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RPLC of intact proteins using sub-0.5 μm particles and commercial instrumentation

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Abstract

This paper addresses whether one can gain an improvement in speed or resolution with a silica colloidal crystal (SCC) of nonporous 470 nm particles when using a commercial nano-UHPLC. Compared to a capillary packed with nonporous 1.3 μm particles and the same C4 bonded phase, the peak width for BSA is decreased by a factor of 6.8 for the SCC. Some of this improvement is attributable to slip flow since the ratio of particle diameters is only 2.8. Resolution in protein separations was compared for a 2-cm capillary of SCC vs. a 5-cm column of porous 1.7 μm particles. Both used a C4 bonded phase, and on-column fluorescence detection was used for the SCC. Split flow (5:1) before the SCC decreased the gradient delay time to 0.4 min and the injected volume to 0.4 nL. For variants from the labeling of BSA, the SCC had a five-fold higher speed and two-fold higher resolution than did the commercial column. For a monoclonal antibody and its aggregates, the SCC had a three-fold higher speed and a three-fold higher resolution compared to the commercial column. The SCC gave baseline resolution of the monomer, dimer and trimer in 5 min. The results show that a significant advantage can be gained using a commercial instrument with the SCC, despite the instrument not being designed for use with such small particles.

INTRODUCTION

Therapeutic monoclonal antibodies are one of the major growth areas in the pharmaceutical industry. These drugs benefit from the highly specific targeting afforded by the complementarity determining region of the antibody, reducing negative side effects of treatment. The market for protein drugs is rapidly expanding, with a yearly growth rate of over 10%.[1] The efficacy of monoclonal antibodies relies on having the correct sequence, as well as the correct tertiary structure and post-translational modifications.[2] Protein aggregation is one of most pressing problems because of the high concentration of formulations. Aggregation proceeds by a number of different mechanisms,[3] and the rate is accelerated by increasing temperature,[4] ionic strength,[5] differential glycosylation,[6] acidic pH,[7] and exposure to adsorptive surfaces such as stainless steel.[8] Structural variations, particularly aggregation, can reduce the efficacy of the therapeutic protein, and can also induce an immunogenic response in the patient.[9]

In drug development, separations play as key of a role for protein drugs as they do for small-molecule drugs. Current methods of antibody analysis include size exclusion chromatography (SEC),[10] ion exchange chromatography,[11-12] and RPLC.[13] RPLC is attractive because of its speed and efficiency, which has made it the primary separation method for small-molecule drugs. Recent literature has shown that high temperatures (80 °C) are required for RPLC of monoclonal antibodies to achieve adequate peak shape and sample recovery, apparently owing to the high molecular weight of antibodies.[13-14] These

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high temperatures cause sample composition changes, such as aggregation, decomposition, as well as conformational changes.[15] Chromatographic columns that allow rapid, room temperature separation of antibodies would be valuable. The advantage of smaller particles for higher efficiency is well known,[16] and using nonporous instead of porous particles give more efficient protein separations.[17] High pressure gives better recovery of proteins in RPLC.[18] Recently, we reported high efficiencies for protein separations with submicrometer nonporous particles, which give radially homogeneous packing,[19] plate heights as low as 15 nm,[20] and enhanced flow rates due to slip flow.[20-21] An ELISA monoclonal antibody and its dimer were shown to separate in about 1 min at room temperature.[20] While these results are quite promising, offline electrokinetic injection was used, and separations were performed using an isocratic pump. To be applicable to routine protein separations, injection needs to be performed using an autosampler, and gradient elution needs to be used. The purpose of this paper is to determine how much of an improvement in the separation of intact proteins is accrued using a commercial instrument for injection and gradient elution, despite commercial instruments being designed for columns with much larger particles and lower efficiencies.

EXPERIMENTAL SECTION

Proteins

Bovine serum albumin, soybean trypsin inhibitor, human recombinant insulin, bovine erythrocyte carbonic anhydrase (Sigma, St. Louis, MO) and a pharmaceutical grade monoclonal antibody (Eli Lilly, Indianapolis, IN) were used. The antibody had undergone drug development but is no longer in the development pipeline. The antibody was fluorescently labeled with Alexaflour 546 (Invitrogen, Carlsbad, CA) following the instructions provided in the labeling kit. A separate BSA sample was also labeled with Alexaflour 647 (Invitrogen, Carlsbad, CA), and commercial BSA that had been over-labeled with Alexaflour 647 was purchased (Invitrogen, Grand Island, NY). Monoclonal antibody aggregation was induced by heating a 1 mg/mL solution of protein in PBS to 80 °C for 60 min. Proteins were diluted to concentrations of 5 μg/mL for chromatography.

Preparation of silica particles

Nonporous silica of nominal diameters of 0.5 μm and 1.3 μm were purchased from NanoGIANT, LLC (Phoenix, AZ), and Fiber Optic Center, Inc. (New Bedford, MA). The same preparation was used for both particle sizes. Silica particles were calcined at 600 °C in a furnace (Lindberg/BlueM). After calcination the particles were sonicated in 250 mL of 200-proof ethanol at a concentration of 4% wt using a 500 mL round bottom flask. After 5 hours of sonication the suspension was centrifuged to form a pellet and dried under vacuum at 60 °C for 5 hours. The same process was repeated two more times. Prior to capillary packing, the surface of silica particles was rehydroxylated by refluxing in a 50:50 (v/v) mixture of 18 MΩ water and HNO_3 at 150 °C for 24 hours. Particles were rinsed and centrifuged with 18 MΩ water four times and dried under vacuum at 60 °C. Silica slurries were prepared in a 1-dram vial at a 30 % (wt./wt.) concentration in 18 MΩ water and sonicated in a water bath.

Capillary packing

Fused silica capillary was purchased from Polymicro (Phoenix, AZ). Silanes were purchased from Gelest, Inc. (Morrisville, PA). Again, the same packing procedure was used for both particle sizes. Capillaries were cleaned by 0.2 M HNO_3 solution flowed by a syringe pump (Harvard Apparatus Holliston, MA) through the capillary at a rate of 200 μL/min for 10 min, followed by ultrapure water, and then ethanol, for 10 and 20 min, respectively. The capillaries were dried under vacuum for 1 hr at 60 °C. Silica slurries were prepared at a 20

% (w/w) concentration in water and sonicated in a water bath. Slurries were wicked into capillaries of 12 cm in length to fill the entire capillary, and the slurry was allowed to gravity settle for 48 hr to form a seed crystal a few mm long at the end of the capillary. After 48 hr, a 0.5 μm PEEK frit (IDEX Health & Science LLC Oak Harbor, WA) was attached to the end of the capillary, and the slurry was then compressed under pressure at 700 bar while sonicating (VWR, model 75T) for 1 hr to eliminate void spaces. After packing, the frit was removed and the capillary was allowed to dry in a desiccator prior to modification. The capillaries were cut to lengths of typically 2.2 cm, and a head space of just over 1.5 cm was allowed for monitoring of injection. Figure 1A shows a photograph of the capillary taken using a zoom microscope with attached digital camera (Nikon, SMZ-1500, Melville, NY). The brilliant colors are opalescence due to Bragg diffraction from the ordered material.[22] SEM images of column cross-sections demonstrated the high order of the material, as detailed in earlier work.[19, 23] The hydrophilic silica was chemically modified by horizontal polymerization using a mixture of trifunctional silanes bearing methyl and butyl groups, as detailed by the previously published method.[23]

Chromatographic Conditions

LC-MS grade acetonitrile and water, as well as trifluoroacetic acid, were purchased (Sigma Aldrich, St. Louis, MO). Nano-UHPLC for the silica colloidal crystal was performed using a Thermo-Dionex UltiMate3000 (Sunnyville, CA). The nano-UHPLC was modified to include a post-valve 5:1 split, as shown in the photograph of Figure 1B, to reduce the gradient delay time and provide a smaller injection volume, 400 pL, which is 0.5% of the free volume in the capillary. Detection was carried out with a fluorescence microscope, as described previously.[20] A commercial reversed phase column was purchased from Waters, Inc. (Milburn, MA): BEH300 C4, 1.7 μm, 2.1 × 50 mm UHPLC with the commercial column was performed using a Thermo Accela UHPLC equipped with a PDA detector, and a 5 μL injection volume was used, which is 4.5% of the free volume in the column. For gradient elution separations, details for mobile phase composition during gradient elution can be found in each figure caption. A commercial size exclusion column was purchased from Sepax, Inc. (Newark, DE): Zenix SEC-300, 3μm, 300Å, 4.6 × 250 mm, and separations were performed using the Thermo-Accela UHPLC and PDA detector. For the SCC pressures were typically on the order of 600 bar.

RESULTS AND DISCUSSION

For all separations, the same protein concentration of 5 μg/mL was used, and similar ratios of injected volume to column volume were used. Figure 2A shows a comparison of chromatograms for capillaries packed with nonporous silica spheres of 1.3 μm vs. 470 nm in diameter, each with 1 cm separation lengths. The sample was BSA labeled with Alexaflour-546. The peaks elute at nearly the same position in the gradient, and the discrepancy is apparently due to a small gradient delay for the 470 nm particles. Peak widths are much narrower in the 470 nm column (σ=0.6 s) compared to the 1.3 μm column (σ=4.1 s), which is a factor of 6.8 in peak width. Since the velocities are close to optimal, one would expect the peak width to be narrowed by a factor of 1.7, which is the square root of the ratio of particle diameters. The peaks are thus four-fold narrower than normally expected. Factors that might contribute to the unduly sharp peaks for the 470 nm particles are higher capacity, lower radial heterogeneity, which is unlikely since the capillaries were packed the same way, and slip flow to reduce the mass transport term. Slip flow is likely a factor because of the extreme peak sharpness. The smaller nonporous particles clearly provide a significantly higher efficiency compared to the larger nonporous particles. This advantage becomes even more pronounced when compared to the chromatogram of the commercial column having porous silica particles of 1.7 μm diameter and a length of 5 cm, as shown in Figure 2B. The peak width (σ=11 s) is nearly 20 times wider, and it elutes five-

fold more slowly for the same gradient. Overall, there is a large increase in the speed and efficiency of RPLC when using the SCC with the commercial instrument.

Efficiency does not necessarily translate to resolution, and speed does not necessarily translate to resolution per time, since the C_4 bonded phases are different. Therefore, separations of mixtures must be studied to compare performance. BSA itself is known to be a mixture of monomer and dimer, as well as other variants, which causes the appearance of tailing.[24] The peak in Figure 2A for the 470 nm particles is actually 20% more asymmetric than that for the 1,300 nm particles, suggesting that BSA variants are resolved somewhat further. Simple protein mixtures that are more amenable to resolution with reversed-phase columns are used for the next comparisons of the SCC having 470 nm particles at room temperature *vs.* the commercial column at 40 °C, again each with C_4 modification. Our previous paper had shown baseline resolution of two differentially labeled forms of BSA, showing a resolution of R_s=2 using isocratic elution,[20] and here we present separations of the same species using gradient elution. The sample was prepared as before by mixing two BSA samples, one that was under-labeled in-house to bear no more than one dye, and the other that was a commercial over-labeled BSA sample, presumably bearing the maximum number of dyes. Figure 3A shows the chromatograms for the SCC with the same gradient as in Figure 2. The two BSA components are resolved. The expanded time scale in the inset of Figure 3A shows R_s=1. Figure 3B shows the chromatogram for the same sample using the commercial column, where one broad peak is seen. The expanded time scale in the inset of Figure 3B reveals that the two components are evident, but are not resolved. The resolution for the commercial column is estimated to be 0.6 from summing two Gaussians to fit the peak. The comparison between SCC and the commercial column shows that the SCC provides two-fold higher resolution, combined with the more than four-fold higher speed, for this gradient-elution separation of intact proteins.

The gradient delay can be estimated by varying flow rate, with the approximation that the protein elutes at a fixed mobile phase composition, and the peak then moves through the capillary in the time t_0. The eluting mobile phase composition would be reached at time t_e if there were no gradient delay. With this idea, the observed net elution time, t_{net}, would be the sum of the theoretical elution time if there were no gradient delay, plus the gradient delay time and t_0, where t_d and t_0 vary with flow rate.

$$t_{net} - t_0 = t_d + t_e \quad (1)$$

Since $t_d \rightarrow 0$ as $t_0 \rightarrow 0$, a plot of t_{net}-t_0 *vs.* t_0 would have an intercept that is t_e. , allowing calculation of t_d. To vary t_0, Figure 4A shows chromatograms for the separation of the two labeling variants of BSA at varying flow rate, and Figure 4B shows that a plot of t_{net}-t_0 *vs.* t_0. The data fit well to a line, and the intercept is 1.99 min. For the highest flow rate, therefore, the gradient delay time is 0.4 min or 24 s. This agrees well with the 27 s that one estimates from the length of tubing from the pump to the splitter (50 cm in length, 20 μm i.d.), plus the 1.5 cm of head space in the SCC capillary. By this calculation, the head space in the capillary contributed most of the gradient delay, ~20 s, and this portion can be eliminated in practical applications since there is no need to monitor injection routinely. The results thus show that the reasonable split ratio of 5:1 would allow a negligibly short gradient delay time, thereby making use of the fast separation times when using commercial instrumentation.

We had previously shown an isocratic separation of a monoclonal antibody made for ELISA, with well resolved monomer and dimer peaks.[20] We now use a highly purified monoclonal antibody provided by Eli Lilly, and in addition, aggregates were generated by heating a portion of the sample to promote aggregation. Figure 5A shows the chromatogram

of the monomer for the SCC, where a single narrow chromatographic peak (σ=1 s) elutes in just under 3 min for a separation length of 0.5 cm. For the Waters column at 40 °C and the same gradient, Figure 5B shows that the monomer elutes with a wider peak (σ=6 s) in just under 10 min. The size exclusion chromatogram of Figure 5C indicates the presence of just a single protein size, as expected. For the aggregated sample, Figure 5D shows three chromatograms for the SCC, each for a different separation length along the same capillary, and three peaks are shown to be baseline resolved for a separation length of 2 cm at room temperature. The first peak lies up with the monomer peak, and the other two peaks are presumably dimer and trimer. The monomer peak is now twice as wide and tailing, indicating that the heat treatment made the monomer heterogeneous, likely by denaturing. The aggregate peaks also tail, perhaps also due to heterogeneity. The commercial RPLC column shows evidence of aggregates in the 10 min separation at 40 °C in Figure 5E, but one cannot quantitate the species. The size-exclusion chromatogram of Figure 5F confirms the presence of aggregates, but it was not able to resolve aggregates from the monomer. For the SCC, the baseline resolution of monomer, dimer and trimer on a time scale of less than 5 min is markedly better than the 30 min time scale for resolution by long SEC columns.[25] Aggregation of proteins is the most pressing problem in the formulation of therapeutic proteins,[25-26] and the ability to separate covalent aggregates faster can facilitate optimization in the formulation of therapeutic proteins.

Separations with more complex protein mixtures were used to estimate the peak capacity. Figure 6 shows a separation of nominally three commercial proteins, trypsin inhibitor, insulin and carbonic anhydrase, each labeled with Alexafluor 546. The gradient was the same as those of the earlier figures. The top panel shows the mixture of the three proteins, and the subsequent three panels show the individual proteins. These commercial proteins are each mixtures, and the basic condition used for labeling likely increases the sample complexity, giving over two dozen peaks plus a baseline of unresolved protein. Based on a peak width (4σ) of 7.2 s, which is the average for the peaks at 6 min and 8 min, the peak capacity is 54. This alone is not an unusual peak capacity, but what is unusual is how short the separation time is. The peaks elute from 4 to 10 min, giving the peak capacity of 54 in only a 6 min time period. Of course, a longer gradient is expected to increase the peak capacity, but the short separation time is valuable because real protein samples require multiple dimensions of separations, and each dimension adds times to the analysis. Future work will address the peak capacity using mass spectrometry for detection, for which peak capacity is an important issue.

CONCLUSIONS

Higher speed and resolution in RPLC is obtainable for protein separations with a silica colloidal crystal when using a commercial nano-UHPLC instrument. Split flow of 5:1 gives fast separations of intact proteins with a gradient delay of <0.5 min using pressures that remain below the maximum of 800 bar (11,200 psi) for a particle diameter of 470 nm and column length of up to 2 cm. Fluorescence detection was used because that was what was available, and on-column absorbance detection could be used to make the system fully integrated. A peak capacity exceeding 50 is obtained in less than 10 min.

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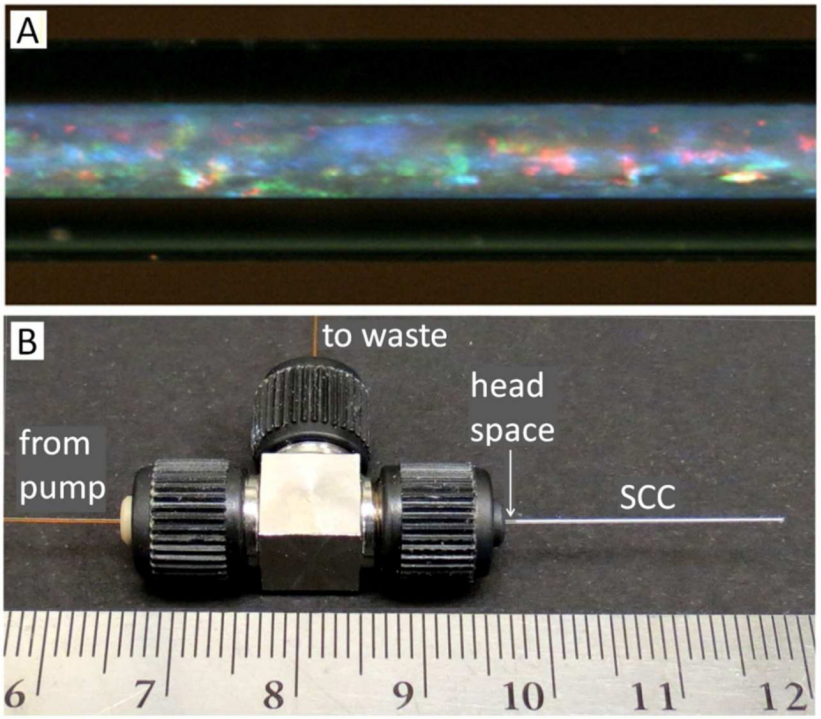

Figure 1.

A) Photograph of a segment of the capillary (75 μm i.d.) packed with SCC, obtained using a zoom microscope. The colors are indicative of high order due to Bragg diffraction. B) Photograph of the flow splitter with a capillary similar to the ones used in the experiments, showing the short section of visible head space before the SCC. The major divisions of the ruler scale are in cm.

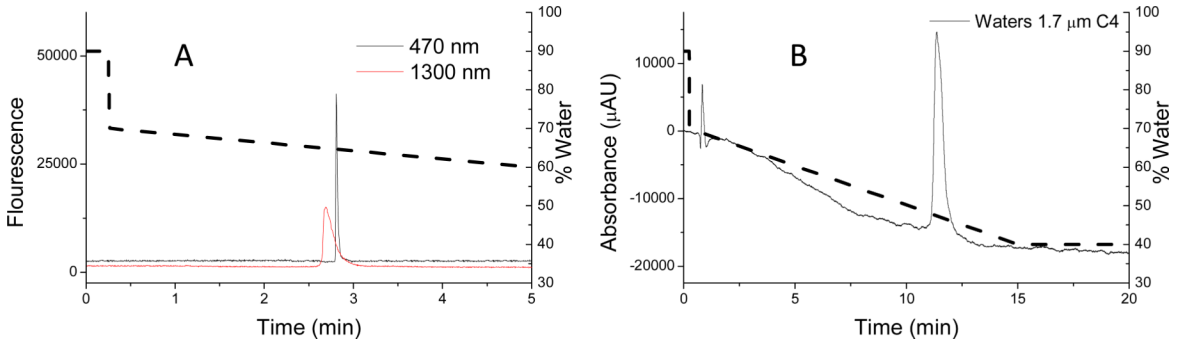

Figure 2.

Chromatograms for BSA labeled with Alexaflour-546. A) Chromatogram for capillaries packed with nonporous 1.3 μm and nonporous 470 nm particles are compared. Each had a C4/C1 bonded phase, and the position for detection was 1 cm from the beginning of the capillary. Flow rates though the capillaries were 200 nL/min, and a volume of 400 pL of 0.01 mg/mL BSA was injected onto the column. The separations were carried out at room temperature (22 °C). B) Chromatogram of the same sample using a Waters BEH C4 column, 2.1 mm id, with a particle diameter of 1.7 μm and separation length of 0.5 cm. Detection used a PDA detector at the dye absorbance wavelength of 546 nm. For B, flow rate was 200 μL/min, and 5 μL of 0.01 mg/mL BSA was injected onto the column. The separation was carried out at 40 °C. The gradients for A and B are the same, and these are indicated by the dashed lines.

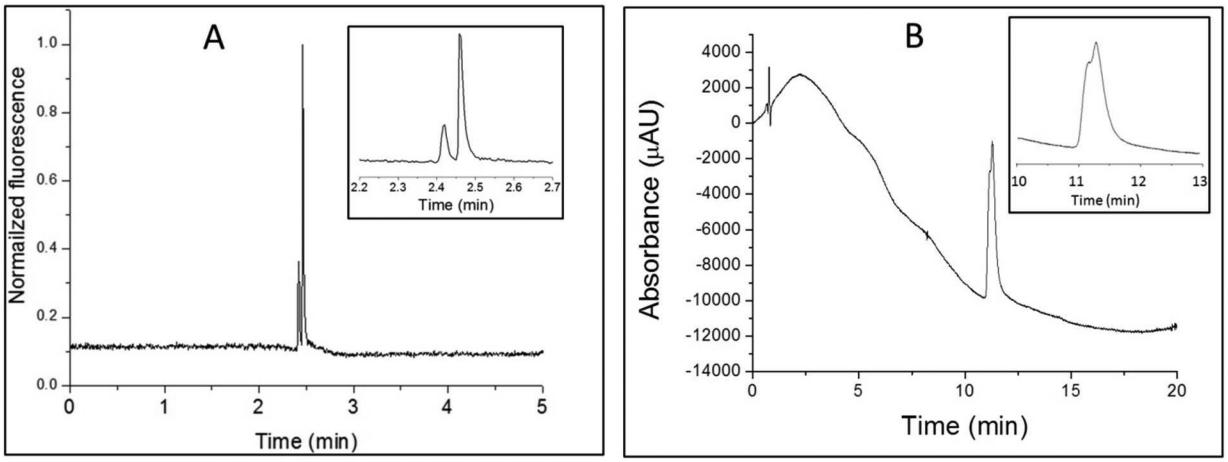

Figure 3.

Chromatograms for the separation of under-labeled from over-labeled BSA. A) SCC with 470 nm particles and pump flow rate of 1.5 μL/min. Inset shows the chromatogram on an expanded time scale. B) Waters 1.7 μm, C4 BEH column. The conditions are the same as those of Figure 2. Inset shows the chromatogram on an expanded time scale.

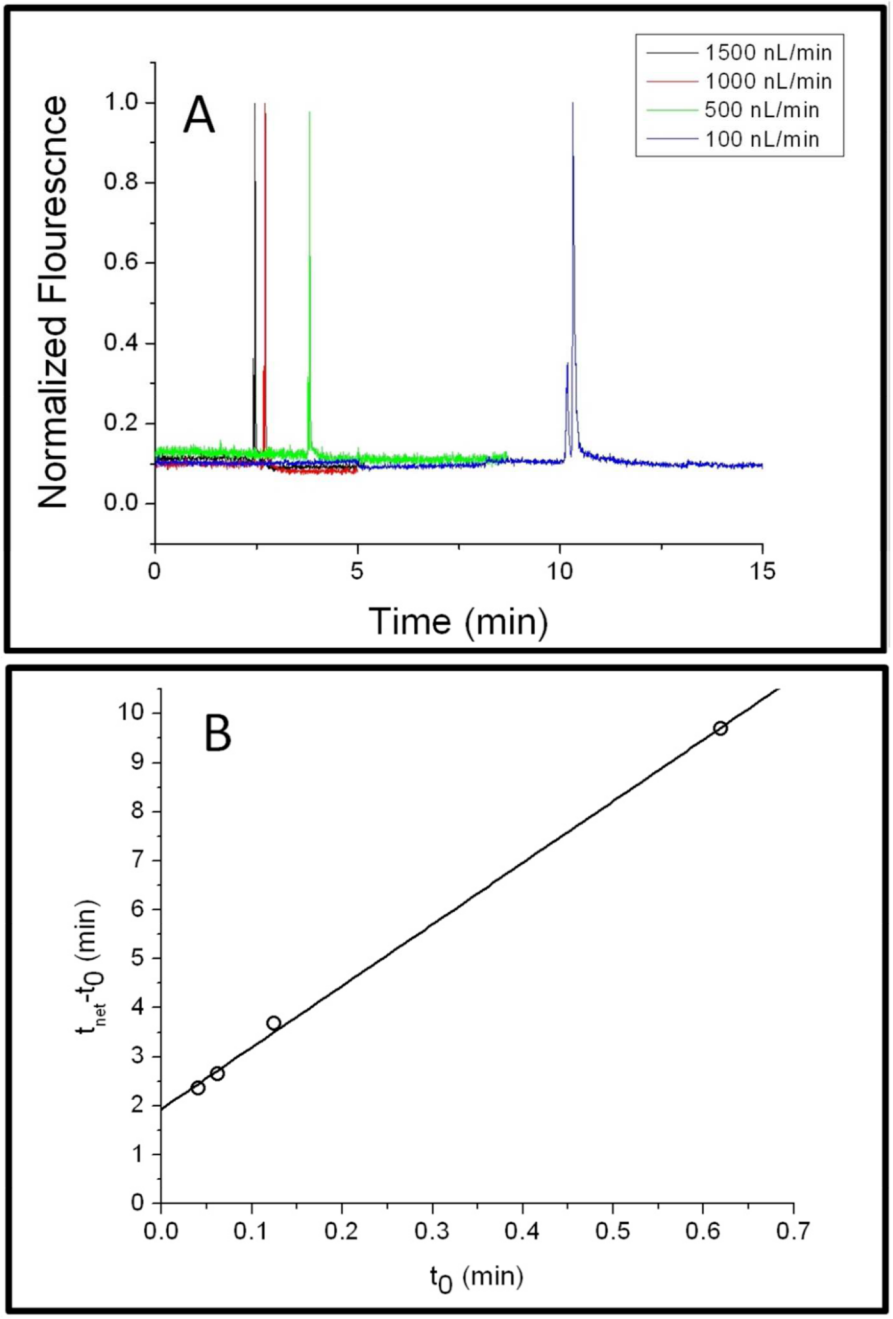

Figure 4.

Determinationj of gradient delay. A) Chromatogrms for the BSA mixture at four different volume flow rates from the pump, and B) plot of t_0 vs.observed elution time minus t_0,where the intercept is the gradient elution time at zero gradient delay. Highest flow rate gives gradient delay tme of 0.4 min.

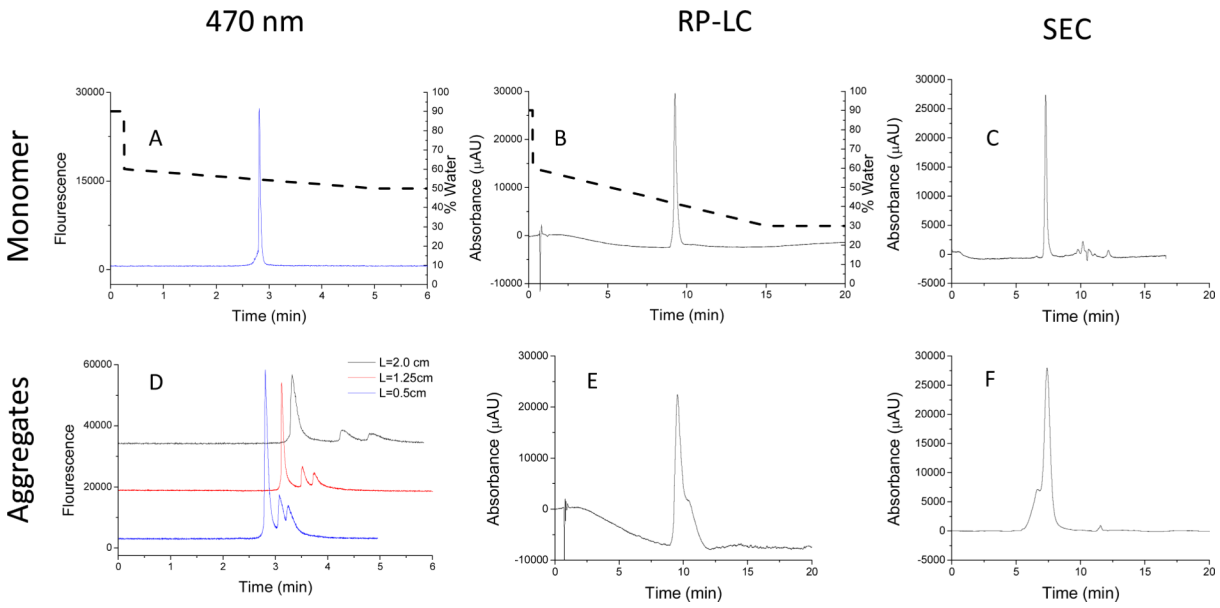

Figure 5.

Separations of monoclonal antibody monomers: A) SCC column with separation length of 0.5 cm, where the gradient is indicated by the dashed line. Flow rate though the column was 200 nL/min, with an overall flow rate of 1000 nL/min. 400 pL of 0.01 mg/mL protein was injected onto the column. The separation was carried out at room temperature (22 °C). B) RPLC was carried out with a PDA detector at 546 nm at 40 °C. Flow rate was 200 μL/min and the 3μL of 0.01 mg/mL protein was injected onto the column, and the gradient is indicated by the dashed line. C) SEC was carried out with a flow rate of 200 μL/min and a 20 μL injection. Chromatograms for aggregated sample of monoclonal antibody: D) SCC, E) RPLC, and F) SEC.

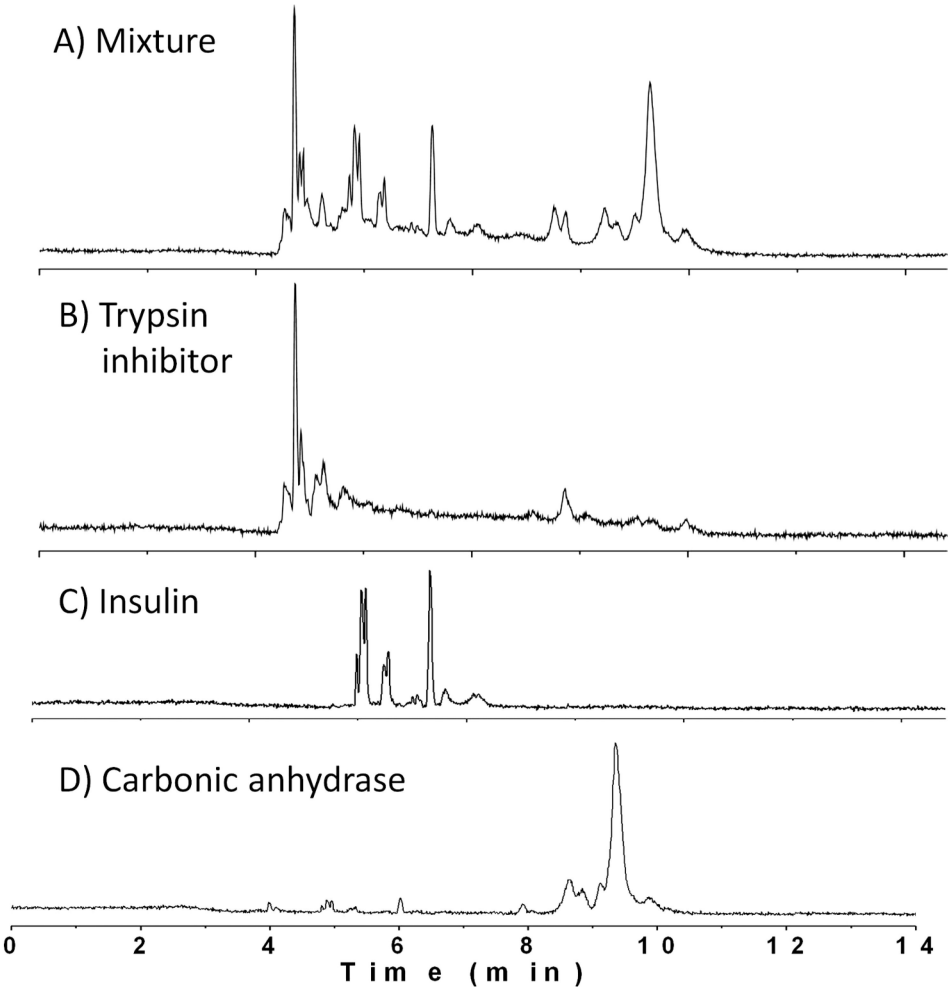

Figure 6.

Separation of a protein mixture, for which each protein was labeled by Alexafluor 546. A) Chromatogram for gradient elution of a mixture of trypsin inhibitor, insulin and carbonic anhydrase. Also shown are chromatograms for gradient elution of the labeled protein injected individually: B) trypsin inhibitor, C) insulin and D) carbonic anhydrase. Gradient is the same as that of Figure 5.