Towards a better understanding of ion exchange materials for purification of proteins

Author(s): Franke, Agnes

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Agnes Franke

Towards a better understanding of ion exchange materials for the purification of proteins

Diss. ETH Nr. 18474
The title page shows four graphs that represent the four main chapters of this thesis. They are shortly explained in the following.

(Top left). This graph shows the dynamic binding capacity $DBC$ for the protein IgG as a function of the ligand density $\rho_{lig}$ for three different flow rates. The data is used for the design of a new stationary phase for the chromatographic purification of proteins in collaboration with an industrial partner.

(Top right). The graph shows the Henry coefficient $H$ as a function of pH and modifier concentration for four different proteins (top left: Serono mAb, top right: Erbitux, bottom left: Avastin, bottom right: Lysozyme) on the mixed mode stationary phase Capto adhere. The high values for the Henry are caused by the interplay of anion exchange and hydrophobic interaction functionalities. Therefore, this kind of stationary phase can be used for the design of an effective polishing step for the downstream processing of antibodies.

(Bottom left). The chromatogram shows the behavior of the protein human serum albumin on the strong cation exchange resin Fractogel EMD SE Hicap (M): the protein elutes with two peaks in the modifier gradient. The elution time of the second peak is a function of the applied temperature. The peculiar elution pattern is analyzed in detail in this thesis.

(Bottom right). The behavior of the human serum albumin on Fractogel EMD SE Hicap (M) is modeled with a general rate model. The proposed binding mechanism is shown in this graph. The protein in the bulk adsorbs on the stationary phase through site 1 with a possible transition to site 2.
Towards a better understanding of ion exchange materials for the purification of proteins

A dissertation submitted to
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for the degree of
Doctor of Sciences

presented by

Agnes Franke
Dipl.-Ing. Universität Stuttgart, Germany
born January 7th, 1980 in Reutlingen, Germany
citizen of Germany

accepted on the recommendation of
Prof. Dr. Massimo Morbidelli, examiner
Prof. Dr. Marco Mazzotti, co-examiner

Zurich 2009
Von den Gleichnissen

Viele beklagen sich, daß die Worte der Weisen immer wieder nur Gleichnisse seien, aber unverwendbar im täglichen Leben, und nur dieses allein haben wir. Wenn der Weise sagt: „Gehe hinüber“, so meint er nicht, daß man auf die andere Seite hinübergehen solle, was man immerhin noch leisten könnte, wenn das Ergebnis des Weges wert wäre, sondern er meint irgendein sagenhaftes Drüben, etwas, das wir nicht kennen, das auch von ihm nicht näher zu bezeichnen ist und das uns also hier gar nichts helfen kann. Alle diese Gleichnisse wollen eigentlich nur sagen, daß das Unfaßbare unfaßbar ist, und das haben wir gewußt. Aber das, womit wir uns jeden Tag abmühen, sind andere Dinge.
Darauf sagte einer: „Warum wehrt ihr euch? Würdet ihr den Gleichnissen folgen, dann wäret ihr selbst Gleichnisse geworden und damit schon der täglichen Mühe frei.“
Ein anderer sagte: „Ich wette, daß auch das ein Gleichnis ist."
Der erste sagte: „Du hast gewonnen."
Der zweite sagte: „Aber leider nur im Gleichnis."
Der erste sagte: „Nein, in Wirklichkeit; im Gleichnis hast du verloren."

Franz Kafka, 1922
Acknowledgments

I wish to express my gratitude to my supervisor, Professor Massimo Morbidelli, for the opportunity to work in his research group and for his support during this thesis. I am especially thankful that he shared his vision about the future of biochromatography with me and that he was also giving me the freedom to carry out my own ideas.

Professor Marco Mazzotti has accepted to co-examine my thesis. His input in the final version of my work is gratefully acknowledged.

For his great scientific support during this thesis I also thank Alessandro Butté. He was guiding me in the beginning into biochromatography and supporting me with his knowledge in the end.

Many thanks go to Thomas Müller-Späth for his scientific support within the exploitation of mixed mode resins.

Božidar Cvjetić was contributing with his master thesis on the characterization of cation exchange materials to my work.

Lena Melter and Miriam Bechtle spent countless coffee breaks with me discussing all important work and non-work related topics.

Thanks to all members of the Morbidelli group who contributed to this work and to my friends in Zurich who made my time here enjoyable.

A big hug goes to my parents, my brother and Tobias. Without the security of their continuous support and faith in me, my way would have not lead me that far.

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Abstract

In search of new active ingredients for drugs, proteins, especially antibodies, have already proven a great potential. However, as they are sensitive to heat, the standard purification scheme includes several chromatographic steps, whereas one of those is usually an ion exchange step. In this work several examples are shown that enlighten some phenomena occurring during the downstream processing of proteins with ion exchange chromatography. Based on fundamental insights, procedures are established for the characterization of proteins on chromatographic materials. By this systematic analysis, the behavior of proteins on stationary phases is linked to material properties. The adsorption and diffusion behavior of a polyclonal antibody is studied in Chapter 2 on a set of custom made cation exchange resins. The performance of functionalized materials, such as cation exchange resins, is not only dependent on the ligand type and ligand density, but also on the pore accessibility of the target molecule. In the case of large molecules such as antibodies this latter parameter becomes crucial, because the size of such molecules falls somewhere inside the pore size distribution of the resin. The influence of the ligand density and accessibility on the overall performance of the material is explored systematically. Five different materials, having the same chemistry as the strong cation exchange resin Fractogel EMD SO₃⁻ (M), have been analyzed. These materials only differ in the ligand density. It is shown that the ligand density directly influences the porosity of the materials as well as the pore diffusivity and the dynamic binding capacity. For a given purification problem an optimal ligand density can be found. Based on the above results a new material is proposed, showing superior properties in terms of dynamic binding capacity. This is achieved by an optimization of the ligand density and by a decrease of the particle size of the stationary phase. The material properties are modeled with a general rate model. Further simulations were conducted to evaluate the performance of the new material in comparison with a conventional resin.

A new class of stationary phases is analyzed in Chapter 3. Mixed mode resins are ma-
terials that exhibit more than one functionality. This feature can be utilized to reduce the number of chromatographic steps in the downstream processing of proteins, while keeping the same purity constraints. This approach might lead to a significant cost reduction in the downstream processing. A promising mixed mode resin, Capto adhere, is therefore studied for the application in the polishing step of the downstream processing of monoclonal antibodies from cell culture supernatant. Capto adhere is a strong anion exchange resin with additional hydrophobic interaction functionalities. The Henry coefficient, which quantifies the adsorption strength, was measured for the full working range of the stationary phase as a function of the sodium chloride concentration and the pH. The results are compared to a conventional anion exchange resin and a hydrophobic interaction resin. Furthermore, Capto adhere is applied for the polishing step of an antibody from an industrial clarified cell culture supernatant. Finally, it is shown that this resin can be used for the separation of IgG subclasses.

In Chapter 4, experiments with human serum albumin on the strong cation exchange resin Fractogel EMD SE Hicap (M) are described. Even though human serum albumin at high purity is used, two peaks in gradient elution experiments occur. It is shown that human serum albumin binds to Fractogel EMD SE Hicap (M) in two different binding conformations: site 1 adsorbs instantaneously, while site 2 adsorbs with a kinetic limitation. The two peak behavior of human serum albumin is analyzed in detail, especially at various gradient lengths, concentrations and temperatures. Breakthrough curves are performed at four modifier concentrations and three velocities. The characteristic behavior, as described for gradient experiments, is confirmed for the breakthrough curves. The two peak elution pattern of human serum albumin is also found for other strong cation exchange resins, but not for weak cation exchange resins. It is concluded that the described behavior is peculiar for the interaction of human serum albumin with the strong cation exchange ligand of the resin.

The adsorption behavior of human serum albumin on Fractogel EMD SE Hicap (M) is modeled with a general rate model in Chapter 5, whereas the two elution peaks are modeled with two binding sites. As the adsorption process is not in equilibrium for this system, the model also includes an approach for the kinetics of adsorption. Isocratic experiments under nonadsorbing conditions were used to characterize the mass transfer of this system. The isotherm of both adsorption sites, as well as the kinetic of adsorption and desorption for the second site, that were modeled with a first order kinetic, are functions of the modifier. The kinetic ansatz is evaluated with linear gradient experiments and step experiments with various adsorption times. It is possible to simulate the characteristics of the experiments with the proposed modified general rate model. The behavior of the system at conditions, that are not considered for the regression, can be predicted with the proposed model.
Zusammenfassung


Simulationen werden durchgeführt, um die Güte des neuen Materials mit konventionellen Stationärphasen zu vergleichen.


Das Verhalten von Humanalbumin auf Fractogel EMD SE Hicap (M) wird mit einem Porendiffusionsmodell in Kapitel 5 beschrieben, wobei die zwei Humanalbumin-Peaks durch zwei verschiedene Adsorptionsstellen modelliert werden. Da der Adsorptionsprozess sich für das beschriebene System nicht im Gleichgewicht befindet, wird in das Modell ein Ansatz für die Adsorptionskinetik eingearbeitet. Isokratische Experimente unter nichtadsorbierenden Bedingungen wurden benutzt, um den Stofftransport des Systems zu charakterisieren. Die Kinetik für Adsorption und Desorption für die zweite Bindungsstelle wird mit einem Ansatz erster Ordnung beschrieben. Sowohl die Kinetik für Adsorption und Desorption, als auch die Isothermen beider Adsorptionsstellen sind Funktionen der Salzkonzentration. Die beschriebenen Experimente können mit dem mo-
difizierten Porendiffusionmodell erfolgreich simuliert werden. Darüber hinaus wird das Verhalten des Systems unter Bedingungen, die nicht für die Regression berücksichtigt wurden, vorhergesagt.
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# Nomenclature

## Arabic letters

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<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
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<tbody>
<tr>
<td>$A$</td>
<td>cross section of the column</td>
<td>[cm$^2$]</td>
</tr>
<tr>
<td>$A$</td>
<td>peak area</td>
<td>[mg]</td>
</tr>
<tr>
<td>$A$</td>
<td>parameter for the calculation of the HETP-value</td>
<td>[cm]</td>
</tr>
<tr>
<td>$B$</td>
<td>parameter for the calculation of the HETP-value</td>
<td>[cm]</td>
</tr>
<tr>
<td>$C$</td>
<td>parameter for the calculation of the HETP-value</td>
<td>[s/cm]</td>
</tr>
<tr>
<td>$c$</td>
<td>concentration of the solute</td>
<td>[g/l]</td>
</tr>
<tr>
<td>$c_{eq}$</td>
<td>equilibrium concentration</td>
<td>[g/l]</td>
</tr>
<tr>
<td>$c_m$</td>
<td>concentration of the modifier</td>
<td>[mol/l]</td>
</tr>
<tr>
<td>$c_p$</td>
<td>concentration of the solute in the liquid phase of the particle</td>
<td>[g/l]</td>
</tr>
<tr>
<td>$c_0$</td>
<td>feed concentration</td>
<td>[g/l]</td>
</tr>
<tr>
<td>$cCCS$</td>
<td>clarified cell culture supernatant</td>
<td></td>
</tr>
<tr>
<td>$CV$</td>
<td>column volume</td>
<td>[ml]</td>
</tr>
<tr>
<td>$D_{ax}$</td>
<td>axial dispersion coefficient</td>
<td>[cm$^2$/s]</td>
</tr>
<tr>
<td>$D_m$</td>
<td>molecular diffusion coefficient</td>
<td>[cm$^2$/s]</td>
</tr>
<tr>
<td>$D_{p,eff}$</td>
<td>pore effective diffusion coefficient</td>
<td>[cm$^2$/s]</td>
</tr>
<tr>
<td>$d$</td>
<td>diameter</td>
<td>[mm]</td>
</tr>
<tr>
<td>$d_p$</td>
<td>particle diameter</td>
<td>[$\mu$m]</td>
</tr>
<tr>
<td>$DBC$</td>
<td>dynamic binding capacity</td>
<td>[g/l]</td>
</tr>
<tr>
<td>$ELISA$</td>
<td>enzyme linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>$F$</td>
<td>phase ratio</td>
<td>[-]</td>
</tr>
<tr>
<td>$FDA$</td>
<td>US Food and Drug Administration</td>
<td></td>
</tr>
<tr>
<td>$H$</td>
<td>Henry coefficient</td>
<td>[-]</td>
</tr>
<tr>
<td>$HCP$</td>
<td>host cell proteins</td>
<td></td>
</tr>
<tr>
<td>$HETP$</td>
<td>height equivalent to a theoretical plate</td>
<td>[cm]</td>
</tr>
<tr>
<td>$HSA$</td>
<td>human serum albumin</td>
<td></td>
</tr>
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</table>
\[ I_{gG} \text{ immunoglobulin G} \]
\[ I_{SEC} \text{ inverse size exclusion chromatography} \]
\[ K_{d, IgG} \text{ pore accessibility for IgG [-]} \]
\[ k_{ad, i} \text{ kinetic parameter of adsorption of site } i [-] \]
\[ k_{de, i} \text{ kinetic parameter of desorption of site } i [-] \]
\[ \tilde{k}_{ad, i} \text{ nondimensional kinetic parameter of adsorption of site } i [-] \]
\[ \tilde{k}_{de, i} \text{ nondimensional kinetic parameter of desorption of site } i [-] \]
\[ k_f \text{ film mass transfer coefficient [cm/s]} \]
\[ L \text{ column length [cm]} \]
\[ m \text{ mass [mg]} \]
\[ mAb \text{ monoclonal antibody} \]
\[ MW \text{ molecular weight [Da]} \]
\[ P_e \text{ Peclet number [-]} \]
\[ P_e_{az} \text{ axial Peclet number [-]} \]
\[ pI \text{ isoelectric point} \]
\[ Q \text{ flow rate [ml/min]} \]
\[ q \text{ capacity [g/l]} \]
\[ q_{eq} \text{ equilibrium capacity [g/l]} \]
\[ q_m \text{ equilibrium capacity of the modifier [mol/l]} \]
\[ q_\infty \text{ saturation capacity [g/l]} \]
\[ R_p \text{ particle radius [cm]} \]
\[ r \text{ radial position in the particle [cm]} \]
\[ r_h \text{ hydrodynamic radius [nm]} \]
\[ r_p \text{ pore radius [Å]} \]
\[ Re \text{ Reynolds number [-]} \]
\[ Sc \text{ Schmidt number [-]} \]
\[ Sh \text{ Sherwood number [-]} \]
\[ St \text{ Stanton number [-]} \]
\[ T \text{ Temperature [K]} \]
\[ t \text{ temperature [°C]} \]
\[ t \text{ time [min]} \]
\[ u \text{ interstitial velocity } (u = Q/\epsilon_{bed} A) \text{ [cm/s]} \]
\[ u_{lin} \text{ linear velocity } (u = Q/A) \text{ [cm/min]} \]
\[ V \text{ volume [ml]} \]
\[ z \text{ axial position in the column [cm]} \]
### Greek letters

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>parameter for the modifier dependence of the Henry coefficient $[1/M^3]$</td>
</tr>
<tr>
<td>$\alpha_{k,ads}$</td>
<td>parameter for the correlation of the kinetic of adsorption of site 2 $[1/M^3]$</td>
</tr>
<tr>
<td>$\alpha_{k,des}$</td>
<td>parameter for the correlation of the kinetic of desorption of site 2 $[1/M^3]$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>parameter for the modifier dependence of the Henry coefficient [-]</td>
</tr>
<tr>
<td>$\beta_{k,ads}$</td>
<td>parameter for the correlation of the kinetic of adsorption of site 2 [-]</td>
</tr>
<tr>
<td>$\beta_{k,des}$</td>
<td>parameter for the correlation of the kinetic of desorption of site 2 [-]</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>parameter for the modifier dependence of the saturation capacity $[g/l]$</td>
</tr>
<tr>
<td>$\gamma_1$</td>
<td>parameter for the calculation of $D_{ax}$ [-]</td>
</tr>
<tr>
<td>$\gamma_2$</td>
<td>parameter for the calculation of $D_{ax}$ [-]</td>
</tr>
<tr>
<td>$\delta$</td>
<td>parameter for the modifier dependence of the saturation capacity $[1/M]$</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>parameter for the modifier dependence of the saturation capacity [-]</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>porosity of a tracer [-]</td>
</tr>
<tr>
<td>$\eta$</td>
<td>dimensionless axial position [-]</td>
</tr>
<tr>
<td>$\eta_s$</td>
<td>solvent viscosity $[\text{mPas}]$</td>
</tr>
<tr>
<td>$\nu_s$</td>
<td>kinematic viscosity of the solvent $[\text{cm}^2/\text{s}]$</td>
</tr>
<tr>
<td>$\rho$</td>
<td>dimensionless radial position [-]</td>
</tr>
<tr>
<td>$\rho_{lig}$</td>
<td>ligand density $[\mu\text{mol}/\text{g}]$</td>
</tr>
<tr>
<td>$\rho_{load}$</td>
<td>loading density (mass antibody per volume stationary phase) $[\text{g/l}]$</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>variance of the peak $[\text{min}]$</td>
</tr>
<tr>
<td>$\tau$</td>
<td>dimensionless time [-]</td>
</tr>
<tr>
<td>$\Phi_B$</td>
<td>association factor of the solvent in the Wilke-Chang equation [-]</td>
</tr>
</tbody>
</table>
1 Introduction

Monoclonal antibodies (mAbs), that are charged macromolecules of biological origin, are purified with a number of chromatographic steps. This purification task is of high academic and industrial relevance, because monoclonal antibodies are the most important therapeutic proteins. In order to understand the fundamental behavior of monoclonal antibodies from clarified cell culture supernatant (cCCS) on chromatographic stationary phases, the system must be simplified. Therefore, often only the adsorption of pure components on stationary phases is studied. Furthermore, monoclonal antibodies are often replaced by polyclonal antibody mixtures or other proteins, such as human serum albumin, myoglobin or lysozyme, because of limited availability and high costs of monoclonal antibodies. Even though human serum albumin is often used as a model protein, it is purified by Cohn fractionation [1], which is a precipitation process, and not by chromatography. Myoglobin is crystallized, while lysozyme is purified by crystallization and chromatography [2-4].

Monoclonal antibodies are a new class of drugs that help to treat widespread diseases such as cancer, rheumatoid arthritis, multiple sclerosis and asthma [5]. Furthermore, they are an important tool in biological diagnostics already. In the latter field they are typically used to detect other proteins in a very specific way: the antigen antibody reaction is used routinely in tests such as the enzyme linked immunosorbent assay (ELISA) or Western Blot.

The production principle for monoclonal antibodies was developed by Köhler and Milstein in 1975 [6]. Nine years later, they were awarded the nobel prize together with Jerne ‘for theories concerning the specificity in development and control of the immune system and the discovery of the principle for production of monoclonal antibodies’ [7].

The first monoclonal antibody, Orthoclone OKT3 was approved by the US Food and Drug Administration (FDA) for the treatment of transplant rejection another two years later, in 1986 [3,6]. In the last two decades, the development of new therapeutic monoclonals was exploding. Until 2002, FDA has approved eleven monoclonal antibodies
for therapeutic usage \cite{10}. Their main range of application is in the field of cancer treatment, transplant rejection and autoimmune diseases \cite{10,11}. For 2009, an annual production of 14 t of monoclonal antibodies is forecasted \cite{12}.

Antibodies are produced by mammalian cell fermentation. As the titers have increased up to 5 g/l, the major cost driver for the drug production is now the downstream part of the production train \cite{13}. Roque et al. reported in 2004 that downstream processing of antibodies creates 50 to 80\% of the total manufacturing costs \cite{14}. A typical purification process consists of a chromatographic capture step where the antibody is selectively bound to a Protein A affinity resin. The ligand of this stationary phase is obtained from the bacterium Staphylococcus aureus that protects itself from the (human) immune defence by binding of the Fe-region of antibodies with Protein A \cite{15}. For polishing and removal of leaked Protein A, two more chromatographic steps follow. It is required by the authorities that virus inactivation and removal is proven in two orthogonal steps \cite{16}. As the product must be eluted at low pH from the Protein A column, this also serves as a pH-inactivation of viruses. Virus removal is obtained with a virus filtration step. Finally, the product is treated by ultra- or diafiltration \cite{17,18}.

**Table 1.1:** Allocation of purification costs for monoclonal antibodies. This table considers only the direct materials costs of goods and raw materials, but not depreciation and labor costs according to Kelley \cite{13}.

<table>
<thead>
<tr>
<th>purification step</th>
<th>cost share (%)</th>
</tr>
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<tbody>
<tr>
<td>harvest</td>
<td>25</td>
</tr>
<tr>
<td>Protein A chromatography</td>
<td>51</td>
</tr>
<tr>
<td>anion exchange chromatography</td>
<td>9</td>
</tr>
<tr>
<td>virus retaining filtration</td>
<td>12</td>
</tr>
<tr>
<td>ultra-/diafiltration</td>
<td>3</td>
</tr>
</tbody>
</table>

The main advantage of Protein A is the extremely high selectivity towards antibodies. However, Protein A is not stable under alkaline conditions which necessitates the usage of expensive denaturing agents such as guanidine chloride for sanitization. Another disadvantage of the Protein A step is the high resin price. Kelley has calculated the cost for the purification of monoclonal antibodies according to Table 1.1. This example, which is based on industrial practice, shows clearly that the most important cost driver in downstream processing is the Protein A step. A replacement of this step, even with two other chromatographic steps (e.g. cation exchange chromatography, hydrophobic interaction chromatography), would cut the costs by 25\%. Moreover, Protein A is highly
toxic \[19\]. Therefore, the removal of leaked Protein A must be proven in subsequent purification steps.

In order to overcome this problem, other purification strategies have been proposed \[20\]. Cation exchange resins present an interesting alternative for the capture step, especially because of their high binding capacity for proteins and their low cost in comparison to Protein A resins \[18, 21, 22\]. Moreover, ion exchange materials have the ability to both capture and resolve different proteins \[23\].

Other promising options include hydrophobic interaction chromatography \[24\]. A rather new approach is made with the so-called mixed mode resins that exhibit more than one functionality for the purification of proteins. Apatite resins offer ionic as well as chelating interactions. Schubert and Freitag showed the application of this kind of stationary phase for the capturing of antibodies from cell culture supernatant \[25\]. Another type of mixed mode resins is made by the introduction of aromatic rings into the ligand of anion exchange resins \[26\]--\[30\]. This modification promotes binding of proteins at elevated salt concentrations due to non-electrostatic interactions.

The replacement of the Protein A affinity resin presents an interesting approach to cut costs in downstream processing of antibodies, because of the extremely high price of these stationary phases. However, a cost reduction can be achieved by other means. Aumann and Morbidelli introduced a continuous process for the purification of antibodies from clarified cell culture supernatant \[31, 32\]. In an example for this purification task, a final host cell protein content of 2 ppm is reached in just two chromatographic steps without Protein A \[21\]. Also the successful separation of antibody variants, which differ only slightly in charge, was performed with this purification scheme \[33\]. Moreover, the continuous process typically reaches a high yield and productivity at low solvent consumption.

In order to better understand the separation process, many authors evaluated and compared stationary phases in order to find the best material for a given purification problem. Staby et al. measured the properties of anion as well as cation exchange resins extensively \[34, 39\]. Ghose et al. compared the properties of hydrophobic charge induction materials to Protein A mimetic and Protein A resins \[40\]. Pore size and retention factors of hydrophobic interaction materials were studied for a set of proteins by To and Lenhoff \[41\]. However, materials from different suppliers were compared. As differences in the synthesis and manufacturing of the resins might lead to great differences in the performance for a certain kind of application, a fair comparison of these materials is difficult. In particular, it is hard to link certain performance data directly to a material property, because the information about each material is incomplete.

The understanding of the adsorption process of proteins on stationary phases gets more
and more important. The general rate model treats the adsorption of proteins on stationary phases in a comprehensive way. Mass transfer effects such as axial diffusion, pore diffusion and molecular diffusion are described with literature correlations. They are not lumped into few variables that only give the possibility to model the system in a very narrow range. As shown by Kaczmarski and Antos, the general rate model is mandatory for systems that have significant mass transfer hindrances. Macro-molecules such as proteins always travel through chromatographic columns with mass transfer limitations. Furthermore, the general rate model provides useful insights into the diffusion and adsorption process of the protein on the stationary phase. As the parameters are not lumped, dominating effects can be easily monitored. With increasing computational power in the last couple of years, it is possible to solve the general rate model fast enough in order to use it for parameter studies.

In Chapter 2 of this thesis, a set of custom made cation exchange resins is analyzed in order to develop a superior stationary phase for the purification of proteins. Therefore, transport parameters as well as the isotherm are analyzed for immunoglobulin G on those materials.

In Chapter 3 a new mixed mode resin is tested for its application in the downstream processing of monoclonal antibodies. The mixed mode functionalities enable binding even at conditions where proteins do not adsorb on comparable anion exchange resins. It is shown that the mixed mode resin can be used for the polishing step of a monoclonal antibody from clarified cell culture supernatant, as well as for the separation of IgG subclasses in a polyclonal antibody mixture.

In Chapter 4 the behavior of human serum albumin on a strong cation exchange resin is studied systematically. The properties of the protein on the stationary phase are analyzed at diluted and at preparative, i.e. overloaded conditions.

Chapter 5 is dedicated to the simulation of the behavior of human serum albumin on Fractogel EMD SE Hicap (M) with a general rate model. Besides a good description of the experimental data, the model predicts data, that was not used for the regression, sufficiently well.
2 Role of the ligand density in cation exchange materials for the purification of proteins

2.1 Introduction

Historically, ion exchange resins were developed for the purification and analysis of small molecules \(45\). Their application to the purification of proteins introduces a manifold of new problems due to the fact that antibodies such as IgG have dimensions comparable to the pore sizes. This leads not only to high mass transfer resistances, but also to limited accessibility of the protein into the pores of the stationary phase. As it will be discussed in detail in this work, some other factors, such as the particle size and especially the ligand density of the support, play an important role here. The influence of the latter on the performance of ion exchange materials has already been studied by Wu and Walters for silica supports in 1992 \(46\). However, they chose rather small proteins such as lysozyme and cytochrome c. Zhang and Sun studied the behavior of bovine serum albumin and bovine hemoglobin on affinity resins with different ligand densities \(47\). Langford et al. studied the mass transfer of lysozyme on a set of cation exchange resins with varying ligand density \(48\).

In this work, a set of custom made cation exchange resins is studied regarding pore size distribution, ligand density and particle size in order to find the effect of those parameters on mass transfer resistances and static and dynamic binding capacity for IgG. To our knowledge, these parameters have not been analyzed systematically for large biomolecules such as IgG. In the first part of this chapter, all analyzed materials have the same polymeric support as the commercial material Fractogel EMD SO\(_3\)\(^-\) (M). Based on the obtained results, a new cation exchange material, tailor made for the purification of IgG, i.e. FractoAIMs, is developed in the second part of this chapter.
The behavior of this material and the benchmark material Fractogel EMD SO$_3^-$ (M) are simulated with a general rate model. This model considers the concentration distribution of the solutes in the axial direction along the chromatographic column as well as along the radial direction in the stationary phase [43]. Even though the model needs to solve a large number of differential equations, its application is needed in the case of systems with dominating mass transfer resistances [43]. The objective here is to verify the possibility of such a model to predict the dynamic binding capacity of a given stationary phase. This could help tremendously in the screening phase of operating conditions and stationary phases by avoiding the dynamic column breakthrough experiments which require time and materials not always available in the early stage of process development. The combination of a rational design of a new material, based on experimental data, with the simulation of the behavior of IgG on this material gives new insights in the mode of operation of ion exchange resins for preparative protein purification. Accordingly, this chapter provides a new design strategy for preparative stationary phases for the purification of large molecules.

2.2 Experimental

2.2.1 Materials

Strong cation exchange resin Fractogel EMD SO$_3^-$ (M), that was chosen as a benchmark material, was provided by Merck (Darmstadt, Germany). It has a crosslinked polymethacrylate matrix with sulfonated butyl as functional groups. These are bound to the matrix with linear polymer chains, the so-called ‘tentacles’ [49]. The particle size of Fractogel EMD SO$_3^-$ (M) is $d_p = 40 \div 90\ \mu m$ (average $d_p = 65\ \mu m$) with a pore size of about $r_p = 400$ Å. For large scale operation, Fractogel EMD SO$_3^-$ (M) is operated at velocities up to $u_{tin} = 350 \text{cm/h}$. As the columns used in this work are much shorter, it was possible to flow pack the resin at a velocity of $u_{tin} = 800\ \text{cm/h}$. The maximum velocity for chromatographic experiments is set to a linear velocity of $u_{lin} = 400\ \text{cm/h}$. Based on the same support used for Fractogel EMD SO$_3^-$ (M), Merck (Darmstadt, Germany) synthesized materials with different ligand densities (Series 1, see Table 2.1). Furthermore, a new material called FractoAIMs, was developed by Merck within this work. This material has an average particle size of 40µm. It was designed in order to have a high rigidity which leads to a much higher operating flow rate than Fractogel EMD SO$_3^-$ (M), also at preparative conditions. Therefore, FractoAIMs was flow packed with a velocity of $u_{tin} = 1100\ \text{cm/h}$ in this work. The ligand density of all materials was
Table 2.1: Synthesized Fractogel materials and FractoAIMs and corresponding ligand density. Series 1 includes Fractogel A-E.

<table>
<thead>
<tr>
<th>material</th>
<th>$\rho_{\text{lig}}$ [nmol/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractogel EMD SO$_3^-$ (M)</td>
<td>380</td>
</tr>
<tr>
<td>Fractogel A</td>
<td>144</td>
</tr>
<tr>
<td>Fractogel B</td>
<td>236</td>
</tr>
<tr>
<td>Fractogel C</td>
<td>338</td>
</tr>
<tr>
<td>Fractogel D</td>
<td>400</td>
</tr>
<tr>
<td>Fractogel E</td>
<td>485</td>
</tr>
<tr>
<td>FractoAIMs</td>
<td>385</td>
</tr>
</tbody>
</table>

evaluated by the supplier by titration as summarized in Table 2.1.

Gammanorm, which is human normal immunoglobulin G (IgG $> 95\%$) in solution ($c_{\text{IgG}} = 165 \text{ g/l}$), has been chosen as a model protein to investigate the behavior of antibodies on ion exchange materials. The polyclonal antibody mixture was purchased at Octapharma (Lachen, Switzerland). Human serum albumin and myoglobin from equine skeletal muscle were purchased at Sigma-Aldrich (Buchs, Switzerland). Sodium chloride and acetic acid (glacial) were purchased at J. T. Baker (Deventer, the Netherlands), sodium acetate trihydrate and sodium hydrogen phosphate dihydrate were purchased at Merck (Darmstadt, Germany). Sodium phosphate dibasic dodecahydrate was obtained by Acros Organics (Geel, Belgium). Pullulan standards were purchased at Polymer Standards Service (Mainz, Germany). Dextran standards were purchased at Fluka (Buchs, Switzerland). Deionized water was purified with a Simpax 2 unit by Millipore (Zug, Switzerland). All used chemicals were analytical grade. All buffer solutions were prepared using a precision balance METTLER AT250 (Mettler-Toledo, Greifensee, Switzerland). The buffer composition was calculated according to the recipes by Beynon and Easterby [50].

2.2.2 Instrumentation

For chromatographic measurements, a HPLC 1100 Series by Agilent Technologies (Santa Clara, CA, USA) was used. The instrument is equipped with an UV detector and a refractive index detector.

Resins were packed into Tricorn columns purchased at GE Healthcare (Chalfont St
Giles, United Kingdom) and Superformance columns by Goetec Labortechnik (Mühltal, Germany). Tricorn columns have a volume of \( V \approx 2 \text{ml} \) and a diameter of \( d = 5 \text{mm} \). Superformance columns have a volume of \( V \approx 20 \text{ml} \) and a diameter of \( d = 16 \text{mm} \). 20\% mechanical compression of the bed was applied to the resin in those columns. Furthermore, columns by Infocroma (Zug, Switzerland) with a diameter of \( d = 4.6 \text{mm} \) and a volume of \( V = 1.0 \text{ml} \) and columns by YMC (Kyoto, Japan) with a diameter of \( d = 7.5 \text{mm} \) and a volume of \( V = 2.2 \text{ml} \) were used. The latter two columns could not be compressed mechanically.

### 2.2.3 Methods

**Pore size distribution.** The pore size distribution was measured by inverse size exclusion chromatography (ISEC) \([51, 52]\). In comparison with other techniques for the determination of the pore structure, such as mercury porosimetry or BET measurements, this method has a number of advantages. Particularly, drying of the sample is not necessary. Furthermore, the measurements can be performed directly in the packed column.

ISEC measurements with various dextran and pullulan tracers, as well as protein tracers, were executed under nonadsorbing conditions. The ratio of the accessible liquid volume \( V_{t,i} \) for a generic tracer \( i \) of specific molecular weight and the total column volume \( V \) is used to calculate the total porosity \( \epsilon_{t,i} \) accessible to the tracer on the resin:

\[
\epsilon_{t,i} = \frac{V_{t,i}}{V}
\]

For dextran and pullulan, the average retention volume is estimated from the maximum of the elution peak, because these tracers are actually constituted by a mixture of molecules with different molecular weight. This causes a broadening of the chromatographic peak and therefore an artificial increase of the first moment of the peak. Since protein tracers on the other hand are monodisperse molecules, the retention volume is calculated from the first order moment of the peak.

The bed porosity \( \epsilon_{\text{bed}} \) is estimated by a tracer with a molecular weight that is large enough to be excluded from all pores of the stationary phase. The particle porosity \( \epsilon_{p,i} \) is linked to the total porosity \( \epsilon_{t,i} \) and the bed porosity \( \epsilon_{\text{bed}} \) by Equation (2.2):

\[
\epsilon_{t,i} = \epsilon_{\text{bed}} + (1 - \epsilon_{\text{bed}})\epsilon_{p,i}
\]

By measurement of the particle porosity \( \epsilon_{p,i} \) of appropriate tracers, it is possible to gather information about the pore size distribution of the stationary phase.
2.2 Experimental

**HETP values.** The concept of the height equivalent to a theoretical plate (HETP) divides the chromatographic column into a number of equilibrium plates. Their height characterizes the separation efficiency of the column and the mass transfer properties of the porous material.

Especially for large molecules such as proteins, diffusion is influencing the path of the tracer through the column. At faster velocities, the chromatogram shows highly asymmetric peaks. Therefore, the HETP values must be calculated from the moments of the peak as follows \[44, 53\]:

\[
HETP = L \frac{\sigma^2}{t_R}
\]  

(2.3)

\(L\) is a function of the length of the column, \(\sigma\) is the variance and \(t_R\) is the average retention time of the peak. Higher moments, such as the variance \(\sigma\), are very sensitive to measurement errors and noise. In order to minimize this influence, each peak was fitted with a perturbed gamma distribution as proposed by Hulbert and Katz \[54\]. The fitted peak was then used to calculate the HETP according to Equation (2.3).

With the van Deemter equation, the column efficiency HETP is related to axial dispersion \(D_{az}\), pore diffusion \(D_{p,\text{eff}}\) and film mass transfer coefficient \(k_f\) of the solute in the column as follows \[55\]:

\[
HETP = \frac{2 D_{az}}{u} + \frac{2 u d_p}{F} \left( \frac{\epsilon_{p,i} F}{\epsilon_{p,i} F + 1} \right)^2 \left( \frac{d_p}{60 D_{p,\text{eff}}} + \frac{1}{6 k_f} \right)
\]  

(2.4)

which applies to the case of a nonadsorbing species. Its derivation as well as a more general representation is given by Guiochon \[42\]. According to Forrer et al. \[56\], the term for axial dispersion might be neglected for large molecules such as IgG. The van Deemter equation is a function of the particle diameter \(d_p\), the phase ratio \(F\), defined as

\[
F = \frac{1 - \epsilon_{\text{bed}}}{\epsilon_{\text{bed}}}
\]  

(2.5)

and the particle porosity \(\epsilon_{p,i}\) of the used tracer. The film mass transfer coefficient \(k_f\) was calculated according to the equation of Wilson and Geankoplis \[57\],

\[
k_f = 1.09 \sqrt{u} \left( \frac{D_m}{\epsilon_{\text{bed}} d_p} \right)^{2/3}
\]  

(2.6)

where \(D_m\), the molecular diffusion coefficient, is given by:

\[
D_m = 8.34 \cdot 10^{-8} \frac{T}{\eta_s \sqrt{MW}}
\]  

(2.7)
in the particular case of proteins \cite{58}. The viscosity of the solvent (assumed to be the same as 0.1 M sodium chloride solution at 20 °C) is estimated to be $\eta_S = 1.001 \text{ mPas}$ \cite{59}. The molecular diffusion coefficient for acetone is calculated with the Wilke-Chang equation \cite{60},

$$D_m = 7.4 \cdot 10^{-5} \frac{T \sqrt{\Phi_B MW}}{\eta_S V_n^{0.6}}$$

(2.8)

where the solvent is assumed to be pure water ($MW_{H_2O} = 18.02 \text{ Da}$). The association factor $\Phi_B$, that accounts for hydrogen bonding of the solvent, was set to $\Phi_B = 2.26$ \cite{61}. The molecular volume $V_n$ of the solute is calculated according to Perry's ($V_n = 73.31 \text{ cm}^3/\text{mol}$ for acetone) \cite{61}.

**Static binding capacity.** Static capacity was measured offline. This procedure is convenient, since it allows gaining information about the equilibrium capacity of a material $q_{eq}$ with very little amount of protein and stationary phase. The resin $V_{sol}$ is mixed with a known amount of protein and buffer $V_{liq}$. Then, the mixture is stirred for two days. After that period of time it is presumed that adsorption equilibrium is reached. The equilibrium capacity $q_{eq}$ is calculated with the initial concentration of protein $c_0$ and the equilibrium concentration $c_{eq}$ in the supernatant:

$$q_{eq} = \frac{V_{liq} (c_0 - c_{eq})}{V_{sol}}$$

(2.9)

Under the selected conditions, the isotherm was rectangular for all materials, i.e. a small concentration of protein ($c_0 < 1 \text{ g/l}$) in the liquid phase is sufficient to reach the saturation capacity $q_\infty$.

**Dynamic binding capacity.** Dynamic binding capacity was measured at 10% breakthrough, i.e. when the outlet protein concentration is 10% of the feed concentration. This was evaluated by testing the feed signal in the UV-detector at a wavelength of 280nm and then running the breakthrough curve up to a signal of 10% of the original signal. The protein feed concentration was approximately $c_0 \approx 1.7 \text{ g/l}$. The dynamic binding capacity at 10% breakthrough was calculated as follows:

$$DBC_{10\%} = \frac{t A u_{in} c}{V}$$

(2.10)

Due to the typically large capacity of these columns and the relatively small feed concentration, the amount of IgG in the liquid phase of the column can be neglected. Under ideal conditions, that is in the absence of mass transfer resistances, the dynamic
2.2 Experimental

binding capacity should be equal to the static binding capacity. Thus, the difference between the two is a way to analyze the extend of mass transfer resistances under loading conditions.

Modeling approach. For the simulation of breakthrough curves, a general rate model was used [23, 62]. The mobile and the stagnant phases are treated separately. All mass balances were set up in dimensionless form. The mass balance for the liquid phase consists of four terms:

\[ \frac{\partial c}{\partial \tau} + \frac{\partial c}{\partial \eta} + \epsilon_{p,t} \frac{\partial q}{\partial \tau} \frac{1 - \epsilon_{bed}}{\epsilon_{bed}} St(c - c_{p|\eta=1}) = \frac{1}{Pe_a} \nabla_{\eta,2} c \]

(2.11)

accumulation of the solute in the mobile phase of the column, convection in the axial direction and flux from the mobile to the stagnant phase in the column. The last term on the right hand side represents axial dispersion along the column. The Stanton number \( St \) is defined as the ratio between the characteristic time for convection and the characteristic time for film mass transport \( d_p/(6 k_f) \):

\[ St = \frac{6 k_f}{u d_p} \]

(2.12)

The axial Peclet number \( Pe_a \) gives the ratio between the times for axial dispersion \( L^2/D_{az} \) and convection in the column as follows:

\[ Pe_a = \frac{u L}{D_{az}} \]

(2.13)

Danckwerts conditions [63] were used as boundary conditions for Equation (2.11):

if \( \tau = 0 \), then \( c = c(0, \eta) \)

if \( \eta = 0 \), then \( \frac{\partial c}{\partial \eta} = Pe_a(c - c_0) \)

(2.14)

if \( \eta = 1 \), then \( \frac{\partial c}{\partial \eta} = 0 \)

The mass balance for the stagnant phase in the general rate model is given by:

\[ \frac{\partial c_p}{\partial \tau} + \frac{1 - \epsilon_{p,t}}{\epsilon_{p,t}} \frac{\partial q}{\partial \tau} = \frac{1}{Pe} \nabla_{\rho,2} c_p \]

(2.15)

where the first term represents accumulation in the stagnant phase with \( c_p \) being the concentration of the solute in the stagnant phase and the second represents accumulation of the solute in the solid phase, whereas \( q \) is the concentration of adsorbed protein on the
stationary phase. The phase ratio is defined through the total porosity of the particle $\epsilon_{p,t}$ (as measured with a small tracer). The term on the right hand side represents diffusion of the solute across the adsorbent particle. The corresponding particle Peclet number $Pe$ is defined as:

$$Pe = \frac{\epsilon_{p,lg} u d_p^2}{4 L D_{p,eff}}$$

(2.16)

The boundary conditions of Equation (2.15) are given by:

- if $\tau = 0$, then $c_p = c_p(0, \rho)$
- if $\rho = 0$, then $\frac{\partial c_p}{\partial \rho} = 0$
- if $\rho = 1$, then $\frac{\partial c_p}{\partial \rho} = Sh (c - c_p(\rho = 1))$

(2.17)

The Sherwood number is defined as the ratio between the characteristic times for pore diffusion and film mass transfer as follows:

$$Sh = \frac{\epsilon_{p,lg} d_p k_f}{2 D_{p,eff}}$$

(2.18)

### 2.2.4 Results and discussion

**Pore size distribution.** ISEC measurements for Fractogel EMD SO$_3^-$ (M), Fractogel C and FractoAIMs were performed with dextran tracers, while for all other materials pullulan was used. The measurements were performed under nonadsorbing conditions, namely 50 mM phosphate buffer pH 7 with 0.5 M sodium chloride. Nonadsorbing conditions have been checked by repeating the same experiment at different salt concentrations. This check, however, can not fully exclude the presence of some unspecific binding (e.g. hydrophobic interaction).

**Table 2.2:** Porosity accessible by various tracers for Fractogel EMD SO$_3^-$ (M) packed in different columns.

<table>
<thead>
<tr>
<th>column</th>
<th>$V$</th>
<th>$L$</th>
<th>$D$</th>
<th>$L/D$</th>
<th>$\epsilon_{bed}$</th>
<th>$\epsilon_{lg}$</th>
<th>$\epsilon_{p,lg}$</th>
<th>$\epsilon_t$</th>
<th>$\epsilon_{p,t}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goetec</td>
<td>20.0</td>
<td>100.0</td>
<td>16.0</td>
<td>6.3</td>
<td>0.32</td>
<td>0.54</td>
<td>0.33</td>
<td>0.73</td>
<td>0.61</td>
</tr>
<tr>
<td>YMC</td>
<td>2.2</td>
<td>50.0</td>
<td>7.5</td>
<td>6.7</td>
<td>0.41</td>
<td>0.62</td>
<td>0.35</td>
<td>0.76</td>
<td>0.59</td>
</tr>
<tr>
<td>GE Healthcare</td>
<td>1.6</td>
<td>82.0</td>
<td>5.0</td>
<td>16.4</td>
<td>0.35</td>
<td>0.56</td>
<td>0.32</td>
<td>0.77</td>
<td>0.65</td>
</tr>
<tr>
<td>Infochroma</td>
<td>1.0</td>
<td>60.5</td>
<td>4.6</td>
<td>13.2</td>
<td>0.44</td>
<td>0.61</td>
<td>0.30</td>
<td>0.80</td>
<td>0.64</td>
</tr>
</tbody>
</table>
2.2 Experimental

![Graph showing the relationship between total porosity and molecular weight](image)

**Figure 2.1:** Comparison of ISEC-measurements with dextran tracers for Fractogel EMD SO$_3^-$ (M): Goetec column, 20 ml (■), GE Healthcare column, 1.6 ml (▲) and YMC column, 2.2 ml (◆).

The bed porosity was determined with a tracer with a molecular weight of 2000 kDa (hydrodynamic radius $r_h = 37.2$ nm, calculated according to DePhillips and Lenhoff [64]) that is totally excluded from the pores. The total porosity of each material was calculated with a dextran tracer with a molecular weight of 1.2 kDa or a pullulan tracer with a molecular weight of 1.1 kDa, respectively. It is assumed that a molecule of this size can penetrate all relevant pores of the stationary phase.

For all materials, protein tracers, especially IgG and HSA, were also tested. Those measurements were performed under nonadsorbing conditions, namely 20 mM acetate buffer pH 5 with a minimum of 0.5 M sodium chloride.

The pore size distribution was measured for the benchmark material Fractogel EMD SO$_3^-$ (M) with various columns, as shown in Table 2.2 and Figure 2.1. In all cases we observe a characteristic trend, i.e. with increasing tracer size the corresponding porosity decreases from the total porosity value $\epsilon_{t,i}$ to the bed porosity $\epsilon_{bed}$ when the tracer is so large that it cannot enter any of the particle pores. It is found that even though the same material was packed in all columns, the porosities differ significantly. As shown in Figure 2.1 the largest differences can be observed for the largest tracers, i.e. in the size range of interest for IgG. Here, a major role is played by mechanical compression. As the Fractogel particles are known to be rigid, compression is mainly decreasing the bed
porosity which explains why YMC and Infochroma columns (without axial compression) exhibit significantly larger bed porosities. As shown in Table 2.2 also the column geometry has a slight influence on the measured porosity. Columns with a high ratio $L/D$ (slim columns) have a larger bed porosity. This effect is due to stronger wall effects in these columns which lead to looser packing.

![Graph](image)

**Figure 2.2:** Comparison of ISEC-measurements for Fractogel A, $\rho_{bg} = 144\,\mu\text{mol/g}$ (◇), Fractogel B, $\rho_{bg} = 236\,\mu\text{mol/g}$ (◆), Fractogel C, $\rho_{bg} = 338\,\mu\text{mol/g}$ (□), Fractogel D, $\rho_{bg} = 400\,\mu\text{mol/g}$ (●) and Fractogel E, $\rho_{bg} = 485\,\mu\text{mol/g}$ (❖).

In Figure 2.2 the pore accessibilities for different materials (Series 1, see Table 2.1) measured in the Goetec column are shown as a function of the dextran/pullulan molecular weight. As all materials are based on the same support, they should have the same pore structure. However, since the ligands block part of the pores, it follows that pore accessibility decreases with ligand density. In particular, Figure 2.2 shows that the transition region of the ISEC curve is shifted depending on the ligand density. We see for example that Fractogel E will not show a good performance with respect to IgG purification, since the porosity of the tracer with a molecular weight of 46 kDa is already equal to the bed porosity, meaning that IgG, which has a molecular weight of 144 kDa, is probably totally excluded from the pores and therefore does not access the ligands in the pores. In all cases a plateau is reached for the largest tracers, thus confirming the choice of the 2000 kDa tracer for the estimation of the bed porosity.
The porosities of IgG for the different materials discussed above are compared in Figure 2.3 as a function of the ligand density. It is seen that the porosity for IgG is highly influenced by the ligand density, as the porosity changes from 42% to 5% for IgG in the analyzed range.

A comparison of the porosity measurements for Fractogel C (Series 1) and the benchmark material Fractogel EMD SO$_3^−$ (M), which both have a similar ligand density (see Table 2.1), is shown in Table 2.3. It is seen that the bed porosity of the two materials is nearly the same. However, the porosity of IgG on Fractogel EMD SO$_3^−$ (M) is slightly higher, indicating either a slightly different pore structure of the two materials or a slightly different ligand distribution in the pores or a combination of the two effects.

Table 2.3: Porosity of various tracers for Fractogel EMD SO$_3^−$ (M) and Fractogel C measured on column Goetec.

<table>
<thead>
<tr>
<th>material</th>
<th>$\epsilon_{\text{bed}}$</th>
<th>$\epsilon_{t,IgG}$</th>
<th>$\epsilon_{p,IgG}$</th>
<th>$\epsilon_t$</th>
<th>$\epsilon_{p,t}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractogel EMD SO$_3^−$ (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.73</td>
<td>0.61</td>
</tr>
<tr>
<td>Fractogel C</td>
<td>0.31</td>
<td>0.50</td>
<td>0.28</td>
<td>0.76</td>
<td>0.66</td>
</tr>
</tbody>
</table>
**HETP values.** Measurements for the estimation of the HETP were conducted under nonadsorbing conditions with 20 mM acetate buffer pH 5 with a minimum of 0.5 M sodium chloride. As for the ISEC measurements, the Goetec column with a volume of 20 ml was used. The HETP was measured in the range of linear velocity values of $u_{in} = 9 - 90 \text{ cm/h}$, whereas the latter value corresponds to the maximum flow rate for the HPLC pump. Figure 2.4 shows HETP of various tracer as a function of the linear velocity for Fractogel C. It is seen that the van Deemter curve is almost flat with small values of HETP for the smallest tracer, i.e. acetone. As expected, there is only little pore diffusion limitation in the column for this small molecule. For proteins, the influence of pore diffusion becomes significant. The largest protein IgG has the highest HETP values for all measured velocities. The number of theoretical plates is highly dependent on the velocity ranging from 20 to 103 plates for the used column. These results confirm the need for optimization of mass transport effects in such columns and explain the large dependence of the dynamic binding capacity on the linear velocity that is typically observed for large proteins.

As described in Equation (2.4), the information from the van Deemter plot can be used to estimate the effective pore diffusion. Note that Figure 2.4 shows the HETP values as a function of the linear velocity $u_{in}$, whereas the values of the pore effective diffusivity $D_{p, eff}$ (Equation (2.4)) are calculated with the interstitial velocity $u (u_{in} = \epsilon_{bed} \bar{u})$. 

![Figure 2.4: HETP values for Fractogel C: acetone (▲), myoglobin (□), HSA (●) and IgG (●).](image-url)
Table 2.4: Film mass transfer coefficient $k_f$, molecular diffusivity $D_m$, pore effective diffusivity $D_{p,eff}$ and ratio between molecular and pore effective diffusivity $D_m/D_{p,eff}$ for selected tracers on Fractogel C. The film mass transfer coefficient was calculated at a velocity of $u = 0.04 \text{cm/min}$. As a comparison, the last column shows the pore effective diffusivity $D_{p,eff}$ as measured by Forrer et al. on Fractogel EMD SE Hicap (M) \[50\].

<table>
<thead>
<tr>
<th>tracer</th>
<th>$k_f$</th>
<th>$D_m$</th>
<th>$D_{p,eff}$</th>
<th>$D_m/D_{p,eff}$</th>
<th>$D_{p,eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetone</td>
<td>$1.14 \times 10^{-2}$</td>
<td>$1.05 \times 10^{-5}$</td>
<td>$1.63 \times 10^{-6}$</td>
<td>6.46</td>
<td>$1.09 \times 10^{-6}$</td>
</tr>
<tr>
<td>myoglobin</td>
<td>$2.27 \times 10^{-3}$</td>
<td>$9.38 \times 10^{-7}$</td>
<td>$1.58 \times 10^{-7}$</td>
<td>5.95</td>
<td>$1.60 \times 10^{-7}$</td>
</tr>
<tr>
<td>HSA</td>
<td>$1.69 \times 10^{-3}$</td>
<td>$6.04 \times 10^{-7}$</td>
<td>$3.82 \times 10^{-8}$</td>
<td>15.80</td>
<td>$5.69 \times 10^{-8}$</td>
</tr>
<tr>
<td>IgG</td>
<td>$1.42 \times 10^{-3}$</td>
<td>$4.66 \times 10^{-7}$</td>
<td>$1.85 \times 10^{-8}$</td>
<td>25.23</td>
<td>$2.31 \times 10^{-8}$</td>
</tr>
</tbody>
</table>

The obtained results are reported in Table 2.4 for different molecules. As expected, it can be observed that the pore effective diffusivity decreases with increasing molecular weight of the tracer. Note that the ratio between molecular and pore effective diffusivity increases with the protein size, thus indicating that the transport becomes more and more hindered with increasing protein size. For a comparison, in the last column of Table 2.4, the pore effective diffusivity values measured by Forrer et al. on Fractogel EMD SE Hicap (M) are reported \[50\]. These numbers correspond rather well with the values measured in this work.

For each of the Series 1 materials, the van Deemer plot has been produced (not shown) and the corresponding slope $HETP/u$ measured as reported in Table 2.5. From such val-

Table 2.5: Slope of the van Deemer curve $HETP/u$ and pore effective diffusivity $D_{p,eff}$ for the benchmark material Fractogel EMD SO$_3^-$ (M) and Series 1 for IgG.

<table>
<thead>
<tr>
<th>material</th>
<th>$\rho_{lig}$</th>
<th>$HETP/u$</th>
<th>$D_{p,eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{\mu mol/g}$</td>
<td>$[\text{s}]$</td>
<td>$[\text{cm}^2/\text{s}]$</td>
</tr>
<tr>
<td>benchmark</td>
<td>380</td>
<td>3.61</td>
<td>$3.28 \times 10^{-8}$</td>
</tr>
<tr>
<td>Fractogel A</td>
<td>144</td>
<td>3.42</td>
<td>$4.69 \times 10^{-8}$</td>
</tr>
<tr>
<td>Fractogel B</td>
<td>236</td>
<td>2.87</td>
<td>$5.12 \times 10^{-8}$</td>
</tr>
<tr>
<td>Fractogel C</td>
<td>338</td>
<td>5.20</td>
<td>$1.85 \times 10^{-8}$</td>
</tr>
<tr>
<td>Fractogel D</td>
<td>400</td>
<td>2.72</td>
<td>$2.25 \times 10^{-8}$</td>
</tr>
<tr>
<td>Fractogel E</td>
<td>485</td>
<td>0.40</td>
<td>$3.05 \times 10^{-8}$</td>
</tr>
</tbody>
</table>
ues, the pore diffusivity has been computed using Equation (2.4) and the porosity values measured in Figure 2.3. It appears that most of the variation of the slope $HETP/u$ is due to different porosity values while the pore diffusivities exhibit a not always coherent trend. Note, that the van Deemter equation (Equation 2.4) is in fact a strong function of the porosity and not only of the pore diffusivity. Indeed, it is seen that Fractogel A and B have larger pore effective diffusivities than the benchmark material Fractogel EMD $SO_3^-$ (M). As these two materials have a significantly lower ligand density than the benchmark material, the corresponding accessible pores are wider which leads to pore effective diffusivities that are approximately 50\% larger. However, when looking at higher ligand density values, this trend is not confirmed. It should be noted however, that for example for Fractogel E, the ligand density is so large and the accessible porosity so low (Figure 2.3) that talking about pore diffusivity is hardly possible and therefore the effect of measurement errors becomes strong.

**Static binding capacity.** Static capacity was measured offline in stirred beakers. A mixture of 50\% resin and 50\% buffer (20 mM acetate buffer with 30 mM sodium chloride

![Figure 2.5: Adsorption equilibrium isotherm for IgG with 20 mM acetate buffer pH 5 with 0.03 M sodium chloride for Fractogel A, $\rho_{lig} = 144\mu mol/g$ (♦), Fractogel B, $\rho_{lig} = 236\mu mol/g$ (○), Fractogel C, $\rho_{lig} = 338\mu mol/g$ (■), Fractogel D, $\rho_{lig} = 400\mu mol/g$ (●) and Fractogel E, $\rho_{lig} = 485\mu mol/g$ (△). The dashed lines represent Langmuir isotherm fits of the data.](image-url)
at pH 5) was agitated for two days with a known amount of IgG. The IgG concentration of the supernatant was then analyzed and the corresponding adsorbed equilibrium concentration was calculated according to Equation 2.19.

The static binding capacity of IgG for the materials of Series 1 is shown in Figure 2.5 in terms of the adsorption equilibrium isotherm. For all measured materials it exhibits a characteristic rectangular shape, i.e. saturation conditions are achieved already with very small IgG concentrations in the liquid phase.

![Graph](image)

**Figure 2.6:** Saturation capacity $q_\infty$ as a function of the ligand density for Series 1 ($\diamond$). Fractogel EMD SO$_3^-$ (M) (■) is also shown.

The saturation capacity $q_\infty$ as a function of the ligand density is shown in Figure 2.6. The binding capacity reaches a maximum at a ligand density of approximately $\rho_{lg} \approx 400 \mu$mol/g. This result is discussed in detail later in the context of the dynamic binding capacity data.

It is worth mentioning that since this method uses only very small amounts of protein and resin, the error of the absolute values for the saturation capacity could be large. However, part of the dataset was measured in duplicate and a maximum error of 15% was measured.

Forrer et al. measured the saturation capacity for Fractogel EMD SE Hicap (M) with GammaNorm with 20 mM acetate buffer with 0.05 M sodium chloride [5.6], hence with a modifier concentration slightly higher than in the experiments presented here. However,
Forrer calculated a saturation capacity of $q_{\infty} = 156 \, \text{g/l}$. This value is 22% higher than the value measured in this paper for Fractogel EMD SO$_3^-$ (M) ($q_{\infty} = 128 \, \text{g/l}$). A similar difference of about 20% in the capacities of these two resins was reported earlier in the literature [22].

![Graph](image)

**Figure 2.7:** Breakthrough curves for different flowrates for Fractogel C: $u_{lin} = 361 \, \text{cm/h}$ (left), $u_{lin} = 181 \, \text{cm/h}$ (right). The feed concentration was $c_{IgG} = 1.46 \, \text{g/l}$.

**Dynamic binding capacity.** These experiments were conducted using 20 mM acetate buffer with 30 mM sodium chloride at pH 5 which are the same conditions used above for the determination of the static binding capacity. The protein was eluted with 20 mM acetate buffer with 1 M sodium chloride at pH 5. After each breakthrough curve cleaning in place (CIP) was done with 0.25 M sodium hydroxide solution.

Dynamic binding capacity at 10% was measured for all materials listed in Table 2.1 at two different velocities ($u_{lin} = 181 \, \text{cm/h}$ and $u_{lin} = 361 \, \text{cm/h}$). The corresponding breakthrough curves are shown in Figure 2.7 for Fractogel C only. The obtained values of $DBC_{10\%}$ computed through Equation (2.10) for the Series 1 materials and for Fractogel EMD SO$_3^-$ (M) at the two selected velocities are shown in Figure 2.8 together with the static binding capacity values discussed above. In all cases a maximum of the dynamic or static binding capacity is seen as a function of the ligand density. This maximum is influenced by the amount of available ligands on the one hand and by the accessible surface area for IgG on the other hand. If a material is
Figure 2.8: Dynamic binding capacity at 10% breakthrough $DBC_{10\%}$: $u_{lin} = 181 \text{ cm/h}$ (>) and $u_{lin} = 361 \text{ cm/h}$ (○). The saturation capacity $q_{\infty}$ is measured in batch mode (□). The empty symbols represent Series 1, whereas the data for Fractogel EMD SO₃⁻ (M) is shown as filled symbols.

Functionalized with a large amount of ligands, the number of possible binding sites is increased, while at the same time, the number of accessible binding sites is decreased, because the ligands block part of the pores that IgG cannot enter anymore.

It is worth noting, that the maximum dynamic binding capacity is not necessarily at the same ligand density value for all flow rates and in particular at static conditions. Figure 2.8 shows that the maximum for the static binding capacity is at a ligand density of $\rho_{lig} \approx 400 \mu\text{mol/g}$, whereas it shifts to lower ligand densities for dynamic conditions. This behavior can be explained as the trade-off between the static capacity and the diffusion limitations that are becoming more pronounced at larger flow rates, thus hindering the access to all the available capacity. This is further complicated by the fact that as discussed above, the ligand density has an independent direct effect in lowering the pore diffusivity. This means that it is important to tune the ligand density for the targeted loading velocity in order to reach an optimal performance.

A simple way to compute the fraction of ligands which is actually accessible under certain conditions is to assume that this is equal to the fraction of pore volume which is
actually accessible under the same conditions that is

\[
\frac{\rho_{\text{acc}}}{\rho_{\text{lg}}} = \frac{\epsilon_{I,G} - \epsilon_{\text{bed}}}{\epsilon_{t} - \epsilon_{\text{bed}}}
\]  \hspace{1cm} (2.19)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_9.png}
\caption{Dynamic binding capacity at 10\% breakthrough as a function of the accessible ligand density $\rho_{\text{acc}}^{\text{lg}}$: $u_{\text{lin}} = 181 \text{ cm/h} \ (>)$ and $u_{\text{lin}} = 361 \text{ cm/h} \ (\circ)$. The empty symbols represent Series 1, whereas the data for Fractogel EMD $\text{SO}_4$ (M) is shown as filled symbols.}
\end{figure}

If we now replot the $\text{DBC}_{10\%}$ values as a function of the so converted accessible ligand density $\rho_{\text{acc}}^{\text{lg}}$, as shown in Figure 2.9, we find the expected trend of the dynamic binding capacity increasing linearly with the ligand density. This is because we consider only the fraction of ligands here which is accessible and not their total number (as in Figure 2.8). The slope of the straight line increases when the velocity decreases due to the presence of mass transport resistances.

Note on the other hand that such a linear behavior is not exhibited by the static binding capacity data in Figure 2.6. This should not be surprising since the concept of accessibility has to be understood in the time frame of a chromatographic run, i.e. some minutes. On the contrary, in static experiments we wait days in order to reach equilibrium conditions and therefore some pores which were not accessible under dynamic conditions may become accessed.
2.2.5 Development of a new ion exchange resin: FractoAIMs

Based on the understanding derived from the analysis of the Series 1 materials, a new stationary phase was designed. The following criteria were set:

- the material should have an optimal ligand density to maximize the dynamic binding capacity.
- the material should have a large pore accessibility to IgG, in order achieve fast mass transport rates.
- the material should have a high rigidity so as to tolerate high eluent velocities (i.e. productivities).

FractoAIMs is the material, that was developed in order to meet those criteria. Mass transfer resistances have been minimized first by a reduction of the particle size from \( d_p = 65 \mu m \) to \( d_p = 40 \mu m \). All the other properties are reported in Section 2.2.1. In order to operate the material with high flow rate also under preparative conditions, the particles are designed to be especially rigid. The ligand density of FractoAIMs (listed in Table 2.1) is selected to lead to high dynamic biding capacities. In the following, FractoAIMs is compared to the benchmark material Fractogel EMD SO\(_3^-\) (M). Both materials were packed into YMC columns.

**Pore size distribution.** The pore size distribution is shown in Figure 2.10 in terms of total accessible porosity as a function of the tracer size for FractoAIMs and Fractogel EMD SO\(_3^-\) (M). They exhibit the same bed porosity and very similar total porosity, i.e. at very low and very high molecular weight of the tracer, respectively. However, the accessibility of the pores is better in the case of FractoAIMs for almost all tracers. In particular, it is seen that for protein tracers, FractoAIMs has a particle porosity that is about 10 \% larger than that of Fractogel EMD SO\(_3^-\) (M).

**HETP values.** The slope of the van Deemter curve is significantly higher for Fractogel EMD SO\(_3^-\) (M) compared to FractoAIMs (see Figure 2.11). However, calculation of the pore effective diffusivity gives \( D_{p,eff} = 3.63 \cdot 10^{-8} \text{cm}^2/\text{s} \) for FractoAIMs and \( D_{p,eff} = 4.28 \cdot 10^{-8} \text{cm}^2/\text{s} \) for Fractogel EMD SO\(_3^-\) (M). These values are close, indicating that the difference in HETP is not due to different pore diffusivity values, but rather to the different accessible porosities. The overall mass transfer is significantly faster for FractoAIMs, mostly because of the smaller particles which lead to a significantly shorter characteristic time for diffusion and again because of the larger pore accessibility. Note for a comparison that Forrer et al. found a pore effective diffusivity of \( D_{p,eff} = \)}
Figure 2.10: ISEC experiments with dextran tracers (empty symbols) and protein tracers (filled symbols) for FractoAIMs (□) and Fractogel EMD SO₃⁻ (M) (◊).

Figure 2.11: HETP measurements as a function of linear velocity for IgG under non-adsorbing conditions for FractoAIMs (■) and Fractogel EMD SO₃⁻ (M) (◊).
2.31 \cdot 10^{-8} \text{cm}^2/\text{s} for IgG on Fractogel EMD SE Hicap (M) with \( d_p = 65 \mu\text{m} \). Karlsson et al. measured a pore effective diffusivity of \( D_{p,\text{eff}} = 3.5 \cdot 10^{-8} \text{cm}^2/\text{s} \) for IgG for the preparative strong anion exchange resin Resource 15Q (Amersham Biosciences, Uppsala, Sweden), which is a monodisperse material with a particle size of \( d_p = 15 \mu\text{m} \).

**Static binding capacity.** Under static conditions the saturation capacity for FractoAIMs is estimated as \( q_\infty = 111\,\text{g/l} \), whereas the binding capacity of Fractogel EMD SO_3^- (M) is \( q_\infty = 110\,\text{g/l} \) in the same experiment. The two materials have in fact very similar ligand densities and this explains the very similar saturation capacities.

![Graph](image_url)

**Figure 2.12:** Dynamic binding capacity at 10% breakthrough for IgG on FractoAIMs (○) and Fractogel EMD SO_3^- (M) (□) measured on the YMC column. The data set from the previous section on the Infochroma column for Fractogel EMD SO_3^- (M) (■) is also shown.

**Dynamic binding capacity.** As shown in Figure 2.12, the dynamic binding capacity is two times larger for FractoAIMs than for Fractogel EMD SO_3^- (M) for the fastest velocities. As it was shown previously, the ligand density and the static binding capacity as well as the pore effective diffusivity of the two materials are the same. Therefore this result underlines the importance to reduce the particle size and increase the pore accessibility in order to achieve larger mass transfer rates and thus better values for the dynamic binding capacity.
Note that the lines in Figure 2.12 serve to guide the eye and do not represent the actual dependence of the dynamic binding capacity on the velocity. As the static capacity is nearly the same for both materials, the two curves must converge to the same value at a velocity of $u_{in} = 0$. The diverging behavior of the lines is therefore an artefact caused by the small number of measured points. The true dependence of the dynamic binding capacity on the velocity is shown later with simulations.

### 2.3 Modeling

The behavior of the two materials under examination, i.e. Fractogel EMD SO$_3^-$ (M) and FractoAIMs, has been investigated also using the general rate model presented in Section 2.2.1. One important application of the developed model is to predict values of the dynamic binding capacity. This would provide a very valuable tool for screening stationary and mobile phases for a given industrial purification process. For this goal, it is a prerequisite to find a procedure for the quick estimation of model parameter values that requires very small amounts of protein and stationary phase. A possible strategy is presented in the following.

Parameter values were fitted to the general rate model in multiple separate regressions. All regressions were done for both Fractogel EMD SO$_3^-$ (M) and FractoAIMs. First, the bed porosity was fitted to ISEC data (Figure 2.10). Then, the porosity for IgG and the

<table>
<thead>
<tr>
<th>parameter</th>
<th>Fractogel EMD SO$_3^-$ (M)</th>
<th>FractoAIMs</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon_{bed}$</td>
<td>[-]</td>
<td>0.43</td>
</tr>
<tr>
<td>$\epsilon_t$</td>
<td>[-]</td>
<td>0.76</td>
</tr>
<tr>
<td>$\epsilon_{i, IgG}$</td>
<td>[-]</td>
<td>0.63</td>
</tr>
<tr>
<td>$Pe_{ax}$ at $u_{in} = 361 \text{ cm}/\text{h}$</td>
<td>[-]</td>
<td>555</td>
</tr>
<tr>
<td>$St$ at $u_{in} = 361 \text{ cm}/\text{h}$</td>
<td>[-]</td>
<td>39</td>
</tr>
<tr>
<td>$k_f$ at $u_{in} = 361 \text{ cm}/\text{h}$</td>
<td>$[\text{cm/s}]$</td>
<td>$1.97 \cdot 10^{-3}$</td>
</tr>
<tr>
<td>$P e$ at $u_{in} = 361 \text{ cm}/\text{h}$ (from HETP)</td>
<td>[-]</td>
<td>4.15</td>
</tr>
<tr>
<td>$P e$ at $u_{in} = 361 \text{ cm}/\text{h}$ (from simulation)</td>
<td>[-]</td>
<td>6.26</td>
</tr>
<tr>
<td>$q_{\infty}$</td>
<td>$[\text{g/l}]$</td>
<td>110</td>
</tr>
</tbody>
</table>
axial diffusion coefficient in the column (in terms of axial Peclet number $P_{e_{ax}}$) were regressed with experimental data under nonadsorbing conditions (Figure 2.10). Literature correlations (Equation (2.6)–(2.7)) were used to estimate the mass transfer coefficients and the Stanton number $St$ (Equation (2.12)). The particle Peclet number $Pe$ was calculated according to Equation (2.10), whereas the pore effective diffusivity $D_{p,eff}$ was estimated from the van Deemter plot (Figure 2.11) according to Equation (2.4). All relevant parameters for both materials are listed in Table 2.6.

![Figure 2.13: Experimental breakthrough curve at $u_{lin} = 361$ cm/h for IgG on Fractogel EMD SO₃⁻ (M) (solid black line), prediction (dashed grey line) and with fit of Peclet number (dashed black line).](image)

Using these parameter values together with values for the static binding capacity from batch experiments, the breakthrough curves, shown in Figure 2.13 and 2.14 for the two materials, have been predicted and compared with the corresponding experimental data. Considering the uncertainties behind such an entirely predictive procedure, the comparison is satisfactory.

In order to improve the fit, the Peclet number (and therefore the pore effective diffusion coefficient $D_{p,eff}$) was used as an adjustable parameter in the general rate model. A better simulation result is achieved with $D_{p,eff} = 2.32 \cdot 10^{-8}$ cm$^2$/s for FractoAIMs and $D_{p,eff} = 2.72 \cdot 10^{-8}$ cm$^2$/s for Fractogel EMD SO$_3^-$ (M), as also shown in Figure 2.13 and 2.14 respectively. This represents a reduction of about 36% in both cases with
Figure 2.14: Experimental breakthrough curve at \( u_{\text{in}} = 361 \text{ cm/h} \) for IgG on FractoAIMs (solid black line), prediction (dashed grey line) and with fit of Peclet number (dashed black line).

This can be explained by the fact that porosities (and therefore mass transfer parameters) can be reduced by other factors that are not included in the model such as the ionic strength of the mobile phase and the protein loading on the stationary phase. In particular, Forrer et al. found that a decrease in the modifier concentration (i.e. sodium chloride) leads to a decrease in the particle porosity \([60]\). In this work the porosities as well as the mass transfer resistances have been measured using at least 0.5 M sodium chloride in the buffer in order to ensure nonadsorbing conditions. On the other hand, the ionic strength was much lower in the breakthrough experiments in Figure 2.13 and 2.14.

More important, Forrer et al. and Melter et al. have shown that the pore accessibility is strongly decreasing as a function of loading, i.e. in conditions typical of our breakthrough experiments \([23, 62]\). Both effects are neglected in the model used here which assumes a constant pore diffusion rate coefficient. These aspects need to be addressed in future work in order to improve the prediction capabilities of this model.

A possible application of the model developed above for process development is shown in Figure 2.15 where the breakthrough curves are predicted for both materials at two different velocity values \( u_{\text{in}} = 181 \text{ cm/h} \) and \( u_{\text{in}} = 475 \text{ cm/h} \). There is an acceptable agreement between simulation and experimental data, although the prediction of the
**Figure 2.15:** Experimental breakthrough curve for IgG (solid line) and prediction (dashed line): Fractogel EMD SO$_3^-$ (M) at $u_{lin} = 181$ cm/h (top left), Fractogel EMD SO$_3^-$ (M) at $u_{lin} = 475$ cm/h (top right), FractoAIMs at $u_{lin} = 181$ cm/h (bottom left), FractoAIMs at $u_{lin} = 475$ cm/h (bottom right).

$DBC_{10\%}$ based on these simulations would not be fully satisfactory. For this, the model improvements discussed above are probably necessary.

Finally, in order to analyze the influence of the particle size on the dynamic binding capacity more closely, the dynamic binding capacity of both materials is simulated for 14 different velocities in the range of $u_{lin} = 15 - 3000$ cm/h (Figure 2.16). This study is hardly possible experimentally, because of high protein consumption of each breakthrough curve and because there are no materials available that can be operated at velocities significantly higher than $u_{lin} = 400$ cm/h. As expected, a strong influence of the particle size on the dynamic binding capacity as a function of the applied flow rate is observed. At very slow loading velocities ($u_{lin} \ll 100$ cm/h), the dynamic binding capacity is almost independent of the particle size. However, already at a linear velocity of $u_{lin} = 181$ cm/h, the dynamic binding capacity of FractoAIMs is 35% higher than that of Fractogel EMD SO$_3^-$ (M). With increasing loading velocities (above 500 cm/h), this difference increases even further.
Figure 2.16: Simulation of dynamic binding capacity for Fractogel EMD SO₅⁻ (M) (▼) and FractoAIMs (□).

2.4 Conclusion

It has been shown that the ligand density is an important parameter in determining the performance of a chromatographic material. It affects not only the saturation capacity of the material but also its pore structure, thus rendering the pores more or less accessible to large molecules and changing also the corresponding pore diffusivities. All these factors play a strong role for the determination of the dynamic binding capacity and in general, they conflict with each other. An optimal ligand density arises as the compromise between a high ligand density for large saturation capacities and a low density for well accessible pores and large mass transfer rates. However, this optimum is a function of the target molecule as well as process conditions such as the loading velocity.

As an example of the procedure for the design of an improved stationary phase for protein purification, a new material was presented that has a high pore accessibility for IgG together with a small particle size. Because of the rigidity of the particles, the material can be operated at high flow rates. Therefore, mass transfer resistances can be reduced while keeping high flow rates. This procedure increases the productivity of the stationary phase and speeds up the downstream process.

A chromatographic column model has been developed and the corresponding parameters have been evaluated using chromatographic experiments under nonadsorbing conditions
and simple batch experiments which need very small amounts of IgG and other tracer molecules. The obtained results are encouraging, although in order to obtain quantitative results which can be used for process screening, the model would require some further improvement. In particular, the effect of protein loading on pore diffusivity should be accounted for. The objective of this model is to predict the column behavior and hence the dynamic binding capacity. This would provide a useful tool in the phase of process screening at the beginning of the development of protein purification processes.
3 Application of mixed mode resins for the purification of monoclonal antibodies

3.1 Introduction

Recently Capto adhere, a new mixed mode resin, was launched by GE Healthcare (Chalfont St Giles, United Kingdom). Even though Capto adhere should be used as an anion exchange resin in flowthrough mode according to the supplier, Müller-Späth et al. have shown that Capto adhere can also be used in bind and elute mode as a polishing step in antibody purification [21, 61]. They reached a final purity with only 2 ppm remaining host cell proteins with a purification scheme that consisted of only two orthogonal chromatographic steps.

The current chapter explores systematically the possibilities of this stationary phase for the polishing of antibodies. The experimental data is compared to anion exchange and hydrophobic interaction chromatography, in order to better evaluate the various effects that occur in mixed mode chromatography. The Henry coefficient, which quantifies the adsorption strength, was measured for the full working range of the stationary phase as a function of the sodium chloride concentration and the pH. Furthermore, several application examples for the stationary phase are shown. The separation of an artificial mixture was predicted by the analysis of the Henry coefficients of the involved proteins. Additionally, Capto adhere was applied for the polishing step of an antibody from an industrial clarified cell culture supernatant. Finally, it is shown that this resin can be used for the separation of IgG subclasses. All those examples prove the necessity to use this stationary phase in the proposed bind and elute mode, as this ensures also the separation of weakly adsorbing impurities, which significantly improves the purity reached in this step.
3.2 Materials and instrumentation

Monoclonal and polyclonal antibodies were purchased from a pharmacy. Erbitux (Merck, Darmstadt, Germany), which is a chimeric IgG1 contains the monoclonal antibody Cetuximab (pI = 8.5) [68, 69]. Avastin (Roche, Welwyn Garden City, United Kingdom) is the brand name of the monoclonal antibody Bevacizumab, which is a humanized IgG1 (pI = 8.3÷8.6) [69, 70]. Gammanorm (Octapharma, Lachen, Switzerland) is human normal immunoglobulin G in solution. This polyclonal antibody consists of the IgG subclasses IgG1 (59%), IgG2 (36 %), IgG3 (4.5 %) and IgG4 (0.5 %) [71]. The mixture of different IgGs covers a pI-range from 6.5 to 10, as determined by Forrer et al. by isoelectric focusing [50].

Clarified cell culture supernatant was kindly donated by Merck Serono (Fénil-sur-Corsier, Switzerland). The antibody (referred to as ‘Serono mAb’ in the following), that is an IgG2 (pI = 7.35 ÷ 8.15), was captured with the strong cation exchange resin Fractogel EMD SO₃⁻ (M) with the continuous MCSGP technology [21]. The product, that was used in the current work as feed for polishing experiments, had a concentration of cIgG = 4.7 g/l of monoclonal antibody with a purity of 96 % according to Protein A analysis. This corresponds to a host cell protein concentration of 360 ppm (measured by ELISA).

Lysozyme from hen egg white was obtained from Fluka (Buchs, Switzerland). The pI of lysozyme is 11.35 [72].

Sodium chloride and acetic acid (glacial) were purchased at J. T. Baker (Deventer, the Netherlands), sodium acetate trihydrate, sodium hydrogen phosphate dihydrate and sodium sulfate were purchased at Merck (Darmstadt, Germany). Sodium phosphate dibasic dodecahydrate was obtained from Acros Organics (Geel, Belgium). Citric acid monohydrate was purchased at Brenntag Schweizerhall (Basel, Switzerland). Deionized water was purified with a Simpax 2 unit by Millipore (Bedford, MA, USA). All used chemicals were analytical grade.

The mixed mode resin Capto adhere was purchased from GE Healthcare (Chalfont St Giles, United Kingdom). Its ligand is N-benzyl-N-methyl ethanol amine. The matrix of the stationary phase is highly crosslinked agarose, with a particle size of dₚ = 75 μm [67]. Strong anion exchange resin Fractogel EMD TMAE Hicap (M) was kindly donated by Merck (Darmstadt, Germany). It has a particle size of dₚ = 40 ÷ 90 μm. The ligand, which is a trimethylammoniummethyl group, is bound to the matrix (crosslinked poly-methacrylate) via linear polymer chains, the patented ‘tentacles’, in order to provide better access of the proteins to the ligand [49].

Hydrophobic interaction resin Octyl Sepharose 4 Fast Flow was bought from GE Health-
care (Chalfont St Giles, United Kingdom). The matrix is highly crosslinked 4\% agarose. The octyl ligand is coupled to the matrix via ether bonds, thus avoiding charges on the stationary phase. The mean particle size is $d_p = 75\mu m$.

Resins were packed into Tricorn columns purchased at GE Healthcare (Chalfont St Giles, United Kingdom). Tricorn columns have a volume of $V \approx 1\text{ml}$ and a diameter of $d = 5\text{mm}$.

The purity of monoclonal antibodies was determined with a Protein A column POROS A/20 (Applied Biosystems, Foster City, CA, USA). Buffer A was 10 mM phosphate buffer pH 7.5, buffer B was 40 mM citric acid pH 2.2. Each sample was eluted with a step gradient from buffer A to buffer B. The purity of a sample according to Protein A analysis was calculated with the following equation:

$$P = \frac{A_{280,IgG}}{A_{280,impurities} + A_{280,IgG}}$$  \hspace{1cm} (3.1)

As Protein A selectively binds IgG, all other compounds elute in the flowthrough. The area of the IgG-peak $A_{280,IgG}$ was measured at a wavelength of 280 nm. The area of the nonbound impurities $A_{280,impurities}$ was measured accordingly. For all runs, a blank run with the same buffer composition as the sample was substracted before calculating the purity. Furthermore, the antibody purity was determined with a commercially available ELISA kit [23].

TSKgel G3000SWXL (7.8 x 300 mm) from Tosoh Bioscience (Stuttgart, Germany) was used for size exclusion experiments. Running buffer of size exclusion experiments was 25 mM sodium phosphate with 0.1 M sodium sulfate at pH 7 and 20°C.

The IgG variant profile of GammaNorm was determined with the analytical weak cation exchange column Propac WCX-10 (4 x 100 mm) from Dionex (Sunnyvale, CA, USA). Therefore, a gradient analysis (0.05 M to 0.5 M sodium chloride in the buffer) was applied using 20 mM acetate buffer pH 5.

For chromatographic measurements a HPLC 1100 Series by Agilent Technologies (Santa Clara, CA, USA) was used. The instrument is equipped with an UV detector, a conductivity meter CDD-10Avp by Shimadzu (Kyoto, Japan) and a pH sensor by Sensorex (Garden Grove, CA, USA). Absorption was monitored at a wavelength of 280 nm.

Preparative studies were conducted with an Äkta Basic (GE Healthcare, Chalfont St Giles, United Kingdom) equipped with a gradient pump, a UV-, a conductivity- and a pH-sensor. All experiments were carried out at $20^\circ C \pm 2^\circ C$. After each run, cleaning in place was performed with 0.5 M sodium hydroxide.
3.3 Measurement of Henry coefficients

Many authors found that simple Langmuir isotherms display the adsorption behavior of proteins in chromatography [23, 50, 74, 76]. Assuming this type of isotherm, a low Henry coefficient is found under conditions, where binding is almost not possible, while a high Henry coefficient and therefore long retention times characterize adsorption. Therefore, Henry coefficients provide a simple method to test the adsorption strength of a stationary phase. A comprehensive overview on isotherm determination was presented by Seidel-Morgenstern [77]. In the current work, Henry coefficients $H$ were measured under isocratic conditions. They were calculated according to Equation (3.2).

$$H = \left( \frac{t Q}{\epsilon_{prot} V} - 1 \right) \frac{1}{F}$$  \hspace{1cm} (3.2)

The Henry coefficient is a function of the retention time $t$ of the protein in the column, the flowrate $Q$, the column volume $V$ and the porosity of the protein $\epsilon_{prot}$ that was measured under nonadsorbing conditions according to

$$\epsilon_{prot} = \frac{V_{prot}}{V}$$ \hspace{1cm} (3.3)

whereas $V_{prot}$ is the accessible liquid volume for the protein in the column. The phase ratio is defined as ratio between the non-accessible and the accessible volume of the column.

$$F = \frac{1 - \epsilon_{prot}}{\epsilon_{prot}}$$ \hspace{1cm} (3.4)

3.3.1 Henry coefficients of proteins on Fractogel EMD TMAE Hicap (M)

In order to have a benchmark, Henry coefficients for lysozyme and the IgGs (Serono mAb, Erbitux and Avastin) were measured on the conventional anion exchange resin Fractogel EMD TMAE Hicap (M). As shown in literature, the porosity of Fractogel resins is a strong function of the ionic strength [74]. Therefore, the protein porosities were determined at pH 4 at various salt concentrations. The low pH ensures ionic repulsion for the tested proteins that all have a basic pI. As shown in Table 3.1, the porosity for lysozyme doubles in the measured range of the salt concentration. At a concentration of $c_{NaCl} = 0.0 \text{ M}$, the porosity for lysozyme is only $\epsilon_{lys} = 0.28$. Besides, the porosity for lysozyme (molecular weight: 14.3kDa) is the same as the porosity for IgG (molecular weight: 144kDa), even though the molecular weight is ten times smaller.
Table 3.1: Porosity $\epsilon_{prot}$ for lysozyme and the IgGs on Fractogel EMD TMAE Hi-cap (M) at various concentrations of sodium chloride in the buffer.

<table>
<thead>
<tr>
<th>$\epsilon_{NaCl}$ [M]</th>
<th>$\epsilon_{Iys}$</th>
<th>$\epsilon_{IgG}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.59</td>
<td>0.39</td>
</tr>
<tr>
<td>0.3</td>
<td>0.52</td>
<td>0.37</td>
</tr>
<tr>
<td>0.1</td>
<td>0.41</td>
<td>0.34</td>
</tr>
<tr>
<td>0.0</td>
<td>0.28</td>
<td>0.28</td>
</tr>
</tbody>
</table>

This is a hint that the Fractogel pores are blocked at low salt concentration and are no longer accessible for proteins.

The Henry coefficients for the Serono mAb, Erbitux, Avastin and lysozyme are shown in Figure 3.1 and 3.2 as a function of the pH and the sodium chloride concentration. In both cases, the dependence of the Henry coefficient can be described with power functions sufficiently well. Note that the Henry coefficient is always below $H = 1$ which is a consequence of the high pI of all measured proteins. An operation of this stationary phase in bind and elute mode is therefore not possible in the examined pH-range. As a matter of fact, anion exchange resins are typically employed in flowthrough mode in the downstream processing of antibodies in order to remove impurities with a low pI.

3.3.2 Henry coefficients of proteins on Octyl Sepharose 4 Fast Flow

It was not possible to find truly nonadsorbing conditions for the selected proteins on the HIC stationary phase. Even without sodium chloride in the buffer, some adsorption of the proteins occurred making it impossible to measure the column porosity for those molecules. Therefore it was assumed that the porosity is similar to the porosity of the other columns characterized in this paper, setting the porosity for the mAbs to $\epsilon_{mAb} = 0.5$ and for lysozyme to $\epsilon_{Iys} = 0.6$. Note that a mismatch of this number shifts all Henry coefficients for the respective molecule, but the trend is kept.

Most often high concentrations of ammonium sulfate are used in hydrophobic interaction chromatography [78]. However, in order to compare the results to anion exchange and mixed mode stationary phases, only moderate concentrations of sodium chloride are explored in this work. Figure 3.3 shows the Henry coefficients for the Serono mAb, Erbitux, Avastin and lysozyme as a function of the salt concentration for pH 4 and pH 8.
Figure 3.1: Henry coefficients for Serono mAb (top left), Erbitux (top right), Avastin (bottom left) and lysozyme (bottom right) as a function of the pH on Fractogel EMD TMAE Hicap (M). The Henry coefficient for each protein was measured at 0.3 M NaCl (●), 0.1 M NaCl (■) and 0.0 M NaCl (▲). The dashed lines show fits with power functions of the according experimental sets.
Figure 3.2: Henry coefficients for Serono mAb (top left), Erbitux (top right), Avastin (bottom left) and lysozyme (bottom right) as a function of the sodium chloride concentration on Fractogel EMD TMAE Hicap (M). The Henry coefficient for each protein was measured at pH 8.0 (△), pH 7.3 (♦), pH 6.1 (●) and pH 4.5 (□). The dashed lines show fits with power functions of the according experimental sets.
Figure 3.3: Henry coefficients for Serono mAb (top left), Erbitux (top right), Avastin (bottom left) and lysozyme (bottom right) as a function of the sodium chloride concentration on Octyl Sepharose 4 Fast Flow. The Henry coefficient for each protein was measured at pH 8.0 (△) and pH 4.0 (◆). The dashed lines show fits with power functions of the according experimental sets.
3.3 Measurement of Henry coefficients

The Serono mAb has small Henry coefficients over the whole measured range. On the contrary, the two other mAbs as well as lysozyme show an increasing Henry coefficients with increasing salt concentration. Surprisingly, also the pH has an important influence on the Henry coefficient of those molecules, whereas at pH 4, that means far from the protein pI, adsorption is favored. To and Lenhoff found the same behavior for various proteins and stationary phases and explained this by a thermodynamic analysis of the solution properties of the proteins \[79\]. Müller and Faude showed that the reason for this pH dependence might be the pH dependent size of the hydration shell of the salt \[80\]. In the best case, that is Avastin, Henry coefficients of almost \( H = 100 \) are reached. However, for most conditions, Henry is more in the range of \( H = 1 \div 10 \), confirming the need for higher salt concentrations and other modifiers in order to reach strong adsorption.

3.3.3 Henry coefficients of proteins on Capto adhere

Table 3.2: Porosity \( \epsilon_{prot} \) for lysozyme and the IgGs on Capto adhere.

<table>
<thead>
<tr>
<th>tracer</th>
<th>( \epsilon_{prot} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysozyme</td>
<td>0.52</td>
</tr>
<tr>
<td>IgG</td>
<td>0.47</td>
</tr>
</tbody>
</table>

Porosities were measured under nonadsorbing conditions with 20 mM acetate buffer pH 4 with 0.0 M sodium chloride (see Table 3.2). As shown later, a high salt concentration induces binding of the proteins even at pH 4 on this stationary phase. Note that the porosity for lysozyme is lower compared to Fractogel EMD TMAE Hicap (M) (at 1.0 M NaCl), but higher for IgG.

The Henry coefficients for the same proteins as in the previous sections were also measured on Capto adhere. As shown in Figure 3.4 and 3.5 the Henry coefficients are significantly higher on this material than on the previous two ones. The Henry coefficient as a function of ionic strength and pH can be fitted with power functions in most cases. Note that it was not possible to measure a dependence for the Henry coefficient of the sodium chloride concentration at pH 8 for the monoclonal antibodies, because this would have required an extremely long experimental time.

Lysozyme, which has a very high pI and should therefore not bind on an anion exchange resin, has a Henry coefficient of \( H = 3.5 \) at \( c_{NaCl} = 1.0 \) M over the whole pH-range on
Figure 3.4: Henry coefficients for Serono mAb (top left), Erbitux (top right), Avastin (bottom left) and lysozyme (bottom right) as a function of the pH for Capto adhere. The Henry coefficient for each protein was measured at 1.0 M NaCl (●), 0.3 M NaCl (▲), 0.1 M NaCl (■) and 0.0 M NaCl (▼). The dashed lines show fits with power functions of the according experimental sets.
Figure 3.5: Henry coefficients for Serono mAb (top left), Erbitux (top right), Avastin (bottom left) and lysozyme (bottom right) as a function of the sodium chloride concentration for Capto adhere. The Henry coefficient for each protein was measured at pH 8.0 (△), pH 7.0 (♦), pH 6.1 (●), pH 4.7 (▷), pH 4.5 (□) and pH 4.2 (◆). The dashed lines show fits with power functions of the according experimental sets. For the sake of visibility, for Serono mAb and lysozyme only the data set at pH 4.2 was fitted with a power function.
Capto adhere (Figure 3.4 (bottom right)). Furthermore, Figure 3.3 (bottom right) reveals that the Henry coefficient of lysozyme is practically independent of the applied pH and therefore only a function of the sodium chloride concentration. In contrast to the behavior on ion exchange resins, the Henry coefficient of the protein is increasing with increasing salt concentration. This effect must be due to the hydrophobic interactions of the protein with the stationary phase.

Henry coefficients for Avastin and Erbitux increase with increasing pH and increasing ionic strength. According to Figure 3.4 the Henry coefficient of Avastin and Erbitux is the same at low concentrations of sodium chloride ($c_{NaCl} = 0.0$ to $0.1$ M). This might be explained with the very similar isoelectric points that those monoclonal antibodies are exhibiting. At a salt concentration of $c_{NaCl} = 0.3$ M, the Henry coefficients for Avastin are significantly higher which might be caused by a different hydrophobicity distribution of Avastin in comparison to Erbitux.

![pH graph](image_url)

**Figure 3.6:** Schematic representation of overlap of hydrophobic (dashed line) and anionic interactions (dash-dotted line) for the Capto adhere (solid line).

The dependence of the Henry coefficient of the Serono mAb on Capto adhere is unique. Figure 3.3 (top left) shows that the Henry coefficient is independent of the pH up to pH 5. A further increase of the pH leads to a drastic increase of the Henry coefficient, especially at low ionic strength. This induces a Henry function of the pH that has a minimum. This behavior might be explained by the overlap of ionic and hydrophobic interactions.
of Capto adhere under these conditions as shown in Figure 3.6. The contributions of the ‘pure’ hydrophobic and anionic stationary phases were shown in the previous sections and are depicted in Figure 3.6. While the Henry coefficient increases with increasing pH on Fractogel EMD TMAE Hicap (M), it decreases on Octyl Sepharose 4 Fast Flow. On the contrary, the Henry coefficient of the selected proteins decreases with increasing salt concentration on Fractogel EMD TMAE Hicap (M), while it increases on Octyl Sepharose 4 Fast Flow. Note that on both of these stationary phases, all Henry coefficients show linear dependencies in the logarithmic representation. An overlap of the described effects leads to the observed behavior for the Serono mAb on Capto adhere as as function of the pH at high salt concentrations (Figure 3.4) and as a function of the salt concentration at high pH (Figure 3.5). Note that also the Henry coefficient of lysozyme as a function of the pH shows an abnormality that is that the slope of the Henry coefficient is changing from positive slope for small salt concentrations to a slightly negative slope at high salt concentrations. This might be due to the same mechanism of overlapping interactions on Capto adhere. Even though this concept offers an explanation for the observed effects, it can only be a qualitative explanation.

Müller-Späth et al. have measured Henry coefficients in the range of $H = 1 \div 100$ for the Serono mAb on two different strong cation exchange resins at pH 6 [21]. Capto adhere offers the possibility to reach the same order of magnitude for the Henry coefficients on a (mixed mode) strong anion exchange resin under physiological conditions.

### 3.4 Separation of proteins

The analysis of the Henry coefficients for various stationary phases can be used to separate mixtures of the model proteins. As discussed above, the Henry coefficients are very different for the tested proteins as a function of the pH and salt concentration (see Figure 3.4 and Figure 3.5).

In order to separate a mixture of two proteins on Capto adhere, a gradient elution was set up, whereas the column was washed for 5 min after injection of the mixture, then a pH gradient was run in 20 min, followed by a final elution at pH 4. After each run the column was cleaned with 0.5 M NaOH. The applied flowrate was $u_{lin} = 764 \text{ cm/h}$ ($Q = 1 \text{ mL/min}$). The buffers were prepared in analogy to the experiments carried out in the previous section.

As shown in Figure 3.7, it is possible to separate Avastin from lysozyme with a pH gradient from pH 8 to pH 4 (concentration of sodium chloride in the buffers was $c_{NaCl} = 0.0 \text{M}$).
on Capto adhere. While the flowthrough peak contains only lysozyme, the peak in the gradient contains about 80% Avastin and 20% lysozyme (calculated from the elution pattern of the pure components). Note that the success of this separation can be predicted from the great difference of the measured Henry coefficients (one order of magnitude) of the two proteins at pH 8 with $c_{NaCl} = 0.0\, M$.

Even though this is a rather simple separation, the two proteins cannot be separated with the anion exchange resin Fractogel EMD TMAE Hicap (M) in the investigated pH range, even though the salt concentration of the experiment was chosen as low as possible in order to reach the best possible adsorption of Avastin. Despite some tailing, the proteins are not retained and none of the two proteins elutes in the pH-gradient, making a separation impossible.

On hydrophobic interaction resin Octyl Sepharose 4 Fast Flow a separation seems possible as most proteins are retained at least to some extend. However, as the Henry coefficients are in the range of $H = 1 \div 10$ and the capacity of hydrophobic interaction resins under the selected conditions is rather low, a separation is neither easy nor efficient.
This comparison shows therefore very nicely the advantages of an operation of Capto adhere in bind and elute mode: the mixed mode functionalities allow to bind antibodies on the stationary phase under physiological conditions. Therefore it is possible to separate the bound antibody from impurities that adsorb weaker on this resin. This mode is lost on conventional anion exchangers that are operated in flowthrough mode. A separation on an hydrophobic interaction resin would require a very high sodium chloride concentration or even ammonium sulfate in the buffer which leads to high buffer costs and expensive waste streams which are the well known drawbacks of those stationary phases.

A second gradient elution was set up, in order to separate a mixture of monoclonal antibodies (gradient conditions were the same as explained previously in this section). Figure 3.8 shows that it is almost not possible to separate the monoclonal antibodies

![Figure 3.8: Separation of mAbs on Capto adhere. The top left graph shows the elution of Erbitux (green line) and Avastin (red line) as obtained in separate experiments. The bottom left graph shows the elution of a 1:1 (mass) mixture of the two proteins (blue line). The top right graph shows the elution of Serono mAb (green line) and Avastin (red line) as obtained in separate experiments. The bottom right graph shows the elution of a 1:1 (mass) mixture of the two proteins (blue line). The pH was measured during the experiment (dash-dotted line). The applied gradient is shown as a dashed line, whereas the used buffers were 20 mM phosphate buffer pH 8 with 0.0 M NaCl and 20 mM acetate buffer pH 4 with 0.0 M NaCl.](image)
Figure 3.9: Separation of mAbs on Capto adhere. The top left graph shows the elution of Erbitux (green line) and Avastin (red line) as obtained in separate experiments. The bottom left graph shows the elution of a 1:1 (mass) mixture of the two proteins (blue line). The top right graph shows the elution of Serono mAb (green line) and Avastin (red line) as obtained in separate experiments. The bottom right graph shows the elution of a 1:1 (mass) mixture of the two proteins (blue line). The pH was measured during the experiment (dash-dotted line). The applied gradient is shown as a dashed line, whereas the used buffers were 20mM phosphate buffer pH 8 with 0.3 M NaCl and 20mM acetate buffer pH 4 with 0.3 M NaCl.

Erbilux or Serono mAb from Avastin without sodium chloride in the buffer. This is a direct consequence of the measured Henry coefficients of the mAbs that are in the range of $H = 0 \div 6$ for pH 4 to 6 (Figure 3.8) which is the elution pH according to Figure 3.8. However, at elevated salt concentration a good separation is reached, as shown in Figure 3.9. Again, this is justified by the Henry coefficients for pH 4 to 6 at $c_{NaCl} = 0.3$ M (Figure 3.9); whereas they stay roughly constant for the Serono mAb, the Henry coefficients are one order of magnitude higher for Erbitux and two orders of magnitude higher for Avastin.

The elution order is Serono mAb, Erbitux, Avastin for both salt concentrations. Note that this is also the order of the isoelectric points of the three proteins. At high salt concentration all proteins elute later, most probably because of hydrophobic interactions at that condition. This comparison shows very clearly the new dimension that can be used
with mixed mode stationary phases: for anion exchange resins (as well as hydrophobic interaction resins), the only degree of freedom is the selected pH, whereas the sodium chloride concentration is fixed as low as possible (or as high as possible for HIC resins) in order to ensure binding. The mixed model resin Capto adhere shows a broad range of Henry coefficients: high Henry coefficients are reached at high salt concentrations, but also without salt and elevated pH. Therefore binding conditions might be selected from a wider range of conditions compared to conventional stationary phases, leading to a greater flexibility to optimize the separation and purification of proteins.

![Graph](image)

**Figure 3.10:** Isocratic separation of Serono mAb (green line), Avastin (red line) and a of the two proteins (blue line) on Capto adhere. The top graph shows the elution at pH 7 with 0.0 M NaCl in the buffer, the bottom graph shows the elution at pH 7 with 0.9 M NaCl in the buffer.

From Figure 3.10 another interesting phenomenon can be predicted. As shown there, the Henry coefficients for the Serono mAb show a pronounced minimum at elevated pH. As the Henry coefficient for Avastin and Erbitux are monotonically increasing at these conditions, a behavior shown experimentally in Figure 3.10 is observed: while the Serono mAb elutes at roughly the same time at a salt concentration of 0.0 M NaCl and 0.9 M NaCl at pH 7 (iso-Henry conditions, because of the minimum), Avastin is eluted ahead of the Serono mAb at 0.0 M NaCl and after the Serono mAb at 0.9 M NaCl. Note that at the latter conditions, it was only possible to elute Avastin by a salt step back to 0.0 M
NaCl, because of the very high Henry coefficient of Avastin at 0.9 M NaCl. This result has to be seen as the need to always measure the complete range of Henry coefficients for proteins, especially on resins with more than one functionality, in order to find all possible separation options for a specific task.

### 3.5 Application examples with Capto adhere

#### 3.5.1 Polishing of a monoclonal antibody from cCCS

As described in Section 3.2, the feed for this study was produced from a clarified cell culture supernatant by purification on a cation exchange resin with the MCGGP process. The feed for the second step (the polishing step, that is analyzed here) was adjusted to pH 7.5 ± 8.5, the conductivity was at least 13 mS/cm, which corresponds to a concentration of $c_{NaCl} = 0.1$ M. The feed contains 2.5% impurities with higher molecular weight than IgG and 1.3% impurities with lower molecular weight according to size exclusion analysis. The applied methods and the used buffers for all analytics were already described in Section 3.2.

The purity of protein samples according to Protein A analysis correlates linearly with the purity measured by ELISA (data not shown). For the used supernatant, this correlation is valid in the range of 90 ± 99% Protein A purity, which covers almost the full working range for the polishing step. Therefore, the purity according to Protein A analysis serves as an appropriate replacement for the expensive and time consuming ELISA test. However, the amount of host cell proteins in very pure samples (Protein A purity > 99%) is overestimated. Note that it was shown elsewhere that a purity of 99.7% corresponds to an HCP content below 5 ppm [21].

Müller-Späth et al. have used Capto adhere successfully for the polishing step of the Serono mAb [21]. With binding at 10 mM phosphate buffer pH 8 and elution with 10 mM phosphate / 10 mM citrate buffer pH 4, a final product purity higher than 99.7% (HCP content lower than 3 ppm) was reached. Therefore these conditions were used as a benchmark.

All experiments described in the following were done at a flowrate of $u_{kn} = 764$ cm/h ($Q = 1$ ml/min). After loading of the column with undiluted feed (with adjusted pH), the column was washed with the respective loading buffer (20 mM phosphate buffer pH 7.5 ± 8.5 with 0 ± 0.15 M NaCl) for 4.4 column volumes. Then a pH gradient from pH 7.5 ± 8.5 to pH 4 was run in 17.6 column volumes. Buffer B was 20 mM acetate buffer pH 4 with the same sodium chloride concentration as the loading buffer. After final elu-
3.5 Application examples with Capto adhere

**Figure 3.11:** Polishing of Serono mAb on Capto adhere with a loading of $\rho_{\text{load}} = 15.3 \text{ g/l}$ Serono feed at 20 mM phosphate buffer pH 8 with 0.0 M NaCl. The UV signal is shown as a solid line and the pH signal is shown as a dash-dotted line. The gradient conditions are shown as a dashed line.

For 4.4 column volumes with buffer B, the column was cleaned with 0.5 M NaOH for 8.8 column volumes. The chosen selection of buffer A and buffer B leads to a relatively smooth pH gradient as shown in Figure 3.11.

**Influence of the loading amount and the salt concentration on the product purity.**

In order to evaluate the influence of the salt concentration on the purification, the above described gradient was run from pH 8 to pH 4 with a concentration of $c_{\text{NaCl}} = 0.0 \text{ M}$ or $c_{\text{NaCl}} = 0.1 \text{ M}$ in both buffer A and buffer B. Both conditions were tested with low loading ($\rho_{\text{load}} = 6.8 \text{ g/l}$) and high loading ($\rho_{\text{load}} = 15.3 \div 15.5 \text{ g/l}$). An example of the preparative run is shown in Figure 3.11. Each run was fractionated and the fractions were analyzed offline by Protein A and SEC analysis. As shown in Table 3.3 it is possible to reach a product fraction with a purity higher than 99% for all preparative runs. Figure 3.12 shows the concentration of the mAb, the purity of the mAb and the concentration of the high and low molecular weight impurities as a function of the elution volume. It can be seen that an increase of the salt concentration decreases the total capacity of the stationary phase (comparison of the two runs at high loading, but with different binding buffers). As a matter of fact, the high loading run at high ionic...
Figure 3.12: Preparative runs with Capto adhere with $p_{\text{load}} = 6.8 \, \text{g/l}$ Serono feed (□) and with $p_{\text{load}} = 15.3 \, \text{g/l}$ Serono feed (●) with loading buffer 20 mM phosphate buffer pH 8 with 0.0 M NaCl and preparative runs with Capto adhere with $p_{\text{load}} = 6.8 \, \text{g/l}$ Serono feed (●) and with $p_{\text{load}} = 15.5 \, \text{g/l}$ Serono feed (◆) with loading buffer 20 mM phosphate buffer pH 8 with 0.1 M NaCl. The Figure shows the concentration of mAb (top graph), the purity of the product according to Protein A analysis (second graph), the concentration of high molecular weight impurities HMW (third graph) and the concentration of low molecular weight impurities LMW (bottom graph) according to size exclusion analysis. The applied gradient is shown as a dashed line.
Table 3.3: Purest fraction of mAb (according to Protein A analysis), purity of pool according to Protein A analysis, purity of pool according to size exclusion analysis and share of high and low molecular weight impurities according to size exclusion analysis. The analysis was done for preparative runs according to Figure 3.12 setting the yield for the pool higher than 95\% according to Protein A analysis.

<table>
<thead>
<tr>
<th></th>
<th>purity purest fraction</th>
<th>purity pool</th>
<th>share</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[%]</td>
<td>[%]</td>
<td>[%]</td>
</tr>
<tr>
<td>0.0 M NaCl, high loading</td>
<td>99.96</td>
<td>99.8</td>
<td>99.2</td>
</tr>
<tr>
<td>0.0 M NaCl, low loading</td>
<td>99.97</td>
<td>99.7</td>
<td>99.1</td>
</tr>
<tr>
<td>0.1 M NaCl, high loading</td>
<td>99.98</td>
<td>97.7</td>
<td>98.0</td>
</tr>
<tr>
<td>0.1 M NaCl, low loading</td>
<td>100.00</td>
<td>99.1</td>
<td>99.2</td>
</tr>
</tbody>
</table>

strength is already overloaded which leads to a pollution of the product pool with low molecular weight impurities due to the peak shift to earlier elution for the product peak (Figure 3.12 Table 3.3). Therefore, the purity of the product is significantly worse than in the other three cases. Note however, that even if most of the low molecular weight impurities elute in the flowthrough, some of the low molecular weight impurities elute in the product peak. Their concentration is $a_{\text{LMW}} \approx 250$ mAU, independent of the product loading. In order to reach a high product purity, it is therefore necessary to reach a high concentration of mAb in the product peak. Besides a high loading, this constraint requires a high capacity of the stationary phase. Therefore the best clearance of the product pool from low molecular weight impurities was reached with high loading at low salt concentration (Table 3.3).

As shown in Figure 3.12, the high molecular weight impurities elute later than the product. Their breakthrough time is independent of the ionic strength. In contrast, the product peak elutes earlier at higher salt concentrations (compare the runs at low loading at different salt concentrations in Figure 3.12). Therefore, if the purification requires a good separation of the high molecular weight impurities from the product, an elevated salt concentration is required.

The overall best results for the polishing of the tested Serono mAb were reached at high loading with low salt concentration in the buffer. Note that this result has to be understand as the highest product purity achieved, without weighting the possibly different importance (e.g. toxicity) of high and low molecular weight impurities. Table 3.3 reveals that it was possible to reach a product pool with a purity of 99.7\% at low ionic
strength with both loading amounts while keeping a yield of 95 %. According to Müller-Späth et al., this purity corresponds to a host cell protein content below 5 ppm in the product [21].

**Study of the loading pH.** Preparative runs on Capto adhere were done in analogy to the previous section. In order to evaluate the influence of the loading pH on the purification, three different loading conditions were tested. Approximately $\rho_{\text{load}} = 13 \text{g/l}$ of feed were loaded onto the Capto adhere column at pH 8.5, pH 8 and pH 7.5. The binding buffer was always 20 mM phosphate buffer with 0.15 M sodium chloride, while buffer B was 20 mM acetate buffer pH 4 with 0.15 M sodium chloride. A pH gradient was run as described in the previous section. The feed had the same conditions as the loading buffer. As shown in Table 3.4, an increase of the pH leads to a higher product yield, while the purity according to Protein A remains above 99 %. This is due to the fact that the binding capacity increases with increasing pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>share [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>38</td>
</tr>
<tr>
<td>8.0</td>
<td>62</td>
</tr>
<tr>
<td>8.5</td>
<td>69</td>
</tr>
</tbody>
</table>

**Table 3.4:** Fraction of Serono mAb with a purity higher than 99 % for the polishing step on Capto adhere.

### 3.5.2 Separation of subclasses for a polyclonal IgG

It was shown by Forrer et al. that Gammanorm, which is a polyclonal antibody product, can be separated into subclasses with salt gradients on cation exchange resins [56]. As shown in Figure 3.13, a shoulder is formed in the salt gradient on the Propac column which is an analytical weak cation exchange column. For an analogous salt gradient separation on the strong cation exchange resin Fractogel EMD SO₄ (M), it was shown with a subclass-specific ELISA test, that this shoulder (denoted as P1) mainly consists of IgG₂, while IgG₁ is accumulated in the main peak (denoted as P2).

In order to separate the IgG subclasses on Capto adhere, Gammanorm was loaded onto the column, as shown in Figure 3.14. After equilibration of the Capto adhere column with 20 mM phosphate buffer pH 8 with 0.1 M sodium chloride, the column is loaded
3.5 Application examples with Capto adhere

Figure 3.13: Elution of Gammanorm on Propac. The sodium chloride gradient is shown as a dashed line. The peak forms a shoulder that is denoted as P1, whereas the main peak is denoted as P2. P1 mainly consists of IgG₂, while IgG₁ is accumulated in P2 (as shown with an subclass-specific ELISA test on Fractogel EMD SO₃⁻ (M)).
Figure 3.14: Separation of Gammanorm on Capto adhere. The UV signal is shown as a solid line and the pH signal is shown as a dash-dotted line. The experimental conditions are shown as a dashed line. Fraction F1 and F2 were taken during the experiment and analyzed with Propac column as shown in Figure 3.15.
with Gammanorm IgG ($\rho_{\text{load}} = 18.8 \text{ g/l}$) dissolved in buffer A. The conditions of the preparative run (buffers, gradient length, flowrate) were done as described in the previous section. With this method it was possible to elute 98% of the loaded protein. According to Figure 3.14 two fractions F1 and F2 were taken during the experiment. Those fractions were analyzed with the Propac column. As shown in Figure 3.15, P1 is enriched in fraction F1, while fraction F2 is almost free of P1. If a separation of the variants on Capto adhere because of charge differences of the IgG variants (using therefore only the anionic functionality of Capto adhere) is assumed, P1 should have been enriched in the late eluting fractions. However, the opposite happens. Therefore, the full mixed mode functionalities of Capto adhere are used in this separation.

Figure 3.16 shows the analysis of the complete fractionation of the preparative experiment (Figure 3.14). As a matter of fact, P1 is eluting preferentially early. As shown exemplary in Figure 3.15, the main IgG peak contains a significantly lower amount of P1. However, the amount of P1 is always above 10%, whereas the feed contains 14% of P1. This behavior is an artefact: it is not possible to separate P1 completely from the remaining peak. Therefore, the split between P1 and P2 was set arbitrarily at $t = 4.8$ column volumes. Consequently, also a pure IgG$_1$ sample would contain a certain amount of P1.

![Figure 3.15](image-url)

**Figure 3.15:** Analysis of fraction F1, F2 and feed with Propac column. The main peak of fraction F1, feed, and fraction F2 is shown from top to bottom. The sodium chloride gradient of the analysis is shown as a dashed line.
**Figure 3.16**: Separation of Gammanorm variants on Capto adhere for the experiment described in Figure 3.14. The share P1 (□) according to Propac analysis and the total concentration of IgG (▲) are shown.

### 3.6 Conclusion

Mixed mode resins such as Capto adhere, present a new class of stationary phases for the polishing of antibodies. Even though they are primarily anion exchange resins, their other modes of interaction are strong enough in order to provide the possibility to use them in bind and elute mode. As shown in this work, they are an interesting alternative for the polishing step in the downstream processing of antibodies. Furthermore, the mixed mode functionalities can be employed also to separate IgG subclasses, which is an extremely difficult separation.

Future efforts should focus on the modeling of the effects described here. It might be interesting to study, whether the adsorption of antibodies on stationary phases with mixed mode functionalities can be described by a combination of the models for anion exchange and hydrophobic interaction chromatography.
4 Behavior of human serum albumin on strong cation exchange resins

4.1 Introduction

Human as well as bovine serum albumin are often used as model proteins to investigate the behavior of proteins on chromatographic materials, because of their good availability in pure state. As the isoelectric point (pI) of both human and bovine serum albumin is reported to be in the range of 4.7 to 4.9 \cite{81-86}, both proteins were mainly used for the characterization of anion exchange resins \cite{34,35,39,87}, but also of cation exchange and mixed mode resins \cite{88-90}. Norde and Lyklema found a maximum of the binding capacity for human serum albumin on a strong cation exchange resin close to the protein pI \cite{88}. Using pH gradients for the protein elution, Pabst et al. recently found that albumins start to elute at about 1.5 orders of magnitude above their pI \cite{89}. Therefore, the pI alone is not sufficient to determine the applicability of cation or anion exchange resins for the chromatographic purification of these proteins.

It has been reported that proteins often exhibit unexpected elution patterns. Jungbauer et al. have found a two peak elution pattern for various pure proteins on hydrophobic interaction phases \cite{91}. The two peaks are caused by partial unfolding of proteins on hydrophobic interaction phases. The authors claim that the ratio folded/unfolded protein is a function of the salt concentration, namely ammonium sulfate, in the buffer. However, they do not analyze the presence of a kinetic process for the unfolding in this paper. Jaulmes et al. observed such a two peak elution of human serum albumin on a reversed phase and an anion exchange material \cite{92}. They have modeled this behavior with two kinetic sites and one bilangmuir isotherm. Furthermore, Hunter and Carta observed a two peak elution of bovine serum albumin on an anion exchange resin \cite{93}. 
In this case, the presence of dimers in the bovine serum albumin was responsible for the two peak elution behavior.

In this work, we show that pure human serum albumin elutes with two peaks in a modifier gradient on a strong cation exchange resin. Based on a variety of experimental studies, the hypothesis is made that the two peaks correspond to two different binding sites or configurations with different properties. In particular, the peculiar behavior of human serum albumin is explained by a kinetic transition of one binding conformation into the other one.

4.2 Materials

4.2.1 Stationary phases and columns

Strong cation exchange resins Fractogel EMD SO₃⁻ (M) and Fractogel EMD SE Hicap (M), as well as weak cation exchange resin Fractogel EMD COO⁻ (M) were kindly donated by Merck (Darmstadt, Germany). All resins have a crosslinked polymethacrylate matrix with functional groups bound to the matrix with linear polymer chains (tentacles). Functional groups are sulfosobutyl groups for Fractogel EMD SO₃⁻ (M), sulfoethyl groups for Fractogel EMD SE Hicap (M) and carboxyethyl groups for Fractogel EMD COO⁻ (M). All three stationary phases have a particle size of \( d_p = 40 \div 90 \mu m \) with a pore size of about \( r_p = 400 \text{Å} \). All Fractogel resins are approved up to a velocity of \( u_{kin} = 800 \text{cm} / \text{h} \), which is the one used for column packing, \( u_{in} = 400 \text{cm} / \text{h} \) is therefore the maximum velocity recommended for column operation.

Resins were packed into Tricorn columns purchased at GE Healthcare (Chalfont St Giles, United Kingdom). Tricorn columns have a volume of \( V \approx 1 \text{ml} \) and a diameter of \( d = 5 \text{mm} \). 20% mechanical compression of the bed was applied to the resin in the column. The mechanical compression makes this small lab scale column comparable to large preparative columns that are self-compressed by their own weight.

Concentration of proteins was determined using the analytical weak cation exchange column Propac WCX-10 (4 x 100 mm) from Dionex (Sunnyvale, CA, USA). The sample concentration was correlated to the peak area under nonadsorbing conditions, namely 20mM acetate buffer pH 5 with 0.5 M NaCl. For more detailed analysis, a gradient analysis (0 M to 1 M sodium chloride) was applied, using the same buffer.

TSKgel G3000SWXL (7.8 x 300 mm) from Tosoh Bioscience (Stuttgart, Germany) was used for size exclusion experiments. Running buffer of size exclusion experiments was 25mM sodium phosphate with 0.1 M sodium sulfate at pH 7, as recommended by the supplier.
4.2.2 Chemicals and buffers

Human serum albumin (HSA) was purchased at Sigma-Aldrich (Buchs, Switzerland). It is delivered as a lyophilized powder with a purity of approximately 99\% (analyzed by agarose gel electrophoresis). The HSA powder is essentially globulin free. Sodium chloride and acetic acid (glacial) were purchased at J. T. Baker (Deventer, the Netherlands), sodium acetate trihydrate, sodium hydrogen phosphate dihydrate and sodium sulfate were purchased at Merck (Darmstadt, Germany). Sodium phosphate dibasic dodecahydrate was obtained by Acros Organics (Geel, Belgium). Deionized water was purified with a Simpak 2 unit by Millipore (Bedford, MA, USA). All used chemicals were analytical grade. All buffer solutions were prepared using a precision balance METTLER AT250 (Mettler-Toledo, Greifensee, Switzerland). The buffer compositions were calculated according to the recipes by Beynon and Easterby [59]. If not otherwise stated, all experiments were carried out with 20mM acetate buffer pH 5 with 0 to 1 M sodium chloride used as modifier. The experiments were carried out at 20°C, if not stated otherwise.

4.2.3 Instrumentation

For chromatographic measurements a HPLC 1100 Series by Agilent Technologies (Santa Clara, CA, USA) was used. The instrument is equipped with an UV detector and a conductivity meter CDD-10Avp by Shimadzu (Kyoto, Japan). Absorption was monitored at a wavelength of 280nm.

4.3 Peak splitting in pure HSA gradient chromatography

In the following, we discuss a series of experiments intended to elucidate the adsorption behavior of human serum albumin on a strong cation exchange resin, i.e. Fractogel EMD SE Hicap (M) and in particular the occurrence of peak splitting. It is worth noting that the used human serum albumin has a guaranteed purity of approximately 99\%. This was verified by a gradient analysis with the analytical cation exchange column Propac which gave a purity of 99.16\%. The impurities are not adsorbing on this column and elute therefore at the column residence time, t₀.

In the elution chromatogram from the analysis of the size exclusion column, three peaks can be identified (not shown here): human serum albumin with a weight percentage of
92.81%, 6.47% of a dimer of human serum albumin and 0.72% of other high molecular weight impurities, most probably oligomers of human serum albumin. This analysis corresponds quantitatively to the analysis on the analytical cation exchange column, assuming that monomer and dimer have the same retention time.

![Graph](image)

**Figure 4.1**: Gradient chromatogram of pure human serum albumin at $u_{\text{lin}} = 153 \, \text{cm} / \text{h}$. The modifier gradient is shown as a dashed line.

A typical elution chromatogram is shown in Figure 4.1 where the occurrence of two peaks is demonstrated. In the following, the first eluting peak will be referred to as ‘peak 1’, and the second as ‘peak 2’. This elution experiment consists of an initial loading time corresponding to $\Delta V = 2.7$ column volumes, followed by a gradient from 0 to 1 M sodium chloride in $\Delta V = 10.6$ column volumes.

In order to guide the discussion that follows, we formulate the hypothesis up-front: let us consider that the presence of two peaks for the same component is due to the presence of two different binding sites. Note that this would be equivalent to the case where the protein exhibits two different binding conformations, in the sense that the effect of these two situations on the behavior of the chromatographic column would not be distinguishable. In addition, we can immediately state that the adsorption process for at least one of the two sites must exhibit a slow kinetics. The presence of an instantaneous equilibrium between two binding sites, as described e.g. by a bilangmuir isotherm, would in fact lead under no circumstances to two peaks in the elution chromatogram [42].
4.3.1 Role of the washing step

![Graph](image)

**Figure 4.2:** Area $A$ of peaks 1 (□) and 2 (●) as a function of the initial washing volume in the gradient experiments.

In order to investigate the behavior of the two peaks, an ‘initial washing’ step has been introduced just after loading the protein and before starting the gradient which ranged from 0 to 127 column volumes (corresponding to 0 to 240 min, respectively). During such a ‘washing’ step, buffer A, which is 20 mM acetate buffer pH 5, was pumped through the column at constant flow rate. It is found that for increasing ‘initial washing’ volumes, the first peak decreases with time, while the second peak gets larger. This is shown in Figure 4.2, where the peak areas of peak 1, $A_{p1}$, and peak 2, $A_{p2}$, are plotted versus the initial washing volume. For longer washings (i.e. time), the peak area of peak 2 becomes dominant. This indicates a slow transition from the first site to the second site in controlling the process which is incompatible with the assumption of two sites having both an instantaneous adsorption equilibrium.

The same trends are observed if the pump is turned off during the ‘initial washing’ (not shown here). This means that after loading, we wait for increasing times without feeding anything to the column before starting the gradient. During the elution gradient, two peaks were always observed and the ratio of peak 1 and peak 2 $A_{p1}/A_{p2}$ was dependent on the waiting time before starting the gradient. This means that the change in ratio $A_{p1}/A_{p2}$ is indeed a function of time and not a function of the buffer volume that is
pumped during the ‘initial washing’ step. This result supports the conclusion that the
timescale for the transition from the first to the second adsorption site is in the order
of minutes (i.e. the elution time of the column).

4.3.2 Role of flow rate and gradient steepness

![Graph showing modifier concentration vs. ΔV at different flow rates](image)

**Figure 4.3:** Modifier concentration $\bar{c}_m$ at elution of human serum albumin at different
gradient steepnesses $\Delta V$: modifier concentration value corresponding to
the elution time of peak 1 at all velocities (○), modifier concentration value corresponding to the elution time of peak 2 at $u_{in} = 76 \text{ cm/h}$ (■), $u_{in} = 153 \text{ cm/h}$ (●) and $u_{in} = 229 \text{ cm/h}$ (▲).

The influence of different flow rates on the elution behavior was tested in the range of
$u_{in} = 76 - 229 \text{ cm/h}$ which covers almost the complete working range of the resin. Four
different gradient lengths were tested, ranging from 10 to 40 column volumes.

The obtained results shown in Figure 4.3 indicate that the modifier concentration cor-
responding to the elution time of the first peak is not a function of the flow rate, but
only of the gradient length. This corresponds to the typical elution behavior of solutes
characterized by local equilibrium adsorption conditions which means that only the peak
broadness changes as a result of different flow rates, but not the retention volume.

On the other hand, the elution time of the second peak depends upon the flow rate (Fig-
ure 4.3). This is another indication for the presence of a kinetically limited process. On
the first site, instantaneous adsorption equilibrium is reached and the elution behavior
is driven by the modifier concentration only, as well described by Yamamoto in the case without mass transfer resistances [43]. On the other hand, the second site, characterized by some kinetic limitation (influencing both the adsorption and the desorption behavior of the solute), is eluted with different kinetics, depending on the applied flow rate. As expected, larger flow rates favor the later elution of the peak and therefore an increase of the modifier concentration at peak elution.

Note that the elution position in Figure 4.3 is expressed in terms of the modifier concentration $c_m$ corresponding to the elution position of the peak (an illustration is shown in Figure 4.1). This representation was selected, because the elution position of the protein in the modifier gradient, assuming instantaneous adsorption equilibrium, is independent of the other operating conditions, as it is the case here for peak 1, but not for peak 2.

### 4.3.3 Role of temperature

![Diagram](image)

**Figure 4.4:** Elution chromatograms of human serum albumin at the same operating conditions as in Figure 4.1 and three temperature values: 10, 20 and 30 °C.

In Figure 4.4 the elution chromatograms at the same operating conditions used in Figure 4.1 but for three different temperature values, are shown. It is seen that the first peak is independent of temperature within the working temperature range of $\Delta t = 10 - 30$ °C. This behavior confirms an adsorption/desorption process that is close to equilibrium and without large enthalpy effects [94].
On the other hand, the second peak moves by almost one column volume in the examined temperature range. This again points at an activated kinetic process which gets faster at larger temperatures.

### 4.3.4 Role of the injection amount

![Graph](image)

**Figure 4.5**: Peak areas $A_{p1}$ (☐), $A_{p2}$ (●) and their ratio $A_{p1}/A_{p2}$ (▲) as a function of the injected mass $m$ of human serum albumin (chromatograms in Figure 4.6).

As shown in Figure 4.5, each injection of a larger amount of human serum albumin resulted in a larger pair of peak 1 and peak 2. The elution chromatograms obtained for the same operating conditions as in Figure 4.1 but with increasing injection amounts, are shown in Figure 4.6. It is seen that the peak maxima do not shift to the left which indicates that the column is not overloaded, i.e. the maximum injection amount is well below the column capacity under these conditions. In fact, peak 1 and peak 2 start to elute at constant times.

As shown in Figure 4.5, the areas of both peaks increase linearly with increasing injection amount. The relative amount of human serum albumin eluting through peak 2 is almost constant, suggesting that that the second peak is generated by the first one through a positive order kinetic process.
4.3 Peak splitting in pure HSA gradient chromatography

Figure 4.6: Elution chromatograms of the same operating conditions as in Figure 4.1 and different injection volumes: $V = 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 \mu l$ of $c_{HSA} = 91 \text{ g/l}$.

4.3.5 Role of the stationary phase

In order elucidate the mechanism leading to the two peaks, various cation exchange resins were considered. In particular, we intended to investigate the role played by:

- the matrix of the material which is crosslinked polymethacrylate for Fractogel EMD SE Hicap (M).
- the ligands that are sulfoethyl groups for Fractogel EMD SE Hicap (M).
- the way the ligand is attached to the matrix which is done by the so called tentacle technology [49].

Fractogel EMD SO$_3^-$ (M), which is another strong cation exchange resin by Merck, differs from Fractogel EMD SE Hicap (M) only in the ligand which are sulfoisobutyl instead of sulfoethyl groups. This resin was tested with various gradients under diluted conditions and in all cases it was found that human serum albumin leads to the formation of two peaks, just like in the case of Fractogel EMD SE Hicap (M) discussed above.

Furthermore, Fractogel EMD COO$^-$ (M) was tested with gradients in the range of pH 5 to pH 7. This material is a weak cation exchanger that has again the same matrix and spacer, but has carboxyethyl groups as functional groups. For all tested gradients
on this stationary phase, no peak splitting could be observed. This indicates that the occurrence of two peaks is related to the chemical nature of the ligands and specifically to their strong interaction with human serum albumin and not to the type of matrix and spacer, at least for the systems examined in this work. While weak cation groups such as COO\(^{-}\)-groups result in fact in a single peak elution, the strong SO\(_3\)-groups produce two human serum albumin peaks.

### 4.3.6 Reinnjection of eluted fractions

In order to exclude the presence of two different components or partial protein unfolding as the reason for the two peak behavior, the eluted peaks shown in Figure 4.1 were fractionated and different fractions were reinjected into the column. It was found that reinjection of a fraction taken from peak 1 leads again to the formation of two separate peaks. The same result is obtained for the reinjection of a fraction taken from peak 2. Furthermore, both fractions were analyzed with size exclusion analysis (method described in Section 4.3.2) and it was found that both of them contain the monomeric as well as the dimeric forms of human serum albumin. Also the injection of pure monomer or dimer (fractionated with the size exclusion column) leads to two peaks in elution chromatogram on Fractogel EMD SE Hirap (M). Note that pure monomer and dimer were also reinjected into the size exclusion column so as to confirm that they were still constituted of only the monomeric or the dimeric form, respectively, and no dimerization reaction is taking place at least in this time scale.

These findings clearly rule out any effect related to the presence of different product states (e.g. different foldings, presence of aggregates, etc.), unless these 'states' are reversible in the time frame typical for HPLC operations (i.e. tens of minutes). Note that also the presence of ion pairs of the protein with different counterions can be excluded, since this is not compatible with the results of the reinnjection experiments as well as of the experiments with very long washing times discussed above.

We can conclude at this point that the only possible explanation for the observed behavior is the presence of either two adsorption sites or two adsorption conformations which cannot be distinguished with a macroscopic analysis of the column adsorption behavior. A microscopic analysis of the observed behavior is not possible, because Fractogel particles are not transparent. Besides, these methods usually require labeling of the protein which influences its adsorption behavior.

In addition, the explanation of the experimental findings requires that the first adsorption site (or conformation) is characterized by a very fast kinetics (at least much faster than the characteristic elution time of the column). The elution behavior of this site is
in fact not affected by any of the investigated operating conditions (washing time and volume, flow rate and temperature). On the contrary, the adsorption on the second site clearly shows a kinetic limitation whose characteristic time is similar or larger than the characteristic elution time of the column. In addition to this, it appears that in all cases the increase of the adsorbed amount on one site occurs at the expenses of the adsorbed amount on the other one.

4.4 Adsorption isotherm

In order to further elucidate the adsorption behavior of human serum albumin on strong cation exchange resins, also the adsorption isotherm of HSA is studied. In this work, the adsorption isotherm for HSA is approximated with a Langmuir isotherm [93]:

\[ q_{eq} = \frac{H c_{eq}}{1 + H c_{eq}/q_\infty} \]  

(4.1)

The equilibrium binding capacity \( q_{eq} \) is a function of the Henry coefficient \( H \), the concentration of the solute at equilibrium \( c_{eq} \) and the saturation capacity \( q_\infty \).

4.4.1 Static binding capacity

<table>
<thead>
<tr>
<th>tracer</th>
<th>( \epsilon_i )</th>
<th>( \epsilon_{p,i} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>blue dextran</td>
<td>0.414</td>
<td>0</td>
</tr>
<tr>
<td>HSA</td>
<td>0.630</td>
<td>0.367</td>
</tr>
<tr>
<td>dextran 1.2kDa</td>
<td>0.832</td>
<td>0.721</td>
</tr>
</tbody>
</table>

Batch experiments are a simple and cheap tool to determine protein isotherms at various conditions. A slurry with 50% resin (\( V_{sol} \)) and 50% buffer (\( V_{aq} \)) with a defined modifier concentration was mixed with a known amount of human serum albumin. After reaching
equilibrium conditions, the human serum albumin concentration \( c_{eq} \) of the supernatant was measured according to the above described analytical method. The equilibrium capacity \( q_{eq} \) is then calculated using the initial concentration of human serum albumin \( c_0 \) and the equilibrium concentration as follows:

\[
q_{eq} = \frac{V_{eq}(c_0 - c_{eq})}{V_{sol}(1 - \epsilon_t)}
\]  

(4.2)

Note that using this equation requires an estimation of the total porosity \( \epsilon_t \) of the resin which correlates the total volume of the resin \( V_{sol} \) to the solid volume of the material \( V_{sol}(1 - \epsilon_t) \). In the following we assume that the total porosity for the batch experiments is equal to that measured in the packed column (values according to Table 4.1). The experimental setup and discussion of the porosity data is presented in the second part of this work [96]).

The experimental data from batch experiments are shown in Figure 4.7. Information about the equilibrium capacity \( q_{eq} \) as a function of the equilibrium concentration \( c_{eq} \) was fitted to Langmuir isotherms with a least square algorithm. For each measured modifier concentration, a separate isotherm was determined (Figure 4.7). It is shown very clearly that an increase in the modifier concentration decreases the capacity and

\begin{figure}[h]
\centering
\includegraphics[width=0.6\textwidth]{binding_capacity.png}
\caption{Static binding capacity for human serum albumin at \( c_m = 0 \text{ M (□)} \), \( c_m = 0.05 \text{ M (●)} \), \( c_m = 0.08 \text{ M (▲)} \), \( c_m = 0.11 \text{ M (★)} \) and \( c_m = 0.13 \text{ M (♦)} \) with 20 mM acetate buffer pH 5.}
\end{figure}
the Henry coefficient that is given by the initial slope of the isotherm. As illustrated in Figure 4.7, the experimental values fit very nicely to Langmuir type isotherms. It is important to notice that the equilibrium capacity measured by these experiments refers to both adsorption sites or adsorption conformations, since the selected equilibration time (two days) is long enough to reach equilibrium on both sites.

4.4.2 Experiments under diluted isocratic conditions

![Graph](image)

*Figure 4.8:* Isocratic experiments for human serum albumin under diluted conditions with a modifier concentration of 1 M, 0.2 M, 0.19 M, 0.18 M and 0.17 M (from left to right).

Isocratic experiments allow measuring of the Henry coefficients as a function of the modifier concentration. Figure 4.8 shows the elution chromatograms of human serum albumin at high to moderate modifier concentrations, i.e. 1 M to 0.17 M sodium chloride. It is seen that the protein elutes with a single peak as it would be expected for a pure component. In particular, at 1 M sodium chloride, the protein does not adsorb on the resin and therefore elutes with a ‘sharp’ peak at the dead volume of the column. At lower modifier concentrations, the protein adsorbs more and more and therefore the peak shifts to the right, exhibiting more pronounced tails.

At modifier concentrations lower than 0.15 M, human serum albumin elutes with two peaks (Figure 4.9), also under isocratic conditions. This indicates that also in this
Figure 4.9: Isocratic experiments for human serum albumin under diluted conditions with low modifier concentration.

In the case we have the presence of two adsorption sites or conformations. However, at higher modifier concentrations, the second site does not result in a second peak (see Figure 4.8), because it overlaps with the more pronounced peak 1. In any case these data do not allow an estimation of the Henry coefficient for peak 2. However, for the elution of the first peak, adsorption equilibrium might be assumed, as shown before. Therefore, Henry coefficients $H$ can be calculated from isocratic pulse experiments under diluted conditions as a function of the salt concentration as follows:

$$H = \frac{\tau_i - 1}{\epsilon_{p,i}} \frac{\epsilon_b}{1 - \epsilon_b} \frac{\epsilon_{p,t}}{1 - \epsilon_{p,t}} - \frac{\epsilon_{p,t}}{1 - \epsilon_{p,t}}$$  \hspace{1cm} (4.3)

whereas $\epsilon_{p,i}$ is the particle porosity of the selected tracer $i$, $\epsilon_{p,t}$ is the total particle porosity, as measured with a small tracer and $\epsilon_b$ is the bed porosity that is measured with tracer larger enough to enter none of the pores of the stationary phase. The dimensionless time $\tau$ is defined by:

$$\tau = \frac{t u}{L}$$  \hspace{1cm} (4.4)

where $t$ denotes the measured retention time, $L$ the column length and $u$ the interstitial velocity.

From the presented isocratic experiments, the Henry coefficients for peak 1 are estimated using Equation (4.3), assuming that for these conditions the effect of the second peak
Figure 4.10: Henry coefficients for human serum albumin on Fractogel EMD SE Hi-cap (M) for peak 1 (□). Henry coefficients as calculated from batch experiments (▲) are also added.

on the elution process is negligible. The necessary porosity data is listed in Table 4.1. In the logarithmic plot in Figure 4.10, the Henry values as a function of the modifier concentration from isocratic experiments (□) are shown together with the values estimated from the uptake of the isotherm of the batch experiments (▲) discussed in the context of Figure 4.7. The following functional dependency on the modifier (salt) concentration \( c_m \) was used for the Henry coefficient \( H \):

\[
H = \alpha c_m^\beta
\]  

Equations (4.3), that is dependent on the fitting parameters \( \alpha \) and \( \beta \), could be justified theoretically starting from the mass action law, as shown by Yamamoto [44]. The regression of the two data sets shown in Figure 4.10 leads to \( \alpha = 3.2008 \cdot 10^{-5} \, \text{M}^{-7.1764} \) and \( \beta = -7.1764 \).

This latest experiment allows to speculate about the kinetic scheme behind the observed adsorption behavior. The simplest option compatible with the experiments shown above would be to suppose that there are two parallel adsorption mechanisms, the second of which is regulated by a slow kinetics. Note that two parallel adsorption mechanisms (both without kinetic limitation), would lead to the so called bilangmuir adsorption
isotherm which does not result in double peaks for single component elutions. In particular, we have observed in the context of Figure 4.2 that the characteristic time of the second site adsorption kinetics has to be larger than the elution time that is $1/k_{ads,2} > 1 \text{ min} \approx 100 \text{s}$. On the other hand, the first site, which has a much faster kinetics (in the limit, instantaneous), has very large Henry coefficients at modifier concentrations typically used for column loading ($H \approx 1 \cdot 10^6$ for $c_m = 0.03 \text{M}$ as seen in Figure 4.10). Combining these two information, it can be foreseen that, as a result of the equilibrium conditions on the first site, the resulting concentration of human serum albumin in the liquid phase is extremely low ($c = q/H$ under diluted conditions). Supposing that the concentration of human serum albumin on the first adsorption site is in the order of $q_1 = 1 \text{ g/l}$ and that the adsorption on the second site has a first order kinetics, it can be written:

$$\frac{d q_2}{dt} = k_{ads,2} \cdot c = \frac{k_{ads,2} \cdot q_1}{H_1} \approx 1 \cdot 10^{-2} \frac{1}{s} \cdot \frac{1 \text{ g}}{10^6 \text{ l}} \approx 1 \cdot 10^{-8} \frac{\text{ g}}{\text{l s}}$$

(4.6)

This means that in order to build up a protein concentration on the second site $q_2$, which is similar to that of the first one (about $1 \text{ g/l}$), as observed experimentally, it would take approximately $10^8 \text{s} \approx 3 \text{ years}$.

![Diagram](image)

**Figure 4.11:** Schematic representation of the protein human serum albumin adsorbing on site 1 from the liquid phase with a possible transition to site 2 through the solid phase.

The simple example above suggests that a different kinetic scheme must be proposed to explain the observed behavior of human serum albumin. In order to explain the accumulation of human serum albumin on the second site, in spite of the fact that the
liquid concentration of human serum albumin becomes rapidly very small due to the fast adsorption on the first site, we need to postulate that the adsorbate state on the second site is generated by the one on the first site and not directly by the liquid state. Such a situation is depicted by the kinetic scheme as shown in Figure 4.11. This scheme is better explained by the presence of a second adsorption conformation for human serum albumin on the surface of the cation exchange resin: human serum albumin adsors rapidly in the first conformation and then changes slowly to the second, more stable conformation, where the protein exhibits a stronger affinity to the stationary phase and in fact, it elutes later. Independently of the mechanistic interpretation of this phenomenon, in the second part of this work, it will be shown that the suggested kinetic scheme (Figure 4.11) is quantitatively compatible with all observed experimental behaviors by the use of a suitable numerical model [66].

4.4.3 Breakthrough curves

From breakthrough experiments, the equilibrium capacity $q_{eq}$ can be determined as a function of the protein solution concentration. This was calculated from the area of adsorbed protein $m_{ads}$ of the breakthrough curve and the solid volume of the stationary phase (in analogy to Equation (4.2) for the static binding capacity), as follows:

$$q_{eq} = \frac{m_{ads}}{V(1 - \epsilon_t)} \quad (4.7)$$

In addition, the elution fraction of the breakthrough curve was collected and analyzed. The two independently measured values for the equilibrium capacity from breakthrough experiments should be identical and in fact they did not differ more than 10%, typically by only 1%.

Breakthrough experiments were conducted for four different modifier concentrations from $c_m = 0.05$ to $0.13 \text{M}$ and different linear velocities. The initial concentration of human serum albumin was $c_0 \approx 3 \text{g/l}$. The presence of the two adsorption sites or conformations discussed above manifests itself also in this kind of experiments as it can be seen in Figure 4.12 at the smallest salt concentrations, typical S-shaped curves are observed during breakthrough elution. These are rather flat, indicating the presence of severe mass transfer limitations. As the modifier concentration is increased and, according to Figure 4.7, the column capacity is reduced, a more and more pronounced shoulder (highlighted in Figure 4.12) in the front of the elution curve can be observed. This is probably due to the limited capacity of the first site. Most likely, the adsorption on the second site is responsible for the behavior of the breakthrough curve after the
Figure 4.12: Breakthrough curves for human serum albumin at $u_{lin} = 229 \text{cm/h}$ and $u_{lin} = 153 \text{cm/h}$ (human serum albumin loaded with slower velocity breaks through later) for four modifier concentrations from $c_m = 0.05$ to 0.13 M. The third curve at $c_m = 0.13$ M corresponds to $u_{lin} = 76 \text{cm/h}$. The feed concentration of human serum albumin is represented by the dashed line.
Figure 4.13: Binding capacity $q$ as a function of modifier concentration $c_m$: data for the equilibrium capacity $q_{eq}$ by breakthrough curves (□) and for the saturation capacity $q_\infty$ by batch experiments (●).

shoulder. It can be seen that the shoulder is more pronounced for larger flow rates, as a possible consequence of the kinetic limitation in the adsorption of the second site. It is clear, that from these data, it is not possible to measure the equilibrium adsorption concentration of the two sites separately. In the following, we estimate the overall saturation capacity $q_\infty$ as given by the sum of the two adsorption sites. For this, we consider first the overall saturation capacity (values shown in Figure 4.13) as a function of the modifier concentration. A possible functional dependency might be:

$$q_\infty = \frac{\gamma}{1 + \exp (\delta c_m + \epsilon)}$$

(4.8)

which is fully empirical and requires the estimation of three fitting parameters $\gamma$, $\delta$ and $\epsilon$. However, many authors have observed a similar behavior [23, 36, 75, 87, 97, 100]. Basically, this equation describes a reversed S-shaped curve of the saturation capacity as a function of the modifier concentration. At low modifier concentration, the protein adsorbs on all available adsorption sites, thus approaching full surface coverage. On the other hand, at high modifier concentrations, the adsorptivity of human serum albumin is strongly reduced and therefore the saturation capacity tends to zero. By fitting the static saturation capacity values, the following numbers for the fitting parameters in
Equation (1.3) are obtained: $\gamma = 732.36 \text{g/l}, \delta = 63.641 /\text{M}$ and $\epsilon = -5.86$. and the corresponding calculated versus experimental comparison is shown in Figure 4.13.

By considering only the breakthrough experiments where the feed is protein concentration was sufficiently high, the determined equilibrium capacity (Equation 4.17) can be regarded as good approximation of the saturation capacity (Figure 4.13). It can be seen that the so obtained data are in good agreement with the saturation capacity measured by static experiments at low modifier concentrations. On the other hand, at higher modifier concentrations ($c_m > 0.11 \text{M}$), the values differ quite significantly. This is probably due to the fact that at those modifier concentrations, the adsorption isotherm has already quite low Henry coefficients (Figure 4.7 and 4.10), and the protein feed concentration ($c_0 \approx 3 \text{ g/l}$) was not high enough to reach the saturation capacity during the breakthrough experiments.

### 4.5 Conclusion

It was shown that pure human serum albumin elute with two peaks on a strong cation exchange resin under certain conditions. The elution time of the second peak changes with flow rate and temperature. Furthermore, the ratio between peak 1 and peak 2 changes with time and injection amount. All these mechanisms indicate the presence of a kinetic limitation in the adsorption behavior of the second site.

As the ratio between peak 1 and peak 2 is not constant, the reason for the two peaks cannot be found in the presence of dimers in the protein solution as it was discussed above. However, a fractionation of the gradient experiment of Figure 4.13 showed the presence of monomer and dimer in peak 1 as well as in peak 2 according to size exclusion analysis. A reinjection of those fractions into the Fractogel EMD SE Hicap (M) column lead again to the two-peak behavior of the standard experiment. We can therefore exclude that two different components are responsible for the described behavior.

Based on the comparison of various cation exchange resins, it is concluded that the two peak elution of human serum albumin in modifier gradients is caused by the strong interaction of human serum albumin with the $\text{SO}_3^-$-ligand of the stationary phase. This result is in agreement with the proposed kinetics of the second site, whereas a direct transition of human serum albumin from the first to the second conformation is postulated.

Despite the peculiar elution pattern in the gradient, experiments under static conditions show that the isotherm of the overall system is described nicely with a Langmuir isotherm. Even if the ‘true’ isotherm might be more complex, this would not lead to a
possible explanation for the previously mentioned behavior. 
In this work, it was possible to characterize the behavior of both binding sites and to show a kinetic limitation for the second site. This needs to be confirmed by a quantitative model.
5 Simulation of human serum albumin on strong cation exchange resins

5.1 Introduction

In the previous chapter it was shown that human serum albumin exhibits a peculiar elution pattern on Fractogel EMD SE Hicap (M), which is a preparative strong cation exchange resin from Merck (Darmstadt, Germany) [4]. When running a modifier gradient from 0 to 1 M NaCl with 20 mM acetate buffer pH 5, human serum albumin elutes with two peaks. It was proven that the two-peak elution pattern is not caused by conformational changes of the protein or by the presence of two components. In contrast, the characteristic two peak elution in the modifier gradient is caused by an interaction of the protein with the stationary phase at the used conditions. Besides, it was shown experimentally that the second peak has a kinetic limitation, while the first elution peak can be described assuming adsorption equilibrium.

For further understanding, the behavior of human serum albumin on Fractogel EMD SE Hicap (M) is modeled with a general rate model in the current chapter [42]. As shown by Kaczmarski and Antos, the general rate model is mandatory for systems that have significant mass transfer hindrances [43]. Macromolecules such as proteins always travel through chromatographic columns with mass transfer limitations [44]. Furthermore, the general rate model provides useful insights into the diffusion and adsorption process of the protein on the stationary phase. As the parameters are not lumped, dominating effects can be easily monitored. With increasing computational power in the last couple of years, it is possible to solve the general rate model fast enough in order to use it for parameter studies. Melter et al. used a multicomponent general rate model to study the separation of monoclonal antibody variants on a weak cation exchange resin, while
Forrer et al. used a similar model for the separation of a polyclonal antibody mixture on a strong cation exchange resin \(^{62, 101}\).

### 5.2 Model

The system was modeled with a general rate model. This model seems sufficient as it takes mass transfer resistances into consideration which highly influence the behavior of the protein on the stationary phase.

In order to account for the peculiar elution behavior of human serum albumin on Fractogel EMD SE Hicap (M), the general rate model was extended to account for two different binding sites. For the so called ‘site 1’ it is assumed that adsorption equilibrium is reached fast. Adsorption of the solute from the liquid phase is expected to be always at site 1. For the other site (‘site 2’), the adsorption process is significantly slower as for site 1 \(^{74}\). The adsorption of site 2 is assumed to be a transition of the adsorbed molecule from site 1. The adsorption isotherms of the two sites are independent of each other.

The model is proposed in dimensionless form, whereas the dimensionless time \(\tau\) is coupled to the time \(t\) through the ratio of the interstitial velocity \(u\) and the length of the column \(L\).

\[
\tau = \frac{\text{time}}{\text{convection}} = \frac{tu}{L} \quad (5.1)
\]

The interstitial velocity \(u\) is defined as

\[
u = \frac{Q}{\varepsilon_{\text{bed}} A} \quad (5.2)
\]

which is the ratio of the volumetric flow rate \(Q\) with the product of the cross sectional area of the column \(A\) and the bed porosity \(\varepsilon_{\text{bed}}\). The porosity of the bed is defined as the porosity obtained with a tracer large enough to enter none of the pores of the resin. According to the definition of the dimensionless time in Equation (5.1), a tracer, that does not enter the pore, leaves the column at \(\tau = 1\) under nonadsorbing conditions.

In analogy to Equation (5.1), axial and radial position \(\eta\) and \(\rho\) are dimensionless though the following equations:

\[
\eta = \frac{z}{L} \quad (5.3)
\]

\[
\rho = \frac{r}{R_p} \quad (5.4)
\]
$R_p$ is the particle radius, $z$ and $r$ are the axial position in the column and the radial position in the particle.

### 5.2.1 Mass balances in the mobile phase and in the pore

The general rate model treats the mobile and the stagnant phase separately. First of all, the mass balance for the solute is set up, whereas $c$ is the concentration of the solute in the liquid phase and $c_p|_{\rho=j}$ the concentration of the solute in the liquid phase of the particle at the position $\rho = j$. In dimensionless parameters, the mass balance for the liquid phase is as following:

$$\frac{\partial c}{\partial \tau} + \frac{\partial c}{\partial \eta} + \epsilon_{p,HSA} \frac{1 - \epsilon_{bed}}{\epsilon_{bed}} St (c - c_p|_{\rho=1}) = \frac{1}{Pe_{ax}} \nabla_{\eta,2} c$$  \hspace{1cm} (5.5)

The first term treats the accumulation of the solute in the mobile phase of the column, while the second term describes the convection in axial direction. The third term gives the flux from the mobile to the stagnant phase in the column. On the right hand side of Equation (5.5), the diffusion of the solute through the column is given. Axial Peclet number $Pe_{ax}$ is defined by

$$Pe_{ax} = \frac{u L}{D_{ax}}$$  \hspace{1cm} (5.6)

which is the quotient of the time for axial dispersion $L^2/D_{ax}$ and convection. The Stanton number $St$, that is used in Equation (5.5), is defined as the quotient of time for convection and film mass transport $\frac{L k_f}{u d_p}$:

$$St = \frac{L k_f}{u d_p}$$  \hspace{1cm} (5.7)

The boundary conditions for Equation (5.5) are the classical Danckwerts conditions:

if $\tau = 0$, then $c = c(0, \eta)$

if $\eta = 0$, then $\frac{\partial c}{\partial \eta} = Pe_{ax}(c - c_0)$ \hspace{1cm} (5.8)

if $\eta = 1$, then $\frac{\partial c}{\partial \eta} = 0$

The general rate model does no longer assume that the stagnant and the solid phase are in equilibrium. Therefore, the stagnant phase is treated separately from the liquid phase (Equation (5.5)).

$$\frac{\partial c_p}{\partial \tau} + \frac{1 - \epsilon_{p,t}}{\epsilon_{p,t}} \frac{\partial q}{\partial \tau} = \frac{1}{Pe} \nabla_{\rho,2} c_p$$  \hspace{1cm} (5.9)
On the left hand side, the first term treats the accumulation in the stagnant liquid in the pores with \( c_p \) being the concentration of the solute inside the pore. The second term gives the accumulation of solute in the solid phase, whereas \( q \) is the total amount of adsorbed protein on the stationary phase. The phase ratio is defined through the total porosity of the particle \( \epsilon_{p,t} \) (as measured with a small tracer). Diffusion of the solute is given on the right hand side of Equation (5.9). The particle Peclet number \( P_e \) in Equation (5.9) is defined as:

\[
P_e = \frac{\epsilon_{p,HSA}}{4} \frac{u d_p^2}{L D_{p,eff}}
\]

whereas \( D_{p,eff} \) is the pore effective diffusion coefficient. The boundary conditions of Equation (5.9) are listed in Equation (5.11).

if \( \tau = 0 \), then \( c_p = c_p(0, \rho) \)

if \( \rho = 0 \), then \( \frac{\partial c_p}{\partial \rho} = 0 \) \hspace{1cm} (5.11)

if \( \rho = 1 \), then \( \frac{\partial c_p}{\partial \rho} = Sh (c - c_p (\rho = 1)) \)

Sherwood number is defined as the ratio of the characteristic times for pore diffusion and film mass transfer:

\[
Sh = \frac{\epsilon_{p,HSA} d_p k_f}{2 \frac{D_{p,eff}}{D_{p,eff}}} \hspace{1cm} (5.12)
\]

The mass balance for the modifier, that is in this case sodium chloride, can be further simplified. As the modifier is more than 1500 times smaller than the protein, its diffusion coefficient into the pores is much higher. Therefore, it is assumed that the mobile and the stagnant phase are in equilibrium for the modifier. Film mass transfer resistances are not considered. The porosity of the modifier is defined as the total porosity of the stationary phase. The mass balance for the modifier is described with the equilibrium-dispersive model of chromatography:

\[
\left(1 + \frac{1 - \epsilon_{bed}}{\epsilon_{bed}}\right) \frac{\partial c_m}{\partial \tau} + \frac{\partial c_m}{\partial \eta} + \left(1 - \frac{1 - \epsilon_{bed}}{\epsilon_{bed}}\right) \frac{\partial q_m}{\partial \eta} = \frac{1}{P e_{\alpha x}} \nabla_{\eta, \tau} c_m \hspace{1cm} (5.13)
\]

whereas \( c_m \) is the concentration of the modifier in the liquid phase and \( q_m \) is the amount of modifier adsorbed on the stationary phase. Simplifications of Equation (5.13) were done according to Guiochon [12].
Figure 5.1: Schematic representation of the protein human serum albumin adsorbing on site 1 from the liquid phase with a possible transition to site 2 through the solid phase.

5.2.2 Mass balances in the adsorbed phase and isotherm definition

Two different binding sites are considered. As shown in Figure 5.1, human serum albumin on the first site $q_1$ is adsorbed through the liquid phase. Adsorption on site 2 happens through a transition of site 1. For the mass balance of the pore (Equation 5.9), only the time derivative of the adsorption capacity $q$ must be known that is a function of the adsorption capacities of site 1 and site 2:

$$\frac{\partial q}{\partial \tau} = \frac{\partial q_1}{\partial \tau} + \frac{\partial q_2}{\partial \tau}$$

(5.14)

As shown in the previous work, the kinetics of site 1 is fast, at least compared to site 2 that has a much slower kinetics of adsorption. Therefore we might simply assume that the total derivative of site 1 is very close to the actual time derivative of site 1 $\frac{\partial q_1}{\partial \tau}$:

$$\frac{\partial q_1}{\partial \tau} = \frac{\partial q_1}{\partial c_p} \frac{\partial c_p}{\partial \tau} + \frac{\partial q_1}{\partial c_m} \frac{\partial c_m}{\partial \tau} + \frac{\partial q_1}{\partial q_2} \frac{\partial q_2}{\partial \tau}$$

(5.15)

This has two reasons: a (slow) transition of one molecule from site 1 to site 2 (or back) is not moving site 1 out of equilibrium, because this can be compensated for by a fast transition of another molecule to or from the stagnant phase. In case a molecule is still adsorbed on site 2, while site 1 has already eluted through peak 1, the desorption of this molecule is proceeding through a (slow) transition of site 2 to site 1. As soon as
the molecule can be associated to site 1, it desorbs very fast to the bulk. Again, the
equilibrium assumption for site 1 is generally true, because the variations are small and
are compensated fast.
For the time derivative of site 2, the kinetic of adsorption and desorption must be
considered.
\[
\frac{\partial q}{\partial \tau} = \tilde{k}_{ads,2} q_{1,eq} - \tilde{k}_{des,2} q_2
\] (5.16)
This derivative is set up according to Figure 5.11 which leads to a first order kinetics for
the second site. Note that it is not possible to treat the second peak behavior simply
by the introduction of a term for solid diffusion. This would lead to a very long tailing
of one single peak, but not to the two peaks that are observed for the current system.
Note that \(k_{ads,2}\) and \(k_{des,2}\) are nondimensional kinetic parameters. The according dimen-
sional parameters are converted with the term for convection \(L/u\).
\[
\tilde{k}_{ads,2} = \frac{L}{u} k_{ads,2}
\]
\[
\tilde{k}_{des,2} = \frac{L}{u} k_{des,2}
\] (5.17)
As described above, adsorption through site 1 is assumed to be fast and therefore close to
equilibrium. Contrarily, the adsorption process of site 2 is significantly slower, therefore
a kinetic ansatz is necessary. Under these assumptions, an elution path from site 2 to the
bulk with \(k_{des,2}\) and \(k_{des,1}\) is mathematically equivalent to an elution path through \(k_{des,2}^a\)
(see Figure 5.11).
The kinetic parameters of adsorption \(k_{ads,2}\) and the kinetic parameter of desorption
\(k_{des,2}\) are described with power functions (in analogy to the well known dependence
of the Henry coefficient of proteins in ion exchange chromatography according to Ya-
mamoto [41] that is discussed later):
\[
k_{ads,2} = \alpha_{k_{ads}} c_{m,ads}^{\beta_{k_{ads}}}
\]
\[
k_{des,2} = \alpha_{k_{des}} c_{m,des}^{\beta_{k_{des}}}
\] (5.18)
In order to calculate the total derivative of \(q\) according to Equation 5.14, information
about the isotherm of the first site must be provided. As the behavior of the system
at overloaded conditions is even more complicated, the adsorption is modeled only for
diluted protein concentrations in this publication. It has been shown previously that a
linear isotherm fits to the experimental data at diluted conditions very good [49]:
\[
q_{i,eq} = H_i c_p
\] (5.19)
whereas the adsorbed amount on site $i = 1$ is described by its Henry coefficient $H_1$ and the concentration of the solute in the liquid phase inside the pores of the particle $c_p$. In ion exchange chromatography, the Henry coefficient is a function of the modifier concentration. As proposed by Yamamoto, the dependence of the Henry coefficient is described with a power function:

$$H_1 = \alpha_1 c_p^{\beta_1} \quad \text{with } \alpha_1 > 0, \beta_1 < 0$$

(5.20)

whereas $\alpha_1$ and $\beta_1$ are constant parameters.

Furthermore, a linear isotherm, as given in Equation (5.19), is also describing the adsorption behavior of the salt. It is assumed, that the salt and the protein are not competing with each other for binding sites.

It is possible to define also a correlation for the Henry coefficient of the second site. Therefore, a mass balance for site 1 and site 2 is set up, assuming equilibrium. According to Figure 5.1, this would lead to

$$k_{ads,1} c_p + k_{des,2} q_2 - (k_{des,1} + k_{ads,2})q_1 = 0$$
$$k_{ads,2} q_1 - k_{des,2} q_2 = 0$$

(5.21)

Equation (5.21) leads to an expression for $q_2$, that is

$$q_2 = \frac{k_{ads,2}}{k_{des,2}} q_1$$

(5.22)

Equation (5.21) 1) and Equation (5.22) define $q_1$ in the well known way (Equation (5.19)):

$$q_1 = \frac{k_{ads,1}}{k_{des,1}} c_p$$

(5.23)

This leads to expressions of the Henry coefficients for site 1 and site 2 as a function of the kinetic parameters:

$$H_1 = \frac{k_{ads,1}}{k_{des,1}}$$
$$H_2 = \frac{k_{ads,2}}{k_{des,2}} \frac{k_{ads,1}}{k_{des,1}} = \frac{k_{ads,2}}{k_{des,2}} H_1$$

(5.24)

As proven experimentally in the previous work, the first site is close to equilibrium. Therefore it is not necessary to determine $k_{ads,1}$ and $k_{des,1}$, because under (close to) equilibrium conditions, the Henry coefficient alone can describe the system.
The Henry coefficient for site 2 is calculated with Equation (5.18) and (5.24):

\[
H_2 = \frac{\alpha_{k, ads}}{\alpha_{k, des}} \alpha_1 c_m \left( \beta_{k, ads} - \beta_{k, des} + \beta_1 \right)
\]

with \( \frac{\alpha_{k, ads}}{\alpha_{k, des}} \cdot \alpha_1 > 0, \ (\beta_{k, des} - \beta_{k, ads}) > \beta_1 \) (5.25)

It must be considered for the determination of \( k_{ads,2} \) and \( k_{des,2} \) that the Henry coefficient of site 2 is a monotonically decreasing function in analogy to the Henry coefficient of site 1.

### 5.2.3 Literature correlations

The film mass transfer coefficient \( k_f \) was calculated according to the equation of Wilson and Geankopolis \[47],

\[
k_f = 1.09 \sqrt{u} \left( \frac{D_m}{\epsilon_{bed} d_p} \right)^{2/3}
\]

which is valid for Reynolds numbers between 0.0015 and 55. The film mass transfer coefficient \( k_f \) is a function of the molecular diffusion coefficient \( D_m \):

\[
D_m = 8.34 \cdot 10^{-8} \frac{T}{\eta_S \sqrt{MW}}
\]

which has been shown to apply for most proteins \[58\]. The viscosity of the solvent is estimated as \( \eta_S = 1.001 \text{mPas} \) which is the viscosity of 0.1 M sodium chloride solution at 20°C according to Afzal et al. \[59\]. The molecular weight of human serum albumin is assumed to be \( MW = 66 \text{kDa} \) \[82, 83, 102\].

The axial dispersion \( D_{ax} \) is calculated according to Perry’s with a correlation for packed beds that neglects nonuniformities in the flow \[61\].

\[
D_{ax} = \gamma_1 D_m + \gamma_2 d_p u
\]

\[
\gamma_1 = 0.45 + 0.55 \epsilon_{bed}
\]

\[
\gamma_2 = 0.5 \left( 1 + \frac{13 \gamma_1 \epsilon_{bed}}{Re Sc} \right)^{-1}
\]

This correlation, that was first presented by Langer et al., considers molecular diffusion and mixing \[103\]. The parameter \( \gamma_1 \) is calculated according to Wicke \[104\]. An expression for \( \gamma_2 \) was proposed by Edwards and Richardson \[105\]. Reynolds number \( Re \) is

\[
Re = \frac{\epsilon_{bed} d_p u}{\nu_s}
\]
and Schmidt number is a function of the molecular diffusion coefficient $D_m$ and the kinematic viscosity of the solvent $\nu_s$.

$$Sc = \frac{\nu_s}{D_m}$$  (5.30)

### 5.3 Materials and Instrumentation

Strong cation exchange resin Fractogel EMD SE Hicap (M) was kindly donated by Merck (Darmstadt, Germany). The resin was packed into Tricorn columns purchased at GE Healthcare (Chalfont St Giles, United Kingdom). Tricorn columns have a volume of $V \approx 1$ ml and a diameter of $d = 5$ mm. 20% mechanical compression of the bed was applied to the resin in the column.

Human serum albumin was purchased at Sigma-Aldrich (Buchs, Switzerland). It is delivered as a lyophilized powder with a purity of approximately 99% (analyzed by agarose gel electrophoresis). The human serum albumin powder is essentially globulin free. Dextran standards were purchased at Fluka (Buchs, Switzerland). Sodium chloride and acetic acid (glacial) were purchased at J.T. Baker (Deventer, the Netherlands), sodium acetate trihydrate, sodium hydrogen phosphate dihydrate and sodium sulfate were purchased at Merck (Darmstadt, Germany). Sodium phosphate dibasic dodecahydrate was obtained by Acros Organics (Geel, Belgium). Deionized water was purified with a Simpakt 2 unit by Millipore (Bedford, MA, USA). All used chemicals were analytical grade. For chromatographic measurements a HPLC 1100 Series by Agilent Technologies (Santa Clara, CA, USA) was used. The instrument is equipped with an UV detector, a refractive index detector and a conductivity meter CDD-10Avp by Shimadzu (Kyoto, Japan). Absorption was monitored at a wavelength of 280 nm.

### 5.4 Parameter determination

The coupled partial differential equations of the general rate model cannot be solved analytically. Finite differences are used to solve this system numerically. The used method was proposed by LeVeque and Strikwerda [106, 107]. It transforms the radial and axial derivatives into algebraic equations within a small discretization interval. The discretization used in this work has 12 radial and 99 axial grid points. This leads to a system of $(12 \times 2 + 2) \times 99$ ordinary differential equations (12 equations each for the stagnant phase of site 1 and site 2, plus one equation each for the mobile phase and the salt) that have to be solved at each point of the parameter regression. Note that always more
grid points for the model were checked, in order to avoid numerical diffusion. The code for the general rate model was written in Fortran 95, whereas the system of ordinary differential equations was solved using the DLSODI package \[108\]. The code was run on a Pentium 4 Dual Core 3.2 GHz computer. Parameters used in the general rate model are regressed, if not otherwise stated. Adaptive simulated annealing was used to find the optimum of the regressed function \[109\]. An appropriate selection of the objective function as well as the determination of the confidence interval give important information on the quality of the regression. For the sake of space, these topics will not be discussed in detail in the current work. It was possible to reach convergence for all discussed simulations.

5.5 Results and discussion

If not otherwise stated, all experiments were carried out with 20mM acetate buffer pH 5 with 0 to 1 M sodium chloride used as modifier. The experiments were performed at 20°C.

5.5.1 Experimental characterization of the column

The adsorption behavior of human serum albumin was characterized on a Tricorn column packed with the strong cation exchange resin Fractogel EMD SE Hicap (M). The column had a length of \(L = 4.8\) cm with a diameter of \(d = 5\) mm (\(V = 0.94\) ml). The column was compressed mechanically by 20%.

Pore size distribution. Pore size distribution was measured through inverse size exclusion chromatography (ISEC) \[51, 52\]. This method has a number of advantages compared to other techniques such as mercury porosimetry or BET measurement: it is not necessary to dry the sample, the measurements can be performed directly in the packed column and there is no need to assume a priori a certain form for the distribution, such as log normal distribution or others. Nevertheless, ISEC measurements give a direct estimation of the pore distribution \[64, 110\]. ISEC was performed with various dextran tracers as well as protein tracers under nonadsorbing conditions. The total porosity \(\epsilon_{t,i}\) measured by a tracer of specific molecular weight on the resin is given by the ratio of the accessible liquid volume \(V_{t,i}\) for the specific tracer and the total column volume \(V\):

\[
\epsilon_{t,i} = \frac{V_{t,i}}{V} \tag{5.31}
\]
The dextran tracers are not monodisperse, even though they were purchased in high purity. Accordingly, part of the peak broadness is due to the tracer molecular weight distribution, rather than to mass transport resistances inside the column. Therefore, the average retention volume of a dextran peak was arbitrarily calculated with the maximum of the elution peak. On the contrary, for the protein tracers the retention volume is calculated with the first order moment of the peak.

If the bed porosity $\epsilon_{bed}$, that is the porosity measured by a very large tracer, which is excluded from all pores, is correctly estimated, the particle porosity $\epsilon_{p,i}$ of the tracers can be calculated as:

$$\epsilon_{p,i} = \frac{\epsilon_{t,i} - \epsilon_{bed}}{1 - \epsilon_{bed}}$$

(5.32)

The ISEC curve shows the characteristic distribution of the pores which goes from the total porosity $\epsilon_t$, that is the porosity measured by the smallest tracer, entering all (relevant) pores of the stationary phase, to the bed porosity $\epsilon_{bed}$, corresponding to the porosity measured by the largest tracer, excluded from all particle pores.

The pore size distribution was measured at three different modifier concentrations, namely 0.05 M, 0.25 M and 0.5 M sodium chloride with 50 mM phosphate buffer at pH 7. Under these conditions most proteins, including human serum albumin are not adsorbing on cation exchange resins. Various dextran tracers with a molecular weight from

![Figure 5.2: Pore size distribution for Fractogel EMD SE Hicap (M) at 0.5 M sodium chloride (□), 0.25 M sodium chloride (♦) and 0.05 M sodium chloride (♦).](image-url)
1.2 kDa (dextran 1.2) to 2000 kDa (dextran 2000) were used to determine the pore size distribution under the same conditions.

Figure 5.2 shows that the porosity accessible to large tracers, which do not enter the pores (and therefore can be used to estimate the bed porosity $\epsilon_{\text{bed}}$), is not a function of the modifier concentration. The assumed porosity of the bed, that was measured with blue dextran ($MW=2000\text{kDa}$), is $\epsilon_{\text{bed}} \approx 0.40$ (measured at $c_m = 0.5\text{ M}$ sodium chloride). The picture changes for smaller tracers. The smaller the tracer, the higher the dependence on the modifier concentration, as shown in Figure 5.2. Larger modifier concentrations result in wider pores.

The pore accessibility values for human serum albumin are listed in Table 5.1. According to Figure 5.2, it is estimated that the accessibility of human serum albumin, that has a molecular weight of 66 kDa, would correspond to a dextran tracer of 30 kDa [82,83,102]. This is reasonable since the globular protein human serum albumin must have a better pore accessibility than an equally sized dextran tracer that consists of branched glucose chains.

**Table 5.1:** Total porosity $\epsilon_{t,HSA}$ and pore accessibility $\epsilon_{p,HSA}$ for human serum albumin on Fractogel EMD SE Hicap (M) at various concentrations of sodium chloride in the buffer.

<table>
<thead>
<tr>
<th>$c_m$ [M]</th>
<th>$\epsilon_{t,HSA}$</th>
<th>$\epsilon_{p,HSA}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.60</td>
<td>0.34</td>
</tr>
<tr>
<td>0.25</td>
<td>0.58</td>
<td>0.31</td>
</tr>
<tr>
<td>0.05</td>
<td>0.53</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Adsorption equilibria of the modifier.** The modifier isotherm was determined by frontal analysis. The modifier concentration was increased stepwise from 0 to 1 M sodium chloride and from 0 to 2 M sodium chloride in two different sets of experiments where no buffer was used. In addition, the study was repeated with 20 mM acetate buffer pH 5 with 0 to 1 M sodium chloride, in order to check the effects of buffering salts on the adsorption of sodium chloride. The breakthrough of each salt step was detected by online conductivity measurement. As shown in Figure 5.3, the obtained equilibrium isotherm is linear over the whole range of concentration both for buffered and non-buffered systems. The Henry coefficient, that is the slope of the linear isotherm, is $H = 0.25$ for all the buffering conditions considered in this work.
Figure 5.3: Isotherm of the modifier analyzed by frontal analysis. Data set measured with sodium chloride from 0 to 2 M (□), measured with sodium chloride from 0 to 1 M (●) and measured with 20 mM acetate buffer pH 5 and sodium chloride from 0 to 1 M (◇).
5.5.2 Characterization of the column with the general rate model

Parameters used in the general rate model have been regressed in multiple separate optimization procedures. First of all, experiments under nonadsorbing conditions were evaluated in order to find parameters that reproduce the column characteristics. In a second loop, parameters regarding the isotherm of human serum albumin on the stationary phase are regressed with the general rate model.

Porosities. Experimental ISEC data as described in Section 5.5.1 was used to fit the porosities of the bed and of human serum albumin of the column. As the proposed numerical model does not account for changes in the porosity due to changes of the modifier concentration, fixed values were assumed for the simulation, as listed in Table 5.2. The slight difference in the values of Table 5.1 and 5.2 originates from different calculation methods: the numbers in Table 5.1 were calculated by moment analysis according to Section 5.5.1 whereas data from Table 5.2 represents a fitting of the chromatograms of ISEC experiments with the numerical model.

Table 5.2: Porosity $\epsilon_i$ and pore accessibility $\epsilon_{p,i}$ for blue dextran and human serum albumin for Fractogel EMD SE Hicap (M). Measurements were carried out with 50 mM phosphate buffer pH 7 with 0.5 M sodium chloride.

<table>
<thead>
<tr>
<th>tracer</th>
<th>$\epsilon_i$</th>
<th>$\epsilon_{p,i}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>blue dextran</td>
<td>0.414</td>
<td>0.367</td>
</tr>
<tr>
<td>HSA</td>
<td>0.630</td>
<td>0.367</td>
</tr>
</tbody>
</table>

Characterization of the mass transport. Experimental data measured under non-adsorbing conditions was used to fit the mass transfer parameter with the numerical model, i.e. axial Peclet $Pe_{ax}$ and Peclet number $Pe$.

Axial Peclet was regressed to $Pe_{ax} = 582$ at $u = 0.10 \text{cm/s}$, meaning that convection is dominating over axial diffusion in this system. According to Equation (5.6), the axial diffusion coefficient is therefore calculated to $D_{ax} = 8.4 \cdot 10^{-4} \text{cm}^2/\text{s}$. This is in agreement with the literature correlation (Equation (5.28)) where $D_{ax} = 3.2 \cdot 10^{-4} \text{cm}^2/\text{s}$ is estimated.

The Peclet number $Pe$ is regressed to $Pe = 1.51$ at $u = 0.10 \text{cm/s}$. The characteristic
time of diffusion into the pores is therefore significantly longer than the characteristic
time for convection. That means that the mass transport into the pores is hindered,
because of the large size of the protein compared to the size of the pores. The effective
pore diffusivity is calculated from the Peclet number to \( D_{p,\text{eff}} = 4.9 \cdot 10^{-8} \text{cm}^2/\text{s} \)
(Equation (5.10)). This is in good agreement with literature data: Forrer et al. found
\( D_{p,\text{eff}} = 5.7 \cdot 10^{-8} \text{cm}^2/\text{s} \) for human serum albumin on Fractogel EMD SE Hicap (M),
Stone and Carta measured \( D_{p,\text{eff}} = 4.7 \cdot 10^{-8} \text{cm}^2/\text{s} \) for bovine serum albumin on SP
Sepharose FF and Boyer and Hsu got \( D_{p,\text{eff}} = 5.7 \cdot 10^{-8} \text{cm}^2/\text{s} \) for human serum albu-
min on Sepharose CL-6B [56, 75, 111].
Stanton number, that gives the ratio of the convective term versus the film mass transfer,
was calculated with literature correlations according to Equation (5.7). For a velocity
of \( u = 0.10 \text{cm/s} \), Stanton number is \( St = 86 \). As Stanton number is larger than \( St = 54 \)
for all studied velocities, the convective term is dominant over the film mass transfer at
least 50 times.

5.5.3 Regression of the isotherm for the first site

Characteristics of the obtained human serum albumin have been checked extensively in
the first part of this work [74]. As shown there, the used human serum albumin is of
high purity. The effects modeled in this publication are not related to any impurity of
the used human serum albumin or to a malfunctioning of the chromatographic column.
Because of the peculiar behavior of human serum albumin on Fractogel EMD SE Hi-
cap (M), it is not possible to treat the two binding sites separately. Gradient experiments
were run with 20 mM acetate buffer with a modifier gradient from 0 to 1 M NaCl in ap-
proximately 10 to 40 column volumes at a velocity of \( u = 0.1 \text{cm/s} \). These experiments
always lead to two elution peaks. In this section, only the first peak is regressed. As
shown in the previous work, the first site is close to equilibrium under the selected con-
ditions and can therefore be described with the Henry coefficient alone [74]. According
to Yamamoto, the Henry coefficient, which is determined by equilibrium conditions,
is only a function of the modifier concentration in ion exchange chromatography [14].
Therefore it is possible to regress the parameters \( \alpha_1 \) and \( \beta_1 \) (Equation (5.2)) from gra-
dient experiments. Experimental data and simulation are compared in Figure 5.3, where
an excellent agreement between experiment and regression of the first peak is shown.
Parameters of the regression were found to be \( \alpha_1 = 0.00181/M^{1.566} \) and \( \beta_1 = -5.66 \).
According to Figure 5.3, the numbers of \( \alpha \) and \( \beta \) for the modifier dependence are in
good agreement with other experimental data that is presented in the previous publica-
tion [74].
Figure 5.4: Regression of the parameters of the isotherm for site 1. The figure shows the experimental data (solid line) and the simulation (dashed line) for modifier gradients of 11 (top left), 21 (top right), 32 (bottom left) and 42 (bottom right) column volumes. The modifier gradient is shown as a dash-dotted line.
**Figure 5.5:** Henry coefficients for human serum albumin on fractogel EMD SE Hicap (M) for peak 1 (□) from isocratic experiments and from batch experiments (◆) according to Franke et al. [74]. The fitting of the general rate model of the Henry coefficients with gradient experiments is shown as a dashed line.
Figure 5.6: Elution time of first peak (left) and modifier concentration at elution of peak 1 (right) for the gradient experiments (□) and for the according simulation (dashed line). Parameters of the simulation were set according to Table 5.3.
5.5 Results and discussion

Figure 5.6 shows the elution time (left) and modifier concentration at peak maximum of peak 1. As already shown in Figure 5.4, there is a very good agreement between experiment and simulation. Furthermore, the experimental set with four different gradient lengths (as shown in Figure 5.4) was run at various flowrates ($u = 0.05 \text{ cm/s}$ and $u = 0.15 \text{ cm/s}$). The elution position of a peak, which is described by adsorption equilibrium, is constant when measured in nondimensional time. Therefore, the result of an experimental set run at a velocity of $u = 0.05 \text{ cm/s}$ or $u = 0.15 \text{ cm/s}$ exactly overlaps with the datapoints found for a velocity of $u = 0.1 \text{ cm/s}$, as shown in Figure 5.6. As the simulation treats the first site as an equilibrium adsorption site, also the simulation produces overlapping results at those flowrates. This agreement is a strong indication that the neglect of a kinetic ansatz for site 1 is well justified by experimental results.

5.5.4 Regression of the kinetic parameters and the parameters of the second isotherm

In order to fit the modifier dependence of the kinetic parameters ($\alpha_{k,ads}$, $\alpha_{k,des}$, $\beta_{k,ads}$, $\beta_{k,des}$), two set of experiments were considered: on the one hand, the experimental series with various gradient lengths, as described in the previous section. Furthermore, a second set of experiments was designed, in order to better evaluate the time frame of the kinetics. The so called 'step experiments' have a variable time to adsorb human serum albumin on the stationary phase at 0 M sodium chloride (from 2.5 to 120 column volumes). Then, the modifier concentration is elevated stepwise to 0.25 M sodium chloride which elutes only the first peak. The second peak is eluted with a second modifier step at 1 M sodium chloride. The step experiments were run at the same flowrate as the gradient experiments ($u = 0.1 \text{ cm/s}$).

Using parameters according to Table 5.3, both experimental sets can be described well as shown in Figure 5.7 and 5.8. In the step experiments, experiment and simulation match nicely for the second peak. However, peak 1 is eluting slightly to early in the simulation (Figure 5.7). This is due to a slight mismatch of the Henry coefficient of site 1 that was regressed with modifier gradients. Furthermore, the simulation shows a first peak that is too narrow. As the throughput matches, the first peak must be too high in the simulation.

The gradient experiments (Figure 5.8) show a good agreement between experiment and simulation. For shallow gradients, the size of the second peak is overestimated. Nevertheless, the broadness of the peak and the elution time of the peak maximum are matching.

Figure 5.9 depicts the dependence of the kinetic parameters and the Henry coefficients
Table 5.3: Set of fitted parameters for the simulations with the modified general rate model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\epsilon_b$</td>
<td>[-]</td>
<td>0.41</td>
</tr>
<tr>
<td>$\epsilon_p$</td>
<td>[-]</td>
<td>0.37</td>
</tr>
<tr>
<td>$P_{e_{ex}}(u = 0.10 \text{cm/s})$</td>
<td>[-]</td>
<td>581.66</td>
</tr>
<tr>
<td>$P_e(u = 0.10 \text{cm/s})$</td>
<td>[-]</td>
<td>1.51</td>
</tr>
<tr>
<td>$St(u = 0.10 \text{cm/s})$</td>
<td>[-]</td>
<td>86.34</td>
</tr>
<tr>
<td>$H_m$</td>
<td>[-]</td>
<td>0.25</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>$[1/\text{M}^{(-5.66)}]$</td>
<td>$1.79 \times 10^{-3}$</td>
</tr>
<tr>
<td>$\beta_1$</td>
<td>[-]</td>
<td>-5.66</td>
</tr>
<tr>
<td>$\alpha_{k,\text{ads}}$</td>
<td>$[1/\text{M}^{1.63}]$</td>
<td>1.18</td>
</tr>
<tr>
<td>$\beta_{k,\text{ads}}$</td>
<td>[-]</td>
<td>1.63</td>
</tr>
<tr>
<td>$\alpha_{k,\text{des}}$</td>
<td>$[1/\text{M}^{7.02}]$</td>
<td>23.93</td>
</tr>
<tr>
<td>$\beta_{k,\text{des}}$</td>
<td>[-]</td>
<td>7.02</td>
</tr>
</tbody>
</table>

Figure 5.7: Regression with parameters according to Table 5.3. The figure shows the experimental data (solid line) and the simulation (dashed line) for step experiments with 2.7 (top left), 32 (top right), 64 (bottom left) and 127 (bottom right) column volumes of initial adsorption time. The modifier step is shown as a dash-dotted line.
Figure 5.8: Regression with parameters according to Table 5.3. The figure shows the experimental data (solid line) and the simulation (dashed line) for modifier gradients of 11 (top left), 21 (top right), 32 (bottom left) and 42 (bottom right) column volumes. The modifier gradient is shown as a dash-dotted line.
from the modifier concentration. Note, that the kinetics of desorption is drastically increasing with increasing modifier concentration. This explains the sharp second peak in the gradient elution. Also the Henry coefficient of the second site is larger than the Henry coefficient of the first site under binding conditions (up to 0.6 M NaCl). This result is consistent with the experimental data that shows that human serum albumin, which is bond on the second site, is eluting always last.

Because the mass transport parameters are not lumped in the general rate model, it is possible to predict the behavior of the column at various flowrates. Therefore the apparent kinetic parameters $\tilde{k}_{\text{ads,2}}$ and $\tilde{k}_{\text{des,2}}$ must be adjusted according to the change in velocity. This is shown in Figure 5.10 and 5.11. Note that for fast flow rates, also the size of the second peak is predicted more accurately.

In analogy to the analysis of the first peak in Figure 5.6 the elution pattern of the of the second peak is analyzed for various flow rates. As shown in Figure 5.12, the elution time is predicted accurately also for the second peak at all flow rates. However, it was not possible to simulate the modifier concentration at elution of the second peak as precise as for peak 1. While the simulation gives accurate modifier concentrations for the elution of shallow gradients, the result differs significantly for fast gradients. This might be due to the simple first order kinetic ansatz that is not describing the system.
Figure 5.10: Prediction of the gradient experiments at a flow rate of $u = 0.05 \text{cm/s}$. The figure shows the experimental data (solid line) and the simulation (dashed line) for modifier gradients of 11 (top left), 21 (top right), 32 (bottom left) and 42 (bottom right) column volumes. The simulation was done with the set of parameters as described in Table 5.3, adjusted for the velocity change according to Equation (5.17). The modifier gradient is shown as a dash-dotted line.
Figure 5.11: Prediction of the gradient experiments at a flow rate of $u = 0.15 \text{cm/s}$. The figure shows the experimental data (solid line) and the simulation (dashed line) for modifier gradients of 11 (top left), 21 (top right), 32 (bottom left) and 42 (bottom right) column volumes. The simulation was done with the set of parameters as described in Table 5.3 adjusted for the velocity change according to Equation (5.17). The modifier gradient is shown as a dash-dotted line.
**Figure 5.12:** Elution time of second peak (left) and modifier concentration at elution of peak 2 (right) for the gradient experiments (points) and for the according simulation (lines).

accurate enough. However, this very simple kinetic approach has the ability to describe all experimentally observed effects, especially the increase of the second peak due to time and the movement of the peak maximum of the second peak due to changes in gradient length and flow rate.

Furthermore, a series of experiments was conducted, where the initial adsorption time was varied, while the flow rate was kept constant at \( u = 0.1 \text{cm/s} \). The elution was accomplished with a modifier gradient in 11 column volumes. As shown in Figure 5.13, it is possible to predict the behavior of the column with the same set of parameters, that was presented in Table 5.3. Especially the size of peak 1 and peak 2 is predicted accurately.

### 5.6 Conclusion

It was possible to describe the behavior of human serum albumin with a general rate model with two binding conformations. It was found that the second site has an adsorption as well as a desorption kinetic, whereas both are functions of the modifier concentration in the system. Considering the simple first order kinetics, which intro-
Figure 5.13: Prediction of gradient experiments (modifier gradient in 11 column volumes) with varying initial adsorption times at a flow rate of $u = 0.1 \text{cm/s}$ (Parameters according to Table 5.3). The figure shows the experimental data (solid line) and the simulation (dashed line) for gradient experiments with 2.7 (top left), 32 (top right), 64 (bottom left) and 127 (bottom right) column volumes of initial adsorption time. The modifier step is shown as a dash-dotted line.
duces only four additional parameters to the classical one component general rate model, the model describes a variety of experiments in an accurate way. Future analysis should try to find a more precise description of the isotherms and the kinetic. Furthermore, the system should also be modeled at overloaded conditions that have already been described experimentally in the previous work.
6 Conclusion and outlook

In this thesis various studies were made in order to achieve a better understanding of a number of phenomena occurring in protein chromatography. In Chapter 2 a set of custom made cation exchange materials was analyzed regarding structure and performance parameters. This data was evaluated in detail in order to propose a new material by design that has superior properties. As shown experimentally and with simulations with a general rate model, the new material has indeed superior properties. In the future, analogous studies should be carried out with other types of stationary phases such as anion exchange, hydrophobic interaction, mixed mode and Protein A. The diffusion hindrances, which are discussed in the current study, might be overcome by the application of monoliths in biochromatography [112].

In Chapter 3 a new commercially available stationary phase was tested for the polishing step of a monoclonal antibody from cell culture supernatant. From the investigation of the dependence of the Henry coefficient as a function of salt concentration and pH of the buffer, it was proven that Capto adhere with its multiple functionalities has a great potential for the application in a two step purification of a monoclonal antibody. The proposed downstreaming scheme is a true alternative to the common process that uses Protein A affinity chromatography. Future work should focus further on the investigation of the mechanisms in mixed mode chromatography. Furthermore, a numerical model must be developed that describes the observed phenomena with special attention to the interplay of anion exchange and hydrophobic interactions of the resin.

In Chapter 4 of this thesis, the elution of human serum albumin was studied on a strong cation exchange resin. This behavior is unique, because the pure protein human serum albumin elutes with two peaks in the modifier gradient. This effect was studied in detail experimentally. It was revealed that the elution pattern is driven by a kinetic transition. In Chapter 5, the studied behavior of human serum albumin was modeled numerically with a modified general rate model. The two peak elution pattern was reflected in the simulation with only few additional and well justified parameters.
This study is an important contribution in order to better understand the mechanisms in protein chromatography. Large, charged molecules such as antibodies do not necessarily elute as single peaks, even if they are available in pure state. Therefore, efforts are taken to describe the behavior of antibodies on stationary phases with a model that calculates electrostatic interactions during binding [113]. As a matter of fact, also the authorities have pointed out the importance of process understanding and modeling for a safe and reliable production (bio)-pharmaceuticals [114].
Bibliography


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Curriculum vitae

06/2009  PhD at the Institute of Chemical and Bioengineering
         ETH Zurich, Switzerland

05/2005  Graduation in process engineering (Verfahrenstechnik)
         Universität Stuttgart, Germany
         Degree: Diplom-Ingenieur

06/1999  Graduation from High School (Abitur)
         Friedrich-Schiller-Gymnasium, Fellbach, Germany

07/01/1980  Born in Reutlingen, Germany
Publications


Conference proceedings


