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Modeling of dual gradient elution in ion exchange and mixed-mode chromatography

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ABSTRACT

Protein retention using dual gradient elution in ion exchange- and mixed-mode chromatography can be modeled using the combination of a modified Yamamoto's LGE model and a conversion term to correlate the elution salt concentration and pH at any given gradient slope. Incorporation of the pH dependence of the binding charges into the model also provides some insights on the dual effects of salt and pH in protein–ligand interaction. The fitted thermodynamic parameters ($\Delta G_P^0/RT$, $\Delta G_S^0/RT$, number of charged amino acids involved in binding) of the dual gradient elution data using lysozyme and mAbs on SP Sepharose® FF, Eshmuno® HCX, and Capto® MMC ImpRes were consistent to the results of mono gradient data. This gives rise to an approach to perform thermodynamic modeling of protein retention in ion exchange- and mixed-mode chromatography by combining both salt and pH gradient into a single run of dual gradient elution which will increase time and cost efficiency. The dual gradients used in this study encompassed a wide range of pH (4–8) and NaCl concentrations (0–1 M). Curve fits showed that $\Delta G_P^0/RT$ is protein type and ligand dependent. $\Delta G_S^0/RT$ is strongly dependent on the stationary phase but not the protein. For mAb04 on mixed-mode resin Capto® MMC, $\Delta G_S^0/RT$ is 5–6 times higher than the result reported for the same protein on cation exchanger Fractogel® EMD SO_3^- (S).

1. Introduction

In order to comply with the guidelines of the FDA for industrial production of pharmaceutical monoclonal antibodies (mAbs), process related—as well as product related impurities have to be removed from the mAbs to ensure biological safety, bioavailability and therapeutic efficacy [1,2]. Gold standard for mAb purification in the downstream process (DSP) often involves multiple chromatographic steps which include affinity (AC), ion exchange (IEC), and hydrophobic interaction chromatography (HIC) [3–6]. While process related impurities (e.g. virus, DNA, host cell protein (HCP)) have been significantly reduced via well-established purification steps, separation of product related impurities (e.g. charge isoforms, soluble fragments, and aggregates) remains a challenge in the DSP. Efficient and robust separation methods for the mAb isoforms are required for preparative processes as well as profiling and characterization of the mAb in analytical processes [7].

Due to minor differences in charge heterogeneity of the mAb isoforms, protein elution via salt gradient appears to be insufficient to achieve highly resolved peaks. Alternatively, pH gradient elution is becoming popular in analytical and preparative chromatographic processes not only for mAb isoforms separation but also for closely related protein variants separation [8–11].

In gradient chromatofocusing (CF) a linear pH gradient is generated by mixing two appropriate buffer solutions of different pH values at the inlet of the column [12–15], which is then travelling through the column. Separation of proteins is achieved by the changing pH values of the mobile phase [12–17]. By increasing or decreasing the pH value, bound proteins lose their net charges and desorb from the stationary phase when the pH of the mobile phase approaches their respective p*I*s [12–17]. Since different mAb isoforms have different p*I* values, it is possible to separate these proteins in the sample mixture using pH gradient [12–15]. For modeling purposes, it is important to generate a linear and well controlled pH gradient. This can be achieved by carefully selecting buffer components which provide stable buffering capacity over a wide pH range (e.g. pH 4–11), preferably with low ionic strengths and which do not interact with the stationary phase [10].

Current trends show increasing demand in mixed-mode chromatography for protein separations especially under elevated salt

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concentration conditions [18,19]. In mixed-mode chromatography, multiple modes of interactions like ionic, hydrophobic interactions and hydrogen bonding are involved in the protein-ligand binding [20]. Over the years, many mathematical models were developed to systematically characterize and elucidate mixed-mode protein retention based on different theories like solvophobic theory, counter-ion condensation theory [21], patch-controlled protein binding theory [18], or by using thermodynamic approaches [22–24]. Majority of these models utilize the change in salt concentration to activate or deactivate the mixed-mode interactions [21–24]. Owed to the presence of variety modes of interactions on a single mixed-mode resin, the binding specificity towards a particular protein species can be strengthened or weakened by manipulating the salt concentration and/or pH value. Xia et al. have reported that simultaneous control of pH and salt concentration is important for the protein separation in mixed-mode chromatography [25]. Although dual salt-pH gradient systems have been used for analytical and preparative mAb purification in cation exchange chromatography (CEX) to remove HCP, leached protein A, DNA, and mAb isoforms [4,26], its application in mixed-mode chromatography is still lacking.

In this study, we show that dual salt-pH gradient system can be also used in mixed-mode chromatography for modeling and separation purposes. We also demonstrate that Yamamoto's LGE model [27–29] which is widely used to describe protein retention with linear isotherm in IEC using salt [30] or pH gradient [31] can also describe protein-ligand binding in mixed-mode chromatography. By using Mollerup's thermodynamic definition of Gibbs free energy, the pH dependency of the equilibrium constant is introduced to the stoichiometric displacement (SD)-based-LGE model [22,32]. The pH dependency of the stoichiometric number of electrostatic interactions is expressed as a function of protein net charge correlated to the number of amine groups (N_{Lys}, N_{Arg} and $N_{N\text{-term}}$), carboxyl groups ($N_{Carboxyl}$), cysteines (N_{Cys}), tyrosines (N_{Tyr}) and histidines (N_{His}) which are involved or affect protein-ligand bindings [31,33,34]. Furthermore, the LGE model is extended to describe protein separation using dual gradient system in which the salt concentration and pH change simultaneously. The fitted model parameters from the dual salt-pH gradients are compared to that of the salt- and pH mono gradient. An example of mAb isoforms separation using dual gradient elution on mixed-mode chromatography is also performed with increasing salt concentration and simultaneously decreasing pH gradient.

2. Theoretical consideration

Yamamoto's LGE model [27–30] is widely used to illustrate protein binding in IEC using linear gradient and step elution with changing salt concentration. The basic differential equation is defined as:

$$\frac{dGH_{salt}}{dI_R} = \frac{1}{K(I_R) - K'} \tag{1}$$

with the normalized gradient slope GH, the elution salt concentration of the protein I_R, the distribution coefficient of the protein K and the distribution coefficient of the salt K'. According to the SD model [27–30], K in the linear range of the protein adsorption isotherm can be defined as a function of the counter-ion concentration in the mobile phase as:

$$K(I_R) = \frac{C_q}{C} = K_E \times \Lambda^{\nu} \times {I_R}^{-\nu} + K_{crt} \tag{2}$$

with the protein concentration in the stationary phase and mobile phase C_q and C, respectively, the equilibrium constant K_E, the ionic capacity of the resin Λ, the number of binding charges for protein retention ν and the distribution coefficient of the protein under non-binding conditions K_{crt}.

Schmidt et al. [31] adapted Eqs. (1) and (2) to describe protein binding in IEC using linear pH gradient, which is given by the following expression:

$$\frac{dGH_{salt}}{dI_R} = \frac{dGH_{pH}}{dpH_R} = \frac{1}{K_E(pH_R)\Lambda^{\nu(pH_R)}(I_R + X)^{-\nu(pH_R)} + K_{crt} - K'} \tag{3}$$

with the elution pH of the protein pH_R and the correction factor due to different pH adjustment methods used during buffer preparations X.

Schmidt et al. [31] also suggested that both ν and K_E are dependent on the pH of the mobile phase. To describe the CF effects of proteins in IEC, they introduced a pH dependence of the protein binding charge ν based on the protein net charge model [31,33–35], which is:

$$\nu = \sum_i - \frac{N_{-i}}{1 + 10^{pK_{a_i} - pH_R}} + \sum_i \frac{N_{+i}}{1 + 10^{pH_R - pK_{a_i}}} \tag{4}$$

with the number of acidic amino acids N_{-i} ($N_{Carboxyl}$, N_{Tyr}) and the number of each basic amino acid N_{+i} (N_{Arg}, N_{Lys}, N_{His}, $N_{N\text{-Term}}$) involved in protein-ligand binding. With the assumption that there is no dependency of the pK_a values of each identical amino acid on the position in the amino acid sequence, the respective pK_a values can be estimated from the values given by Creighton [36].

According to Mollerup's [22] thermodynamic framework, the equilibrium constant is defined as:

$$RT \ln K_E = \nu \cdot \Delta G_S^0 - \Delta G_P^0 \tag{5}$$

with the universal gas constant R, the absolute temperature T and the difference between the standard Gibbs energy ΔG^0 in the absorbed and the solute state for protein (index P) and counter-ion (index S). Both Gibbs energy parameters are assumed to be pH independent.

By substituting Eqs. (4) and (5) into Eq. (3) gives the following expression:

$$\frac{dGH_{salt}}{dI_R} = \frac{dGH_{pH}}{dpH_R} = \frac{1}{\exp\left(\left(\sum_i - (N_{-i}/1 + 10^{pK_{a_i} - pH_R}) + \sum_i (N_{+i}/1 + 10^{pH_R - pK_{a_i}})(\Delta G_S^0/RT) - (\Delta G_P^0/RT)\right)\right)} \cdot \frac{1}{\Lambda\left(\sum_i - N_{-i}/(1 + 10^{pK_{a_i} - pH_R}) + \sum_i N_{+i}/(1 + 10^{pH_R - pK_{a_i}})\right)} \times \frac{1}{(I_R + X)^{-\left(\sum_i - N_{-i}/(1 + 10^{pK_{a_i} - pH_R}) + \sum_i N_{+i}/(1 + 10^{pH_R - pK_{a_i}})\right)} + K_{crt} - K'} \tag{6}$$

The normalized gradient slope GH is defined by Yamamoto [27–29] as:

$$GH_{salt,pH} = g_{salt,pH} \times V_0 \left(\frac{V_C - V_0}{V_0}\right) = g_{salt,pH} \times (V_C - V_0) \tag{7}$$