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Fluorescence recovery after photobleaching investigation of protein transport and exchange in chromatographic media

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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 multi-component 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. Finally, 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 numerous manufacturers,are distinguished by their high static binding capacities that result from the extension of protein sorption from 2D adsorption on a surface to 3D partitioning into the polymer [10,12]. Despite the additional barrier to transport presented by the polymer layer, dynamic binding capacities (DBC) are often high [2,13–17], 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 [8,20,21], 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.

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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 [32–36]. Kinetically-controlled models have also been adopted [37–40], some of which also considered diffusive contributions [24,37]. In the past 10 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 [24,32,33,35]. While irreversibly bound protein may be a relevant concern in cellular mechanisms and processes, there is less fundamental basis to assume its 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 [35,38,42,47].

Applying FRAP to analyzing sorption and transport in chromatographic media adds the additional complication that the measurements and hence the transport are inherently in 3D. However, for conventional media the system is still one of coupled diffusion and adsorption, so a similar approach is feasible to that in 2D. 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 [49,50], and Maxwell–Stefan surface diffusion [51]. Fickian pore [21,48,49,52] and Fickian homogeneous [21,24,25,53–55] 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 [1,18]. 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 ($23 \pm 3\,^{\circ}C$) using deionized water from a Millipore (Bedford, MA) Milli-Q system (> 18.2 MΩ cm) and filtered with 0.22 μm 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 μm 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 cm × 1.6 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 replaced by 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 μm 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.

Table 1
Summary of relevant protein properties.

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

[a] Provided by Amgen Inc.
[b] Radius of a sphere of equivalent volume.