Advanced Strategies for Characterizing
Molecular Imprinted Polymers

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Tag der Promotion: 21.05.2014
“Oh, you can’t always get what you want
Oh, you can’t always get what you want
Oh, you can’t always get what you want
But if you try sometimes, you just might find
You get what you need”
The Rolling Stones (Mick Jagger & Keith Richards)
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Peer-reviewed research articles
Stefan Eppler, Michael Stegmaier, Florian Meier and Boris Mizaikoff; A novel extraction device for efficient clean-up of molecularly imprinted polymers, Analytical Methods, 2012, 4, 2296 - 2299.

Conference presentations

Stefan Eppler, Jenna Bowen, Adrian Porch, Chris Allender and Boris Mizaikoff; Why MIPs are better than we thought! A closer look at the rebinding process of synthetic receptors. Graduate Student Symposium on Molecular Imprinting, 15. - 17. August 2013, Belfast.

Conference posters

A complete set of publications is provided within the curriculum vitae (page 177).
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<th>Description</th>
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<tbody>
<tr>
<td>4VP</td>
<td>4-vinylpyridine</td>
</tr>
<tr>
<td>AA</td>
<td>Acrylic acid</td>
</tr>
<tr>
<td>AAm</td>
<td>Acrylamide</td>
</tr>
<tr>
<td>ABCN</td>
<td>1,1’-azobis-(cyclohexanecarbonitrile)</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AIBN</td>
<td>2,2’-azobis-(isobutynitrile)</td>
</tr>
<tr>
<td>ATR</td>
<td>Attenuate total reflection</td>
</tr>
<tr>
<td>BET</td>
<td>Brunauer-Emmett-Teller</td>
</tr>
<tr>
<td>B&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Binding capacity</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
<tr>
<td>DVB</td>
<td>Divinylbenzene</td>
</tr>
<tr>
<td>EGDMA/EDMA</td>
<td>Ethyleneglycoldimethacrylate</td>
</tr>
<tr>
<td>EGME</td>
<td>Ethyleneglycolmethylether</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transformation</td>
</tr>
<tr>
<td>FIB</td>
<td>Focused ion beam</td>
</tr>
<tr>
<td>GIF</td>
<td>Generic imprinting factor</td>
</tr>
<tr>
<td>GIF&lt;sub&gt;(%)&lt;/sub&gt;</td>
<td>percentage generic imprinting factor</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl-methacrylate</td>
</tr>
<tr>
<td>HOAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IF</td>
<td>Imprinting factor</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International union of pure and applied chemistry</td>
</tr>
<tr>
<td>K&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Equilibrium association constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LF</td>
<td>Langmuir-Freundlich</td>
</tr>
<tr>
<td>MAA</td>
<td>Methacrylic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MIP</td>
<td>Molecularly imprinted polymer</td>
</tr>
<tr>
<td>NIP</td>
<td>Non imprinted polymer</td>
</tr>
<tr>
<td>NTBA</td>
<td>N-tertbutylacrylamide</td>
</tr>
<tr>
<td>PRO</td>
<td>Propranolol</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
</tr>
<tr>
<td>RAFT</td>
<td>Reversible addition fragmentation chain transfer</td>
</tr>
<tr>
<td>SAM</td>
<td>Self-assembling monolayers</td>
</tr>
<tr>
<td>SCF</td>
<td>Supercritical fluid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TENAX</td>
<td>Poly(2,6-diphenyl-p-phenylene oxide)</td>
</tr>
<tr>
<td>TOL</td>
<td>Toluene</td>
</tr>
<tr>
<td>TRIM</td>
<td>Trimethylolpropane trimethacrylate</td>
</tr>
<tr>
<td>ULEX</td>
<td>Ulm extractor</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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1. Introduction, motivation and scope of the work

Molecular imprinted polymers (MIPs) are a success story since over 20 years, and have been subject of extensive research. First studies published by Linus Pauling in the 1940’s describing the synthesis of artificial antibodies\(^1,2\) are considered among of the very first publications concerning an imprinting process of molecules. Later, the basic concepts and the termini in MIP technology were mainly influenced by the groups of Wulff\(^3\) and Mosbach\(^4\) during the 1970’s and 1980’s resulting in consistently expanding research activities within the field.

Continuous research improving and advancing synthesis strategies for MIPs have opened the applicability of these polymers to a wide variety of analytical scenarios by enabling nowadays synthesizing MIPs for small target molecules as well as large biomolecules or even entire cells.\(^5-9\)

The attraction of imprinting strategies is undoubtedly based on the - at first sight - simplicity and effectiveness of self-assembly and free-radical polymerization for preparing synthetic receptors providing impressive affinity and selectivity.\(^10\) Perhaps surprisingly, whilst support for application-driven research has exceeded expectations, resulting commercial success has proved to be elusive. This may be attributed in part to the absence of standardised and robust methodologies for assessing and reporting on the performance of molecular imprinted polymers. While some progress has been made to align the reporting of equilibrium indicators, significantly less data is reported on methodologies addressing the binding kinetics and dynamic binding properties of such systems.\(^11,12\)

The performance of molecularly imprinted polymers (MIPs) is generally studied via the generation of binding isotherms, which are more often than not produced via batch analysis experiments, i.e., by incubating a known mass of polymer with different concentrations of selected analytes for a defined period of time, and comparison thus obtained data to a non-
imprinted polymer (NIP) synthesized with the same procedure yet in absence of the template.\textsuperscript{[13]} Since the adsorption of an analyte onto a solid sorbent material from a surrounding liquid is in fact a highly complex process, a variety of isotherms have been used to describe the binding behaviour of MIPs.\textsuperscript{[14–16]} Alternatively, the binding behaviour of MIPs may be chromatographically assessed (e.g., via liquid and frontal chromatography) or by evaluating distribution ratios, as reported by Horvai and others; a more detailed evaluation of methods describing the binding behaviour is provided in chapter 2.1.5 of this thesis.\textsuperscript{[17–20]}

Chromatography is a particularly useful method, since in theory one may probe both equilibrium and kinetic characteristics of a sorbent. However, packing columns with non-uniform or deformable particles renders reproducible packing of chromatographic columns difficult, and usually requires more complex non-linear chromatography theory for subsequently analysing the sorbent behaviour, therefore resulting in only few reported approaches in routine MIP characterization.\textsuperscript{[18]}

As shown in the present thesis, in order to fully realise the potential of MIPs within applications where the speed of binding and the dynamics of the binding process are essential (e.g., chem/bio sensing), it is important that MIP characterization reproducibly covers both dynamic/kinetic performance and equilibrium behaviour, ideally via transferable and unified analysis protocols applicable to all kinds of MIPs. Consequently, a more detailed analysis of the post-synthesis treatment of MIPs is necessary to evaluate and fundamentally understand the potential of MIPs serving as adsorbing media correctly, which summarizes the motivation for the studies performed within this thesis.

Advanced strategies were developed for the treatment of MIPs immediately after the synthesis of the polymer by introducing innovative clean-up methods ensuring a more exhaustive extraction of template and impurities prior to the analysis of the polymer. For obtaining equilibrium and kinetic data on thus synthesized MIPs and NIPs, a flow-cell-based setup was
developed, and a theoretical basis facilitating the understanding on equilibrium and kinetic data was developed. Furthermore, high-throughput methods for the generation of sufficient quantities of equilibrium binding data have been investigated along with potential problems and limitations occurring during the comparison of different experimental methods. Finally, this thesis is concluded with a proposed redefinition of one of the key parameters commonly used during MIP/NIP evaluation – the so-called *imprinting factor* – to more precisely render the significance of this figure of merit.
2. Theory and state of the art

2.1 Molecularly imprinted polymers

2.1.1 Basic considerations on molecularly imprinted polymers

Molecular recognition via MIPs is frequently explained by the lock and key mechanism known in biochemistry, as these synthetic polymers ideally mimic the recognition properties of their biological analogs offering highly selective binding sites. This is achieved by the usage of functional monomers that offer interactions with a template molecule. Typical interactions are hydrogen bonding, electrostatic interactions, π-π stacking or van-der-Waals interactions. These functional monomers along with appropriate cross-linking molecules are polymerized in the presence of the template molecule for creating a synthetic polymer matrix offering recognition sites based on the interactions between template molecule and functional monomers. In addition, also the steric information of the template molecules may be imprinted into the polymer, as the steric conformation and the location of the functional groups used for recognition of the template molecule is introduced into the polymer matrix, thus even enabling to separate chiral molecules in mixtures.\textsuperscript{[21,22]} Prior to usage of the polymer, the template molecule applied during the synthesis has to be removed in a clean-up step. The entire imprinting process is schematically illustrated in Figure 1 for the example of non-covalently imprinted materials. More information on MIP theories and their application for explaining the binding behavior are briefly summarized within the following chapters, and are extensively covered within various reviews, books, and book chapters.\textsuperscript{[10,23–34]}
2.1.2 Fundamental synthesis concepts of molecular imprinting

The synthesis of MIPs is typically classified into two approaches - the covalent and the non-covalent imprinting method. Beside, also combinations such as semi-covalent imprinting or solid phase approaches with reusable templates are used nowadays, as detailed in the following sections.

2.1.2.1 Non-covalent imprinting

Non covalent-imprinting - also called self-assembling approach - is the most commonly used imprinting method, and was mainly devised by Mosbach et al. in the 1980's.\textsuperscript{[4,35–37]} Driven by molecular interaction forces such as hydrogen bonding, electrostatic or hydrophobic interactions, the functional monomers and template molecules form a stable prepolymerization complex in solution.\textsuperscript{[38,39]} By adding cross-linking molecules and polymerization initiators the complex is “secured” by the formation of a
polymer matrix around the complex. Afterwards, extraction processes remove the template molecules. Polymers synthesized with this approach most closely resemble the recognition principles occurring in nature described as the “lock-and-key” mechanism by Emil Fischer in 1894 as the main interaction mechanism between (bio)molecules in biological systems. The stability of the prepolymerization complex is crucial for this method, as it defines the binding sites available after the imprinting procedure. Therefore, functional monomers are frequently used in excess to shift the equilibrium to the complex site. However, a disadvantage of this method is the formation of less- or non-specific binding sites, as functional monomers can be randomly embedded into the polymer matrix. The amount of non-specific binding and the corresponding loss in selectivity usually increases with decreasing prepolymerization complex stability, and thus limits the quality of the obtained imprint. Hence, if the strength and number of interactions between the functional monomers and the template molecules are limited, the resulting MIP usually offers similar binding properties as a blank/control polymer, i.e., the non-imprinted polymer (NIP) that was prepared by exactly the same synthesis strategy yet in absence of the template molecules within the reaction mixture.

2.1.2.2 Covalent imprinting

The covalent approach – also called pre-organized or stoichiometric approach - was developed by Wulff and coworkers. In contrast to the non-covalent approach, a prepolymerization compound consisting of functional monomers covalently bound to the template molecule is literally synthesized, and then added to the prepolymerization solution. After polymerization into a matrix, the covalent bond between the functional monomers and the template molecules are chemically cleaved, and the template molecule may be removed. The rebinding interactions are usually
based on reestablishing a (weak) covalent bond again.\cite{29} While the synthesis of such a prepolymerization compound may be complex, it offers distinct advantages justifying the additional synthesis efforts. As no “free” functional monomer is co-polymerized, less- and non-specific binding is usually reduced compared to non-covalent imprinting. Furthermore, harsh conditions have only minor influence on the entire imprinting process, as no prepolymerization complex has to be formed but is provided as a pre-synthesized compound. While the binding strength due to the nature of the covalent bonds appears superior, the kinetic binding properties of such MIPs may be limited.

Hence, the needs of the application and the synthetic efforts largely drive the selection of the imprinting strategy, whereby covalent imprinting despite being the first approach developed within the concept of molecular imprinting appears less applied nowadays compared to non-covalent or semi-covalent approaches.

2.1.2.3 Semi-covalent imprinting and other strategies

The semi-covalent approach is a combination of the covalent and the non-covalent approach using the advantage of a covalently bonded template-functional monomer compound along with the advantageous kinetics of non-covalent interactions.\cite{46, 47} Typically, the template molecule is modified with a cleavable functional monomer. After polymerization and extraction of the template molecule, the remaining part of the functional monomer is able to interact with the template molecule via non-covalent interactions. This approach likewise leads to polymers with almost no unspecifically arranged functional monomers and therefore reduced non-specific binding in comparison with the non-covalent approach.

A rather new synthesis method is the so-called solid phase assisted synthesis of MIP nanoparticles via a reusable template.\cite{48} In this approach the
template molecules are immobilized at solid surface e.g. chromatographic particles, which are packed into UV-transparent columns and applied to a flow system. The prepolymerization solution is pumped in and the column is irradiated with UV light to initiate the polymerization leading to imprinted nanoparticles with high affinity. If the solution is kept under flow, low affinity particles are washed out at the beginning and high affinity particles are retained longer within the column by separating the resulting polymer particles respective their selectivity. Using this approach, polymers with unique properties almost providing the binding characteristics of antibodies are obtained. However, in contrast to conventional MIP approaches, it is not possible to synthesize a NIP at the same conditions. So a detailed investigation of the imprinted nanoparticle has to be performed in absence of a suitable NIP.

2.1.3 Polymerization strategies

In literature, a wide variety of polymerization methods have been published and compared for synthesizing MIPs.\textsuperscript{[49]} Therefore, in the following sections only the most commonly used approaches relevant to this thesis are described in more detail. A selection of the most commonly used cross-linkers, functional monomers, and radical initiators is shown in Figure 2.
Figure 2. Functional monomers: \( \textbf{a}) \) – acrylic acid (AA), \( \textbf{b}) \) – methacrylic acid (MAA), \( \textbf{c}) \) – acrylamide (AAm), \( \textbf{d}) \) – N-tertbutylacrylamide (NTBA), \( \textbf{e}) \) – 4-vinylpyridine (4VP), \( \textbf{f}) \) – 2-hydroxyethyl-methacrylate (HEMA); cross-linker: \( \textbf{g}) \) – divinylbenzene (DVB), \( \textbf{h}) \) – ethylenglycoldimethacrylate (EGDMA or EDMA), \( \textbf{i}) \) - trimethylolpropane trimethacrylate (TRIM); radical initiator: \( \textbf{j}) \) – 2,2’-azobisisobutyronitrile (AIBN), \( \textbf{k}) \) - 1,1’-azobis(cyclohexanecarbonitrile) (ABCN).

2.1.3.1 Bulk polymerization

Bulk polymerization is the most common method applied in non-covalent imprinting due to the comparatively simple experimental execution.\textsuperscript{[50–53]} The template molecules, cross-linkers, functional monomers and the radical initiator are dissolved in a suitable solvent within a reaction container and the reaction mixture is purged with nitrogen gas to remove dissolved oxygen, which could delay or prohibit the polymerization. The
polymerization is then thermally initiated or by irradiation with UV light and kept for an optimized period of time at these conditions until a solid block co-polymer is obtained. Afterwards, the reaction container is removed and the solid block co-polymer is crushed and sieved into particulate fractions (typically between 10 – 100 μm) in dependence on the desired application. For most applications ideally monodisperse particles with a limited distribution of diameters are advantageous. However, bulk polymerization usually leads to a heterogeneous size distribution, and thus, significant waste of polymer resulting from the grinding process of the solid polymer block yielding a substantial amount of fine particles (< 1 μm). Furthermore, it is not possible to inherently create a defined particle shape via bulk polymerization, which is advantageous e.g., in chromatographic applications.

However, bulk polymerization is frequently the method of choice if new templates, synthesis designs or analysis methods for MIPs are initially investigated, which is in part the case within the present thesis. As an example, polymer particles generated via bulk polymerization for the template propranolol are shown in Figure 3.
2.1.3.2 Precipitation polymerization

As a modification of the bulk synthesis, the precipitation polymerization method is applied for creating micro- and nanospheres. The experimental difference between the two methods is the considerably larger amount of solvent used for precipitation polymerization, and continuous stirring during the polymerization process. Consequently, the polymerization reaction is more controlled leading to oligomers, which then grow into polymers at extended reaction times leading to the precipitation
of polymer particles rather than a solid polymer block. The size of thus obtained particles – usually of spherical shape based on minimization of the surface energy during precipitation - is defined by the solubility of the growing oligomers within the solvent of choice, i.e., when the particles become insoluble and precipitate. The reaction is usually initiated by heat or UV irradiation like during bulk polymerization; however, also the reversible addition fragmentation chain transfer (RAFT) polymerization approach was used in precipitation polymerization for even more control on the progression of the synthesis.\textsuperscript{[56–58]}

The main advantage of this method is the control on particle size and shape. Consequently, precipitation polymerization is the method of choice when generating particles for usage e.g., in chromatographic or solid-phase extraction applications.\textsuperscript{[59,60]}

2.1.3.3 Suspension and emulsion polymerization

A suspension is defined as a heterogenic mixture of a liquid and a solid phase, e.g., fine polymer particles in all kinds of solvents, while an emulsion is a mixture of two immiscible liquids, e.g., water in oil.\textsuperscript{[61]}

In MIP literature these two physical definitions are not always consistently used, as at the starting point of the polymerization there is always an emulsion present, and after the synthesis a suspension of polymer particles in a solvent is given. Suspension polymerization is usually applied to create spherical microparticles. Therefore, a reaction solution consisting of template molecule, functional monomer, cross linker and radical initiator is mixed into an immiscible solvent phase. Typically by stirring, small droplets are created and polymerized by irradiation with UV light. The particle size is controlled by the stirring rate, temperature, nature of the solvent and the immiscible secondary solvent phase. The success of the polymerization is mainly influenced by the stability of the emulsion before the reaction is
started. If the emulsion is not sufficiently stable or some reaction components diffuse from the droplets into the secondary solvent, the resulting particles may not be spherical or the resulting size distribution is heterogeneous; in a worst case, no particles are obtained. Stable suspension systems are obtained using organic solvents in mixtures of e.g., polyvinyl alcohol/water, acetonitrile in mineral oil, and acetonitrile in silicon oil.[62-65] Nanoparticles are frequently synthesized using miniemulsion polymerization strategies. For the creation of a stable miniemulsion, homogenizers or ultrasonic probes are used to create the miniemulsion providing nanoscale droplet dimensions, which is stabilized via the addition of surfactants like sodium dodecyl sulfate (SDS).[66,67] An exemplary SEM image of oxybutynin imprinted polymers, synthesized via suspension polymerization using an acetonitrile in silicon oil system, are shown in Figure 4.
2.1.3.4 Core-shell polymerization

The concept of core-shell imprinting is based on the usage of a particle as a core and the creation of an imprinted polymer shell around or grafted onto that particle. Using substrate particles not involved into the imprinting process provides several benefits. As the creation of the imprinted polymer shell can be well controlled, the obtained particles have a narrow size distribution and the shape of the core particle is conserved. Hence, using this technique spherical particles for chromatographic applications can be
created and there is virtually no size limitation as it is the case e.g., for precipitation polymerization.\[72\] Typical polymer core particles are made from styrene or methyl methacrylate and are usually synthesized via emulsion polymerization methods.\[73\]

Modifying e.g., metal core particles with a MIP coating offers the advantage of imprinted polymers with a magnetic core, thereby facilitating molecular imprinted polymers for a wide variety of assay applications were the particles have to be removed after binding occurs.\[74,75\]

Using modified silica particles as core with a MIP film grafted at the surface is also reported in literature.\[72,76\] The imprinted shell can be created using e.g., emulsion polymerization methods\[73,77\] or further precipitation polymerization\[72,76\] of the seed core particles.

The following Table 1 summarizes some key aspects of the presented polymerization methods.

<table>
<thead>
<tr>
<th>polymerization method</th>
<th>particle shape</th>
<th>particle size</th>
<th>size distribution</th>
<th>experimental effort</th>
</tr>
</thead>
<tbody>
<tr>
<td>bulk</td>
<td>irregular</td>
<td>micro</td>
<td>broad</td>
<td>low</td>
</tr>
<tr>
<td>precipitation</td>
<td>regular</td>
<td>micro/nano</td>
<td>broad</td>
<td>low</td>
</tr>
<tr>
<td>suspension/ emulsion</td>
<td>regular</td>
<td>micro/nano</td>
<td>broad</td>
<td>high</td>
</tr>
<tr>
<td>core-shell</td>
<td>regular</td>
<td>depending on seed particle</td>
<td>narrow</td>
<td>high</td>
</tr>
</tbody>
</table>

*Table 1. Key aspects of the different polymerization methods.*
2.1.4 Polymer clean-up

After successful synthesis of a molecularly imprinted polymer the embedded template molecule and other impurities such as unreacted cross-linker molecules or functional monomers have to be removed in order to avoid leaching effects during subsequent applications. For covalently imprinted materials the chemical bond between template molecule and polymer has to be cleaved prior to removal of the template molecules. Common techniques include the elution of these components from the polymer via suitable solvents by soxhlet extraction\[^78\] or by packing HPLC columns with the polymer and flushing.\[^79\] A summary of techniques and extraction methods along with a thorough evaluation and comparison was published by the research group of B. Sellergren.\[^80\] While these commonly applied techniques may be well established they also have some shortcomings such as the requirement of packing HPLC columns, which is difficult especially for soft materials potentially damaging the particles. Also, classic soxhlet extraction usually does not work properly if e.g., solvent mixtures are applied.

Consequently, a remaining challenge in MIP technology is the exhaustive clean-up of the obtained MIP material ensuring sufficient removal of the template molecule to avoid compromising the evaluation of the polymer properties or even its subsequent analytical or biomedical application by template leaching.\[^81,82\]

Hence, an ideal extraction procedure should be effective yet avoiding extreme conditions negatively affecting the MIP matrix, and should remove the entirety of embedded template molecules in a short time period with using a minimal solvent volume.

In order to improve the clean-up procedure of MIPs, an efficient continuous extraction method was developed within this thesis and already published.\[^83\] For the so called ULEX (UlExtraction) method a specialized device was designed that significantly reduced the extraction time and
enabled the use of solvent mixtures in an easy-to-handle procedure.\cite{83} Details of this method are summarized in chapter 3 of the present thesis. Though, as shown by F. Meier et al. even after exhaustive extraction procedures template molecules may still be found embedded within the polymer matrix.\cite{84} However, it was apparent that these template molecules were completely embedded into the polymer network, i.e., without access to pores or channels, and thereby will not leach into the surrounding environment at conventional application circumstances that do not purposefully disintegrate the polymer matrix. Recently, Lorenzo et al. started the discussion how the template molecule removal influences the binding pockets e.g., by inducing collapsing of the pockets.\cite{85} Hence, the extraction process of template molecules and impurities is apparently subject of on-going research and remains a crucial step during the generation of imprinted materials of any kind.

\section*{2.1.5 Figures of merit for MIPs and evaluation methods}

The evaluation of the quality of the synthesized molecularly imprinted polymers is a crucial step for describing the MIP properties, and for the comparison between different polymers. Conventionally applied figures of merit and associated evaluation methods are summarized within the following chapters.

\subsection*{2.1.5.1 Imprinting factor, binding affinity, binding capacity and binding selectivity}

The \textit{imprinting factor} (IF) is among the main characteristic parameters applied in molecular imprinting and is reported in most publications for describing the quality of a MIP compared to its NIP. Before the expression
imprinting factor became prevalent, the separation factor of a MIP column compared to a blank or NIP column as derived from chromatographic theory was considered by comparing the capacity factor of the target analyte at both materials for discriminating between acceptable or limited imprinting efficiency.\textsuperscript{[86–88]} The separation factor was called imprinting factor for the first time in the late 1990’s and early 2000’s introduced by the research groups of I. Karube,\textsuperscript{[89,90]} C. Baggiani,\textsuperscript{[91,92]} and J. A. Tarbin\textsuperscript{[93]}, and calculates as shown in Equation 1.

\[
\text{separation factor (α) ≈ imprinting factor (IF) = } \frac{k'_{\text{MIP}}}{k'_{\text{NIP}}} \quad (1)
\]

\(k'_{\text{MIP}}\) - capacity factor of the MIP column

\(k'_{\text{NIP}}\) - capacity factor of the NIP column

This concept of describing the imprinting efficiency for analytical columns was then transferred to other applications such as batch incubation experiments where the imprinting factor is typically calculated by dividing the amount of template bound by the MIP to the amount of template bound by the NIP at a certain concentration, which could be individually selected.\textsuperscript{[94]} Therefore, it is readily evident that several different imprinting factors could be calculated for the same polymer by calculating the imprinting factor at different incubation concentrations. Consequently, for an exhaustive characterization of the MIP binding behavior isotherms, e.g., bound/free isotherms should be reported, as proposed by the research group of C. Allender.\textsuperscript{[13]} Also, during chromatographic evaluations the imprinting factor may in fact be unsuitable as a result of nonlinear chromatographic theory, which should be used for the evaluation of chromatographic MIP columns.\textsuperscript{[18]} Despite these considerations, the imprinting factor is still most commonly applied for providing a first impression on the quality of a newly synthesized
polymer and is considered the most relevant figure of merit related to MIPs in a wide variety of publications.\cite{95-98}

Within the present thesis, the concept of *imprinting factor* was rigorously reconsidered given the theoretical description of batch analysis experiments and a so-called *percentage generic imprinting factor* (GIF(\%)) was defined, which considers the entire binding behavior of a MIP. A detailed description of this concept is given in chapter 6 within this thesis.

The *binding affinity* of a polymer describes the binding strength between an analyte molecule and the polymer, and is related to the adsorption equilibrium, as shown in Equation 2. The association constant $K_a$ describes the formation of the association, and the desorption constant $K_d$ describes the desorption of the template molecule from the place of adsorption, respectively.\cite{10}

\[
\text{template molecule + adsorption place} \quad \xrightleftharpoons{K_a \quad K_d} \quad \text{bound template molecule} \quad (2)
\]

The *binding capacity* $B_{\text{max}}$ describes the maximum amount of analyte that can be bound usually per gram or milligram of dried polymer. The *binding capacity* is not necessarily equal to the amount of template molecules used for generating the imprint, as not every template molecule creates a specific adsorption location thus giving rise to non-specific binding sites within every polymer matrix.\cite{10}

*Binding affinity* and *binding capacity* are typically determined by chromatography\cite{20,99-101} or batch experiments,\cite{102-104} which are described in more detail in the following chapter.

The *binding selectivity* of a MIP describes its recognition ability, i.e., how well it discriminates between the template molecule used during the synthesis and other analyte molecules with different or similar chemical structures (i.e., structural analogs). Typically, the MIP is exposed to a mixture of analyte
molecules (template and structural analogs) while monitoring the binding process, which could be done via batch or chromatographic experiments for assessing the selectivity of the polymer.\cite{9,105,106}

### 2.1.5.2 Chromatographic and batch evaluation methods

Batch experiments and chromatographic evaluation methods are the most common and widely used techniques during MIP evaluation.\cite{107}

For a batch experiment certain known amounts of polymer are incubated with different analyte concentrations for several hours under continuous shaking or stirring to achieve adsorption equilibrium. Afterwards, the polymer slurry is centrifuged or filtered and the supernatant is analyzed for the remaining analyte concentration. Similar results are obtained by using MIP-columns and flushing the column with different analyte concentrations in frontal chromatography methods for the determination of $B_{max}$ and $K_d$ values.\cite{20,21,108,109}

Both methods also enable determining the \textit{binding selectivity} of a MIP. For that purpose, structural analogs of the template molecules are mixed with the template molecule itself and the polymer properties are investigated with the molecular mixture. In a batch experiment the supernatant is analyzed via the rebinding properties of the template molecule compared to the analogs after incubation.\cite{110} For HPLC experiments the column is tested with mixture samples and for a selective MIP the retention times of the initial template molecules are compared to its structural analogs.\cite{111,112}
2.1.6 Applications of molecularly imprinted polymers

In the following section the main application areas of MIPs in chromatographic and solid phase extraction applications as well as their usage within biomimetic sensors and the utilization of MIPs as plastic antibodies are outlined.

2.1.6.1 Stationary phase in chromatographic columns & solid phase extraction

As previously mentioned, MIPs are frequently used as stationary phase in chromatographic applications due to their specific binding affinity enabling the separation of analyte molecules even in complex mixtures of structural analogs. In order to provide reproducible properties of chromatography columns using MIPs as stationary phase it is desirable that the material provides a controlled particle size, uniformity of the particles, stability and chemical resistance. Currently, the main focus for MIPs in separation sciences is the usage as solid phase extraction (SPE) material (e.g., packed into SPE cartridges or columns) applied prior to HPLC analysis. Considering which polymerization method should be used for the preparation of chromatographic MIP materials, bulk polymerization methods are of limited value, as even after sieving the ground bulk polymer into fractions with a narrow size distribution the resulting non spherical particles may lead to broad and tailing chromatographic peaks.\cite{113,114} However, there are many successful reports in literature demonstrating the use of bulk polymers, e.g., the separation of (±)-ephedrine by supercritical fluid (SCF) chromatography,\cite{115} the purification of vanilla from soda black liquor,\cite{116} the separation of testosterone glucuronide from other drugs in urine,\cite{117} the separation of the structural isomers of nitrophenol\cite{9} and the extraction and enrichment of caffeine.\cite{51} Nowadays, stationary phases applied in analytical
MIP columns are usually prepared as spherical particles via appropriate polymerization methods, as described in chapter 2.1.3. Such materials have successfully been used also in SPE applications including e.g., the extraction of parabens from river water samples, the ultratrace level determination of six fluoroquinolone antimicrobials in drinking and aquaculture water by combination of online SPE with liquid chromatography, the extraction of penicillin from milk and the extraction of thiabendazoles from citrus fruits.

2.1.6.2 Biomimetic sensors

Utilizing the specific recognition of analyte molecules MIPs appears to be ideal for the design of sensors mimicking biological binding events within so-called biomimetic devices, i.e., in lieu of conventional biosensor technology. However, the application of MIPs for sensor purposes has been proven less useful compared to chromatography. One reason may be the more complex polymerization techniques required for immobilizing or grafting MIPs at the active transducer surface of a sensor compared to just packing the synthesized material into a column for chromatographic applications. S. Piletsky and A. Turner reported three critical issues for designing MIP-based sensors:

- A highly sensitive transducer has to be developed able to monitor the binding process and the transformation into a measureable analytical signal.
- For selective recognition a high-affinity MIP has to be designed.
- Transducer and MIP have to be combined.

The most commonly used signal transducing methods reported in literature are electrochemical, mass sensitive and optical transducers. Electrochemical transducers are widely used, as electrochemistry not only offers mature amperometric or potentiometric detection schemes received
from electrochemical chem/bio sensing technology, but also conductivity or impedance schemes for analyzing binding events.[123] Due to its robustness and low price the quartz crystal microbalance (QCM) is another commonly applied mass-sensitive transducer, which may used in combination with MIPs for detecting the adsorbed analyte molecules by a shift in the resonance frequency.[8,125] Optical detection schemes take advantage of a change in optical properties upon binding between template and MIP giving rise to a quantitative analytical signal. For example, fluorescence may be quenched or enhanced by appropriate analyte-polymer interactions[126,127] and even fluorogenic monomers can be used for the MIP synthesis.[128] It is also possible to directly determine target-molecule-specific signatures e.g., in the mid-infrared spectral range by depositing MIPs onto an attenuate total reflection (ATR) waveguide and measuring the analyte spectrum by evanescence wave absorption spectroscopy[129] or by combining the MIP polymer with modified gold nanoparticles facilitating surface plasmon resonance (SPR) studies.[130,131]

Synthesis strategies used for combining MIP and transducer can derive from spin-coating of MIP particles onto e.g., an electrode or QCM surface[132] or in situ grafting or synthesis of the MIP at the transducer surface, e.g., via electrochemical polymerizations, which is frequently used for conducting surfaces such as gold.[122] The main electopolymerizable systems used for the synthesis of imprinted polymers are polypyrrole[133,134]; however, also MIPs based on phenols or thiophene derivatives are reported.[122] Suitable for biomimetic sensors is also the synthesis of MIP membranes[28] or the modification of electrodes with self-assembling monolayers (SAMs) providing a molecular architecture for the subsequent synthesis of thin MIP films.[135]
2.1.6.3 Plastic antibodies

In a seminal contribution, the research group of K. Mosbach mentioned the use of MIPs as antibody-mimicking material, which was later termed plastic antibodies.\textsuperscript{136,137} The concept of utilizing polymers for purposes that have been previously been exclusively dominated by antibodies was enticing, yet, while MIPs offer selective binding sites similar to antibodies they also have disadvantages such as their insolubility.\textsuperscript{138} Comparing the two main parameters - binding affinity and solubility - for a successful usage of MIPs instead of antibodies it appears obvious how MIPs have to be optimized for providing a competitive alternative to their natural analogs. The first challenge is to achieve binding affinities comparable to antibodies, which are reported in the picomolar range, while MIPs are frequently characterized by low micro- to nanomolar dissociation constants.\textsuperscript{139} The second challenge is to obtain the same binding properties that MIPs usually show in organic solvents also in aqueous and complex biological matrices. Selected examples have already demonstrated that MIPs may indeed serve as plastic antibodies. The research group of K. Shea has shown that MIPs could be used in vitro for the recognition, neutralization and clearance of target peptides.\textsuperscript{140} Especially imprinted nanoparticles offer potential as synthetic analog in lieu of antibodies\textsuperscript{141} and therefore polymerization techniques for nanoparticles are extensively researched already demonstrating remarkable results for the synthesis of highly selective MIP nanoparticles (e.g., solid phase assisted synthesis of MIP nanoparticles with an reusable template), as described in chapter 2.1.2.3.\textsuperscript{48} Consequently, using appropriate synthesis strategies the benefits of MIPs serving as plastic antibodies could be the beginning of a new era for MIPs serving as synthetic receptors, as outlined by K. Haupt in a recent review.\textsuperscript{142}
2.2 Adsorption and kinetic theory for molecularly imprinted polymers

2.2.1 General considerations on adsorption

Adsorption occurs whenever a solid surface is exposed to a gas or a liquid and is defined as the enrichment of material or liquid at the solid surface. Adsorption phenomena to solid surfaces are ubiquitous ranging from biological mechanisms to catalytic reactions and remain a subject of vital research. One of the first applications of adsorption was the use for separation techniques already known by the ancient Egyptians, Greeks and Romans e.g., for the desalination of water. The most relevant terms for describing adsorption are listed in Table 2.

<table>
<thead>
<tr>
<th>Adsorption</th>
<th>Enrichment of material or liquid onto a solid layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorptive</td>
<td>Material or liquid that is adsorbed to the solid layer</td>
</tr>
<tr>
<td>Adsorbent</td>
<td>Solid layer that adsorbs the adsorptive</td>
</tr>
<tr>
<td>Desorption</td>
<td>Process where the amount of adsorptive decreases from the adsorbent</td>
</tr>
<tr>
<td>Absorption</td>
<td>The adsorptive penetrates the solid surface and enters the structure of the material</td>
</tr>
<tr>
<td>Sorption</td>
<td>Generic term covering adsorption and absorption processes</td>
</tr>
</tbody>
</table>

Table 2. General terms used for the description of adsorption phenomena derived from literature (IUPAC).

The forces involved in adsorption are classified into physisorption and chemisorption. The chemisorption or chemical adsorption uses chemical (covalent) bonds to bind the adsorptive to the adsorbent. The physisorption or physical adsorption is based on Van-der-Waals forces and is a general
phenomenon with a lower specificity compared to chemisorption, which is related to the activity of adsorbent and adsorptive. In adsorbing processes both phenomena may also appear together. Chemisorption typically forms a monolayer of adsorbed material and onto that layer more material may build up a layer via physisorption, which itself may form monolayers or – usually at high concentrations - multilayers of adsorbed material. The energy of the chemical (covalent) bond is higher than in Van-der-Waals forces; therefore, chemisorbed material is considered bound “stronger” to a surface compared to physisorption. Frequently, a certain activation energy is needed for chemisorption, whereas the activation energy for physisorption is very low. All adsorption processes are temperature dependent with chemisorption typically being somewhat slower than physisorption processes.

For describing the amount of adsorbed material onto a surface the so-called surface coverage $\theta$ is used, as described in Equation 3.

$$\theta = \frac{\text{number of occupied adsorption places}}{\text{total amount of adsorption places}} = \frac{B}{B_{\text{max}}}$$  (3)

$\theta$ - surface coverage
$B$ - adsorbed amount
$B_{\text{max}}$ - maximum adsorbed amount

$\theta$ is used to express the fraction of occupied places ranging from no adsorbed material at the surface ($\theta = 0$) to full monolayer coverage of the surface ($\theta = 1$) and to multilayer adsorption ($\theta > 1$).

### 2.2.2 Adsorption isotherms

After exposing an adsorptive to an adsorbent a dynamic equilibrium is established. Monitoring the coverage of the surface in relation to the
concentration at a fixed temperature is described by so-called adsorption isotherms. According to the shape of the function describing the isotherms, these are classified into four distinct groups. Hence, this classification introduced by Giles et al. is only based on the shape and not on the chemical or physical processes leading to a certain behavior.\textsuperscript{[14,145,146]} In Figure 5 the shapes of the different classifications are illustrated. The first adsorption isotherms reported were describing the adsorption of gases onto surfaces, and the associated fundamental isotherms are the Langmuir, Freundlich, and Brunauer-Emmett-Teller (BET) isotherms.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{isotherms.png}
\caption{The four main isotherm shapes and their classification into C-, L-, H- and S-shaped isotherms after Giles et al.\textsuperscript{[145,146]} (adopted from Alberti et al. with permission from Elsevier\textsuperscript{[14]})}
\end{figure}
2.2.2.1 The Langmuir isotherm

The Langmuir isotherm was reported by I. Langmuir in 1918, and explained the modeled behavior of gas adsorption on glass, mica and platinum. Based on the assumption that all adsorption places are equal and provide the same binding feasibility, the Langmuir isotherm models the adsorption until every place at the surface is occupied considering that no further binding (i.e., multilayer binding) is possible ($\theta = 1$). The Langmuir equation is given as Equation 4.

$$B = \frac{B_{\text{max}} \cdot K \cdot c}{1 + K \cdot c} \quad (4)$$

- $B$ - adsorbed amount
- $B_{\text{max}}$ - maximum adsorbed amount (monolayer)
- $K$ - Langmuir constant
- $c$ - equilibrium concentration

An exemplary Langmuir plot is shown in Figure 6 for arbitrary generated data points with a Langmuir curve fit using Equation 4. The Langmuir isotherm was initially established for gas-solid adsorption, yet is nowadays widely used for all kinds of binding phenomena including adsorption from liquids to solids.
2.2.2.2 The Freundlich isotherm

The Freundlich isotherm was reported by H. M. F. Freundlich in 1906, and is the earliest known sorption model. This empirical model describes non-ideal sorption and also multilayer sorption following Equation 5. An exemplary Freundlich plot is shown Figure 7 for arbitrary generated data points with a Freundlich curve fit using Equation 5.

\[ B = K \cdot c^{1/n} \]  

\( B \) - adsorbed amount  
\( K \) - Freundlich constant  
\( c \) - equilibrium concentration  
\( n \) - empirical parameter

Figure 6. Exemplary Langmuir fit to arbitrary generated binding data.
2.2.2.3 The BET isotherm

The BET isotherm was reported in 1938 by S. Brunauer, P. H. Emmett, and E. Teller, and describes the multilayer adsorption of gases.\cite{149} The BET isotherm essentially derives from the Langmuir isotherm, and includes the fact that the first adsorbed layer could also be used for further adsorption processes, which is ignored by the Langmuir theory. Hence, even at very high pressures there will be no saturation of the material obtained. The BET equation is shown by Equation 6, and an exemplary plot is given in Figure 8 for arbitrary generated data points with a BET curve fit using Equation 6.

\[
\nu = \frac{v_m \cdot c \cdot p}{(p_0 - p) \cdot (1 + (c - 1)(p/p_0))}
\]  

(6)

- \(v\) - adsorbed gas quantity
- \(v_m\) - monolayer adsorbed gas quantity
- \(c\) - BET constant
- \(p\) - equilibrium pressure
- \(p_0\) - saturation pressure
Figure 8. Exemplary BET fit to artificial generated binding data.

The BET isotherm has a substantial impact on the determination of surface areas, as it provides the basis of the so-called BET method, a technique where a surface is completely adsorbed and desorbed using nitrogen gas. By analyzing the adsorbed amount of nitrogen, the surface area can be determined.

2.2.3 Adsorption at molecularly imprinted polymers

The adsorption of analyte molecules at a MIP or any other solid from solution is far more complex than gas adsorption, and in particular adsorption of inert gases such as N₂. However, the binding behavior of MIPs is frequently analyzed using already known isotherms for modeling the adsorption behavior. Besides the classic isotherms mentioned in the previous chapters, a wide variety of additional isotherm theories have been used, however, not one single isotherm has been determined thus far exhaustively and accurately describing the binding behavior of all MIPs. The typical isotherms most commonly used in literature are still the Langmuir and Freundlich isotherms or combinations of the two.[150-153] There are many variations and combinations of the Langmuir isotherm known, as listed by P.
G. Grant et al. in a comparison in 1998 for the modeling of S-shaped and multi-site adsorption plots.\[154\] For MIPs, especially the Langmuir-Freundlich (LF) equation was made popular via publications by the group of K. D. Shimizu.\[15,155,156\] The Langmuir-Freundlich equation is also called the Sips equation - named after R. Sips - or the exponential Langmuir model, as referred to in the summary of P. G. Grant.\[154,157\] At low concentrations, this model follows the Freundlich equation, and at high concentrations the Langmuir equation; therefore, the Langmuir-Freundlich is also based on the assumption that a monomolecular layer is formed.\[14\] The general equation is shown by Equation 7, and the equation K. D. Shimizu introduced for the analysis of MIPs by Equation 8.

\[
B = \frac{B_{\text{max}}(K \cdot c)^{1/n}}{1 + (K \cdot c)^{1/n}}
\]

\(B\) - adsorbed amount

\(B_{\text{max}}\) - maximum adsorbed amount

\(K\) - constant

\(c\) - equilibrium concentration

\(n\) - constant
\[ B = \frac{N_t \cdot a \cdot F^m}{1 + a \cdot F^m} \]  

(8)

with

\[ K_0 = a^{\frac{1}{m}}. \]  

(9)

B - bound amount

N_t - total number of binding sites

a - variable

F - free equilibrium concentration

m - heterogeneity index \((m = 1\) homogeneous material; \(m < 1\) heterogeneous material)

K_0 - median binding affinity

In summary, a general recommendation for an ideal isotherm sufficiently describing the binding behavior of MIPs has not been established to date. Hence, for every particular polymer the commonly used isotherms – or combinations thereof – have to be tested for their utility.

2.2.4 Kinetic aspects related to adsorption at molecularly imprinted polymers

Using MIPs in time-critical or time-dependent applications such as biomimetic sensors or dialysis renders the kinetic evaluation of the rebinding behavior pivotal for ensuring further progress in the field. Consequently, the time necessary for establishing equilibrium between adsorptive and adsorbent has to be determined, thereby describing the dynamic/kinetic binding behavior in addition to equilibrium binding. Typically, adsorption kinetics are analyzed by considering the adsorption process as a chemical reaction sequence, and the step with the highest "resistance" is determined as the time depending step largely determining
the overall behavior. The four “reaction” steps for adsorption from solution to a bulk material were described by G. Alberti et al., and are defined as follows:[14]

1. Transport within the incubation solvent.
2. Diffusion through the solvent film adjacent to the particle surface (external mass transfer).
3. Diffusion within the particle to the adsorption site (intra-particle diffusion).
4. Adsorption at the binding site by chemisorption or physisorption via functional groups.

In comparison, the first two processes occur very fast at typical incubation conditions, i.e., stirring or shaking the polymer incubated within an analyte solution. Hence, the last two steps largely determine the binding kinetics. This circumstance also explains the increased interest in polymerization methods creating (nano)thin MIP layers (i.e., surface imprinting) for reducing the rather slow intra-particle diffusion, and the use of non-covalent imprinting methods taking advantage of physisorption processes, which are generally considered faster than the formation of a chemical bond in covalent imprinting.

Common methods for analyzing the temporal adsorption behavior are methods based on the reaction order. Especially, pseudo-first (Equation 10) and pseudo-second (Equation 11) order equations are used in literature for the description of adsorption processes.[158-160]

\[ B_t = B \times (1 - e^{-k_1 \times t}) \]  
(10)

\[ B_t = \frac{B^2 \times k_2 \times t}{(1 + B \times k_2 \times t)} \]  
(11)

- \( B_t \) - adsorbed amount at time \( t \)
- \( B \) - adsorbed amount at equilibrium
A theoretical analysis of these kinetic equations and their theoretical derivation was performed by S. Azizian, and has been published in detail.\cite{161} 

In the pertinent MIP literature, these equations have predominantly been used for the kinetic characterization of polymers\cite{162-164}, however, the kinetic analysis of MIPs is not a routine procedure to date and therefore frequently not exhaustively evaluated. The pseudo-first and pseudo-second order description of the dynamic MIP binding behavior was also used in chapter 4.3.4 of this thesis as the basis for further advancing knowledge on the kinetic binding behavior of MIPs.

### 2.3 Template molecules used within this thesis

Given that this thesis focuses on advancing fundamental understanding on the binding behavior of MIPs, template molecules that have already successfully been imprinted and characterized were studied rather than novel target molecules.

#### 2.3.1 Iohexol

Iohexol is a x-ray contrast agent with the registered trade name Omnipaque from Nycomed (Norway); its chemical structure is shown in Figure 9.\cite{165} Fundamental imprinting of this polar template molecule was investigated during the PhD thesis of F. Meier within our research team at the Institute of Analytical and Bioanalytical Chemistry, University of Ulm, Germany.\cite{166} Iohexol was used as an exemplary template molecule representing modern
iodinated x-ray contrast media for the synthesis of imprinted polymers in chapter 3.

Figure 9. Chemical structure of iohexol.

2.3.2 Propranolol

Propranolol, a beta-blocker developed by J. Black in the 1960’s, represents the evolution of the drug pronethalol, and became one of the most successful beta-blockers applied in medicine. Propranolol and its medical effectiveness is still under research such as its use against hemangiomas of infancy. Propranolol is a widely used template molecule in molecular imprinting, and is frequently applied for developing new applications or imprinting systems as an exemplary template molecule. Therefore, propranolol (structure shown in Figure 10) was used for the synthesis of MIPs in chapter 4 & 5.
Figure 10. Chemical structure of propranolol.
3. ULEX - A novel extraction strategy for efficient MIP clean-up

The measurements and findings presented in this chapter are in part adapted from the article “A novel extraction device for efficient clean-up of molecularly imprinted polymers” by S. Eppler, M. Stegmaier, F. Meier and B. Mizaikoff published in Analytical Methods in 2012 with permission from The Royal Society of Chemistry.[83] The ULEX design and the extracted polymers were in part also described in the thesis of M. Stegmaier published in 2011 at the Institute of Analytical and Bioanalytical Chemistry, University of Ulm.[171]

3.1 Introduction

The extraction of template molecules and other impurities from MIPs after polymerization is a crucial step for the evaluation of MIPs, and their utility in analytical applications, as already highlighted in chapter 2.1.4. As the typical clean-up procedures have several shortcomings, a new extraction method was developed within this thesis to improve the effective clean-up of MIPs and other polymers or binding materials.

An ideal extraction method should allow the application of all kinds of extraction conditions and a wide variation of solvents and solvent mixtures, yet ensuring a minimum of solvent and polymer waste. Additional opportunities for online monitoring of the extraction process are also desired for optimizing and particularly minimizing the clean-up time.

Based on these requirements for an ideal extraction method, the most beneficial properties of conventional extraction methods were combined into a novel clean-up strategy discussed in detail within this chapter. The developed extraction device - ULEX = Ulm Extractor - was compared against a classical batch extraction procedure using daily solvent exchange, and against the most commonly applied soxhlet extraction.
3.2 Experimental

3.2.1 MIP synthesis

An iohexol MIP was synthesized via radical polymerization in a synthesis approach using 40 mg azobisisbutyronitrile (AIBN), 164 mg iohexol as template molecule, 172 mg methacrylic acide (MAA) as functional monomer, 3171 mg ethyenglycoldimethacrylate (EGDMA) as cross-linker, and 7 mL ethyenglycolmethylether (EGME) as porogen. After 17 h at 60°C, the obtained MIP block co-polymers were crushed and grinded in a mill to obtain polymer particles, and was used without further sieving, as sieving – especially wet sieving – could negatively influence the extraction analysis.

3.2.2 ULEX design

For a rapid and efficient MIP extraction under a wide range of extraction conditions at minimal reagent consumption, an extraction device was constructed. The schematic experimental setup is illustrated in Figure 11a, and a variation without the use of a stirrer in Figure 11b (i.e., shaking setup). The extraction device was manufactured from glass – although any other material can be used in future modifications - and is shown in Figure 11c. The ULEX mounted within the shaking setup is shown in Figure 11d. A comparable device was developed by Wulff et al., and was used as a cleavage apparatus, however, not for the extraction of polymers.[43]
Figure 11. **a)** Experimental setup for the extraction of polymers with the developed ULEX device. Suspensions were generated with **a)** a stirring plate, and **b)** with a shaking plate. **c)** Extraction device made from glass. **d)** Experimental setup of the glass extraction device with a shaking plate and a flexible-tube pump. Reproduced from Ref. [83] with permission from The Royal Society of Chemistry.
3.2.3 Extraction procedure

The polymer particles are filled into the extraction device together with a stirring bar and the device is placed onto a magnetic stirrer. For ensuring continuous extraction, the device is connected to a flexible-tube-pump by combining the glass thread at the end of the riser with the pump tubing. Detailed information about the connector can be found in appendix 10.3.1 (Appendix 1). At the top of the extractor a storage vessel is placed in an airtight fashion containing a single solvent or solvent mixtures that have been determined suitable for optimal extraction. After opening the storage vessel valve, the solvent level inside the extraction device can be adjusted by opening the venting valve. If the venting valve is closed, the solvent level inside the extracting device remains constant regardless how much solvent is extracted via the flexible-tube-pump, as the inner pressure inside the device is constant, and every amount of solvent extracted is instantaneously and continuously replaced from the storage vessel. The extraction process of the polymer is initiated by starting the magnetic stirrer to generate a homogeneous polymer slurry, and by activating the pump. The continuously added fresh solvent extracts the polymer like in a classical soxhlet extraction procedure. The extraction progress can be analyzed via continuous monitoring of the analyte concentration within the solvent exiting the ULEX or by collecting samples from the extracted solvent.

3.2.4 Extraction conditions

3.2.4.1 Extraction device

2 g of polymer were loaded into the device and stirred in an initial solvent volume of 30 mL. The MIP was extracted at room temperature with a solvent mixture of 50% acetonitrile and 50% H₂O (v/v), which was adapted from
previous studies by F. Meier. The stirring rate was set to 250 rpm, and the flow rate was set to 1.1 mL/min. The glass frit used in the extraction device had a pore size of 10-16 µm (P4, European standard). Approximately 1 mL samples were collected and analyzed using a HPLC system equipped with a Phenomenex Luna cyano column (20 µL, 245 nm, 2mL/min 75% acetonitrile / 25% H₂O (v/v) as mobile phase). The corresponding calibration plots can be found in appendix 10.3.1 (Appendix 2).

3.2.4.2 Batch extraction procedure with daily solvent exchange

2 g of the polymer were suspended in a glass beaker containing 40 mL of extraction solvent (50% acetonitrile and 50% H₂O (v/v)) and stirred at 250 rpm. The beaker was sealed, stirred over night, centrifuged and the supernatant solution was removed prior to the addition of 40 mL of fresh solvent mixture. The procedure was repeated several times, and the analyte concentration in the supernatant was likewise determined using the procedure described above.

3.2.4.3 Soxhlet extraction

2 g of polymer were filled into soxhlet extraction tubes and extracted in a soxhlet extractor for 4 h with a solvent mixture of 50% acetonitrile and 50% H₂O (v/v) at 130°C. After the extraction, the polymer was dried and 100 mg were resuspended in 1 mL fresh solvent mixture and shaken for 1.5 h in an Eppendorf tube. Afterwards, the sample was centrifuged and the analyte concentration in the supernatant was analyzed using the procedure described above.
3.3 Results and discussion

The conventional MIP extraction via daily solvent changes is illustrated in Figure 12a, and shows that a minimum of 3 days is required for the exhaustive extraction of the iohexol template from the MIPs. After stirring the particles for another 72 h, no measureable iohexol concentration was observed and indicates that the extraction was complete. However, the handling steps using this procedure are tedious. The vial containing the polymer slurry has to be centrifuged, and the supernatant has to be removed, which may lead to polymer loss. This polymer loss rises with diminishingly fine particles, and also increases the centrifugation time. Another disadvantage of long extraction times is the mechanical stress applied to the polymer particles, which could further ground the particles or negatively affect their functionality e.g., for surface imprinted polymers.

The results illustrated in Figure 12b show the extraction of the same amount of polymer performed with the ULEX extraction device. The obvious main advantage is the substantially reduced extraction time, as within less than 3 h the iohexol concentration in the extracted solvent dropped to almost zero indicating a complete clean-up of the MIP. With the applied flow rate, a total volume of 230 mL of the solvent mixture was used, in contrast to only 160 mL for the conventional MIP extraction. Hence, a reduction of solvent waste was not achieved yet, however, by evaluating improved extraction conditions (e.g., adjusting temperature or the flow rate) the solvent waste could be further reduced.
Figure 12. **a)** Conventional solvent exchange - iohexol extraction with a daily solvent change. After extracting for 3 days, the MIPs were extracted 3 days without solvent change, in addition. **b)** Iohexol MIP extraction with the developed ULEX extracting device. Reproduced from Ref. [83] with permission from The Royal Society of Chemistry.

Soxhlet extraction of polymers is also frequently used for the extraction of MIPs, as the experimental effort is minimal. Hence, for the extraction of iohexol-imprinted polymers this method was compared to the developed ULEX extraction strategy. After a soxhlet time of 4 h the polymer was dried, resuspended and the supernatant solution showed a substantial amount of leaching iohexol, as illustrated in Figure 13.
Figure 13. MIP supernatant solution chromatogram extracted with soxhlet extraction. A HPLC system equipped with a Phenomenex Luna cyan column (2 mL/min; 75 % acetonitrile / 25 % H₂O (v/v) as mobile phase) was used analyzing 20 µL of sample with an UV-detector at 245 nm. The retention time of 0.984 min and a peak area of 30.8 mAU*min yields an iohexol concentration of 81 µg/mL, and indicated insufficient extraction of iohexol from the MIP matrix. Reproduced from Ref. [83] with permission from The Royal Society of Chemistry.

The key feature of the developed ULEX extraction method is its universal applicability to optimize the extraction of MIPs. From solvent mixtures to aggressive solvents all kinds of extraction conditions may be applied under stirring, shaking and temperature control. Furthermore, it is possible to place the device into an ultrasonic bath to achieve e.g., ultrasonic supported extraction conditions. By monitoring the exiting analyte concentration, it is
finally possible to accurately determine the end of the extraction. Given that these measurements provide time-resolved information, information on the release kinetics of the extracted polymers may in addition be obtained.

3.4 Conclusions and outlook

The efficient extraction of MIPs is a crucial step during their synthesis and has vital impact on their performance in analytical and biomedical applications. As the typical extraction procedures suffer from individual shortcomings, the developed ULEX-based extraction procedure could be used as an alternative facilitating a wide range of controlled extraction conditions paired with exceptionally easy experimental handling. Especially the use of almost any kind of solvent or solvent mixture is a key benefit of the developed procedure. The extraction performance was demonstrated for the clean-up of iohexol-imprinted polymers. Based on the obtained results it should be possible to also regenerate MIPs with this method for extended and repetitive usage as binding material. Especially if MIPs based upon expensive template molecules are used, the recovery of the template molecule could be useful and should be taken into consideration.

The demonstrated ULEX prototype made from glass is a comparatively cheap device, however, based on the desired extraction concept and conditions also other materials can be used for tailoring the ULEX method to almost any extraction problem. Next to adapting the device to other extraction conditions, up- or downscaling of the device for accommodating either large or minute quantities of polymers, as well as adaptation to any kind of particle size by selection of the corresponding pore dimensions for the retention frit is facilitated. Another improvement of the setup could be the implementation of an in-line detection device for further investigation of the kinetics of the extraction process not only for MIPs but also for other
extraction problems. Last but not least, it should be noted that this device not only works for MIPs but also could be used for any kind of particulate binding and/or recognition material.
4. Advanced equilibrium and kinetic evaluation of molecularly imprinted polymers

Typically, MIPs are evaluated via binding experiments using e.g., frontal chromatography or batch experiments modeled by conventional binding isotherms, as shown in chapter 2.1.5. As both methods offer some disadvantages a novel evaluation method was devised within this thesis combining both approaches.

4.1 Introduction

In classical frontal chromatography evaluation, a MIP column using the imprinted polymer as stationary phase is flushed with different analyte concentrations, thereby leading to a multi-step chromatogram, as schematically shown in Figure 14a for 4 different concentrations.
Figure 14. **a)** Theoretical chromatogram obtained via frontal chromatography using 4 different analyte concentrations. **b)** Frontal chromatogram over the same concentration range but using 11 different analyte concentrations. **c)** Frontal chromatogram of a sample providing a continuous concentration change covering the same concentration range as in **a)** and **b)**. **d)** Typical bound-vs-free isotherm.

To improve the significance of an evaluation, an isotherm should always be calculated using as many experimental data points as possible. Therefore, the frontal evaluation shown in Figure 14a has to be performed with a variety of different concentrations, thereby substantially increasing also the experimental efforts. Assuming that a large number of different concentrations are added to the experiment but the highest concentration will remain the same, the chromatogram in Figure 14a will change to a chromatogram with many small concentration steps (as shown in Figure
If the number of steps is even further increased, the chromatogram will approach the shape of a continuous concentration increase. This is illustrated by the line in Figure 14c where every point on that line represents an infinitesimal small concentration step, which leads to an isotherm created with a massively increased number of data points (Figure 14d). In consequence, an ideal frontal analysis experiment would be based on a continuous concentration change of the analyte rather than only few discrete concentrations. This could be theoretically achieved with a mixing device prior to the chromatographic column to successively increase the concentration of analyte. However, such a concentration change would also be present inside the chromatographic column, thereby resulting in a concentration slope within the analyzed polymers in flow direction inside the column, thus massively complicating the evaluation.

A closer look at a classic batch incubation experiment reveals that in a vial filled with a known amount of polymer surrounded by an analyte solution, the concentration of the supernatant should always be the same at equilibrium conditions regardless at which location within the vial the supernatant concentration is analyzed. Hence, if the analyte concentration could be measured in real time directly in the batch incubation vial, it would be possible to design an experiment where the concentration is instantaneously changed, and the binding process by the polymer is directly analyzed. Consequently, a setup was designed to achieve this type of detailed analysis by combining the benefits of batch and frontal analysis.

**4.1.1 Preliminary results**

Based on the ideas outlined in chapter 4.1, the developed ULEX extraction device (chapter 3) was modified for facilitating the analysis of polymers, and to provide a first impression whether the ideas conceptualized herein are
indeed viable. For that purpose, the storage vessel was filled with an analyte solution - here, sodium benzoate - and for an adsorbing material TENAX (poly(2,6-diphenyl-p-phenylene oxide); an air adsorbing material) was selected for this proof-of-concept. The polymer was filled into the ULEX extractor device – like the MIP particles - with a solvent added to achieve a homogeneous slurry at continuous stirring, as illustrated in Figure 15.

![Figure 15. Modified ULEX extraction system (as described in chapter 3). Instead of an extraction solvent, an analyte stock solution was provided in the storage vessel, and the increase of analyte concentration was analyzed using a diode-array UV detector. Adapted from Ref. [83] with permission from The Royal Society of Chemistry.](image-url)
As illustrated in chapter 3, the solvent amount within the ULEX device remains constant while pumping. Consequently, the analyte concentration inside the extracting device increases by pumping fresh analyte stock solution from the storage vessel into the polymer/pure solvent slurry. The concentration increase was continuously analyzed using an in-line diode-array UV detector during the experiment. Afterwards, the entire experiment was repeated with the same amount of solvent inside the ULEX device, however, without polymer (sorbent) present. From these first experiments, a distinct difference was evident between a polymer(TENAX)/pure solvent slurry, and the same experiment with only pure solvent inside the ULEX device in absence of the sorbent particles, which could therefore only be explained by adsorption of analyte molecules onto the TENAX particles. This preliminary result is illustrated in Figure 16. Based on these preliminary findings, a novel flow-cell setup was developed to investigate the observed adsorbing phenomena in more detail. This was necessary as the initial glass ULEX device had significant dead volumes (e.g., in the riser), and also the flexible-tube-pump at the distal outlet of the system did not provide sufficient control on the flow rate at a precision level needed for reproducible experimental conditions. Details on the flow cell setup along with the associated theoretical considerations and analysis results are discussed in the following chapters.
Figure 16. Difference between a blank measurement (i.e., pure solvent in the ULEX device) vs. a TENAX slurry present with the ULEX. 11 mL solvent in the device (82% H2O, 18% MeOH, pH 2 (HCl)) and 2g of TENAX polymer were used. Sodium benzoate as analyte; 81 µg/mL determined at 225 nm with a Gynkotek HPLC diode-array UV detector. Applied flow rate: 2.4 mL/min via a peristaltic pump.

4.2 Experimental

4.2.1 Flow cell design and analysis setup

For the present studies, a custom flow cell for dynamic sorbent analysis was designed and built from stainless steel, as illustrated in Figure 17. Commercial internal reducer fittings from VALCO (IZR41F; 1/4” to 1/16”) were used as inlet and outlet enabling usage of conventional HPLC tubing for connection to the pump and detector; 2 µm frits were inserted for retaining particulate polymer or sorbent materials within the cell during analysis. The cell is sealed with a removable lid and provides a total volume of 19.1 cm³ after subtracting the volume of the triangular stirring bar (6x12 mm) used
during experiments. The lid facilitates the addition of washers for volume variation (not shown in detail within Figure 17), which was not used for the measurements performed herein.

![Diagram of flow cell components](image)

*Figure 17. Construction scheme of the flow cell developed for dynamic binding studies at particulate sorbents and a stainless steel prototype.*

The entire measurement setup shown in Figure 18 comprises solvent reservoir, HPLC pump, Rheodyne HPLC-switch, magnetic stirrer, HPLC diode-array (DAD) UV detector, and waste reservoir. The UV detector was connected via an analog digital converter to a computer for data acquisition (software: Lab Chart 6 – sampling rate 4/s). For measurements, the solvent reservoir was filled with propranolol solutions, which are pumped into the measurement cell. The pump may be switched directly to the waste reservoir for flushing to avoid dilution effects inside the pump.
Figure 18. Flow-chart of the entire analysis setup including the measurement cell.

4.2.2 Calibration of the flow cell setup

For calibration, the measurement cell was removed from the setup and the tubing from the switching unit was directly connected to the UV detector. 0, 1, 5, 10, 50, 100, 200, and 250 µM propranolol calibration solutions in 70% acetonitrile : 30% methanol were prepared, and the setup was flushed with the 0 µM solution and analyzed at 290 nm until a stable baseline was recorded. The data recording was then reset, the flow rate was set to 0.5 mL/min, and the measurement was started with the 0 µM solution. After 2 min, the pump was switched to the waste and flushed for 1 min with the 1 µM solution. After flushing, the previous flow rate was again established and switched back to the UV detector analyzing the solution for 3 min before the channel was again switched to flush with the 0 µM solution. This procedure was uniformly repeated for all calibration solutions.

4.2.3 Synthesis of propranolol-imprinted polymer particles

Propranolol (PRO) MIPs were synthesized using a bulk preparation scheme. Propranolol hydrochloride was converted to the free base by titrating an aqueous solution with concentrated sodium hydroxide solution and collecting the precipitate and after drying Propranolol free base was
dissolved in acetonitrile (ACN) and Toluene (TOL). Methacrylic acid (MAA) or acrylamide (AAm) as functional monomer, different ratios of divinylbenzene (DVB) and ethylene glycol dimethacrylate (EGDMA) were used as cross-linkers and 1,1'-azobis(cyclohexanecarbonitrile) (ABCN) as radical initiator were added. The entire composition and labeling of all prepared MIPs is summarized in Table 3. The mixture was degassed with nitrogen for 4 min and polymerized for 18 h at 95°C. After polymerization, the monolith was crushed and sieved, and the fraction of 25 - 63 µm was separated for analysis. The template was removed by excessively washing the polymer with 90% methanol and 10% acetic acid, and finally with pure methanol in tubes under gentle rocking. The washed particles were dried in a vacuum oven at 40°C prior to usage. The non-imprinted (NIP) particles were prepared in the same way without adding propranolol free base to the reaction mixture.
### Table 3. MIP and NIP compositions.

<table>
<thead>
<tr>
<th></th>
<th>Prop</th>
<th>MAA</th>
<th>AAm</th>
<th>DVB</th>
<th>EGDMA</th>
<th>ABCN</th>
<th>ACN</th>
<th>TOL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIP 0</strong></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>418 mg</td>
<td>546 µL</td>
<td>-</td>
<td>4406 µL</td>
<td>30.93 mmol</td>
<td>67.3 mg</td>
<td>0.275 mmol</td>
<td>5.2 mL</td>
</tr>
<tr>
<td><strong>NIP 0</strong></td>
<td></td>
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<tr>
<td></td>
<td>-</td>
<td>546 µL</td>
<td>-</td>
<td>4406 µL</td>
<td>30.93 mmol</td>
<td>67.3 mg</td>
<td>0.275 mmol</td>
<td>5.2 mL</td>
</tr>
<tr>
<td><strong>MIP 10</strong></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>418 mg</td>
<td>546 µL</td>
<td>-</td>
<td>3964 µL</td>
<td>27.83 mmol</td>
<td>583 µL</td>
<td>3.09 mmol</td>
<td>67.3 mg</td>
</tr>
<tr>
<td><strong>NIP 10</strong></td>
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<tr>
<td></td>
<td>-</td>
<td>546 µL</td>
<td>-</td>
<td>3964 µL</td>
<td>27.83 mmol</td>
<td>583 µL</td>
<td>3.09 mmol</td>
<td>67.3 mg</td>
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<tr>
<td><strong>MIP 20</strong></td>
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<tr>
<td></td>
<td>418 mg</td>
<td>546 µL</td>
<td>-</td>
<td>3525 µL</td>
<td>24.75 mmol</td>
<td>1166 µL</td>
<td>6.19 mmol</td>
<td>67.3 mg</td>
</tr>
<tr>
<td><strong>NIP 20</strong></td>
<td></td>
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<td></td>
<td>-</td>
<td>546 µL</td>
<td>-</td>
<td>3525 µL</td>
<td>24.75 mmol</td>
<td>1166 µL</td>
<td>6.19 mmol</td>
<td>67.3 mg</td>
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<tr>
<td><strong>MIP 40</strong></td>
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<tr>
<td></td>
<td>418 mg</td>
<td>546 µL</td>
<td>-</td>
<td>2644 µL</td>
<td>18.56 mmol</td>
<td>2334 µL</td>
<td>12.37 mmol</td>
<td>67.3 mg</td>
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<tr>
<td><strong>NIP 40</strong></td>
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<td></td>
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<tr>
<td></td>
<td>-</td>
<td>546 µL</td>
<td>-</td>
<td>2644 µL</td>
<td>18.56 mmol</td>
<td>2334 µL</td>
<td>12.37 mmol</td>
<td>67.3 mg</td>
</tr>
<tr>
<td><strong>MIP AA</strong></td>
<td>190 mg</td>
<td>-</td>
<td>205 mg</td>
<td>2004 µL</td>
<td>14.07 mmol</td>
<td>-</td>
<td>30.6 mg</td>
<td>0.125 mmol</td>
</tr>
<tr>
<td><strong>NIP AA</strong></td>
<td>-</td>
<td>-</td>
<td>205 mg</td>
<td>2004 µL</td>
<td>14.07 mmol</td>
<td>-</td>
<td>30.6 mg</td>
<td>0.125 mmol</td>
</tr>
</tbody>
</table>

#### 4.2.4 Polymer hydrolysis

400 mg fractions of all synthesized methacrylic acid polymers were hydrolyzed by adding 20 mL of a 3.66 M KOH solution (MeOH / H₂O; 2:1 (v/v)) and heating to 60°C over night in sealed vials. Afterwards, the particles were exhaustively washed with 50% methanol 50% water followed by 80% methanol 20% acetic acid, and finally, by pure methanol.
4.2.5 Static batch experiments

Polymer (MIP0 or NIP0, 30 mg) was suspended in 15 mL of a 70% acetonitrile 30% methanol solution and stirred at high speed. Aliquots of the polymer suspension (500 µL) were pipetted into Eppendorf tubes, and made up to 1 ml to the relevant concentration of propranolol solution (0, 2.5, 5, 10, 25, 50, 75, 100 µM), also prepared in 70% acetonitrile 30% methanol solution. For each concentration two polymer samples were incubated. The tubes were sealed, located onto a shaker, and incubated over night until they were centrifuged, the supernatant filtered with a syringe filter, and the samples analyzed with a HPLC system at 290 nm using a C18 column (RP18, 5 µm, 25 cm x 4.6 cm) and the ChromQuest software for data evaluation. For measurement, all samples were diluted 1:1 with water to optimize interactions with the column. As mobile phase 70% acetonitrile and 30% of a 10 mM sodium dodecylsulfate – 10 mM disodium hydrogen orthophosphate buffer adjusted to pH 2.3 with concentrated phosphoric acid was used. A calibration curve was created using propranolol standard solutions (50, 20, 10, 5, 2.5, 0 µM), and is shown in appendix 10.3.2 (Appendix 3).

4.2.6 Kinetic batch experiments

For kinetic batch evaluations, 50 mg MIP0 polymer was suspended in 25 mL of a 70% acetonitrile 30% methanol solution and stirred at high speed. Aliquots of the polymer suspension (500 µL) were pipetted into a 10 mL syringe tube after removing the plunger and after the syringe tube was plugged into a PTFE syringe filter to suppress leakage of the solution. 500 µL of a 100 µM propranolol solution (70% acetonitrile 30% methanol) were added, the syringe was gently shaken, and then covered with a plastic paraffin film. After a defined time (1, 2, 3, 5, 10, 30 min), the plastic paraffin film was removed and the plunger put back into the syringe tube to filter the
solution into Eppendorf tubes. For every measured time point, 3 samples were prepared and all samples were analyzed using the HPLC method and sample preparation described in chapter 4.2.5.

A second kinetic test was performed using the flow cell as a stirring reservoir without the lid. The inlet and outlet was sealed with screws and 21.6 mg MIP0 were suspended in 21.6 mL of a 100 μM propranolol solution (70% acetonitrile 30% methanol) and stirred at 700 rpm with a triangular stirring bar. Samples were collected after a defined time (1, 5, 10, 15, 20, 30 min), filtered, and then analyzed using the HPLC method and sample preparation described in chapter 4.2.5.

4.2.7 Flow-cell data handling.

The analyzed measurement curves were used without further manipulation. However, for fitting theoretical curves or isotherms to the data, only values with a minimum signal of 5 mV (maximum signal for 250 μM – approx. 1400 mV) were taken to determine the fitting constants. Therefore the first seconds of the measurements were not taken for fitting to reduce the influence of noise.

4.3 Results and discussion

4.3.1 Calibration

An exemplary calibration graph is illustrated in Figure 19a, and shows the signals of each individually applied calibration solution. For each concentration, a rectangular peak is resulting comprising 4 segments. The first segment is a steep rise of the signal once the pump is switched to the detector. The second segment is a plateau once only the calibration solution
is pumped through the detector. By switching back the pump to the waste for flushing, the detector remains filled with calibration solution without flow. This defines the third segment. Finally, after switching back again to the 0 µM solution the fourth segment characterizes the decline to the baseline. For calibrating the system, only the second segment of the peak (plateau) providing a consistent signal under flow was used during data analysis. With this data, a calibration function was created, which is shown in Figure 19b. The obtained data shows a nearly perfect straight line indicating an excellent goodness-of-the-fit ($R^2$) for the regression function. Therefore, all measurements were calibrated using the described procedure. Each data point has been determined by taking the data from the second peak segment, as described above, resulting in several hundred values (sampling rate 4/s); the associated error bars have been calculated by taking the drift of the second peak segment during the measurement but are too small to be evident in Figure 19b.

Figure 19. **a)** Exemplary calibration studies. The periods between injections were varied such that a stable baseline was achieved for minimizing errors. The 1 µM solution was not used for establishing the calibration function shown in **b**), as the signal was only twice as strong as the baseline drift between start and end of the calibration.
4.3.2 Equilibrium and kinetic evaluation of MIPs

The approach developed within this thesis relies on the incessant measurement of a continuously varied analyte concentration, i.e., a concentration gradient is applied to the polymer, using a specifically designed flow-through cell containing the polymer particles of interest (Figure 17). The developed measurement setup could be considered a variation of frontal chromatography by replacing the analytical column with a flow cell.

The inlet of the flow cell is connected to a HPLC pump, while the outlet directly feeds into an in-line UV detector. The flow cell itself has a defined and precisely known volume, and the MIP particles inside are continuously stirred throughout the experiment to prevent sedimentation and to generate a homogeneous polymer slurry. If no sorbent is present and the cell is completely filled with pure solvent, the introduction of analyte solution increases the concentration of analyte within the cell following Equation 12 (derivation of Equation 12 can be found in Appendix 10.4).

\[
c(t) = c_0 \star \left(1 - \exp \left(-\frac{F \star t}{V_{\text{cell}}} \right) \right)
\]  
(12)

\[
c(t) = c_0 \star \left(1 - \exp \left(-\frac{F \star t \star \theta}{V_{\text{cell}}} \right) \right)
\]  
(13)

\[
\theta = -\left(\frac{V_{\text{cell}}}{t \star F} \right) \star \ln \left(1 - \left(\frac{c(t)}{c_0} \right) \right)
\]  
(14)

\[
\theta = \frac{K \star c}{1 + K \star c}
\]  
(4)

- \text{c(t)} \quad \text{– concentration at time t}
- \text{c}_0 \quad \text{– stock concentration}
- \text{F} \quad \text{– flow rate}
- \text{t} \quad \text{– time}
\( V_{\text{cell}} \) – solvent volume inside cell
\( \theta \) – fractional surface coverage
\( c \) – concentration
\( K \) – Langmuir constant

Equation 12 therefore describes a blank measurement generating a blank curve that is used for theoretical calculations. If a polymer - in our case a propranolol MIP or NIP - is added to the flow cell, the obtained signal deviates from the blank curve, as analyte molecules adsorb to the polymer, as already fundamentally shown within the preliminary TENAX experiments in chapter 4.1.1. An exemplary MIP curve along with the corresponding theoretical blank curve is shown in Figure 20a. The fundamental processes occurring in this scenario are schematically illustrated in Figure 21a/b.
Figure 20. **a**) Experimentally obtained binding behavior of a propranolol MIP and calculated blank curve using Equation 12; flow rate 0.3 mL/min, propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), stirring rate 700 rpm, measured at 290 nm. **b**) $\theta$ vs. free isotherm using Equation 14 to calculate $\theta$ values from the data points of the experimental curve shown in Figure 20a. **c**) Experimental binding curve and fitted simulated MIP curve; integration of the area between the curves leads to a $B_{\text{max}}$ of 47.7 nmol/mg. **d**) Bound-vs-free isotherm using the calculated $B_{\text{max}}$ value multiplied with the $\theta$ values shown in Figure 20b for MIP and NIP (NIP not shown in Figure 20a-c).
Figure 21. **a)** Fundamental processes occurring inside the flow-through cell without adsorbing media, and **b)** with adsorbing media (e.g., MIP particles).

While the analytical signal remains to be the increase in analyte concentration within the flow cell, due to the introduction of analyte solution this process is now delayed due to binding of analyte molecules to the MIP/NIP – or to phrase in terms of frontal chromatography, the signal is retained. For a theoretical understanding of this adsorption behaviour, Equation 12 needs to be modified taking into account this binding process. Initially, the binding process is dominant, as all binding sites at the sorbent are freely available. Following adsorption theory describing a fixed number of binding sites as a model for the behaviour of MIPs, the relation between occupied and initially available binding sites is expressed as the fractional
coverage of the surface $\theta$, as explained in chapter 2.2.1. As $\theta$ increases, the rate of adsorption decreases until the rates for adsorption and desorption are equal, $(d\theta/dt = 0)$ i.e., equilibrium has been reached. By adding $\theta$ into the exponential part of Equation 12 yields Equation 13, and the increase in concentration observed within the flow cell is influenced by the binding process until adsorption and desorption are equal and Equation 13 becomes again Equation 12. By rearranging Equation 13 for $\theta$ as shown in Equation 14 and using the experimental data in Figure 20a, the value of $\theta$ is calculated. As the measured concentration directly reflects the free concentration, an isotherm is generated and illustrated in Figure 20b. In order to establish conventionally used bound-vs-free isotherms facilitating performance estimates, the binding capacity ($B_{\text{max}}$) of the MIP has to be determined. The area between a complete blank and MIP curve represents the amount of bound analyte such that the $B_{\text{max}}$ value may be directly determined by integration, as derived from frontal chromatography data analysis theory.\textsuperscript{[172]}

For integration from the beginning to a theoretical point where both curves merge, an appropriate function has to be fitted to the experimental data using Equation 13 and the Langmuir Equation 4, as shown in Figure 20c to simulate the entire curve. By variation of the Langmuir K parameter using least square regression via the Microsoft Excel Solver Tool, the fitting is optimized. The Langmuir isotherm was selected as a simple model of the adsorption process for the first demonstration and evaluation of the flow cell method. In chapter 4.3.3.1, it is shown that alternative, i.e., more suitable isotherms can be used for fitting the obtained data. Knowing $B_{\text{max}}$, bound values can be calculated by multiplication with the $\theta$ value, thus producing a typical bound-vs-free isotherm, as shown in Figure 20d.

During typical MIP rebinding studies, the polymers are incubated from several up to 24 h to achieve maximum binding at equilibrium conditions.\textsuperscript{[53,173,174]} Hence, considering the general perception that the binding kinetics of bulk MIPs are slow, a strong flow rate dependence of the
flow cell measurement principle was anticipated, but also that resulting bound-vs-free concentrations of the ligand would approach flow-rate independence, if equilibrium conditions are rapidly attained. Therefore, the measurement could be used as a method for the more detailed analysis of the rebinding kinetics of MIPs. Resulting, binding isotherms established prior to equilibrium should be notably different from those obtained at equilibrium conditions.

The experimental data shown in Figure 20 was produced at a flow rate of 0.3 mL/min within a total measurement time of approximately 2 h. By varying the flow rate, the concentration change within the cell and the incubation time will also vary. Therefore, different isotherms were anticipated for different flow conditions. Consequently, the obtained data shown in Figure 22a were indeed surprising, revealing that the isotherms obtained at four distinctly different flow rates were very similar, thus leading to the unexpected conclusion that in fact the rebinding behaviour of MIPs may be significantly faster than initially expected. Hence, to experimentally achieve non-equilibrium conditions, and as a proof of the postulated behaviour, the rate of concentration change inside the flow cell had to be dramatically increased using higher concentrated stock solutions. Thereby, the applied concentration gradient is significantly boosted in order to distinctly shift the isotherms apart from equilibrium behaviour by challenging the kinetics to keep up.

Using stock solutions at significantly elevated concentrations, it was possible to study MIPs at non-equilibrium conditions, as shown in Figure 22b. Only under conditions of rapid concentration changes the obtained isotherms could be differentiated from isotherms at equilibrium conditions, which in turn indeed confirms the predicted behaviour.

These observations are of utmost importance above and beyond the MIP community – but certainly especially important for using MIPs as plastic antibodies, as such synthetic receptors appear to perform significantly better than equilibrium studies to date may have suggested, when taking particular
advantage of their dynamic binding properties. Furthermore, the developed method for analyzing concentration changes within a flow cell along with appropriately designed binding isotherm models facilitates the characterization of essentially any kind of synthetic receptor or sorbent material with respect to their kinetic behaviour.

Figure 22. a) $\theta$ vs. free isotherm for a propranolol bulk MIP analyzed at four different flow rates; propranolol stock solution 250 $\mu$M in acetonitrile/methanol 70/30 % (v/v), stirring rate 700 rpm, detection at 290 nm. b) $\theta$ vs. free isotherm for three different propranolol stock solutions in acetonitrile/methanol 70/30 % (v/v), flow rate 0.5 mL/min, stirring rate 700 rpm, detection at 290 nm. The equilibrium isotherm represents the 0.8 mL/min isotherm also shown in Figure 22a.

Methacrylic acid (MAA) is the monomer of choice when imprinting propranolol. To demonstrate the displacement of the isotherms in the presence of the MIP was due to a specific recognition/binding process, an imprinted polymer with acrylamide (AAm) as functional monomer (p(AAm-co-DVB)) was synthesized to create a negative test system for the measurement method, as a reduced binding performance with this MIP compared to the p(MAA-co-DVB) MIP was anticipated. From Figure 23a it is
evident that virtually no displacement of the MIP curve from the blank curve was observed with isotherms similar for MIP and NIP performance at a significantly decreased $B_{\text{max}}$ value of 1.12 nmol/mg polymer, as shown in Figure 23b. These results clearly indicate that the results obtained for the MAA-MIP system were indeed a consequence of specific binding processes, thereby further substantiating the utility of the developed method for differentiating the binding performance of different sorbents.

*Figure 23. a) Experimental binding curve of a propranolol MIP imprinted with acrylamide instead of methacrylic acid along with the calculated blank curve; propranolol stock solution 250 $\mu$M in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm. b) Bound vs. free isotherm for the acrylamide MIP and NIP (NIP data not shown in Figure 23a).*
4.3.3 Comparing the flow cell with batch measurements – alternative isotherms, individual kinetic experiments, reproducibility and troubleshooting

4.3.3.1 Fitting of alternative isotherms

In chapter 2.2.3, the Langmuir-Freundlich isotherm was described as the most frequently used isotherm for describing the binding behavior of MIPs. Yet, also other isotherms may be utilized for modeling the binding behavior. Hence, the first evaluation of the flow cell measurement principle was performed using the Langmuir equation. To analyze the MIP binding experiments in more detail also other isotherms were used and introduced into Equation 13, after transposing them for the surface coverage \( \theta \) for enabling fitting. For evaluation of suitable isotherms, the measured curve was simulated by fitting the respective model parameters to the experimental data via least square regression, as shown in Figure 24a for the Langmuir equation (Equation 4). Also, a deviation profile of the fit was created by plotting the squared residual of the different isotherms, as illustrated in Figure 24b. For an illustration of different isotherms and their fit to the experimental data the Langmuir-Freundlich isotherm (Equation 7), the Jovanovic isotherm (Equation 15), and the Tóth isotherm (Equation 16) were selected with the resulting curves shown in appendix 10.3.2 (Appendix 4, Appendix 5, Appendix 6) and the relevant parameters summarized in Table 4.
**Figure 24.** a) Langmuir simulation fitted to the experimental data; propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm. b) Squared residuals of the fitting (Figure 24a) plotted against time.

<table>
<thead>
<tr>
<th>Isotherm</th>
<th>Equation</th>
<th>EqNr.</th>
<th>$B_{\text{max}}^{\text{nmol/mg}}$</th>
<th>Pearson coefficient</th>
<th>Sum $\Delta^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Langmuir</td>
<td>$B = \frac{B_{\text{max}} \cdot K \cdot c}{1 + K \cdot c}$</td>
<td>4</td>
<td>46.90</td>
<td>0.99992</td>
<td>14261.3</td>
</tr>
<tr>
<td>Langmuir-Freundlich</td>
<td>$B = \frac{B_{\text{max}} \cdot (K \cdot c)^{1/n}}{1 + (K \cdot c)^{1/n}}$</td>
<td>7</td>
<td>48.52</td>
<td>0.99995</td>
<td>3912.4</td>
</tr>
<tr>
<td>Jovanovic$^{[14]}$</td>
<td>$B = B_{\text{max}} \cdot (1 - \exp(-K \cdot c))$</td>
<td>15</td>
<td>35.10</td>
<td>0.99897</td>
<td>623006.3</td>
</tr>
<tr>
<td>Tóth$^{[14]}$</td>
<td>$B = \frac{B_{\text{max}} \cdot K \cdot c}{[1 + (K \cdot c)^{n}]^{1/n}}$</td>
<td>16</td>
<td>48.56</td>
<td>0.99994</td>
<td>4221.1</td>
</tr>
</tbody>
</table>

**Table 4.** Fitting parameters of the analyzed isotherms – binding capacity $B_{\text{max}}$, Pearson correlation coefficient and squared residual $\Delta^2$ (fitting to experimental data; propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm).

In principle, any isotherm model could be used that allows the calculation of the surface coverage $\theta$. By comparing the selected isotherms, the Langmuir-Freundlich isotherm proved to be the isotherm of choice for the analyzed propranolol MIPs followed by the Tóth and the Langmuir isotherm. The Jovanovic isotherm revealed only poor matching with the experimental
data. The isotherm plots are summarized in Figure 25 along with selected binding points obtained by classical batch binding experiments. The graphs show that Langmuir, Langmuir-Freundlich, and Tóth isotherms are almost coincident, while the Jovanovic isotherm yields a significantly different isotherm, as suggested by the fitting parameters in Table 4.

A possible explanation for the deviation between the flow cell isotherms and the batch experimental points could be found in the experimental design, as the batch experiment was carried out with 1 mg polymer per 1 mL of solvent, and the flow cell experiments had a higher polymer-to-volume ratio by a factor of 1.1. Also, only 7 data points of the static batch studies are compared to over 10,000 data points for the flow cell measurements. However, it is also possible that multi-layer binding occurs introduced by the concentration change using the flow cell method, which could explain the increased binding. The influence of the polymer-to-volume ratio is investigated in more detail in chapter 5, and solutions for a comparison of different experimental designs are suggested.

![Figure 25. Bound-vs-free isotherm illustrating all analyzed equations and some data points obtained from batch experiments.](image)
4.3.3.2 Kinetic batch experiments

In order to compare and confirm the kinetic behavior of MIPs analyzed with the flow cell setup two - more batch-like - kinetic tests were performed. For slow adsorption kinetics, an increase of adsorbed propranolol per time was expected. However, the results of both experiments show almost no time dependence and maximum binding after one minute, as illustrated in Figure 26. These experiments support the previously proposed exceptionally rapid binding kinetics of the propranolol MIPs, as observed using the flow cell method, which underlines the utility of the system. The observed error range for these kinetic experiments are mainly associated with the manual handling procedures, as 3 samples per data point have to be consecutively prepared.

![Figure 26. Kinetic batch studies performed within a) syringes and b) in the flow cell as a container.](image)

4.3.3.3 Reproducibility and limitations of the method/system

The experiments listed in Table 5 were performed two times and the reproducibility was analyzed using Pearson’s correlation coefficient. In
addition, the squared residual of the two measurements was calculated and plotted against the measurement time for the 0.3 mL/min (250 µM) and the 0.5 mL/min (2500 µM) experiments, respectively, as illustrated in Figure 27. For further studies on the reproducibility, the Pearson correlation coefficient was calculated for all hydrolyzed measurements (chapter 4.3.4), as listed in appendix 10.3.2 (Appendix 7).

<table>
<thead>
<tr>
<th>Flow rate [mL/min]</th>
<th>Stock concentration [µM]</th>
<th>Pearson [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>250</td>
<td>0.99999</td>
</tr>
<tr>
<td>0.5</td>
<td>500</td>
<td>0.99997</td>
</tr>
<tr>
<td>0.5</td>
<td>1000</td>
<td>0.99993</td>
</tr>
<tr>
<td>0.5</td>
<td>2500</td>
<td>0.99584</td>
</tr>
</tbody>
</table>

Table 5. Pearson correlation coefficients for two measurements analyzed at the same conditions.

Figure 27. Squared residual plots illustrating the differences between two measurements analyzed at the same conditions for **a)** 0.3 mL/min; 250 µM, and **b)** 0.5 mL/min; 2500 µM. (Applied factor 10 Fast Fourier Transformation (FFT) smooth to both graphs to reduce noise).
As the Pearson correlation coefficient shows only a slight difference, the squared residuals between two measurements indicate the limitations of the systems for experiments characterized by a rapid concentration change. The squared residuals plotted in Figure 27a illustrate an almost perfect fit, in particular since the two compared experiments were analyzed with a time gap of 59 days. However, for rapid experiments the difference between two measurements is larger, as illustrated in Figure 27b. Hence, to increase the accuracy and to calculate quantitative values for rapid concentration change measurements more experiments have to be performed or the cell has to be redesigned to minimize errors caused by the setup. Especially, increasing flow rates lead to increasing backpressure in the presented setup (e.g. 1.8 mL/min – 260 bar). As the entire experimental design and analysis is based on the assumption that the flow is applied nearly instantaneously, high backpressures could delay the set flow rate at the outlet side, and thus, influence the measurement. If in addition high concentrations are investigated, those influences may become more critical for quantitative analysis. This limitation is illustrated in Figure 28 for a very fast experiment with a very fast concentration change (0.5 mL/min; 5000 µM) where the delay covers almost the entire measurable range, thereby preventing correct analysis of the measurement via integration. On the other hand, experiments with measurement times covering several hours could be negatively influenced by e.g., temperature changes or detector variations comparable to any conventional chromatographic experiment. Implementing temperature control and UV detector lamps with a constant output could optimize these factors; however, during the experiments presented in this thesis such influences were not observed.
4.3.4 Kinetic evaluations of hydrolyzed MIPs

MIP performance is mainly influenced by the interactions between functional monomers and the template molecule. However, also the used cross-linker has a key role, as it influences parameters like stability or pore-volume of the synthesized polymer. Altering this backbone of the polymer could therefore change the performance of the characterized propranolol MIP used in chapter 4.3.2. Hence, the divinylbenzene (DVB) backbone was altered by adding ethyleneglycoldimethacrylate (EGDMA) to the polymerization mixture, which could be hydrolyzed for obtaining changes in pore structure, and for improved accessibility of the binding sites. The polymers, which are described in the experimental section of chapter 4.2.3 were analyzed for their binding behavior at equilibrium and kinetic conditions. For the kinetic analysis, all polymers (MIP0, NIP0; MIP10, NIP10; MIP20, NIP20; MIP40, NIP40; and all their hydrolyzed counterparts) were measured at the same conditions using a 1000 µM propranolol stock solution.
at a flow rate of 0.5 mL/min, and the data analysis was done by applying the pseudo first order and the pseudo second order kinetic theory. Fitting theoretical curves to the measured data as described in chapter 4.3.2 could lead to errors in the calculation of the actual bound amount based on the part that has to be simulated for integration and calculation of the binding capacity at kinetic conditions. Therefore, the kinetic evaluation was performed by calculating $\theta$ values from the experimental data using Equation 14, and then fitting a pseudo first order, and a pseudo second order curve for calculating the velocity constants. Hence, the pseudo first (Equation 10) and pseudo second order (Equation 11) equations had to be transposed for $\theta$ values instead of actual bound values to allow fitting.

Based on Lagergren's rate equation, the pseudo first order equation was therefore modified not using the actual bound amount $B_t$ and the binding capacity $B$, but the surface coverage $\theta$ and the equilibrium surface coverage $\theta_E$. This yields Equation 17.

$$\frac{d\theta}{dt} = k_1(\theta_E - \theta) \quad (17)$$

After integration with the boundary conditions $t = 0$ to $t = t$ and $\theta = 0$ to $\theta = \Theta$, the exponential solution already shown in chapter 2.2.4 is obtained (Equation 18).

$$\theta = \theta_E * (1 - e^{-k_1*\theta}) \quad (18)$$

The same was done for the second order rate equation.

$$\frac{d\theta}{dt} = k_2(\theta_E - \theta)^2 \quad (19)$$
After integration using the same boundary conditions \((t = 0 \text{ to } t = t\) and \(\theta = 0 \text{ to } \theta = \theta)\), again the solution already shown in chapter 2.2.4 is obtained (Equation 20).

\[
\theta = \frac{\theta_E^2 * k_2 * t}{1 + \theta_E^2 * k_2 * t}
\]  

(20)

- \(\theta\) : surface coverage
- \(\theta_E\) : equilibrium surface coverage
- \(k_1\) : first order rate constant
- \(k_2\) : second order rate constant
- \(t\) : time

All polymers were analyzed at the same kinetic conditions two times, and the equilibrium data for the determination of the binding capacity was analyzed via batch analysis. The results are listed in Table 6, and an exemplary fit of the pseudo first and second order equations to the experimental data and the equilibrium fits is given in appendix 10.3.2 (Appendix 8).

<table>
<thead>
<tr>
<th>Sample</th>
<th>(\theta_E) [1]</th>
<th>(k_1) [min(^{-1})]</th>
<th>Pearson 1</th>
<th>(\theta_E) [1]</th>
<th>(k_2) [min(^{-1})]</th>
<th>Pearson 1</th>
<th>(B_{\text{max}}) [nmol*mg(^{-1})]</th>
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</thead>
<tbody>
<tr>
<td>MIP 0</td>
<td>0.7949</td>
<td>0.02305</td>
<td>0.9523</td>
<td>0.8520</td>
<td>0.04557</td>
<td>0.9415</td>
<td>63.95 ± 19.18</td>
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<tr>
<td></td>
<td>0.8202</td>
<td>0.02509</td>
<td>0.9432</td>
<td>0.8708</td>
<td>0.05142</td>
<td>0.9312</td>
<td></td>
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<tr>
<td>NIP 0</td>
<td>0.8617</td>
<td>0.02305</td>
<td>0.9644</td>
<td>0.9183</td>
<td>0.04535</td>
<td>0.9441</td>
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</tr>
<tr>
<td></td>
<td>0.8405</td>
<td>0.02065</td>
<td>0.9727</td>
<td>0.9065</td>
<td>0.03837</td>
<td>0.9588</td>
<td></td>
</tr>
<tr>
<td>MIP 0H</td>
<td>0.8276</td>
<td>0.00975</td>
<td>0.9896</td>
<td>0.9618</td>
<td>0.01279</td>
<td>0.9930</td>
<td>118.1 ± 87.69</td>
</tr>
<tr>
<td></td>
<td>0.7981</td>
<td>0.00774</td>
<td>0.9955</td>
<td>0.9522</td>
<td>0.00942</td>
<td>0.9908</td>
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<tr>
<td>NIP 0H</td>
<td>0.8985</td>
<td>0.02026</td>
<td>0.9700</td>
<td>0.9646</td>
<td>0.03589</td>
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<tr>
<td></td>
<td>0.8838</td>
<td>0.01542</td>
<td>0.9820</td>
<td>0.9794</td>
<td>0.02326</td>
<td>0.9580</td>
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</tbody>
</table>
Table 6. Results of all analyzed MIPs at kinetic conditions (0.5 mL/min; 1000 µM stock solution) analyzed with pseudo first and pseudo second order kinetics and equilibrium binding data for calculating the binding capacity.

<table>
<thead>
<tr>
<th>MIP 10</th>
<th>MIP 10H</th>
<th>NIP 10</th>
<th>NIP 10H</th>
<th>MIP 20</th>
<th>MIP 20H</th>
<th>NIP 20</th>
<th>NIP 20H</th>
<th>MIP 40</th>
<th>MIP 40H</th>
<th>NIP 40</th>
<th>NIP 40H</th>
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<tbody>
<tr>
<td>0.7742</td>
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<td>0.8573</td>
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<td>0.7761</td>
<td>0.7485</td>
<td>0.8437</td>
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</tr>
<tr>
<td>0.8085</td>
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<tr>
<td>0.9623</td>
<td>0.9835</td>
<td>0.9745</td>
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<td>0.9519</td>
<td>0.9648</td>
<td>0.9599</td>
<td>0.9817</td>
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From a theoretical perspective, the measured results fit to the pseudo first order kinetic, as well as to the second order kinetic according to the Pearson coefficients. Also, the arithmetic mean values of the Pearson coefficients for first and second order kinetics are both the same (1. - 0.9686; 2. - 0.9688). Hence, a clear discrimination between the two theoretical kinetic approaches is not possible, however, the 0 % EGDMA polymers appear to fit
better to the pseudo first order kinetic, and the 40 % EGDMA polymers to the pseudo second order kinetic.

The evaluation of the obtained kinetic values indicates only a few trends regardless of which kinetic order. For the 0 % EGDMA polymer, the kinetic constants of MIP and NIP are equal, yet, after hydrolysis the kinetic constant of the NIP slightly and for the MIP significantly dropped. As KOH in the Methanol/Water mixture should only hydrolyze EGDMA as shown in Figure 29, the pure DVB MIP and NIP should not be affected. These lower kinetic constants are consistent for all analyzed materials after hydrolysis regardless of their percentage content of EGDMA. An explanation for the lower kinetic constants for MIP 0H and NIP 0H could be an additional washing effect including swelling of the polymers by different chemical conditions, which may release non-extracted and embedded template molecules in the MIP. Also, a mechanical alteration of the polymer by stirring and heating over night could lead to changes of the binding properties.

The hydrolysis of the polymer leads to two main processes gaining more binding sites by additional carboxylic acid groups, as illustrated in Figure 29, and to provide additional access to embedded specific binding sites, which were unable to bind analyte molecules prior to hydrolysis. The equilibrium data for the non-hydrolyzed and the hydrolyzed MIPs indicate that indeed a higher binding capacity was achieved. Especially MIP 10H and MIP 20H show higher binding capacities compared to their non-hydrolyzed counterparts.

The increase in binding capacity of MIP 0H compared to MIP 0 is mainly based on the discrepancy in the last binding data point, and also leads to the high standard deviation (see also appendix 10.3.2(Appendix 9)). Therefore, the observed increase in binding capacity may be misleading. The highest binding was observed for MIP 40H, which appears plausible, as the high EGDMA amount leads to significantly more carboxylic groups after hydrolysis compared to the other polymers. Hence, the binding capacity value could not be compared to the non-hydrolyzed MIP 40, as no binding plateau could be analyzed with the experimental data.
An explanation for the general trend of reduced kinetic constants for the hydrolyzed polymers could be the formation of multiple layer binding or weak unspecific binding occurred by the increased number of unspecific binding places. Anticipating that the formation of additional layers or additional unspecific binding takes longer than binding to specific binding places could cause lower binding constants. This additional slow binding corresponds also with the increased binding capacity of the hydrolyzed polymers.

\[\text{Figure 29. EGDMA hydrolysis protocol.}\]

In order to investigate the structural changes occurring due to the hydrolysis, the polymers were analyzed using BET studies (appendix 10.3.2 (Appendix 10)). The results indicate that the surface area remains the same for all polymers beside MIP 40H and NIP 40H, which was anticipated as at this composition the structural backbone of the polymer is significantly weakened by hydrolysis potentially leading to collapse of the polymer structure. However, for all other polymers the results may be compared excluding structural changes.

In conclusion, the kinetic analysis of the hydrolyzed MIPs and NIPs yields a reduced kinetic performance compared to non-hydrolyzed polymers. However, a content of 10% or 20% EGDMA significantly increased the binding capacity, which can be seen as a benefit of the hydrolysis procedure.
4.4 Conclusions and outlook

In summary, an analytical method was developed and was exemplarily applied for the characterization of propranolol-imprinted polymers. The theoretical basis along with suitable isotherm models was analyzed in detail, and the system was evaluated for the application of studying binding materials at equilibrium and at kinetic conditions. The results for the propranolol MIP clearly indicate that the kinetic binding properties of this bulk-polymerized MIP are highly underrated, as surprisingly rapid binding kinetics have been observed. The detailed kinetic behavior of 4 different MIP/NIP compositions for propranolol, and their partially hydrolyzed backbone structure was also investigated using the method developed herein. The results for those polymers did not yield a clear trend to date in their kinetic binding behavior independent of the kinetic model – pseudo first or second order – applied.

Clearly, a wide range of different polymers and binding materials could be analyzed with the developed system, yet for a more detailed understanding on the kinetic behavior of MIPs more experiments have to be performed enabling variation of more parameters e.g., concentration changes, etc.. Especially, the structural changes after hydrolysis should be studied in more detail, as the BET analysis is only a bulk method that does not reveal in depth information on the detailed pore structure, which in turn is crucial for understanding adsorption phenomena in MIPs and NIPs.

An entirely novel approach is currently in development within our research group for visualizing the actual pore nanostructure of MIPs and NIPs in three dimensions using a focused-ion-beam (FIB) slice-and-view technique, which could help to visualize the structure of the synthesized polymers.

As the applicability of the concepts developed herein were corroborated using the flow cell prototype devised in the course of this study, alternative designs of the flow cell should be taken into consideration for future work. Miniaturization of the setup would for example facilitate analyzing smaller
amounts of particles. For the analysis of nanoparticles, nanoporous frits or membranes have to be used or developed for retaining the particles yet facilitating acceptable flow rates at acceptable backpressures. In addition, positioning of the inlet and outlet may become critical to prevent polymer nanoparticles clogging the frits. Also multi constituent detection could be applied to the system by using a DAD-UV detector or infrared (IR) detection methods, for studying the polymers binding selectivity under equilibrium and kinetic parameters.

In conclusion, the developed methodology provides a powerful approach for rapidly and reproducibly generating equilibrium and kinetic data for characterizing the binding performance of a wide range of sorbent materials including synthetic polymers such as MIPs, as well as biological receptors such as antibodies.
5. High-throughput generation of MIP isotherms and their mass-independent evaluation

5.1 Introduction

In chapter 4, the detailed analysis of MIPs with a newly developed flow cell method was demonstrated for the generation of detailed binding information based on a very large number of data points. However, for some considerations such a complete evaluation is not necessary, and methods for rapid isotherm evaluation based on fewer data points would be sufficient. As explained in chapter 2, the evaluation of binding materials based on isotherms is the method of choice for MIPs, and typically performed via chromatographic or batch analysis. As the packing of columns with MIPs and NIPs may be problematic (e.g., irregularly shaped polymer particles, high back pressures, etc.), the number of polymers that can be analyzed in a short period time is somewhat limited, as for each polymer a separate column has to be packed. Hence, for a rapid analysis of different polymer particles bulk evaluation methods are favored. Therefore, in order to improve the data acquisition rate for MIPs and NIPs, a high-throughput batch method based on a 96 well plate format has been developed. This format is readily established in bioanalysis, and offers rapid analysis of 96 samples at moderate costs.

In this thesis, three methods – an Eppendorf tube incubation, a filter plate incubation, and an incubation based on the commercial available ResiQuot system – were established, evaluated and compared using the propranolol MIP/NIP polymer system already discussed in chapter 4. Theoretical considerations on the analyzed data complete this fundamental study.
5.2 Experimental

5.2.1 MIP synthesis

The evaluated MIP/NIP system was derived from chapter 4.2.3 (labeled as MIP40 & NIP40), and was used throughout this study. A propranolol MIP was synthesized using 418 mg (1.61 mmol) propranolol free base as template molecule (Propranolol hydrochloride was converted to the free base by titrating an aqueous solution with concentrated sodium hydroxide solution and collecting the precipitate), 547 µL (6.44 mmol) methacrylic acid as functional monomer, 2644 µL (18.56 mmol) divinylbenzene and 2334 µL (12.37 mmol) ethyleneglycoldimethacrylate as cross linkers and 67.3 mg (0.28 mmol) 1,1’-azobis-(cyclohexanecarbonitrile) as radical initiator. A non-imprinted polymer (NIP) was synthesized in the absence of propranolol. All components were dissolved in 5.2 mL acetonitrile and 1.3 mL toluene in a glass freeze-drying vial, sparged with nitrogen for 5 minutes and heated in an oil bath at 95°C for 18h. The solid bulk polymer was crushed in a mortar and the particles sieved into a 25 - 63 µm fraction. The polymer particles were washed extensively with 90% methanol / 10% acetic acid (v/v) and pure methanol.

5.2.2 Rebinding studies

5.2.2.1 Eppendorf tube incubation

Polymer (MIP or NIP, 100 mg) was suspended in 10 mL of a 70% acetonitrile 30% methanol solution and stirred at high speed. Aliquots of the polymer suspension (500 µL) were pipetted into Eppendorf tubes and made up to 1 ml providing the required concentration of the propranolol solution (0, 25,
50, 125, 300, 400, 500 µM) also prepared in 70% acetonitrile 30% methanol solution. For every concentration, two polymer samples were incubated. The tubes were sealed, located onto a shaker and incubated over night until they were centrifuged, the supernatant filtered with a syringe filter, and samples analyzed in a 96 UV well plate with an UV plate reader at 290 nm.

5.2.2.2 Filter plate incubation

Polymer (100 mg) was suspended in 10 mL of a 70% acetonitrile 30% methanol solution and stirred at high speed. Aliquots of the polymer suspension (500 µL) were pipetted into a 2 mL PTFE filter plate (0.2 µm), and filled with 500 µL of the propranolol incubation concentrations (0, 25, 50, 125, 250, 300, 400, 500 µM) also prepared in 70% acetonitrile 30% methanol solution. The plate was sealed and placed onto a shaker for 8 h, and afterwards mounted into a MultiScreen Vacuum Manifold + deep well collar to transfer the solutions into a 2 mL deep well plate. Then, the samples were transferred into a 96 well UVstar plate and analyzed with an UV plate reader at 290 nm, as described above.

5.2.2.3 ResiQuot incubation

MIP polymer was suspended in a 80% water 20% ethanol solution (for preparation not for incubation) and mixed with a shaker to establish a homogeneous polymer slurry, which was pipetted into the upper holes of the ResiQuot, as shown in Figure 30. By applying vacuum to the bottom part of the ResiQuot for 5 min, the particles were sucked into the removable 96-hole slider. Retained by the frit, the holes were completely filled with particles and the slider could be removed and placed above a 2 mL PTFE filter plate. The still wet particles stick to the holes and are plucked out into
the plate for further incubation studies. By weighing the empty plate and the filled plate after drying the particles, the amount of polymer is determined. Afterwards, 1 mL of propranolol incubation solution (0, 25, 50, 125, 250, 300, 400, 500 µM) prepared in 70% acetonitrile 30% methanol solution, was added and the plate sealed. After 7 h of shaking, the plate was placed into a MultiScreen Vacuum Manifold + deep well collar to transfer the solutions into a 2mL deep well plate before the samples were transferred into a 96 well UVstar plate and analyzed at 290 nm, as described above.

Figure 30. ResiQuot system with the main body (a), the removable 96-hole slider (b), and the stamps to remove the polymer from the holes (c). The frit underneath the slider is shown in the inset as a schematic cross section.
5.2.2.4 Control batch incubation (HPLC)

Polymer (30 mg) was suspended in 15 mL of a 70% acetonitrile 30% methanol solution and stirred at high speed. Aliquots of the polymer suspension (500 µL) were pipetted into Eppendorf tubes and filled with 500 µL of the propranolol incubation concentrations (0, 5, 10, 25, 50, 75, 100 µM). For each concentration, two polymer samples were incubated. The tubes were sealed, mounted onto a shaker and incubated over night until they were centrifuged, the supernatant filtered with a syringe filter and the samples analyzed with a HPLC system at 290 nm using a C18 column (Gracesmart RP18, 5µm, 25 cm x 4.6 cm) and the ChromQuest software for data evaluation. For measurement, all samples were diluted 1:1 with water to optimize interactions with the column. As mobile phase 70% acetonitrile and 30% of a 10 mM sodium dodecylsulfate– 10 mM disodium hydrogen orthophosphate buffer adjusted to pH 2.3 with concentrated phosphoric acid was used at a flow rate of 1 mL/min.

5.3 Results and discussion

5.3.1 High-throughput studies

The methods developed in the present thesis offer rapid, time saving, and readily applicable strategies for generating data on MIP binding isotherms in a high-throughput fashion, which is essential for screening a wide variety of binding materials e.g., for applications in biotechnology. All experiments were designed fitting the format of 96 well plates, and may readily be analyzed using a conventional UV plate reader. Using UV well plates significantly accelerates the sample throughput compared to other methods such as HPLC measurements, as up to 96 samples could be simultaneously
analyzed thereby saving analysis time and consumables (i.e., elution solvents).

Figure 31. Results of **a) Eppendorf tube incubation, b) Filter plate incubation,** and **c) ResiQuot incubation.** All data was obtained using a conventional UV well plate reader at 290 nm. Isotherms fitted using Prism 5 software and the implemented analyzing tool with the “one site – specific binding” (equates Langmuir isotherm) model.

The results in Figure 31 clearly indicate the possibility of creating isotherms using the presented methods. However, the obtained results are not as comparable as initially expected, as they do not yield similar isotherm for the same material.

From an experimental point of view, the Eppendorf tube incubation is the easiest one to handle, however, the required number of pipetting steps could contribute to errors. The incubation method using the filter plates offers the same principle procedure as the Eppendorf tube, but allows transferring the incubation step completely into the 96 well plate format. The crucial step for this method is the accurate alignment of the frit plate and the deep well plate inside the vacuum manifold avoiding mixing of different solutions. Using the ResiQuot system reduces the error created within the other two methods by pipetting polymer slurry. With the ResiQuot system, a constant polymer volume is deployed into the incubation frit plate, while thereafter the remaining incubation procedure is similar to the
previously discussed methods. The major drawback of the ResiQuot approach is the usage of fixed volumes of polymer, instead of a fixed polymer mass. Therefore, an additional gravimetric step is necessary to calculate the typical bound-per-mg values for comparison. Also, fill height differences within the 96-hole slider may occur by removing and placing the filled slider onto the well plates. Such fill height errors and gravimetric errors evidently sum up yielding the error bars shown in Figure 31c.

Although, the obtained results evidence an imprinting effect for the analyzed materials, the comparison of the isotherms proves more complex than evident at first sight. Consequently, interpretation and isotherm data presentation has been investigated in more detail within the next chapter.

5.3.2 Comparing isotherms – the mass dependence of binding studies

Figure 32 shows the data points from Figure 31a using 5 mg polymer per mL, and a second experimental data set of the same MIP prepared with 1 mg polymer per mL, incubated with a 5 times lower incubation concentration (experimental details can be found in chapter 5.2.2.4). By plotting the bound-per-mg polymer value on the ordinate, and the measured concentration of the supernatant on the abscissa - as it is typically done in MIP evaluations - two different isotherms are yielded indicating in fact two different materials. At first sight, this is confusing as the same isotherm is ad hoc expected for the same material, regardless if 1 mg or 5 mg are incubated per milliliter. However, the typically reported bound-vs-free isotherms are apparently mass-dependent, and therefore, comparing materials isotherms are only apparently useful, if the experimental procedures including the sorbent material mass are exactly the same. If not, one may obtain different values for e.g., affinity constants, for one and the same material simply by using a different experimental preparation each experiment. Therefore, the results obtained via the ResiQuot approach cannot be directly compared to
the other two methods, as they decidedly analyze a different polymer mass. In order to facilitate a comparison of the data obtained with different methods (and in particular, with different polymer masses), the polymer mass has to be factored into a standardization of all data.

Normalizing the entire binding data set such that the graphs are identical has to be performed by appropriately adapting the abscissa, as the ordinate is already normalized to the amount of incubated polymer.

One potentially useful normalization strategy is shown in Figure 33 for the 5 mg and the 1 mg incubation. The supernatant concentration, which usually serves as the free concentration on the abscissa, was multiplied with the volume-to-mass ratio of the polymer. It is important to note that the supernatant concentration is not only divided by the amount of applied polymer, as this would lead to errors if for example 5 mg polymer were

Figure 32. Experimental bound-vs-free data sets for the same MIP incubating 1 mg/mL (squares) and 5 mg/mL (triangles) (incubated with a 5 times higher concentration compared to the 1 mg/mL experiment).
incubated in 5 mL of solvent. Therefore, the abscissa is now labeled as “free template per mg polymer [nmol/mg].”

![Graph](image)

*Figure 33. Normalization of the data sets shown in Figure 32 by multiplying the free concentration with the volume-to-mass ratio of the polymer.*

Consequently, with this proposed normalization strategy it is possible to compare two differently obtained experimental data sets yielding largely similar isotherm data sets, rather than indicating two different materials, which is the case prior to the normalization. The results now appear clearly in the same range, and the volume to mass ratios or experimental handling errors may readily explain the remaining differences.

The normalization method is particularly crucial when applying the ResiQuot approach, as this device divides up the polymer fraction only by volume, and not by mass. Hence, even if MIP and NIP are compared, both may have a different polymer mass per well ranging here from 4.74 mg (MIP) to 6.07 mg (NIP), and therefore rendering a normalization inherently necessary. In Figure 34, all MIP data sets obtained via the three different methods are normalized, thereby almost entirely collapsing onto the same data range for all experimental preparation methods - despite their differences - within an
acceptable error range. The ResiQuot data remains an exception, as albeit the device is an easy-to-handle tool and beneficial for generating data in a 96 well format, the current drawback of a comparatively large error range remains an issue. Considering the facts herein, the filter plate incubation method appears most suitable for further high-throughput analysis of MIPs at the current stage of development.

![Figure 34](image.png)

*Figure 34. Summary graph of all measured data sets after normalization indicating the comparability of the selected methods except for the large error bars associated with the ResiQuot method emerging as the major drawback of this technique.*

### 5.3.3 Theoretical reconsideration of the assumptions for the applied normalization

The typical binding isotherm evaluation with the free concentration on the abscissa and the bound analyte per mass on the ordinate serves well for experiments that are either performed at exactly the same conditions or if a scale-up of mass and volume is ensured. To fully elucidate the aspect of mass-dependence during the performed binding studies along with the
proposed normalization strategy, detailed theoretical considerations are discussed herein.

There are two plausible explanations for the observed difference in isotherms prepared using a different mass of polymer within the same incubation volume (Figure 32). Either there is a massive change in the binding properties of the polymer or the data evaluation method is not suitable for comparing data obtained at different experimental conditions in the typical bound-vs-free format.

As two different isotherms indicate different materials with different binding properties, it is helpful to initially focus just on a largely simplified binding concept for explaining the problem in more detail using a theoretical batch binding experiment for explaining the considered assumptions behind the normalization.

We consider a theoretical polymer with a maximum binding capacity of 1 analyte molecule per mg polymer, which is incubated at starting conditions of 3 mg polymer present with 3 analyte molecules added within 1 mL of solvent. After equilibrium is reached, it is considered that 2 analyte molecules are bound occupying 2 binding sites. Therefore, 1 mg of polymer remains unoccupied, and 1 analyte molecule is still in solution. This theoretical binding experiment is schematically illustrated in Figure 35a.

If this theoretical experiment is now partially scaled-up (incubation vial volume stays constant) to e.g., 9 mg polymer and 9 analyte molecules added again within 1 mL of solvent, it is assumed that 6 analyte molecules should occupy 6 binding sites, and 3 mg polymer are unoccupied while 3 analyte molecules remain in solution, respectively. This theoretical binding experiment is schematically illustrated in Figure 35b. Both theoretical experiments were constructed by adopting the behavior observed within data presented in Figure 32 (raw data shown in appendix 10.3.3 (Appendix 11) for clarity).
Hence, for the first experiment 2 analyte molecules are bound (NOTE: not per mg), and 1 analyte molecule remains free in solution. For the scaled-up experiment, correspondingly 6 analyte molecules are bound (NOTE: not per mg), and 3 analyte molecules remain free in solution. By plotting those values in the typical bound-vs-free format, the bound analyte amount is normalized to the amount of polymer used in the experiment. Hence, for both experiments a value of 2/3 analyte molecules per mg polymer is obtained; however, the free value is not normalized (i.e., 1 vs. 3 free analyte
molecules), and consequently, two different points are obtained. The whole problem is illustrated in Figure 36 for the artificial created binding experiment described above and for one experimental data point from Figure 32 (Incubation concentration: 1 mg – 25 µM; 5 mg – 125 µM. The raw data is also provided in appendix 10.3.3 (Appendix 11)). For both experiments (artificial and experimental data) the raw data is presented in the bound-vs.-free format and in the proposed normalized format.

Figure 36. Illustration of the raw data presentation for artificial and experimental data using the conventional bound-vs-free format, and in comparison the herein proposed normalized format.

The initially confusing aspect of the typical bound-vs-free format – i.e., different “free” values at approximately the same “bound” value (Figure 36) – could be explained by a substantial change in the binding behavior resulting from the polymer-to-volume ratio. This is the only parameter that has significantly changed during the scale-up within the virtual experiment. However, in a “virtual experiment” this should have no discernable effect.
More likely is the fact though that plotting the data using an abscissa that is not normalized yields erroneous results (see also Fig. 36).

The proposed normalization strategy (i.e., multiplying the measured free concentration with the volume-to-mass ratio) apparently worked well for the data presented in Figure 32 resulting in comparable data points, as shown in the normalized representation of the same data in Figure 33. Therefore, appropriate data plotting appears a plausible explanation compared to a complete change in the binding behavior of the same polymer. However, while a certain influence of the mass-to-volume ratio cannot be excluded and will definitely have an influence on the binding equilibrium within real-world experiments, yet the main difference between the two isotherms presented in Figure 32 has to be generated by the partially scaling-up and representing the data without useful normalization, causing the misleading data plotting for partially up-scaled experiments i.e., in the conventionally applied typical bound-vs.-free format.

5.4 Conclusions and outlook

Isotherms are probably the most appropriate representation of MIP data for illustrating the binding properties, and there is a wide variety of experimental variations reported how data for the calculation of isotherms may be analyzed. The methods developed within this chapter are based on a 96 well plate design, thereby offering the advantage of high-throughput analysis using e.g., a UV well plate reader, and the possibility to analyze many samples at the same time without generating sizeable amounts of solvent waste compared to HPLC methods. By using more automated methods such as the ResiQuot approach or the filter plate incubation, even more time may be saved avoiding additional pipetting steps. However, the error range of the ResiQuot system and the associated procedure at the
current stage remains too high for a useful comparison with the other methods tested herein.

Next to the development of the experimental methods, the theoretical data treatment and representation was investigated revealing that a normalization of the isotherms appears mandatory, if different experimental methods should be compared. In particular, if individual methods require a partial scale-up of the experimental design (i.e., by mass, volume, stock concentration, etc.) it is mandatory to implement appropriate data normalization routines, as otherwise erroneous data interpretation may result indicating e.g., different binding behavior of yet the same materials just by evaluating e.g., a different mass. So the presented normalization of the abscissa by multiplying the measured free concentration with the volume to polymer ratio together with the typical presentation of the bound amount of analyte molecules per mass of polymer on the ordinate may be used generically for experiments performed with discrepancies in the analyzed polymer mass. As the polymer to volume ratio is not negligible and will have an influence on the binding behavior this proposed normalization may also be used to study this influence by monitoring the discrepancies of the data points with increasing polymer mass.
6. Redefining the imprinting factor – a single value for the evaluation of MIP performance based on isotherm integration

6.1 Introduction

The imprinting factor (IF) is the most commonly used figure-of-merit in the MIP literature for describing the performance of an imprint, and have their origin in the evaluation of MIP chromatographic columns, as described in chapter 2.1.5.1. Even though the IF provides a first impression on the performance of a MIP, the randomness of the calculation of the commonly used IF prevents its useful transpose to batch incubation studies, as detailed in the following.

Typically MIP and NIP are incubated with a certain concentration of the target analyte, and the ratio in binding between MIP and NIP is expressed as imprinting factor. This method can be used as a first indication of an imprinting effect during MIP synthesis, however, the comparison of different experimental preparations may yield misleading values for the imprinting factor, as the instantaneous IF is in fact related to an undefined point along the binding isotherm, which could lead to a wide range of imprinting factors for a single material (i.e., MIP). This circumstance was readily demonstrated in a seminal contribution by C. Allender and coworkers in 2011.[13]

However, the general concept of an imprinting factor remains appealing, as it is much more handy comparing two discrete values rather than two isotherms. In order to advance the applicability of the concept of imprinting factors derived from batch experiments, a redefinition termed a percentage generic imprinting factor (GIF(%) is proposed herein and argued in detail throughout the following chapters.
6.2 Definition of the percentage generic imprinting factor (GIF\(^{(\%)}\)) based on isotherm integration

As the calculation of an imprinting factor strongly depends on the selection of a specific point along a binding isotherm, redefining the theory of imprinting factor calculations should be based on utilizing the information within the entire binding isotherm for enhancing the comparability of imprinting factors. Furthermore, the concept of a redefined imprinting factor taking into account the entire information of the binding isotherm would in essence compress the exhaustive description of the binding behavior of a polymer (i.e., here, a MIP) into a single value. With experimentally obtained MIP and NIP isotherms, this may be accomplished by calculating the ratio of the respective binding isotherm areas derived by for obtaining a generic imprinting factor (GIF), as shown in Figure 37. Hence, by dividing areas instead of individual concentrations, the information of the entire binding isotherm is used to calculate a modified imprinting factor considering the entire binding behavior of the MIP.

The critical step of this procedure is to appropriately define the integration boundaries, as those are crucial for the future generic comparison of generic imprinting factors. Especially the upper integration limit requires careful consideration for ensuring comparability of thus obtained generic imprinting factors.

It is easily derived that the lower boundary of integration for binding isotherms should be the point of origin; yet, the upper integration boundary is more complex to define. Defining the upper integration boundary at \( B_{\text{max}} \) is one possibility, and readily applicable for all MIPs following e.g. the Langmuir isotherm. However, this would exclude MIPs showing a more Freundlich-type behavior. As there are all kinds of isotherms reported for MIPs, it appears therefore impossible to define the upper integration boundary using a certain location along an isotherm shape. Consequently, a
A novel concept is proposed defining the upper integration boundary via the experimental synthesis protocol of the MIP. Typically, MIPs are synthesized using a defined amount of template molecules within the prepolymerization solution. Therefore, a maximum of possibly imprinted binding pockets per mg of final polymer could be calculated by dividing the amount of template used by the amount of crosslinking molecules and functional monomers applied (assuming 100% of polymer product).

Evaluation of the polymer after successful clean-up is frequently performed by incubating the polymer at different target analyte concentrations during a batch study. Hence, the isotherm used for integration should be established by using the incubation concentrations covering the region from the point of origin to an upper integration boundary, which is defined by the theoretically calculated maximum number of imprinted binding pockets. Consequently, if a MIP has e.g., a theoretical number of 10 nmol of possibly imprinted binding pockets per mg polymer, and 1 mg of MIP is incubated in 1 mL of solvent, the highest concentration required for integration will be a 10 µM analyte solution, as this concentration provides exactly 10 nmol of analyte molecules within 1 mL of incubation solution. The integration is than executed from the point of origin to the respective free concentration of the 10 µM solution, which will be the upper maximum integration boundary. The same integration limits for the MIP are of course also used for integration of the NIP isotherm. Here, for the same example discussed above, the 10 µM target analyte solution used to incubate 1 mg of NIP in 1 mL of solvent likewise provides the respective free concentration applied as upper maximum integration boundary, as schematically illustrated in Figure 37. After integrating both isotherms, their areas are divided to calculate an imprinting factor termed generic imprinting factor (GIF), now describing the MIP binding behavior and quality of the imprinting process considering the entire selective binding range of the synthesized MIP.
Figure 37. Illustrated integration areas for the calculation of the GIF for MIP and NIP using the origin and the maximum incubation concentration, which is based on the amount of template molecules used during synthesis, as the respective lower and upper integration boundaries.

So far, this new definition works well for MIPs that can be characterized over their whole binding range. However, if a significant amount of template is needed during synthesis to generate binding sites (i.e., template molecules in excess during synthesis), the upper maximum integration boundary may be far too high for calculating a significant generic imprinting factor, as it might be not possible to generate an isotherm covering the whole theoretically needed concentration range. Hence, MIPs with a lower density of imprinted sites in comparison to the amount of template used for the synthesis, and MIPs where only the very first segment of the isotherm is significant for a comparison would be attributed a disadvantageous GIF, if still the entire integration range would be applied.

Taking this into account, the upper integration boundary may therefore be set to a lower point along the binding isotherm. However, it is absolutely essential that in such cases a so-called percentage generic imprinting factor
GIF(%) is reported, i.e., considering that only a fraction of the binding isotherm vs. the earlier defined upper maximum integration boundary is used for the calculation expressed as a percentage value. With this additional information – i.e., describing how far a real MIP evaluation deviates from a theoretical maximum of 100% imprinting binding sites, the *generic imprinting factor* becomes even more universal, and offers advanced information for the comparison of polymers. A summary of the entire procedure is illustrated in Figure 37 for artificially generated binding data, and in chapter 6.3 the calculation is exemplified for experimentally obtained data.

Using this newly defined *percentage generic imprinting factor*, it is anticipated that this figure-of-merit provides an advanced and independent comparison parameter between different polymers, i.e. for the comparison of MIP vs. NIP and for the comparison of different MIPs. It should be noted that the calculation of the *percentage generic imprinting factor* using the method developed in this thesis will only cover the first segment of a binding isotherm, as there are of course not only imprinted binding sites generated, but also unspecific binding sites, which could be studied by analyzing an isotherm across an extended concentration range. It is anticipated that the more specific imprinted binding sites are favored (given their higher affinity to the template molecule), and should be occupied with analyte molecules first due to the higher binding affinities, i.e., prior to or at least faster than unspecific binding occurs. Consequently, analyzing a binding isotherm the imprinting effect will be predominantly evident in the first segment, whereas at higher concentrations unspecific binding or multiple layer binding will increasingly occur. Hence, any *imprinting factor* should in fact only describe the binding behavior resulting only or at least predominantly from imprinted binding sites, as this mostly defines the quality and utility of a particular MIP. Considering these aspects, higher concentrations as the earlier defined upper maximum integration boundary, which essentially defines the GIF(100) - even for almost perfect polymers, should not be included into *imprinting*
factor calculation as illustrated in Figure 38. In conclusion, the final percentage generic imprinting factor is now defined by Equation 21.

\[ \text{GIF}(\%) = \frac{\int_0^{a_{MIP}} f(x)dx}{\int_0^{a_{NIP}} g(x)dx} \]  

(21)

**GIF(\%)**  Percentage generic imprinting factor

\%  Fraction of the used upper integration boundary from the theoretical maximum integration boundary expressed in percentage

\(a_{MIP/NIP}\)  Upper integration boundary for MIP/NIP

\(f(x)\)  Function (isotherm) describing the MIP binding gradient

\(g(x)\)  Function (isotherm) describing the NIP binding gradient
Figure 38. MIP and NIP analysis also considering unspecific binding at higher concentrations. Hence, for the calculation of a final percentage generic imprinting factor the integration of the data is only performed to the upper integration boundary.
6.3 Exemplary calculation of the percentage generic imprinting factor for the results obtained by the flow cell method (chapter 4.3.2)

In Figure 20d, the isotherms of a propranolol MIP and NIP are shown. For the calculation of the newly defined percentage generic imprinting factor, first the upper integration boundary of the MIP has to be calculated. The experimental details are listed in Table 3 illustrating that 4026.78 mg of DVB and 554.42 mg of MAA were used to synthesize the polymer together with 1.61 mmol propranolol. Hence, the theoretical maximum mass of the polymer is 4581.2 mg, and therefore 1 mg of polymer could imprint a maximum of 351.44 nmol. As for the flow cell based analysis 21.6 mg MIP were used, the maximum uptake of propranolol by imprinted sites would be 7.591 µmol. To calculate the upper integration maximum, the theoretical free concentration providing exactly 7.591 µmol has to be determined. Considering a cell volume of 19.1 mL, the resulting upper integration boundary would be 397.44 µM. As the highest free concentration analyzed during the experiment with the flow cell was 197.6 µM, the respective incubation concentration was 209.96 µM (as obtained from the blank curve), which represents 52.8% of the upper integration limit. At the same point, a free concentration of 205.23 µM was obtained for the NIP defining its upper integration limit at 52.8%.

Subsequently, the integration of the MIP isotherm yields an area of 5829 µM*nmol/mg, and the integration of the NIP isotherm an area of 2904 µM*nmol/mg (integration performed using GraphPad Prism 5 software and the implemented area under curve analyzing tool). Therefore, the calculated percentage generic imprinting factor for the data presented in Figure 20d is $G_{IF}^{(52.8)} = 2.01$. 

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Consequently, for analyzing approximately half of the polymers theoretical binding range, almost twice the amount of propranolol could be adsorbed by the MIP compared to the NIP.

6.4 Conclusions and outlook

With the proposed redefinition of the imprinting factor (IF), the randomness of imprinting factors obtained during batch incubation experiments may be eliminated and replaced by a set of defined conditions for the calculation of the newly defined generic imprinting factor (GIF). Further extending and even more generalizing this concept, the percentage generic imprinting factor (GIF_{%}) enables the direct comparison of imprinting factors between any kind of MIP (or NIP), which was to date limited using existing calculation concepts. A substantial benefit is the additional information provided by the added percentage, which indicates how many template molecules were in fact used for generating the imprint represented by the single value. Using the percentage generic imprinting factor, it should be possible to compare - in any respect - different experimental preparations without any restrictions providing a universal figure-of-merit for describing the binding properties of MIPs using a single value.
7. Summary

Molecularly imprinted polymers are synthetic binding and/or scavenger media, which are increasingly researched and used in recent decades in a wide variety of applications. As a wide range of synthesis methods for a substantial variety of template molecules has been reported in literature to date, this thesis was aiming at focusing on advanced strategies for the post-synthesis treatment of MIP materials and their fundamental characterization for enhancing the knowledge on MIPs with particular emphasis on their binding behavior. Hence, the main focus of this thesis was the development of novel strategies for an effective characterization and evaluation of MIPs prior to their analytical usage. Based on the generic scheme for the preparation/synthesis of MIPs (Figure 1), three crucial steps were identified after the synthesis: (i) efficient MIP clean-up strategies, (ii) characterization of MIPs under equilibrium and kinetic aspects and (iii) the improved characterization and figures-of-merit calculation for describing the binding properties of MIPs.

The first step after the synthesis of a MIP or NIP is the clean-up of the polymer, i.e., the efficient extraction of the template molecules. Furthermore, impurities such as unreacted cross-linking molecules and functional monomers have to be removed. This step is absolutely crucial, as any non-extracted impurity may leach from the polymer matrix e.g., within an analytical application of the MIP, and thus, may influence the obtained results. This extraction problem remains one of the most prominent drawbacks of MIP technology, as the highest affinity binding pockets could still contain template molecules, which will not only block the binding site for recognizing analyte molecules this reducing the MIP binding capacity, but also may give rise to template leaching. Template leaching is particularly problematic in analytical MIP applications, as a higher analyte concentration may be suggested, and in biotechnological MIP applications, as
contamination may occur. Given these requirements, exhaustive MIP clean-up using conventional strategies such as soxhlet extraction or batch multiple solvent change extraction methods are frequently time-consuming and solvent-extensive.

In order to maximize the efficiency of the extraction process, a device was developed (see chapter 3) that facilitates a possibly rapid extraction of MIPs with constantly refreshed solvent at a wide variety of extraction conditions, yet keeping solvent consumption and extraction time at a minimum. Figure 11 shows the developed ULEX device made from glass, which has been tested in the course of this thesis for the extraction of iohexol-imprinted polymers, and favorably compares with conventional extraction techniques.

A substantial benefit of the ULEX device along with the established extraction strategy is its versatility, (i) as the design allows the usage of solvents or solvent mixtures, (ii) that temperature-control is enabled, and (iii) that the device is adaptable to any kind of particles in terms of dimension and functionality and any extraction volume (i.e., ULEX may be miniaturized). Finally, the ULEX may be combined with any online/inline detection method facilitating a detailed analysis of the extract for optimized control/monitoring of the extraction efficiency. Thereby, optimal extraction conditions for a certain kind of recognition matrix or particle may be established, thus further reducing the extraction time. In addition, ULEX may also be used for other applications including monitoring catalytic reactions at particles, delivery and release properties of particles or carriers, etc..

In summary, the extraction method developed herein provides enhanced MIP clean-up strategies not only facilitating usage of MIPs in analytical and biotechnological applications, but also supports an improved characterization of MIPs and related binding matrices.

A correct and detailed performance evaluation is the next crucial step for analyzing synthesized MIPs prior to usage, as the binding behavior of these polymers largely determines their applicability. A variety of characterization methods are reported in literature with the most commonly used techniques
summarized in chapter 2.1.5. However, the most commonly applied methods for MIP characterization are based on the generation of equilibrium binding isotherms. These isotherms are typically obtained via chromatographic studies or batch incubation methods. As both methods have their own drawbacks, another aim of this thesis was the development of a generic MIP evaluation method particularly focusing on the kinetic/dynamic binding behavior of MIPs, which has received significantly less attention despite its potential utility.

Based on the ULEX device (chapter 3), in chapter 4 a flow-cell method was developed by combining the data evaluation concept of frontal chromatography with the fundamentals of conventional batch analysis via in situ generation of incubation concentration gradients within a polymer slurry. The presented flow-cell setup illustrated in Figure 17 and Figure 18 was experimentally and theoretically fully characterized and enables fundamentally understanding all relevant processes occurring during a binding experiment (chapter 4.3). In essence, a sample solution subject to a continuous concentration change is applied to the polymer material, and the difference between an experiment with and without polymer present (i.e., polymer vs. blank measurement) yields precise information on the polymer binding properties. Advantageously, the developed method enables analyzing equilibrium binding properties for very slow concentration changes, and kinetic binding properties for very fast concentration changes. Hence, MIPs may be readily and fully characterized at any kind of subsequent application conditions. This was proven within chapter 4 for the example of propranolol-imprinted bulk polymers revealing yet unprecedented data on the dynamic binding characteristics of bulk-imprinted material, which was significantly faster than anticipated. Thus obtained data was also applied to investigate several isotherm models for describing the MIP binding behavior with the Langmuir-Freundlich isotherm serving as a best fit for the investigated propranolol MIP.
The potential of kinetic evaluations was further used on propranolol-imprinted bulk polymers using different cross-linker backbones to evaluate whether a post-synthesis modification of the MIPs structural backbone could enhance the binding kinetics. For that purpose, MIPs and NIPs with 0, 10, 20, and 40% EGDMA serving as an additional cross-linking molecule next to the already used DVB were synthesized. By applying a KOH induced hydrolysis of the EGDMA molecules, the backbone of the obtained polymers was altered post synthesis, and the binding behavior of the polymers was analyzed at kinetic conditions for the non hydrolyzed and hydrolyzed polymers. The obtained data was evaluated using pseudo first and pseudo second order binding models, although the results did not indicate a preferred model. While the obtained kinetic data summarized in Table 6 did not indicate an improvement of the kinetic binding behavior, the hydrolysis appears to increase the binding capacity of these polymers.

In summary, the results obtained with this chapter clearly reveal the largely underrated kinetic binding potential of bulk-imprinted MIPs, and confirm the potential of the developed evaluation method. Again, the characterization strategies shown herein may not only be used for the analysis of MIPs, but could be applied to analyze binding phenomena for other recognition materials and even within biological processes.

While the previous studies focus on analyzing individual materials, chapter 5 discusses parallelizing the characterization of MIPs toward high-throughput methods enabling the rapid generation of binding data relevant for MIP characterization based on the 96 well-plate format. Therefore, (i) an Eppendorf tube incubation with an UV 96 well-plate analysis, (ii) a method based on 96 well filter plates, and (iii) the commercially available ResiQuot system were applied to generate associated data for the same propranolol-imprinted polymer. However, as all methods are based on different experimental procedures, treatment of the obtained data enabling a fair comparison appeared problematic. Hence, conventionally applied data representation routines were questioned within this chapter, as data
obtained at different experimental conditions yet for one and the same material were not comparable in the typical bound-vs-free isotherm format. Especially, it was discovered that the mass dependence of this typical isotherm presentation is problematic, and that advanced normalization schemes are required facilitating the comparison of incubation studies experimentally performed using different polymer masses. Finally, this thesis was concluded by critically reconsidering the key parameter usually applied to characterize the quality of a molecular imprint – the so-called imprinting factor (IF), as detailed in chapter 6. Based on the developed isotherm evaluation methods, a redefinition of the imprinting factor is proposed leading to a percentage generic imprinting factor (GIF(%)), which is based on the integration of isotherms rather than at a selected snapshot at a selected discrete concentration along the equilibrium binding isotherm, which is the routine procedure for conventionally calculating an IF. The calculation of a single key value based on the integration of isotherms uses the entire binding information to establish a generic imprinting factor for a MIP/NIP system, thereby relating this figure-of-merit much closer to the actual binding characteristics of the polymers, and significantly reduces the randomness of discretely calculated conventional imprinting factors. The utility of this concept was exemplary demonstrated for theoretical and experimental data within chapter 6.3.

In summary, this thesis illustrates advanced and innovative methods for the post-synthesis treatment of MIPs including more effective clean-up strategies and alternative characterization techniques for developing an enhanced understanding on the kinetic and equilibrium binding behavior of molecularly imprinted polymers. It is anticipated that this knowledge will not only support more detailed characterization strategies for MIPs, but also provides more comprehensive data on analyzing the binding behavior of MIPs, which is essential for achieving the long-term goal of rationally designed binding materials.
8. Zusammenfassung

Molekular geprägte Polymere (MIP) sind einzigartige Adsorptionsmaterialien, deren Erforschung über die letzten Jahrzehnte zu einer Vielzahl an Anwendungsmöglichkeiten führte. Da ein großer Schwerpunkt der MIP-Forschung auf der Synthese dieser Polymere für ein breites Spektrum an Zielmolekülen liegt, wurde das Hauptaugenmerk dieser Dissertation auf die oft vernachlässigte Untersuchung der Polymereigenschaften nach der Synthese gelegt um das Wissen über MIPs, insbesondere in Bezug auf ihr Bindungsverhalten, zu vertiefen. Basierend auf dem klassischen MIP Schema in Abbildung 1 wurden 3 entscheidende Schritte nach der Synthese des Polymers identifiziert: (i) die Aufreinigung der Polymere nach der Synthese, (ii) ihre Auswertung unter Gleichgewichtsbedingungen und kinetischer Analyse sowie (iii) die verbesserte Präsentation der gewonnenen Daten zur Beschreibung des Bindungsverhaltens des Polymers.

Der erste wichtige Schritt nach der Synthese molekular geprägter Polymere ist die effektive Aufreinigung von MIPs und NIPs. Verunreinigungen wie zum Beispiel Eduktmoleküle (Quervernetzer, funktionelle Monomere und insbesondere Templatmoleküle) müssen aus der Polymermatrix entfernt werden. Dieser Aufreinigungsschritt muss mit größtmöglicher Sorgfalt durchgeführt werden, da diese Verunreinigungen im weiteren Verlauf ausgewaschen werden können und somit die Auswertung oder Anwendung der Polymere negativ beeinflussen. Die Extraktionsproblematik ist eine der bekanntesten Nachteile von MIPs, da die Bindungsstellen mit der stärksten Bindungsaffinität in einem geprägten Polymer bei einer unvollständigen Extraktion immer noch Templatmoleküle enthalten können. Diese Templatmoleküle blockieren nicht nur die stärksten Bindungsstellen und reduzieren folglich die Effizienz des Polymers, sondern es ist dadurch auch möglich, dass Templatmoleküle in templatfreie Umgebungen verschleppt
werden. Die Notwendigkeit der Extraktion für die molekular geprägten Polymere macht diese mit den herkömmlichen Aufreinigungsmethoden (Soxhlet Extraktion, wiederholter Lösungsmitteltausch in einer Batch-Extraktion) sehr oft zu einem zeitaufwändigen und kostenintensiven Prozess.

Zur Verbesserung des Extraktionsprozesses wird in Kapitel 3 ein neuer experimenteller Aufbau dargestellt, dessen Kern der neu entwickelte ULEX Extraktionsapparat darstellt und durch den eine kontinuierliche Extraktion unter frei wählbaren Extraktionsbedingungen bei niedrigem Lösungsmittelverbrauch und geringen Aufreinigungszeiten ermöglicht wird. Abbildung 11c zeigt den ULEX Prototyp aus Glas, der sich als das Mittel der Wahl zur effektiven Aufreinigung von Iohexol geprägten Polymeren im Vergleich zu anderen herkömmlichen Aufreinigungsmethoden herausstellte.


Die entwickelte ULEX Methode löst die oben beschriebene Extraktionsproblematik für molekular geprägte Polymere und erlaubt eine effektive weitere Charakterisierung und Anwendung der synthetisierten Polymere oder auch anderer Adsorptionsmaterialien.

Basierend auf dem ULEX Design (Kapitel 3) wurde in Kapitel 4 eine Durchflusszelle entwickelt, die das Konzept der frontalchromatographischen Auswertung mit der konventionellen Batch Analyse verknüpft und eine Untersuchung der Polymere durch das Erzeugen eines Konzentrationsgradienten in situ ermöglicht. Der komplette experimentelle Durchflusszellenaufbau, dargestellt in Abbildung 17 und 18, wurde vollständig theoretisch untersucht und erlaubt somit die Beschreibung aller relevanten Vorgänge während eines Bindungsexperiments (Kapitel 4.3). Im Detail werden die Bindungseigenschaften des Polymers durch das Erzeugen eines Konzentrationsgradienten des jeweiligen Analyten in der mit einer Polymersuspension gefüllten Durchflusszelle im Vergleich zu einer Messung ohne Polymersuspension untersucht (Polymer vs. blank Messung). Für sehr langsame Konzentrationsänderungen während der Messung können die Polymere unter Gleichgewichdsbedingungen untersucht werden und für rasche Konzentrationsänderungen lassen sich Rückschlüsse auf die kinetischen Eigenschaften des zu untersuchenden Materials ziehen. Somit können MIPs unter den jeweiligen Bedingungen analysiert werden, die für
verschiedenste Anwendungen relevant sind. Diese wurde in Kapitel 4 beispielhaft für Propranolol-geprägte Bulk-Polymer gezeigt. Dabei stellte sich heraus, dass diese Bulk-MIPs signifikant schneller Analyten binden als dies bisher angenommen und erwartet wurde. Mit Hilfe der erzeugten Daten wurde ebenfalls eine Reihe von Bindungsisothermen untersucht, wobei sich zeigte, dass die Langmuir-Freundlich Isotherme die Bindungseigenschaften der Propranolol MIPs am Besten beschreibt und für weitere Untersuchungen verwendet werden kann.

Die kinetische Analysenmethode wurde weiter verwendet um Propranolol geprägte Bulk-Polymere, die mit einer unterschiedlichen Zusammensetzung an Quervernetzern synthetisiert wurden zu untersuchen und um zu überprüfen, ob die nachträgliche Modifizierung des Polymergerüstes die Bindungseigenschaften verbessert. Hierzu wurden MIPs und NIPs mit 0, 10, 20, und 40% EGDMA-Gehalt als Quervernetzer zusätzlich zum bereits verwendeten DVB synthetisiert. Die nachträgliche Hydrolyse des EGDMA Polymergrundgerüstes mittels KOH wurde unter dynamischen Bedingungen untersucht, um die kinetischen Eigenschaften der Materialien vor und nach der Hydrolyse zu erfassen. Es wurden kinetische Modelle pseudo erster und pseudo zweiter Ordnung zur Analyse der Bindungseigenschaften verwendet, jedoch konnten die Ergebnisse keinen Aufschluss über das tatsächliche kinetische Modell liefern. Auch konnte kein verbessertes kinetisches Verhalten der hydrolysierten Polymere festgestellt werden, allerdings zeigten die hydrolysierten Polymere im Vergleich zu ihren nicht hydrolysierten Vergleichspolymeren eine erhöhte Bindungskapazität.


Zusammenfassend zeigt diese Dissertation neue Methoden und Konzepte für die Handhabung von molekular geprägten Polymeren nach der Synthese. Beginnend mit einer verbesserter Aufreinigung wurden neue
Analysemethode zur differenzierteren Charakterisierung der Polymere unter Gleichgewichtsbedingungen wie auch der kinetischen Untersuchung der Polymere entwickelt. Somit werden die gewonnenen Erkenntnisse in dieser Dissertation nicht nur die Charakterisierung von MIPs unterstützen, sondern auch helfen, das langfristige Ziel eines rational designten Bindungsmaterials zu erreichen.
9. References


10. Appendix

10.1 List of chemicals

10.1.1 Chapter 3

• Iohexol (Histodenz™) – Sigma Aldrich (St. Louis, MO, USA)
• Ethylenglycolmonomethylether; ≥ 99.5% - Merck KGaA (Darmstadt, Germany)
• Ethyleneglycoldimethacrylate; 98% - Sigma Aldrich (St. Louis, MO, USA)
• Azobisisobutyronitrile; ≥98% - Sigma Aldrich (St. Louis, MO, USA)
• Acetonitrile; HPLC grade – Lab-Scan Analytical Sciences (Gliwice, Poland)

10.1.2 Chapter 4

Preliminary results

• TENAX TA, 60 – 80 Mesh – Chrompack (Middelburg, The Netherlands)
• Sodium benzoate; ≥99.5% - Fluka Biochemika, Sigma Aldrich (St. Louis, MO, USA)
• Methanol; HPLC grade – Lab-Scan Analytical Sciences (Gliwice, Poland)
• Hydrochloric acid; extra pure – Merck KGaA (Darmstadt, Germany)

• (±) Propranolol hydrochloride; ≥ 99% - Sigma Aldrich (St. Louis, MO, USA)
• 1´,1´-Azobis-(cyclohexanecarbonitrile); 98% - Sigma Aldrich (St. Louis, MO, USA)
• Divinylbenzene; technical grade, 80% - Sigma Aldrich (St. Louis, MO, USA)
• Ethyleneglycoldimethacrylate; 98% - Sigma Aldrich (St. Louis, MO, USA)
• Methacrylic acid; 99% - Sigma Aldrich (St. Louis, MO, USA)
• Acrylamide; 99%- USB Corporation (Santa Clara, CA, USA)
• Sodium hydroxide; ≥ 98% - Sigma Aldrich (St. Louis, MO, USA)
• Potassium hydroxide; ≥ 98% - Sigma Aldrich (St. Louis, MO, USA)
• Acetonitrile; HPLC grade – Fisher chemicals (Loughborough, United Kingdom)
• Methanol; HPLC grade – Fisher chemicals (Loughborough, United Kingdom)
• Ethanol; absolute, analytical reagent grade – Fisher chemicals (Loughborough, United Kingdom)
• Acetone; Laboratory reagent grade – Fisher chemicals (Loughborough, United Kingdom)
• Toluene; HPLC grade – Acros Organics (Loughborough, United Kingdom)
• Sodiumdodecylsulfate – BDH Chemicals (Poole Dorset, United Kingdom)
• Disodium hydrogen orthophosphate dihydrate; Analytical reagent - Fisher chemicals (Loughborough, United Kingdom)

10.1.3 Chapter 5

• (±) Propranolol hydrochloride; ≥ 99% - Sigma Aldrich (St. Louis, MO, USA)
• 1´,1´-Azobis-(cyclohexanecarbonitrile); 98% - Sigma Aldrich (St. Louis, MO, USA)
• Divinylbenzene; technical grade, 80% - Sigma Aldrich (St. Louis, MO, USA)
• Ethyleneglycoldimethacrylate; 98% - Sigma Aldrich (St. Louis, MO, USA)
• Methacrylic acid; 99% - Sigma Aldrich (St. Louis, MO, USA)
• Sodium hydroxide; ≥ 98% - Sigma Aldrich (St. Louis, MO, USA)
• Potassium hydroxide; ≥ 98% - Sigma Aldrich (St. Louis, MO, USA)
• Acetonitrile; HPLC grade – Fisher chemicals (Loughborough, United Kingdom)
• Methanol; HPLC grade – Fisher chemicals (Loughborough, United Kingdom)
• Ethanol; absolute, analytical reagent grade – Fisher chemicals (Loughborough, United Kingdom)
• Acetone; Laboratory reagent grade – Fisher chemicals (Loughborough, United Kingdom)
• Toluene; HPLC grade – Acros Organics (Loughborough, United Kingdom)
• Sodiumdodecylsulfate – BDH Chemicals (Poole Dorset, United Kingdom)
• Disodiumhydrogenorthophosphosphate dihydrate; Analytical reagent - Fisher chemicals (Loughborough, United Kingdom)

All chemicals not mentioned have been of analytical- or laboratory standard quality.

10.2 List of equipment, instrumentation and software used

10.2.1 Chapter 2 (SEM images)

• Helios Nanolab 600, FEI (Eindhoven, Netherlands)

10.2.2 Chapter 3

• IKA Combimag Ret magnetic stirrer (Staufen, Germany)
• ALITEA C8 MIDI 2.5 – 50 RPM flexible tube pump (Stockholm, Sweden)
• Tygon MHLL (black/black) tube from IDEX/Ismatec (Wertheim, Germany)
• Retsch RM100 automated mill (Haan, Germany)
• Thermo Scientific Heraeus vacuum oven (Hanau, Germany)
• Thermo Scientific Heraeus Megafuge 16 (Hanau, Germany)
• IKA MS3 digital shaker (Staufen, Germany)
• MilliQ Academic from Millipore (Darmstadt, Germany)
Dionex HPLC system (Thermo Fisher Scientific; Waltham, MA, USA):

- UVD340S detector
- P580 pump
- ASI-100 Automated Sample Injector
- Chromeleon 6 software
- Luna 5 µm CN HPLC column (150 x 4.6 mm) from Phenomenex (Aschaffenburg, Germany)

10.2.3 Chapter 4

Preliminary results

- IKA Combimag Ret magnetic stirrer (Staufen, Germany)
- ALITEA C8 MIDI 2.5 – 50 RPM flexible tube pump (Stockholm, Sweden)
- Tygon MHLL (black/black) tube from IDEX/Ismatec (Wertheim, Germany)
- Gynkotek UVD 340S DAD Detektor (Thermo Fisher Scientific; Waltham, MA, USA)

Flow-cell setup

- Pharmacia LKB HPLC Pump 2248 (Uppsala, Sweden)
- Rheodyne 7725i HPLC switch (Rohnert Park, CA, USA)
- Fisher Scientific magnetic stirrer (Loughborough, United Kingdom)
- SpectraSeries UV 100 Thermo Seperation Products detector (Waltham, MA, USA)
- AD Instruments PowerLab 2/26 AD-converter (Oxford, United Kingdom)
- AD Instruments Lab chart 6 software (Oxford, United Kingdom)

HPLC setup

- Thermo Separation Products (Waltham, MA, USA):
  - Spectra Series P100 pump
• Spectra System AS3000 autosampler
• Spectra System UV2000
• ChromQuest software

• BD Plastipak 1 mL syringes (Oxford, United Kingdom)
• Kinesis KX Syringe Filters 13 mm, 0.22 µm nylon (Malta, NY, USA)
• Gilson Pipetman pipets (Middleton, WI, USA)
• Gallenkamp vacuum oven (Loughborough, United Kingdom)
• Corning 50 mL centrifuge tubes (Corning, NY, USA)
• BRAND Micro-tubes 2 mL (Wertheim, Germany)

10.2.4 Chapter 5

• ResiQuot Atoll GmbH (Weingarten, Germany)
• Fluostar Optima BMG Labtech (Ortenberg, Germany)
• Greiner UV-Star Microplate 96 well (Kremsmünster, Austria)
• MultiScreen Vacuum Manifold + deep well collar from Millipore (Darmstadt, Germany)
• Pall AcroPrep Advanced 96 Filter Plate, 2 mL, 0.2 µm PTFE (Portsmouth, United Kingdom)
• Beckmann 96 Deep-well Titer Plate (Polystyrene) 1 mL (High Wycombe, United Kingdom)
• BD Plastipak 1 mL syringes (Oxford, United Kingdom)
• Kinesis KX Syringe Filters 13 mm, 0.22 µm nylon (Malta, NY, USA)
• Gilson Pipetman pipets (Middleton, WI, USA)
• Gallenkamp vacuum oven (Loughborough, United Kingdom)
• KNF LaboPort pump (Trenton, NJ, USA)
• Corning 50 mL centrifuge tubes (Corning, NY, USA)
• BRAND Micro-tubes 2 mL (Wertheim, Germany)
10.2.5 Software used within this thesis

- Microsoft Office 2011, Microsoft (Redmond, WA, USA)
- Plot version 0.997 (author: Michael Wesemann, Co-Author: Barend J. Thijsse; plot.micw.eu)
- Corel Graphics 11, Corel Corporation (Ottawa, Ontario, Canada)
- CS Chem Office 2010, Cambridgesoft, Perkin Elmer (Waltham, MA, USA)
- Prism 5, Graph Pad software (La Jolla, CA, USA)
- Zotero version 4.0.8 (developer: Center for History and New Media at George Mason University (Fairfax, VA, USA)
Appendix 1. ULEX connector made from PEEK a) and stainless steel b). Both connectors are equipped with a rubber gasket.
Appendix 2. Calibration plots for the ULEX extraction data a) and b); daily solvent change c) and the soxhlet extraction d).
Appendix 3. Calibration plot for the static batch experiment and the kinetic batch experiments using 50, 20, 10, 5, 2.5, 0 µM propranolol standard solutions.

Appendix 4. a) Langmuir-Freundlich simulation fitted to the experimental data; propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm. b) Squared residuals of the fitting plotted against time.
Appendix 5. a) Jovanovic simulation fitted to the experimental data; propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm. b) Squared residuals of the fitting plotted against time.

Appendix 6. a) Tóth simulation fitted to the experimental data; propranolol stock solution 250 µM in acetonitrile/methanol 70/30 % (v/v), flow rate 0.3 mL/min, stirring rate 700 rpm, measured at 290 nm. b) Squared residuals of the fitting plotted against time.
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Appendix 7. Pearson correlation coefficients for all hydrolyzed measurements (two experiments per polymer)

Appendix 8. Exemplary fit of the pseudo first and second order kinetic equations to the θ vs. time plot of MIP 0 at kinetic conditions (0.5 mL/min; 1000 µM stock solution). Fitting parameters – k1: θE = 0.7949, k1 = 0.02305 min⁻¹, Pearson = 0.9523; k2: θE = 0.8520, k2 = 0.04557 min⁻¹, Pearson = 0.9415.
Appendix 9. Equilibrium batch experiments and isotherm fits using Prism 5 software and the implemented analyzing tool with the “one site – specific binding” (equates Langmuir isotherm) model. **a)** MIP 0 & MIP 0H, **b)** MIP 10 & MIP 10H, **c)** MIP 20 & MIP 20H, **d)** MIP 40 & MIP 40H. Fitting an isotherm to the binding data of polymer MIP 40 was not possible, as no useful fitting parameters was obtained by the Prism 5 software.
<table>
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<tr>
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<th>Surface area [m$^2$/g]</th>
<th>Sample</th>
<th>Surface area [m$^2$/g]</th>
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Appendix 10. BET data measured by Dr. Panagiotis Manesiotis at the Pharmaceutical and Molecular Biotechnology Research Centre, Waterford Institute of Technology, Waterford, Ireland. Using a Micromeritics Gemini VI, 50 to 55 mg of polymer was analyzed for each measurement and Dr. Manesiotis estimated a general error of 5% for the obtained values.
### 10.3.3 Chapter 5

<table>
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<th>Bound molecules (not per mg) [nmol]</th>
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*Appendix 11. Raw data for the 1 mg/mL experiment and the 5 mg/mL batch binding experiment.*
10.4 Derivation of Equation 12

Appendix 12. Fundamental processes occurring inside the flow-through cell without adsorbing media.

The measured concentration increase over time is based on two processes as illustrated in Appendix Figure 12. By applying a flow to the system a volume $\Delta V$ with the stock concentration $c_0$ is added, however also the same volume is flown out from the system to the detector. The two processes can be described with the following equations.

Inlet:

$$c_{\text{inlet}}(t + \Delta t) = c_0 \ast \Delta t + c(t) \quad (i)$$

Outlet:

$$c_{\text{outlet}}(t + \Delta t) = (c_0 \ast \Delta t + c(t)) \ast \frac{\Delta V}{V_{\text{cell}}} \quad (ii)$$

So the total measurable concentration at the detector can be described as follows

$$c = c_{\text{inlet}} - c_{\text{outlet}} \quad (iii)$$

and therefore
\[ c(t + \Delta t) = c_0 \cdot \Delta V + c(t) - \left( c_0 \cdot \Delta V + c(t) \right) \cdot \frac{\Delta V}{V_{cell}} \quad \text{(iv)} \]

As \( \Delta V \) is based on the applied flow rate over time, \( \Delta V \) can be expressed as

\[ \Delta V = F \cdot \Delta t \quad \text{(v)} \]

and therefore Equation (iv) can be expressed as

\[ c(t + \Delta t) = c_0 \cdot F \cdot \Delta t + c(t) - \left( c_0 \cdot F \cdot \Delta t + c(t) \right) \cdot \frac{F \cdot \Delta t}{V_{cell}}. \quad \text{(vi)} \]

Equation (vi) can be expressed for the total number of analyte molecules using the following two equations

\[ n(t + \Delta t) = V_{cell} \cdot c(t + \Delta t) \quad \text{(vii)} \]
\[ n(t) = V_{cell} \cdot c(t). \quad \text{(viii)} \]

Hence,

\[ n(t + \Delta t) = c_0 \cdot F \cdot V_{cell} \cdot \Delta t + n(t) \]
\[ - \left( c_0 \cdot F \cdot V_{cell} \cdot \Delta t + n(t) \right) \cdot \frac{F \cdot \Delta t}{V_{cell}}. \quad \text{(ix)} \]

The constants \( c_0, F \) and \( V_{cell} \) can be combined into a constant \( K \)

\[ K = c_0 \cdot F \cdot V_{cell} \quad \text{(x)} \]

and therefore Equation (ix) reduces to

\[ n(t + \Delta t) = K \cdot \Delta t + n(t) - \left( K \cdot \Delta t + n(t) \right) \cdot \frac{F \cdot \Delta t}{V_{cell}}. \quad \text{(xi)} \]

To calculate the actual amount of added analyte molecules (\( \Delta n \)) it has to be calculated as follows

\[ \Delta n = n(t + \Delta t) - n(t) \quad \text{(xii)} \]

therefore
\[ \Delta n = K \Delta t - \left( K \Delta t + n(t) \right) \frac{F \Delta t}{V_{cell}} \]  \hspace{1cm} (xiii)

and focusing on the amount of analyte molecules over time the equation can be expressed as

\[ \frac{\Delta n}{\Delta t} = K - K \Delta t \frac{F}{V_{cell}} - n(t) \frac{F}{V_{cell}}. \]  \hspace{1cm} (xiv)

Focusing on infinitesimal small time changes (\(\Delta t \rightarrow 0\)) Equation (xiv) can be expressed with the following simplification

\[ \lim_{\Delta t \rightarrow 0} K \Delta t \frac{F}{V_{cell}} = 0 \]  \hspace{1cm} (xv)

yielding for the focus on infinitesimal small time changes

\[ \frac{dn(t)}{dt} = K - n(t) \frac{F}{V_{cell}}. \]  \hspace{1cm} (xvi)

This ordinary differential equation (ODE) can be solved as the following

\[ n(t) = \frac{K V_{cell}}{F} \left( 1 - \exp \left( -\frac{F t}{V_{cell}} \right) \right) \]  \hspace{1cm} (xvii)

and by dividing this equation by \(V_{cell}\) the final equation for the measured concentration increase in derived

\[ c(t) = c_0 \left( 1 - \exp \left( -\frac{F t}{V_{cell}} \right) \right). \]  \hspace{1cm} (xviii)
11. Acknowledgements

Prior to the list of all helping minds, I want to highlight the help of my advisor Boris Mizaikoff for giving me the great opportunity to work on my PhD thesis at the Institute of Analytical and Bioanalytical Chemistry, University of Ulm. I want to thank for all his motivation and support during the last years.

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I would like to express my deepest gratitude to everyone helping and supporting me during this thesis!

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Peer-reviewed Publikationen

Ángela López-Lorente, Bartolomé Simont, Miguel Valcárcel, Stefan Eppler, Roland Schindl, Christine Kranz, Boris Mizaikoff; Characterization of stainless steel assisted bare gold nanoparticles and their analytical potential, Talanta, 2013, 118, 321-327.

Stefan Eppler, Michael Stegmaier, Florian Meier, Boris Mizaikoff; A novel extraction device for efficient clean-up of molecularly imprinted polymers, Analytical Methods, 2012, 4, 2296 - 2299.


Posterpräsentationen


Vorträge


Stefan Eppler, Jenna Bowen, Adrian Porch, Chris Allender, Boris Mizaikoff; Why MIPs are better than we thought! A closer look at the rebinding process of synthetic receptors, Graduate Student Symposium on Molecular Imprinting, 15. - 17. August 2013, Belfast.
Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die in der Arbeit aufgeführten Hilfsmittel verwendet habe.

Ulm, den 30.01.2014  ____________________