Advances in Separation Science

Modeling and Experimental Determination of Chromatographic Parameters by Frontal Chromatography

Molecular Imprinting: Development of Spherical Beads and Optimization of the Formulation by Chemometrics

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To my beloved ones

Would I have done what I did if I had known what I know?

H. Kempe

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Abstract

Chromatography is one of the most commonly used methods to separate molecules of various sizes. The technique is frequently applied to the separation of proteins. Such biotechnical applications require effective tools to predict the chromatographic process in order to avoid tedious and costly laboratory experiments during process development. In this thesis, the use of experiments as well as mathematical models to achieve this goal is demonstrated.

The advantages of using an intrinsic mathematical model for simulation of fixed bed chromatography are demonstrated. The chromatography model includes axial dispersion in the bulk liquid, external and internal mass transfer resistances, and instationary non-linear adsorption. The intrinsic model is compared to simplified lumped models. The former model is able to describe variations in the physical, kinetic, and operating parameters better than the latter ones. This results in a more reliable prediction of the performance of the chromatography process as well as a better understanding of the underlying mechanisms responsible for the separation.

A procedure to determine effective diffusion coefficients of proteins in chromatographic gels, required as model input parameters, is presented. An experimental methodology based on frontal liquid chromatography was combined with a numerical methodology based on a detailed mathematical model describing the chromatographic process including the extra-column dispersion, the dispersion due to the packed bed, the external mass transfer from the bulk phase to the stationary phase, and the diffusive transport within the stationary phase. The procedure has several advantages compared to previously reported methods to determine diffusion coefficients in that no other equipment than an HPLC is required, any class of stationary phases can be investigated as long as the experiments are performed under non-binding conditions, and no modification, e.g., molding of slabs or membranes, to the stationary phase is required. To show the applicability of the procedure, the effective diffusion coefficients of lysozyme, bovine serum albumin, and immunoglobulin γ in Sepharose™ CL-4B were determined and shown to be comparable to those determined by other methods.

Molecularly imprinted polymers (MIPs) are man-made polymeric materials with molecular recognition abilities. They mimic the molecular recognition of naturally occurring molecular recognition elements such as receptors and antibodies by binding target molecules by either non-covalent, covalent, or metal-coordinating interactions.

Traditionally, MIPs are synthesized in the form of monolithic polymers which are subsequently crushed, ground, and sieved to an appropriate size range. In this thesis, a suspension polymerization method to prepare MIPs in the shape of spherical beads is presented. The method involves suspending a pre-polymerization solution in mineral oil, used as the continuous phase. The droplets are transformed into solid spherical beads by free-radical polymerization. The beads have been shown to compare well to the traditional irregularly shaped particles prepared from monoliths. The advantages of the method compared to previously reported methods are the low cost and commercial availability of the continuous phase and the absence of the need for stabilizers for the formation of droplets of pre-polymerization solution in the mineral oil. When compared to the method to prepare particles from monolithic polymers, this method is advantageous due to the spherical shape of the resulting beads and the reduction in time needed to prepare a MIP.

When a new MIP is designed, the traditional approach is to use either a previously reported protocol or rules of thumb based on previous knowledge. This results in non optimized MIPs. The number of possible combinations of monomers, cross-linkers, solvents, and initiators are huge. A full optimization of a MIP formulation therefore requires a large number of experiments. To facilitate the efforts, chemometrics was applied to the work described in this thesis. Three factors (i.e., the amount of monomer, the amount of cross-linker, and the amount of porogen) were chosen as the factors in the model. Multivariate data analysis of the binding to the MIPs indicated how the factors influenced the binding and an optimized MIP composition was identified.

The combined use of the suspension polymerization method to produce spherical beads with the application of chemometrics was shown in this thesis to drastically reduce the number of experiments and the time needed to design and optimize a new MIP.

List of Publications

This thesis is based on the following papers, referred to by their roman numerals in the text and included at the end of the thesis.

Paper I: Henrik Kempe, Anders Axelsson, Bernt Nilsson, and Guido Zacchi, 'Simulation of chromatographic processes applied to separation of proteins', *Journal of Chromatography, A*, 846 (1999) 1-12. *(Republished with the kind permission from Elsevier)*

The author was responsible for all computational work and for writing part of the paper.

Paper II: Henrik Kempe, Patrik Persson, Anders Axelsson, Bernt Nilsson, and Guido Zacchi, 'Determination of diffusion coefficients of proteins in stationary phases by frontal chromatography', *Biotechnology and Bioengineering,* 93 (2006) 656-664. *(Republished with the kind permission from Wiley and Sons)*

The author was responsible for all of the experimental work and for writing the paper.

Paper III: Henrik Kempe, and Maria Kempe, 'Novel method for the synthesis of molecularly imprinted polymer bead libraries', *Macromolecular Rapid Communications*, 25 (2004), 315-320. *(Republished with the kind permission from Wiley and Sons)*

The author was responsible for all of the experimental work and for writing part of the paper.

Paper IV: Henrik Kempe, and Maria Kempe, 'Development and evaluation of spherical molecularly imprinted polymer Beads', *Analytical Chemistry*, 78 (2006) 3659-3666. *(Republished with the kind permission from ACS Publications Division)*

The author was responsible for the major part of the experimental work and for writing part of the paper.

1. Introduction

As more and more biologically active molecules such as proteins, peptides, and antibodies find therapeutic use, the industrial need for rapid and cost efficient means of separation and purification increases. Examples of separation methods, used in laboratory scale as well as in industrial scale, are chromatography, aqueous two-phase systems, filtration, crystallization, precipitation, and extraction.

When new chromatographic purification steps are planned or when an existing step is scaled up, it is common practice to perform numerous tedious chromatographic optimization experiments. As the target product molecules often are valuable and available only in small quantities, these experiments will be expensive to perform. Predicting the performance of the process by mathematical modeling and computer simulation minimizes the amount of experiments required. For this reason, simulation of chromatographic processes is becoming a valuable tool.

The model used in the simulation needs to be detailed enough to respond to changes in the experimental conditions. Advanced numerical methods are required to solve the more sophisticated mathematical models. Along with the development of efficient computer hardware more detailed models can be used. In order to predict the outcome of a separation by simulation, it is essential to have relevant parameters to insert into the model. Those parameters usually need to be determined by carefully designed experiments.

A number of phenomena determine the performance of a chromatography step: (i) the dispersion, (ii) the external mass transfer resistance, (iii) the diffusion within the stationary phase, and (iv) the adsorption/desorption kinetics. The dispersion causes an injected pulse or a step change to broaden. It arises due to the flow of the liquid through the column. In a perfectly packed column, the flow is evenly distributed over the cross section of the column. Deviations from this perfectly packed column give uneven flow distribution that

leads to additional band broadening. In the extreme case this is sometimes referred to as fingering. The external mass transfer resistance influences the rate of transport of molecules from the bulk to the stationary phase. The diffusion governs the rate of the molecules moving through the stationary phase to the sites of interaction. Finally, the adsorption/desorption kinetics determines how fast and strong the molecule will adsorb to the stationary phase. Simplified models, neglecting some of these phenomena, are often applied. The applicability of these simplified models is limited as lumped constants are valid only for the specific case for which they were determined. In this thesis, an intrinsic model is used to describe the chromatography process along with a procedure to determine the parameters. The procedure not only describes what happens in the column, but also describes the connecting tubings and the end pieces. In this way, the dispersive behavior of the whole experimental set-up is taken into account.

The aim of all separations and purifications is to achieve sufficient specificity and selectivity to reach the required purity. One of the most selective separation methods is affinity chromatography. The technique utilizes the interactions taking place in nature between, for example, enzyme–substrate, hormone–receptor, and antibody–antigene. The stationary phase in affinity chromatography contains either the actual biological recognition element or a synthetic equivalent. With biological recognition elements there are several important issues that restrict their general applicability, such as the high cost, the limited shelf life, and problems with regeneration and cleaning. An ingenious invention with more attractive features is molecularly imprinted polymers (MIPs).

MIPs are polymers synthesized in the presence of a template, also sometimes referred to as the print molecule. Removal of the template leaves a polymer selective for the template.

Several variables exist in the design of a MIP. An investigation of the functional groups of the template may give indications facilitating the choice of functional monomer(s). The requirement of degree of cross-linking dictates the choice of cross-linking monomer(s). The choice of solvent may be governed by the solubility of the template. The thermostability and photostability of the template can help in the choice of initiator. This list of variables is not complete and as can be seen the number of variables is large. With such a large set of

variables the number of combinations is enormous. One way to bring order to the problem is to make use of chemometrics. Chemometrics is a method based on intelligent experimental design and statistical evaluation of the results. By using chemometrics, the required number of experiments is drastically reduced while the reliability of the result is maintained.

MIPs are inexpensive, have excellent shelf life and are easily regenerated and cleaned. The polymers can be targeted for small molecules, such as amino acid derivatives or drug molecules, or large molecules, such as peptides or proteins.

MIPs are traditionally synthesized as monoliths, which are subsequently crushed and sieved to an appropriate size range, determined by the intended use. The resulting particles are irregularly shaped and the yield of usable particles is often poor as the losses in each grinding-sieving step are large. In this thesis a novel method to prepare MIPs as spherical beads in good yield is presented. Spherical beads perform better than irregular particles in packed bed applications such as liquid chromatography and solid-phaseextraction. Usually spherical beads are easier to handle than irregular particles. In Paper III and Paper IV this method is combined with a chemometric approach to synthesize and optimize MIPs selective for propranolol.

1.1. Aim

Physical phenomena, such as diffusion, dispersion, and adsorption/desorption kinetics, along with the set-up of the chromatographic system, govern the outcome of a separation. The diffusion of small molecules in the stagnant film surrounding the stationary phase and within the stationary phase itself is often so fast that it can be neglected. However, in the case of larger molecules, such as polypeptides and proteins, the diffusion is substantially slower and the mass transfer resistances have to be accounted for. The aim of the first part of this work was to design experiments and evaluation procedures to determine diffusion coefficients by frontal chromatographic experiments. To accomplish this, each phenomenon influencing the chromatographic process has to be isolated and evaluated separately. By this approach, modular models

taking into account relevant phenomena can be designed, thereby avoiding models based on lumped parameters. This type of intrinsic model is well suited for evaluating the effects of changes in the operating conditions of chromatographic processes, for example, the changes seen at scale-ups. The intrinsic model will be more sensitive to such changes than models involving lumped parameters.

Traditionally the preparation of an optimized molecularly imprinted polymer is a time consuming procedure. The most commonly occurring format to synthesize MIPs in is as bulk polymers that are subsequently crushed, ground and sieved to an appropriate size range. This procedure has to be repeated for each investigated polymer during the optimization. The procedure to crush, ground and sieve bulk polymers result in irregularly shaped particles that perform less good in packed bed applications such as liquid chromatography and solid-phaseextraction. The aim with the second half of this thesis was to develop a method for rapid preparation and screening of molecularly imprinted polymer libraries in the shape of spherical beads.

1.2. Disposition of this thesis

In Chapter 2, an overview of liquid chromatography is given with a historical background, descriptions of different types of columns and matrices, and a presentation of different chromatographic techniques. Chapter 3 and Paper I go into detail on how to model a chromatography column using an intrinsic model as well as the extracolumn volumes. Two analytical solutions are described. In Chapter 4, a description of diffusion is given as well as an overview of methods for the determination of diffusion coefficients. Chapter 5 describes the equipment (hardware and software) that was constructed as part of this work. Chapter 5 and Paper II also describe the procedure of determining diffusion coefficients by frontal chromatography.

Chapter 6 gives a brief introduction to the field of molecular recognition. In Chapter 7 the concept of molecular imprinting are introduced and different approaches of molecular imprinting are presented. Chapter 8 describes different formats that MIPs can be synthesized in and Chapter 9 describes the considerations needed

when a molecularly imprinted polymer is designed. Chapter 10 describes how MIPs are characterized and Chapter 11 describes application areas of MIPs. Paper III and Paper IV detail the development and characterization of a propranolol MIP using chemometrics and the method to synthesize MIPs as spherical beads.

Finally, in Chapter 12 this thesis is concluded.

2. Chromatography

2.1. Historical background

Even though some people claim that there is evidence of chromatography already in the Old Testament (Exodus 15:25; Moses purifies water using a piece of wood), the general opinion is that the story of liquid chromatography starts with the Russian botanist M. S. Tswett (1872-1919). Tswett separated different plant pigments using glass columns packed with calcium carbonate. During the separation, bands of different colors traversed the column and Tswett coined the term 'chromatography' which is derived from the Greek word '*chroma*' meaning '*color*' and '*graphia*' meaning '*to write*', that is, '*to write with colors*' (n.b., 'tswett' is Russian for color). Most of the investigators that tried to duplicate the work of Tswett failed, probably due to poor understanding of his work. Tswett identified several parameters of importance when working with packed columns, including particle size, particle size distribution, packing homogeneity, and the purity of the packing material.

After the work by Tswett there was not much activity in the area until the early 1940s when Archer Martin and Richard Synge published a study on liquid chromatography that resulted in the Nobel Prize in chemistry in 1952. As a spin-off from the Manhattan project (i.e., the development of the nuclear bomb during World War II) large-scale purification of several rare earth element oxides was carried out by ion exchange chromatography during the 1940s. Another large-scale project was conducted by the American Petroleum Institute during the late 1940s and early 1950s when crude oil was fractionated using silica gel chromatography. In 1963, the first commercial liquid chromatograph, the Model GPC 100 Liquid Chromatograph, was introduced by Waters. In 1967, the Waters ALC 100 HPLC made chromatography a tool that was affordable for most laboratories. The evolution and the use of liquid chromatography from the 1970s until today have been explosive. It is nowadays often

the method of choice for analytical and preparative purposes and the equipment is highly automated and offers high throughput. Even though the techniques have evolved, the basic concepts remain unchanged.

2.2. High and low pressure chromatography

The pressure required to achieve a flow through a packed bed depends, among other things, on the size and shape of the particles that constitutes the packing material. If the bed is packed with large particles, sufficient flow rates can be achieved using gravity as the driving force. In the case of small particles, it is necessary to use pumps to overcome the pressure drop over the bed. Quite often, especially in laboratory scale set-ups, gravity or simple peristaltic pumps are used to feed columns with mobile phase. However, if higher separation efficiencies are needed, packing materials with smaller bead sizes are required. In analytical high pressure liquid chromatography, HPLC, pressures up to 300 bars are common and high pressure pumps are mandatory.

2.3. Column chromatography

2.3.1. Packed bed chromatography

In packed bed chromatography, separation is performed using columns packed with the separating stationary phase. The column consists of a tube fitted with end pieces containing filters that retain the packing material. The up-stream end piece distributes the incoming feed over the entire cross section area of the column while the down-stream end piece retains the bed in the column. Packed bed chromatography can be fed either using gravity or by using pumps.

2.3.2. Moving bed chromatography

Regular column chromatography is a batch process and has shorter or longer down-times when the column is regenerated. One way of getting pass this is to use moving bed chromatography. In this case, the packing material is moving in the opposite direction to the mobile phase. A true moving bed (TMB) chromatography process, referred to as the Hypersorption™ process, was commercialized in the early 1950s by the Union Oil Company of California. Apart from the difficulties of working with a solid-phasethat is moving, the drawback with TMB is that only binary or pseudo binary mixtures can be separated. Another way to achieve the performance of a continuous moving bed is to use a number of columns that are connected to each other by a rotary valve. In this way the columns are simultaneously at different stages of the separation. For example, one column is loaded with sample, while some are in the separating mode, one is eluted, and others are being regenerated and equilibrated. The technique is called simulated moving bed chromatography (SMB). This technique has been used in the petrochemical industry and in the purification of sugars, but only to a minor extent in the pharmaceutical industry (Chin and Wang, 2004).

2.3.3. Monolithic chromatography

Monolithic chromatography is performed using columns filled with a continuous block of material. The material has to be porous and permeable to allow a convective flow through the column. Monolithic materials can either be molded to the shape of the column or made up of small particles that are sintered together or consolidated by a sol-gel process (Kele and Guichon, 2002).

2.3.4. Expanded bed chromatography

One major drawback with the chromatography methods mentioned above is that they impose the need of debris free samples of relatively low viscosity. Such samples are rare in the biotechnical industry. A crude cell extract, for example, can not be directly injected on a column without pre-treatment. The problem with clogging is overcome in expanded bed chromatography (EB) in which columns

are partially filled with the adsorbent phase. An expanded bed column consists of a column with a fixed bottom end piece and a moving upper end piece. The expanded bed is run under fluidized conditions during adsorption, that is, with the upper end piece in its upper position, and as a packed bed during elution, washing, and equilibration, that is, with the upper end piece in contact with the adsorbent phase. In contrast to fluidized beds, expanded beds do not suffer from internal mixing of the particles due to differences in the density and/or the hydrodynamic diameters of the particles. This keeps the back-mixing of the stationary phase at a low level (Anspach et al., 1999).

2.4. Positive and negative chromatography

All adsorption chromatography techniques can be run in either the positive mode or in the negative mode. In the positive mode, the solute of interest is adsorbed while contaminants are eluted directly. Positive chromatography usually leads to an increase in concentration of the solute when eluted. In the negative mode, the contaminants are adsorbed while the solute of interest passes directly through the column. Negative chromatography does not give an increase in concentration. If the desired solute is more abundant than the contaminants or if the product is difficult to adsorb, negative rather than positive chromatography may still be preferable to use.

2.5. Matrices

Stationary phases for chromatography can be either soft or rigid. The soft matrices are best suited for low pressure chromatography as they will collapse at high pressures. Examples of soft matrices are agarose, dextran, and low cross-linked synthetic polymers. The ability to withstand slightly higher pressures can be achieved by higher degree of cross-linking of the polymeric network. Rigid matrices are made of materials that can withstand high pressures. Such materials can be mineral based, for example, silica and alumina, or based on rigid polymeric materials such as highly cross-linked acrylates and styrenes.

2.6. Chromatography techniques

2.6.1. Gel permeation chromatography

Gel permeation chromatography (GPC), also known as gel filtration chromatography or size exclusion chromatography, is based on a sieving effect emanating from the network in the column packing material. Due to the porous nature of the network of the stationary phase, molecules of different sizes will experience different volumes inside the solid phase, that is, the material will show different apparent porosities depending on the size of the molecules. If a solute is sufficiently small to enter all pores, it will experience the entire volume of the stationary phase, excluding only the volume of the material itself. The fraction of the stationary phase available to a solute decreases with increasing size of the solute. If a mixture of molecules with different sizes is applied to a GPC column, the molecules will elute at different times due to the differences in apparent porosities. Large molecules will elute first and successively smaller molecules thereafter (Porath and Flodin, 1959).

2.6.2. Affinity chromatography

Affinity chromatography is a method based on the non-covalent bonds formed between biologically active molecules, for example, hormones–receptors, antibodies–antigens, and enzymes–inhibitors. Most often, the smaller molecule, referred to as the affinity ligand, is bound to the stationary phase while the larger one is captured during the chromatography. The method is often used in laboratory scale, but more seldom in larger scale due to the risk of leakage of the affinity ligand. Contamination of the product with ligand residues is especially critical in the production of proteins for therapeutic use. Tedious and costly qualifications and validations would be required. When the purity demands are less stringent, affinity chromatography can be a viable choice. Elution is initiated by changing the mobile phase so that the binding between the adsorbed molecules and the affinity ligand is weakened. This can be achieved, for example, by changing the pH, the ionic strength, or by adding the affinity ligand to the mobile phase (Wilchek and Chaiken, 2000).

2.6.3. Chromatography using molecularly imprinted polymers

MIPs are tailor-made polymers capable of selective binding (Sellergren, 2001). This selectivity makes MIPs suitable as stationary phases in affinity chromatography. MIPs are prepared by mixing the molecule for which specificity is wanted (the template) with cross-linkers and functional monomers. The monomer mixture is allowed to polymerize, the template is extracted and the resulting MIP then contains sites that are complementary to the template. The technique is detailed in Chapter 7. Until recently, the common practice was to prepare MIPs in the shape of monoliths which were crushed, ground and sieved to the desired particle size. The disadvantage with this method is that the resulting particles are irregularly shaped and pack poorly in columns. Paper III and Paper IV describe a novel method to synthesize MIPs in the shape of spherical beads.

An attractive feature of MIPs is their ability of chiral discrimination when an optically active compound has been used as the template. Many therapeutically active substances are racemic and efficient methods for chiral resolution are therefore needed. MIP stationary phases have been applied mainly for the separation of molecules with low molecular weight, although the imprinting of macromolecules such as proteins has also been reported (Kempe et al., 1995; Hjertén et al., 1997; Hawkins et al., 2005; Shiomi et al., 2005).

2.6.4. Ion-exchange chromatography

Ion-exchange chromatography (IEX) was introduced during the 1960s and has since then played a major role in the separation of macromolecules. In IEX, a charged stationary phase is used. The stationary phase is loaded with a counter ion during regeneration and equilibration. The solutes in the feed stock compete with the counter ion for the charged sites of the stationary phase during the adsorption step. The strength of the binding to the stationary phase depends on the net charge of the solute. This enables elution on the basis of the net charge by varying the ionic strength or the pH of the mobile phase, either stepwise or using a gradient. IEX can be run in either the anionic or the cationic mode depending on the sign of the charges of the molecule/molecules of interest. Anionic ion exchange

stationary phases carry positive charges and adsorb negatively charged molecules while cationic ion exchange stationary phases carry negative charges and thus adsorb positively charged molecules (Fritz, 2004).

2.6.5. Immobilized metal affinity chromatography

With the possibility to genetically modify proteins and include extra sequences of amino acids the method of immobilized metal affinity chromatography (IMAC) opened up. The inserted amino acid sequence is often a poly-histidine tag, added to either the carboxylic or the amino terminus. The terminus of choice is the one that exposes the tag on the exterior of the molecule. IMAC stationary phases contain chelating groups that bind metal ions. The method was presented in a paper by Porath and coworkers in 1975 (Porath et al., 1975) where they separated proteins from human serum using IMAC with immobilized zinc and copper ions. The method is not limited to only poly-histidine tags or to zinc and copper as the metal ions (Chaga, 2001).

2.6.6. Hydrophobic interaction chromatography

Introduced in 1948 by 1948-years Nobel Prize laureate in Chemistry, Arne Tiselius, hydrophobic interaction chromatography (HIC) is maybe one of the most widely used chromatographic techniques (Tiselius, 1948; Queiroz et al., 2001). In analytical scale it is most often referred to as reversed-phase chromatography (RPC). HIC/RPC is the chromatographic counterpart to liquid-liquid extraction using liquids of different polarity. In the HIC/RPC technique, non polar hydrocarbon chains are attached to the stationary phase. RPC phases usually have a higher degree of substitution than HIC phases, which makes the former phases less suitable for separation of proteins due to increased risk of denaturation. The nature of the mobile phase is often hydrophilic so that the partitioning of hydrophobic substances is driven towards the hydrophobic stationary phase. The elution is initiated and carried out either by the addition of more hydrophobic components to the

mobile phase or simply by running the separation long enough to let all analytes elute.

3. Modeling chromatography

This chapter presents the chromatography models used in this thesis. In section 3.2, different models for the extracolumn volume are presented. The first model is an ordinary tanks-in-series model that can be used to model simple dispersion behavior; the second model is an extended tanks-in-series model that beside dispersion also can account for a diffusion behavior. In section 3.1, a detailed model for the column is presented together with two simplified models with analytical solutions. In Paper I, a comparison is made between the intrinsic model and the two simplified models.

3.1. Modeling the column

3.1.1. Intrinsic model

The intrinsic model is made up of two parts: the first part accounts for the packed column and the second part for the beads. In order to reduce the model complexity a number of assumptions were made:

- Non-ideal behavior of the column is due to axial dispersion and the axial dispersion coefficient is constant throughout the column.

- The mass transfer from the mobile phase to the stationary phase can be described using a mass transfer coefficient, which is constant throughout the column.

- The bed is packed with mono-disperse spherical beads resulting in constant bed porosity throughout the column.

- The effective diffusion coefficient describing the mass transport within the stationary phase is constant.

- The process is considered to be isothermal.

Under these assumptions, a mass balance over a section of the column results in Equation 1 describing the change in protein concentration over time. In Equation 1, C_L is the concentration of protein in the liquid bulk phase, l is the length coordinate in the axial direction, D_{ax} is the dispersion coefficient, v_{INT} is the interstitial flow velocity, K is the mass transfer coefficient between the bulk liquid and the stationary phase bead, a is the specific area of the stationary phase, ε_c is the porosity of the packed bed, and $C_{Pr=R}$ is the protein concentration at the surface of the bead.

$$\frac{\partial C_L}{\partial t} = D_{ax} \cdot \frac{\partial^2 C_L}{\partial l^2} - v_{INT} \cdot \frac{\partial C_L}{\partial l} - K \cdot a \cdot \frac{1-\varepsilon_C}{\varepsilon_C} \cdot \left(C_L - C_{p_{r=R}}\right) \tag{1}$$

The first term in Equation 1 on the right hand side describes the band broadening due to dispersion in the axial direction, the second describes the convective flow, and the third describes the depletion of the substance in the mobile phase due to the uptake by the beads.

Two boundary conditions are used to describe the column inlet (Equation 2) and the column outlet (Equation 3).

$$\left.\frac{\partial C_{inlet}}{\partial l}\right|_{l=0} = \frac{v_{INT}}{D_{ax}} \cdot \left(C_{inlet} - C_0\right) \tag{2}$$

$$\left.\frac{\partial C_{outlet}}{\partial l}\right|_{l=L} = 0 \tag{3}$$

The mass transport within the bead is described as a diffusive transport process with D_{Eff} as the effective diffusion coefficient. Equation 4 describes this process in spherical co-ordinates. In Equation 4, r is the coordinate in the radial direction, C_P the concentration of protein in the liquid within the bead, and ε_P the porosity within the bead. In this equation r_P is the kinetic expression

for the adsorption/desorption process that takes place within the bead.

$$\frac{\partial C_P}{\partial t} = \frac{D_{Eff}}{\varepsilon_P} \cdot \left(\frac{\partial^2 C_P}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial C_P}{\partial r} \right) + \frac{r_P}{\varepsilon_P} \tag{4}$$

If the adsorption follows the Langmuir isotherm (Equation 5), the adsorption rate is given by Equation 7. In Equations 5 and 7, C is the concentration at time t, q is the adsorbed amount, and q_m is the maximum amount that can adsorb. K_a is defined by Equation 6 as the ratio between the adsorption and desorption coefficients k_{ads} and k_{des}.

$$q = \frac{K_a \cdot q_m \cdot C}{1 + K_a \cdot C} \tag{5}$$

$$K_a = \frac{k_{ads}}{k_{des}} \tag{6}$$

$$-r_p = \frac{\partial q}{\partial t} = k_{ads} \cdot C \cdot (q_m - q) - k_{des} \cdot q \tag{7}$$

The boundary condition used for a bead is given in Equation 8.

$$\left. \frac{\partial C_P}{\partial r} \right|_{r=R} = \frac{K}{D_E} \cdot (C_L - C_{\mathrm{Pr=R}}) \tag{8}$$

In order to numerically solve these three differential equations, several methods can be used, for example, orthogonal collocation (used in Paper I) or finite differences (used in Paper II). Both methods convert partial differential equations into ordinary differential equations. This conversion greatly simplifies the solving of the equations.

3.1.2. Analytical solutions

The physical description of the process can be simplified by certain assumptions that lead to more simple differential equations that can be solved analytically. These are often referred to as Arnold's model (Arnold et al., 1985) and Thomas' model (Thomas, 1944; Chase, 1984).

3.1.2.1. Arnold's model

In order to obtain Arnold's model the following assumptions are made:

- The column is considered to be ideal, that is, the flow profile is plug shaped. This implies that the axial dispersion is negligible ($D_{ax} = 0$).
- The mass transport in the fluid film around the bead can be described with a mass transfer coefficient K.
- The mass transport resistance within the stationary phase is described by an effective diffusion coefficient D_{Eff}.
- There is no accumulation of adsorbate in the pore liquid.
- The adsorption process is instantaneous compared to the mass transfer, that is, the kinetic is negligible. Thus, the adsorbed quantity is given by the adsorption equilibrium isotherm, for example, the Langmuir isotherm (Equation 5).
- The process is considered to be isothermal.

The mass transfer in the bulk is described by Equation 9.

$$\frac{\partial C_L}{\partial \theta} = -v_{INT} \cdot \frac{\partial C_L}{\partial l} - \frac{1-\varepsilon_C}{\varepsilon_C} \cdot \frac{\partial q}{\partial \theta} \tag{9}$$

θ is the time t, corrected with respect to the dead volume, that a bead at position z has been in contact with the bulk liquid flowing with the superficial velocity v_{sup} (Equation 10).

$$\theta = t - \frac{\varepsilon_C \cdot z}{v_{\sup}} \tag{10}$$

Equation 11 describes the mass transport within the stationary phase.

$$\frac{\partial C_P}{\partial t} = \frac{D_{Eff}}{\varepsilon_P} \cdot \left(\frac{\partial^2 C_P}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial C_P}{\partial r} \right) - \frac{1}{\varepsilon_p} \cdot \frac{\partial q}{\partial t} \tag{11}$$

The initial values for the system are summarized in Equation s 12 and 13. Equation 12 describes that the concentration, C, in the bulk liquid is zero at the time zero. Equation 13 shows that the adsorbed amount in the stationary phase, q, is zero at time zero.

$$C(0 < z < L, \theta = 0) = 0 \tag{12}$$

$$q(0 < z < L, \theta = 0) = 0 \tag{13}$$

Two boundary conditions are present when solving the system for a step change. The first is that the concentration at the column inlet is constant over the time (Equation 14).

$$C(z = 0, \theta \geq 0) = C_0 \tag{14}$$

The second boundary condition is, according to the condition stated earlier that there is no accumulation in the film surrounding the beads. This implies that the transport from the bulk to the surface of the bead is equal to the transport from the surface of the bead to the pore liquid within the beads (Equation 15).

$$\left.\frac{\partial C_p}{\partial r}\right|_{r\to R} = \frac{K}{D_E}\cdot\left(C - C_p\big|_{r=R}\right) \tag{15}$$

Taking into account the mass transfer resistances in the film surrounding the beads and the diffusion in the pore liquid within the beads, an implicit solution, Equation 16, is obtained (Arnold et al., 1985).

$$T - 1 = \left(\frac{1}{N_{pore}} + \frac{1}{N_f}\right)\cdot\left(\phi(X) + \frac{N_{pore}}{N_f}(\ln X + 1)\right)\cdot\left(\frac{N_{pore}}{N_f} + 1\right)^{-1} \tag{16}$$

The parameter ϕ in Equation 16 is defined by Equation 17 below.

$$\phi(X) = 2.44 - 3.66\cdot(1 - X)^{1/2} \tag{17}$$

In Equations 16 and 17 X is the concentration, C, normalized against the inlet concentration, C_0 (Equation 18).

$$X = \frac{C}{C_0} \tag{18}$$

A dimensionless parameter, T, can be calculated according to Equation 19, to describe the volume that the concentration front has passed at time t.

$$T = \frac{C_0\cdot v_{\sup}}{(1-\varepsilon_C)\cdot q_m\cdot L}\left(t - \frac{\varepsilon_C\cdot L}{v_{\sup}}\right) \tag{19}$$

The number of transfer units due to pore diffusion, N_{pore}, can be expressed according to Equation 20.

$$N_{pore} = \frac{15 \cdot D_{Eff} \cdot (1 - \varepsilon_C) \cdot L}{R^2 \cdot v_{sup}} \tag{20}$$

The number of transfer units due to the mass transport in the film, N_f, can be expressed according to Equation 21.

$$N_f = \frac{K \cdot a_p \cdot L}{v_{sup}} \tag{21}$$

From the number of transfer units, given by Equations 20 and 21, it can be deduced which of the two phenomena, either the pore diffusion or the external mass transfer through the stagnant liquid film, is governing the overall rate. The rate determining phenomenon is the one with the lowest number of transfer units. If the mass transfer through the stagnant film is rate determining, Equation 16 can be simplified to Equation 22.

$$X = \exp(N_f \cdot (T - 1) - 1) \tag{22}$$

If the pore diffusion governs the overall rate, Equation 16 can be simplified to Equation 23.

$$X = 1 - \left(\frac{2}{3} - \frac{N_{pore} \cdot (T - 1)}{3.66} \right)^2 \tag{23}$$

3.1.2.2. Thomas' model

In order to obtain Thomas' model the following assumptions are made:

- The column is considered to be ideal, that is, the flow profile is plug shaped. This implies that the axial dispersion is negligible ($D_{ax} = 0$).

- The mass transport resistance between the bulk liquid and the stationary phase is instantaneous and negligible, that is, *K* equals infinity.

- The mass transport resistance within the stationary phase is negligible, that is, the effective diffusion coefficient is "large".

- A non-linear instationary Langmuirian kinetic relationship can describe the adsorption/desorption kinetics.

- The process is considered to be isothermal.

Under the given assumptions the expression for the bulk liquid, Equation 1, changes to Equation 24.

$$\frac{\partial C_L}{\partial t} = -v_{INT} \cdot \frac{\partial C_L}{\partial l} - \frac{1-\varepsilon_C}{\varepsilon_C} \cdot \frac{\partial q}{\partial t} \tag{24}$$

If Equation 24 is to be solved for a step change the initial value values for the concentration, *C*, and the adsorbed amount, *q*, are that *C* is zero at all places in the column (Equation 25) and nothing is adsorbed, that is, *q* equals zero throughout the column (Equation 26).

$$C(0 < z < L, t = 0) = 0 \tag{25}$$

$$q(0 < z < L, t = 0) = 0 \tag{26}$$

The boundary condition for the system is that the concentration at the inlet is always constant (Equation 27).

$$C(z = 0, t \geq 0) = C_0 \tag{27}$$

Given that the adsorption/desorption process can be described with a non-linear instationary Langmuir expression (Equation 7), the

initial values (Equation 25 and 26) and the boundary condition (Equation 27), the analytical solution is given by Equation 28.

$$\frac{C}{C_0} = \frac{J\left(\frac{n}{r}, n\Gamma\right)}{J\left(\frac{n}{r}, n\Gamma\right) + \left[1 - J\left(\frac{n}{r}, n\Gamma\right)\right] \exp\left[\left(1 - \frac{1}{r}\right)(n - n\Gamma)\right]} \tag{28}$$

The parameters r, n and Γ in Equation 28 are dimensionless parameters defined by Equations 29-31.

$$r = 1 + \frac{C_0 \cdot k_{des}}{k_{ads}} \tag{29}$$

$$n = q_m \cdot k_{ads} \cdot L \cdot \frac{A}{Q} \cdot (1 - \varepsilon_c) \tag{30}$$

$$\Gamma = \left(\frac{K_d + C_0}{q_m}\right)\left(\frac{Q \cdot t}{A \cdot L} - \varepsilon_c\right)\left(\frac{1}{1 - \varepsilon_c}\right) \tag{31}$$

J is a function of α and β given by Equation 32.

$$J(\alpha, \beta) = 1 - e^{-\beta} \int_0^{\alpha} e^{-\xi} I_0\left(2\sqrt{\beta\xi}\right) d\xi \tag{32}$$

In Equation 32, I_0 is a modified zeroth order Bessel function of the first kind (Equation 33).

$$I_0(x) = \sum_{n=0}^{\infty} \frac{\left(\frac{1}{2}x\right)^{2n}}{(n!)^2} \tag{33}$$

3.2. Modeling extracolumn volumes

An important matter to consider when modeling and simulating chromatography is the extracolumn volume, originating from tubings, end pieces, etc. This is especially important when the column volume is small and the extracolumn volume constitutes a large portion of the total volume. Ideally, the transport of the mobile phase through the extracolumn volume should occur with a plug shaped flow profile. It is, however, more or less inevitable that the extracolumn volume will contribute to the spreading of the injected sample, that is, cause band broadening. If the model is to be used for parameter estimations and the effect of extracolumn volume is neglected, the determined parameters will also include contributions from the dispersion in the extracolumn volume and will thus not represent what they were intended to do. However, by representing the extracolumn volume as a series of fictive tank volumes or by an open dispersion column the additional dispersion can be modeled separately (Levenspiel, 1972).

3.2.1. Dispersion behavior

Figure 1 shows the concept of modeling the extracolumn volume as fictive tanks-in-series is shown. The inlet part of the extracolumn volume is represented with one series of tanks containing three fictive tanks and the outlet part as another series of tanks containing three fictive tanks. The tanks are considered ideal, that is, they are completely mixed.

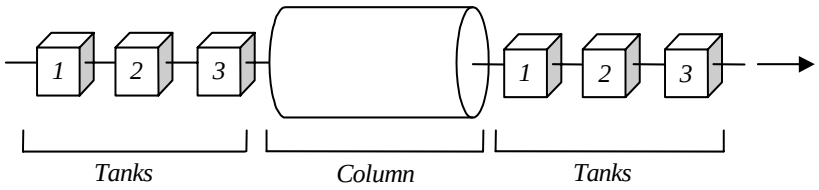


Figure 1. *The concept of a column model with the extracolumn volume modeled as fictive tanks in series.*

In Figure 2 the *i'th* tank in Figure 1 is shown with entering and exiting flow rates F_t and with incoming concentration $C_{t(i-1)}$ and outgoing concentration $C_{t(i)}$.

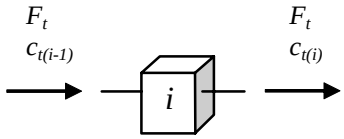


Figure 2. *Schematic representation of tank segment number i in the tank series with flows (F_t) and concentrations ($C_{t(i-1)}$ and $C_{t(i)}$, respectively).*

The mathematical description for the *i'th* tank is given in Equation 34 where V_t is the volume of the tank.

$$\frac{dc_{t(i)}}{dt} = \frac{F_t}{V_t}\left(c_{t(i)} - c_{t(i-1)}\right) \tag{34}$$

By using this kind of tank series it is possible to capture the dispersive behavior of the extracolumn volume. The number of tanks determines the extent of the dispersive behavior that the tank series accounts for.

3.2.2. Dispersion with some diffusive behavior

Figure 3 shows the concept of a column model taking into account diffusive behavior in the connecting tubings and end pieces. The model is based on the one described above but with the addition of a smaller tank attached to each tank in the tanks-in-series. The tanks are thought of as being ideal, that is, they are completely mixed.

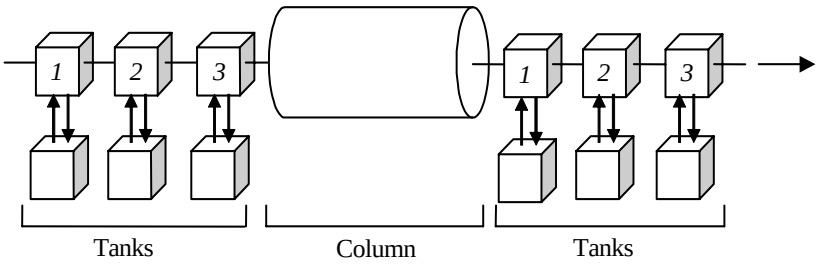


Figure 3. *The concept of a column model taking into account extracolumn dispersion with diffusive behavior modeled as fictive tanks in series.*

In Figure 4 the *i'th* tank pair from Figure 3 is shown.

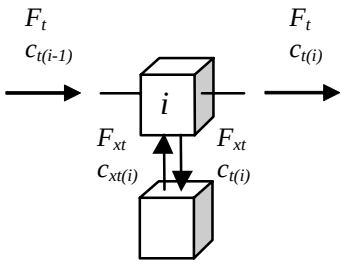


Figure 4. *Schematic representation of tank segment number i in the tank series with flows (F_t and F_{xt}) and concentrations ($C_{t(i-1)}$, $C_{t(i)}$, and $C_{xt(i)}$, respectively).*

Each tank in the main tank series has an exchange of liquid with one extra tank. The flow rate between the tanks in the main tank series is F_t while the flow rate to the extra tanks is F_{xt}. The concentration in the flow entering the main tank is $c_{t(i-1)}$ and the concentration in the tank and the flows leaving the tank is $c_{t(i)}$. The concentration in the extra tank and the flow leaving it is $c_{xt(i)}$. All the tanks in the main tank series have the same volume V_t and the extra tanks are all of volume V_{xt}. If the needed number of tanks is N_t then the total extracolumn volume, V_{ec}, is given by Equation 35.

$$V_{ec} = N_t \cdot (V_t + V_{xt}) \tag{35}$$

The mathematical description of this kind of tank series is given in Equations 36 and 37.

$$\frac{dc_{t(i)}}{dt} = \frac{F_t}{V_t}\left(c_{t(i-1)} - c_{t(i)}\right) + \frac{F_{xt}}{V_t}\left(c_{xt(i)} - c_{t(i)}\right) \tag{36}$$

$$\frac{dc_{xt(i)}}{dt} = \frac{F_{xt}}{V_{xt}}\left(c_{t(i)} - c_{xt(i)}\right) \tag{37}$$

By using this kind of tanks-in-series it is not only possible to capture the dispersive behavior but also some diffusive behavior. The extent of the diffusive behavior that the tank series accounts for is determined by the flow rate between the main tanks and the extra tanks as well as by the volume of the extra tanks.

3.3. Plate height theory

The concept with tanks-in-series introduced in section 3.2 to model extra-column volumes can be extended to model an entire chromatography column.

3.3.1. Plate number from experimental data

The evaluation of the number of theoretical plates is a traditionally used system suitability test for chromatographic systems. Each plate, or tank, represents one separation stage within which equilibrium is reached. The theoretical plate number is calculated from elution data according to Equation 38 where V_e is the elution volume (determined as the apex of an eluted pulse or the inflexion point of an eluted step) and $W_{1/2}$ is the width of the peak at half of the maximum height. The number of plates is measure of the chromatographic efficiency, directly related to the band broadening in the chromatographic system.

$$n = 5.54 \cdot \left(\frac{V_e}{W_{1/2}}\right)^2 \tag{38}$$

Column efficiency can also be determined as the number of effective theoretical plates (N_t) by Equation 39 where V_0 is the column dead volume. N_t measures the band broadening taking place only in the stationary phase and is therefore considered more fundamentally important than n. N_t and n converge at high values of V_e.

$$N_t = 5.54 \cdot \left(\frac{V_e - V_0}{W_{1/2}} \right)^2 \tag{39}$$

3.3.2. Dispersion coefficients from plate number

If a pulse or step change is still symmetric after passage through the column, the number of tanks (or plates) can be recalculated to a dispersion coefficient (D_{ax}) according to Equation 40. If the pulse or step instead is unsymmetrical the dispersion coefficient can be calculated using Equation 41 (Levenspiel, 1972).

$$\frac{1}{N_t} = 2 \cdot \frac{D_{ax}}{v_{\text{int}} \cdot L} \tag{40}$$

$$\frac{1}{N_t} = 2 \cdot \frac{D_{ax}}{v_{\text{int}} \cdot L} - 2 \cdot \left(\frac{D_{ax}}{v_{\text{int}} \cdot L} \right)^2 \left(1 - \exp\left(- \frac{v_{\text{int}} \cdot L}{D_{ax}} \right) \right) \tag{41}$$

3.3.3. van Deemter theory

The 'height equivalent to a theoretical plate' (*HETP*) is easily calculated from the number of theoretical plates (n) using Equation 42 where L is the length of the column.

$$HETP = \frac{L}{n} \tag{42}$$

The plate height can also be related to physical parameters. The van Deemter equation (Equation 43) gives the contribution to the total plate height (*HETP*) from eddy diffusion (*A*), longitudinal diffusion (*B*), mass transfer in the stationary and mobile phases ($C_{stationary}$ and C_{mobile}), and the average linear velocity of the mobile phase (*u*).

$$HETP = A + \frac{B}{u} + (C_{stationary} + C_{mobile})u \tag{43}$$

The term eddy diffusion, or axial dispersion as referred to earlier in this thesis, is a result of inhomogeneous packing of the column. It is proportional to the diameter of the stationary phase particles. In order to minimize the eddy diffusion, the diameter of the stationary phase should hence be as small as possible and the packing as homogenous as possible. The longitudinal diffusion term *B* describes the contribution to the total plate height from diffusion of the solutes in the mobile phase in the axial direction of the column. In liquid chromatography, the contribution from diffusion of the solutes in the mobile phase is often negligible except at low flow rates. The $C_{stationary}$ term describes the diffusion within the stationary phase while the C_{mobile} term describes the radial diffusion in the mobile phase.

Thus, the van Deemter equation includes more or less the same mass transfer hindrances as discussed in this thesis, but lacks a detailed description of the relation to adsorption kinetics and adsorption equilibrium.

4. Diffusion

4.1. General

The term 'diffusion' comes from '*diffundo*' which is latin for 'to spread'. Diffusion is a spontaneous mass transport phenomenon that can be observed when a concentration gradient exists in a matrix. The transport is due to random motions in three dimensions. These random motions are called Brownian motions when they occur in a liquid. The net result of diffusion is that concentration gradients are evened out.

4.2. Diffusion in free solution

When a concentration gradient of a substance exists in a solution, the diffusion can be described by Fick's first law of diffusion (Equation 44).

$$J = -D\frac{\partial C}{\partial x} \tag{44}$$

In Equation 44, J is the diffusive flux of the solute per unit area in the x direction, D is the diffusion coefficient of the solute in the solvent and C is the concentration of the solute. Fick's first law describes steady state diffusion, that is, the concentration gradient is constant and hence also the driving force for the diffusion.

4.3. Diffusion in porous materials

In a porous material, the material act as obstacles and hinders the diffusion. Two properties can effect the diffusion, namely the tortuosity and the porosity of the material.

4.3.1. Tortuosity

The tortuosity, τ, describes the prolonged length (L_e) of an actual pore compared to the length (L) of a hypothetical straight pore (Equation 37).

$$\tau = \frac{L_e}{L} \tag{45}$$

Since a solute has to diffuse a longer distance in a tortuous pore than in a straight pore, the apparent diffusivity is lower in a tortuous pore (Equation 46).

$$D_g = \frac{D_0}{\tau^2} \tag{46}$$

In Equation 46, D_g is the gel diffusion coefficient, D_0 the diffusion coefficient in the pure solvent at infinite dilution and τ the tortuosity. Typical values for tortuosity in agarose gels are in the range 1.2-1.4 (Gustavsson et al., 1998)

4.3.2. Porosity

The ratio between the available volume within a porous material and the total volume of the material is called the porosity, ε, of the material. Typical values for agarose based materials are 0.94-0.96. In other words, this is the volume fraction that small solutes and solvent molecules experience within the material. However, if the diffusing molecules are larger they might experience a much smaller "free" volume within the material. This difference in apparent porosities for

molecules of different sizes is the basis for the separation effect of gel filtration phases. Molecules larger than a certain threshold size are completely hindered from entering the porous material and will elute with the elution front. The effective diffusion coefficient, D_{eff}, can be calculated according to Equation 47.

$$D_{eff} = \varepsilon \frac{D_0}{\tau^2} \tag{47}$$

The tortuosity is however difficult to measure (Gustavsson et al., 1998). Normally the effective diffusion coefficient is determined experimentally.

4.4. Diffusion of proteins

Determination of diffusion coefficients of proteins is a difficult task due to the complex nature of these macromolecules. Proteins are made up of amino acids. The basic structure of an amino acid is an amino group and a carboxylic acid group coupled to an α-carbon. The α-carbon also carries a hydrogen atom and a variable side chain. The amino acids are linked together via amide bonds. The amide bonds of peptides are referred to as peptide bonds. The side chain determines the nature of the amino acid. The 20 natural amino acids can be divided into hydrophobic and hydrophilic residues. The hydrophobic residues include those with aliphatic side chains (i.e., alanine, valine, leucine, isoleucine, and methionine) and those with aromatic side chains (i.e., phenylalanine, tyrosine, and tryptophan). The hydrophilic amino acids can be further divided into neutral polar (i.e., serine, threonine, asparagine, and glutamine), acidic (i.e., aspartic acid and glutamic acid), and basic (i.e., histidine, lysine, and arginine). Cysteine and proline confer special properties to polypeptides. Cysteine contains a thiol group, which normally is oxidatively coupled to another thiol from another cysteine to form a disulfide bond. These bonds are important for the three-dimensional structure of proteins. The secondary amino acid proline affects the protein conformation due to its cyclic structure and alkylation of the amino group.

The neutral polar, acidic and basic amino acids are often found on the surface of the proteins while the non-polar ones are located in the hydrophobic core. The basic and acidic side chains on the surface give proteins complex charge patterns. The net charge of a protein depends on the pH of the environment. The pH where the net charge of a protein is zero is called the isoelectric point of the protein, denoted pI.

The diffusion of a charged protein in a gel is depending on the pH and the ionic strength of the solution (Raj and Flygare, 1974; Gaigalas et al., 1992; Mattisson et al., 2000). This influence can partially be overcome by increasing the ionic strength (in the range of 0.05 to 0.1 M salt concentration) of the solution to shield the charged groups on the protein. The choice of the salt strongly influences the determined diffusion coefficients.

4.5. Predicting diffusion coefficients

One of the most commonly used expressions to estimate diffusion coefficients in liquids is the Stokes-Einstein equation (Equation 48) (Bird et al., 1960), where k_B is Boltzmanns constant, T is the absolute temperature, μ is the viscosity of the fluid, R_S is the Stokes radius of the molecule, R is the ideal gas constant, and N_A is Avogadro's number.

$$D_0 = \frac{k_B \cdot T}{6\pi\mu R_s} = \frac{RT}{6\pi\mu R_s N_A} = 7.32 \cdot 10^{-25} \frac{T}{\mu R_s} \tag{48}$$

From Equation 40 it can be seen that the diffusion coefficient is linearly proportional to the temperature. However, the diffusion coefficient is also dependent on the viscosity, which in turn depends on the temperature exponentially. Thus the diffusion coefficient has a very strong temperature dependency.

It can also be seen that the diffusion coefficient is inversely proportional to the size of the molecule. One problem with using the Stokes radius as a measure of the protein size is that proteins seldom are spherical. They are usually more ellipsoidal. The Stokes radius entered into Equation 48 needs to be an average of the dimensions

of the protein. Furthermore, if the protein is hydrated the radius must be that of the hydrated complex, not the protein itself (Cussler, 1997).

4.6. Methods to determine diffusion rates

In the following section an overview of some common methods to determine diffusion rates are given.

4.6.1. Diffusion cell

The diffusion cell (Westrin, 1991; Gutenwik et al., 2004), also known as diaphragm cell or Ussing cell (Dunson, 1969; Brasselman et al., 1980), consists of two well-mixed compartments separated from each other by a film or membrane (Figure 5).

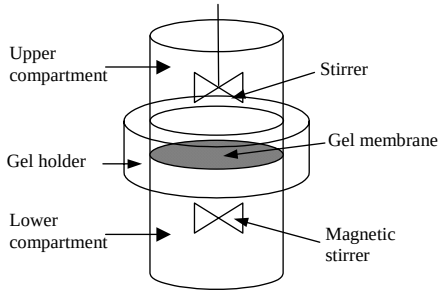


Figure 5. *The concept of the diffusion cell*

The film is made of the material that the molecules are to diffuse through. The cell can be operated in two modes, in steady-state or pseudo-steady-state. When operated in the steady-state mode, the concentrations on both sides of the membrane are kept constant by continuous pumping of the fluid in both compartments. This gives a real steady-state diffusional flux through the membrane. Concentration measurements are made on the solutions leaving the diffusion cell. When operated in the pseudo-steady-state mode, the compartments of the cell are loaded with solutions with known initial concentrations of the diffusant. The concentrations and hence the diffusional driving force are thus not constant over the time. The

diffusion rate is calculated from the concentration change as a function of time.

The effective diffusion (D_{eff}) can be described using Fick's first law rewritten in the following way (Equation 46). A represents the area of the film.

$$V \cdot \frac{\partial C}{\partial t} = -D_{eff} \cdot A \cdot \frac{\partial C}{\partial x} \tag{49}$$

This form of Fick's first law implies that D_{eff} and the volume, V, are constant. The disadvantage of this method is that the investigated material must be in the form of a film.

4.6.2. Confocal laser scanning microscopy

Diffusion measurements with confocal laser scanning microscopy (CLSM) (Moussaoui et al., 1992; Cutts et al., 1995; De Smedt et al., 1997; Pal et al., 2000; Schröder et al., 2005) is based on the visualization of diffusing molecules tagged with fluorescent groups. Confocal microscopy can be used in two different modes, differing in how the fluorescence is used, namely Fluorescence Recovery After Photo bleaching (FRAP-CLSM) (Moussaoui et al., 1992; Cutts et al., 1995; De Smedt et al., 1997) and the traditional way (CLSM) (Pal et al., 2000). When used in the CLSM mode the diffusant is added at the start of the experiment and is therefore initially distributed unevenly in the system. In a completely non-adsorbing system, diffusion will eventually even out the concentration gradient. When operated in the FRAP-CLSM mode, the diffusant is initially distributed evenly in the system. At the start of an experiment, the fluorescence within a small volume is irreversibly bleached with the laser and the recovery of fluorescence is considered to be due to diffusion of unbleached molecules into the bleached area.

The concept of confocal laser scanning microscopy is shown in Figure 6.

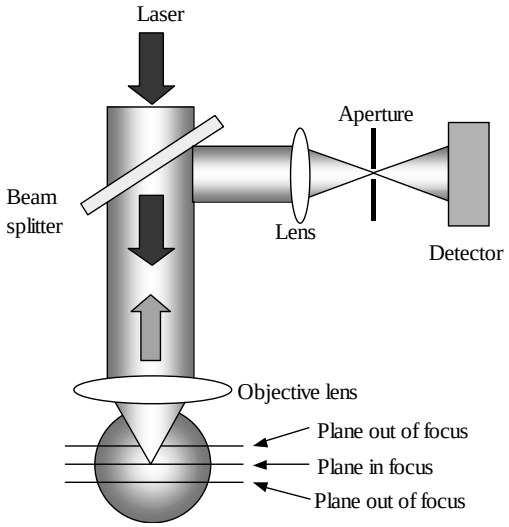


Figure 6. *The concept of confocal laser scanning microscopy.*

A laser beam passes through a beam splitter and is focused in a thin plane, that is, the focal plane, in the object that is studied. The returning light passes the same way back as the incident light but is deflected by the beam splitter and focused on an aperture that precedes the detector. The aperture guarantees that only the light from the focal plane reaches the detector. Light coming from planes above and below the focal plane falls outside the aperture. This set-up allows for the instrument to scan a sample in three dimensions and can thus produce a 3-D representation of the sample. Normally when used to determine diffusion coefficients, the sample is only scanned in two dimensions, usually through the central plane of the sample.

One of the advantages of confocal microscopy is that diffusion measurements can be performed on single beads. Provided that the concentration outside the bead can be considered constant and that the experiment is performed under non binding conditions, the only process observed is the diffusion. The concentration change is followed by measurement of the fluorescence from the tagged molecules. Confocal microscopy requires expensive equipment and trained personnel.

4.6.3. Holographic laser interferometry

Holographic laser interferometry (HLI) is a variant of traditional interferometry. In both traditional interferometry and HLI, two matched identical cuvettes, one reference cuvette and one sample cuvette, are required. In HLI, the reference cuvette is a hologram, instead of a real physical cuvette, which assures a pair of perfectly matched cuvettes. The holographic reference cuvette is a picture of the sample cuvette taken prior to the addition of the diffusant. The diffusive process is monitored photographically and a concentration profile is constructed from the interference pattern that occurs due to the change in refractive index, which results in a change in the optical pathway in the sample cuvette. The interference pattern is seen as a set of bands of light and dark fringes. Knowledge about the refractive index change as a function of the concentration is required. The concept of holographic laser interferometry is shown in Figure 7.

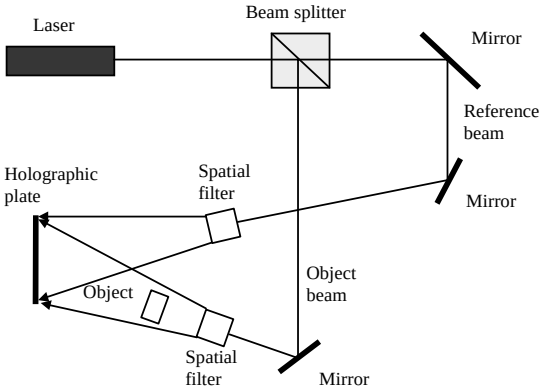


Figure 7. *The concept of holographic laser interferometry.*

A disadvantage of this method is that the object must be transparent at the wavelength of the laser light. Another disadvantage is that the solid material must be cast to the shape of the cuvette. This can give the material different properties than those present in beads. The HLI method can be used to determine diffusivities in liquids (Bochner and Pipman, 1976; Szydlowska and Janowska, 1981; Fenichel et al., 1984; Ruiz-Beviá et al., 1985; Gierow and Jernqvist, 1993) and in gels (Ruiz-Beviá et al., 1989; Gustafsson et al., 1993; Mattisson et al., 1996; Kong et al., 1997; Mattisson et al., 2000; Roger

et al., 2000). HLI requires special equipment and trained personnel for its execution. It is of great importance that the set-up is built in a stable and chock resistant way, for example, on a heavy slab of stone. The slightest vibration can cause the optical equipment to shift out of place.

4.6.4. Electronic speckle interferometry

Electronic speckle interferometry (ESPI) (Karlsson et al., 2002) is in principle the same method as HLI. The difference between the two methods is that while the interference pattern in HLI is created using a holographic image of the sample cuvette in its original state, the interference pattern in ESPI is calculated using two pictures of the sample cuvette, one in the original state (prior to the addition of the diffusant) and one at a later time. The way of evaluation is the same as for HLI and so is the need for the investigated gel to be transparent.

4.6.5. Dynamic light scattering

During the determination of diffusion coefficients by dynamic light scattering (DLS) (Sellen, 1986; Sellen, 1987; Fang and Brown, 1990; Placidi and Cannistraro, 1998; Grigsby et al., 2000; Annunziata et al., 2005) monochromatic coherent laser light passes through a cuvette containing a sample solution. As the light passes the cuvette some of the light will be scattered (Figure 8).

The wavelength of the scattered light will be slightly different than that of the laser light due to the fact that the molecules in the cuvette are moving. This wavelength shift is correlated to the rate of the molecules, that is, the diffusion rate in the case of an unstirred system. The equipment for DLS is expensive and requires trained personnel.

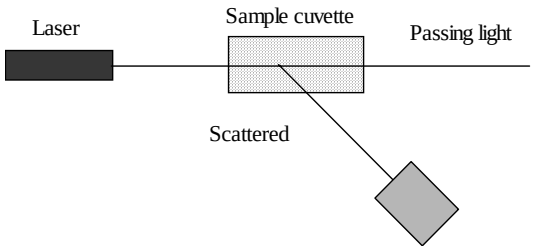


Figure 8. *The concept of dynamic light scattering.*

4.6.6. NMR

Several methods based on nuclear magnetic resonance (NMR) to determine diffusion rates have been reported in the literature. Electron paramagnetic resonance spectroscopy (EPR spectroscopy) (Khramtsov and Marsh, 1991) is an NMR method that detects spin-state changes in substances containing unpaired electrons. Another method used is pulsed magnetic field gradient NMR (PMFG-NMR). This method has been applied to determine diffusion rates in aqueous solutions (Krishnan, 1997; Nesmelova and Fedotov, 1998:1; Nesmelova and Fedotov, 1998:2) as well as in porous media (Gibbs et al., 1992; Coffman et al., 1997). These methods require expensive equipment and trained personnel for its execution.

4.6.7. Batch experiments

Stirred tank experiments can be used to study either the release or the uptake of substances (Tanaka et al., 1984; Chevalier et al., 1987; Merchant et al., 1987; Pu and Yang, 1988; Scott et al., 1989; Scott et al., 1989; Nguyen and Loung, 1986; Melick et al., 1987; Hulst et al., 1989; Merril et al., 1993; Bautista et al., 1998). If an uptake process is studied, a known amount of the solid-phasematerial investigated is added to a solution of known concentration of the diffusant. The decrease in concentration in the bulk liquid is then recorded. If a release process is studied, a know amount of the preloaded material is added to a known volume of solvent. The increase in concentration is then recorded. The equipment can be as simple as a test tube to more sophisticated systems with on-line detection. The rate of the stirring is important to minimize the effect of external

mass transfer resistance. The equipment for batch experiments can be found in any laboratory and does not require any special training by its performer.

4.6.8. Column experiments

Diffusion rate determinations using column experiments (Bautista et al., 1998; Larew and Walters, 1987; Bosma and Wesselingh, 2000; Paper II) have several advantages compared to other methods. The basic experimental set-up consists of a pump, a column, a detector, and a data recording system. This means that these experiments can be performed on a regular HPLC system. The choice of pumps and pump heads depends on the size of the column. More sophisticated set-ups can contain multi port-valves, injection valves, fraction collectors, and other components (Figure 9, Chapter 5).

The advantage of column experiments over batch experiments is that the environmental parameters (i.e., the mass transfer from bulk to bead surface and the dispersion in the mobile phase due to the convective flow around the beads) are identical to the parameters in a real separation. However, these parameters must either be determined in separate experiments or be calculated using empirical correlations in order to determine the diffusion rate. Key to the success of the method is that the experiments are carried out under non-adsorbing conditions. This is achieved by either using a non-functionalized stationary phase (i.e., the stationary phase is void of interacting ligands), or the mobile phase composition prevents adsorption. Determination of diffusion coefficients using column experiments can be performed either as a pulse response experiment or as a step response experiment, the latter also known as frontal chromatography. In a pulse response experiment, a finite amount of sample is injected as a plug and the eluted peak is evaluated by fitting of a suitable model. In frontal chromatography experiments, the mobile phase is changed to one containing the investigated solute, so that a steep step change is applied to the column. The resulting front eluting from the column is evaluated by fitting of a suitable model. Frontal chromatography experiments are detail in Chapter 5.

5. Determination of diffusion coefficients using frontal chromatography

Frontal chromatography can be used to determine diffusion coefficients. Equipment designed for this purpose and used in Paper II is shown in Figure 9.

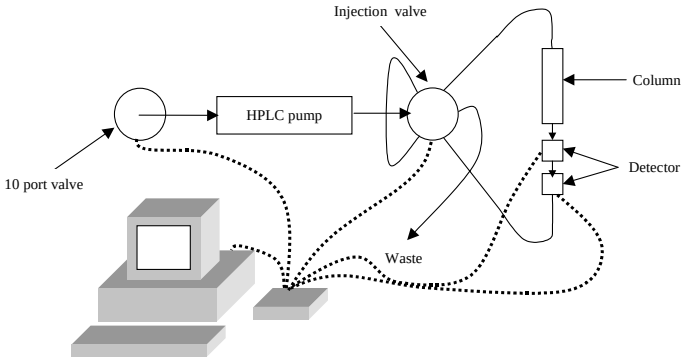


Figure 9. *Schematic representation of the experimental set-up used for determination of diffusion coefficients using frontal chromatography. The equipment consisted of a double piston pump (2248 HPLC pump, LKB, Bromma, Sweden), a ten-port valve (C15-6110EMH, Valco Instruments Co. Inc., Houston, TX, USA) used as a solution selection switch valve, a six-port injection valve (C2-1036H, Valco Instruments Co. Inc., Houston, TX, USA) was used to assure that the concentration change entering the column was as close to a step change as possible. Monitoring of the concentration of the liquid flow leaving the column was achieved with two UV-detectors (UV-MII, Pharmacia Biotech). A DAQPad-1200 (National Instruments, Austin, TX, USA), connected to the computer's parallel port, was used as an interface between the control program running on the computer and the valves and to collect the signals from the detectors.*

Frontal chromatography is a chromatographic method where a large volume of solute is applied to the column. The volume is large

enough to allow for the concentration applied to the column to equal the concentration at the column outlet. This assures that the concentration of solute in the mobile phase is constant throughout the column. This technique is often used to fully load affinity chromatography columns.

To fully characterize a gel filtration system, four different breakthrough curves need to be generated to determine the relevant volumes of the system. The elution volume (V) is determined from the frontal chromatogram by integration of the area above and to the left of the breakthrough curve. Figure 10 shows three theoretical breakthrough curves: (a) one generated with the column end pieces adjusted to be in contact with each other, (b) one for a molecule or particle not penetrating the stationary phase, and (c) one for a molecule species that fully penetrates the stationary phase.

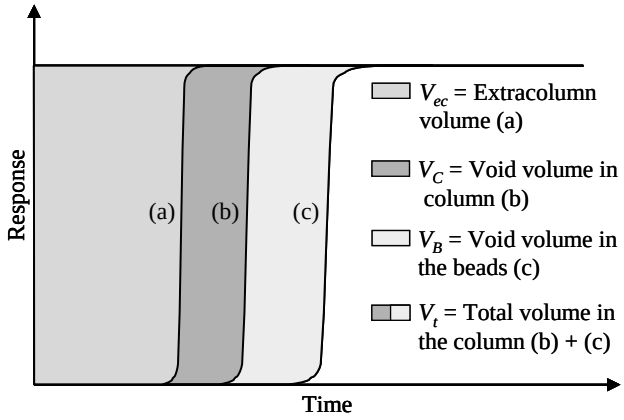


Figure 10. *A schematic representation of elution volumes, V, in frontal chromatograms, (a) empty column with the end pieces adjusted to be in contact with each other, (b) and (c) packed column.*

The extracolumn volume (V_{ec}) is determined from the breakthrough curves of acetone or polystyrene latex particles on an empty column with the flexible end pieces adjusted so that they are in contact with each other (breakthrough curve (a) in Figure 10). In order to check the column for unexpected dispersion behavior it is advisable to record breakthrough curves for acetone as well as polystyrene latex particles. The void volume of the packed column (V_C) is determined from the breakthrough curves of polystyrene latex particles not penetrating the stationary phase (breakthrough curve (b) in Figure

10). The total volume in the column (V_t) is determined with acetone. Acetone can fully penetrate the packing material and thus gives the total volume in the packed bed excluding the volume of the solid material in the packing material (breakthrough curve (c) in Figure 10). Finally, the breakthrough curves for the substances of interest are determined.

The breakthrough curves obtained for acetone and polystyrene latex particles with the column end pieces adjusted so that they are in contact with each other is used to determine the extracolumn volume along with an appropriate number of fictive tanks in the tanks-in-series model (Equation 34, or Equation 36 and Equation 37) by the means of least square fitting.

The volume in the bed external to the packing material, that is, the bed void, can be determined by two approaches from the breakthrough curve of polystyrene latex particles. In the first approach, the area left of and above the breakthrough curve is integrated (as shown in Figure 10). The other is to fit Equation 50 (which is Equation 1 without the transport to the beads) to the breakthrough curve by varying the volume as well as the axial dispersion coefficient in the bed. The advantage with the latter approach is that the dispersion coefficient is found at the same time as the volume. The two approaches should ideally give the same result. If the first approach is applied, an empirical correlation should be used to calculate a value for the dispersion coefficient.

$$\frac{\partial C_L}{\partial t} = D_{AX} \cdot \frac{\partial^2 C_L}{\partial l^2} - v_{INT} \cdot \frac{\partial C_L}{\partial l} \tag{50}$$

The easiest way to determine the total bed volume is to integrate the area above and left of the breakthrough curve for acetone. The difference between the volumes determined from the acetone breakthrough curve and the breakthrough curve for polystyrene latex particles represents the maximum accessible volume (V_B) inside the beads excluding the volume of the polymer network constituting the stationary phase.

The final step in this procedure is to record a breakthrough curve for the investigated substance. By integration of the area above and to

the left of the breakthrough curve the accessible part, that is, ε_p, of the packing material for the substance can be determined.

The effective diffusion coefficient is determined by fitting the complete model accounting for the extracolumn volume (Equation 34, or Equation 36 and Equation 37), the model for the column (Equation 1) and the model for the packing material (Equation 51) to the breakthrough curve of the substance by varying the effective diffusion coefficient until the best fit is obtained.

$$\frac{\partial C_P}{\partial t} = \frac{D_{Eff}}{\varepsilon_P} \cdot \left(\frac{\partial^2 C_P}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial C_P}{\partial r} \right) \tag{51}$$

6. Molecular recognition

One of the corner-stones of life is recognition. Living cells and organisms need to be able to distinguish between good and evil, friend and foe, and food and poison. The phenomenon of recognition occurs on a macroscopic level as well as on a microscopic one. For example, the ability to recognize a familiar face is practical in our everyday life and the recognition of a transmitter substance by its receptor is essential for the function of the nervous system.

Molecular recognition is the creation of a complex between a host molecule and a guest molecule. The interactions involved in the complex formation can be either covalent or non-covalent. The latter interactions include hydrophobic interactions, van der Waals interactions, hydrogen bonds, and ionic interactions.

A sophisticated example of molecular recognition is the ability of the immune system to distinguish between self and non-self. The antibodies play a crucial role in this process and are produced as a result of stimuli in the form of antigens, such as bacteria, viruses, pollens, or foreign molecules. A theory for the process of formation of antibodies was reported by Linus Pauling in 1940 (Pauling, 1940). He suggested that the antigen acts as a template during the folding of the polypeptide chain constituting the antibody. Although Pauling's theory was incorrect, his ideas are strikingly similar to the concept of molecular imprinting.

Closely related to the work of Pauling on antibodies is the study by his student Frank Dickey published in 1949 (Dickey, 1949). In this paper Dickey, presented a method to polymerize sodium silicate in the presence of dye molecules. After removing the dyes, the silica materials bound preferentially the respective dye present during the polymerization.

Molecular recognition elements find use in a broad range of applications, for example, analysis, diagnosis, sensing, monitoring,

drug delivery, trace enrichment, purification, and separation. The need for molecular recognition elements can to a certain degree be satisfied by naturally occurring species, for example, enzymes, antibodies, and receptors. Naturally occurring molecular recognition elements do, however, suffer from limitations, such as, low stability in organic media, extreme pHs, and elevated tempteratures, they may be difficult and/or expensive to isolate, or simply do not exist for the intended target molecule. Since the papers by Pauling and Dickey, large efforts have been put into the development of synthetic receptors and molecular recognition elements. These efforts have resulted in molecular recognition elements based on supramolecular assemblies such as crown ethers, cyclophanes, and molecular clefts and cavities (Lehn, 1988; Rebek, 1990; Cram, 1992; Webb and Wilcox, 1993; Conn and Rebek, 1997); de novo designed peptides and proteins (Baltzer, 1998); aptamers (Osborne et al., 1997; Celia, 2004); ligands obtained through combinatorial methods (Lowe et al., 2001; Labrou, 2003; Srinivasan and Kilburn, 2004; Schmuck and Wich, 2006); and molecularly imprinted polymers (MIPs).

7. The concept of molecular imprinting

Molecular imprinting is a technique to synthesize polymers capable of selective molecular recognition and binding. The polymers are synthesized in the presence of a template, sometimes referred to as the print molecule. Figure 11 shows the procedure, starting with dissolution of template, functional monomers, cross-linking monomers, and initiator in a porogenic solvent. The functional monomers are chosen so that they complement the functionalities of the template molecule. The monomers will arrange themselves spatially around the template (step 1 in Figure 11). The positions of the monomers are made permanent by copolymerization with cross-linking monomers (step 2). The polymerization is most often carried out as a free-radical polymerization initiated by photolytic or thermolytic homolysis of peroxide or azo compounds. The polymerization runs through the chain-reaction steps, that is, initiation, propagation, and termination.

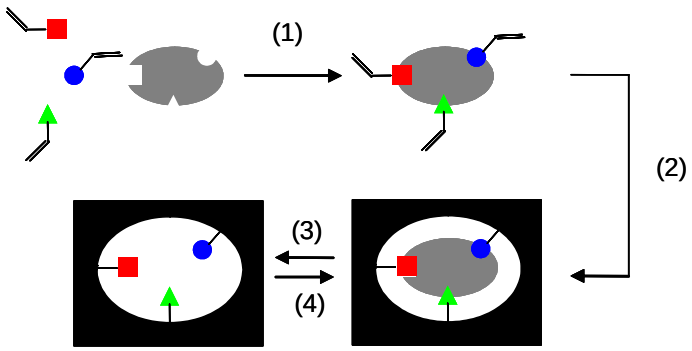


Figure 11. *The principle of non-covalent molecular imprinting: (1) arrangement of monomers around the print molecule, (2) cross-linking of monomers, (3) removal and (4) rebinding of print molecule.*

After the completion of the polymerization step, the template is removed from the polymer by extraction (step 3). The resulting polymer is then able to selectively rebind the template molecule (step 4).

Traditionally, molecular imprinting is classified according to the nature of the interactions between the monomers and the template during polymerization, i.e., non-covalent, covalent, or metal ion mediated. Semi-covalent molecular imprinting is a special case of covalent imprinting where the interactions during the imprinting procedure are covalent while the interactions during the rebinding are non-covalent.

The different classes of molecular imprinting are detailed in this chapter. The following chapters exemplify the formatting (Chapter 8), the design (Chapter 9), and the characterization (Chapter 10) of MIPs. Finally, Chapter 11 gives examples of applications of MIPs.

7.1. Non-covalent molecular imprinting

The non-covalent MIPs (Arshady and Mosbach, 1981) utilize the same kind of interactions as those present in biological recognition systems. The monomers interact with the print molecules via non-covalent interactions, for example, hydrogen bonding, ion-pairing, hydrophobic interactions, and dipole-dipole interactions. The interactions are of non-covalent nature during the synthesis of the polymer as well as during the subsequent rebinding. The monomers can be acidic, basic, or neutral. The most widely applied monomer in non-covalent imprinting so far is methacrylic acid. An overview of other commonly used monomers is given in Section 9.1.

The non-covalent molecular imprinting approach has been applied to a broad range of templates, including free amino acids (Vidyasankar et al., 1997; Arnold, 1997), protected amino acids (Sellergren et al., 1985; O'Shannessy et al., 1989a; O'Shannessy et al., 1989b; O'Shannessy et al., 1989c; Sellergren, 1989b; Andersson et al., 1990a; Andersson et al., 1990b), herbicides (Piletsky et al., 1994; Muldoon and Stanker, 1995; Matsui et al., 1995a; Matsui et al., 1995b; Siemann et al., 1996; Baggiani et al., 2000a), pesticides (Baggiani et al., 1999), fungicides (Liu et al., 2005), narcotics (Andersson et al., 1995), antibiotics (Levi et al., 1997; Senholdt et al., 1997; Siemann and

Andersson, 1997; Skudar et al., 1999; Lai et al., 2002; Cederfur et al., 2003; Urraca et al., 2006), barbiturates (Tanabe et al., 1995), and steroids (Ramström et al., 1996; Sreenivisan, 1998; Baggiani et al., 2000b; Kugimiya et al., 2001). The non-covalent approach is not restricted to small molecules but has also been applied to the imprinting of larger molecules such as proteins (Hjertén et al., 1997; Liao et al., 1996; Shi et al., 1999) and even bacteria (Aherne et al., 1996).

Since the interactions between the monomers and the templates are of weak nature, several combinations of monomer-template complexes exist in the pre-polymerization mixture. In addition, the monomers are often present in an excess, which will result in randomly distributed non-specific binding sites. After polymerization this plurality will also exist in the binding sites of the polymer; the sites are heterogeneous. This heterogeneity can be observed as a distribution of affinities for rebinding of the template.

7.2. Covalent molecular imprinting

In the covalent approach of molecular imprinting, one or more polymerizable groups are coupled covalently to functionalities on the template to form a polymerizable template-monomer complex. Upon completion of the polymerization, the template is cleaved from the resulting polymer and extracted. This leaves a polymer with positioned functional groups capable of re-forming the covalent bonds under the correct conditions. Theoretically, this approach will give recognition sites with very similar affinity for the template throughout the polymer. The recognition sites are thus more homogenous than those formed during non-covalent molecular imprinting. The kinetics of re-binding is often quite slow. In order to be a practical method, the cleavage and condensation reactions should occur under rather mild conditions.

The most succesfull approach in covalent molecular imprinting is probably the coupling of boronic acids to hydroxyl groups on the template to form boronate esters (Figure 12). This approach has, for example, been applied to the imprinting of glyceric acid (Wulff and Sarhan, 1972) and sugars (Wulff et al., 1973; Wulff et al., 1977; Wulff and Schauhoff, 1991).

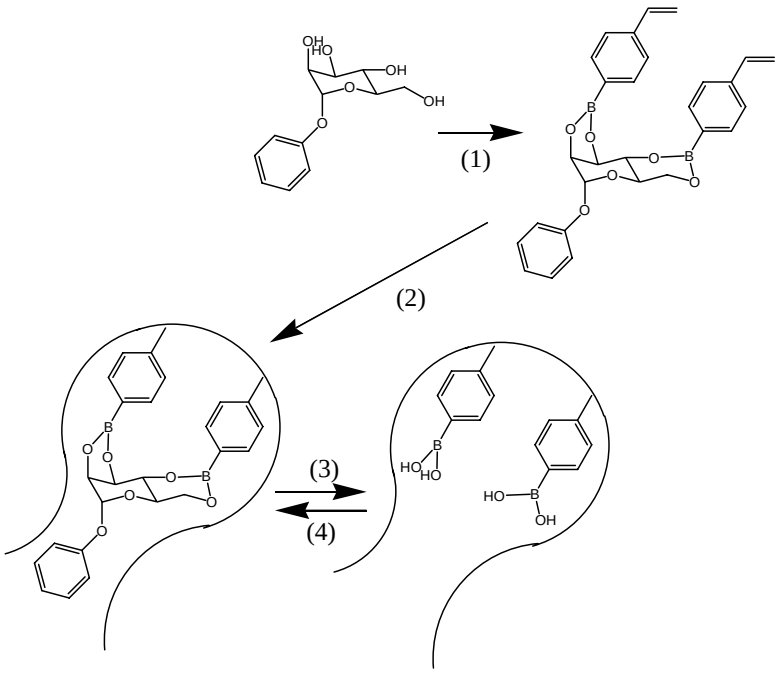


Figure 12. *Imprinting of phenyl α-D-mannopyranoside using (4-vinylphenyl)-boronic acid. Formation of monomer-template complex (1), polymerization (2), cleavage and extraction (3), and rebinding (4).*

Templates containing diols can be reacted with monomers carrying a carbonyl functionality to form acetals or ketals. In the same way, carbonyl containing templates can be derivatized with polymerizable groups by reaction with diol containing monomers. This approach has, for example, been applied to the imprinting of mono- and di-ketones (Shea and Dougherty, 1986; Shea and Sasaki, 1989; Shea and Sasaki, 1991; Marty et al., 1999) and alcohols (Wulff and Wolf, 1986; Reppy and Gin, 1998).

Schiff's bases contain an amide bond that is readily reversible. Schiff's bases are formed between primary amines and carbonyl compounds. The formation of Schiff's bases has been utilized in the imprinting of, for example, amino acid derivatives (Wulff et al., 1984; Wulff and Vietmeier, 1989), mono- and di-aldehydes (Wulff et al., 1986; Shea et al., 1990), and aromatic compounds (Katz and Davis, 2000).

7.3. Semi-covalent molecular imprinting

In semi-covalent molecular imprinting, the imprinting procedure is accomplished in the same way as in the covalent approach. The rebinding, however, relies on non-covalent interactions (Sellergren and Andersson, 1990). The semi-covalent approach has successfully been used for the preparation of MIPs selective for p-aminophenyl-alanine ethyl ester (Sellergren and Andersson, 1990) and triazines (Cacho et al., 2006). When the template moiety is cleaved off from the polymer, the resulting space might be smaller than what would be optimal for the formation of non-covalent interactions during the rebinding. This problem was addressed by Whitcombe in 1994 when the sacrificial spacer approach to semi-covalent molecular imprinting was introduced (Whitcombe et al., 1994).

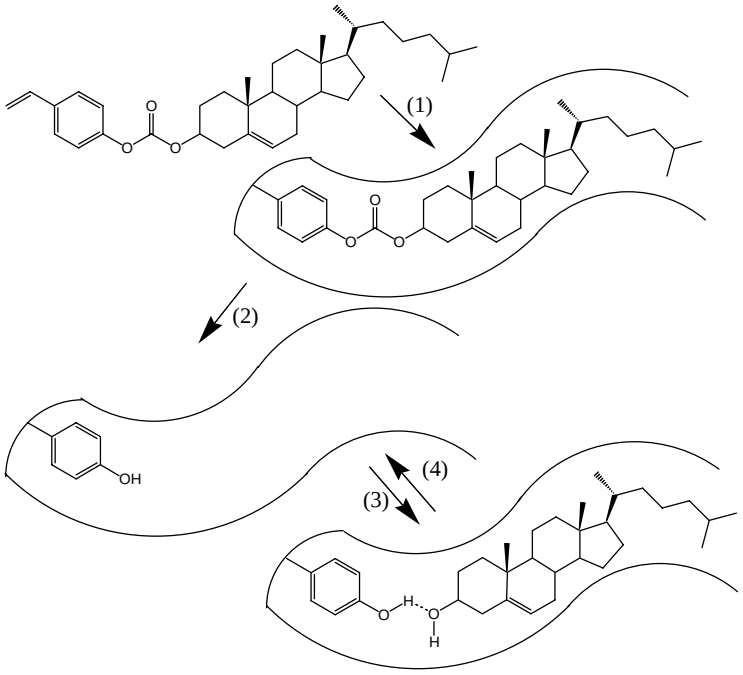


Figure 13. *Semi-covalent molecular imprinting of cholesterol using the sacrificial spacer approach. Polymerization (1), cleavage and extraction (2), rebinding (association) (3), and dissociation (4).*

In this approach, part of the monomer is also cleaved when the template is cleaved from the polymer (Figure 13). The sacrificial

spacer approach has been applied to the imprinting of cholesterol (Whitcombe et al., 1995), DDT (Graham et al., 2002), and heterocyclic aromatic compounds (Kirsch et al., 2004).

7.4. Metal ion mediated molecular imprinting

The majority of molecular imprinting polymerizations described so far are performed in organic solvents and the resulting polymers thus perform best in organic solvents. Template molecules insoluble in organic solvents are not readily imprinted by conventional methods. A feasible method for such templates is the use of metal coordinating (chelating) monomers. Figure 14 shows the use of the monomer [N-(4-vinylbenzyl)imino]-diacetic acid for the imprinting of histidine containing molecules (Dhal and Arnold, 1992; Kempe et al., 1995).

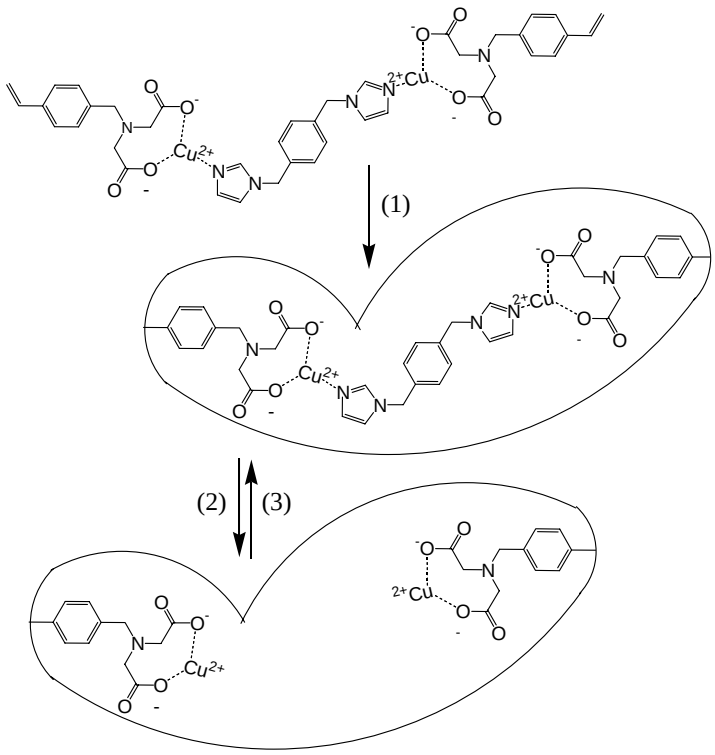


Figure 14. *Example of metal ion mediated molecular imprinting, Cross-linking and polymerization (1), extraction (2), and rebinding of template (3).*

8. Formats of molecularly imprinted polymers

The most common procedure to prepare MIPs is to synthesize a network in which functional monomers arranged around a template have been cross-linked. The spatial arrangement of the functional monomers creates binding sites capable of rebinding the template. This method requires that the template can diffuse within the network. If the template has restricted mobility in the network, imprinting of it is not a viable option. An option is imprinting on surfaces. The approach with imprinted surfaces has for example been used for enzymes and proteins such as ribonuclease A (Kempe et al., 1995) and lysozyme (Hirayama et al., 2001). Surfaces imprinted with ampicillin have been shown to demote bacterial growth (Sreenevisan, 2005).

8.1. Irregular particles

The traditional way to synthesize MIPs is via, what in the molecular imprinting world is called, bulk polymerization. This is the method conceived by the pioneers Wulff, Mosbach, and Shea (Wulff and Sarhan, 1971; Arshady and Mosbach, 1981; Shea and Dougherty, 1986). The template, the functional monomer(s), the cross-linker, and the initiator are dissolved in the porogen. This mixture is hereafter referred to as the pre-polymerization solution or pre-polymerization mixture. The polymerization is carried out in a sealed container. After completion of the polymerization, the container is broken and the polymer monolith is coarsely crushed, then ground to particles, and sieved. This procedure often needs to be repeated several times to transform the entire polymer to particles of the appropriate size range. After sieving the particles, the template molecules are extracted from the polymer. Finally, the polymer particles are dried. The drawbacks with bulk polymerization include the time consuming procedure of grinding and sieving, the substantial loss of polymer due to the repeated grindings, and not least the fact that the shape of the resulting particles are unpredictable.

In the strictest polymer chemistry sense this is not a real bulk polymerization but rather a solution polymerization since the pre-polymerization mixture contain a solvent, the porogen. The porogen is present in order to make the polymer porous enough for easy mass transfer within the polymer. In Figure 15 a scanning electron micrograph of irregular particles prepared in this way is shown.

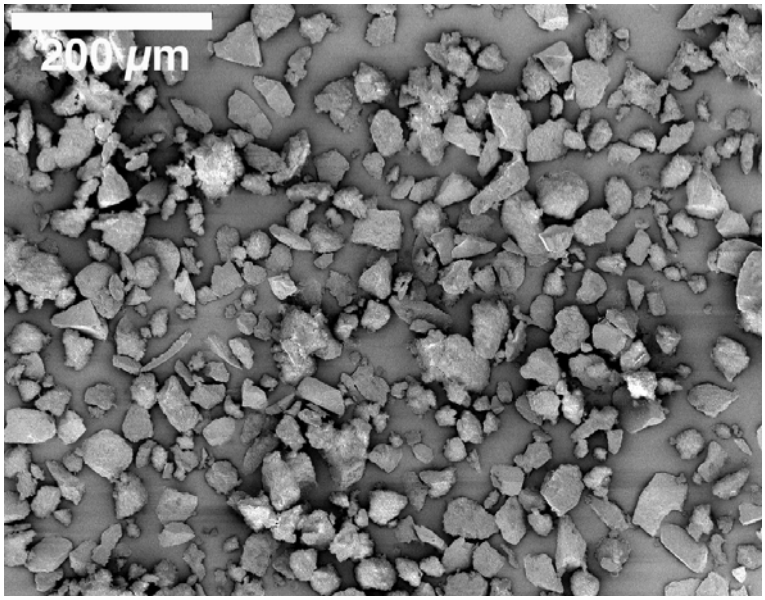


Figure 15. *Scanning electron micrograph of irregular particles (illustration from Paper IV). Accelerating voltage: 5 kV; Working magnification 150×.*

8.2. Beads

Spherical beads have superior hydrodynamic and diffusion properties compared to irregularly shaped particles. It is hence of great interest to synthesize MIPs in the shape of spherical beads, especially for applications where the beads are to be used in flow-through applications. Several methods exist to synthesize polymers in the shape of spherical beads. The techniques are often classified according to the initial state of the polymerization mixture, i.e., whether it is homogeneous (precipitation polymerization and dispersion polymerization) or heterogeneous (emulsion polymerization and suspension polymerization). There are several other techniques to prepare spherical MIP beads, of which the

techniques of two-step swelling, core-shell polymerization, and the preparation of composite beads will be exemplified.

8.2.1. Polymerization of beads from a homogeneous mixture

When beads are synthesized by polymerization from a homogeneous mixture, a pre-polymerization mixture is prepared in the same way as during a bulk polymerization, that is monomer(s), cross-linker(s), initiator and template molecule are dissolved in a solvent. The pre-polymerization mixture is, however, in this case further diluted with the solvent so that polymer beads are formed rather than a bulk polymer during the polymerization. In that sense, it is more reasonable to use the term 'polymerization medium' instead of 'porogen' when referring to the solvent.

Two different polymerization methods for beads from homogeneous mixtures exist; dispersion polymerization and precipitation polymerization. In both of the methods, the initiation and nucleation takes place in the polymerization medium. The difference between the two methods emanates from how good a solvent the polymerization medium is for the resulting polymer nuclei. In precipitation polymerization, the nuclei do not swell in the polymerization medium. The particle growth proceeds via coagulation of the nuclei into larger particle aggregates. The resulting particles will thus be irregularly shaped and polydisperse. MIP beads by precipitation polymerization were pioneered by the Mosbach group (Ye et al., 1999a).

The nuclei produced during dispersion polymerization swell in the polymerization medium and the particle growth takes place to a large extent in the swollen particles. The resulting particles are spherical and can be made monodisperse by the addition of stabilizers to the polymerization medium. Criteria for stabilizers used in dispersion polymerization are that they should have low solubility in the polymerization medium and moderate affinity for the polymer particles.

8.2.2. Polymerization of beads from a heterogeneous mixture

Two liquids that are immiscible or nearly immiscible with each other can be made to form an emulsion by mixing. The liquid forming the droplets is called the dispersed phase and the surrounding liquid is referred to as the continuous phase. Emulsions can be of the oil-in-water or water-in-oil types depending on the characteristics of the two liquids. Emulsions are by nature often unstable and hence often needs to be stabilized by the use of an emulsifier. The emulsifier is absorbed at the interface between the two liquids and forms a protective layer around the droplets. The layer lowers the rate with which the droplets coalesce and thus effectively stabilize the emulsion. Other ways to stabilize emulsions are by increasing the viscosity of either the continuous or the dispersed phase. Increased viscosity of the continuous phase reduces the movement of the droplets and thus lowers the rate of coalescence. This can in general be achieved by addition of viscosity modifiers such as xanthan gums, clays, or gelatin. Increased viscosity of the dispersed phase results in more rigid droplets that are less prone to coalesce.

Heterogeneous polymerization can be further divided into emulsion and suspension polymerization. In both cases the monomers are dissolved in the dispersed phase. The initiator is dissolved in the dispersed phase in suspension polymerization, whereas the initiator is dissolved in the continuous phase in emulsion polymerization. This leads to nucleation and growth of the beads taking place in the continuous phase in the case of emulsion polymerization and in the droplets in the case of suspension polymerization. Suspension polymerization was described already in 1909 in a patent by Hoffman and Delbruch. The technique has been used by several investigators for preparation of MIPs, for example Mayes and Mosbach (1996), Lai et al. (2002), and Strikovsky et al. (2003).

In Paper III and IV a suspension polymerization method for the synthesis of MIPs is described. The method uses mineral oil as the continuous phase and acetonitrile as the porogen. No addition of stabilizing agents or emulsifiers is needed due to the high viscosity of the mineral oil. In Figure 16 a scanning electron micrograph of beads prepared by this method is shown.

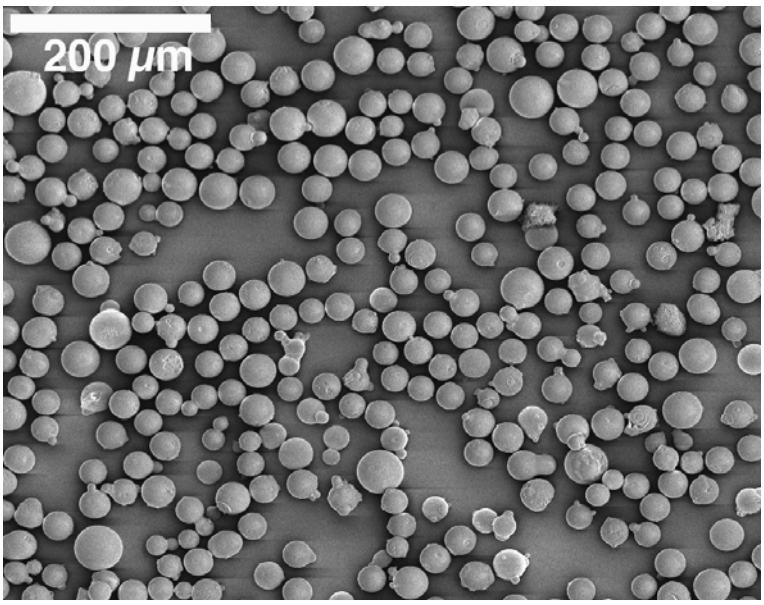


Figure 16. *Scanning electron micrograph of beads prepared by the method described in Paper III and Paper IV (illustration from Paper IV). Accelerating voltage: 5 kV; Working magnification 150×.*

8.2.3. Two-step swelling polymerization

Two-step swelling polymerization is the first of two seed particles methodologies that well be described in this thesis. It is a method to produce monodisperse particles. Sub-micron sized non cross-linked seed particles, prepared by emulsion polymerization, are added to a pre-polymerization mixture. The seed particles swell in the pre-polymerization mixture and the polymerization takes place within the volume of the swollen seed particle (Hosoya et al., 1994).

8.2.4. Core shell polymerization

Core shell polymerization is a seed particle polymerization variation of emulsion polymerization. The seed particles are suspended in the continuous phase. The pre-polymerization solution of monomer(s), cross linker(s), template, and initiator is added to the particle suspension as an emulsion prepared in the continuous phase. The mixture is stirred until the polymerization has completed. The addition of pre-polymerization solution is repeated several times so

that the size of the resulting spheres reaches the desired range (Pérez et al., 2000; Pérez et al., 2001).

8.2.5. Composite beads

Composite MIP beads can be synthesized in the pores of pre-made porous particles. Porous silica particles are often used for this purpose. To ensure that the MIP forms a unit with the particle, the particles are often first chemically modified by coupling of polymerizable groups or initiator molecules to the particle surface (Norrlöv et al., 1984; Otsu et al., 1986; Sulitzky et al., 2002). The use of immobilized initiators is often referred to as the iniferter (initiator-transfer agent-terminator) approach. The iniferter approach for molecularly imprinted polymer beads was pioneered by the Sellergren group (Rückert et al., 2002). It has for example been used for the imprinting of phenylalanine anilide (Sellergren et al., 2002), theophyliine (Hattori et al., 2004), pyrimethanil (Baggiani et al., 2005), and proprazine (Tamayo et al., 2005). When MIPs are polymerized in silica particles, it is possible to remove the silica after polymerization by dissolution with hydrofluoric acid, resulting in porous molecularly imprinted polymer beads (Yilmas et al., 2002, Titirici et al., 2002). MIP composites have also been made using for example chitosan as the support (Guo et al., 2004; Guo et al., 2005).

8.3. Films and membranes

MIPs can be synthesized as films and membranes. Films are polymerized on flat surfaces with or without polymerizable groups attached to them. The advantage with attached polymerizable groups on the surface is that the film will be covalently bound to the surface. MIP films are usually used as sensing elements in MIP based sensors. An example of this was reported by Jakush et al. (Jakush et al., 1999) where a thin MIP film selective for the herbicide 2,4-dichloro-phenoxyacetic acid (2,4-D) was synthesized on a zinc selenide crystal. Other examples of this will be given in Chapter 11 where applications of MIPs are presented.

Molecularly imprinted membranes can either be prepared as thicker ffilms or as composites in the pores of base-membranes. Preparation of membranes as composites combines the selective properties of the

imprinted material with the properties of the base-membrane. Membranes can also be prepared by phase inversion polymerization. Utilizing the selective nature of MIPs membrane can be made selectively permeable (Silvestri et al., 2004; Wang et al., 2004).

9. Design of molecularly imprinted polymers

When a molecularly imprinted polymer is to be designed only one component is given at the start, namely the template. The other components that can be varied in the system are the choice of the monomer(s), the cross-linker(s), the porogen, and to some extent the initiator.

9.1. Functional monomers

The selectivity of a MIP arises from the interactions between the template and the functional monomer(s). The functional monomer(s) are chosen so that their functionality complements the functionalities of the template. Table 1 summarizes some commonly used monomers. The most commonly used functional monomer is meth-acrylic acid.

Table 1. *Functional monomers used in non-covalent molecular imprinting.*

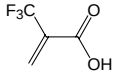
Name	Structure	References
Acrylic acid (AA)		Andersson et al., 1985 Sellergren et al., 1985
Methacrylic acid (MAA)		Sellergren et al., 1988 O'Shannessy et al., 1989 Sellergren, 1989a Matsui et al., 1995 Ramström et al., 1996
(2-Trifluoro-methyl)acrylic acid (TFMAA)		Matsui et al., 1995b Matsui et al., 1997

Table 1. (Continued)

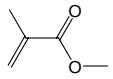
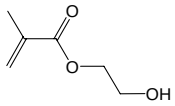
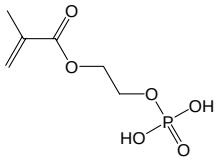
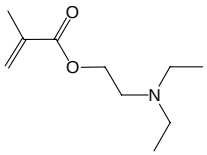
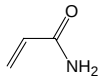
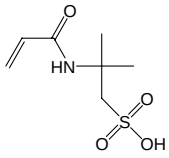
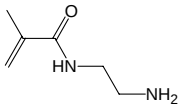
Name	Structure	References
Methyl methacrylate (MMA)		Andersson and Mosbach, 1989
2-Hydroxyethyl methacrylate (HEMA)		Sreenivisan, 1998
2-(Methacryloyl-oxy)ethyl phosphate (MEP)		Kugimiya et al., 2001
N,N-diethylamino-ethyl methacrylate		Levi et al., 1997
Acrylamide		Yu and Mosbach, 1997 Yu et al., 1997 Liu et al., 2006
2-Acrylamido-2-methyl-1-propane-sulphonic acid (AMPSA)		Dunkin et al., 1993
N-(2-aminoethyl) methacrylamide		Spivak and Shea, 1999

Table 1. (Continued)

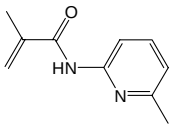
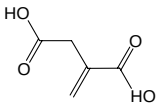
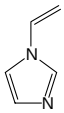
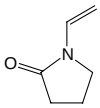
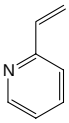
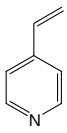
Name	Structure	References
2-Methyl-N-(6-methyl-pyridine-2-yl)-acrylamide (MAP)		Ju et al., 1999a
Allyl alcohol		Joshi et al., 1999
Itaconic acid		Fischer et al., 1991
1-Vinylimidazole		Kempe et al., 1993
Vinyl pyrrolidone		Takagishi et al., 1982
2-Vinylpyridine		Sarhan and El-Zahab, 1987 Ramström et al., 1993
4-Vinylpyridine		Kempe et al., 1993

Table 1. (Continued)

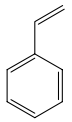
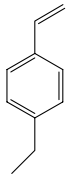
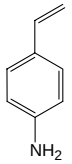
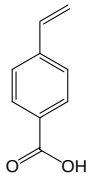
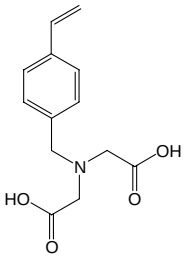
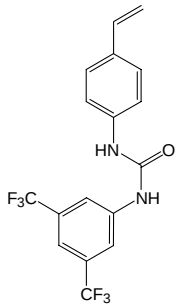
Name	Structure	References
Styrene		Andersson, 1988
4-Ethyl styrene		Andersson et al., 1984
4-Amino styrene		Ju et al., 1999a Ju et al., 1999b
p-Vinyl benzoic acid		Andersson et al., 1984
[N-(4-vinyl-benzyl)imino]-diacetic acid		Dhal and Arnold, 1992 Kempe et al., 1995

Table 1. (Continued)

Name	Structure	References
1-(3,5-Bis-trifluoro-methyl-phenyl)-3-(4-vinyl-phenyl)-urea		Hall et al., 2005 Urraca et al., 2006

9.2. Cross-linking monomers

Several molecules containing two or more polymerizable groups have been used as cross-linking monomers in molecular imprinting. The purpose of the cross-linking monomer is to lock the position of the functional monomers relative to the template in order to form the recognition sites. A cross-linking monomer is usually chosen so that it does not interact with the template. Table 2 summarizes some commonly used cross-linking monomers. Among the cross-linking monomers given in Table 2, ethylene glycoldimethacrylate (EDMA) is so far the most commonly used.

Table 2. *Cross-linking monomers.*

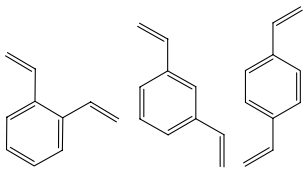
Name	Structure	Reference
Divinylbenzene (DVB)		Andersson et al., 1984

Table 2. (Continued)

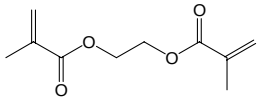
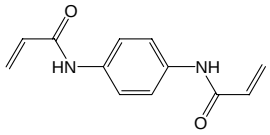
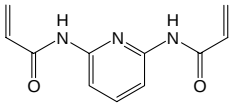
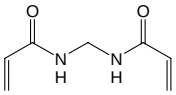
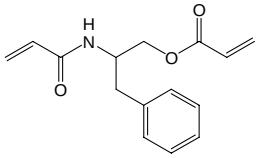
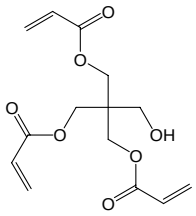
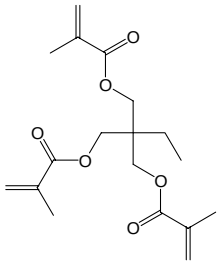
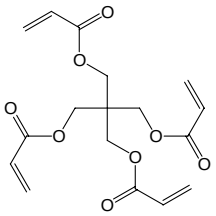
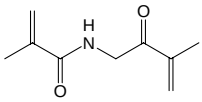
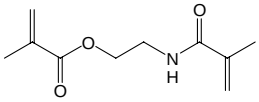
Name	Structure	Reference
Ethylene glycol-dimethacrylate (EDMA)		Sellergren et al., 1985 Sellergren et al., 1988 O'Shannessy et al., 1989
N,N'-1,4-phenylenedi-acrylamide		Norrlöw et al., 1984
2,6-Bis(acryl-amido)pyridine		Tanabe et al., 1995
N,N'-methylenedi-acrylamide		Norrlöw et al., 1984
N,O-bisacryloyl-L-phenylalaninol		Andersson et al., 1985
Pentaerythritol triacrylate (PETRA)		Kempe, 1996

Table 2. (Continued)

Name	Structure	Reference
Trimethylol-propane trimeth-acrylate (TRIM)		Kempe and Mosbach, 1995 Kempe, 1996 Ramström et al., 1996
Pentaerythritol tetraacrylate (PETEA)		Kempe, 1996
N,α-bis-methacryloyl glycine (NAG)		Sibrian-Vazquez and Spivak, 2003 Sibrian-Vazquez and Spivak, 2004a
N,O-bis-methacryloyl ethanolamine (NOBE)		Sibrian-Vazques and Spiva, 2003 Sibrian-Vazquez and Spivak, 2004a Sibrian-Vazquez and Spivak, 2004b

As stated earlier, the cross-linking monomer(s) are usually chosen so that they do not interact with the template. N,α-bismethacryloyl glycine (NAG) and N,O-bismethacryloyl ethanolamine (NOBE), however, has been shown to possess interesting qualities in forming interactions with templates (Sibrian-Vazquez and Spivak, 2003; Sibrian-Vazquez and Spivak, 2004a; Sibrian-Vazquez and Spivak, 2004b).

9.3. Porogen

The porogen is the solvent used for dissolving the monomers, the template, and the initiator. The porogen is also responsible for the creation of the pores in the polymer, and thus also affects the surface area. In non-covalent molecular imprinting, the porogen is usually chosen so that it promotes the interactions between the functional monomer(s) and the template. Ideally it should also demote non-specific interactions so that high specificity is obtained in the affinity between the final polymer and the template. Aprotic organic solvents as acetonitrile, chloroform and toluene are often chosen as the porogen. In covalent molecular imprinting, the template has been coupled covalently to the monomer beforehand to form a template-monomer complex that is co-polymerized with the cross-linker during the imprinting. The nature of the porogen, is therefore less important in this case than in non-covalent imprinting. The only restrictions are that the monomer-template complex and the cross-linker must be soluble in the porogen and that the porogen should be inert to the polymerization reactions.

9.4. Initiation of polymerization

Most of the functional monomers and the cross-linking monomers mentioned contain one or more vinyl functionalities. The polymerization of this type of compounds is in molecular imprinting traditionally performed as a free-radical polymerization, initiated via either thermolytic or photolytic homolysis of an initiator. One of the most commonly used free radical initiators is 2,2'-azobis(isobutyronitril) (AIBN). Other examples of free-radical polymerization initiators are phenyl-azo-triphenylmethane, tert-butyl peroxide (TBP), acetyl peroxide, benzoyl peroxide (BPO), lauroyl peroxide, tert-butyl hydroperoxide, and tert-butyl perbenzoate.

9.5. Optimization of imprinting conditions

For a newcomer to the field of molecular imprinting, the easiest way to approach the task of preparing a molecularly imprinted polymer is to search the literature and simply copy an existing protocol developed for a similar or non-similar template molecule. The

resulting molecularly imprinted polymer will probably be useful but it will not necessarily be optimal in binding capacity and selectivity.

In the beginning of the molecular imprinting era, polymers were mainly developed by a trial-and-error approach. Optimization studies were often limited to variations of one parameter at a time. Later on, the empirical knowledge gained formed the basis of a number of rules of thumb. For example, one rule says that the content of the cross-linking monomer in the monomer mixture should be above 50 mol% (Sellergren, 1989a; Wulff et al., 1982; Wulff, 1986). Several promising methods to approach the optimization problem in a more systematic way have been carried out, for example as described in Paper III by using statistical methods to do the variations and evaluations (the chemometrical approach) or by investigating the molecular interactions either experimentally or by computational methods. More recently combinatorial synthesis approaches combined with high-throughput screening have been applied. Molecularly imprinted polymer libraries have been synthesized in several different formats, for example on filter membranes in single use modules (El-Toufaili et al, 2004) and on the bottom of HPLC sample vials (Takeuchi et al., 1999; Lanza and Sellergren, 1999, Lanza et al., 2001) and the wells of 96-well micro titer plates (Chassaing et al., 2004).

By the experimental approach, using spectroscopic methods it is possible to study the complex-formation between the template and the functional monomer(s). The experiments are often performed as titrations where the template is titrated with the functional monomer(s). The formation of the interactions between the template and the functional monomer(s) is followed by recording the spectra for the mixture during the titration. The spectroscopic titration approach has been used successfully in NMR spectroscopy (Sellergren et al., 1988; Karlsson et al., 2004; Farrington et al., 2006; O'Mahony et al., 2006), and UV spectroscopy (Andersson and Nicholls, 1997).

The computational approach involves evaluation of the interactions between the template and the functional monomer(s) using computer programs. The procedure often starts with a screening of a virtual library of monomers against the template molecule to decide the appropriate monomer(s). An iterative routine places a monomer molecule at different positions around the template molecule and the

interactions are calculated. During a later stage of the procedure, the number of functional monomer molecules needed per template molecule in order to get the optimal interaction is determined. This approach has successfully been used by a number of investigators (Piletsky et al., 2001; Subrahmanyam et al., 2001; Chianella et al., 2002; Wu et al., 2003; Meng et al., 2004; Piletsky et al., 2004; Diñeiro et al., 2005; Dong et al., 2005; Pavel and Lagowski, 2005a,b; Chianella et al., 2006).

The final approach that will be discussed here for the optimization of MIPs is chemometrics. Chemometrics utilizes statistical experimental design and multivariate data analysis. Statistical experimental design is a tool used to make rational decisions during the design of an optimization experiment. In chemometrics, the number of experiments needed to draw statistically significant conclusions about a problem is minimized. The reduction in the number of experiments is achieved by co-variation of parameters in the model. Depending on the nature of the problem, models with different degrees of details can be chosen. Models can be chosen for screening, optimization, or robustness testing purposes. The parameters in the model chosen are called factors and the measures studied in the experiments are called responses. Depending on the model, different combinations of the factors are tested against the responses.

The investigator chooses the model, the factors, and the responses to be included in the model. The ranges the factors are varied within are also determined by the investigator. The design tool then creates an appropriate experimental design containing the minimal number of experiments needed. Most chemometrics tools also include an evaluation tool based on multivariate data analysis. The optimal composition of the molecularly imprinted polymer will be based on results from the multivariate data analysis of the screening experiment. Figure 17 shows a surface plot visualizing the relationship between the factors and the response in an optimization experiment (Paper III).

Several investigators have successfully used chemometrics to design and evaluate MIPs (Paper III; Vicente et al., 2004; Navarro-Villoslada et al., 2004; Davies et al., 2004; Baggiani et al., 2004; Greene et al., 2004; Navarro-Villoslada and Takeuchi, 2005; Rosengren et al., 2005; Mijangos et al., 2006).

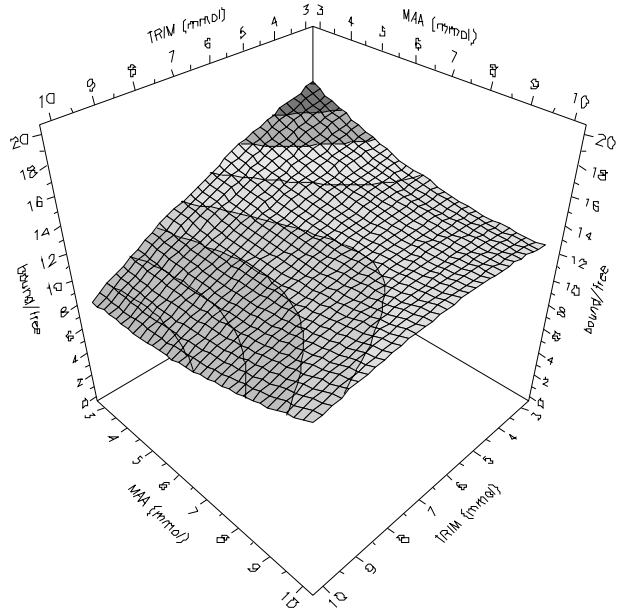


Figure 17. *Surface plot showing the influence of the factors MAA (methacrylic acid, mmol) and TRIM (trimethylolpropane trimethacrylate, mmol) at 3 mL of acetonitrile on the response (bound/free) for MIP beads imprinted with propranolol.*

10. Characterization of molecularly imprinted polymers

10.1. Methods to study binding

After designing and preparing a novel MIP, the next step is to elucidate whether or not the polymer possesses the wanted binding properties; in other words if the imprinting has worked. The basis for all binding studies is the comparison of the MIP with a control polymer (CP). The CP is prepared in the same way as the MIP but in the absence of the template or with a different template present during polymerization. The binding can be evaluated by several different methods. So far, the most common ways to study the binding properties of MIPs are in batch wise mode and in the packed column mode. The final use of the polymer determines which mode is preferred. If the intended use of the polymer is as a separation media in chromatography, it is suitable to perform the binding studies in the chromatographic mode (that is, in the packed column mode). If the polymer is to be used in a sorbent binding assay, it is more suitable to apply a batch wise evaluation of the binding.

In batch wise binding studies, the template is incubated with the MIP and the CP, respectively, in a suitable solvent for a certain time. The most appropriate and reliable approach is to allow the binding to reach equilibrium. Figure 18 shows the time dependency for the binding of propranolol to a propranolol MIP and a CP, calculated as the percentage of propranolol bound. The bound fraction (B/T, where B is the bound amount and T is the total initial amount added), the percentage bound or the bound amount (B) are often used as parameters for the comparison of the efficiency of imprinted polymers.

Single point binding investigations give only limited information about the binding characteristics of the polymer. The equilibrium concentrations at which the binding are compared are not the same for the MIP and the CP even though the same initial concentration

was used. The comparison is thus made at different concentrations on the binding isotherms of the polymers being compared. An alternative parameter, which to some degree considers the concentration differences, is the imprinting factor (calculated as the amount template bound per gram MIP divided by the free concentration of the template).

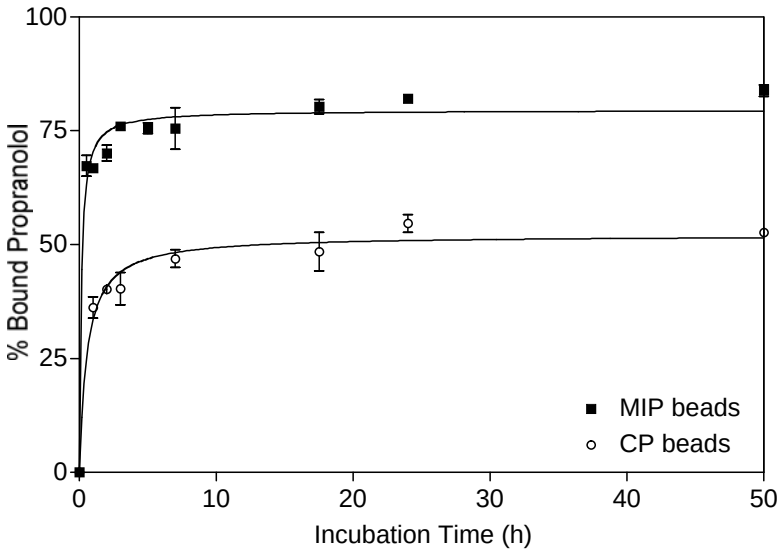


Figure 18. *Time course of the binding of ^{3}H-labelled propranolol (1 nM) to beads of MIP and CP (0.1 mg/mL; Ø 25-50μm) in acetonitrile (Paper IV). Data points are the mean values of three replicates and standard deviations are indicated with error bars.*

The best way of characterizing the binding is to determine the complete binding isotherm (Tóth et al., 2006). This is carried out by studying the binding at a range of concentrations for both the MIP and the CP. By adjusting the parameters in an expression describing a binding isotherm to the experimental data (i.e., the bound amount and the equilibrium concentration), the parameters in the isotherm expression are obtained. Three commonly used isotherms are described in Appendix A. Data for isotherm determinations can be obtained by either batch wise experiments or by frontal chromatographic experiments (Kempe and Mosbach, 1991; Ye et al., 1999b; Szabelski et al., 2002). Information gained from binding isotherms are, for example, the dissociation constants and the

number of binding sites (i.e., the maximum binding capacity of the polymer). Representative binding isotherms to a MIP and a CP are shown in Figure 19.

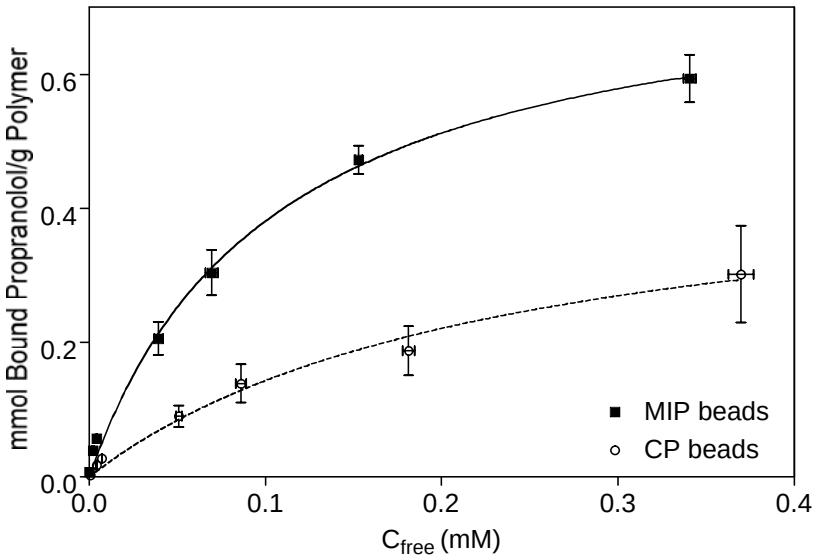


Figure 19. *One-site Langmuir binding isotherms of propranolol to MIP and CP beads (Ø 25-50 μm) (Paper IV). Data points are the mean values of three replicates and standard deviations are indicated with error bars.*

The cross reactivity of a MIP is a measure of the selectivity of the polymer. The cross reactivity is calculated as the ratio of the EC_{50} value of the template to that of a competitor. The EC_{50} value (effective concentration 50%) is the concentration of a competitor needed to occupy half of the binding sites in competition with the template (Motulzky, 1999). Typical competition curves are shown in Figure 20.

Parameters of interest to study for MIPs intended to be used as stationary phases in chromatography are the capacity factors ($k' = (t-t_0)/t_0$, where t is the retention of the analyte and t_0 the void), the separation factors ($\alpha = k'_1/k'_2$, where k'_x is the capacity factor of compound x) and the resolution (R_s, calculated according to Wulff et al., 1986 or Meyer, 1987). If the polymer is to be used as stationary phase in solid-phaseextraction, the recovery is also of interest.

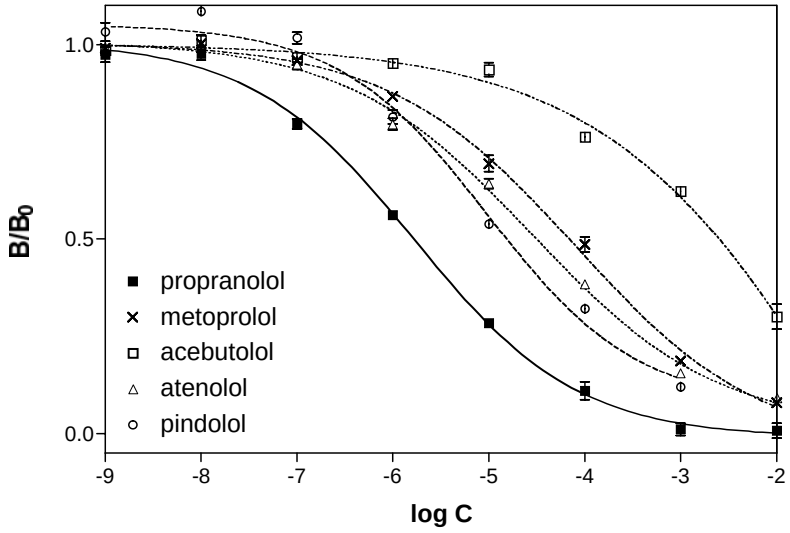


Figure 20. *Displacement of ^{3}H-labeled propranolol (1 nM) binding to propranolol MIP beads in acetonitrile–acetic acid (199:1) by increasing concentrations of competing ligands (Paper IV). B/B_0 is the ratio of the amount of ^{3}H-labeled propranolol bound in the presence of displacing ligand to the amount bound in the absence of displacing ligand. Samples were prepared in triplicate and data were fitted by GraphPad Prism software (San Diego, CA) to a sigmoidal dose-response curve with variable slope model. Data points are the mean values and standard deviations are indicated with error bars.*

Various sensors can be used to study the binding properties of MIPs. Examples of optical sensors, mass sensitive sensors, and electrochemical sensors are given in section 11.4.

10.2. Physical Characterization

In a flow-through application such as chromatography, it is favorable if the stationary phase consists of spherical and monodisperse beads. The particle size distribution of a particulate sample can be determined in several ways, for example by fractionation of the sample by sieving and gravimetric determination of the fractions, by the use of laser diffraction instruments as in Paper IV (e.g., the Malvern Mastersizer instrument or the Sympatec Helios instrument),

by the use of a Coulter Counter, or by direct measurement in micrographs.

Since adsorption and binding usually take place on surfaces, the surface area, pore size distribution, and pore diameter are valueable to determine. They can be determined using gas sorption measurements. The pore size distribution can also be determined using mercury penetration.

10.3. Chemical Characterization

To ascertain that the composition of the molecularly imprinted polymer is the expected, an elemental analysis can be carried out. This is usually done by pyrolysis of a known amount of polymer. Comparison of the analytically determined amounts of various elements with the theoretical values, gives an indication of the outcome of the polymerization. An estimation of the success of the removal of the template can also be obtained by elemental analysis, especially if the template contains elements not present in the monomers, for example, halogens, sulphur, phosphorous, or nitrogen which are not present in the most common monomers and cross-linkers used for MIP synthesis. Other chemical characterization methods that give information on the composition of a polymer are infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR). The information gained from infrared spectroscopy emanates from vibrating bonds in the polymer while NMR gives information on the atoms in the vicinity of, usually, protons or carbons in the polymer.

11. Applications of molecularly imprinted polymers

11.1. Solid-phase extraction

A rapidly growing application area for MIPs is what has become known as molecularly imprinted polymer solid-phaseextraction (MISPE). The use of MIPs as the stationary phase in solid-phaseextraction makes it possible to perform specific enrichments to facilitate analysis of substances available only at trace levels in samples. Obvious areas of interest for this technique are the analysis of drugs and environmental pollutions. Examples of the prior species are caffeine (Theodoridis and Manesiotis, 2002), scopolamine (Theodoridis et al., 2003), naproxen (Caro et al., 2004b), tetracycline (Caro et al., 2005), cholesterol (Shi et al., 2006), and local anaesthetics (Andersson et al., 2006). Examples of the latter species are organophospate flame retardants (Möller et al., 2001; Möller et al., 2004a; Möller et al., 2004b), triazines in soil and vegetable samples (Cacho et al. 2006), and naphthalene sulfonates in river water (Caro et al., 2004a).

11.2. Liquid chromatography

In the beginning of the era of molecular imprinting, the method of choice for analysis and characterization of the binding was liquid chromatography. Even though an immense amount of work has been performed using MIPs as stationary phases, not many applications has found practical use outside academia. This is mainly because of the poor binding characteristics of MIPs. MIPs as stationary phases generally suffer from severe tailing effects. The tailing arises from the fact that the binding isotherms of most MIPs are non-linear (Tóth et al., 2006). The non-linearity of the binding isotherms is due to the heterogeneity of the binding sites. One of the strengths of MIPs is the possibility to tailor make the selectivity and achieve chiral resolution when the template is an optically reactive

compound. For this reason a tremendous number of reports on MIP chiral stationary phases have appeared (Sellergren, 1994; Kempe and Mosbach, 1995; Kempe, 2000; Kempe, 2001; Sellergren, 2001).

11.3. Solid-phase binding assays

Solid-phase binding assays using MIPs, also know as molecularly imprinted sorbent assays (MIA), is an equivalent to the immunoassays with antibodies. The strategy is to use a reporter molecule that is displaced from the MIP or is competing with the analyte during binding. The reporter molecule can be radioactively labeled or possess optical properties that allow easy detection. An alternative route is to include a fluorescent moiety in the polymer network that either start to fluoresce or is quenched upon the binding of the analyte. MIA has been developed for a broad range of compounds present in a variety of matrices (Vlatakis et al., 1993; Andersson, 2000; Ansell, 2002). In Paper IV, a 96-well plate format assay using MIP beads for the analysis of propranolol in serum is described.

11.4. Sensors

11.4.1. Optical sensors

The majority of the MIP optical sensors reported in the literature utilize fluorescence as the detectable signal (Gao et al., 2005). This can be achieved by the use of a fluorescent molecule either competing with or being displace by the analyte, or by the incorporation of a fluorescent monomer in the polymer network that either start to fluoresce or is quenched upon binding of the analyte. The simplest case is if a fluorescent molecule is the target molecule, in this case it can be expected that the fluorescence of the MIP increases upon binding of the analyte. Another popular approach is sensors based on surface plasmon resonance (SPR). These are based on MIPs synthesized on a gold surface. When the MIP binds the analyte, the reflective properties of the gold surface changes and is recorded.

11.4.2. Mass sensitive sensors

The quartz crystal microbalance (QCM) is a mass sensitive sensor (Marx, 2003). The sensor is based on a piezoelectric crystal covered with an adsorbing agent. When applying an electric field across a piezoelectric quartz crystal it starts oscillating with a frequency characteristic for the crystal, usually in the range from 5 MHz to 20 MHz. When an analyte adsorbs to the crystal surface a decrease in the oscillation frequency can be detected. The decrease is ideally directly proportional to the oscillating mass. By polymerizing a thin film of a molecularly imprinted polymer on the crystal surface the rebinding of the template can be studied. The QCM have, for example, been used to study polymers imprinted with tryptophan (Liu et al., 2006).

11.4.3. Electrochemical sensors

MIPs have proven to be valuable as sensing elements in electrochemical sensors. The first report of an electrochemical sensor based on MIPs was published in 1993 (Hedin et al., 1993). The sensing device was a field-effect capacitor with a thin polymer membrane imprinted with L-phenylalanine anilide as the sensing layer. The shift in the capacitance-voltage was taken as a measure for the amount of bound substance. Differences could also be observed when different substances were investigated. In 1995, Kriz and Mosbach demonstrated a morphine sensitive amperometric sensor based on competitive binding of codeine (Kriz and Mosbach, 1995). In 1996, a sensor based on conductometric measurements was reported for the first time (Kriz et al., 1996). In 1999, a novel approach was introduced by the group of Piletsky (Panasyuk et al., 1999). By the use of electropolymerization, a capacitive sensor consisting of a thin layer of phenylalanine imprinted polyphenol polymerized on a gold surface was created. The same group reported the same year on a cholesterol sensitive amperometric sensor fabricated by spontaneous self-assembly of hexadecyl mercaptan on gold surfaces (Piletsky et al., 1999) and atrazine sensitive conductometric sensors utilizing imprinted membranes (Sergeyeva et al., 1999a; Sergeyeva et al., 1999b). Several investigators have reported glucose sensitive sensors (Chen et al., 1997; Cheng et al., 2001; Seong et al., 2002). An amperometric sensor for

fructosylamines based on catalytic molecularly imprinted polymer was reported by Yamazaki et al., (2003).

11.5. Synthetic enzymes

The possibility to tailor make MIPs towards the desired selectivity together with the high stability of the materials under a broad range of conditions render MIPs attractive as synthetic enzymes (Ramström and Mosbach, 1999; Severin, 2005). A strategy that has been successfully applied to reduce the activation energy of reactions has been to imprint a transition state analogue. Furthermore, the synthesis of synthetic enzymes is realized by the inclusion of catalytic active groups in the polymer network.

12. Conclusions

In the first part of this work an intrinsic model describing chromatography is compared to two commonly used simplified models (Paper I). The low complexity of the simplified models allows analytical solutions to be obtained while the detailed intrinsic model needs to be solved numerically. It is shown that the intrinsic model is more versatile and sensitive to changes in the simulated experimental running conditions than the simplified models. A model was designed to describe a complete chromatographic separation step including the intrinsic model accounting for the chromatography column, and tanks-in-series models accounting for the inlet and outlet volumes of the system (Paper II). This model was subsequently used to determine the diffusion coefficients for three proteins (lysozyme, BSA, and IgG) in Sepharose™ CL-4B. Different experiments were designed to determine the parameters needed in the model. The experiments were designed so that parameters were obtained one at a time. The determined diffusion coefficients correlated well to those previously reported in the literature.

Designing MIPs is not an easy task. Optimization of the formulation and synthesis protocol is required. The number of possible combinations of monomers, cross-linkers and porogens are nearly endless. In order to screen a large number of MIP recipes, rapid methods to synthesize MIPs are required. In the second part of this thesis, a novel method to prepare MIPs in the form of spherical beads is described (Paper III and IV). The method is used together with chemometrics to prepare an optimized MIP selective for propranolol (Paper III). In the experimental design, three factors (parameters), that is, the amount of monomer, the amount of cross-linker, and the volume of the porogen, were varied. The number of MIPs needed to be prepared was drastically reduced by the use of chemometrics. It was shown that the combination of chemometrics and the novel method to prepare spherical MIP beads is very well suited to rapidly design and optimize a new MIP. I believe that this strategy should be helpful for future developments of well optimized

MIPs for various applications. Given that the strategy is easy and rapid there should no longer be any reason to use non optimized protocols for the synthesis of MIPs.

The method to prepare spherical beads is a suspension polymerization method with mineral oil as the continuous phase and the pre-polymerization mixture as the dispersed phase. The suspension is created, without the need of addition of stabilizers, by vigorous mixing with a mixing device. The resulting suspension is stable during the entire polymerization. Recovery of the beads is easily performed by removal of the mineral oil by filtration. The advantages of this method to prepare spherical MIP beads over other methods described in the literature are the low cost and commercial availability of the continuous phase and the absence of the need for stabilizers due to the viscosity of the oil.

In my opinion the future for MIPs looks bright. The industrial need for more and more selective separation methods has increased with time and the demands from regulatory authorities for safer products are not likely to be lowered. Therefore, MIPs should be useful for highly selective chromatographic separations as well as solid-phase extractions for purification and trace enrichment. However, in order to meet these demands and to convince those still not believing in the MIP technology, it becomes more and more vital that the methods to design and synthesize MIPs are improved. Other intriguing applications for MIPs are as sensing elements in sensors, as recognition elements in solid-phase binding assays, and as synthetic enzymes. For all applications of MIPs, it is important that the problems with the 'polyclonality' and the difficulty of applying MIPs in aqueous media are addressed and solved.

Appendix A – Isotherms

Many different expressions have been used to describe the equilibrium between adsorption and desorption to surfaces. In this section, three of the most common expressions, the Langmuir, the bi-Langmuir, and the Freundlich isotherms, will be discussed.

Langmuir

The most commonly used isotherm in the literature is the Langmuir isotherm (equation 52).

$$q = \frac{C_p \cdot q_m}{C_p + K_d} \tag{52}$$

The Langmuir isotherm describes an adsorption/desorption process that is based on the following assumptions (Langmuir, 1916): every site is equivalent, and the probability that a molecule will adsorb is independent of whether or not the sites around are occupied or not. Further, all adsorption occurs through the same mechanism and the adsorption only occurs in a single finite layer with a finite capacity q_m, which is also known as the saturation capacity for a certain molecule. The dissociation constant, K_d, is the ratio between the rate coefficient for desorption and the adsorption rate coefficient.

The Langmuir isotherm was originally developed for adsorption of gases on solid phases but can also be applied to liquid-solid systems with high specificity where no unspecific adsorption occurs, for example, some systems for purification of immunoglobulins. However, most often, the systems used are of a lower specificity that actually, in a way, disqualifies this isotherm. All the same, this is the most commonly used isotherm.

Bi-Langmuir

Often the stationary phases are not homogeneous. This can be the result of different orientations of ligands or non-specific adsorption sites. The Langmuir isotherm can be extended to account for either different type of sites with different affinities for adsorption or adsorption in multiple layers. This extended isotherm is called the bi-Langmuir isotherm if two equilibriums are included (equation 53).

$$q = \frac{C_p \cdot q_{m,1}}{C_p + K_{d,1}} + \frac{C_p \cdot q_{m,2}}{C_p + K_{d,2}} \tag{53}$$

This isotherm describes two superimposed ordinary Langmuir isotherms, each with its own saturation level and adsorption/desorption kinetics.

Freundlich

There are several expressions used to describe more complex adsorption/desorption behaviours. One is the Freundlich isotherm (equation 54). This isotherm is an attempt to describe adsorption to sites that are not energetically equal. Since nature always tends to move towards as low energy as possible, the sites where the energy gain is the highest are occupied first. In the Freundlich isotherm, the energy change is logarithmic (Freundlich, 1906).

$$q = b \cdot C_p^n \tag{54}$$

A disadvantage with the Freundlich isotherm, compared to the Langmuir and the bi-Langmuir isotherms, is that at first sight the parameters do not have any physical interpretation. It is simply an exponential expression that has been found suitable especially for adsorption in liquid systems.

Symbols and abbreviations

a	specific area [m^2/m^3]
C_0	inlet concentration to column [mol/m^3 solution]
C_L	concentration in mobile phase [mol/m^3 solution]
C_P	concentration in pore liquid [mol/m^3 pore liquid]
$C_{Pr=R}$	concentration at surface of particle [mol/m^3 pore liquid]
C_{inlet}	concentration in mobile phase entering the column [mol/m^3 solution]
C_{outlet}	concentration in mobile phase leaving the column [mol/m^3 solution]
D_{AB}	diffusion coefficient in free liquid [m^2/s]
D_{ax}	axial dispersion coefficient [m^2/s]
D_{Eff}	effective diffusion coefficient [m^2/s]
d_c	column diameter [m]
d_p	bead diameter [m]
HETP	height equivalent to a theoretical plate [m]
K	mass transfer coefficient [m/s]
K_a	association constant [g/m^3]
k'_1, k'_2	capacity factors for substance 1 and 2
k_{ads}	adsorption rate coefficient [$m^3/(mol \cdot s)$]
k_B	Boltzmann's constant
k_{des}	desorption rate coefficient [s^{-1}]
L	column length [m]
l	length coordinate in column [m]
n	number of tanks/plates
N_A	Avogadro's number
N_t	number of effective tanks/plates
q	adsorbed adsorbate concentration in beads [mol/m^3 gel]

q_m	maximum adsorbed adsorbate concentration in beads [mol/m^3 gel]
Q	volumetric flow through column [m^3/s]
R	bead radius [m]
R_S	Stokes radius [m]
r	length coordinate in bead [m]
r_P	negative adsorption rate [mol/m^3 gel·s]
t	time [s]
T	absolute temperature [K]
V_B	bead void volume [m^3]
V_C	column void volume [m^3]
V_e	elution volume [m^3]
V_{ec}	extracolumn volume [m^3]
V_t	total column volume [m^3]
v_{sup}	superficial velocity [m/s]
v_{INT}	interstitial velocity [m/s]
$W_{1/2}$	peak width at half height [m^3]

Greek symbols

α	separation factor
ε_c	void in bed [m^3 mobile phase/m^3 column]
ε_p	void in bead [m^3 pore liquid/m^3 bead]
μ	dynamic viscosity [Pa s]

Abbreviations

AA	acrylic acid
AIBN	2,2'-azobis(isobutyronitril)
AMPSA	2-acrylamido-2-methyl-1-propane-sulphonic acid
CLSM	confocal laser scanning microscopy
CP	control polymer
DLS	dynamic light scattering
EB	expanded bed
EC_{50}	effective concentration 50%
EDMA	ethylene glycoldimethacrylate

EPR	electron paramagnetic resonance spectroscopy
ESPI	electronic speckle interferometry
DVB	divinylbenzene
FRAP	fluorescence recovery after photo bleaching
FTIR	fourier transform infrared spectroscopy
GPC	gel permeation chromatography
HEMA	2-hydroxyethyl methacrylate
HIC	hydrophobic interaction chromatography
HLI	holographic laser interferometry
HPLC	high pressure liquid chromatography
IEX	ion exchange chromatography
IMAC	immobilized metal ion affinity chromatography
MAA	methacrylic acid
MAP	2-methyl-N-(6-methyl-pyridine-2-yl)-acrylamide
MEP	2-(methacryloyloxy)ethyl phosphate
MIP	molecularly imprinted polymer
MISPE	molecularly imprinted polymer solid-phase extraction
MMA	methyl methacrylate
NAG	N,α-bismethacryloyl glycine
NMR	nuclear magnetic resonance
NOBE	N,O-bismethacryloyl ethanolamine
PETEA	pentaerythritol tetraacrylate
PETRA	pentaerythritol triacrylate
PMFG-NMR	pulsed magnetic field gradient NMR
QCM	quartz crystal microbalance
RPC	reversed phase chromatography
SMB	simulated moving bed
SPR	surface plasmon resonance
TFMAA	(2-trifluoromethyl)acrylic acid
TMB	true moving bed
TRIM	trimethylolpropane trimethacrylate

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Populärvetenskaplig sammanfattning

Inom läkemedelsbranschen och finkemikalieindustrin ställs mycket höga krav på god separationsförmåga och hög produktrenhet. Dessa är några av skälen varför separationsteknik och kunskap om separationer är viktiga. Det traditionella tillvägagångssättet vid utveckling av ett nytt separationssteg och uppskalning från laboratorieskala till full produktionsskala har varit att antingen förlita sig på tumregler baserade på gamla kunskaper eller helt enkelt genomföra en stor mängd försök. Speciellt det sistnämnda kan leda till mycket stora kostnader, särskilt om produkten som ska renas upp är dyrbar.

Utfallet av en kromatografisk separation beror på en mängd variabler såsom koncentration av det som ska separeras, flödeshastighet samt salthalt och pH (surhetsgrad) hos lösningen. Ju fler variabler det finns desto fler försök behövs för att optimera separationsprocessen. För att förenkla optimeringen kan datorbaserade simulerings- och modelleringsverktyg tillämpas. Dessa programvaror är baserade på matematiska modeller med vars hjälp utfallet av en separation under givna betingelser kan beräknas. Den första delen av denna avhandling behandlar just olika modeller som kan användas för att simulera och modellera en typ av kromatografisk separation, nämligen gelfiltreringskromatografi.

I gelfiltreringskromatografi används en kolonn packad med ett poröst material i form av kulor. De molekyler som ska separeras tillsätts i ena änden av kolonnen och får därefter strömma genom kolonnen i ett flöde av vätska. Beroende på storleken hos molekylerna som separeras upplever de volymen i kulmaterialet som olika stor. Om en molekyl är tillräckligt stor kommer den inte att kunna tränga in i porerna i kulmaterialet medan riktigt små molekyler tar sig in i kulornas alla skrymslen och vrår. Detta gör att en blandning av stora och små molekyler kommer att separeras utifrån storlek. Stora molekyler kommer att passera kolonnen fort medan mindre molekyler behöver längre tid på sig.

Den matematiska modell som förespråkas i den här avhandlingen tar hänsyn till hur fort en lösning pumpas genom kolonnen samt hur snabbt de olika molekylerna förflyttar sig via diffusion i kulmaterialet. Modellen tar även hänsyn till hur snabbt molekylerna transporteras

från den flödande vätskan till kulmaterialets yta. Alla omströmmade kroppar omges av en orörlig vätskefilm. Tjockleken på denna vätskefilm bestäms av hur snabbt omgivande vätska strömmar förbi. Genom filmen sker transporten av molekylerna via diffusion. Beroende på filmtjockleken kommer diffusionen av molekylerna från den flödande vätskan till kulmaterialet att ta olika lång tid. Modellen beaktar dessutom den flödande vätskans omblandning som uppkommer vid passage genom den packade kolonnen. Modellen tar även hänsyn till vad som händer i de slangar och rör som är kopplade till kolonnen.

Den andra delen av avhandlingen handlar om 'molecularly imprinted polymers' eller vad som översatt till svenska brukar kallas för polymerer framställda med molekylär avtrycksteknik – eller kort och gott 'MIPar'.

Plaster är uppbyggda av byggstenar som kallas monomerer, från grekiskans 'mono' (en) och 'meros' (del). Då dessa kopplas samman bildas en polymer (grek. poly, många). För att öka styrkan hos polymera material kan dessa tvärbindas med hjälp av tvärbindande monomerer. MIPar är plastmaterial som har förmågan att känna igen olika molekyler på samma sätt som till exempel antikroppar känner igen antigen eller receptorer känner igen hormon och signalsubstanser. Denna egenskap får MIPen genom att den tillverkas i närvaro av det molekylslag, det så kallade templatet, som MIPen ska känna igen.

Sedan tekniken att framställa MIPar uppfanns har dessa material ofta tillverkats som större block som sedan krossats, malts och siktats till partiklar i lagom storlek. Partiklarna har oftast väldigt oregelbunden form. Generellt sett så brukar en stor del av materialet gå till spillo då det vid malningen bildas en hel del mycket små partiklar som inte går att använda. Ett mer ekonomiskt sätt är att redan från början tillverka MIParna i form av sfäriska småkulor. Dessa är dessutom lättare att hantera och fungerar bättre än de oregelbundna partiklarna i alla tillämpningar där de befinner sig i flödande vätskor.

Det finns flera olika metoder för att framställa polymera material i form av småkulor. Exempel på sådana metoder är suspensionspolymerisation och emulsionspolymerisation. Båda dessa metoder bygger på att en lösning av polymerens byggstenar finfördelas i droppform i en annan vätska, den kontinuerliga fasen.

Denna avhandling beskriver en suspensionspolymerisationsmetod där smådroppar av monomerer, tvärbindare och templatmolekyler, lösta i ett lösningsmedel, skapas genom snabb omrörning i mineralolja. Monomerer och tvärbindare i dropparna sammankopplas, polymeriseras, och bildar fasta småkulor. Oljan avlägsnas genom filtrering och kulorna tvättas så att templatmolekylerna avlägsnas. Kvar i kulorna finns hålrum som är avgjutningar av templatmolekylerna. Kulorna har nu fått förmågan att känna igen templatmolekylerna och binda in dem.

Det finns inte något givet recept på hur MIPar ska tillverkas utan det varierar beroende på templatmolekyl och slutlig användning. Antalet monomerer, tvärbindare och lösningsmedel som kan användas är stort. Mångfalden ökar ytterligare av valet av koncentrationer och mängder av ingående komponenter vid tillverkningen. Ska alla kombinationer undersökas är detta ett ytterst tidskrävande arbete. Ett sätt att bringa ording i oredan och påskynda arbetet är att ta hjälp av kemometri. Kemometri är en metod som baseras på rationell design av experiment och statistiska utvärderingsmetoder. Istället för att variera en parameter åt gången varieras flera parametrar samtidigt. Detta gör att samverkanseffekter beaktas samtidigt som antalet nödvändiga experiment reduceras. I denna avhandling beskrivs hur MIPar riktade mot beta-blockeraren propranolol framställs och optimeras med hjälp av kemometri och den tidigare nämnda kultillverkningsmetoden.