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Influence of protein and stationary phase properties on protein–matrix-interaction in cation exchange chromatography

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ABSTRACT

A large number of different stationary phases for ion-exchange chromatography from different manufacturers are available, which vary significantly in a number of chemical and physical properties. As a consequence, binding mechanisms may be different as well. In the work reported here, the retention data of model proteins (lysozyme, cytochrome c and two monoclonal antibodies) were determined for nine commercially available cation-exchange adsorbents. The linear gradient elution model in combination with a thermodynamic approach was used to analyse the characteristic parameters of the protein–stationary phase-interactions. Based on the pH dependency of the characteristic charge and the equilibrium constant for binding the differences between the standard Gibbs energies in the adsorbed and the solute state for the protein ΔG_P° and the salt ΔG_S° were calculated. The characteristic charge B of the proteins strongly depends on the molecular mass of the protein. For small proteins like lysozyme there is almost no influence of the stationary phase chemistry on B, while for the Mabs the surface modification strongly influences the B value. Surface extenders or tentacles usually increase the B values. The variation of the characteristic charge of the MABs is more pronounced the lower the pH value of the mobile phase is, i.e. the higher the negative net charge of the protein is. The standard Gibbs energy changes for the proteins ΔG_P° are higher for the Mabs compared to lysozyme and more strongly depend on the stationary phase properties. Surface modified resins usually show higher ΔG_P° and higher B values. A correlation between ΔG_P° and B is not observed, indicating that non-electrostatic interactions as well as entropic factors are important for ΔG_P° while for the B values the accessibility of binding sites on the protein surface is most important.

1. Introduction

A series of liquid chromatography steps are involved in separation and purification processes of biological products such as protein-based drugs [1–3]. Purification of recombinant proteins makes high demands on downstream processes as the target proteins have to be separated from very similar protein variants to very high purities. Amongst different separation modes such as size exclusion, hydrophobic interaction or affinity chromatography, ion-exchange chromatography (IEC) is commonly used and is therefore a major unit operation in purification processes of therapeutic proteins [2–6].

As design and optimization of IEC unit operations require consideration of many operating and chromatographic parameters one of the main challenges in biochromatography is predicting protein elution behaviour under various conditions based on a limited set of experimental data. In ion-exchange chromatography protein adsorption depends on the composition and concentration of the protein sample, on operating conditions such as buffer composition and pH, flow rate and sample load and on the physical properties of the adsorbent matrix [7].

Prediction is usually done using different mathematical chromatography models, which are based on the equilibrium model, the plate model and the rate model, for instance. They describe the retention behaviour of a protein dependent on the relevant separation parameters. For IEC several publications show the applicability of the models for different elution modes [4,8–14].

As IEC is widely used in protein drug purification a large variety of stationary phases is offered by different manufacturers. To obtain the benefits of ion-exchange chromatography, which are high selectivity, capacity and throughput, the evaluation and selection of a suitable adsorbent is very important [15]. Various resins, differing in chemical and physical properties, such as base matrix composition, stability regarding chemicals, pH and pressure, density of charged groups and ligand chemistry.

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In this study, several strong cation-exchange chromatography (CEX) resins were examined. Special attention was paid to the influence of surface modifications on the interaction between protein and matrix. Therefore several non-modified resins were compared to surface modified ones.

Source 30S, Toyopearl SP-650M and SP Sepharose FF are conventional resins whose functional groups are attached to the matrix by spacer arms. Source 30S and Toyopearl SP-650M have a polymeric base matrix while the bead of SP Sepharose FF consists of cross-linked agarose. Mean particle diameters range from 30 μm (Source 30S) to 65 μm (Toyopearl SP-650M) and 90 μm (SP Sepharose FF).

Toyopearl GigaCap S-650M is an improved ion-exchange resin consisting of the same polymeric base matrix as Toyopearl SP-650M except for a slightly larger average particle size of 75 μm, which leads to an improvement of pressure-flow characteristics. Furthermore, optimized ligand attachment chemistry leads to higher protein dynamic binding capacities, improved resin binding and desorption as the functional groups are preferentially placed in the larger pores which are more accessible for proteins.

Fractogel EMD SO_3^- (M) and Fractogel SE Hicap (M) have a similar base matrix as the conventional Toyopearl SP-650M but are surface modified by polyelectrolyte "tentacles". These long, linear polymer chains are attached to the beads and carry the functional ligands. As the tentacles are highly flexible the accessibility of the ligands without steric hindrance is improved and this results in a tighter binding of biomolecules. The polyelectric chains also allow additional interaction between proteins and ligands. Fractogel media was developed to afford high capacities at high flow rates and therefore allow higher yields at reduced throughput times compared to conventional resins. Due to the strong binding of proteins to the ligands higher salt concentrations in the sample have a minor effect on the binding capacity. As the Fractogel and Toyopearl resins are developed from the same beads, data for these materials allow immediate comparison to examine the effect of the tentacles.

Capto S consists of a highly cross-linked agarose matrix with similar bead and pore sizes as SP Sepharose FF. Dextran surface extenders link the functional group to the agarose matrix which increases capacities and mass transfer properties.

Poros 50 HS consist of polymeric particles with a mean diameter of 50 μm. The beads are coated with a proprietary hydrophilic polymer onto which the functional groups are covalently attached. Flow-through pores (500–10,000 Å) allow rapid mass transport.

S Ceramic HyperD F ion-exchangers combine characteristics of a soft, high capacity hydrogel with the stability of a rigid ceramic bead. The large pores of a rigid ceramic bead are filled with a functionalized hydrogel.

The selected set of strong cation-exchangers offers, apart from a variety of base matrices, particle and pore sizes, different ligand chemistries and this, therefore, allows the investigation of surface modified resins in comparison to conventional non-modified resins. Although many studies deal amongst other aspects with dynamic and static binding capacities, binding strength, ionic capacities, efficiency, resolution, adsorption isotherms, mass transfer, selectivity and protein recovery for conventional as well as surface modified resins [15–27], and several studies focus on properties of modified stationary phases [15,21,28–32], the impact of modifications is not yet well understood. Especially in the case of tentacle resins some studies show results regarding the influence of tentacles on the strength of protein retention which are contradictory to theoretical considerations. As tentacles offer an additional contact area between the charged sites of the proteins and the charged groups of the stationary phase this should lead to a significant increase in retention, which, without exception, was not reported for anion-exchange resins [33,34]. Contrary to these results, Bruch et al. observed increased numbers of interactions for tentacle modified resins [29]. DePhillips and Lenhoff tried to explain their sometimes ambiguous results for tentacle resins with differences in pore structure and tentacle length [15]. The examination of a broad variety of different stationary phases in this study should allow a better understanding of the influence of surface modifications on protein–matrix-interactions in ion-exchange chromatography and facilitate a target-oriented selection of chromatographic resins. GH-I_R-curves were calculated by the application of a linear gradient elution model [35,36]. Apart from data for the prediction of protein elution behaviour the applied model delivers information about stationary phase properties and electrostatic as well as non-electrostatic protein–matrix-interactions.

2. Theoretical considerations

2.1. Electrostatic interactions in ion exchange chromatography

In ion-exchange chromatography the separation mechanism is based on electrostatic interactions between the sample proteins and the functional groups of the resins. The interaction between a protein P with z_P charged sites and the ion exchange group in the presence of a counterion S according to the law of mass action or the stoichiometric displacement model (SDM) is described in the following equation [37]:

$$P + Z_P \cdot \bar{S} \Leftrightarrow \bar{P} + Z_P \cdot S \tag{1}$$

where $\bar{S}$ and $\bar{P}$ represent the salt and protein in the stationary phase. The equilibrium constant K_{eq} based on activities is given by:

$$K_{eq} = \frac{a_{\bar{P}} \cdot a_S^{Z_P}}{a_P \cdot a_{\bar{S}}^{Z_P}} \tag{2}$$

Under the assumptions that the activity coefficients are constant and close to unity Eq. (2) can be rewritten into the following equation:

$$K_{eq} = \frac{C_q}{C}\left(\frac{I}{I_q}\right)^{Z_P} \tag{3}$$

with the concentration of bound protein C_q, the concentration of protein in the mobile phase C, the salt concentration in the mobile phase I and the salt concentration in the stationary phase I_q.

The ion-exchange capacity Λ is given by:

$$\Lambda = I_q + z_P C_q \tag{4}$$

The distribution coefficient K_q describes the ratio of protein bound to ion-exchange groups to unbound protein and is given by combining Eq. (3) and (4):

$$K_q = \frac{C_q}{C} = K_{eq}\left(\frac{I}{I_q}\right)^{-Z_P} = K_{eq} \cdot \Lambda^{Z_P} \cdot I^{-Z_P} \tag{5}$$

with $\Lambda = I_q$ valid for low protein concentrations.

2.2. Linear gradient elution model

Yamamoto et al. established a simple graphical model based on the equilibrium and the plate model for the determination of elution characteristics from linear gradient elution (LGE) experiments in IEC [35,36]. This model (here called LGE-model) delivers data concerning the salt concentration at the peak position and the peak width as a function of gradient slope or flow rate. This information can be used for the design and optimization of linear gradient elutions or step elutions and for scale-up. Further this model delivers information about stationary phase properties and protein–matrix-interactions.

By performing linear gradient elutions with different gradient slopes g [M/mL] and determining the ionic strength at the peak