and visible light illumination when analyte release and uptake was observed, respectively. Binding isotherms fitted by the Freundlich model revealed the photomodulation of the number of binding sites and their average affinity. We envisage that this approach may give an attractive starting point to endow currently existing highly selective MIPs with photoswitchable properties, thereby extending the scope of spiropyran-based photoresponsive smart materials.

In a part of my research work I have extended the scope of a modified precipitation polymerization method earlier described in our group. Molecularly imprinted polymer (MIP) microparticles have been prepared by precipitation polymerization using high monomer loadings (≥25 v/v %) which generally lead to bulk monoliths. The microparticle format was achieved by the use of a non-solvating diluent, for example paraffin oil, in combination with a co-solvent. We observed two distinct morphologies; monodisperse smooth, microspheres were obtained using a thermodynamically good co-solvent whereas segmented irregular particles were formed with poorer co-solvents. It has been found that during polymerization the forming polymer particles were enriched in the co-solvent. This effect was morepronounced using good co-solvents. The particle morphology could be tuned from segmented microparticles to uniform smooth microspheres by changing the co-solvent/paraffin oil ratio. Initiator concentration, type and relative amount of functional monomer and crosslinker and type of co-solvent have been varied and their effect on the particle size and morphology were examined. With the proposed methodology molecularly imprinted microparticles have been prepared successfully for two acidic templates, naproxen and diclofenac using a basic functional monomer, 4-vinylpyridine. The technique avoids large solvent-waste and can use a much larger selection of polymerization solvents as opposed to conventional precipitation polymerization. The technique can provide a synthesis alternative even for nonimprinters, and
can offer a cost-effective way for the generation of micron-sized polymer particles which is a desired polymer format in many applications.

In a project utilizing our group’s earlier expertise with MIP composite filterplate membranes I have synthesized molecularly imprinted polymers in 24-well glass fiber membrane filterplates to obtain a novel type of solid phase extraction device for the cleanup of propranolol. Sample processing parameters like residence time during sample loading, sample volume, pH, sample solvent, type and amount of washing and elution solvents have been investigated and optimized. Important differences from the conventional molecularly imprinted solid phase extraction (MISPE) cartridges were identified. The MIP modified composite membrane suits well the sample preparation of low volume biological samples. A protocol has been elaborated for the quantitation of propranolol from urine and plasma samples in the clinically relevant concentration range demonstrating the applicability of MIM adsorbers for the sample preparation of real samples for the first time. Preliminary validation results indicated that the composite MIP membrane filterplates offer a viable alternative to existing MISPE cartridges and at the same time have advantages like much easier and faster synthesis method and high-throughput sample preparation.

As a further extension of the abovementioned work we have introduced a novel approach to prepare MIP particle membrane adsorbers incorporating molecularly imprinted micro/nanoparticles into commercially available macroporous filtration membranes. The polymerization was carried out in nonsolvating polymerization solvents and the particles were formed in situ in the pores of the support membrane. MIP particle composite membranes selective for terbutylazine were prepared and characterized by scanning electron microscopy and \( \text{N}_2 \) porosimetry. By varying the polymerization solvent micro/nanoparticles with diameters ranging from several hundred nanometers to 1 micrometer could be embedded into the support. The imprinted composite membranes showed high MIP/NIP selectivity for the template in organic media both in equilibrium rebinding measurements and in filtration experiments. Solid phase extraction of a mixture of the template, its analogs and a non-related compound demonstrated MIP/NIP selectivity and substance selectivity of the new molecularly imprinted membrane. The synthesis technique offers a potential for the cost-effective production of selective membrane adsorbers with high capacity and high permeability.
The most important findings of my dissertation can be concluded in the following thesis points (the ordinal number of paper in which the results were published can be found in square brackets vide infra p. 90)

1. I have synthesized spiropyran-based MIP microspheres exhibiting photoswitchable template binding for the first time. [3]

2. The spiropyran-based MIP microparticles were the proof of a novel concept for the design of photoswitchable molecularly imprinted polymers. The selective interaction between the template and the polymer is ensured by a commonly used functional monomer whereas the spiropyran-based co-monomer is responsible solely for the photoswitching of the binding event because it makes possible the rearrangement of the binding sites by the photomodulation. [3]

3. The modified precipitation polymerization technique for the synthesis of monodisperse microspheres has been used only for one type of copolymer and one solvent composition so far. I have proven that the method can be extended to a wide variety of monomers commonly applied in molecular imprinting and particles of various morphology and polydispersity can be obtained depending on the applied solvent mixture, the type and ratio of the monomer. To obtain particulate polymers the solvent has to be thermodynamically incompatible with the polymer to a large extent. I have determined that how the particle morphology can be influenced with the variation of parameters: monodisperse microspheres of smooth surface can be obtained by using a co-solvent which is a good, solvating medium for the polymer. [5]

4. I have applied MIP composite membranes in multiwell filterplates as a high-throughput solid phase extraction media for the first time. I have identified specific operational conditions that are different from common SPE protocols using the cartridge format. The feasibility of molecularly imprinted membrane adsorbers for the sample pretreatment of real samples has been proven for the first time by the selective binding of β-blockers from biological samples. [1]

5. I have introduced a new approach for the synthesis of MIP nano/microparticle-composite membranes. In contrast with previous methods that incorporate preformed MIP particles into support membranes, I have created MIP nano/microparticles in situ in a support membrane in one step. This was achieved using the modified precipitation polymerization technique described in Thesis Point 3. [2]
9. Publications

Papers


Other papers not related to this thesis


Oral presentations


[3] Tibor Renkecz, Krisztina László, Viola Horváth:
Different MIP formats using highly viscous solvents: from composite membranes to nanogels
4th Graduate Student Symposium, London, UK, 28-30 September 2011

[4] Renkecz Tibor, Horváth Viola:
Új eljárások molekuláris lenyomatú polimerek előállítására
IX. Oláh György Doktori Iskola Konferenciája, 17 May 2012

[5] Tibor Renkecz, Giorgio Ceolin, Viola Horváth:
Molecularly imprinted polymer composites for biological sample preparation

[6] Viola Horváth, Tibor Renkecz, Krisztina László, George Horvai:
Spherical MIP particles obtained by polymerization in highly viscous porogens
6th International Conference on Molecular Imprinting, New Orleans, USA, 9-12 August 2010

**Posters**

[1] Tibor Renkecz, Zsolt Szemők, Giorgio Ceolin, Viola Horváth:
Multiwell membrane filterplates modified with MIPs – A novel format for solid phase extraction
6th International Conference on Molecular Imprinting, New Orleans, USA, 9-12 August 2010
and VIII. Oláh György Doktori Iskola Konferenciája, 3 February 2011

[2] Tibor Renkecz, George Horvai, Viola Horváth:
Synthesis of MIP microparticles for different targets using high viscosity solvents
7th International Conference on Molecular Imprinting, Paris, France, 27-30 August 2012
## 10. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-VPy</td>
<td>4-vinylpyridine</td>
</tr>
<tr>
<td>ABDV</td>
<td>2,2’-azo-bis-(2,4-dimethyl-valeronitrile)</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-azo-bisisobutryonitrile</td>
</tr>
<tr>
<td>BEE</td>
<td>benzoin ethyl ether</td>
</tr>
<tr>
<td>BMIM(^{+})BF(_{4}^{-})</td>
<td>1-Butyl-3-methylimidazolium tetrafluoroborate</td>
</tr>
<tr>
<td>CAP</td>
<td>caprylonitrile</td>
</tr>
<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
</tr>
<tr>
<td>DVB</td>
<td>divinylbenzene</td>
</tr>
<tr>
<td>EGDMA</td>
<td>ethyleneglycol dimethacrylate</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HSP</td>
<td>Hansen solubility parameter</td>
</tr>
<tr>
<td>IF</td>
<td>imprinting factor</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MAAm</td>
<td>methacrylamide</td>
</tr>
<tr>
<td>MC</td>
<td>merocyanine</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
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<tr>
<td>MIA</td>
<td>molecularly imprinted sorbent assay</td>
</tr>
<tr>
<td>MIM</td>
<td>molecularly imprinted membrane</td>
</tr>
<tr>
<td>MIP</td>
<td>molecularly imprinted polymer</td>
</tr>
<tr>
<td>MISPE</td>
<td>molecularly imprinted solid-phase extraction</td>
</tr>
<tr>
<td>MRPL</td>
<td>minimum required performance limit</td>
</tr>
<tr>
<td>MRM</td>
<td>multireaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>tandem mass spectrometry</td>
</tr>
<tr>
<td>NIP</td>
<td>nonimprinted polymer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>QCM</td>
<td>quartz crystal microbalance</td>
</tr>
<tr>
<td>PO</td>
<td>paraffin oil</td>
</tr>
<tr>
<td>PH3 T FAP</td>
<td>trihexyl(tetradecyl) phosphonium tris(pentafluoroethyl) trifluorophosphate</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly(methylmethacrylate)</td>
</tr>
<tr>
<td>RAFT</td>
<td>reversible addition-fragmentation chain transfer</td>
</tr>
<tr>
<td>RTIL</td>
<td>room temperature ionic liquid</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>SP</td>
<td>spiropyran</td>
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<tr>
<td>SPE</td>
<td>solid phase extraction</td>
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<tr>
<td>SPMA</td>
<td>1’-(2-methacryloyloxyethyl)-3’,3’-dimethyl-6-nitrospiro(2H-1benzopyran-2,2’-indoline)</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TOL</td>
<td>toluene</td>
</tr>
<tr>
<td>TRIM</td>
<td>trimethylolpropane trimethacrylate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>WADA</td>
<td>World Anti-Doping Agency</td>
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</table>
11. References


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11. References


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11. References


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DECLARATION

I, the undersigned Tibor Renkecz hereby declare, that I prepared the present Ph.D. thesis by myself and I used only the given sources. Every part that I literally adapted or rephrased with the same content, I cited unambiguously with the indication of the source.

Budapest, 21 November 2013

Tibor Renkecz