Characterization of Lysozyme Adsorption in Cellulosic Chromatographic Materials using Small-Angle Neutron Scattering

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Abstract

Measurements of the nanoscale structure of chromatographic adsorbents and the associated distribution of sorbed protein within the media can facilitate improvements in such media. We demonstrate a new technique for this purpose using small-angle neutron scattering (SANS) to characterize the nano- to microscale structure of the chromatographic media and sorbed protein under conditions relevant for preparative chromatographic separations. The adsorption of lysozyme on cellulosic S HyperCel™ (Pall Corporation), a strong cation exchanger, was investigated by SANS. The scattering spectrum is reduced to three contributions arising from (1) the chromatographic medium, (2) discrete protein molecules, and (3) the distribution of sorbed protein within the medium. These contributions are quantified for a range of protein loadings. The total concentration of protein in the chromatographic media can be quantified from the SANS spectrum and the protein is observed to retain its tertiary structure upon adsorption, within the resolution of the method. Further analysis of the SANS spectra shows that protein adsorption is uniform in the media. These measurement techniques provide new and valuable nanoscale information about protein sorption in chromatographic media.

1. Introduction

The nanoscale architecture of chromatographic media has proven to be challenging to characterize using conventional techniques. Imaging techniques such as optical microscopy are capable of visualizing the macro- and microstructure of these materials, but they lack the required resolution to expose the structural information on the length scale of the analyte. Other methods with nanoscale resolution, such as electron microscopy and atomic force microscopy, require special sample preparation techniques, such that the images may not reflect the behavior *in situ*. In particular, the natural and synthetic polymers widely used especially as base matrices for preparative chromatography are susceptible to structural changes as a result of drying and other steps necessary for sample preparation. Conversely, while characterization techniques that measure surface area or pore distribution, such as inverse size-exclusion chromatography (ISEC), are capable of providing structural information at these length scales, they do not provide a detailed image of the material's architecture. However, knowledge of the architecture of chromatographic media is critical to the mechanistic understanding of their performance. Consequently, both modeling of existing chromatographic media and the design of new media will benefit from novel techniques that reveal the media's nanoscale structure and location of protein within the media under typical operating conditions.

Better architectural understanding is especially useful in the case of stationary phases for protein chromatography. Chromatography is the dominant large-scale separation technique for proteins, such that improvements in efficiency and the development of non-chromatographic alternatives are a subject of current research [1–4]. Virtually all protein purification processes are developed around at least one chromatographic step [5], with an average of roughly three chromatographic steps per process. About 40 % of these steps are ion-exchange chromatography [6]. There is a wide variety of available chromatographic media for protein separation, ranging from conventional porous beads to polymer-

derivatized materials [1,7]. These materials have evolved to accommodate the protein's macromolecular nature, which leads to requirements for the pore accessibility, surface area, and binding characteristics [8]. Indeed, the media's architecture can greatly influence the adsorption, distribution, retention, stability, and transport rates of the protein during the chromatographic process. Hence, there is not only an interest in characterizing the material's architecture, but also in investigating its effect on protein behavior within the material. For example, advances in protein chromatography will benefit from quantitative measurements of protein adsorption internal to the chromatographic materials. Consequently, there is a need for new characterization techniques able to measure the intrinsic structure of the materials as well as the nanoscale distribution of protein in the stationary phase under practically relevant physiochemical conditions and preferably, without altering the protein and its adsorption characteristics by chemical labeling.

Here, we investigate a novel application of a nanoscale characterization technique, namely small-angle neutron scattering (SANS), to measure (1) the structure of chromatographic media and (2) protein adsorption in these chromatographic media *in situ* with protein-scale resolution [9–12]. Although neutron scattering techniques are well-established in the scientific community, to our knowledge they have never been demonstrated in the literature previously to study protein adsorption in chromatographic media. SANS is particularly well suited for this application as it is capable of resolving spatial features from the nanometer to the micrometer length scale and can penetrate sufficiently through opaque materials to quantitatively determine the internal structure [9]. In addition, neutron scattering techniques are minimally disruptive and allow the study of both static and dynamic aspects of the structure [13]. SANS experiments can also exploit contrast-matching by isotope substitution to improve resolution and isolate individual components in a mixture, which is especially valuable when probing samples comprised primarily of carbon and elements with similar atomic numbers for which there is very weak contrast in X-rays. However, a comparative

disadvantage of SANS is the limited access to, and comparatively low fluxes of,
neutron sources.

In this work, we validate SANS as a feasible technique to study protein
adsorption in chromatographic media by investigating the adsorption of lysozyme
in S HyperCel™ (Pall Corporation). This chromatographic medium shows
excellent performance, presumably partly as a consequence of its distinctive
structure when compared to conventional or polymer-derivatized media [14,15].

However, methods such as electron microscopy show relatively featureless
protein distributions locally within the resin, prompting an interest in alternative
characterization methods. To this end, we present a framework for the
quantitative analysis of protein adsorption in these systems by direct comparison
of the scattering spectra with and without protein adsorption. Clear changes in
the scattering spectrum are observed upon protein adsorption. Analysis of the
spectra yields quantitative knowledge about the extent and distribution of protein
adsorption in the chromatographic particles.

2. Materials and Methods

2.1. Buffers

Monobasic sodium phosphate (NaH_2PO_4), sodium chloride (NaCl), and
deuterium oxide (D_2O) at 99.8 atom % D were purchased from Fisher Scientific
(Fair Lawn, NJ) and used to prepare 10 mM sodium phosphate buffer solutions in
both deionized (DI) water (H_2O) and D_2O at pH 7. The total ionic strength (TIS) in
the H_2O solutions was adjusted using NaCl to 10 mM, 50 mM, and 100 mM TIS.
In addition, unbuffered solutions of 20 mM, 100 mM, and 200 mM TIS NaCl in
 D_2O were prepared and used to manipulate contrast in the scattering
experiments.

2.2. Protein Solutions

Solutions were prepared by dissolving lyophilized hen egg white lysozyme (molecular weight [MW] 14.3 kD, Sigma-Aldrich) in the desired TIS-controlled buffer and concentrated using 10K Amicon centrifugal filters. Concentrated samples were diluted with H₂O-based buffer and reconcentrated three times for the 10 mM TIS solution and twice for the 50 mM and 100 mM TIS solutions. Protein concentrations were determined using UV spectrophotometry (Shimadzu UV-1700 or Thermo Scientific NanoDrop 2000). The protein concentrations in stock solutions were 108.2 mg/mL for the 10 mM TIS solution, 100.7 mg/mL for the 50 mM TIS solution, and 115.0 mg/mL for the 100 mM TIS solution.

2.3. Chromatographic Media

S HyperCel™ (lot AU31072012-4) was provided by Pall Corporation (Northborough, MA). The chromatographic particles are synthesized from a cellulosic base matrix with a sulfonate ligand for cationic exchange (CEX). The average particle diameter is reported to be 75-80 µm. The chromatographic particles were washed three times by suspending in DI H₂O and then centrifuging and decanting. This was followed by solvent exchange with D₂O by repeating this procedure twice by suspending in D₂O and letting the solution equilibrate for a twelve-hour interval.

2.4. Sample Preparation

For measurement of the chromatographic particle structure, a sample was prepared by equilibrating 0.5 mL hydrated particle volume (hvp) of chromatographic particles with a 20 mM TIS solution in 100 mole % D₂O.

For measurement of protein adsorption, a series of samples was prepared for different TIS conditions and protein concentrations. Each sample was prepared with (1) 0.5 mL hvp of chromatographic particles equilibrated with pure D₂O, (2) a volume of protein solution calculated to yield the desired final protein

concentration, and (3) D₂O solution to bring the total sample volume to 10 mL. As the D₂O solutions were not buffered, the pH of these samples was not controlled.

Samples were equilibrated by gentle rotation over a 48-hour period, after which protein concentrations in the supernatant solutions were measured using UV spectrophotometry. The settled chromatographic particle slurry, consisting of approximately 10 vol % protein, 20 vol % cellulosic particles, and 70 vol % water, depending on the sample, was separated from the supernatant to use in the neutron scattering experiment.

The adsorbed amount q (mg/mL hpv) was determined by mass balance

$$q = \frac{V}{V_m} (C_0 - C), \quad (1)$$

where V (mL) is the total solution volume, V_m (mL hpv) is the hydrated particle volume (hpv), C_0 (mg/mL) is the initial protein concentration, and C (mg/mL) is the final protein concentration in the supernatant.

Six samples were prepared at three different total ionic strengths (TIS) of nominally 40 mM, 100 mM, and 200 mM, with the actual sample compositions given in Table 1. The D₂O:H₂O fraction in the solvent varied among the samples as H₂O is introduced into the samples in proportion to the protein concentration via the protein solution. The amount of H₂O in the sample has an important effect on the background scattering and scattering intensity and Table 1 lists the D₂O fractions for the samples.

The six samples varied in initial protein concentration C_0 , ranging from 3.68 g/mL to 22.7 g/mL. These values, along with the measured final protein concentration in the supernatant after equilibration C , enable calculation of the adsorbed protein concentration q via Equation 1. The values for q range from 81.6 mg/mL hpv to 109.7 mg/mL hpv, as included in Table 1. In general, the extent of protein adsorption is expected to increase with protein concentration and decrease with TIS [15]. Previous reports for this system show somewhat higher adsorbed amounts than measured here [15]. The lower adsorption might be due to several factors, including the lack of pH control and D₂O being a major

component of the solvent. D₂O has stronger hydrogen bonding than H₂O, which
186 may affect the adsorption strength [16]. Because of the variations in solvent
composition and TIS, the q values in Table 1 do not represent a true isotherm.
188 However, neither this nor such variations as that of pH is significant for the
present work, as our main goal is to obtain samples differing in protein
190 concentration in the stationary phase for subsequent SANS characterization. The
range of conditions explored was sufficiently limited such that we do not
192 anticipate qualitative changes in the nature of adsorption.

The total protein concentration in the particle slurry is determined by both the
194 adsorbed protein in the particles as well as the protein in the surrounding
solution. The values, ranging from 83.5 mg/mL hpv to 117.5 mg/mL hpv, were
196 calculated from the protein concentrations in the cellulosic particles (q) and the
surrounding water (C) in each sample and are included in Table 1.

2.5. Small-Angle Neutron Scattering

Small-angle neutron scattering (SANS) arises from fluctuations in the
202 scattering length density, which generally reflects structural heterogeneities [9–
11]. The scattered intensity of neutrons is collected as a function of their
204 deflection from the incident beam, measured as the scattering angle θ , which is
typically reported as the magnitude of the momentum transfer vector or
206 scattering vector Q , given by [11]

$$Q = \frac{4\pi}{\lambda} \sin \frac{\theta}{2}, \quad (2)$$

210 in which λ is the wavelength of the neutrons. The momentum transfer vector $\mathbf{Q}$
has dimensions of inverse length, e.g., $\text{\AA}^{-1}$, and experimental data given as a
212 function of $\mathbf{Q}$ are appropriately termed as being in reciprocal space. The
magnitude Q is directly related to the length scale being probed, L , by Bragg's
214 law [9], i.e.,

$$L = \frac{2\pi}{Q}. \quad (3)$$

Hence, structures observed in SANS spectra are inversely related to real-space features in the sample.

The measured intensity $I(Q)$ is proportional to the measured count rate of neutrons at a certain deflection or momentum transfer Q . The measured intensity provides information about the sample structure at the length scale being probed. For simple systems, the intensity can be written as

$$I(Q) \propto (\Delta\rho)^2 \cdot P(Q) \cdot S(Q) + B, \quad (4)$$

in which the factors in the first term on the right-hand side are the contrast factor, the form factor, and the structure factor, respectively. The contrast factor represents the difference in scattering length density (SLD) ρ of the different components in a heterogeneous system, in which the SLD is physically calculated by summing the coherent cross section of all the atoms present in a specific volume [17,18]. The intensity of deflected neutrons is proportional to the squared difference of the SLD between the components. The form factor $P(Q)$ represents the contribution of the shape of the particles or building blocks in the system, while the structure factor $S(Q)$ represents the contribution of the interaction among these components.

The second term on the right-hand side of Equation 4 is the background scattering value B , which is generally independent of Q and provides no structural information, but depends on sample composition. It is typically observed in the high- Q region, where the first term of the equation becomes small. The background scattering is often dominated by the large incoherent scattering cross-section of the hydrogen atom. Hence, the background is primarily dependent on the H (or H_2O) concentration in the system.

The experiments were carried out on the 30 m NG3 SANS instrument at the NIST Center for Neutron Research (NCNR), National Institute of Standards and

Technology (NIST), Gaithersburg MD [19]. The tests were run at three diffractometer settings:

- high Q: 1 m sample-to-detector distance (SDD) with 6 Å neutrons for a 360 s count time,
- intermediate Q: 4 m SDD with 6 Å neutrons for 600 s count time, and
- low Q: 13 m SDD with lenses with 8 Å neutrons for a 900 s count time.

These yield a scattering variable range $0.001 \text{ \AA}^{-1} < Q < 0.4 \text{ \AA}^{-1}$, with a wavelength spread $\Delta\lambda/\lambda$ of 0.15. Demountable quartz window sample cells with a path length (thickness) of 1 mm were used for all samples. All experiments were performed in a 10-cell sample holder at 25 °C using a temperature bath.

2.6. Data Reduction

Standard data reduction procedures were followed using the program IGOR Pro to obtain corrected and radially averaged SANS scattering spectra [20]. Importantly, the varying H₂O content in the samples led to differences in both the background scattering and the scattering intensity. Hence, the data were further reduced in two fundamental ways: (1) removal of the background scattering and (2) scaling of the scattering spectra to account for variation in the contrast factor.

The background scattering (Equation 4) was removed from all samples by fitting the intensity to the expected Porod behavior at large Q [10]:

$$I(Q) \cdot Q^4 \propto A + B \cdot Q^4, \quad (5)$$

in which A is a constant proportional to the surface-to-volume ratio in the sample. This value of the background scattering B was subtracted from the scattering intensity.

The differences in scattering intensity, or contrast, in Equation 4 for the samples probed here are primarily affected by the samples' H₂O concentration (Equation 4). For reasons that are explained and validated in detail later, the spectra were scaled by assuming that the adsorbed protein does not affect the intermediate-Q region, such that intensity changes observed in this Q region can be fully attributed to the difference in scattering contrast. This enabled the quantitative determination of a scaling factor for each scattering spectrum with reference to the spectrum of the pure chromatographic particles via least-squares fitting.

2.7. Chromatographic Particle Modeling

The SANS spectra from the chromatographic particles were modeled by standard expressions to obtain quantitative structural information. Prior work on similar systems, including polymer gel systems [24], has successfully used the generalized Guinier-Porod model [21]. This Guinier-Porod model was shown to be the best representation of the scattering from the chromatographic particles used here [22]. Although this is a generic model, its structure is suitable for the goals of this paper, in which we need only a descriptive model of scattering from the resin.

The generalized Guinier-Porod model is given by

$$I(Q) = \frac{G}{Q^s} \exp\left[\frac{-Q^2 R_g^2}{3-s}\right] + B \quad \text{for } Q \leq Q_1 \quad \text{and}$$

$$I(Q) = \frac{D}{Q^m} + B \quad \text{for } Q > Q_1, \quad (6)$$

in which,

$$Q_1 = \frac{1}{R_g} \sqrt{\frac{(m-s)(3-s)}{2}} \quad \text{and}$$

$$D = G \exp\left[\frac{-Q_1^2 R_g^2}{3-s}\right] Q_1^{m-s}, \quad (7)$$

306 based on continuity constraints. In these expressions, B is the background
308 scattering, G is a scaling coefficient, R_g is the radius of gyration, and m and s are
the Porod exponent and the dimension variable, which are related to the fractal
310 dimensions of the microstructure on short and long real-space length scales,
respectively. The radius of gyration can be obtained from the Q -value at the
312 inflection point Q_1 between these two fractal regions. This radius of gyration is a
characteristic average length scale within the particle structure. Note that the size
314 of the chromatographic particles themselves is of the order of $80\text{ }\mu\text{m}$ and hence,
is not observable with the instrument used.

316 **2.8. Protein Modeling**

318 Protein monomers have a characteristic scattering pattern that is due to their
320 shape, termed the protein form factor contribution. In addition, proteins can have
a structure factor contribution due to local interactions in solution, clustering, and
322 aggregation. Both of these can have an effect on the total scattering spectrum,
but for the analysis used here only the form factor is of importance.

324 The form factor for the protein in its native state can be calculated directly
from knowledge of the protein crystal structure using the program CRYSON [23].
326 CRYSON calculates the form factor of a protein from its atomic structure as
available from the Protein Data Bank (PDB) [24], and can take several effects
328 such as the solvation shell (not applied here) and the solvent composition (100
mole % D_2O) into account. The PDB file used to model lysozyme has the PDB ID
330 6LYS [25].

The CRYSON output is the form factor of a single protein monomer with
332 scattering cross-section expressed in units of barns ($1\text{ b} = 10^{-28}\text{ m}^2$). The
scattering spectrum scales linearly with the protein concentration, so the
334 concentration of the protein was taken into account by multiplication of the
calculated scattering spectrum by the protein concentration. Thus, the protein
336 monomer contribution can be predicted from the protein concentration in the

system, or conversely, the protein concentration can be determined from its contribution to the scattering intensity.

3. Results and Discussion

3.1. Scattering from the Components

Figure 1 shows the scattering spectra obtained from the pure components: the cellulosic chromatographic particles (top panels) and a lysozyme solution (bottom panels). The scattering spectrum for the pure cellulosic particles (Figure 1a) shows a monotonically decreasing profile as a function of the magnitude of the scattering vector Q . The spectrum is continuous and smooth without maxima, which indicates that the structure contains a spectrum of length scales rather than a few well-defined structural length scales. The spectrum can be divided into three regions. The first two exhibit power-law scattering typical of a self-similar or fractal structure, with the fractal dimension related to the slope of the logarithmic plot. In the third region, the spectrum converges to a background, constant value at high Q . After removal of the background value, the power-law slope of the second region is observed to extend into the third region, as shown by the open symbols in Figure 1a.

Qualitatively similar spectra have been reported for hydrogels [26–28] and polymer gel systems. Several studies have been conducted on agarose [13,29] and other polymer gels [30–35]. Indeed, the Q -ranges of the three regions, and hence the corresponding length scales, correspond well to those observed in agarose gels [29].

Using Bragg's law (Equation 3) as an estimate of the length scales of the structures enables comparison of the scattering spectra for the chromatographic particles to structures observed in an SEM image [14] of a related Q HyperCel™ in Figure 1b. The SEM image was obtained using chemical fixation by OsO_4 for contrast. Q and S HyperCel™ are assumed to have almost identical architecture, as the only fundamental difference is in the ion-exchange ligands. The fibrous

network shown is characteristic of natural carbohydrate polymer adsorbents, and appears to contain “pores” on the order of 100-500 Å in characteristic dimension. The low-Q fractal region from 420-6300 Å corresponds to the fractal dimension of the total particle porous structure, while the intermediate-Q fractal region from 160-420 Å corresponds approximately to the denser fractal structure inside of the fibrous network.

Modeling of the chromatographic particle spectrum using the generalized Guinier-Porod model (Equation 5) through least-squares fitting reinforces this interpretation. The fitted model, also shown in Figure 1a, has a slope in the low-Q region of $s = 1.8$, which typically corresponds to a swollen polymer network [10]. The slope of the intermediate-Q region is $m = 2.9$, which is indicative of an almost fully collapsed polymer coil. This corresponds to the observed structures at these length scales in the SEM image. The radius of gyration R_g found for these chromatographic particles is 35 Å. Importantly, this value corresponds well to the mean pore radius as determined via inverse size-exclusion chromatography (ISEC) [14]. ISEC yields a mean pore radius for the cylindrical pore model of 44 Å, and for the Ogston model [36] of 36 ± 4 Å. This demonstrates that SANS provides a direct method to characterize the internal structure of the chromatographic particle.

Panel c in Figure 1 shows the scattering from an unbuffered 11 mg/mL solution of lysozyme in D₂O at 25 °C (data courtesy of P. Douglas Godfrin) compared with the CRYSON prediction. At this low protein concentration, protein-protein interactions are negligible, so that measurements directly reveal the form factor. The CRYSON predictions for scattering from the protein structure are in good agreement with the experimental data.

The corresponding structure of lysozyme, presented using PyMOL from the same PDB file, is shown in Figure 1d [37]. Lysozyme is globular and has approximately the shape of a prolate ellipsoid, with major and minor axes of 48.0 and 27.0 Å, respectively [38]. These real-space length scales correspond well with the decrease in the scattering pattern around 0.15 Å^{-1} , which by Equation 3 corresponds to a characteristic real-space correlation length of 42 Å.

3.2. Scattering from Protein-Laden Chromatographic Particles

3.2.1. Scattering Spectra

The scattering spectra of the chromatographic particles with adsorbed protein (Table 1) are shown in Figure 2, in which Figure 2a depicts the scattering spectra after basic reduction. All the spectra show a monotonically decreasing intensity similar to that for the pure chromatographic particles and the spectra vary systematically with protein concentration. Part of this variation is due to differences in scattering contrast arising from different solvent compositions for different samples. As noted previously, the primary difference is the presence of H₂O introduced during the sample preparation, which leads to varying contrast factors as well as incoherent scattering. To better resolve differences due to protein adsorption in the chromatographic particles, the background is subtracted as described in the Methods section and the intensity is scaled by matching the intermediate-Q region, which, because it is primarily due to the dense backbone of the chromatographic particles, is not directly affected by the presence of protein. These fitted background and scaling values are reported in Table 2.

The result of this advanced reduction is shown in Figure 2b, which compares the reduced spectra before and after protein adsorption. From inspection of this figure, it is clear that protein adsorption changes the scattering spectrum in the low- and high-Q regions (regions I and III). The scattering in the intermediate-Q region is not affected by protein adsorption (region II), confirming the hypothesis that this region corresponds to the scattering from the inner cellulose fibrous structure. The result is similar to the findings of Yadav *et al.*, in which silica nanoparticles are aggregated in the presence of lysozyme [38]. In that work, the SANS also shows that the presence of protein changes the scattering in the low- and high-Q regions, but not the intermediate-Q region. In their case, this intermediate region is dominated by the silica structure.

From these observations, the scattering spectrum can be decomposed into
430 three independent contributions [39], *i.e.*,

$$432 \quad I(Q) = I_p(Q) + I_m(Q) + I_\Delta(Q), \quad (8)$$

434 representing the scattering from the pure chromatographic particles, the protein
monomers, and the change in structure due to the adsorption of the protein,
436 respectively.

It is assumed that protein adsorption does not affect the internal structure of
438 the chromatographic particles. Therefore, the scattering contribution from the
pure chromatographic particles, $I_p(Q)$, in the mixed system is given by the
440 measured spectrum for the media in the absence of protein, as represented by
the generalized Guinier-Porod model.

442 Subtraction of this contribution from the total scattering spectrum reveals the
changes due to the addition of protein to the system, which is shown in Figure
444 2c. Figure 2c shows that, after subtraction of the scattering due to the pure
chromatographic particles, two contributions to the total scattering remain. The
446 change in scattering upon protein adsorption in the high- Q region (region III) is
attributed to the scattering from the protein monomers, $I_m(Q)$. The contribution to
448 scattering in the low- Q region (region I), which corresponds to the fractal region
of the particle structure, $I_\Delta(Q)$, is due to the adsorption of protein in the particle's
450 internal microstructure. The two contributions are discussed in greater detail in
the subsequent subsections.

452 **3.2.2. Region III: Protein Monomers**

454 Figure 2c shows a clear contribution to the scattering intensity from
lysozyme in the high- Q region (Figure 2c) as a shoulder, starting at high Q
456 around $0.3 \text{ \AA}^{-1}$ and increasing with decreasing Q up to a plateau value around
458 $0.07 \text{ \AA}^{-1}$. The shape and location of this feature in the scattering corresponds to
the characteristic lysozyme monomer form factor (Figure 1c) [38,40–42].

An important consideration is the possible presence of a protein structure factor contribution, in addition to the form factor (Equation 4). The presence of a structure factor deviating from unity may interfere with the protein concentration measurement. The structure factor for concentrated lysozyme solutions has been reported as a peak around $Q \approx 0.07 \text{ \AA}^{-1}$ [40,41,43–45]. However, the structure factor peak has been shown to disappear at the higher TISs used here [40,41], and can be safely ignored in this analysis.

Figure 2c compares the measured scattering after subtraction of the protein monomer form factor as obtained by CRYSON, scaled to a lysozyme concentration of $7.0 \times 10^{-6} \text{ mole/mL}$ or 100 mg/mL (MW of lysozyme 14.3 kD). As seen, the model calculation represents the experimental data well, showing the presence of protein in the scattering volume. In fact, the protein concentration in the scattering volume can also be determined by the plateau value evident at the lowest Q values in this region of scattering.

Small deviations in the scattering in the high- Q region as compared to the protein's calculated form factor are not considered critical and may be caused by several factors, including the amount of deuterium-hydrogen exchange on the protein, the temperature [43], the solvation shell, and lysozyme structural dynamics, leading to deviations from the structure in the PDB file. Furthermore, the generalized Guinier-Porod model may not fully capture the data at high Q , so some of the deviation may arise from the model subtraction. Such issues could result in small deviations in the scattering at these high- Q values where the intensity is also close to the background and therefore has a higher uncertainty.

To obtain quantitative protein concentrations from the SANS measurements, the CRYSON curve is fit to the fully subtracted spectra with least-squares fitting, for $Q \geq 0.05 \text{ \AA}^{-1}$. The scaling factor should yield the protein concentration, which includes both the adsorbed protein and the protein in solution. The results are compared in Figure 3 to the protein concentrations as obtained from UV spectrophotometry during sample preparation (Table 1) and are quantitatively accurate, with a ratio between predicted and actual values of 1.03 ± 0.04 . This

has several important consequences. Firstly, this demonstrates that the protein concentration in the system can be calculated from the scattering spectrum. Further, this validates the scaling method used to analyze the scattering spectra, which in turn validates the assumption that the scattering in the intermediate-Q region is independent of the protein adsorption. Finally, it is clear that the adsorbed protein exhibits a scattering spectrum consistent with the folded protein in solution, suggesting that the lysozyme retains its tertiary structure while adsorbed.

3.2.3. Region I: Protein Adsorption

The final contribution to the total scattering spectrum is the apparent change in particle structure due to protein adsorption. This change can result from both an actual change in the cellulosic backbone of the particle, or, because the scattering length density of the protein and the cellulosic media are comparable, simply the filling of the particle's void volume with adsorbed lysozyme.

Figure 2b (region I) shows that protein adsorption slightly increases the fractal dimension the particle's internal structure. However, this change in fractal dimension is relatively small among the different samples, ranging from 2.5 to 2.8, as shown in Table 2, and consequently, is not significant enough to discern a relationship with the amount of adsorbed protein. ~~However, it~~ is statistically meaningful that protein adsorption increases the apparent fractal dimension significantly above that of the pure media itself, which has an apparent fractal dimension of 1.8 in the low-Q region.

~~Furthermore~~Because, this increase in scattering with protein adsorption is uniform in that there are no peaks or other qualitative changes to the scattering spectrum, and, ~~Because~~ the protein and the cellulosic backbone of the particle have a very similar scattering length density, adsorption of the protein can be viewed as a uniform densification of the media, Thus, we interpret the scattering results to indicate that there is no change in the chromatographic media itself, which is consistent with the relatively uniform structure of protein-loaded

HyperCel™ reported previously based on electron microscopy [15]. Consequently, protein loading of these particles can be thought of as a partitioning into the three-dimensional cellulosic structure, instead of as adsorption at an extended two-dimensional surface. This structural similarity to protein sorption in polymer-derivatized materials supports the high static binding capacity and rapid uptake of protein observed in HyperCel™ [5,14,15].

4. Conclusions

Small-angle neutron scattering (SANS) measurements of protein sorption in chromatographic media provide both new and complementary nanoscale to mesoscale structural information that increases our understanding of chromatographic materials and their adsorption characteristics. For the particular system investigated here, lysozyme adsorption on S HyperCel, protein adsorption leads to two major individual contributions to the scattering spectrum. The first, at high-Q values, demonstrates the presence of protein monomers. The second, at low-Q values, demonstrates the uniform adsorption of the protein inside the particle structure. Consequently, protein adsorption into this chromatographic media can be considered to be partitioning into the three-dimensional cellulosic structure, consistent with the rather uniform distribution of protein seen previously by electron microscopy [15].~~In the intermediate-Q region, protein adsorption has no effect on the scattering spectrum, which is dominated by the dense structure of the cellulosic backbone.~~

We also demonstrate that the total concentration of protein in the sample can be accurately determined from the absolute SANS spectrum. In general, the results support the idea that lysozyme adsorbs uniformly in the S HyperCel™ structure, and that this leads to an effective densification of the cellulosic gel-like particle structure. Furthermore, SANS measurements confirm that, to within resolution, the protein retains its tertiary structure during sorption.

These novel measurements demonstrate some of the quantitative capabilities of neutron scattering to resolve protein sorption in chromatographic media.

Further studies will explore how neutron scattering methods can elucidate differences in protein sorption with different proteins and chromatographic media, particularly for systems in which sorption is less uniform. We also anticipate that these methods can be combined with advances in the ability to study protein structure and hydration [12,23,44] to learn more about protein-adsorbent interactions and protein-protein interactions within the chromatographic media.

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710

712 **Figure Captions**

714 Figure 1: Typical SANS spectra and their analyses, applied to the scattering from
S HyperCel™ particles without adsorbed protein (top panels) and lysozyme in
716 solution (bottom panels) in 100 % D₂O solutions: a) Scattering spectrum obtained
from S HyperCel™ particles, fitted by the generalized Guinier-Porod (GGP)
718 model. Corresponding length scales are indicated on top, and the spectrum is
divided into three regions. The spectrum after background removal is shown by
720 the open symbols. b) SEM image of Q HyperCel™ [14], which has the same
microstructure as S HyperCel™. c) Scattering spectrum obtained from a 11
722 mg/mL lysozyme solution, compared to the spectrum for the same concentration
calculated by CRYSON [23] from the 6LYZ Protein Data Bank structure. d)
724 Image of lysozyme drawn using PyMOL [37] from the same PDB file.

726 Figure 2: SANS spectra obtained for cellulosic particles with different amounts of
adsorbed protein (see Table 1 for sample compositions): a) Experimental data
728 after basic reduction using the program IGOR Pro [20], together with the pure-
particle scattering model as shown in Figure 1a. Similarly to that figure, the
730 spectrum is divided into three regions and the real-space length scales are
indicated. b) Spectra after advanced reduction, which takes differences in
732 background and scaling into account. All spectra have been given the same
background for display purposes. c) Spectra after subtraction of the pure-particle
734 model (multiplied by a factor of 0.9 for display purposes). The two different
contributions of protein adsorption are clearly visible. For comparison, the pure
736 lysozyme scattering spectrum as predicted by CRYSON (Figure 1c) is included.

738 Figure 3: Comparison between the protein concentrations in the particle slurry as
determined by (1) UV spectrophotometry during sample preparation (Table 1)
740 and (2) SANS measurements.

Figure 1

Figure 2

Figure 3

Table 1: Composition of the six SANS samples containing a chromatographic particle slurry with adsorbed protein. The total protein concentration in the particle slurry is determined by both the equilibrium concentration of protein adsorbed in the particles q (Equation 1) as well as the equilibrium concentration of protein in the surrounding solution C . In addition, the table shows the total ionic strength (TIS) and the D₂O fraction in the solvent for each sample.

Sample	Protein in particle slurry [mg/mL hpv]	q [mg/mL hpv]	C [mg/mL]	TIS [mM]	D ₂ O fraction in solvent [mole % D ₂ O]
1	83.8	81.6	2.6	183.7	94.1
2	95.3	91.8	4.2	181.8	92.2
3	110.5	105.2	6.3	88.8	88.1
4	116.7	104.6	14.4	84.5	80.0
5	116.9	109.7	8.6	35.2	86.7
6	117.5	102.7	17.6	33.5	78.5

Table 2: Fitted parameters for the SANS spectra of the protein-laden chromatographic particles, with the background B obtained from a Porod analysis, the scaling factor from scaling of the intermediate- Q region (region II) with the pure particle spectrum (Figure 2a and 2b), and the power-law slope from the low- Q region (region I) in the reduced spectrum (Figure 2b).

Sample	Background B [cm ⁻¹]	Scaling factor [-]	Power-law slope (region I) [-]
1	0.21	2.04	-2.51
2	0.24	2.24	-2.55
3	0.25	3.08	-2.70
4	0.32	4.31	-2.78
5	0.28	3.85	-2.81
6	0.32	4.69	-2.83