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Self-interaction chromatography of proteins on a microfluidic monolith

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Abstract

A novel miniaturized system has been developed for measuring protein-protein interactions in solution with high efficiency and speed, and minimal use of protein. A chromatographic monolith synthesized in a capillary is used in the method to make interaction measurements by self-interaction chromatography (SIC) in a manner that, compared to column methods, is more efficient as well as more readily practicable even if only small amounts of protein are available. The microfluidic monolith requires much less protein for both column synthesis and the chromatographic measurements than a conventional SIC system, and in addition offers improved mass transfer and hence higher chromatographic efficiency than for previous SIC miniaturization systems. Protein self-interactions for catalase as a model protein, quantified by measurement of second virial coefficients, B_{22} , were determined by SIC and follow trends that are consistent with previously reported values. Different column derivatization conditions were studied in order to optimize the chromatographic behavior of the microfluidic system for SIC measurements. Chromatographic sensitivity can be further increased by using different column synthesis conditions.

Keywords

protein-protein interactions; protein solution thermodynamics; osmotic second virial coefficient; catalase; chromatographic monolith

1. Introduction

Measurements of protein-protein interactions have great potential value due to their relevance in several research fields. In fundamental biophysical research, in particular in structural biology, protein-protein interactions are used to seek suitable conditions for protein crystallization [1]. In applied industrial research and development, protein interaction measurements can guide characterization and optimization of downstream processing and formulations [2–4]. For example, in the pharmaceutical industry protein-based therapeutic formulations usually require high concentrations of protein to minimize the volume per dose. However, at such high concentrations proteins tend to aggregate and therefore lose their potential as a therapeutic. Pharmaceutical applications require finding optimal conditions where drug formulations are physically stable, that is, solutions without protein aggregates, protein crystals or amorphous protein with a sufficient shelf-life.

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Interaction measurements are also correlated with the formation of other industrially useful protein dense phases, such as precipitates and gels [5].

Protein interaction measurements are most frequently made in terms of the second osmotic virial coefficient, B_{22} , which provides a direct but highly averaged measure of pairwise protein-protein interactions in solution. Such measurements are most commonly made by static light scattering (SLS) [1,6–12], but other methods are sometimes used as well, including osmometry [13,14] and small-angle neutron and X-ray scattering [12,15–18]. While some of these methods have advantages, most of them are relatively slow and require relatively large amounts of protein, making extensive exploration of B_{22} dependence on solution conditions problematic.

A more recent method for measuring B_{22} is self-interaction chromatography (SIC), in which the protein of interest is immobilized on a chromatographic column. The retention time in the column of a pulse of the same protein under isocratic elution conditions then provides a measure of its retardation due to interactions between the free and immobilized protein. The method is a variant of affinity chromatography, and its initial use in characterizing protein-protein interactions was mainly to show interaction trends [19]. However, a detailed statistical mechanical analysis, including consideration of molecular anisotropy, led to a simple relation between B_{22} and the chromatographic retention factor k' in SIC [20]:

$$B_{22} = B_{HS} - \frac{k'}{\rho_s \varphi} \quad (1)$$

Here B_{HS} is the hard-sphere or excluded volume contribution to B_{22} , ρ_s is the immobilized protein concentration and φ is the phase ratio, i.e., the accessible surface area per mobile phase volume; the product $\rho_s \varphi$ represents the overall concentration of immobilized protein in the column. Since B_{HS} is directly related to the size of the protein and ρ_s and φ can be measured directly [20–22], a particularly important feature of Eq. (1) is that B_{22} can be determined, in principle, without the use of adjustable parameters. Alternative relations between chromatographic retention and B_{22} have been derived [23–25], but they are of essentially the same form as Eq. (1).

Where comparisons have been performed, SIC has been shown to yield generally good agreement with SLS measurements of B_{22} , both in terms of the general trends and the quantitative values [23,24,26]. The advantage of SIC lies in the much smaller amounts of protein and time needed for the measurements. Reported immobilized protein concentrations are typically of order 10 mg/ml, and for analytical column volumes of order 1 ml, a column holds of order 10 mg protein. Each injection of tens of μ l in volume, containing 1–10 mg/ml protein, comprises less than 1 mg of protein. These amounts are significantly smaller than those typically used in SLS, where multiple samples are needed to obtain a single B_{22} data point. SIC is also much faster than SLS, as tens of values can be determined in the course of a day, and the measurements can be automated using widely available chromatographic workstations. Consequently SIC allows relatively high throughput measurement of B_{22} , and it has been used quite extensively on a variety of protein and peptide solutions containing different additives [2,20,24,27–33].

Despite the smaller amount of protein required for SIC relative to standard methods, the amount of protein used for on-column immobilization and, to some extent, injection remains excessive for some situations, especially in structural biology applications. Efforts have therefore been made to develop miniaturized or microfluidic implementations. The most straightforward route is to use microcolumns with internal diameters of mm dimensions or

even smaller [27–29,34–36], and the first microfluidic SIC device [37,38] was in fact an extension of this in comprising normal preparative chromatographic particles packed into a poly(dimethylsiloxane) (PDMS) microchip-based channel. A device reported more recently is an open-tube chromatography column synthesized on a commercial microfluidic microreactor chip with a channel cross-sectional dimension of about 150 μm [39]. In both cases the data were analyzed using Eq. (1) or equivalents, but the parameters in the equation were not determined independently; instead the equation was calibrated relative to previously reported SLS measurements, effectively introducing adjustable parameters and requiring at least some SLS measurements to be performed. Furthermore, both of the previously described microfluidic SIC systems have features that may hinder the efficiency of the chromatographic measurements. In the open-tube microfluidic system [39] the tube cross-sectional dimension of 150 μm makes the phase ratio extremely low, so that ideal monolayer immobilization should make k' measurements difficult; the reported data suggest that multilayer immobilization may in fact occur. In addition, the combination of the open-tube format and laminar flow may lead to significant peak broadening. In the packed-column microfluidic SIC system [37], where 65 μm particles are packed into a channel of cross-section 250 $\mu\text{m} \times 127 \mu\text{m}$, a potential disadvantage is the contribution to peak broadening due to wall effects [40].

In this work, we introduce an alternative microfluidic SIC system by performing SIC in chromatographic monoliths synthesized in 100 μm diameter capillaries. Monoliths are chromatographic stationary phases that are polymerized directly as a single unit with highly interconnected channels. One of the most important advantages of monoliths is that they allow convective mass transport through channels larger than typical intraparticle pores, and polymeric monoliths such as those used here eliminate narrower pores that present the principal diffusional resistance in most systems using porous particles. As a result monoliths allow rapid high-resolution separations and band broadening largely independent of flow rate. Monoliths have phase ratios intermediate between those of the open-tube and packed-column systems but they allow more efficient mass transfer than both of the alternative systems. Furthermore, their synthesis is quite straightforward, reproducible and inexpensive. We demonstrate for the example of catalase, used as a model protein, that such a system allows fast measurements of B_{22} using very little protein, and that independent measurement of column parameters is feasible, which can obviate the need for adjustable parameters.

2. Experimental

2.1. Materials

Butyl methacrylate (BuMA) (product no. 235865), ethylene dimethacrylate (EDMA) (335681), poly(ethylene glycol) methacrylate (PEGMA, average mol. mass 526 g/mol, 10 ethylene glycol units) (409529), 1,4-butanediol (493732), 1-propanol (402893), *t*-butyl alcohol (308250), 2,2'-azobisisobutyronitrile (AIBN) (441090), benzophenone (BP) (B9300), 3-(trimethoxysilyl)propyl methacrylate (440159), and ethanolamine (E9508) were purchased from Sigma-Aldrich (St. Louis, MO, USA). BuMA, EDMA and PEGMA were purified by passing them through an inhibitor-remover preppacked column (Sigma-Aldrich). 2-Vinyl-4,4-dimethylazlactone (VAL) (D2123) was purchased from TCI America (Portland, OR, USA). Catalase from bovine liver (C40) and uridine 5'-monophosphate disodium salt (UMP) (U6375) were obtained from Sigma-Aldrich. Lengths of UV-transparent fused silica capillary (100 μm ID) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Equipment

A Spectroline microprocessor-controlled UV crosslinker, Spectrolinker XL-1500 (Spectronics Corporation, Westbury, NY, USA), was used for UV exposure.

Chromatographic experiments were performed using a liquid chromatography system consisting of a P680A DGP-6 dual low-pressure gradient pump (Dionex Corporation, Sunnyvale, CA, USA), an Accurate pre-column flow splitter (LC Packing, a Dionex company), a Cheminert microbore internal sample injector with a 10 nl sample injection loop (model C4, Valco, Houston, TX, USA), and a UV-vis Ultimate 3000 variable wavelength detector (model VWD-3400) with a 3 nl fused silica flow cell (Dionex).

2.3. Preparation of SIC monoliths

The microfluidic SIC devices consist of porous polymer monoliths prepared *in situ* in fused silica capillaries of 100 μm internal diameter, following procedures described by Svec, Fréchet and co-workers [41–48]. These monoliths are further derivatized to enable protein immobilization. The principal synthesis steps are:

2.3.1. Activation of capillary surface—The inner surface of the fused silica capillary is first activated with 3-(trimethoxysilyl)propyl methacrylate, introducing pendant vinyl groups on the surface of the capillary that subsequently react with monomer molecules to attach the polymer covalently to the capillary surface. The procedure involves rinsing sequentially with acetone, water, 0.2 M NaOH, water, 0.2 M HCl, water and ethanol. Derivatization is performed using a 20 wt% solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol, which is pumped through the capillary at 0.5 $\mu\text{l}/\text{min}$ for 60 min. The capillary is rinsed with acetone, blown dry with nitrogen and left at room temperature for 24 hours.

2.3.2. Synthesis of base monolith—A UV-initiated photopolymerization procedure is used to synthesize porous polymethacrylate monoliths. The polymerization mixture consists of 24 wt% BuMA and 16 wt% EDMA as monomers, 26 wt% 1,4-butanediol and 34 wt% 1-propanol as porogens and 1 wt% (with respect to the monomers) AIBN as the initiator. This mixture is ultrasonicated for 15 min to remove any air and then loaded into an activated capillary, which is placed under a UV light source and irradiated for 30 minutes. The porogenic solvents are flushed out of the monolith by pumping methanol at 0.5 $\mu\text{l}/\text{min}$ overnight.

2.3.3. Photografting of PEGMA—The surface of the polymethacrylate monolith is hydrophilized by photografting poly(ethylene glycol) methacrylate. The procedure uses benzophenone (BP) as an initiator, introduced by rinsing the methacrylate monolith with a 5 wt% BP solution in methanol, filling the monolith with the same solution and irradiating the monolith with UV light for about 3 minutes. After rinsing with methanol to remove unbound initiator, a 0.1 M solution of PEGMA in water is pumped through the monolith at 0.5 $\mu\text{l}/\text{min}$. The monolith containing the PEGMA solution is exposed to UV irradiation for about 3 minutes, following which the monolith is rinsed with water at 0.5 $\mu\text{l}/\text{min}$ rate overnight in order to remove unreacted PEGMA monomer.

2.3.4. Photografting of 4,4-dimethyl-2-vinylazlactone (VAL)—The azlactone functionality is introduced by photografting of the reactive monomer 4,4-dimethyl-2-vinylazlactone. The procedure involves rinsing and filling the PEGMA-grafted monolith with a BP solution in methanol and irradiating the monolith with UV light for a given time, which was varied to explore the effect on monolith performance. After rinsing with methanol to remove unbound initiator, the monolith is filled with a VAL solution in water and irradiated with UV light for about a given time. It is then rinsed with acetone at 0.5 $\mu\text{l}/\text{min}$ to remove the unreacted monomer. Different irradiation times and concentrations of BP and VAL were investigated.

2.3.5. Protein immobilization onto the azlactone-functionalized monolith—The reactive azlactone groups enable the covalent immobilization of proteins onto the monoliths via reaction with amine groups of proteins. After the grafting of VAL, a 2 mg/ml solution of the desired protein is pumped through the monolith at 0.5 $\mu\text{l}/\text{min}$ for 1–2 hours. This solution can be recirculated to economize on the amount of protein used. The monolith is rinsed with 3 M ethanolamine at pH 9.0 to quench unreacted azlactone groups and then rinsed with buffer to remove excess reagents.

2.4. Determination of immobilized protein concentration

The amount of protein immobilized onto the monolith was determined using the Micro BCA assay (Pierce Inc.). A known length of monolith (~ 1 cm) was cut into short sections (< 1 mm) and placed in DI water. This sample was then mixed with the BCA working reagent, incubated at 60 $^{\circ}\text{C}$ for 1 hour, and allowed to cool at room temperature before the absorbance of the supernatant was measured at 562 nm. The protein concentration of the sample was determined by the use of a calibration curve based on the same protein as that immobilized. The nature of the measurement on short column sections is similar to that on porous chromatographic particles, where independent verification could be obtained by mass balance [20]. For such short sections of the monolith and such high temperatures, diffusion is fast enough that the final measurement on the supernatant can be considered as a representative measure of the total protein in the monolith sections, allowing the amount of immobilized protein per unit length of monolith to be calculated. The amount of protein immobilized on a monolith is calculated as an average of the measurements on three 1 cm sections of the column, taken from different parts of the monolith.

2.5. Fluorescent labeling of protein

Lysozyme was labeled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) by adding 50 μl of a 0.25 mg/ml solution of FITC in 0.1 M carbonate-bicarbonate buffer, pH 9, dropwise while stirring to a vial containing 0.2 ml of 5 mg/ml lysozyme solution in the same buffer. The vial was then completely covered with aluminum foil to protect the contents from the light and incubated for 2 hours at room temperature with gentle stirring. The labeled protein was separated from the unreacted label using a Sephadex G-25M size exclusion column running with a 10 mM phosphate buffer saline (PBS), pH 7.4. The fluorescein/protein molar ratio (F/P) was calculated from the absorbance readings at 280 nm and 495 nm of the FITC-protein conjugate sample to be 0.39.

2.6. Self-interaction chromatography and B_{22} calculation

Bis-tris buffer solutions at pH 7.0 were prepared with added concentrations of 0, 0.25, 0.50, 0.75 and 1 M ammonium sulfate. Catalase solutions were then prepared at a concentration of 2 mg/ml using these buffer solutions. The protein solutions were filtered and briefly sonicated to remove any possible air bubbles. A 25 cm monolith with immobilized catalase was equilibrated with 60 μL of a specific buffer, and 10 nl of catalase in that buffer was injected into the monolith. The absorbance at 280 nm was recorded at the column outlet. Different flow rates in the range of 0.4 – 1 $\mu\text{l}/\text{min}$ were used and the retention factor was calculated from the retention times, resulting in good agreement independent of the flow rate used. Accurate measurements of the flow rate were obtained by connecting a 10 μl graduated syringe without the plunger at the outlet of the system and recording readings of the syringe volume filled at different times. All retention time measurements in the monolith were performed in triplicate.

The second virial coefficient, B_{22} , was calculated from the chromatographic retention factor using Eq. (1). No adjustable parameters were needed because k' was measured and all the

other quantities were determined independently, as follows. k' was calculated from the retention volume as

$$k' = \frac{V_r - V_0}{V_0} \quad (2)$$

where V_r is the retention volume and V_0 is the dead volume, which was determined from the retention volume of UMP by subtracting the excluded volume for catalase molecules. Both V_r and V_0 were corrected for extra-column effects. The excluded volume contribution B_{HS} was calculated by assuming the catalase molecule to be a sphere with an equivalent diameter of 7.9 nm [36], giving a value of B_{HS} of 9.91×10^{-6} mol ml/g². The product $\rho_s \phi$ represents the overall concentration of the immobilized protein in the column, per unit accessible pore volume (g/cm³). Since the Micro BCA procedure described above gives the amount of protein immobilized per length of column, $\rho_s \phi$ is found simply by using the column length and V_0 .

3. Results and discussion

3.1. Characterization and optimization of the monoliths

The monolith synthesis conditions were manipulated to obtain monoliths suitable for SIC, a key requirement being that the retention factor k' be accurately measurable. Since the protein-protein interactions to be measured by SIC are weak, relatively low k' values are expected; since k' is proportional to the phase ratio [21,49], it can be maximized by using a stationary phase with a high phase ratio. Monoliths generally have phase ratios intermediate between those of open-tube and packed column systems but they allow more efficient mass transfer than both of the alternative systems. At a given phase ratio, measurement of small k' values may be made feasible by using sufficiently long columns to resolve relatively small differences in retention times or volumes.

The base poly(BuMA-*co*-EDMA) monoliths were synthesized according to previously reported conditions [48], yielding a mean pore size of order 1 μ m and a column porosity of 60–70%. These dimensions are apparent in the scanning electron micrographs at different resolutions shown in Fig. 1. The low-magnification image shows the small internal diameter (100 μ m) of a silica capillary containing the BuMA-*co*-EDMA polymer, while increasing the magnification shows more details of the polymer structure, which comprise aggregates of roughly spherical particles of order 1 μ m in diameter. This microstructure is similar to those previously reported [46], while different reaction mixture compositions can produce smaller polymer aggregates that give rise to higher phase ratios [50]. A phase ratio on the order of 4×10^4 m⁻¹ was estimated from the electron micrographs in Fig. 1.

The porosity of ten synthesized columns was measured after hydrophilization by inverse size exclusion chromatography using uridine 5'-monophosphate disodium salt (UMP), giving an average value of 0.65 ± 0.01 , where the error value reported is the 95% confidence limit. The results show some variability in the porosity of different columns synthesized under the same conditions, indicating that measurement of the porosity of each column should be considered for more accurate calculations. Knowing the porosity of the column is important in order to determine V_0 for the SIC calculations, and is also useful in estimating the coverage of immobilized protein in the monolith.

Reaction conditions were investigated to optimize the two derivatization steps, viz. hydrophilization of the methacrylate surface using PEGMA and introduction of the

azlactone functionality for protein immobilization. In both cases a sequential two-step photografting technique was found to be optimal. In the first step, the monolith was filled with an initiator solution and UV-irradiated, leading to the formation of reactive moieties at the methacrylate surface. In the second step, a solution containing only monomer was pumped through the monolith, which was then UV-irradiated, allowing the monomers to attach to the surface by graft polymerization. An advantage of this two-step polymerization is the reduced formation of nongrafted polymer in solution, which could occlude the pores. Another advantage is better control of the grafting process since the grafting density can be controlled by manipulating the reaction conditions in the first step and the graft chain length by the conditions in the second step [51].

The hydrophilization step, using PEGMA of molecular weight 526, [47] yielded monoliths that were able to resist non-specific adsorption. The success of this hydrophilization step was tested by pumping fluorescently labeled lysozyme through a monolith with half of its length photografted with PEGMA solution and the other half without treatment. After the monolith was rinsed with 0.01 M phosphate buffer, pH 7.4, the untreated portion of the column showed significantly more non-specific adsorption of protein (Fig. 2).

The absence of significant non-specific adsorption was further confirmed by the similarity of the chromatograms obtained when a pulse of catalase was injected in columns without protein immobilized and those with immobilized protein under conditions of minimal interaction.

For the VAL derivatization the goal is to introduce adequate azlactone functionality to produce sub-monolayer protein coverage in the interest of maximizing consistency of the SIC conditions with the assumptions leading to Eq. (1). This too was accomplished using the sequential two-step photografting technique. Experiments with 0.5 and 5 wt% benzophenone solution and a concentration of 0.1 M vinylazlactone were performed with reaction times of 3 min for both steps. Only small differences in the amount of protein immobilized were observed, corresponding to approximately monolayer coverage or higher. Similar results were obtained when the reaction time for the first step was reduced to as low as 20 s. Additional evidence for excessive VAL polymerization was that the pressure drop in the monoliths after protein immobilization increased in all cases, suggesting the possibility of a reduction in void volume. These results suggested that the immobilization should be better controlled, and that this could best be done using the VAL polymerization step. Therefore reaction times of 20 s were used for both steps and the benzophenone concentration kept at 5 wt%, while the concentration of VAL was varied.

The amounts of immobilized protein obtained are shown in Table 1. The VAL concentration is seen to be an effective means of controlling the immobilized protein concentration, with a VAL concentration of 0.05 M providing a catalase concentration corresponding approximately to the sub-monolayer coverage needed for SIC. In this monolith no increase in pressure drop was observed after the protein immobilization, consistent with the similar column porosities obtained by size-exclusion chromatography before and after the protein immobilization. For comparison, the theoretical amount of protein immobilized in monolayer coverage was calculated to be about 1.5 $\mu\text{g}/\text{cm}^2$ based on a column porosity of 0.65, a column phase ratio of $4 \times 10^4 \text{ m}^{-1}$ and a catalase equivalent diameter of 7.9 nm.

3.2. Self-interaction chromatography and B_{22} determination

Catalase was injected into a 25 cm long column with immobilized catalase under isocratic conditions at different ammonium sulfate concentrations. For runs at low salt concentration (0–0.5 M ammonium sulfate), retention time values close to those corresponding to unretained conditions were obtained, with slightly higher values observed for lower salt

concentrations, which is consistent with the salting-in behavior of catalase [36]. At higher salt concentrations, the retention time increases with salt concentration, as shown in Fig. 3; chromatograms at ammonium sulfate concentrations below 0.5 M are not shown in the interest of clarity.

The agreement among the different curves indicates the reproducibility of the SIC retention measurements. In addition, these chromatograms were obtained at different flow rates, which confirms a key benefit of convective mass transfer in monolithic media by showing that the retention measurements are largely independent of flow rate.

The second virial coefficient, B_{22} , was determined using Eq. (1). A value of 9.91×10^{-6} mol ml/g² was obtained for the excluded volume contribution, B_{HS} , calculated using an equivalent diameter of 7.9 nm for catalase. The retention factor, k' , was calculated from the retention volumes obtained in the chromatographic runs (Fig. 3) using Eq. (2). To determine V_0 , or the volume required for a noninteracting molecule the same size as a catalase molecule to pass through the column, the retention volume of uridine 5'-monophosphate disodium salt (UMP), a small-molecule probe expected to be noninteracting, was calculated. To account for the different size between UMP and catalase, the theoretical excluded volume for a catalase molecule was calculated based on the dimensions and porosity of the column, the phase ratio and the amount of protein immobilized onto the monolith, and the diameter of the protein. By inverse size exclusion chromatography using UMP, a porosity value of 0.644 was determined for the microfluidic monolith studied here. The total amount of immobilized protein in the column was calculated by multiplying the length of the column (25 cm) by the value of 0.6 μ g of protein immobilized per cm of column (Table 1); this estimate of 15 μ g indicates the extremely low demand for protein in this system. An estimate of V_0 for the monolith was then found by subtracting the calculated excluded volume from the retention volume obtained with UMP. Finally, the overall amount of immobilized protein per unit mobile-phase volume in the optimized column was calculated by dividing the total amount of immobilized protein by V_0 .

Fig. 4 shows the B_{22} values for catalase for various concentrations of ammonium sulfate at pH 7.0 obtained using an optimized column. That the values shown are lower than those often reported for B_{22} generally is because the conversion to units of ml mol/g² involves division by the square of the molecular weight, and catalase is a much larger protein than those for which B_{22} is often measured, e.g., lysozyme. For catalase specifically, the values in Figure 4 are comparable in magnitude and the trend very similar to B_{22} values for the same conditions measured by SIC in a macroscopic packed column and reported in dimensionless terms [36]. However, the values in the negative B_{22} range shown in Fig. 4 are smaller in magnitude (less attractive) than those found on the macroscopic system. One way to address this discrepancy would be to use one of the published B_{22} values as a reference point, thereby introducing an adjustable parameter into the equivalent of Eq. (1); this approach was used in previous microfluidic SIC devices [37,39].

Alternatively, the possible sources of uncertainty in results such as those in Fig. 4 could be investigated. The error bars in the figure represent the error in the measurements based on the reproducibility of V_r , but of greater interest are systematic errors that may be introduced by uncertainties in quantities used in the SIC calculations, such as the amount of protein immobilized, the porosity of the monolith, and V_0 . Another possible source of error is the estimate of the surface area in the monolith and its effect on the calculated surface coverage of immobilized protein, which, although not used directly in Eq. (1), is a consideration in determining the validity of the underlying SIC theory. If the surface coverage exceeds sub-monolayer values, for example, the immobilized protein will be less accessible to free protein than is implied by the use of Eq. (1).

Although it is unclear which elements of the procedures here might have contributed to the discrepancy between Fig. 4 and the earlier results [36], the latter might also have been affected by systematic errors. In particular, because catalase is a fairly large protein, it is susceptible to pore exclusion effects in the porous packings used for the chromatographic measurements, even for the wide-pore material employed. This would complicate accurate estimation of the effective phase ratio, and it is possible that this contributes to the discrepancy between those results and those in Fig. 4. The minimal size exclusion effects present in the monolithic media may in fact represent another advantage over traditional chromatographic packings for larger proteins.

4. Conclusions

We have shown that protein-protein interactions in solution can be measured by SIC in microfluidic monoliths synthesized in capillary tubes. The synthesis of these microfluidic devices is quite straightforward, reproducible and inexpensive, and allowed fast and accurate measurements of B_{22} using very little protein (tens of μg). More efficient mass transfer processes than in alternative miniaturized SIC implementations are possible, leading to higher chromatographic efficiency. In addition, the method presented does not require the use of an adjustable parameter, avoiding the need for complementary SLS measurements. Beyond the results reported here, the sensitivity of the chromatographic measurements can be increased by using alternative polymerization formulations, which can yield higher phase ratios.

Research Highlights

- The osmotic second virial coefficient B_{22} is a measure of protein interactions
- B_{22} can be measured efficiently by self-interaction chromatography (SIC)
- Microfluidic SIC measurements in 100 μm diameter monoliths are demonstrated
- This miniaturized system makes B_{22} measurement possible with minimal protein consumption

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Fig. 1. Scanning electron micrographs of poly(BuMA-*co*-EDMA) monolith prepared in 100 µm i.d. capillary. Scale bars (from left to right): 200 µm, 50 µm, 5 µm.

Fig. 2. Fluorescence micrograph of a poly(BuMA-*co*-EDMA) monolith showing the resistance to non-specific adsorption due to modification with PEG. The right half was photografted with PEGMA solution and the left half was not. Labeled lysozyme was pumped through the monolith and the monolith was then rinsed with buffer. As observed, non-specific adsorption of protein is observed on the part without grafted PEG.

Fig. 3. Chromatograms for SIC measurements on catalase at pH 7.0 and different $(\text{NH}_4)_2\text{SO}_4$ (AS) concentrations using a 25 cm microfluidic chromatographic monolith.

Fig. 4. Values of B_{22} for catalase in ammonium sulfate at pH 7. Error values represent 95% confidence limits (n=3).

Table 1

Amount of catalase immobilized in microfluidic monoliths derivatized with different azlactone concentration. Each value represents an average of three measurements, with 95% confidence intervals shown.

VAL concentration	Protein immobilized, $\mu\text{g}/\text{cm}$
0.1 M	1.4 ± 0.1
0.05 M	0.6 ± 0.2
0.01 M	0.0 ± 0.2