

Review

# Multimodal chromatography: An efficient tool in downstream processing of proteins

*Kristian Kallberg, Hans-Olof Johansson and Leif Bulow*

Pure and Applied Biochemistry, Lund University, Lund, Sweden

Chromatography has become an indispensable tool for the purification of proteins. Since the regulatory demands on protein purity are expected to become stricter, the need for generating improved resins for chromatographic separations has increased. More advanced scientific investigations of protein structure/function relationships, in particular, have also been a driving force for generating more sophisticated chromatographic materials for protein separations. As a consequence, the development of alternative chromatographic strategies has been very rapid during the last decade and several new ligands have been designed and explored both in the laboratory and in large-scale industrial settings. This review describes some of these efforts using multimodal chromatography, where two or more physicochemical properties are used to enhance the specificity of the interactions between the protein and the ligand on the chromatographic matrix. In addition to experimental studies, computer modeling of ligand-protein binding has improved the design of ligands for protein recognition. The use of descriptors as well as in silico docking methods have been implemented to design multimodal resins in several instances.

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## 1 Introduction

Most, if not all, proteins examined in the laboratory or industrially produced on a large scale need to be purified from host cell proteins (HCPs) and other contaminating agents to allow for their further use or exploitation. The vast majority of today's proteins that require a high purity are isolated by a series of liquid chromatography steps because these procedures are often gentle, reproducible, and can be tailored for automation. Because the requirements for high-quality protein products is expected to be more stringent, more specialized chromatography media will need to be developed for specific uses, such as the

isolation of well-defined glycosylated proteins or the removal of final traces of HCPs, misfolded variants, point mutations, or affinity tags.

The isolation of a protein of interest is normally composed of an extended chromatographic process that can frequently be divided into three steps: capture, purification, and polishing. Each step consists of one or more different chromatography methods and is designed with different goals in mind. In the capture step, the main aim is simply to collect and concentrate most of the protein material on the matrix. It is possible to design this step to include buffer changes, virus inactivation [1], and it may even be possible to combine it with final inactivation of pathogenic bacteria by using hydrophobic ligands [2, 3]. The resin here is often prepared for maximal binding capacity and flow rate. In the purification step, the majority of contaminating proteins is removed and a more pure feedstock is generated for the last step. Finally, in the polishing step, the priority is high resolution to remove impurities that closely resemble the protein of interest, such as misfolded proteins or aggregates [4].

**Correspondence:** Kristian Kallberg, Pure and Applied Biochemistry, Lund University, PO Box 124, 221 00 Lund, Sweden  
**Email:** Kristian.Kallberg@tbiokem.lth.se

**Abbreviations:** HCP, host cell protein; HIC, hydrophobic interaction chromatography; IEX, ion exchange chromatography; IMAC, immobilized metal ion affinity chromatography; MMC, multimodal chromatography; SMA, steric mass action

## 2 Ligand diversity in chromatography

### 2.1 Single-mode chromatography

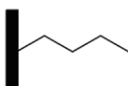
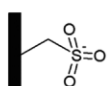
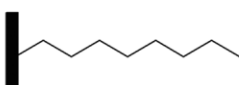
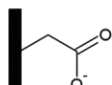
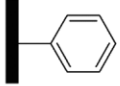
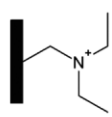
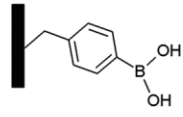

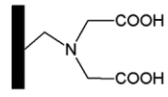
The conventional method of exploring chromatography is based on separating proteins according to a single physical property, such as charge (ion-exchange chromatography, IEX), hydrophobicity (hydrophobic interaction chromatography, HIC), size (size exclusion chromatography, SEC), specific interactions (affinity chromatography, AC), or metal-chelating groups (immobilized metal ion affinity chromatography, IMAC). These methods can therefore be referred to as being single mode. Figure 1 shows a selection of some frequently used and commercially available chromatography ligands. These commonly used resins have been described thoroughly in many excellent reviews and books [5, 6] and are not treated in depth herein. However, it is important to realize that each method has its own distinctive advantages and drawbacks. The strategy is often to combine these methods in a series of steps to overcome the drawbacks.

### 2.2 Multimodal chromatography

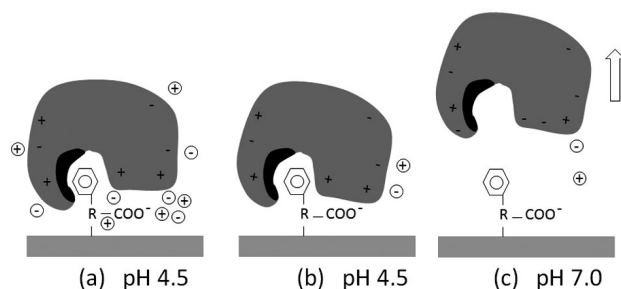
More elegant alternatives to a series of single-interaction steps are multimodal or mixed-mode chromatography

media (MMC) that exhibit multiple binding interactions. For instance, ion and hydrophobic interactions can occur at the same time, which frequently may increase the selectivity and specificity (Fig. 2). MMC has been used for a variety of applications, including separation of phosphorylated and sialylated proteins [7], oligosaccharides [8], antibody purification [9, 10], and separation of nucleotides. It is also possible to use these media as “single-mode” chromatography resins by choosing conditions that minimize or modify one or more of the other normally occurring interactions [11, 12]. This possibility renders these MMC media very flexible and potentially very useful for any separation of biomolecules.

The concept of MMC was first introduced as early as in the 1950s with the use of the mineral hydroxyapatite [13]. However, particularly in the last decade, there has been rapidly increasing interest in developing and using such media. By exploring powerful screening methods, often linked with rational design, it is possible to design MMC media for specific purposes, such as high salt concentrations [14]; pH-tunable hydrophobicity [15, 16]; or capture of specific proteins, for example, IgG from feedstocks [17]. In 1998, Burton and Harding [18] identified and prepared a variety of pyridyl and imidazolyl compounds that were useful as ligands for hydrophobic charge induction chromatography, in which the media

Hydrophobic ligands	Cation-exchanging ligands
 <p>Butyl</p>	 <p>Methylsulfonate</p>
 <p>Octyl</p>	 <p>Carboxymethyl</p>
 <p>Phenyl</p>	
Anion-exchanging ligands	Affinity ligands
 <p>Diethylaminoethane (DEAE)</p>	 <p>Boronate affinity</p>
 <p>Quaternary amine</p>	 <p>IMAC</p>

**Figure 1.** The most commonly used traditional chromatographic ligands. Several different ligands have been developed and successfully explored over the years. Some of the more commonly used ligands for single-mode chromatography are shown.



**Figure 2.** Multimodal chromatographic principle. The binding and elution strategy of a target protein to a chromatographic medium containing a ligand with both hydrophobic and charged groups (adapted from [52]). (A) At pH 4.5 a target protein in a mobile phase with a high salt concentration is bound to the hydrophobic part of the ligand. (B) Upon decreasing the salt concentration, but still maintaining a low pH, the electrostatic part of binding becomes the dominating force. (C) With a pH step increase to 7.0, the protein will have the same charge as the ligand and be repelled by it and is therefore eluted.

was designed to match the  $pK_a$  values of target proteins and protein elutions could be controlled by a pH change. Additionally, Johansson et al. [14] observed that the most useful multimodal cation exchangers were composed of aromatic carboxylic acids with an amide group on the  $\alpha$ -carbon. Media based on these ligands showed breakthrough capacities that were up to 30 times higher than traditional ion exchangers at elevated salt concentrations. Since then, many new ligands have emerged and Fig. 3 shows a list of some commonly used mixed-mode ligands. Zhao et al. [19] have prepared an excellent review on the characteristic principles of multimodal chromatography ligands.

### 3 Examples of using multimodal chromatography

#### 3.1 IgG purification and multimodal chromatography

A very specific affinity system adapted from the strong molecular recognition found in nature is *Streptococcus* protein A affinity chromatography. Since its discovery, this protein ligand has been modified and adapted for better robustness by genetic engineering [20] and a number of protein A mimics have been generated [21–24]. Protein A can be used as a capture step for efficient and selective purification of IgG and is arguably the industry standard today. The almost unlimited access to highly purified IgGs can no doubt be seen as the single largest advance in therapeutic antibody production to date, but it is not without its drawbacks. The resins used are expensive and therefore need to be re-used for multiple purification cycles. This may in turn lead to ligand leakage and degraded capacities, even though protein A is fairly

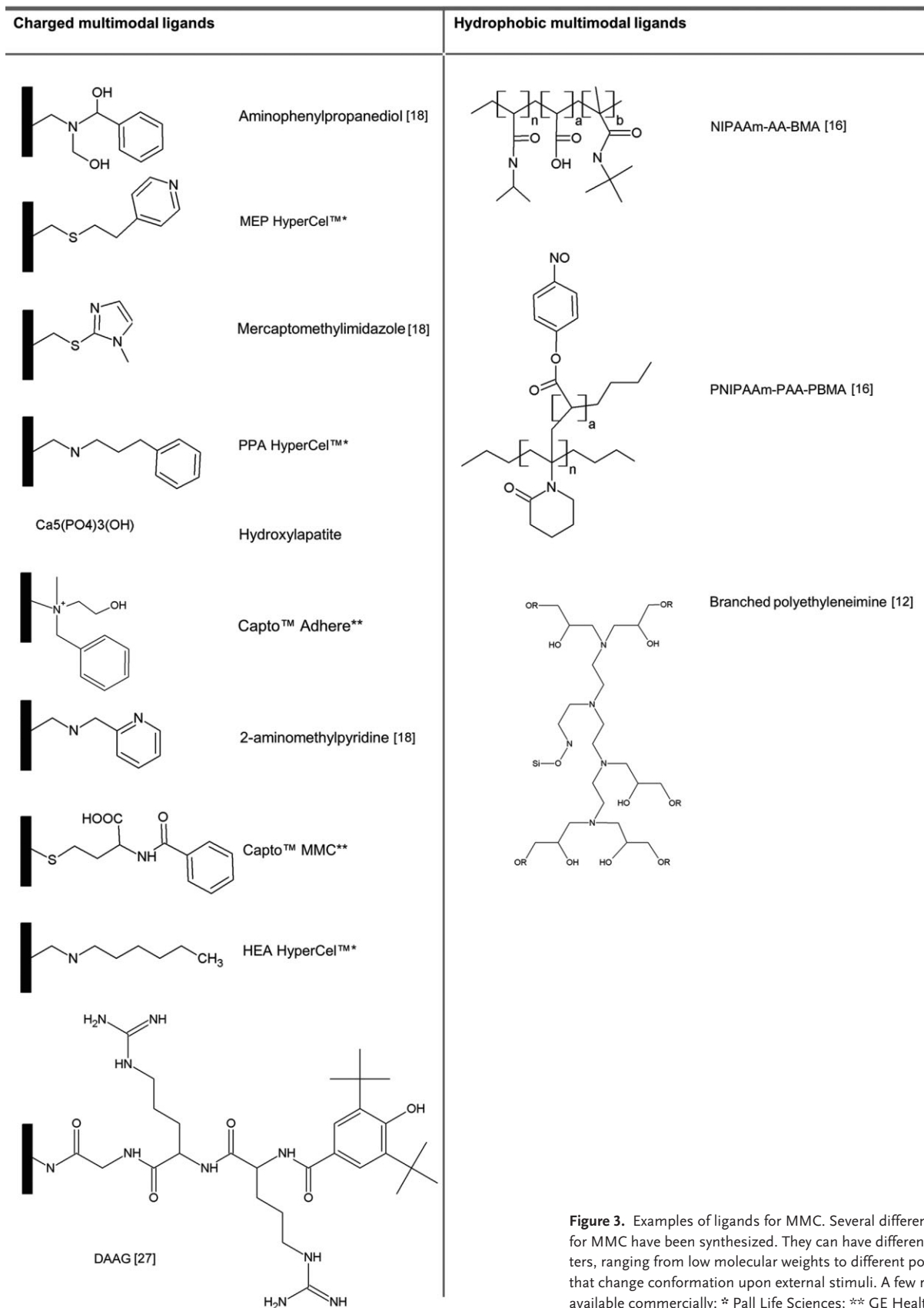
robust [25]. Multimodal media can be a solution to both of these problems, for instance, antibody-selective MEP Hypercel™ media provides similar binding capacities [26] at a fraction of the cost with no ligand contamination. Recently, Lund et al. [27] synthesized a new ligand with multimodal characteristics that provided even higher dynamic binding capacities of up to 48 mg/mL. Another improvement MMC can offer concerns the low pH required for elution from a protein A column, which may cause IgG aggregation [28]. By employing a high-throughput design in their experimental approach, Touille et al. [29] achieved elution at considerably less acidic pH, which improved both elution yield and removal of aggregates, while still keeping contaminating HCPs to a minimum.

In most IgG production processes the protein A step is followed by an MMC step to remove possible IgG aggregates or remaining DNA. However, the most widespread use for these multimodal media has been HCP removal in downstream processing. Here they can serve as a very effective polishing step after protein A chromatography, replacing a series of conventionally used steps [30–32].

#### 3.2 Detection of post-translational modifications

Glycosylated biomolecules, especially glycoproteins, play important roles in several physiological and pathological processes and the majority of all eukaryotic proteins are glycosylated [33, 34]. Glycosylation is thus often an essential post-translational step for the generation of a functional and stable protein [35, 36]. Because of chemical stability and efficient analyte binding, affinity ligands based on boronic acid derivatives have often been used to offer efficient separation of carbohydrates, nucleosides, glycolipids, RNA, and glycoproteins. Various boronic acid ligands were investigated [37, 38]. The common feature of these ligands is that they contain one boronic acid moiety able to bind *cis*-diols, and one functional group (e.g. amino, thiol, or a polymerizable vinyl group) used for immobilization to a solid support. Since an aliphatic arm is often used in these ligands to generate a spacer between the support material and the boronic acid, hydrophobic interactions are often involved, as well giving the final resin MMC character [39, 40]. The chemical reactions used for immobilization often require harsh conditions (non-aqueous solvent, high temperature, etc.), or alternatively, show low selectivity in the coupling reactions. They are therefore often difficult to utilize in the presence of proteins and other biological molecules. Sukrichavalit et al. [37] therefore developed a new type of ligand based on the recently established “click chemistry” methodology. This results in more efficient and truly bio-orthogonal coupling reactions.

Besides developing boronate ligands, it is essential to develop more complex MMC ligands able to recognize several physical properties of the protein on the same



**Figure 3.** Examples of ligands for MMC. Several different ligands for MMC have been synthesized. They can have different characters, ranging from low molecular weights to different polymers that change conformation upon external stimuli. A few resins are available commercially: \* Pall Life Sciences; \*\* GE Healthcare.

time. For instance, the ligand NIPAAm-AA-BMA (Fig. 3) can be composed of three different functional groups, *N*-isopropylacrylamide (N), acrylic acid (A), and *tert*-butylmethylacrylamide (B). The functional group provides temperature-driven self-association and its pH sensitivity can be utilized. The inclusion of the acrylic acid monomer in the ligand thus results in a polymer that can change its hydrophobicity over the pH range of 4–8.

This range of pH sensitivity linked to the ligand makes it ideal for fine-tuning its intrinsic physical properties over a range that also is very useful for most protein separations. The ligand can thereby be utilized as both a hydrophobic and an ion-exchange ligand by controlling the pH during chromatography. Becker et al [41] have used this material to identify very small structural variations on a protein. Small differences in glycosylation could thus easily be detected. Several different proteins have been examined, including monoclonal antibodies with different glycans and ribonucleases [15]. The material has also been used to evaluate the hydrophobicity of single-point mutated proteins [42]. This type of chromatography can be utilized both as a first efficient purification step of a crude cell extract and as a fine-polishing step after, for instance, an ion exchanger. The authors therefore feel that this type of polymer should be further developed for protein chromatography due to excellent selectivity. Such simple polymers, in single- or multimode fashion, can also be very powerful for other separation methodologies. For instance, proteins, DNA, and RNA can be efficiently partitioned in aqueous two-phase systems using these materials [43, 44].

#### 4 Affinity tags based on multimodal recognition

Besides developing new and optimized chromatographic resins, it is also possible to modify proteins themselves to facilitate their purification. A generally applicable and frequent strategy is to add a peptide fragment to the target protein on the DNA level. Such affinity tagging has become very common, especially for small-scale protein isolation, for a review, see [45]. The design and use of these tags bear strong resemblance to the developments in single- and multimodal chromatography. Several alternative tag systems are available, but the most common are peptides appropriate for single-mode interactions, such as hexahistidine-containing tags [46, 47]. In this case, IMAC makes it possible to design a one-step purification scheme using a resin loaded with nickel or zinc ions, for example. Many other tags developed provide additional properties to the protein that may change its retention behavior to better fit the separation profile of the process in question. Such tags could thus involve addition of a single physical property, such as charge or hydrophobicity, to increase retention of the target protein. In anal-

ogy with MMC, it is feasible to design a small and highly expressed tag that offers multimodal functionality. By constructing a single ligand, often in the form of a hexapeptide, which harbors histidine, tyrosine, and aspartic acid residues, the physicochemical behavior of these residues can be explored to purify the protein using IMAC, HIC, and IEX, respectively [48, 49]. It is thereby feasible to prepare a relatively small and readily expressed tag that offers multimodal capabilities in relation to several different physicochemical properties. To date, this was normally achieved by attaching multiple tags to the target protein. Protein tags may influence protein folding and other protein properties. Interestingly, such tagging may also enhance expression levels that favorably affect final yields in a protein production process [50].

### 5 Modeling of multimodal chromatography for proteins

With the increasing importance of MMC, the development of adequate modeling of the separation behavior has become necessary for generating a better understanding and improving its performance [51]. In MMC, the most frequent combination is based on ion-exchange and hydrophobic interactions, which implies that it is obvious to try to combine the mathematical driving forces for these techniques in the first step [52, 53]. Depending on the ligand hydrophobicity, some resins will act more as ion exchangers rather than as HIC media [54]. With easy access to large databases on protein and ligand structures [19], together with free or commercially available software packages, it is now possible to perform *in silico* studies of ligand-protein interactions [55]. This is often performed early in the screening phase to select the appropriate ligand for separation. The ligand in MMC normally carries a hydrophobic and electrostatic property, but alternatively, two different ligands, one hydrophobic and the other a charged ligand, which are equally distributed on the underlying matrix, can be used. In the latter case the matrix is sometimes called multimodal stochastic media [14]. The adsorption of proteins in the MMC media depends on the salt concentration in a more complex way than in IEC or HIC [18]. To elute the protein it is often necessary to change the pH of the media and cause electrostatic repulsion between the protein and ligand, as depicted in Fig. 2.

#### 5.1 Basic chromatographic modeling

The fundamental equations for MMC are based on the basic equations for adsorption to a ligand matrix, as outlined by Chase [56]. Component A, for example, a protein, binds to ligand B with rate constant  $k_1$  to form complex AB, which can disintegrate into separate A and B again with the rate constant  $k_{-1}$  (Eq. 1).



The overall process can also be represented by the equilibrium constant  $K_{eq}$ . By preparing rate mass balances at equilibrium,  $q_{eq}$ , it is possible to derive the Langmuir isotherm for adsorption of the target molecule with concentration  $c_{eq}$  in the mobile phase (Eq. 2):

$$\frac{dq_{eq}}{dt} = k_1 * c_{eq} (q_{max} - q_{eq}) - k_{-1} q_{eq} = 0 \quad (2)$$

$$q_{eq} = \frac{K_{eq} * c_{eq} q_{max}}{(1 + K_{eq} * c_{eq})} \quad (3)$$

The partition coefficient ( $D$ ) is the ratio of the concentrations of the adsorbed and non-adsorbed protein and its definition is often restricted for the linear region of Eq. (3), that is, at low concentrations of the target molecule (Eq. 4).

$$D = \frac{q_{eq}}{c_{eq}} \quad (4)$$

The retention factor,  $k'$ , is related to the mass ratio of protein in the mobile and solid phases [57, 58]; thus giving Eq. (5):

$$k' = \frac{V_R - V_0}{V_0} = \frac{q_{eq}}{c_{eq}} \phi \quad (5)$$

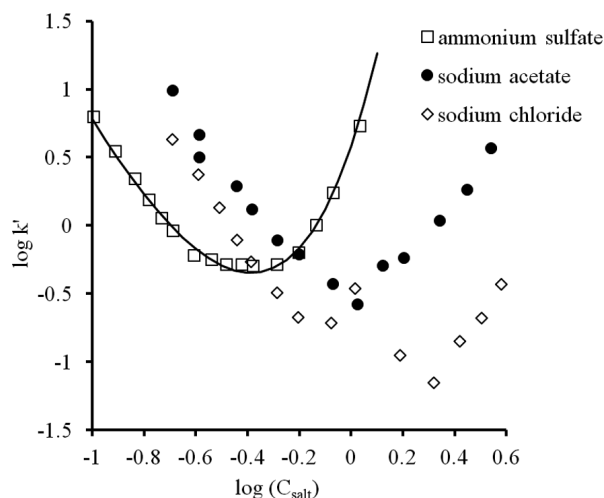
in which  $V_R$  is the volume needed to elute the protein,  $V_0$  is the column void volume, and  $\phi$  is the phase ratio of the adsorbed and mobile phases. Equations (1)–(5) give the relationship between the thermodynamics of adsorption and the chromatographic performance measured in terms of retention volumes. By combining the basic isothermal relationship with kinetic theory of pore and plug flow diffusion, it is thus possible to simulate the elution profile [57, 59–61].

## 5.2 Condensed parameterized modeling of chromatographic retention

The retention factor is, in the case of both IEC and HIC, dependent on the salt concentration of the mobile phase. It has been shown by Melander et al. [58] that the retention in a MMC system is given by Eq. (6):

$$\log(k') = A - B * \log(c_{salt}) + C * c_{salt} \quad (6)$$

in which the parameters  $A$ ,  $B$ , and  $C$  encapsulate properties of the specific system, including electrostatics and hydrophobicity. Equation (6) describes the typical U shape of MMC [12, 52, 58]. At high salt concentration, the proteins are attached to the ligand by hydrophobic forces and by electrostatic forces at low ionic strength. Experimental data, which fits well to Eq. (6), is presented in Fig. 4.



**Figure 4.** Retention factor as a function of salt molarity in MMC. The retention of alpha-chymotrypsinogen A in a weak cation exchanger under three different salt conditions is shown. The solid line is a parameterized curve of Eq. (6), in which parameters  $A$ ,  $B$ , and  $C$  are  $-3.58$ ,  $3.94$ , and  $4.14$ , respectively. Data taken from Melander et al. [58].

For IEC systems the validity of Eq. (6) or similar expressions has been demonstrated in various systems [57, 58, 62–64]. For HIC systems, there are many cases where no simple linear relationship between  $\log(k')$  and salt concentration in the mobile phase can be observed [65, 66]. The linear relationship of  $\log(k')$  is only reasonably valid at high salt concentrations [52, 67]. To understand the complex interplay of system variables governing the separation process, much effort has been focused on the thermodynamics behind the parameters in Eq. (6). In the steric mass action (SMA) theory, the adsorption in IEC is described in terms of stoichiometric exchanges of charges upon binding. The ligand density and steric effects of the protein are accounted for in the SMA formalism [68, 69]. In HIC systems, a similar formalism can be applied. It has been recognized that adsorption to the hydrophobic ligand implies local changes in interactions between the protein surface, ligand, and solvent (including ions); so-called preferential interactions [70]. The role of water in the HIC adsorption process has also been modeled by several groups [53, 67, 71]. For instance, Chen et al. [67] found through modeling that the number of water molecules released was larger in the presence of ammonium sulfate than that with sodium chloride. Using the above theories, Nfor et al. [53] succeeded in modeling MMC and reproducing the typical U-shaped retention behavior mentioned above with a minimum binding capacity at a critical salt concentration. In this study, the mixed mode interactions given in Eq. (7) were considered:



In Eq. (7), protein P interacts both with an ionic ligand (SL) and a hydrophobic ligand (L) to form the protein-ligand complex PL. In the process  $\nu$ S ions are released from the ionic ligand.

The thermodynamic equilibrium constant for the reaction in Eq. (7) can now be written as Eq. (8):

$$K_{eq} = \exp\left(-\frac{\Delta G_p^\circ}{RT} + \nu_i \frac{\Delta G_s^\circ}{RT}\right) \equiv \left(\frac{q_p}{c_p}\right) \left(\frac{c_s}{q_s}\right)^\nu \left(\frac{c}{c_L}\right)^n \left(\frac{1}{\gamma_p}\right) \quad (8)$$

in which  $q_p$  and  $q_s$  are the protein and salt concentrations, respectively, at the adsorbed layer, and  $c_p$  and  $c_s$  are the bulk solution protein and salt concentrations, respectively. The concentration of the free ligand is  $c_L$  and  $c$  is the solution molarity in the pore site. The last equality is obtained by setting all activity coefficients of all components equal to one except for the protein.

In the modeling of Nfor et al., IEX conditions for electroneutrality in terms of IEX ligand density is used in Eq. (9), according to Mollerup [63]:

$$\Lambda_{IEX} = z_s q_s + (z_p + \sigma) q_p \quad (9)$$

in which  $z_s$  is the salt counterion charge and  $\sigma$  is the charge for the sterically shielded ligands on the chromatographic resin. Furthermore, a HIC ligand density term expressing the conservation of the hydrophobic groups on the surface is used in Eq. (10) [53]:

$$\Lambda_{HIC} = c_L + (n + \delta) q_p \quad (10)$$

in which  $n$  is the number of hydrophobic binding sites and  $\delta$  is the number of (sterically) inaccessible hydrophobic ligands. The adsorption thermodynamics of the MMC can now be expressed (Eq. 11) in terms of electrostatic and hydrophobic ligand densities of the chromatographic material by combining Eqs. (9) and (10) into Eq. (8):

$$K_{eq} = \left(\frac{q_p}{c_p}\right) \left(\frac{z_s c_s}{\Lambda_{IEX} - (z_p + \sigma) q_p}\right)^\nu \left(\frac{c}{\Lambda_{HIC} - (n + \delta) q_p}\right)^n \left(\frac{1}{\gamma_p}\right) \quad (11)$$

Nfor et al. [53] derived a relationship connecting the critical salt concentration ( $c_{s,min}$ ) at which the protein adsorption reaches the minimum value (Eq. 12):

$$c_{s,min} = \frac{\nu}{K_s} \quad (12)$$

$K_s$  is a protein-salt interaction parameter and  $\nu$  is the number of ions released from the ionic ligand upon protein binding, also called the characteristic charge [72]. The  $K_s$  parameter is dependent on the three components: (1) aqueous solvent; (2) protein; and (3) salt in terms of pairwise parameters (Eq. 13).

$$K_s = \frac{2}{RT} (a_{12} - a_{32}) \quad (13)$$

in which  $a_{12}$  and  $a_{32}$  are the water-protein and salt-protein interaction parameters, respectively.

### 5.3 Protein properties based on modeling of descriptors

To disentangle the many factors influencing the adsorption process, more detailed and mechanistic approaches have been explored. Herein some recent work on descriptor analysis for IEX and HIC is reviewed. The importance of using descriptor analysis lies in providing more accurate modeling of protein adsorption in dual-mode chromatographic matrices. The properties of the proteins themselves, such as QSPR (quantitative structure-property retention relationship) [41, 73] or QSRR (quantitative structure-retention relationship) [67], are of primary interest in recent modeling. In QSPR modeling, protein physicochemical properties are used as descriptors of chromatographic performance, usually a retention factor. In one study on IEC, no less than 58 different descriptors, ranging from protein size and shape factors to protein electrostatic properties, such as average electrostatic potential and clustering of charged amino acid side chains [73], were used. The data were analyzed with principal component analysis (PCA), which could sort out the variables that had the strongest influence on retention. It was observed that size and average electrostatic potentials were the most important factors for most proteins. A detailed disentanglement of the variables in the SMA formalism was performed by Ladiwala et al. [72]. The steric factor was strongly influenced by the number of aromatic atoms. Similar QSRR studies were performed on HIC by Chen et al. [67] and Ladiwala et al. [69]. They found that most important descriptors for HIC were size, shape, and hydrophobic surface factors. The accessible hydrophobic surface is a key factor in all HIC studies. However, equally sized proteins with similar hydrophobic areas may display different retention values [74]; this observation was explained by considering the shape of the hydrophobic region and its role on effective ligand binding. Thus, concave pockets gave lower retention values than convex regions with the same hydrophobic surface area. The effect of changes on the protein entropy and aggregation were addressed by To and Lenhoff [60, 65, 66] and McCue et al. [59] respectively. It was confirmed that protein size and ligand density were strongly correlated to retention times.

### 5.4 Ligand-protein modeling

The ligand structure is essential in the development of new and improved MMC media. The principles for ligand design were reviewed by Zhao et al. [19]. Much effort has been devoted to elucidating protein-ligand interactions

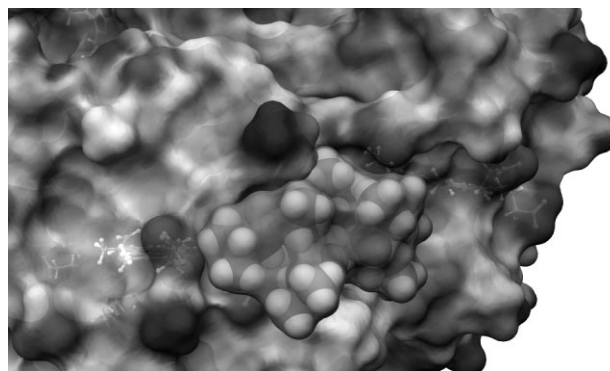
by using a combination of tools: chromatographic retention of site-directed mutated model proteins, NMR spectroscopic studies of free ligands binding to protein, and molecular dynamics (MD) [75, 76]. In these works the protein ubiquitin and its interaction with the analogue Capto MMC ligand (*N*-benzoyl-DL-methionine) was mapped for the wild-type (WT) protein and a mutant. The ligand interacts both electrostatically and hydrophobically and its binding values at different parts of the protein can be mapped with NMR spectroscopy studies. Coherent results from all these techniques were obtained and binding hotspots of the protein were mapped, leading to the conclusion that there was a strong synergy in electrostatic and hydrophobic dual-mode attachment. Data from Holstein et al. [76] supported theories that the long-range electric potential of the protein functions as a guide to the binding site field for the ligand [77, 78].

One fundamental purpose of multimode separation is to attain high specificity and minimize the contamination of HCP. This has been extensively studied and modeled by Pezzini et al. [54], who studied the separation of antibodies from HCP and the influence of the salt concentration (conductivity), pH, and ligand type on the separation efficiency. With strongly hydrophobic ligands, such as phenylpropylamine, acidic and highly hydrophobic HCP could bind more strongly than antibodies and minimal levels of contaminants were detected upon elution at low pH and low conductivity. However, the operational window was small and somewhat compromised the robustness of the system. In the case of phenylpropylamine, there is a significant distance between the charged nitrogen and the main aryl group, which is the hydrophobic part of the ligand, and explains the strong forces for this media (PPA HyperCel™). The example above illustrates the importance of understanding the general underlying effects of ligand docking with the target protein. A large study of both anionic and cationic multimode ligands was performed by Johansson et al. [14, 79], in which the 10% breakthrough capacity,  $Q_b$ , was measured for a large library of ligands. The model proteins were bovine serum albumin, lysozyme, and IgG and the data obtained were evaluated by the aforementioned PCA method. In this multivariate analysis, the ligand data is projected onto score plots, showing what properties of the ligands are similar or correlated and which result in high  $Q_b$  values. It was found that ligands with primary and secondary amines performed better (higher  $Q_b$ ) than tertiary or quaternary amines. One explanation is the higher probability of hydrogen bonding in systems with primary or secondary amines. The hydrophobicity of the ligand must be carefully balanced to facilitate elution and recovery at different salt concentrations. For the anionic MMC ligands, it was found that aromatic ligands resulted in significant lower recoveries than aliphatic ligands, probably due to too strong hydrophobicity. A more complex picture was obtained from MMC cationic exchangers. Non-aromatic

ligands could give high capacity media only if the ligand contained hydrogen-acceptor groups close to the carboxylic groups.

## 6 Concluding remarks

With the combined versatility available today through all the above chromatography options, there is a good possibility of designing a robust and reproducible separation process for virtually any protein from most starting materials. As more advances are being made, the quality of our separation processes needs to be improved in parity with increasing requirements from regulatory agencies and customers. Several tools have become available to facilitate these efforts. In particular, simple computer-based protein-docking software packages (e.g. the Maestro suite, see Fig. 5) have become essential for generating an understanding of chromatographic separations in general and of MMC in particular [51, 53]. Such theoretical studies facilitate and speed up subsequent experimental evaluations. For instance, in a ligand modeling study, a library of arylamines combined with glucuronic acids was generated by virtual docking. In the docking process the receptor was kept fixed, while the ligand was allowed to be flexible. Through visual inspection of the docking results, the total library of 234 compounds could be reduced to 23 lead ligands that were subsequently chemically synthesized [55]. Experiments later verified that the computational docking was indeed useful for ligand design and development. Computational docking may give a more detailed and rapid picture of the nature of binding, which may involve multiple interactions such as direct coulombic, hydrophobic contacts and  $\pi$ - $\pi$  stacking. It can therefore be concluded that the exploration of possible chemical diversity of ligands will be instrumental in creating the



**Figure 5.** Example of a protein–ligand docking study. An MMC ligand (a pentamer of *N*-isopropylacrylamide) is docked to human hemoglobin (PDB code: 1GZX). The protein has been modeled using the Maestro 1.2 package (<http://www.schrodinger.com>) and LYS66 on the beta chain has been mutated to TYR. The docking study shows that the oligomeric ligand position is centered on Tyr 42 on the alpha chain. The default force field was used.



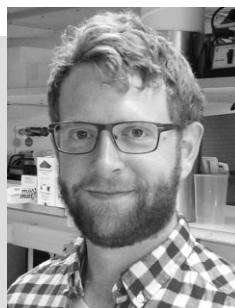
desirable high affinity and unique selectivity of MMC systems.

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## 7 References

- [1] Brorson, K., Sherrie, K., Lee, K., Hamilton, E. et al., Bracketed generic inactivation of rodent retroviruses by low pH treatment for monoclonal antibodies and recombinant proteins. *Biotechnol. Bioeng.* 2003, *82*, 321–329.
- [2] Haldar, J., An, D. Q., de Cienfuegos, L. A., Chen, J. Z., Klibanov, A. M., Polymeric coatings that inactivate both influenza virus and pathogenic bacteria. *Proc. Natl. Acad. Sci. USA* 2006, *103*, 17 667–17 671.
- [3] Hsu, B. B., Wong, S. Y., Hammond, P. T., Chen, J. Z., Klibanov, A. M., Mechanism of inactivation of influenza viruses by immobilized hydrophobic polycations. *Proc. Natl. Acad. Sci. USA* 2011, *108*, 61–66.
- [4] Vazquez-Rey, M., Lang, D. A., Aggregates in monoclonal antibody manufacturing processes. *Biotechnol. Bioeng.* 2011, *108*, 1494–1508.
- [5] Scopes, R. K., *Protein Purification: Principles and Practice*, Springer, New York 1994.
- [6] Janson, J.-C., *Protein purification: Principles, High Resolution Methods, and Applications*, Wiley, Hoboken 2011.
- [7] Gilar, M., Yu, Y. Q., Ahn, J., Fournier, J., Gebler, J. C., Mixed-mode chromatography for fractionation of peptides, phosphopeptides, and sialylated glycopeptides. *J. Chromatogr. A* 2008, *1191*, 162–170.
- [8] Neville, D. C. A., Dwelk, R. A., Butters, T. D., Development of a single column method for the separation of lipid- and protein-derived oligosaccharides. *J. Proteome Res.* 2009, *8*, 681–687.
- [9] Guerrier, L., Flayeux, I., Boschetti, E., A dual-mode approach to the selective separation of antibodies and their fragments. *J. Chromatogr. B* 2001, *755*, 37–46.
- [10] Voitl, A., Muller-Spath, T., Morbidelli, M., Application of mixed mode resins for the purification of antibodies. *J. Chromatogr. A* 2010, *1217*, 5753–5760.
- [11] Hou, Y., Cramer, S. M., Evaluation of selectivity in multimodal anion exchange systems: A priori prediction of protein retention and examination of mobile phase modifier effects. *J. Chromatogr. A* 2011, *1218*, 7813–7820.
- [12] Kennedy, L. A., Kopaciewicz, W., Regnier, F. E., Multimodal liquid-chromatography columns for the separation of proteins in either the anion-exchange or hydrophobic-interaction mode. *J. Chromatogr.* 1986, *359*, 73–84.
- [13] Tiselius, A., Hjerten, S., Levin, O., Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* 1956, *65*, 132–155.
- [14] Johansson, B. L., Belew, M., Eriksson, S., Glad, G. et al., Preparation and characterization of prototypes for multi-modal separation aimed for capture of positively charged biomolecules at high-salt conditions. *J. Chromatogr. A* 2003, *1016*, 35–49.
- [15] Kallberg, K., Becker, K., Bülow, L., Application of a pH responsive multimodal hydrophobic interaction chromatography medium for the analysis of glycosylated proteins. *J. Chromatogr. A* 2011, *1218*, 678–683.
- [16] Van Alstine, J., Larsson, C., Rudstedt, Å., Palmgren, R., *Use of pH-responsive polymers*, International Patent WO2004/082801, 2004.
- [17] Guerrier, L., Girot, P., Schwartz, W., Boschetti, E., New method for the selective capture of antibodies under physiological conditions. *Bioseparation* 2000, *9*, 211–221.
- [18] Burton, S. C., Harding, D. R. K., Hydrophobic charge induction chromatography: Salt independent protein adsorption and facile elution With aqueous buffers. *J. Chromatogr. A* 1998, *814*, 71–81.
- [19] Zhao, G. F., Dong, X. Y., Sun, Y., Ligands for mixed-mode protein chromatography: Principles, characteristics and design. *J. Biotechnol.* 2009, *144*, 3–11.
- [20] Linhult, M., Gulich, S., Graslund, T., Simon, A. et al., Improving the tolerance of a protein analogue to repeated alkaline exposures using a bypass mutagenesis approach. *Proteins: Struct., Funct., Bioinf.* 2004, *55*, 407–416.
- [21] Fassina, G., Verdoliva, A., Palombo, G., Ruvo, M., Cassani, G., Immunoglobulin specificity of TG19318: A novel synthetic ligand for antibody affinity purification. *J. Mol. Recognit.* 1998, *11*, 128–133.
- [22] Liu, Y. C., Lu, Y., Liu, Z., Restricted access boronate affinity porous monolith as a protein A mimetic for the specific capture of immunoglobulin G. *Chem. Sci.* 2012, *3*, 1467–1471.
- [23] Qian, J., El Khoury, G., Issa, H., Al-Qaoud, K. et al., A synthetic protein G adsorbent based on the multi-component Ugi reaction for the purification of mammalian immunoglobulins. *J. Chromatogr. B* 2012, *898*, 15–23.
- [24] Teng, S. F., Sproule, K., Husain, A., Lowe, C. R., Affinity chromatography on immobilized “biomimetic” ligands synthesis, immobilization and chromatographic assessment of an immunoglobulin G-binding ligand. *J. Chromatogr. B* 2000, *740*, 1–15.
- [25] Hale, G., Drumm, A., Harrison, P., Phillips, J., Repeated cleaning of protein A affinity column with sodium hydroxide. *J. Immunol. Methods* 1994, *171*, 15–21.



**Kristian Kallberg** (M.Sc. Eng.) is currently a doctoral student at the Department of Pure and Applied Biochemistry at Lund University. His research is in the area of hydrophobic multimodal chromatography for the separation and detection of small surface changes on proteins. The focus is on the separation of rationally engineered mutants and post-translationally glycosylated proteins for therapeutic purposes. These include hemoglobin variants for blood substitutes and monoclonal antibodies for more efficient drug candidates.



**Leif Bülow** (D.Sc. Eng., Dr. h.c.) is Professor and Head of the Department of Pure and Applied Biochemistry at Lund University. His research involves the engineering of proteins involved in oxygen binding and catalysis. To achieve these goals, several protein separation technologies have been developed, including various aqueous two-phase systems and hydrophobic interaction chromatography (HIC). The development of alternative ligands for HIC has allowed fine-tuning of protein separations, which has permitted the separation of protein variants carrying differences in single amino acid residues or minor differences in post-translational glycosylations.

- [26] Schwartz, W., Judd, D., Wysocki, M., Guerrier, L. et al., Comparison of hydrophobic charge induction chromatography with affinity chromatography on protein A for harvest and purification of antibodies. *J. Chromatogr. A* 2001, *908*, 251–263.
- [27] Lund, L. N., Gustavsson, P. E., Michael, R., Lindgren, J. et al., Novel peptide ligand with high binding capacity for antibody purification. *J. Chromatogr. A* 2012, *1225*, 158–167.
- [28] Sahin, E., Grillo, A. O., Perkins, M. D., Roberts, C. J., Comparative effects of pH and ionic strength on protein–protein interactions, unfolding, and aggregation for IgG1 antibodies. *J. Pharm. Sci.* 2010, *99*, 4830–4848.
- [29] Touelle, M., Uzel, A., Depoisier, J. F., Gantier, R., Designing new monoclonal antibody purification processes using mixed-mode chromatography sorbents. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2011, *879*, 836–843.
- [30] Chen, J., Tetrault, J., Ley, A., Comparison of standard and new generation hydrophobic interaction chromatography resins in the monoclonal antibody purification process. *J. Chromatogr. A* 2008, *1177*, 272–281.
- [31] Eriksson, K., Ljunglöf, A., Rodrigo, G., Brekkan, E., MAb Contaminant removal with a multimodal anion exchanger. *BioProcess Int.* 2009, *7*, 52–56.
- [32] Azevedo, A. M., Gomes, A. G., Borlido, L., Santos, I. F. S. et al., Capture of human monoclonal antibodies from a clarified cell culture supernatant by phenyl boronate chromatography. *J. Mol. Recognit.* 2010, *23*, 569–576.
- [33] Gamblin, D. P., Scanlan, E. M., Davis, B. G., Glycoprotein synthesis: An update. *Chem. Rev.* 2009, *109*, 131–163.
- [34] Krishnamoorthy, L., Mahal, L. K., Glycomic analysis: An array of technologies. *ACS Chem. Biol.* 2009, *4*, 715–732.
- [35] Price, J. L., Powers, D. L., Powers, E. T., Kelly, J. W., Glycosylation of the enhanced aromatic sequon is similarly stabilizing in three distinct reverse turn contexts. *Proc. Natl. Acad. Sci. USA* 2011, *108*, 14127–14132.
- [36] Wang, C. Q., Eufemi, M., Turano, C., Giartosio, A., Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry (Mosc.)* 1996, *35*, 7299–7307.
- [37] Suksrichavalit, T., Yoshimatsu, K., Prachayasittikul, V., Bulow, L., Ye, L., “Clickable” affinity ligands for effective separation of glycoproteins. *J. Chromatogr. A* 2010, *1217*, 3635–3641.
- [38] Weith, H. L., Wiebers, J. L., Gilham, P. T., Synthesis of cellulose derivatives containing dihydroxyboryl group and a study of their capacity to form specific complexes with sugars and nucleic acid components. *Biochemistry (Mosc.)* 1970, *9*, 4396–4401.
- [39] Ren, L. B., Liu, Z., Dong, M. M., Ye, M. L., Zou, H. F., Synthesis and characterization of a new boronate affinity monolithic capillary for specific capture of *cis*-diol-containing compounds. *J. Chromatogr. A* 2009, *1216*, 4768–4774.
- [40] Bailon, P., *Affinity Chromatography: Methods and Protocols*, Humana Press, Totowa 2000.
- [41] Becker, K., Hallgren, E., Carredano, E., Palmgren, R., Bulow, L., Characterization of multimodal hydrophobic interaction chromatography media useful for isolation of green fluorescent proteins with small structural differences. *J. Mol. Recognit.* 2009, *22*, 104–109.
- [42] Becker, K., Grey, M., Bulow, L., Probing protein surface accessibility of amino acid substitutions using hydrophobic interaction chromatography. *J. Chromatogr. A* 2008, *1215*, 152–155.
- [43] Franco, T.T., Galaev, I.Y., Hattikaul, R., Holmberg, N., Bulow, L. et al. Aqueous two-phase system formed by thermoreactive vinyl imidazole vinyl caprolactam copolymer and dextran for partitioning of a protein with a polyhistidine tail. *Biotechnol. Techn.* 1997, *4*, 231–235.
- [44] Johansson, H.-O., Matos, T., Luz, J. S., Feitosa, E., Oliveira, C. C. et al., Plasmid DNA partitioning and separation using poly(ethylene glycol)/poly(acrylate)/salt aqueous two-phase systems. *J. Chromatogr. A* 2012, *1233*, 30–35.
- [45] Fexby, S., Bulow, L., Hydrophobic peptide tags as tools in bioseparation. *Trends Biotechnol.* 2004, *22*, 511–516.
- [46] Carlsson, J., Mosbach, K., Bulow, L., Affinity precipitation and site-specific immobilization of proteins carrying polyhistidine tails. *Biotechnol. Bioeng.* 1996, *2*, 221–228.
- [47] Hanora, A., Bernaudat, F., Plieva, F. M., Dainiak, M. B. et al., Screening of peptide affinity tags using immobilised metal affinity chromatography in 96-well plate format. *J. Chromatogr. A* 2005, *1087*, 38–44.
- [48] Becker, K., Van Alstine, J., Bulow, L., Multipurpose peptide tags for protein isolation. *J. Chromatogr. A* 2008, *1*, 40–46.
- [49] Bernaudat, F., Bulow, L., Combined hydrophobic-metal binding fusion tags for applications in aqueous two-phase partitioning. *Protein Expression Purif.* 2006, *2*, 438–445.
- [50] Svensson, J., Andersson, C., Reseland, J. E., Lyngstadaas, P., Bulow, L., Histidine tag fusion increases expression levels of active recombinant amelogenin in *Escherichia coli*. *Protein Expression Purif.* 2006, *1*, 134–141.
- [51] Chung, W. K., Freed, A. S., Holstein, M. A., McCallum, S. A., Cramer, S. M., Evaluation of protein adsorption and preferred binding regions in multimodal chromatography using NMR. *Proc. Natl. Acad. Sci. USA* 2010, *107*, 16811–16816.
- [52] Gao, D., Lin, D. Q., Yao, S. J., Patch controlled protein adsorption in mixed-mode chromatography with benzylamine as functional ligand. *Biochem. Eng. J.* 2008, *38*, 355–361.
- [53] Nfor, B. K., Noverraz, M., Chilamkurthi, S., Verhaert, P. D. E. M. et al., High-throughput isotherm determination and thermodynamic modeling of protein adsorption on mixed mode adsorbents. *J. Chromatogr. A* 2010, *1217*, 6829–6850.
- [54] Pezzini, J., Joucla, G., Gantier, R., Touelle, M. et al., Antibody capture by mixed-mode chromatography: A comprehensive study from determination of optimal purification conditions to identification of contaminating host cell proteins. *J. Chromatogr. A* 2011, *1218*, 8197–8208.
- [55] Baumann, H., Ohrman, S., Shinohara, Y., Ersoy, O. et al., Rational design, synthesis, and verification of affinity ligands to a protein surface cleft. *Protein Sci.* 2003, *12*, 784–793.
- [56] Chase, H. A., Prediction of the performance of preparative affinity chromatography. *J. Chromatogr.* 1984, *297*, 179–202.
- [57] Jonsson, B., Stahlberg, J., The electrostatic interaction between a charged sphere and an oppositely charged planar surface and its application to protein adsorption. *Colloid Surf. B* 1999, *14*, 67–75.
- [58] Melander, W. R., Elrassi, Z., Horvath, C., Interplay of hydrophobic and electrostatic interactions in bio-polymer chromatography-effect of salts on the retention of proteins. *J. Chromatogr.* 1989, *469*, 3–27.
- [59] McCue, J. T., Engel, P., Thommes, J., Effect of phenyl sepharose ligand density on protein monomer/aggregate purification and separation using hydrophobic interaction chromatography. *J. Chromatogr. A* 2009, *1216*, 902–909.
- [60] To, B. C. S., Lenhoff, A. M., Hydrophobic interaction chromatography of proteins III. Transport and kinetic parameters in isocratic elution. *J. Chromatogr. A* 2008, *1205*, 46–59.
- [61] Borrmann, C., Helling, C., Lohrmann, M., Sommerfeld, S., Strube, J., Phenomena and modeling of hydrophobic interaction chromatography. *Sep. Sci. Technol.* 2011, *46*, 1289–1305.
- [62] Deitcher, R. W., Rome, J. E., Gildea, P. A., O’Connell, J. P., Fernandez, E. J., A new thermodynamic model describes the effects of ligand density and type, salt concentration and protein species in hydrophobic interaction chromatography. *J. Chromatogr. A* 2010, *1217*, 199–208.
- [63] Mollerup, J. M., Applied thermodynamics: A new frontier for biotechnology. *Fluid Phase Equilib.* 2006, *241*, 205–215.
- [64] Mollerup, J. M., Hansen, T. B., Kidal, S., Staby, A., Quality by design—thermodynamic modelling of chromatographic separation of proteins. *J. Chromatogr. A* 2008, *1177*, 200–206.

- [65] To, B. C. S., Lenhoff, A. M., Hydrophobic interaction chromatography of proteins I. The effects of protein and adsorbent properties on retention and recovery. *J. Chromatogr. A* 2007, *1141*, 191–205.
- [66] To, B. C. S., Lenhoff, A. M., Hydrophobic interaction chromatography of proteins II. Solution thermodynamic properties as a determinant of retention. *J. Chromatogr. A* 2007, *1141*, 235–243.
- [67] Chen, J., Yang, T., Luo, Q., Breneman, C. M., Cramer, S. M., Investigation of protein retention in hydrophobic interaction chromatographic (HIC) systems using the preferential interaction theory and quantitative structure property relationship models. *React. Funct. Polym.* 2007, *67*, 1561–1569.
- [68] Brooks C. A., Cramer S. M., Steric mass-action ion-exchange displacement profiles and induced salt gradients. *AIChE J.* 1992, *38*, 1969–1978; *AIChE J.* 1993, *39*, 538.
- [69] Ladiwala, A., Rege, K., Breneman, C. M., Cramer, S. M., A priori prediction of adsorption isotherm parameters and chromatographic behavior in ion-exchange systems. *Proc. Natl. Acad. Sci. USA* 2005, *102*, 11 710–11 715.
- [70] Perkins, T. W., Mak, D. S., Root, T. W., Lightfoot, E. N., Protein retention in hydrophobic interaction chromatography: Modeling variation with buffer ionic strength and column hydrophobicity. *J. Chromatogr. A* 1997, *766*, 1–14.
- [71] Geng, X. D., Guo, L., Chang, J. H., Study of the retention mechanism of proteins in hydrophobic interaction chromatography. *J. Chromatogr.* 1990, *507*, 1–23.
- [72] Ladiwala, A., Rege, K., Breneman, C. M., Cramer, S. M., Investigation of mobile phase salt type effects on protein retention and selectivity in cation-exchange systems using quantitative structure retention relationship models. *Langmuir* 2003, *19*, 8443–8454.
- [73] Malmquist, G., Nilsson, U. H., Norrman, M., Skarp, U. et al., Electrostatic calculations and quantitative protein retention models for ion exchange chromatography. *J. Chromatogr. A* 2006, *1115*, 164–186.
- [74] Mahn, A., Lienqueo, M. E., Asenjo, J. A., Effect of surface hydrophobicity distribution on retention of ribonucleases in hydrophobic interaction chromatography. *J. Chromatogr. A* 2004, *1043*, 47–55.
- [75] Freed, A. S., Garde, S., Cramer, S. M., Molecular simulations of multimodal ligand-protein binding: Elucidation of binding sites and correlation with experiments. *J. Phys. Chem. B* 2011, *115*, 13320–13327.
- [76] Holstein, M. A., Chung, W. K., Parimal, S., Freed, A. S. et al., Probing multimodal ligand binding regions on ubiquitin using nuclear magnetic resonance, chromatography, and molecular dynamics simulations. *J. Chromatogr. A* 2012, *1229*, 113–120.
- [77] Berg, O. G., Vonhippel, P. H., Diffusion-controlled macromolecular interactions. *Annu. Rev. Biophys. Biophys. Chem.* 1985, *14*, 131–160.
- [78] Davis, M. E., Madura, J. D., Sines, J., Luty, B. A. et al., Diffusion-controlled enzymatic reactions. *Methods Enzymol.* 1991, *202*, 473–497.
- [79] Johansson, B. L., Belew, M., Eriksson, S., Glad, G. et al., Preparation and characterization of prototypes for multi-modal separation media aimed for capture of negatively charged biomolecules at high salt conditions. *J. Chromatogr. A* 2003, *1016*, 21–33.