Published in final edited form as:

J Chromatogr A. 2014 May 2; 1340: 33–49. doi:10.1016/j.chroma.2014.02.072.

Fluorescence Recovery after Photobleaching Investigation of Protein Transport and Exchange in Chromatographic Media

Steven J. Traylor[¶], Brian D. Bowes[†], Anthony P. Ammirati, Steven M. Timmick[§], and Abraham M. Lenhoff^{*}

Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, USA

Abstract

A fully-mechanistic understanding of protein transport and sorption in chromatographic materials has remained elusive despite the application of modern continuum and molecular observation techniques. While measuring overall uptake rates in proteins in chromatographic media is relatively straightforward, quantifying mechanistic contributions is much more challenging. Further, at equilibrium in fully-loaded particles, measuring rates of kinetic exchange and diffusion can be very challenging. As models of multicomponent separations rely on accurate depictions of protein displacement and elution, a straightforward method is desired to measure the mobility of bound protein in chromatographic media. We have adapted fluorescence recovery after photobleaching (FRAP) methods to study transport and exchange of protein at equilibrium in a single particle. Further, we have developed a mathematical model to capture diffusion and desorption rates governing fluorescence recovery and investigate how these rates vary as a function of protein size, binding strength and media type. An emphasis is placed on explaining differences between polymer-modified and traditional media, which in the former case is characterized by rapid uptake, slow displacement and large elution pools, differences that have been postulated to result from steric and kinetic limitations. In conclusion good qualitative agreement is achieved predicting flow confocal displacement profiles in polymer-modified materials, based solely on estimates of kinetic and diffusion parameters from FRAP observations.

1 Introduction

Protein transport in chromatographic media has been studied extensively, including the effects of protein size, charge, resin structure, and other parameters [1–9]. Such transport is important during uptake in determining the extent to which the capacity is decreased under dynamic conditions relative to the static capacity. It is also a factor determining resolution in analytical and preparative separations and the pool volume in the preparative case.

Of particular interest for the present work is transport in polymer-functionalized stationary phases [10, 11]. These media, which are produced for large-scale biologics production by

^{*}Corresponding author. Phone +1 302 831 8989. lenhoff@udel.edu.

[¶]Current address: Bristol-Myers Squibb, Hopkinton, MA 01748, USA.

[†]Current address: Eli Lilly and Co., Indianapolis, IN 46209, USA.

[§]Current address: Isermann Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180.

numerous manufacturers, are distinguished by their high static binding capacities that result from the extension of protein sorption from 2-D adsorption on a surface to 3-D partitioning into the polymer [10, 12]. Despite the additional barrier to transport presented by the polymer layer, dynamic binding capacities (DBC) are often high [13–17, 2], indicating that the additional transport resistance during uptake is low.

In contrast, transport limitations in polymer-functionalized media have been noted to be more limiting during elution, whether full elution or displacement. Limitations during elution have been observed using confocal microscopy [11] and during displacement of one monoclonal antibody variant by another using both confocal microscopy and batch displacement measurements [1, 18, 19]. Transport studies are usually performed under dynamic conditions, whether uptake [20, 21, 8], displacement [1, 18, 22, 23] or elution [11]. While a complete macroscopic picture of transport behavior is readily obtainable in these situations, the molecular processes contributing to overall transport, including kinetics, pore and surface or homogeneous diffusion and extraparticle transport resistance are less accessible. As a result, experimental data often provide an insufficient basis to understand molecular behavior completely and discriminate appropriately between models.

Here we measure and compare intrinsic transport rates in a near-equilibrium system in order to seek insights into the relative rates and mechanisms of the different transport and kinetic steps. The method employed, fluorescence recovery after photobleaching (FRAP), has previously been used to study protein transport by surface diffusion and kinetic exchange on flat surfaces [24–26] and to quantify intrinsic protein diffusion under weakly-retained conditions within polymer and gel networks in chromatographic materials [27–30] as summarized by Schroder et al. [31], but has only recently been applied to chromatographic media [11]. FRAP utilizes a confocal laser scanning microscope to observe fluorescently-labeled protein within a cell membrane or other environment in which it can undergo binding or transport. A high intensity laser is used to permanently photobleach fluorophores in a small region, and transport of labeled protein back into the bleached region may then be quantified to yield a kinetic or diffusive rate, as well as any fraction that may be permanently fixed in a binding site or membrane [32, 33].

Although molecular events are not directly observable, appreciable mechanistic information is obtainable by a careful analysis of FRAP data. The recovery of intensity in the bleached region was originally fitted assuming diffusion-controlled recovery within a Gaussian or cylindrical bleach geometry [32, 34–36, 33]. Kinetically-controlled models have also been adopted [37–40], some of which also considered diffusive contributions [24, 37]. In the past ten years detailed diffusion-kinetic modeling has been performed for various geometries [41–44], with three experimental parameters fitted: a diffusivity, an off-rate, and an on-rate [42–44]. Comprehensive numerical analysis has shown that fitting three parameters (diffusion and kinetic) to a single FRAP curve cannot yield unique parameter values, so additional equilibrium isotherm information is used to relate on- and off-rates [42–44].

An additional possible feature of modeling FRAP behavior is accounting for an irreversible fraction, i.e., a fraction of the bleached fluorescence that does not recover within the time scale of the experiment [32, 35, 24, 33]. While irreversibly bound protein may be a relevant

concern in cellular mechanisms and processes, there is less fundamental basis to assume their existence in chromatographic media. To properly determine the fraction of irreversibly bound protein, observations should be made for an order of magnitude longer than the half-time of recovery [32, 45, 46]. In addition to an irreversible fraction, models may utilize multiple diffusion rates and binding sites, which may be mechanistically descriptive, but also introduce additional estimated or fitted parameters [35, 47, 38, 42].

Applying FRAP to analyzing sorption and transport in chromatographic media adds the additional complication that the measurements and hence the transport are inherently in 3-D. However, for conventional media the system is still one of coupled diffusion and adsorption, so a similar approach is feasible to that in 2-D. However, it is necessary to take into account the different models that can be used to describe transport in chromatographic media. Transport and sorption within porous media have historically been described by a variety of models, including Fickian pore diffusion [48, 49], Fickian surface or homogeneous [50, 49], and Maxwell-Stefan surface diffusion [51]. Fickian pore [48, 49, 52, 21] and Fickian homogeneous [24, 25, 53–55, 21] diffusion models are most commonly used.

Similar analyses can be used for polymer-functionalized media, but the interpretation of the data and parameter values may be different. The bulk transport within a chromatographic particle may be analyzed in terms of the standard chromatographic models discussed above. However, the relatively slow elution and the appreciable constriction of the pore space by the polymer layer have led to the use of single-file Maxwell-Stefan diffusion to describe displacement of large proteins such as monoclonal antibodies within these media [18, 1]. Meanwhile, protein entry into and egress from the polymer layer involves both transport and kinetic effects [56–60], but the appreciable difference in characteristic lengths for transport parallel and perpendicular to the polymer layer can make the processes interpretable as kinetic on and off steps, akin to adsorption and desorption on a surface.

The focus of this work is to derive insights into protein transport mechanisms in traditional and polymer-functionalized materials from microscopic FRAP measurements of protein transport and kinetics. These measurements should better elucidate the contributions of pore size limitations and kinetic exchange rates to sluggish rates of displacement and elution from these materials. These exchange kinetics and diffusion measurements made near equilibrium should offer additional insights into modeling macroscopic measurements of protein uptake, displacement and elution.

2 Materials and Methods

2.1 Materials and Solutions

Chemicals were obtained from Fisher Scientific (Fair Lawn, NJ) and used without further purification. Phosphate buffer was prepared using 10 mM sodium phosphate (20 mM ionic strength, I.S.). Acetate buffer was prepared using 10 mM glacial acetic acid by mass (6 mM I.S.). Phosphate and acetate buffers were adjusted to pH 7.0 and 5.0 respectively using a solution of 1 M sodium hydroxide. Appropriate amounts of sodium chloride were added to further adjust the ionic strength. All buffers were prepared at room temperature (23 ± 3 °C)

using deionized water from a Millipore (Bedford, MA) Milli-Q system ($> 18.2 \text{ M}\Omega\cdot\text{cm}$) and filtered with $0.22 \mu\text{m}$ Gelman VacuCap bottle-top filters (Pall Corporation, Ann Arbor, MI).

Deoxyribonucleic acid (DNA) sodium salt from calf thymus (catalog number D1501) was obtained from Sigma (St. Louis, MO) and dissolved directly in 1 M ionic strength buffer. Solutions were filtered prior to use through a $0.45 \mu\text{m}$ Millipore Millex-HV filter to remove any undissolved DNA.

Hen egg white lysozyme (LYS, catalog number L6876) was obtained from Sigma (St. Louis, MO) with a manufacturer-reported purity of 95%. Bovine lactoferrin was donated by DMV-International (Veghel, The Netherlands) and was initially purified on a $27 \text{ cm} \times 1.6 \text{ cm}$ i.d. SP Sepharose XL cation-exchange column using a sodium chloride gradient in 10 mM phosphate buffer at pH 7. MAbs A and B, provided by Amgen Inc. (Seattle, WA), are closely related IgG2s that differ only in that two Arg residues near the CDRs of mAb A are an Ala and a Thr in mAb B. They were provided in formulation buffer at 150 and 32.2 mg/mL, respectively.

Protein solutions were repeatedly concentrated and exchanged into the appropriate buffer using Millipore 10 kDa Amicon Ultracel centrifugal filters. All protein solutions were filtered through $0.22 \mu\text{m}$ Millipore Millex-GV filters to remove any possible aggregates, both after preparation and again after storage at 4°C . Protein concentrations were determined via UV absorbance at 280 nm (UV-1700, Shimadzu, Kyoto, Japan). Extinction coefficients and other relevant protein characteristics are summarized in Table 1.

Fluorescently-labeled protein samples were prepared by reacting concentrated protein stocks prepared in 10 mM phosphate buffer in vials of DyLight 488 or DyLight 650 NHS-Ester (Thermo Fisher Scientific, Waltham, MA) fluorescent dyes, using similar methods to those described previously [13]. After an hour of contact, 10 kDa Amicon Ultracel centrifugal filters were repeatedly used to separate any unreacted label from the labeled protein stock. Spectrophotometry was used to quantify the label concentration, using measurements at wavelengths of 280 and 493, or 280 and 655 nm, respectively. Molar labeling ratios of around 5% were typically observed after separation of unreacted label. Labeled protein stocks were diluted with unlabeled protein stocks to achieve a final molar label ratio of around 1% for all fluorescence experiments [67].

Experiments were performed using three agarose-based strong cation-exchange resins (GE Healthcare, Piscataway, NJ): SP Sepharose FF (lot 10020387); SP Sepharose XL (lot 311563); and Capto S (lot 10061582). Resin properties are summarized in Table 2. The first material is a traditional ion exchanger while the other two contain dextran extenders. Adsorbent particles were washed three times with a 1.0 M sodium chloride solution before column packing. Helium was used to degas the slurry prior to column packing in a high-salt buffer. Resin for equilibrium isotherm and confocal studies was temporarily placed in a 1-2 mL column for washing with 15-20 column volumes of 1 M NaCl followed by 15-20 column volumes of buffer of the appropriate pH and ionic strength.

2.2 FRAP Experiments

Resin samples for FRAP measurements were equilibrated with fluorescently-labeled protein in Eppendorf tubes under gentle agitation for several days to a week or more. Samples were prepared by first making protein stocks and equilibrating resin in the appropriate buffer following the methods outlined above. Lysozyme and lactoferrin samples were prepared at 20 and 100 mM total ionic strength, pH 7.0, while mAb A and B samples were prepared at 6 and 100 mM total ionic strength, pH 5.0. Samples of mAb B were also prepared at 20 and 100 mM total ionic strength, pH 7.0. To obtain fully-loaded particles, Eppendorf tubes were prepared containing protein at a target concentration of 6 mg/mL for low and 5 mg/mL for high ionic strength samples. Resin samples were drawn into Wiretrol II 100 and 200 μ L capillary tubes (Drummond Scientific Company, Broomall, PA) using a plunger and were allowed to gravity settle. The bed height was adjusted until roughly the same amount of resin remained in each capillary, as measured by a digital caliper. Resin was then dispensed from the capillaries into the protein-filled Eppendorf tubes and allowed to equilibrate. For later experiments studying partially-loaded lysozyme and lactoferrin on SP FF and Capto S particles at 20 mM ionic strength, pH 7.0, samples were prepared using the more stringent procedures outlined below for measuring isotherms.

A Zeiss LSM 780 and a Zeiss LSM 5 LIVE laser scanning confocal microscope (Carl Zeiss, Germany), each equipped with a 40x C-Apochromat water-immersion lens (numerical aperture N.A. 1.20), were used to perform FRAP measurements. Equilibrium samples observed on the upright LSM 780 were prepared in an Invitrogen (Eugene, OR) Secure-Seal 0.12 mm spacer affixed to a microscope slide and covered with a Leica Microsystems (Richmond, IL) 22x22 #1.5 cover glass. Samples observed in the inverted LSM 5 LIVE were prepared in a Nunc (Roskilde, Denmark) Lab-Tek II #1.5 Chambered Coverglass and were sealed from air with paraffin oil and a cover glass. Twenty-six microliters of well-mixed and equilibrated resin and labeled protein solution were dispensed in both types of sample chamber.

Fluorescent dyes were excited using a 25 mW, 488 nm argon ion laser, a 20 mW, 561 nm DPSS laser and a 5 mW, 633 nm HeNe laser on the LSM 780. The 488 nm argon ion laser has an additional manual laser power control, which was set to less than 25% for all experiments. A 30 mW, 488 nm argon ion laser and a 5 mW, 633 nm HeNe laser were utilized for imaging on the LSM 5 LIVE. Laser intensity settings of 1-2% were typically sufficient to illuminate the fluorescent label without causing significant photobleaching. Depending somewhat on the laser power and dye, multiple bleaching iterations at 100% intensity were required to achieve 30-70% bleaching of total fluorescence [36]. The typical number of bleaching iterations was 80, and results of experiments varying significantly from this number are specifically noted. Circular regions significantly smaller than the particle diameter were selected for bleaching, with typical diameters ranging from 6-18 μ m.

2.3 FRAP Analysis

The data were analyzed in terms of the normalized intensity after photobleaching, defined here as the ratio of the spatially-averaged fluorescence intensity within the region of interest (t) to the intensity in the region before bleaching $I_0(t)$. However, several factors may

confound this analysis and must be normalized out. These include variations in laser intensity and photobleaching during imaging. Both laser intensity and the dyes used were observed to be very stable, but in a few experiments small fluctuations in each were observed and corrected. Normalization is performed by defining the spatial average within the bleached region as $\bar{I}_B(t)$ and the intensity in the region before bleaching as $\bar{I}_{B0}(t)$. This ratio is divided by the ratio of the intensity within a control region of the particle far away from the bleached region, $\bar{I}_C(t)$, to the initial intensity in the control region, $\bar{I}_{C0}(t)$ [40]:

$$\frac{\bar{I}(t)}{\bar{I}_0} = \frac{\bar{I}_B(t)}{\bar{I}_{B0}} / \frac{\bar{I}_C(t)}{\bar{I}_{C0}}. \quad (1)$$

A final scaling of the bleached region to account for the amount of bleaching must be performed before fitting [32, 35, 33]

$$fl(t) = \frac{\bar{I}(t)/\bar{I}_0 - \bar{I}_U/\bar{I}_0}{1 - \bar{I}_U/\bar{I}_0}, \quad (2)$$

where $\bar{I}_U(t)$ is the “unbleached intensity”, or the average intensity of the bleached region immediately post-bleaching. Following this step, the normalized fluorescence, $fl(t)$, may be fit to an appropriate model. The irreversible-binding fraction in this equation has been set to zero, as is justified later.

2.4 Confocal Microscopy Displacement Experiments

Confocal observation of protein uptake and displacement within chromatographic particles under flow conditions was performed using the LSM 5 LIVE inverted laser scanning confocal microscope and the 40x C-Apochromat lens described above. A flow cell was packed with chromatography resin as described previously [20] and sealed with a Leica Microsystems (Richmond, IL) 22x22 #1.5 cover glass. Protein and buffer solutions were fed continuously through the flow cell at a flow rate of 1 mL/min using a LKB Bromma positive displacement pump setup also described previously [13, 4].

For displacement experiments, Capto S resin was carefully washed at high ionic strength and equilibrated as described above in 10 mM phosphate pH 7.0 buffer. Lysozyme labeled with DyLight 650 was prepared at 2.0 mg/mL in 10 mM phosphate pH 7.0 buffer. Capto S resin was equilibrated for several days in a large volume of the labeled lysozyme before being centrifuged out and placed in fresh lysozyme from the same stock. This process was repeated twice, resulting in a total of three incubation periods in fluorescently-labeled lysozyme stock at 2.0 mg/mL. For displacement, lactoferrin was labeled with DyLight 488 and prepared at 2.0 mg/mL in 10 mM phosphate pH 7.0 buffer. The Capto S resin sample prepared at equilibrium with the 2.0 mg/mL lysozyme was then carefully loaded into the flow cell and equilibrated again briefly under flow in 2.0 mg/mL lysozyme stock, after which flow was switched to the 2.0 mg/mL lactoferrin stock.

2.5 Isocratic Retention Measurements

The isocratic retention of lactoferrin and each of the monoclonal antibodies was measured on an ÄKTA Explorer 10 (GE Healthcare, Piscataway, NJ) and a Waters Alliance 2695 Separations Module (Waters Corporation, Milford, MA). AP Minicolumn glass chromatography columns (Waters Corporation, Milford, MA) were packed with each of the resins used. Bed dimensions were 2.84 cm long $\times$ 0.5 cm i.d. (0.56 mL) packed with SP Sepharose FF; 2.94 cm long (0.58 mL) packed with SP Sepharose XL; and 2.05 cm long (0.40 mL) packed with Capto S. The isocratic retention procedures were the same as those used to obtain corresponding results for lysozyme [12].

Lactoferrin experiments on SP XL and Capto S were performed in 10 mM phosphate buffer at pH 7.0, while mAb experiments were performed on all resins in 10 mM acetate buffer at pH 5.0. Protein concentrations varied from a few tenths to several mg/mL, depending on the expected retention time. Injections of 10-40 μ L were made at a flow rate of 0.2 mL/min. Retention times at high ionic strengths were used to determine the total volume accessible to unretained protein.

The extraparticle porosity was additionally measured for each resin, using 20 μ L injections of 0.1 mg/mL calf thymus DNA dissolved in 1.0 M ionic strength pH 7.0 buffer with 10 mM phosphate. Apparent intraparticle porosities for each protein-resin pair were estimated from

$$\varepsilon_{p,A} = \frac{V_{HS} - V_E}{V_C - (V_E - V_d)}, \quad (3)$$

where V_{HS} is the high-salt retention volume of the protein on the resin, V_E is the extraparticle volume measured by DNA injection, V_C is the geometric column volume, and V_d is the dead volume measured by an injection without the column in place.

2.6 Adsorption Isotherms

Several points in the plateau region of the mAb A and B isotherms were measured on each of the three resins at pH 5.0 in 6 and 100 mM total ionic strength, 10 mM acetate-buffered sodium chloride. A similar protocol to that used for preparing equilibrium confocal samples was utilized for measuring isotherm points. Protein stocks were prepared and resin particles were washed and equilibrated in a column, each as described in Materials and Solutions. Resin samples were drawn into Wiretrol II 100 and 200 μ L capillary tubes, allowed to gravity-settle, and the settled resin height and total liquid height measured with a digital caliper. Resin samples were then dispensed into Eppendorf tubes prepared with known amounts of protein and buffer and equilibrated under gentle agitation for a period of two weeks. After equilibration, the final concentration of protein in the supernatant fluid, c , was measured and the amount of adsorbed protein per hydrated particle volume, q , calculated from a mass balance [12]. Lysozyme and lactoferrin isotherm results, obtained using very similar methods, were adapted by Bowes et al. [12] from Dziennik et al. [70, 20] and were used here without further modification.

3 Theory

3.1 Reaction-Diffusion Modeling

A fluorescence recovery model for proteins on chromatographic media was derived by combining chromatographic and FRAP approaches [11, 42]. Pore diffusion, homogeneous diffusion and kinetic exchange between the pore and surface are allowed in the general formulation of this model, though an assumption of either pore or homogeneous diffusion must be made to avoid overfitting parameters.

The governing equations outlined in Sprague et al. are used here, with several redefinitions. Free protein concentration c is defined as protein mass per solution volume and bound protein concentration q in units of mass per hydrated particle volume. To maintain consistent units, c must be multiplied by the apparent intraparticle porosity, $\varepsilon_{p,A}$, which has been estimated for each protein-resin pair as described in Materials and Methods. The open site concentration s is defined in units equivalent to those for q .

Following the simplifications in Sprague et al., binding sites are assumed not to diffuse and photobleaching is assumed to change only the illuminated state of the free and bound protein, not the number of free sites for binding [42]. Lumping the equilibrium free-site concentration into the on-rate, $k_{on}^* = k_{on}S$, results in the overall governing equations for FRAP:

$$\varepsilon_p \frac{\partial c}{\partial t} = \varepsilon_p D_p \nabla^2 c - \varepsilon_p k_{on}^* c + k_{off} q \quad \frac{\partial q}{\partial t} = D_q \nabla^2 q + \varepsilon_p k_{on}^* c - k_{off} q \quad (4)$$

Fickian pore-diffusion ($J = -\varepsilon_p D_p \nabla c$) and homogeneous-diffusion contributions ($J = -D_q \nabla q$) are each accounted for in the equations above, but the Maxwell-Stefan model ($J = -D_{MS} q/c \nabla q$) is a possible alternative diffusion mechanism [71, 51, 72, 73]. In chromatographic literature, a constant Maxwell-Stefan diffusivity $D_{MS,0}$ has been used, as is consistent for Maxwell-Stefan diffusion in an open porous network [74–77]. For the limiting case of single-file Maxwell-Stefan diffusion, a standard form for the concentration-dependence of the diffusivity for Langmuir adsorption [77, 74, 78] yields a driving force ($J = -D_{MS,0} \nabla q$) identical to that for Fickian homogeneous diffusion [78, 74, 77, 1].

Because of the limitation on the number of unique parameters that can be fit to a single FRAP curve, the full pore and homogeneous diffusion model of Eq. 4 must be restricted to either pore or homogeneous diffusion-kinetic recovery, with concomitant simplification of either equation in Eq. 4. A pore diffusion-kinetic (PDK) model is extensively explored in the FRAP literature [42–44]

$$\varepsilon_p \frac{\partial c}{\partial t} = \varepsilon_p D_p \nabla^2 c - \varepsilon_p k_{on}^* c + k_{off} q \quad \frac{\partial q}{\partial t} = \varepsilon_p k_{on}^* c - k_{off} q \quad (5)$$

In many cases studied here recovery times are observed that are far longer than the characteristic time scale for pore diffusion in the media, which for unhindered diffusion is expected to be on the order of several seconds [40]. An alternative model is therefore considered where pore diffusion is very rapid and recovery is dominated by homogeneous

diffusion and kinetics (*SDK*), with the pore concentration taken as a constant equivalent to its value before bleaching.

$$\frac{\partial q}{\partial t} = D_q \nabla^2 q + \varepsilon_p k_{on}^* c - k_{off} q. \quad (6)$$

3.2 Analytical and Numerical Solutions

Before a solution to the model equations can be obtained, the geometry of the bleached region must be ascertained as an initial condition. Within the focal plane a circular, or disk-shaped, region is bleached, but bleaching also occurs above and below the focal plane. Such bleaching profiles for FRAP experiments have been discussed extensively [79, 80, 42], with significant attention paid to out-of-plane bleaching profiles. Typically out-of-plane profiles have been approximated as cylindrical [44, 42, 79] or more accurately with analytical approximations of the laser point-spread function (PSF) [81, 82, 80]. The bleaching profile in our experimental system presented challenges due to the high numerical aperture of the lens used and the resulting analytically-intractable out-of-plane bleaching profile of the laser beam. Because of this issue, an experimental investigation of the bleaching PSF is presented in Results and Discussion, with the conclusion that a spherical bleaching PSF provides analytical simplicity while remaining sufficiently accurate for the analysis here. Homogeneously-distributed protein binding, homogeneously-distributed fluorescent labeling, and a small bleach region relative to the overall particle size are additionally assumed, allowing simplification to consider transport only in the radial direction.

Analytical and numerical solutions are presented here for the normalized fluorescence intensity defined in Eq. 2. To simplify the solution, the dimensionless relationship $C_{eq} + Q_{eq} = 1$ is defined, where

$$C_{eq} = \frac{\varepsilon_p c_{eq}}{\varepsilon_p c_{eq} + q_{eq}}, \quad Q_{eq} = \frac{q_{eq}}{\varepsilon_p c_{eq} + q_{eq}}, \quad (7)$$

and the equilibrium pore and surface concentrations under the experimental conditions are defined as c_{eq} and q_{eq} respectively.

The model may be solved in full or simplified to one of two limiting regimes: diffusion-only or kinetic-only. Each of these cases is covered at length in the literature, and only relevant results are presented here. Kinetic-only recovery yields the geometry-independent result [42, 38]

$$fl(t) = 1 - Q_{eq} \exp(-k_{off} t). \quad (8)$$

If diffusion is limiting, however, the results are geometry-dependent. Homogeneous diffusion-only recovery in a spherical bleached region within an infinite domain is given by [83]

$$f_l(t, r) = 1 - \frac{1}{2} \left[\operatorname{erf} \frac{w+r}{2\sqrt{D_q t}} + \operatorname{erf} \frac{w-r}{2\sqrt{D_q t}} \right] + \frac{1}{r} \sqrt{\frac{D_q t}{\pi}} \left[\exp \left(-\frac{(w-r)^2}{4D_q t} \right) - \exp \left(-\frac{(w+r)^2}{4D_q t} \right) \right], \quad (9)$$

a result that may be derived analogously to that for the cylindrical case [83, 35, 42]. Here w is the radius of the bleached region and r the radial position. A radially-averaged intensity profile may be obtained from this result by integration. This expression also applies for pore or free-solution diffusion in a system where no binding occurs. As the overwhelming majority of the bleached protein is bound, it would be inapplicable here.

The key distinguishing characteristics between the diffusion- and kinetic-limited regimes are the existence of a radial concentration gradient and the dependence of recovery on the size of the bleached region in the former case. If neither regime is limiting, a full solution of the diffusion-kinetic model is required [42–44].

The method of Sprague et al. [42] to derive various limiting diffusion-kinetic models is followed here. Based on the assumption of near-equilibrium conditions, the pore and surface concentrations within the particle can be related through isotherm relations, for which a Langmuir isotherm was assumed for convenience and because it describes experimental data adequately. For the high-affinity conditions studied and especially for cases where deviations from saturation are small, the precise model used has relatively little effect. The recovery equations were transformed into and solved in Laplace space (see Supporting Information for details), leading to

$$\overline{f_l}(p, r_1, r_2) = \frac{1}{p} - \frac{D_p C_{eq}}{w^2 p^2} \left[\alpha^2 - \frac{2(1+\alpha)\exp(-\alpha)}{\left(\frac{r_2}{w}\right)^2 - \left(\frac{r_1}{w}\right)^2} \left[\cosh \left(\alpha \frac{r_2}{w} \right) - \cosh \left(\alpha \frac{r_1}{w} \right) \right] \right] - \frac{Q_{eq}}{p+k_{off}} \quad (10)$$

$$\alpha^2 = \frac{p w^2}{D_p} \left(1 + \frac{Q_{eq}}{C_{eq}} \frac{k_{off}}{p+k_{off}} \right) \quad (11)$$

for the pore diffusion-kinetic (*PDK*) model and

$$\overline{f_l}(p, r_1, r_2) = \frac{1}{p} - \frac{D_q Q_{eq}}{w^2 (p+k_{off})^2} \left[\alpha^2 - \frac{2(1+\alpha)\exp(-\alpha)}{\left(\frac{r_2}{w}\right)^2 - \left(\frac{r_1}{w}\right)^2} \left[\cosh \left(\alpha \frac{r_2}{w} \right) - \cosh \left(\alpha \frac{r_1}{w} \right) \right] \right] \quad (12)$$

$$\alpha^2 = \frac{w^2}{D_q} (p+k_{off}) \quad (13)$$

for the surface diffusion-kinetic (*SDK*) model. In these expressions integration with respect to the radial position has been left in terms of the limits r_i to allow integration of individual radial slices. The single-file Maxwell-Stefan diffusion-kinetic model (*SFMS*) model yields an identical result to the *PDK* model, but with the pore and Maxwell-Stefan diffusivities, pore and adsorbed concentrations and the on- and off-rate terms exchanged.

The Laplace variable, p , may be inverted to yield time, but because an analytical solution is not available, rapid numerical inversion to real space was performed using the Matlab routine *invlap.m* [42]. This leads to transformed results for the radially-averaged fluorescence profile within the bleached region, determined for each of the limiting assumptions. Model fitting was performed by evaluating a range of all reasonable diffusivity and off-rate values and using the closest fit as the starting point for nonlinear regression in Matlab.

4 Results and Discussion

4.1 Adsorption Isotherms

To aid in a mechanistic understanding of the FRAP results, isotherm points were obtained for each of the experimental conditions studied. Isotherm data for lactoferrin and lysozyme at various ionic strengths at pH 7.0 were obtained from literature measurements [12] and recalibration [12] of literature results [70, 20] in the case of lysozyme on SP Sepharose FF. Selected points from these lactoferrin and lysozyme isotherms are replotted in panels *a* and *b* of Figure 1 to show the experimental conditions used for FRAP experiments. Isotherm points in the plateau region were measured for mAbs A and B on each of the three resins at ionic strengths of 6 and 100 mM in 10 mM acetate buffer at pH 5.0. Large, bold data points in panels *c* and *d* represent FRAP experimental conditions, while small data points show measured isotherm plateau concentrations. The wide error bars on the experimental FRAP conditions in the plateau region of the isotherm result from estimation of the final protein concentration in the sample and include propagated uncertainties from the isotherm fit, initial protein concentration and resin volume.

4.2 Isocratic Retention

Isocratic retention experiments were performed to determine the binding strength of each protein-resin pair under different conditions, expressed as the retention factor $k' = (V_R - V_{HS})/(V_{HS} - V_d)$, with the same variable definitions as in Eq. 3. Large proteins may be excluded from the polymer layer and restricted pore spaces of polymer-modified media under non-binding conditions, in which case the calculated k' value would not be strictly correct if based on a purely empirical V_{HS} . Bowes et al. [12] previously reported only the retention times of lactoferrin and a mAb (different from the mAbs studied here) on polymer-modified resins SP Sepharose XL and Capto S as a result of this limitation. For strongly retained proteins, which represent most of the situations studied, the k' values would be expected to be in error by at most a constant factor, much less than an order of magnitude, and these discrepancies are smaller than the effects that we seek to examine, so we report k' values based on the measured V_{HS} .

Lactoferrin and lysozyme results at pH 7.0 [12] are shown in Figure 2*a*, and mAb A and B results at pH 5.0 in Figure 2*b*. Additional experiments were performed for lactoferrin on SP Sepharose XL at pH 7.0 that agree well with the previously reported data [12]. The data all appear to follow the linear trend usually encountered for k' curves plotted vs. ionic strength on log-log axes, although the different slopes result in crossing of some of the curves.

Though it is difficult to compare across all proteins, in part due to the differences in pH, at low ionic strengths mAb B is observed to bind the most weakly, followed in order by lysozyme, mAb A and lactoferrin. At intermediate to high ionic strengths, lysozyme and mAb A appear to switch places in the binding strength ranking, with mAb A binding more strongly. The binding strength appears to be similar across the three resins for a given protein, probably due to the resins all having the same functional group (SO_3^-). Additionally, the significant difference in the binding strengths of the almost-identical mAbs is explained by the substitution of two amino acids discussed previously.

Extrapolation of the binding strengths to lower ionic strength values was performed using the functional form of the stoichiometric displacement model [84]

$$k' = K_{SD} I^{-z}, \quad (14)$$

which provides a reasonable fit of the measured isocratic retention data without necessarily confirming the validity of the model. Fitted parameters and 95% confidence intervals are presented in Table 3.

4.3 FRAP Experimental Parameters

FRAP experiments were performed using four model proteins on three resins at two ionic strengths. Initially experiments were performed on particles fully loaded with labeled protein. Experiments for all proteins except for mAb A were repeated with two different fluorescent labels (DyLight 488 and 650) to allow identification of specific dye effects. An extensive data set showed no correlation with dye wavelength, so results for both labels are presented here without distinction. Later experiments were performed for lysozyme and lactoferrin partially-loaded on SP Sepharose FF and Capto S to investigate loading effects.

Additional experimental parameters characterizing photobleaching may also affect the results obtained, so their effects were explored to ensure the validity of the interpretation. The bleached intensity profile is a function of the laser point-spread function, image acquisition settings and the z -distance from the bleach plane. The laser beam PSF is characterized by a radial resolution r_0 and vertical resolution z_0 [81, 82, 80]. When the radius w of the bleached region is much larger than the radial resolution of the laser and the lens has a low numerical aperture, the observed fluorophore concentration post-bleaching may be written as [80]:

$$I_b(r, z) = \begin{cases} I_0(r, z) \exp[-K_0 \exp(-2z^2/z_0^2)] & r \leq w \\ I_0(r, z) & r > w \end{cases}, \quad (15)$$

where the imaging constant K_0 is defined as

$$K_0 = \frac{\alpha I_{0b} Z^2}{v_0 \Delta y_0}. \quad (16)$$

The parameter Z is the electronic zoom factor, which may vary between images depending on the particle size. The constant α is defined as the fluorophore bleach rate in a given medium, and constants v_0 and Δy_0 are the line scanning speed and the distance between

scanned lines at an electronic zoom factor of 1. Finally, the parameter I_{0b} is related to the laser intensity.

The intensity profile was explored in detail experimentally by imaging a particle in three dimensions (z -stack of x - y plane images) before and after bleaching. A large particle and a large bleach region (radius $w = 13 \mu\text{m}$) were selected in order to simplify data analysis. Bleaching was performed 80 times with the 488 nm and 561 nm laser lines at 100% power to guarantee significant bleaching. Imaging the entire particle in 3D took roughly 5 minutes, so a protein, resin and buffer condition that would not recover appreciably during imaging were selected: mAb A labeled with DyLight 488 loaded to capacity on Capto S in 10 mM acetate buffer (6.0 mM total ionic strength, T.I.S.) at pH 5.0. After imaging, a 10 px. disk filter was applied in Matlab to each x - y image of the pre- and post-bleach z -stacks (each image in the stack is 512x512 px.) in order to reduce random noise (the images had to be collected rapidly with high scan-speeds and no averaging). Following averaging, the post-bleach stack was subtracted pixel-by-pixel from the pre-bleach stack; the result is shown in Figure 3. Bleached areas appear as regions of light, while regions where no bleaching occurred are dark.

Figure 3*a* and *b* show planar (x - y) and perpendicular (x - z) slices through the bleached region, while panel *c* shows a 3D projection through the particle in the bleach plane (x - y). The conical bleaching pattern in panel *b* is a result of the relatively-high numerical aperture of the lens (N.A. = 1.2) and is similar to z -dimension bleaching profiles obtained previously [81, 82, 46]. Visual inspection validates the assumption of the bleach radius w being significantly greater than the radial resolution r_0 of the laser.

The vertical laser resolution z_0 is investigated in Figure 3*d*, where the normalized average x - y intensity within the bleached region is plotted as a function of distance from the bleached plane in the z direction. The bleached intensity rapidly decreases above and below the bleached plane and appears to be reasonably well-described by a Gaussian intensity profile, Eq. 15, with a z_0 value of 6.5 micrometers and a negligibly-small K_0 value.

Significant tailing is observed outside of the predicted bleached region and may be attributed to several factors. First, because of the relatively-high numerical aperture, the bleached profile is conical instead of the cylindrical profile required by Eq. 15. Therefore, significant additional bleaching occurs in adjacent regions above and below the bleached plane. Second, while recovery is negligible within the bleached plane over the five-minute imaging period, some diffusion will occur and may be more apparent in marginally-bleached regions. Third, a small and unavoidable amount of photobleaching may have occurred while imaging the particle before bleaching. Finally, minor variations may exist between the two pre- and post-bleach images due to fluctuations in laser and fluorescence intensity.

Because of the obvious shortcomings of the Gaussian PSF model illustrated in Figure 3*d* and the unavoidability of such issues because of the high numerical aperture of the lens used experimentally, the disc-Gaussian geometry was undesirable. Instead, values of the bleach radius, w , ranging from 3 to 9 micrometers were used for almost all FRAP experiments, making a simpler spherical bleaching PSF model a reasonable approximation for the bleach

region in the x , y and z dimensions. Theoretical predictions for spherical bleaching PSF models of different bleach radii (w) are shown in Figure 3d. Despite the shortcomings of the spherical model, its bleaching PSF predictions are similar or slightly better than those of the Gaussian PSF model, and are significantly easier to model mathematically. Additionally, as is explored later, recovery observed in chromatographic media tended towards being kinetically-limited, which results in an independence of bleach geometry.

An additional parameter affecting recovery is the extent of bleaching, which was therefore studied systematically on the LSM 780. Bleaching between 30 and 70% of total fluorescence [36] is optimal to maintain a good signal to noise ratio and avoid fitting fractions of small differences in intensity. Generally 80 bleaching iterations were found to be sufficient to achieve the desired amount of bleaching, but the effects of bleaching intensity on recovery rates were also investigated for SP Sepharose FF and Capto S using DyLight 488-labeled lactoferrin at 20 mM total ionic strength, T.I.S., pH 7.0. As shown in Eq. 16, the bleaching amount depends not only on the number of iterations, N , but also the electronic zoom factor of the microscope, Z . All other variables were held constant for the same dye, microscope and laser power. The bleaching results in Figure 4 are plotted as a function of the bleach factor $K_0 = Z^2 N$.

Figure 4 shows the fitted off-rate and diffusivity (PDK model) as a function of the bleach factor. The fitting process is discussed in greater detail below, but of interest here is simply the consistency of the parameter estimates as a function of the bleach factor. For lactoferrin on SP Sepharose FF, both parameters appear to be within expected experimental variability (roughly an order of magnitude), independent of bleach amount. Recovery curves are not shown here, but recovery is very slow and little protein recovers over the course of the experiment, similar to the behavior shown in Figure 6c. In comparison, results for the same protein and dye on Capto S appear to be significantly more scattered as a function of bleach amount, with a possible decrease in diffusivity and off-rate with increasing bleach amount. Less-bleached samples appear to recover somewhat faster and more completely than more-bleached samples, though it is clear that there is significant experimental variability in these results. The proteins, labels, and label ratios (~1%) are the same among these experiments, so it is unlikely that this is a bleaching-dependent effect.

Possible explanations include the unlikely one of inhomogeneity between Capto S particles, or the more likely difference in z -profiles at different bleaching amounts. Less in-plane bleaching also results in less out-of-plane bleaching and additional recovery flux from the z -dimension. The z -plane bleaching profile was measured at the highest bleach setting (right half of the graph in Figure 4), so it is possible that the assumed spherical geometry becomes less valid for smaller bleaching amounts.

The scatter in the data makes it difficult to address this issue completely here, beyond noting that all results reported below were performed using 60-90 bleach iterations, with similar bleach radii and electronic zoom factors. Qualitative and semi-quantitative comparisons among resins and proteins are therefore possible based on these measurements. Possible future improvements to improve confidence in the fitted constants are discussed later.

4.4 FRAP Analysis

In each FRAP experiment, stable measurements of initial intensities were taken for five frames [40, 46] prior to bleaching. A Matlab script utilizing the routines *tiffread.m* [85] and *lsminfo.m* was developed to import and average confocal data. The intensity within the bleached disk was averaged in four concentric radial bands in order to fully capture the spatial profile of fluorescence recovery but to retain the smoothing effect of averaging and not to generate excessive data for fitting. Radially-averaged regions were chosen from fractional radii of 0.00-0.25, 0.25-0.50, 0.50-0.75 and 0.75-0.95 of the bleached region. Figure 5b illustrates the normalized intensity of the radially-averaged regions as coarse grey bars and also investigates the effect of selecting finer radial bands, overlaid in cross-hatched bars. Intensities outside a fractional radius of 0.95 were excluded to eliminate fluorescence from outside the bleached region resulting from spatial resolution limits and slight shifts in particle position. The particle in Figure 5a was imaged immediately after bleaching and illustrates the sizes and locations of the bleached and control regions. The bar chart of the normalized average intensity (Figure 5b) illustrates why these selections are appropriate, with the fine cross-hatched bars illustrating the radial resolution of the microscope and image analysis algorithm.

The normalized intensities were obtained using Eq. 1, and were further normalized before fitting with Eq. 2. The average intensity immediately after bleaching in this case is roughly 0.57. Though an irreversible fraction is frequently imposed at this normalization step, no irreversible fraction was assumed in this work due to the lack of a solid theoretical justification. Further, observation for the length of time necessary to confirm or rule out full recovery in slowly-recovering samples is impractical (hours to days). As a result, irreversible fractions are not accounted for, and full eventual fluorescence recovery is assumed.

While formal fits of the diffusion-kinetic models to the recovery data are discussed below, the spatio-temporal trends can also allow qualitative distinctions to be made among diffusion-controlled, kinetically-controlled and diffusion-kinetic recovery. Diffusion-controlled recovery would be accompanied by development of a gradient in the radial direction, while kinetically-controlled recovery would result in uniform recovery as a function of radial position. Diffusion-kinetic recovery would fall somewhere between the two. A number of FRAP recovery curves and their radially-dependent averages are presented here and in most cases recovery is similarly-rapid across the radial dimension. While accelerated recovery is observed in some cases at the outer radial boundary, the FRAP recoveries observed here appear to be controlled primarily by kinetics with less-significant diffusive contributions.

FRAP results fitted to the pore- (PDK) and surface- (SDK) diffusion kinetic models show recovery falling into one of three categories, illustrated in Figure 6 for three cases on Capto S. Panel *a* shows complete and fairly rapid recovery, and both model fits for these cases are generally excellent. In panel *b*, slow and incomplete recovery was observed. In these cases model fits tended to be less exact, with slight underprediction of recovery immediately after bleaching and overprediction at longer recovery times. Recovery fractions are far more

significant than would be seen for recovery in the pore space alone, so clearly some of the bound protein is exchanging. However, the results clearly suggest a more-mobile fraction of protein that can exchange rapidly and a less-mobile fraction of protein that cannot exchange as rapidly. A more detailed model for this behavior is not pursued here, as there is likely a wide distribution of configuration-dependent binding energetics and steric hindrances contributing to the tailing recovery. Panel *c* shows protein that is almost immobile after bleaching. A small fraction, likely the protein in the pore space, recovers rapidly, while the rest remains immobile. The model fits well, assuming that all protein eventually recovers, though it is clear recovery will not happen on any realistically-observable time scale.

Mechanistically, Figure 6 investigates the effect of protein loading by equilibrating samples of lactoferrin and lysozyme on Capto S and SP Sepharose FF at 20 mM T.I.S., pH 7.0, at multiple points in the linear, shoulder and near-saturation regions of the isotherm. The free and bound protein concentrations in this investigation were very carefully prepared, calibrated, measured and validated against isotherm data [12]. This study is in contrast with many results presented later investigating fully-loaded particles far into the saturation portion of the isotherm, where free and bound concentrations were simply estimated by mass balance. The representative plots in Figure 6 were selected from this set of experiments, and model inputs for these fits, as well as fitted parameter values, are presented in Table 4.

The fitted kinetic off-rate constants are similar for both the PDK and SDK models. As kinetics appears to be the limiting factor in the majority of cases studied, this similarity is consistent with expectations. The fit quality is also similar for the two models and while there is certainly reason to suspect that pore and surface diffusion would contribute differently in different types of media, it is difficult in this case to justify choosing one model exclusively over the other. Because of the simplicity and ease of parameter comparison in the pore diffusion-kinetic (PDK) model, this model is used exclusively from here on, though its use is not intended to be taken as a universal mechanistic interpretation.

For cases where recovery is rapid, such as partially-loaded lysozyme on Capto S, fitted pore diffusivities are occasionally larger than expected in free solution. Due to the low pore concentration and small apparent intraparticle porosity, pore diffusion accounts for a very small fraction of total recovery. Further, most pore recovery occurs during and immediately after bleaching (a process that takes several seconds), making this diffusion rate extremely difficult to estimate. Additionally, bleaching geometry likely plays a role, as some molecules diffusing from above and below the bleached plane travel a shorter distance during recovery than molecules from the edges of the circle in the bleached plane. The general conclusion for these cases remains that while diffusion plays a part in recovery, kinetics occupies a more significant role in the model.

Trends in the recovery curves and fitted models tell a compelling story. Lysozyme recovery kinetics appears to be more rapid and complete at lower loadings, and to decrease at higher loadings. Lactoferrin, a protein that binds more strongly and achieves higher loadings, recovers almost negligibly under the same conditions. Based on these initial results, recovery appears to be significantly impacted by both binding strength and particle loading.

Figure 7 provides a more global picture of the effects of protein, ionic strength and media type on recovery rates, emphasizing the interplay among factors controlling protein mobility. The first row shows results for lysozyme at 20 mM T.I.S., with recovery universally slow and incomplete.

At the more weakly retained condition of 100 mM T.I.S. (second row), rapid recovery is seen on SP Sepharose FF and SP Sepharose XL, while Capto S lags far behind. It is not clear why this trend is observed, since similarities would be expected between the dextran-bearing Sepharose XL and Capto S, which would be expected to differ from the dextran-free Sepharose FF. However, significant differences in some transport characteristics between these two media have been seen by confocal microscopy previously [13]. The two media differ in ligand density and in the nominal size of the residual pore space, but the retention behavior is similar.

The opposite trend is seen for the much larger protein lactoferrin under the same weakly-binding conditions (Figure 7, bottom row): recovery is most rapid on Capto S and slowest, if present at all, on SP Sepharose FF. The most obvious explanation for this trend is the binding strengths of lactoferrin on different resins (Figure 2), with the strongest (SP Sepharose FF) and weakest (Capto S) binding correlated with slow and fast recovery respectively. These trends are further explored and generalized in the following section.

Table 5 summarizes experimental conditions and fitted diffusive and kinetic parameters for the conditions shown in Figure 7. The PDK off-rates fitted as infinity indicate pore diffusion-controlled recovery with very rapid kinetic exchange. SDK kinetic off-rates fitted as approximately zero indicate very slow kinetic exchange and primarily surface-diffusion controlled recovery. As discussed previously and further illustrated in these figures, the PDK model provides the best fit of the experimental data, especially in cases that the SDK model fits as being diffusion-limited. For kinetically-limited cases, both models fit reasonably well and provide similar kinetic parameter values, as would be expected.

4.5 Overview of Model Parameters Fitted to FRAP Results

The kinds of interpretations reflected in the preceding discussion emerge more broadly from a comprehensive analysis of recovery measurements over the complete range of proteins, resins, ionic strengths and particle loadings studied. These results are plotted in Figure 8 as a function of the isocratic retention factor estimated from Eq. 14 and Table 3, and of the bound protein concentration, q . Because the extrapolation to estimate k' is in some cases over several orders of magnitude, there is appreciable uncertainty in the values shown, but the overall trends are pronounced enough to remove any significant ambiguity in the conclusions. The kinetic off-rate generally decreases with increasing binding strength, and to a lesser extent with increasing bound concentration. As the isocratic retention factor is related to the equilibrium binding constant, it is reasonable to expect a significant off-rate dependence on k' , with stronger retention resulting in slower desorption. Similarly, increased particle loading may result in a decreased rate of desorption, particularly for the dextran-modified resins in which sorption occurs by volumetric partitioning; however, this trend is significantly weaker and more scattered. While sufficient data are available to show a

dependence on bound concentration in each case studied, the data are insufficient to make general comparisons between resins.

Fitted pore diffusivities generally fall within the expected range of protein diffusivities in chromatographic media. The few that are greater than physically realistic generally have very large confidence intervals owing to their relative insignificance in a kinetically-controlled recovery model. The fitted pore diffusivity results are fairly scattered and no significant trends are observed.

Some experiment-to-experiment variation is observed in the results and may be attributed to differences between individual particles and samples as well as previously-discussed imperfections in the model and assumptions. Most off-rates can be confidently estimated to within an order of magnitude, and in some cases closer agreement between measurements is observed. An important caveat for the fitted off-rates on the lower end of the observed range is that the time scale of the experimental data was much less than required for significant recovery. As such, the actual off-rate may be slower than measurable over the course of these experiments. Potential improvements in experimental design and computational approaches may further improve accuracy and are discussed briefly later.

As the off-rate appears to be the most important model parameter in most of the experiments, all fitted kinetic parameters are plotted by protein and resin in Figure 9 as a function of the estimated isocratic retention. The overall trend in off-rates as a function of isocratic retention is made more apparent in these results, despite the uncertainties in the exact k' values. As mentioned previously, lysozyme and lactoferrin on SP Sepharose FF and Capto S were studied as a function of particle loading at 20 mM T.I.S., so some additional variation in results for these proteins on these media is visible here. The mAb A results are indicative of the almost-irreversible binding observed for this mAb on strong ion exchangers, while the mAb B results illustrate the significant effect of the mutation of two amino acids on the binding strength and off-rate.

Differences in lysozyme off-rates among the three resins studied are further illustrated in these plots, with all resins recovering similarly at lower ionic strengths, and with off-rates progressively increasing among Capto S, XL and FF at higher ionic strengths. An opposite trend in lactoferrin desorption at higher ionic strengths is further confirmed to depend on k' . Recovery among the monoclonal antibodies is fairly slow for most conditions studied, so comparison between resins is difficult. Some differences between resins are seen at higher ionic strengths, though no conclusive trends are observed. Such a trend would be especially relevant to understanding the differences in kinetics and transport between dextran-modified and traditional media. Slow exchange of lysozyme on Capto S when compared to SP Sepharose FF suggests that desorption kinetics may be retarded to some extent by the polymer layer. However, in the case of lactoferrin, the binding strength appears to play a much more dominant role than possible polymer effects, reversing this trend and resulting in no obvious resolution to this issue. Additional differences between the proteins may play a role as well in these opposing trends, including the small relative size of lysozyme and the non-uniform charge distribution of lactoferrin.

Further confounding the issue is the aforementioned effect of protein loading, which suggests a complex interplay between binding interactions and the extent of protein loading, both of which may be modulated by the presence of the polymer layer. As the polymer layer allows a significantly greater loading density, retardation of protein mobility resulting from steric hindrance by neighboring proteins must also be considered as a factor. Here again, the trend reversal observed between lysozyme and lactoferrin on polymer-modified and traditional materials leaves the issue unresolved, though the protein binding strength, the protein loading and the interactions with the polymer layer are all expected to play an interconnected role.

Individual diffusivity results are not shown here, but the lysozyme and mAb A results are typically scattered within an order of magnitude of 10^{-7} cm²/sec, while the lactoferrin and mAb B results are significantly more scattered and range in extreme cases to values of order 10^{-9} cm²/sec. Given the scatter, it was difficult to identify patterns among resins and proteins, but fairly rapid diffusion rates can be observed under the right conditions for both large and small proteins in all media types studied.

4.6 Modeling of Protein Displacement

The type of exchange seen in FRAP in media with high protein loadings has some similarities to protein displacement under column conditions, which has also been observed to be very slow [11, 1, 18, 19]. One interpretation of this slow displacement, for mAbs on Capto S, is that it is governed by single-file Maxwell-Stefan (SFMS) diffusion within the pore space, which is restricted by the presence of dextran [1, 18] (see apparent porosities in Table 3). The SFMS model, modified to allow kinetic exchange, was also applied to the FRAP data presented above, but the fits obtained were generally poor. This is perhaps not surprising in view of the significant dominance of kinetic effects in most of the data obtained.

The converse exercise was also undertaken of using the PDK model, with the parameter values determined above, to model displacement. Displacement data were obtained for smaller proteins (lysozyme and lactoferrin) on Capto S; these proteins were selected because of the availability of isotherm and FRAP data, and more importantly because their size would allow less restrictive diffusion within the pore space of Capto S than would mAbs. Specifically, the estimated pore accessibilities tabulated in Table 3 are 0.64 for lysozyme, 0.20 for lactoferrin and 0.09-0.15 for mAbs. Confocal concentration profiles from the displacement experiment are shown in Figure 10. Some slight particle movement occurred during the experiment, but it is apparent that displacement of the lysozyme by lactoferrin is significantly hindered, taking 132 minutes to reach a surprisingly small fraction of saturation.

A pore diffusion-kinetic model was developed by applying Eq. 5 to a chromatographic particle, assuming a convective boundary condition, as described in the Supplemental Information. Multicomponent adsorption was accounted for using a multicomponent SMA isotherm [86] for consistency with the original work [1, 19]. Model predictions were calculated based on the averages of FRAP experimental results at similar conditions (Table

6) and SMA isotherm fits of previously published isotherm data [12]; therefore no adjustable parameters were used.

As with the kinetic off-rates, apparent pore diffusivities used in the model were averaged from FRAP measurements of protein diffusion in particles saturated with protein, which suggest a higher apparent pore diffusivity for lactoferrin than for the smaller lysozyme. These diffusivities reflect trends illustrated in Figure 7 and summarized in Table 5, where the more-strongly bound lysozyme appeared to have a smaller pore diffusivity and smaller kinetic off-rate than the more-weakly bound lactoferrin. Interestingly, these values agree with the trend observed for apparent pore diffusivities measured under identical uptake conditions. Accounting for the apparent porosities, the apparent pore diffusivities were 7.4×10^{-7} and 9.0×10^{-7} cm²/sec respectively for lysozyme and lactoferrin in Capto S at 100 mM T.I.S. pH 7.0 [88]. The diffusivities measured by FRAP under fully-loaded conditions are understandably smaller than those observed under uptake conditions, though they should be used with caution, given the measurement uncertainties and the confounding effect of the kinetically-dominated FRAP recovery profiles.

The predicted displacement, shown as simulated confocal images in Figure 10, progresses even more slowly than observed in the experiment, so an unusually slow diffusional contribution, as reflected in the SFMS model, appears not to be a significant factor. There may be several explanations for the discrepancies between prediction and observation. The experimental variability inherent in the fitted FRAP parameters introduces some uncertainty, as does the more diffuse boundary predicted by the model than seen in the experiment. Finally, any possible experimental drift in the z-plane away from the center of the particle will slightly exaggerate uptake. It is heartening to observe that the general form and rate of displacement are captured by this model, as a standard pore diffusion model would predict completion of displacement well within the time frame of this experiment.

Given this success, the similar confocal data for mAb displacement [19] were modeled using the FRAP data on mAbs reported above. These mAbs are not the same as those used by Tao et al., but the FRAP data used were an average of those for mAbs A and B under fully-loaded conditions at similar ionic strengths and equilibrium capacities as reported by Tao et al.; the parameter values are summarized in Table 6. The model predictions in Figure 10 show excellent agreement with the experimental data, and again suggest the possibility that binding kinetics plays a role in the slow displacement rates observed in this media.

4.7 Experimental Modifications

While the geometrical constraints of two-dimensional bleaching provide a less-than-perfect framework for fitting transport parameters, the data presented here provide a good comparison of resin behavior under similar conditions. To improve estimates of parameters for predictive modeling, several improvements could be made, including in the spherical bleaching model necessitated by the relatively-high numerical aperture lens. Ideally, a lens with a much smaller numerical aperture would be used, resulting in a Gaussian bleaching profile and allowing a more accurate recovery fit [80]. Such a change would likely come at the expense of higher magnifications, which are necessitated due to the small particle sizes.

This change would also result in a loss of model simplicity, but would help address issues such as fitted diffusivities faster than the free-solution diffusivity.

Additionally, the possibility of recovery rates depending on bleach amount should be explored in more detail to determine whether this is a real issue or merely an artifact of the model or bleach geometry. Significant scatter is observed in the data reported here, so improvements in data collection and model accuracy would help.

Finally, FRAP measurements may be affected by photobleaching resulting in sample heating, covalent crosslinking of dye molecules, or damage to the proteins or media [46]. Axelrod [89] has addressed sample heating issues and estimated that a temperature change of less than one degree Celsius would occur as a result of laser bleaching. While it is possible that covalent crosslinking of dye molecules and proteins can occur, at the dye concentrations used here (~1% labeling) at most ~2% of the total protein would be irreversibly affected by this damage. The remaining 98% of unbleached and undamaged protein would still be free to exchange and diffuse. Finally, concerns of protein or resin damage are mitigated by the laser wavelengths necessary to excite the selected dye molecules. All bleaching is performed at wavelengths well above 450 nm, and therefore outside the range in which biomolecules tend to absorb light [46].

5 Conclusions

FRAP techniques offer a unique insight into protein mobility and exchange in protein-loaded resin, allowing a wealth of information to be rapidly gathered about transport in chromatographic media. Adaptation of FRAP theory to place transport and kinetics within the framework of established chromatographic models allows kinetic parameters and diffusivities to be determined for proteins on a variety of chromatographic materials. Application of the method to several different protein/media systems shows that protein mobility in chromatographic media may be affected by a number of factors, including the binding strength k' , the protein loading, the presence of polymer modification and the pore architecture, among others. The data show a particularly strong dependence of FRAP-estimated protein binding kinetics on the binding strength k' , with weaker, protein-specific dependence on the protein loading and polymer modification. Studies such as these may yield additional insights into the unique transport properties of polymer-modified materials, especially in the case of slow displacement effects.

Beyond our emphasis here on understanding transport-kinetic mechanisms, the combination of parameter estimates with more extensive distributed chromatographic models can benefit chromatographic practice as well. This is seen most clearly in predictive modeling using the pore diffusion-kinetic model for two-protein displacement in chromatographic media, which compares favorably with experimental data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for financial support from the National Science Foundation under grant CBET-0828590, as well as for the donation of the monoclonal antibodies used in this work by Amgen Inc. The use of confocal microscopes at the Delaware Biotechnology Institute Bioimaging Facility, which is supported in part by NIH grants P30 GM103519 and P20 GM103446 from the IDeA program of the National Institute of General Medical Sciences, is also appreciated.

Bibliography

1. Tao Y, Chen N, Carta G, Ferreira G, Robbins D. *AIChE J.* 2012; 58:2503–2511.
2. Stone MC, Tao Y, Carta G. *J. Chrom. A.* 2009; 1216:4465–4474.
3. Dismer F, Petzold M, Hubbuch J. *J. Chrom. A.* 2008; 1194:11–21.
4. Langford JF, Xu X, Yao Y, Maloney SF, Lenhoff AM. *J. Chrom. A.* 2007; 1163:190–202.
5. Yao Y, Lenhoff AM. *J. Chrom. A.* 2006; 1126:107–119.
6. Dziennik SR, Belcher EB, Barker GA, Lenhoff AM. *Biotechnol. Bioeng.* 2005; 91:139–153. [PubMed: 15889407]
7. Staby A, Jensen IH. *J. Chrom. A.* 2001; 908:149–161.
8. Chang C, Lenhoff AM. *J. Chrom. A.* 1998; 827:281–293.
9. Nash DC, Chase HA. *J. Chrom. A.* 1998; 807:185–207.
10. Müller E. *Chem. Eng. Technol.* 2005; 28:1295–1305.
11. Bowes BD, Traylor SJ, Timmick SM, Czymmek KJ, Lenhoff AM. *Chem. Eng. Technol.* 2012; 35:91–101.
12. Bowes BD, Koku H, Czymmek KJ, Lenhoff AM. *J. Chrom. A.* 2009; 1216:7774–7784.
13. Bowes BD, Lenhoff AM. *J. Chrom. A.* 2011; 1218:4698–4708.
14. Staby A, Sand M-B, Hansen RG, Jacobsen JH, Andersen LA, Gerstenberg M, Bruus UK, Jensen IH. *J. Chrom. A.* 2004; 1034:85–97.
15. Suda EJ, Thomas KE, Pabst TM, Mensah P, Ramasubramanyan N, Gustafson ME, Hunter AK. *J. Chrom. A.* 2009; 1216:5256–5264.
16. Tugcu N, Roush DJ, Go KE. *Biotechnol. Bioeng.* 2008; 99:599–613. [PubMed: 17680666]
17. Shi Q-H, Jia G-D, Sun Y. *J. Chrom. A.* 2010; 1217:5084–5091.
18. Perez-Almodovar EX, Wu Y, Carta G. *J. Chrom. A.* 2012; 1264:48–56.
19. Tao Y, Perez-Almodovar EX, Carta G, Ferreira G, Robbins D. *J. Chrom. A.* 2011; 1218:8027–8035.
20. Dziennik SR, Belcher EB, Barker GA, DeBergalis MJ, Fernandez SE, Lenhoff AM. *PNAS.* 2003; 100:420. [PubMed: 12522150]
21. Carta G, Ubiera AR, Pabst TM. *Chem. Eng. Technol.* 2005; 28:1252–1264.
22. Traylor SJ, Xu X, Lenhoff AM. *J. Chrom. A.* 2011; 1218:2222–2231.
23. Martin C, Iberer G, Ubiera AR, Carta G. *J. Chrom. A.* 2005; 1079:105–115.
24. Burghardt TP, Axelrod D. *Biophys. J.* 1981; 33:455–467. [PubMed: 7194696]
25. Tilton RD, Gast AP, Robertson CR. *Biophys. J.* 1990; 58:1321–1326. [PubMed: 2291948]
26. Yuan Y, Velev OD, Lenhoff AM. *Langmuir.* 2003; 19:3705–3711.
27. Moussaoui M, Benlyas M, Wahl P. *J. Chrom.* 1992; 591:115–120.
28. Johnson EM, Berk DA, Jain RK, Deen WM. *Biophys. J.* 1995; 68:1561–1568. [PubMed: 7787041]
29. Poitevin E, Wahl P. *Biophys. Chem.* 1988; 31:247–258. [PubMed: 2466497]
30. Pluen A, Netti PA, Jain RK, Berk DA. *Biophys. J.* 1999; 77:542–552. [PubMed: 10388779]
31. Schröder M, von Lieres E, Hubbuch J. *J. Phys. Chem. B.* 2006; 110:1429–1436. [PubMed: 16471694]
32. Axelrod D, Koppel DE, Schlessinger J, Elson EL, Webb WW. *Biophys. J.* 1976; 16:1055–1069. [PubMed: 786399]
33. Reits EAJ, Neefjes JJ. *Nat. Cell Biol.* 2001; 3:E145–E147. [PubMed: 11389456]

34. Edidin M, Zagayansky Y, Lardner TJ. *Science*. 1976; 191:466–468. [PubMed: 1246629]
35. Soumpasis DM. *Biophys. J.* 1983; 41:95–97. [PubMed: 6824758]
36. Meyvis TKL, De Smedt SC, Van Oostveldt P, Demeester J. *Pharm. Res.* 1999; 16:1153–1162. [PubMed: 10468014]
37. Thompson NL, Burghardt TP, Axelrod D. *Biophys. J.* 1981; 33:435–454. [PubMed: 7225515]
38. Kaufman EN, Jain RK. *Biophys. J.* 1990; 58:873–885. [PubMed: 2248992]
39. Phair RD, Misteli T. *Nat. Rev. Mol. Cell Biol.* 2001; 2:898–907. [PubMed: 11733769]
40. Phair RD, Gorski SA, Misteli T. *Methods Enzymol.* 2004; 375:393–414. [PubMed: 14870680]
41. Carrero G, McDonald D, Crawford E, de Vries G, Hendzel MJ. *Methods*. 2003; 29:14–28. [PubMed: 12543068]
42. Sprague BL, Pego RL, Stavreva DA, McNally JG. *Biophys. J.* 2004; 86:3473–3495. [PubMed: 15189848]
43. Sprague BL, McNally JG. *Trends Cell Biol.* 2005; 15:84–91. [PubMed: 15695095]
44. Zadeh KS, Montas HJ, Shirmohammadi A. *Theor. Biol. Med. Modell.* 2006; 3:36.
45. Gordon GW, Chazotte B, Wang XF, Herman B. *Biophys. J.* 1995; 68:766–778. [PubMed: 7756543]
46. Goldman, RD.; Swedlow, JR.; Spector, DL., editors. *Live Cell Imaging*. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2010.
47. Elson EL. *Annu. Rev. Phys. Chem.* 1985; 36:379–406.
48. Masamune S, Smith JM. *AIChE J.* 1964; 10:246–252.
49. Ruthven, DM. *Principles of Adsorption and Adsorption Processes*. New York: John Wiley & Sons, Inc; 1984.
50. Barrer RM. *J. Phys. Chem.* 1953; 57:35–40.
51. Wesselingh, JA.; Krishna, R. *Mass Transfer in Multicomponent Mixtures*. Delft: Delft University Press; 2000.
52. Arve BH, Liapis AI. *AIChE J.* 1987; 33:179–193.
53. Yoshida H, Maekawa M, Nango M. *Chem. Eng. Sci.* 1991; 46:429–438.
54. Yoshida H, Yoshikawa M, Kataoka T. *AIChE J.* 1994; 40:2034–2044.
55. Farnan D, Frey DD, Horváth C. *J. Chrom. A.* 2002; 959:65–73.
56. Fang F, Szleifer I. *PNAS*. 2006; 103:5769–5774. [PubMed: 16571661]
57. Genzer J, Arifuzzaman S, Bhat RR, Efimenko K, Ren C-L, Szleifer I. *Langmuir*. 2012; 28:2122–2130. [PubMed: 22181708]
58. Rabe M, Verdes D, Seeger S. *Adv. Colloid Interface Sci.* 2011; 162:87–106. [PubMed: 21295764]
59. Fang F, Satulovsky J, Szleifer I. *Biophys. J.* 2005; 89:1516–1533. [PubMed: 15994887]
60. Satulovsky J, Carignano MA, Szleifer I. *PNAS*. 2000; 97:9037–9041. [PubMed: 10908651]
61. Wetter LR, Deutsch HF. *J. Biol. Chem.* 1951; 192:237–242. [PubMed: 14917670]
62. Plate K, Beutel S, Buchholz H, Demmer W, Fischer-Frühholz S, Reif O, Ulber R, Scheper T. *J. Chrom. A.* 2006; 1117:81–86.
63. Canfield RE. *J. Biol. Chem.* 1963; 238:2698–2707. [PubMed: 14063294]
64. Moore SA, Anderson BF, Groom CR, Haridas M, Baker EN. *J. Mol. Biol.* 1997; 274:222–236. [PubMed: 9398529]
65. Sophianopoulos AJ, Rhodes CK, Holcomb DN, Van Holde KE. *J. Biol. Chem.* 1962; 237:1107–1112. [PubMed: 13915231]
66. Groves ML. *JACS*. 1960; 82:3345–3350.
67. Hubbuch J, Kula MR. *Bioprocess. Biosyst. Eng.* 2008; 31:241–259. [PubMed: 18196281]
68. Ubiera AR, Carta G. *Biotechnol. J.* 2006; 1:665–674. [PubMed: 16892315]
69. Tao Y, Carta G, Ferreira G, Robbins D. *J. Chrom. A.* 2011; 1218:1519–1529.
70. Dziennik, SR. Ph.D. thesis. University of Delaware; 2002.
71. Mattisson C, Roger P, Jönsson B, Axelsson A, Zacchi G. *J. Chrom. B.* 2000; 743:151–167.
72. Wesselingh JA, Bosma JC. *AIChE J.* 2001; 47:1571–1580.

73. Sun Y, Yang K. Sep. Purif. Technol. 2008; 60:180–189.
74. van den Broeke LJP. AIChE J. 1995; 41:2399–2414.
75. Chen YD, Yang RT. AIChE J. 1991; 37:1579–1582.
76. Kapoor, A.; Yang, RT.; Wong, C. Surface Diffusion. New York: Marcel Dekker, Inc.; 1989.
77. Krishna R, Wesselingh JA. Chem. Eng. Sci. 1997; 52:861–911.
78. Riekert L. AIChE J. 1971; 17:446–454.
79. Kubitscheck U, Wedekind P, Peters R. Biophys. J. 1994; 67:948–956. [PubMed: 7811951]
80. Braeckmans K, Peeters L, Sanders NN, De Smedt SC, Demeester J. Biophys. J. 2003; 85:2240–2252. [PubMed: 14507689]
81. Blonk JCG, Don A, Van Aalst H, Birmingham JJ. J. Microsc. 1993; 169:363–374.
82. Kubitscheck U, Wedekind P, Peters R. J. Microsc. 1998; 192:126–138.
83. Crank, J. Mathematics of Diffusion. 1st ed.. London: Oxford University Press; 1956.
84. Boardman NK, Partridge SM. Biochem. J. 1955; 59:543–552. [PubMed: 14363145]
85. Nédélec F, Surrey T, Maggs AC. Phys. Rev. Lett. 2001; 86:3192–3195. [PubMed: 11290140]
86. Brooks CA, Cramer SM. AIChE J. 1992; 38:1969–1978.
87. Tao Y, Carta G, Ferreira G, Robbins D. J. Chrom. A. 2011; 1218:1530–1537.
88. Traylor SJ, Lenhoff AM. 2014 in preparation.
89. Axelrod D. Biophys. J. 1977; 18:129–131. [PubMed: 851570]

Figure 1.

Adsorption isotherms and experimental FRAP conditions for lysozyme in pH 7.0 10 mM phosphate buffer (panel *a*), lactoferrin in pH 7.0 10 mM phosphate buffer (panel *b*), mAb A in pH 5.0 10 mM acetate buffer (panel *c*), and mAb B in pH 5.0 10 mM acetate buffer (panel *d*). Large symbols indicate FRAP experimental conditions, while small symbols indicate individually-measured isotherm points for the mAbs. Ionic strengths are designated by symbol as well as by color, with SP Sepharose FF, SP Sepharose XL and Capto S at 20 mM T.I.S. for lactoferrin and lysozyme and 6 mM T.I.S. for the mAbs indicated respectively by resin with black upright triangles, squares and circles. Results at 100 mM T.I.S. are represented respectively by resin with red upside-down triangles, diamonds, and pentagrams.

Figure 2.

Isocratic retention data for lysozyme, lactoferrin (panel *a*: 10 mM phosphate buffer, pH 7.0) and the two mAbs (panel *b*: 10 mM acetate buffer, pH 5.0) on SP Sepharose FF (triangles), SP Sepharose XL (squares) and Capto S (circles). Some data for lysozyme and lactoferrin are adapted from previously published results [12], as discussed in the text.

Figure 3.

False-color image showing the three-dimensional profile of the LSM 780, 488 and 561 nm laser beams on bleaching DyLight 488-labeled mAb A on Capto S at pH 5.0 10 mM acetate. Panel *a* shows a single x-y plane slice through the bleached region, while panel *c* shows a similar view but looking through a composite projection of the entire particle. Panel *b* shows a single x-z slice through the particle and the bleached region, illustrating the z-depth of the bleached region and the conical bleaching profile. Panel *d* plots the intensity profile from

panel *b* as well as various mathematical approximations of the laser beam point spread function.

Figure 4.

FRAP recovery rates estimated using the pore diffusion-kinetic model (Eqs. 10 and 11) as a function of the bleached amount. Partially-loaded lactoferrin is investigated in each case on Capto S (square symbols) and SP Sepharose FF (circular symbols) at 10 mM phosphate, pH 7.0. Error bars represent the 95% confidence interval of each fitted parameter.

Figure 5.

Confocal image of a particle immediately after bleaching showing the bleached region, the concentric radial bands that are averaged and plotted to measure recovery and the external control regions used for intensity normalization. The bar chart in *b* shows the radially-averaged and normalized intensity profile for two different radial averaging band sizes. The coarse band corresponds to the concentric circles on the confocal image.

Figure 6.

FRAP comparison of partially-loaded lysozyme (panel *a*), more completely-loaded lysozyme (panel *b*) and more completely-loaded lactoferrin (panel *c*) on Capto S in 10 mM phosphate buffer, pH 7.0. Each plot shows a different radial fraction, with the uppermost plot being for the central portion of the bleached region and the lowest plot being for the outermost ring of the bleached region. Pore diffusion-kinetic results were fit using Eqs. 10 and 11, while the surface diffusion-kinetic results were fit using Eqs. 12 and 13. Fitted parameters are summarized in Table 4.

Figure 7.

Comparison of results for fully-loaded lysozyme and lactoferrin on SP Sepharose FF, XL and Capto S in 10 mM phosphate buffer, pH 7.0, adjusted to 20 and 100 mM total ionic strength with NaCl. Each plot shows recovery in a different radial slice, with the uppermost plot being for the central portion of the bleached region and the lowest plot being for the outermost ring of the bleached region. Pore diffusion-kinetic results were fit using Eqs. 10 and 11, while the surface diffusion-kinetic results were fit using Eqs. 12 and 13.

Figure 8.

Pore diffusivities and kinetic off-rates fitted using the pore diffusion-kinetic model (Eqs. 10 and 11) for all proteins on all media as a function of the bound protein concentration q and the isocratic retention factor $\log(k')$, which was estimated from Eq. 14 and the data in Table 3. 3-D plots: lysozyme results are plotted as tetrahedrons and lactoferrin results as stars in 10 mM phosphate, pH 7.0, and 20 and 100 mM T.I.S. MAb A results are plotted as spheres and mAb B results as cubes in 10 mM acetate, pH 5.0, and 6 and 100 mM T.I.S. Results for SP Sepharose FF, SP Sepharose XL and Capto S are shown in red, blue and black, respectively.

2-D projections: lysozyme, lactoferrin, mAb A and mAb B are plotted in blue, black, red and green respectively. Higher ionic strength results are represented by filled symbols and lower ionic strength results by open symbols. SP Sepharose FF, SP Sepharose XL and Capto S results are represented by triangles, squares and circles, respectively. The graph window has been limited to omit several fitted diffusivity and kinetic data points that were unconstrained and had no impact on the fitted recovery curve. Similar unconstrained parameters that remain within the graph window are easily identifiable by their wide error margins.

Figure 9.

Plot of fitted pore diffusion-kinetic (Eqs. 10 and 11) FRAP off-rates as a function of the isocratic retention factor $\log(k')$, which was estimated from Eq. 14 and the data in Table 3. Panel *a* shows lysozyme results and panel *b* shows lactoferrin results at pH 7.0, 10 mM phosphate, 20 and 100 mM T.I.S. Panel *c* shows mAb A results and panel *d* shows mAb B results at pH 5.0, 10 mM acetate, 6 and 100 mM T.I.S. Higher ionic strength results are represented by filled symbols and lower ionic strength results by open symbols. SP Sepharose FF, SP Sepharose XL and Capto S results are represented by triangles, squares and circles, respectively.

Figure 10.

The upper left set of images shows the displacement of lysozyme by lactoferrin in a Capto S particle of diameter 98 μm . Particles were equilibrated at 2.0 mg/mL lysozyme then switched to 2.0 mg/mL lactoferrin at time zero. The upper right set of images is taken directly from [19] and shows displacement of monoclonal antibody charge variants in a Capto S particle of diameter 53 μm . The lower right set of images show the pore diffusion-kinetic displacement model prediction using parameters from Table 3 and Table 6.

Table 1

Summary of relevant protein properties.

	Lysozyme	Lactoferrin	mAb A	mAb B
pI	11.4 [61]	8.8 [62]	8.1 ^(b)	7.9 ^(b)
M _W (kDa)	14.3 [63]	78 [64]	144	144
Radius (nm) ^(a)	1.6	2.8	3.4	3.4
ϵ_{280} (cm ² /mg)	2.64[65]	1.51[66]	1.47 ^(b)	1.47 ^(b)

^(a) Radius of a sphere of equivalent volume^(b) Provided by Amgen Inc.

Table 2

Summary of resin characteristics.

	Base matrix <i>(a)</i>	Polymer modification <i>(a)</i>	Functional group <i>(a)</i>	Mean particle size (μm) <i>(a)</i>	iSEC mean pore size (nm) <i>(b)</i>
SP Sepharose FF	Agarose	None	Sulfonate	90	16 [68]
SP Sepharose XL	Agarose	Dextran	Sulfonate	90	5.1 [68]
Capto S	High-flow agarose	Dextran	Sulfonate	90	4.7–6.2 [69]

(a) Manufacturer-reported information

(b) Performed at 20 mM total ionic strength

Table 3

Summary of fitted isocratic retention parameters and associated 95% confidence intervals using Eq. 14 and isocratic retention data from Figure 2. Additionally shown are the apparent intraparticle porosities for each protein and resin calculated using Eq. 3 from the unretained isocratic retention volumes provided here for completeness as $V_0 = V_{HS} - V_d$.

Protein	pH	Resin	$\epsilon_{p,A}$	V_0 (mL)	$\log(K_{SD})$	Z
LYS	7.0	SP FF	0.96	0.726	-2.01 ± 0.46	4.75 ± 0.76
LYS	7.0	SP XL	0.80	0.841	-1.78 ± 0.30	4.91 ± 0.56
LYS	7.0	Capto S	0.64	0.744	-1.57 ± 0.29	4.40 ± 0.49
LAC	7.0	SP FF	0.65	0.747	-0.45 ± 0.09	9.35 ± 0.65
LAC	7.0	SP XL	0.25	0.107	-2.20 ± 0.18	9.73 ± 0.64
LAC	7.0	Capto S	0.20	0.045	-2.71 ± 0.41	8.88 ± 0.93
mAb A	5.0	SP FF	0.65	0.261	-3.32 ± 0.13	9.18 ± 0.29
mAb A	5.0	SP XL	0.20	0.089	-4.15 ± 0.66	7.83 ± 1.05
mAb A	5.0	Capto S	0.15	0.034	-4.73 ± 0.74	8.73 ± 1.16
mAb B	5.0	SP FF	0.50	0.201	-5.72 ± 1.00	7.57 ± 1.19
mAb B	5.0	SP XL	0.16	0.068	-4.98 ± 1.23	6.01 ± 1.41
mAb B	5.0	Capto S	0.09	0.020	-7.35 ± 1.23	8.98 ± 1.45

Table 4

Summary of FRAP experimental conditions and fitted model results from the representative experiments in Figure 6. The PDK model was fit using Eqs. 10 and 11, while the SDK model was fit using Eqs. 12 and 13. The 95% confidence interval is reported for each fitted parameter, though the experimental variability under the same conditions may be greater or less than this value. The isocratic retention factor $\log(k')$ was estimated from Eq. 14 and the data in Table 3.

Panel	Resin	Protein	T.I.S. (mM)	pH	c (mg/mL)	q (mg/mL)	log(k')	PDK			SDK				
								$k_{off} \times 10^3$ (1/s)	$D_p \times 10^6$ (cm ² /s)	$k_{off} \times 10^3$ (1/s)	$D_s \times 10^{12}$ (cm ² /s)				
a	Capto S	LYS	20	7.0	0.015	89	5.9	70.6	±30.6	13.8	±3.0	20.9	±1.7	9.03	±0.002
b	Capto S	LYS	20	7.0	0.343	276	5.9	2.07	±0.59	0.11	±0.03	0.85	±0.05	2.06	±1.28
c	Capto S	LAC	20	7.0	0.148	312	12.4	0.28	±0.10	0.12	±0.04	0.11	±0.02	0.58	±0.26

Table 5

Summary of FRAP experimental conditions and fitted model results from the FRAP curves shown in Figure 7. The PDK model was fit using Eqs. 10 and 11, while the SDK model was fit using Eqs. 12 and 13. The 95% confidence interval is reported for each fitted parameter, though the experimental variability under the same conditions may be greater or less than this value. Infinity or zero values are reported when the fitted parameters are large or small enough to be insignificant to the model prediction. The isocratic retention factor $\log(k')$ was estimated from Eq. 14 and the data in Table 3.

Panel	Resin	Protein	T.I.S. (mM)	pH	c (mg/ mL)	q (mg/mL)	$\log(k')$	PDK				SDK		
								$k_{\text{off}} \times 10^3$ (1/s)	$D_p \times 10^7$ (cm ² /s)	$k_{\text{off}} \times 10^3$ (1/s)	$D_s \times 10^{12}$ (cm ² /s)			
a	SP FF	LYS	20	7.0	3.8	185	6.1	8.87	±8.14	0.126	±0.102	2.88	±0.53	3.16 ±16.03
b	SP XL	LYS	20	7.0	2.0	320	6.6	7.72	±6.68	0.310	±0.103	2.05	±0.23	0.349 ±0.0002
c	Capto S	LYS	20	7.0	2.4	295	5.9	4.28	±2.86	0.152	±0.072	1.80	±0.16	0.0724 ±0.00003
d	SP FF	LYS	100	7.0	3.5	130	2.8	Inf		0.212	±0.009	~0		502 ±20
e	SP XL	LYS	100	7.0	2.2	220	3.1	30.9	±5.9	5.25	±1.58	17.8	±0.6	2.18 ±0.0002
f	Capto S	LYS	100	7.0	2.1	240	2.8	20.7	±6.7	0.433	±0.053	~0		180 ±14
g	SP FF	LAC	100	7.0	3.1	160	8.9	0.376	±0.136	0.371	±0.491	0.249	±0.022	5.87 ±2.85
h	SP XL	LAC	100	7.0	3.1	150	7.5	7.25	±3.03	0.555	±0.097	~0		204 ±24
i	Capto S	LAC	100	7.0	2.9	170	6.2	37.5	±32.1	2.73	±0.83	~0		711 ±132

Summary of parameters for the pore diffusion-kinetic model used to predict protein displacement in Capto S. SMA isotherm parameters for lysozyme and lactoferrin were fitted to previously-published experimental data [12], while the parameters for the mAbs were used exactly as published in the original work [69]. Off-rates and diffusivities were obtained by averaging FRAP experimental results at similar conditions, while film mass transfer rates have relatively-insignificant effects and obtained directly from ref.[87].

Table 6

SMA Isotherm Parameters									
Protein	q_0 (mM)	C_1 (mM)	K_e	z	σ	k_{off} (1/s)	D_p (cm ² /s)	k_f (cm/s)	
LYS	220	14.8	1.68×10^3	4.4	5.89	3.13×10^{-3}	5.46×10^{-8}	0.0015	
LAC	220	14.8	37.7	8.9	39.5	1.34×10^{-4}	1.73×10^{-7}	0.0015	
mAb	220	20	Obtained from [69]			1.91×10^{-4}	3.21×10^{-8}	0.0015	