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High ionic strength narrows the population of sites participating in protein ion-exchange adsorption: A single-molecule study

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ABSTRACT

The retention and elution of proteins in ion-exchange chromatography is routinely controlled by adjusting the mobile phase salt concentration. It has repeatedly been observed, as judged from adsorption isotherms, that the apparent heterogeneity of adsorption is lower at more-eluting, higher ionic strength. Here, we present an investigation into the mechanism of this phenomenon using a single-molecule, super-resolution imaging technique called *motion-blur Points Accumulation for Imaging in Nanoscale Topography* (mbPAINT). We observed that the number of functional adsorption sites was smaller at high ionic strength and that these sites had reduced desorption kinetic heterogeneity, and thus narrower predicted elution profiles, for the anion-exchange adsorption of α-lactalbumin on an agarose-supported, clustered-charge ligand stationary phase. Explanations for the narrowing of the functional population such as inter-protein interactions and protein or support structural changes were investigated through kinetic analysis, circular dichroism spectroscopy, and microscopy of agarose microbeads, respectively. The results suggest the reduction of heterogeneity is due to both electrostatic screening between the protein and ligand and tuning the steric availability within the agarose support. Overall, we have shown that single molecule spectroscopy can aid in understanding the influence of ionic strength on the population of functional adsorbent sites participating in the ion-exchange chromatographic separation of proteins.

1. Introduction

Ion-exchange chromatography is an essential tool of the modern pharmaceutical industry. The cost of purification of pharmaceutical proteins remains high and may reach up to 50% of the total cost of goods [1]. This expense motivates efforts toward a more fundamental understanding of the complex competitive interactions of mixtures of proteins with heterogeneous polymeric adsorbents [2–4]. Adsorption isotherm measurements have traditionally been used to quantify the relationship between adsorbent properties, such as the dissociation constant and capacity, under varying ionic strength, pH, and other mobile phase conditions. Adsorption data often are fit with empirically-determined constants that modify the Langmuir and other adsorption isotherm models [5–15]. Alternatives to the idealized Langmuir model have been applied to protein adsorption data, both to capture the expected non-idealities of the process, and to better fit the observed heterogeneities of protein adsorption data [10,13,14].

A variety of sources can lead to the observed heterogeneity of protein adsorption, including intrinsic variance of the adsorbate molecule population due to differences in post-translational modifications, translational inaccuracy, and post-synthesis modifications such as deamidation and oxidation [6,16,17], inter-protein

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electrostatic repulsion and steric overlap [6,8], and irregularities in the adsorbent surface [18,19]. Because adsorption heterogeneity is observed even with uniform adsorbate populations and at very low loadings [9], it is likely that inherent heterogeneity of the adsorbent surface commonly plays the larger role. Adsorbent heterogeneity can arise from a range of potential causes, including steric accessibility and constraint, surface entropy and mobility [13], and the stochastic clustering of ligands on the adsorbent surface [20]. We have found that while adsorbents based on pre-organized, pentavalent clustered-charge ligands show higher protein affinity and capacity than adsorbents of the same total density of charge randomly distributed, isotherms for protein adsorption even on these nominally-homogeneous adsorbents show heterogeneity [12]. This observation further implicates adsorbent steric and surface mobility properties as a ubiquitous source of heterogeneity; especially as our recent single-molecule observations highlighted the role of both ligand clustering and steric availability with the porous support [20].

Adsorption in ion-exchange chromatography is the result of interacting influences of adsorbent heterogeneity and mobile phase composition. Ionic strength and (less frequently) pH often are used to tune adsorption characteristics [21], as they can influence the retention, resolution, and recovery of biomolecules [5–15] and can often provide non-denaturing elution. As discussed below, the observed heterogeneity often is lower at less-adsorptive conditions of higher ionic strength, as inferred from adsorption isotherm fit parameters [12,13]. Despite extensive and careful study, only limited mechanistic explanations of adsorption heterogeneity and its modulation by ionic strength are available. Studies using scanning confocal microscopy have shown that ionic strength influences pore diffusivity and capacity within porous agarose-based stationary phases [22,23]. Extension of confocal microscopy to include Raman vibrational spectroscopy has shown that over a range of solution conductivities, sorption can occur with only minimal changes in protein structure [24]. While these studies have offered insights into the role of ionic strength, ensemble averaging complicates approaches to an *a priori* understanding of ion-exchange chromatography [23]. Experiments accessing the molecular scale thus potentially could support a more fundamental understanding of mobile phase selection in biomolecular separations.

Single molecule spectroscopy avoids ensemble averaging to access the underlying molecular processes controlling protein–stationary phase interactions in separations. Foundational single molecule work resolved variability present at heterogeneous ion-exchange and reverse-phase stationary phase interfaces using confocal microscopy combined with fluorescence correlation spectroscopy and blip analysis [25–31] and total internal reflection microscopy [32–36]. We have recently extended single molecule techniques to the super-resolution level (<250 nm, below the diffraction limit of light) to investigate adsorptive separations at the single ligand-single protein level [20]. Super-resolution imaging allows for long-sought direct observations at the nanometer scale of the interfacial interactions that control chromatography. Using improved experimental and analysis methods, we showed the importance of charge-clustering of ligands and the kinetic heterogeneity even of nominally chemically-identical adsorption sites [12,20,25,37]. These results demonstrate the potential of single molecule techniques to advance a mechanistic understanding of chromatography difficult to obtain with ensemble techniques.

In this work, we apply single molecule super-resolution imaging to investigate the influence of ionic strength on the heterogeneity of protein ion-exchange functional adsorption sites, and therefore the heterogeneity of elution profiles. The system studied was α-lactalbumin on an agarose-supported uniformly clustered-charge ligand (penta-argininamide) stationary phase which we recently reported has higher protein capacity and affinity than dispersed-charge adsorbents of identical overall charge density [12,20,25,37]. We show that at high ionic strength, adsorption on lower affinity sites is suppressed, and heterogeneity is reduced, leaving a more uniform population of sites to participate. Circular dichroism spectroscopy shows that tuning the ionic strength does not significantly change the structure of α-lactalbumin, while optical imaging of model agarose microbeads shows a change in support structure. With the addition of single protein adsorption kinetics, our findings support the conclusion that a combination of electrostatic screening between the protein and ligand and the tuning of the steric availability within the agarose support leads to the reduction in heterogeneity. Overall, single molecule spectroscopy offers the potential for a more detailed mechanistic understanding of the influence of ionic strength on the ion-exchange separation of proteins.

2. Experimental methods

2.1. Sample preparation

All materials were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Glass coverslips (22 × 22 mm, No. 1; VWR, Houston, TX) were cleaned for 90 s in a basic TL1 solution (4% (v/v) H_2O_2 [Fisher Scientific, Radnor, PA] and 13% (v/v) NH_4OH) at 80 °C and then with O_2 plasma for 2 min at medium power (PDC-32G; Harrick Plasma, Ithaca, NY). Silicon templates (Grace BioLabs, Bend, OR) were placed on top of the cover slips, and a precision coat-bake system (200CBX; Brewer Science Cee, Rolla, MO) was used at 2000–3000 rpm to spin coat 1 mL of hot 1% (w/w) agarose solution (US Biological, Swampscott, MA) to prepare agarose thin films. Intra and inter-sample agarose film uniformity was investigated by spinning agarose on (opaque) silicon wafers and measuring film thickness by ellipsometry (J.A. Woolam M-2000 spectroscopic ellipsometer); agarose preparation and spinning conditions on silicon were identical to those used for deposition on (transparent) glass, on which ellipsometry was less reliable. Average film thickness of the film was estimated to be 80 nm using a Cauchy dispersion model. Film thickness was not detectably altered by rate of pouring of molten agarose, or by pre-cooling for 10 min before spin-coating (the agarose solution was spin-coated within 2 min of preparation). A custom flow chamber (1 mm height, with an elliptical opening of 12 × 5 mm; part no. 43018C, Grace BioLabs) was then placed over the agarose thin film.

Charged penta-argininamide ligands were immobilized on the agarose surfaces using aldehyde functionalities. As described by Afanassiev et al. [38] the agarose hydroxyl groups were first activated by 30 min treatment with 20 mM $NaIO_4$. The activated surfaces were washed with deionized water and dried of standing droplets under a stream of helium gas. A 43 μM solution of the penta-argininamide peptide (NH_2-GGRRRRRamide, Biomatik, Wilmington, DE) in coupling buffer (100 mM Na_2HPO_4/NaH_2PO_4 [EM Science, Gibbstown, NJ] and 150 mM NaCl [Mallinckrodt Chemical, St. Louis, MO], pH 7.2) was introduced to the activated surface and was coupled to the surface with several drops of 20 mM $CNBH_4$ (Pierce, Rockford, IL) at 4 °C for 30 min. The sample was then rinsed with coupling buffer to remove excess uncoupled peptide. Unreacted aldehyde sites were reduced with 66 mM $NaBH_4$ (in 25% EtOH/75% phosphate-buffered saline) at 4 °C for 5 min. The final surfaces were rinsed with deionized water and stored at 4 °C. The guanidinium side group of arginine (pK_a = 12.5) is protonated at pH 7.2 and thus unreactive toward the agarose aldehyde groups, and coupling through the N-terminal primary amine is strongly favored.

Ca^{2+}-depleted α-lactalbumin (L6010) was prepared from unpasteurized bovine milk and was purified by ion-exchange chromatography on DEAE-agarose. The purity was ≥85% as determined