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Post-capillary affinity detection of protein microheterogeneity in capillary zone electrophoresis

Jean K. Abler, K.R. Reddy, Cheng S. Lee*

Department of Chemistry and Ames Laboratory US Department of Energy, Iowa State University, Ames, IA 50011, USA

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Abstract

Post-capillary affinity detection is developed to selectively evaluate protein microheterogeneity in complex sample matrices. In this scheme, separation of protein variants is accomplished prior to affinity detection. A post-capillary reactor is constructed to allow the transfer of separated variants as well as the addition of a fluorescein-labeled analyte-binding protein into the reaction capillary. Affinity complexes are formed within the reaction capillary and monitored by laser-induced fluorescence detection. Fluorescence enhancement, upon affinity complex formation, allows selective detection of protein variants. Successful resolution of IgG Fc variants is presented and compared with affinity capillary electrophoresis techniques reported in the literature. Additionally, the effects of analyte transfer and affinity complex formation on the resolution and detection limits of IgG Fc variants are discussed.

Keywords: Detection, electrophoresis; Affinity detection; Protein microheterogeneity; Immunoglobulins; Proteins

1. Introduction

Biosynthetic production of therapeutic proteins rarely results in a single protein product. Contaminants due to product variants, in addition to serum and cellular proteins, are common. Sources of variation include expression errors, post-translational modifications, deamination, as well as proteolytic degradation [1]. Thus, on-line evaluation of product microheterogeneity is critical to assess purity and lot-to-lot consistency. Additionally, rapid analysis of bioreactor conditions allows feedback controls to modify process parameters, thereby improving product quality and yield.

The formation of affinity complexes followed by electrophoretic separations were studied by capillary electrophoresis [2–6]. Rapid capillary zone electrophoresis (CZE) separation of complexed and free human growth hormone was presented by Nielsen et al. [2]. Due to the low detection limits obtained with laser-induced fluorescence, other researchers used fluorescently tagged antibody to evaluate the total concentration of antigen by complex formation prior to CZE separation [3–6]. Schultz and Kennedy [3] employed competitive and non-competitive capillary electrophoresis-based immunoassays to quantify insulin. Similarly, Schmalzing et al. [4] and Reif et al. [5] were able to selectively determine serum concentrations of cortisol and IgG, respectively. The complexed and free antibodies were separable, provided that there was a significant mobility difference between free and complexed antibody and minimum

*Corresponding author.

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heterogeneity of antigen and antibody. However, Shimura and Karger [6] achieved resolution of immunocomplexes between antibody and deaminated variants of methionyl recombinant human growth hormone by capillary isoelectric focusing.

Recently, Fishman et al. [7] combined capillary electrophoresis with a cell-based detector for the separation and identification of various receptor antagonists. In this study, a post-capillary affinity detection technique is developed and utilized to selectively examine protein microheterogeneity. Post-capillary affinity detection, shown in Fig. 1, combines the separation power of CZE with the specificity of affinity binding. CZE allows the resolution of protein variants in the separation capillary. Subsequently, the variants are transferred across a 20-μm gap and introduced into the reaction capillary. A fluorescently tagged reporter protein present in the gap is also siphoned into the reaction capillary and selectively binds the protein variants. To accomplish transfer with minimal loss of resolution and efficiency, a post-capillary reactor was developed.

Numerous CZE post-capillary designs have been introduced for analyte transfer and fluorescent derivatization [8–11]. Rose and Jorgenson [8] used a coaxial capillary reactor and raised the height of the reagent reservoir to drive the introduction of *o*-phthaldialdehyde. Other workers focused on different schemes to control transfer and reagent introduction driven by electrophoretic mobility and electroosmotic pumping [9–11]. Kuhr et al. [9] used lower ionic strength buffer in the capillary junction and studied the transfer efficiency of dyes across capillary gaps of 50–200 μm. Albin et al. [10] fixed the capillaries 50 μm apart and used a larger internal diameter reaction capillary to drive reagent delivery. Cassidy et al. [11] employed separate electric field strengths in the separation and detection capillaries, thus allowing electronic control of analyte transfer and reagent introduction. In our study, the capillaries are fixed 20 μm apart and a vacuum is applied to the outlet reservoir (see Fig. 1) to introduce fluorescently tagged reporter protein. The selection and comparison of our post-capillary reactor with the designs reported in the literature [9–11] are discussed.

As a model system for evaluating post-capillary affinity detection, the extent of microheterogeneity within human IgG Fc is assessed. Post-capillary addition of fluorescein-labeled fragment B of protein A results in selective binding with IgG Fc variants as

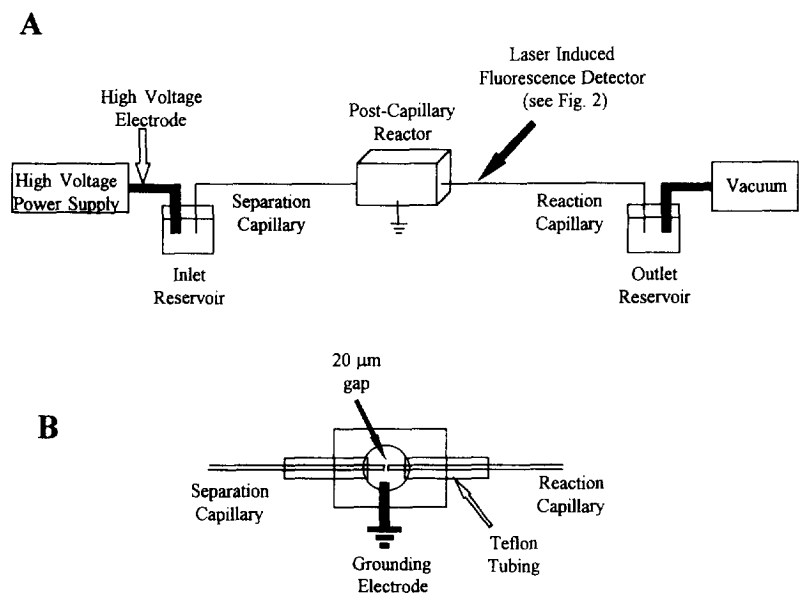

Fig. 1. Schematic diagram of post-capillary affinity detection: (A) Overview of the system and (B) expanded view of the post-capillary reactor. A 20-μm gap between the separation and reaction capillaries is surrounded by solution containing fluorescently labeled reporter protein for post-capillary reaction.

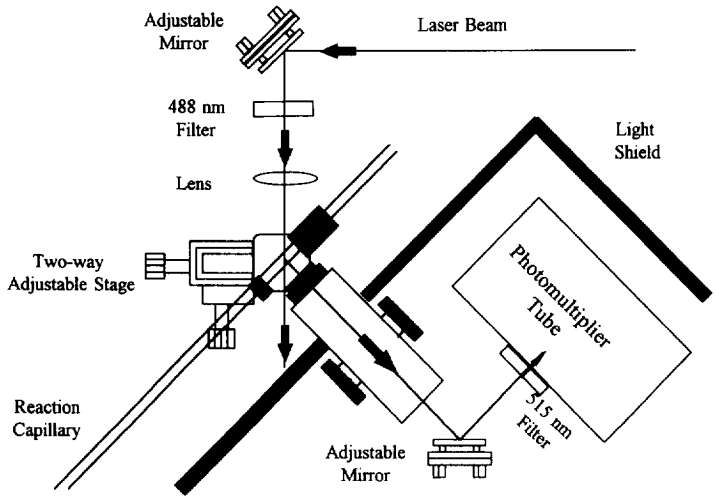

Fig. 2. Optical layout of laser-induced fluorescence detection (LIFD).

they elute from the separation capillary. When IgG binds fragment B–fluorescein (BF), the local pH surrounding the fluorochrome increases. Because the fluorescence intensity of fluorescein rises with increasing pH, the fluorescence intensity of the BF–IgG complex is higher than that of free BF. Mechanistic studies of this phenomenon have been discussed previously and have been used to develop a homogeneous immunoassay for measuring total IgG concentration [12–14]. The fluorescence intensity in the reaction capillary is monitored by laser-induced fluorescence detection (LIFD) (see Figs. 1 and 2), thus allowing selective detection of BF–IgG complexes. The predicted electropherogram consists of a constant background fluorescence from continual siphoning of BF into the reaction capillary. Peaks are observed on top of the background when separated IgG Fc variants form affinity complexes with BF in the reaction capillary. Post-capillary affinity detection is presented and compared with reported affinity capillary electrophoresis techniques [3–6] to assess the microheterogeneity of IgG Fc variants.

2. Experimental

2.1. Fluorescein conjugation

Fragment B of protein A (Sigma, St. Louis, MO, USA) was reacted with 5-carboxyl fluorescein succinimidyl ester (Molecular Probes, Eugene, OR, USA). Subsequently, the mixture was separated by HPLC and fractions were collected as described previously [14]. The fraction corresponding to fluorescein conjugated with lysine 154 of fragment B was diluted in run buffer for use in all studies.

2.2. CZE analysis

Human IgG Fc (CalBiochem, San Diego, CA, USA) was reconstituted with de-ionized water and stored in aliquots at −10°C prior to use. CZE–UV analyses were performed at 200 nm with a linear UVIS 200 multi-wavelength detector (Linear Instruments, Reno, NV, USA). High-voltage power supplies, equipped to deliver 0–30 kV (Spellman, Plainview, NY, USA), were housed in self-contained interlock boxes and used for all CZE experiments.

Run buffer was prepared by titrating solutions containing 20 m*M* monobasic and dibasic phosphate (Fisher Scientific, Pittsburgh, PA, USA) to pH 7.3 and adding neutral and weakly acidic fluorochemical surfactants at a concentration of 0.0025% each. Prior to use, fluorochemically coated capillaries (50 μm I.D. and 375 μm O.D.; J&W Scientific, Folsom, CA, USA) were used as the separation capillaries and were conditioned overnight in buffer containing a ten

times higher concentration of fluorochemical surfactants than in the run buffer.

In transfer experiments, the reaction capillary was fused-silica (25 μm I.D. and 375 μm O.D.; Polymicro Technologies, Phoenix, AZ, USA). Fused-silica capillaries were treated with 100 m*M* sodium hydroxide (Fisher Scientific) followed by de-ionized water and then run buffer, prior to use. UV absorbance and fluorescence detection in the reaction capillary were monitored at 5 and 1.5 cm from the post-capillary reactor, respectively. For UV analysis with transfer, run buffer was placed in the post-capillary reactor and a vacuum was applied to the outlet reservoir via a piper-aid (Drummond Scientific, Broomall, PA, USA). In fluorescence experiments with transfer, conditions were similar, however, BF was diluted to 2 μg/ml in run buffer and placed in the post-capillary reactor.

The 488 nm line of an argon ion laser (Ion Laser Technology, Salt Lake City, UT, USA) was used for LIFD. A LIFD system (see Fig. 2) originally designed by Yeung et al. [15] was geometrically modified to allow the integration of a post-capillary reactor. Specifically, the laser beam, objective and the reaction capillary were positioned in the same plane with a 45° angle between the laser beam and the capillary and a 90° angle between the capillary and the objective. A mirror directed the laser beam through two 488 nm filters, after which a lens focused the beam onto the capillary window (polyamide coating removed by hot sulfuric acid). A 10× objective, mounted on a movable stage, collected the fluorescence and a second mirror directed it through two 515 nm filters and then onto a photomultiplier tube.

2.3. Post-capillary reactor

A rectangular post-capillary reactor was machined out of Plexiglas with dimensions of 2.5×1.3×0.7 cm and a 50-ml reservoir (see Fig. 1). A hole with 1/16 in. (1 in.=2.54 cm) diameter was drilled through the reservoir and PFTE tubing (0.3 mm I.D. and 1/16 in. O.D.) was placed on both sides of the reservoir. The small I.D. of the tubing allowed secure alignment of the separation and reaction capillaries at the same height and lateral position. The end-to-end distance between the capillaries in the post-capillary reactor was adjusted manually and visualized by a custom microscope (Edmund Scientific, Barrington, NJ, USA) equipped with a 10× objective and a 10× eyepiece. The use of a micrometer disc reticle allowed measurement of the 20-μm gap between the capillaries.

2.4. Gel isoelectric focusing

A gel containing 3–10 Bio-Lyte (Bio-Rad, Richmond, CA, USA) was cast and photopolymerized, as described in Bio-Rad's Model 111 Mini IEF instruction manual. Human IgG Fc samples were run in a "Mini-Protean" dual slab cell (Bio-Rad) using an ISCO 453 power supply (ISCO, Lincoln, NE, USA). Solutions of 20 m*M* sodium hydroxide and 10 m*M* phosphoric acid (Fisher Scientific) were added to the cathodic and anodic reservoirs, respectively. A 5-μg sample of IgG Fc was applied to the gel and the voltage was gradually increased to 500 V. This field was maintained until the current dropped to 10% of the initial value, signaling completion of the focusing. Silver staining was performed as described elsewhere [16].

3. Results and discussion

Most affinity capillary electrophoresis techniques reported in the literature [3–6] have focused on complex formation followed by electrophoretic separation. In contrast, our proposed scheme (see Fig. 1) evaluates protein microheterogeneity by resolving protein variants prior to affinity detection. The main advantage of our technique, as demonstrated in this study, is improved resolution of protein variants relative to affinity complex formation prior to CZE analysis.

3.1. CZE–UV studies

The success of our proposed scheme for examining the microheterogeneity of IgG Fc depended on the accomplishment of a number of steps. First and foremost, IgG Fc variants had to be resolved by CZE. To achieve this goal, experimental parameters such as buffer composition, field strength, the type of capillary (coated- vs. fused-silica) and the effective

capillary length were examined. Successful baseline resolution of four IgG Fc isoforms was obtained in CZE by using fluorocarbon-coated capillaries (see Fig. 3A). The separation efficiencies were lower than those commonly expected with CZE and were probably due to a combination of protein–wall interactions and even further microheterogeneity. The variant with the least negative electrophoretic mobility was the most abundant; its peak area accounted for 55% of the total area. Also shown in Fig. 3B, the isoelectric focusing of IgG Fc resulted in separation and detection of four variants with a p*I* range of about 6.2–6.8. At a buffer pH of 7.3, all four variants had a net negative charge, which was consistent with their negative electrophoretic mobilities observed in CZE. In comparison with gel isoelectric focusing, an accurate assessment of the extent of IgG Fc variation was achieved by CZE.

IgG Fc variants resolved by CZE had to be transferred across a 20-μm gap and introduced into the reaction capillary. Minimal loss in separation resolution and efficiency of the variant peaks was critical for maintaining the distinction between variants and for making an accurate measurement of IgG Fc microheterogeneity. Transfer experiments were carried out by applying a vacuum to the outlet reservoir and were monitored with UV detection, to estimate the loss in resolution. In this case, run buffer without BF was placed in the post-capillary reactor and the IgG Fc variants were detected at 200 nm, after they were transferred into the reaction capillary. In comparison with the single capillary separation, the transfer electropherogram (see Fig. 3C) showed somewhat poorer resolution. For example, resolution between peaks 2 and 3 decreased from 1.2 to 1.1.

Resolution changes due to transfer were insignificant, however, peak height differences were notable in the UV analysis. When the IgG Fc variants were transferred across the gap and introduced into the reaction capillary, the peak heights decreased. This decrease, although large, was attributable to our post-capillary reactor design. Because the contents of the separation capillary and the buffer in the post-capillary reactor were both siphoned into the reaction capillary, the concentrations of the IgG Fc variants were decreased by ca. 40%, due to dilution. Also, the UV detection limits were poorer in the transfer studies because the optical pathlength in the reaction capillary was only 25 μm, while that of the separation capillary was 50 μm.

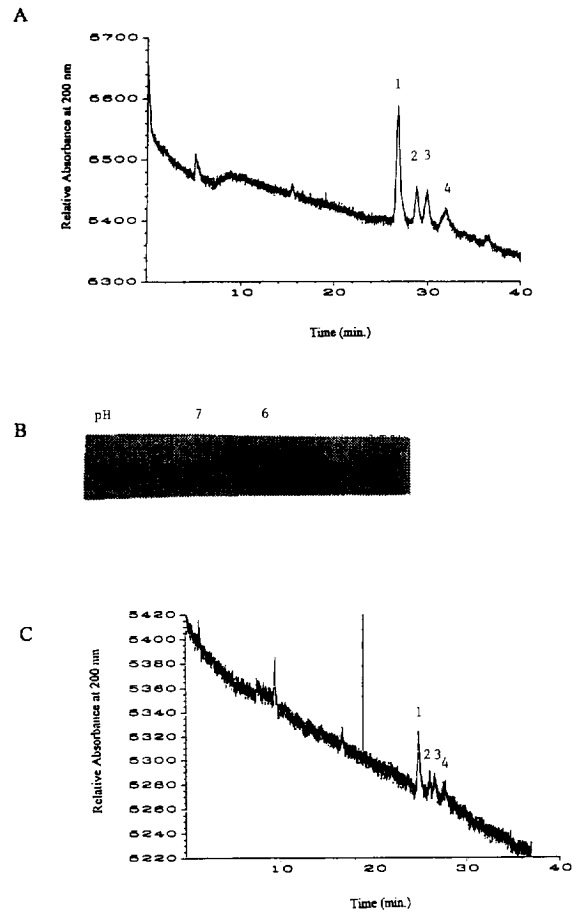

Fig. 3. Analysis of IgG Fc variants. (A) CZE separation of 250 μg/ml IgG Fc in a fluorocarbon-coated capillary. The separation capillary was 50 cm (30 cm to detector)×50 μm I.D.×375 μm O.D.; 10 kV was applied to the inlet reservoir for a 10-s sample injection and was maintained during the separation. The run buffer was 20 m*M* phosphate with 0.005% fluorochemical surfactant at pH 7.3. UV absorbance was detected at 200 nm. (B) Gel isoelectric focusing of IgG Fc. (C) CZE separation and transfer of 500 μg/ml IgG Fc. The separation capillary was fluorocarbon-coated, 30 cm×50 μm I.D.×375 μm O.D.; 7 kV was applied across the separation capillary for a 5-s sample injection and for electrophoresis. The run buffer and the buffer in the post-capillary reactor was 20 m*M* phosphate with 0.005% fluorochemical surfactant at pH 7.3. The UV absorbance at 200 nm in the fused-silica reaction capillary (20 cm×25 μm I.D.×375 μm O.D.) was measured 5 cm from the post-capillary reactor. A vacuum was applied to the outlet reservoir.

3.2. Post-capillary affinity detection of IgG Fc

Transfer of IgG Fc variants across the post-capillary reactor was possible without significant deterioration in resolution. For post-capillary affinity detection of the IgG Fc variants, BF was added to the post-capillary reactor. The fluorescence enhancement upon binding of BF with IgG Fc variants, due to a microenvironmental pH increase, varied with solution conditions such as pH and ionic strength [12–14]. Therefore, fluorescence emission scans of BF in the absence and presence of excess IgG Fc were performed under identical solution conditions (pH, ionic strength and fluorochemical surfactant concentration) as used in the CZE separation. When 16 μg/ml of IgG Fc was reacted with 2 μg/ml of BF, the fluorescence intensity increased by 68% without any observable shift in fluorescence emission wavelength. This increase in fluorescence intensity upon binding was significant and allowed the selective detection of IgG Fc variants in the reaction capillary by on-column LIFD.

In post-capillary affinity detection, IgG Fc variants (with a total concentration of 100 μg/ml) were separated in a fluorocarbon-coated capillary and transferred across a 20-μm gap into the reaction capillary. Simultaneously, BF was siphoned from the post-capillary reactor into the reaction capillary by applying a vacuum to the outlet reservoir. Initially, a large fluorescence front was observed in the electropherogram shown in Fig. 4, due to flow of BF into the reaction capillary. On top of the fluorescence background, a system peak appeared at 17 min, followed by four additional peaks at around 25 min. The pattern of four peaks was similar to that observed in CZE–UV studies; the first peak accounted for 57% of the total area in post-capillary affinity detection and 55% in CZE–UV separations. Furthermore, peak-height ratios between the four components only changed by approximately 5% relative to CZE–UV measurements. These observations indicated that the four IgG Fc variants had comparable binding affinities towards BF, as well as similar local environment changes upon binding.

In comparison with the CZE–UV transfer experiments (see Fig. 3C), resolution of IgG Fc variants in post-capillary affinity detection was poorer, due to the introduction of the affinity reaction. The resolution decreased from 2.0 to 1.3 between peaks 1 and 2 and reduced from 1.1 to 0.8 between peaks 2 and 3. In post-capillary affinity detection, detection limits for IgG Fc variants were superior to those with UV measurements (see Fig. 3). As shown in Fig. 5, IgG Fc variants with a total concentration of 10 μg/ml total IgG Fc were separated and detected. Based on peak-area ratios, the injected concentration of the first variant was estimated to be approximately

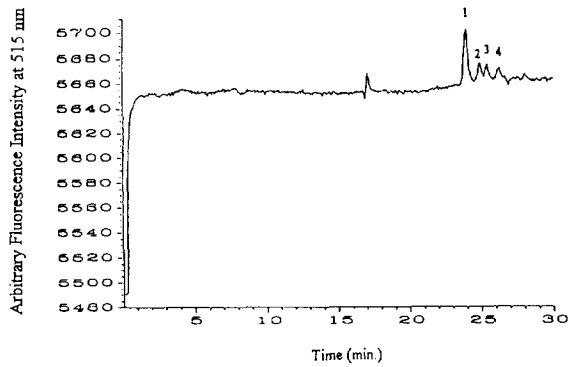

Fig. 4. Post-capillary affinity detection of 100 μg/ml IgG Fc variants. Separation was performed in a fluorocarbon-coated capillary, 30 cm×50 μm I.D.×375 μm O.D.; 6 kV was applied across the separation capillary for the 5-s sample injection and was maintained for separation. The run buffer contained 20 m*M* phosphate and 0.005% fluorochemical surfactant at pH 7.3. The fused-silica reaction capillary was 20 cm×25 μm I.D.×375 μm O.D. LIFD at 515 nm was monitored in the reaction capillary, 1.5 cm from the post-capillary reactor. A vacuum was applied to the outlet reservoir and 2 μg/ml BF was placed in the post-capillary reactor.

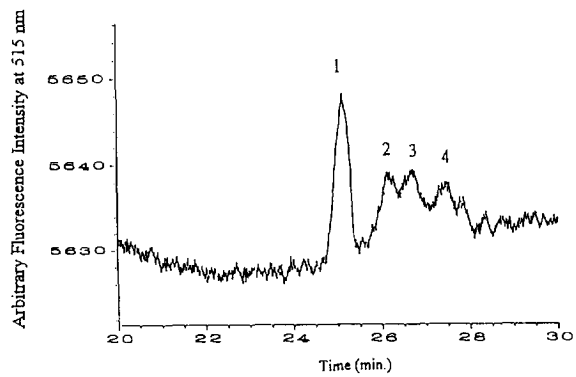

Fig. 5. Post-capillary affinity detection of IgG Fc variants. Conditions were identical to those in Fig. 4, except that the sample contained only 10 μg/ml total IgG Fc.

6 μg/ml. The concentrations of the remaining three variants were slightly greater than 1 μg/ml.

The linearity of post-capillary affinity detection of IgG Fc variants was evaluated. Triplicate injections of IgG Fc at total concentrations of 25, 50, 75 and 100 μg/ml were analyzed and the peak area of the first variant was plotted as a function of the total IgG Fc concentration (see Fig. 6). Regression analysis resulted in a correlation coefficient of 0.997, indicating excellent linearity of IgG Fc microheterogeneity analysis. Therefore, post-capillary affinity detection has the ability to simultaneously analyze both the extent and the quantification of protein microheterogeneity. Day-to-day variation in system conditions could be minimized by spiking samples with a known amount of IgG and thus normalizing the peak-area response.

System peaks have been observed in micellar electrokinetic chromatography with indirect fluorescence detection of alcohols and phenols [17]. Amankwa and Kuhr [17] have attributed the system peaks to excess background fluorophores that were displaced from the micelles by analyte molecules and eluted at the time of the electroosmotic flow. Additionally, both positive and negative system peaks have been observed in CZE–UV analysis and are believed to have physical origins at the capillary inlet, which caused a buffer ion concentration change in the sample plug [18]. In all post-capillary affinity electrophoretic measurements, system peaks were observed at a time corresponding to the electroosmotic flow. It was possible that the system peaks observed in post-capillary affinity detection were due to variation in the ionic strengths of the sample plug and the run buffer, which then altered the fluorescence signal.

The efficient transfer and mixing of IgG Fc variants with BF in the reaction capillary was critical. Transfer efficiency depended on alignment of the separation and reaction capillaries within 20 μm. Post-capillary reactor construction, PTFE tubing alignment, as well as parallel capillary ends, were critical to achieve accurate gap distances. In this study, both BF and IgG Fc were large molecules with small diffusion coefficients in the range of 10^{-6}–10^{-7} cm^2/s. Small diffusion coefficients resulted in longer mixing times in the reaction capillary. Therefore, the I.D. of the reaction capillary was held at 25 μm, to minimize the time requirement. Additionally, the application of a vacuum and the resultant parabolic flow enhanced mixing in the reaction capillary.

Another advantage of using a vacuum in the reaction capillary was that equal flow-rates were achieved for all components. Peak distortions and negative peaks were observed when an electric field was applied to the reaction capillary in place of a vacuum (see Fig. 7). The negative peaks could be explained by examining the migration processes in the reaction capillary. Once BF and IgG Fc variants bond in the reaction capillary, the local concentration of free BF was depleted. Simultaneously, the BF and BF–IgG Fc complexes were migrating at different velocities. The BF–IgG Fc complexes have a higher

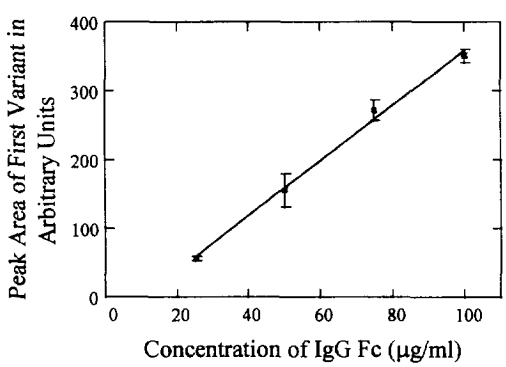

Fig. 6. Peak area of variant 1 as a function of total IgG Fc concentration. Run conditions were similar to those in Fig. 4.

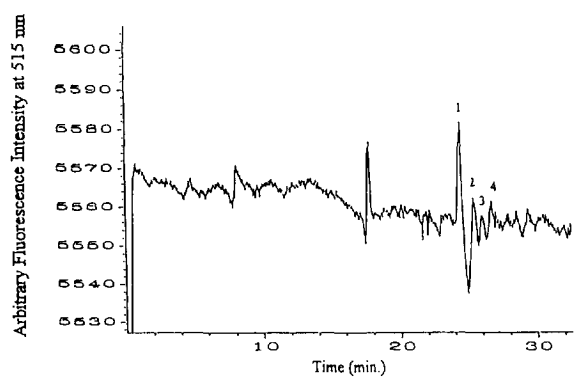

Fig. 7. Post-capillary affinity detection of IgG Fc variants with electric field in the reaction capillary. Conditions were identical to those in Fig. 4, however, 5.5 kV was applied across the reaction capillary in place of the vacuum.

overall mobility and could start to migrate out of the region of depleted BF. Therefore, the elution of the complex peak for each IgG Fc variant was followed by a negative peak as a result of a depleted BF zone.

3.3. Selectivity and generality of post-capillary affinity detection

The key advantage of post-capillary affinity detection was selective detection of protein variants due to affinity interactions. Despite the addition of 1 mg/ml myoglobin and 1 mg/ml serum albumin to a 65 μg/ml IgG Fc sample, only peaks due to IgG Fc were observed by post-capillary affinity detection (data not shown). Because myoglobin and serum albumin lacked affinity binding with BF, their presence did not contribute to any induced changes in the fluorescence intensity. The rapid analysis of IgG Fc microheterogeneity was achieved without any interference from sample components including myoglobin and serum albumin, as presented in this study. Selectivity of affinity interactions is critical for many systems containing complex sample matrices, including cell cultures. Future work is focused on direct cell culture analysis of IgG microheterogeneity.

In order for an affinity system to be studied by post-capillary affinity detection, there must be a significant change (either increase or decrease) in fluorescence intensity of fluorescently tagged reporter protein upon binding with analytes. Although not trivial, this requirement has been accomplished with other analytes. Current studies underway in our laboratory demonstrate the viability of detecting biotin derivatives using post-capillary reactions with fluoroscein-labeled avidin in post-capillary affinity detection.

3.4. Comparison with reported affinity capillary electrophoresis techniques

Affinity capillary electrophoresis techniques reported in the literature [3–5], involving complex formation prior to CZE analysis, were employed to evaluate IgG Fc microheterogeneity. As shown in Fig. 8A, a shoulder on the BF peak was observed in the CZE–LIFD measurement. This observation indicated potential microheterogeneity among BF, even after HPLC purification (see Section 2). When the

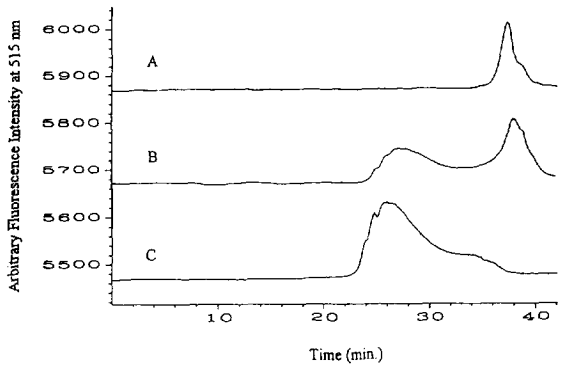

Fig. 8. CZE–LIFD separation of BF–IgG Fc affinity complexes. The fluorocarbon-coated capillary of 50 μm I.D.×375 μm O.D. had a total length of 46 cm and a length of 30 cm to the LIFD at 515 nm; 11 kV was applied for the 5-s sample injection and was maintained for the separation. Run buffer contained 10 m*M* phosphate and 0.005% fluorochemical surfactant at pH 7.0. BF and IgG Fc were prepared in run buffer with (A) 12 μg/ml BF, (B) 12 μg/ml BF and 12 μg/ml IgG Fc and (C) 12 μg/ml BF and 50 μg/ml IgG Fc.

amount of IgG Fc was limiting in the reaction prior to CZE separation, peaks were observed for both the complexed and free BF (see Fig. 8B). The baseline failed to return to background levels between the complexed and free BF peaks, which indicated possible complex dissociation during the CZE analysis. When the amount of IgG Fc was in excess, the complex peak shown in Fig. 8C was broad, with shoulders. Perhaps these shoulders on the complex peak resulted from poorly resolved IgG Fc microheterogeneity. By comparing the results shown in Figs. 4 and 8, post-capillary affinity detection clearly exhibited greater resolving power for examining protein microheterogeneity in complex sample matrices.

Affinity complex formation prior to CZE analysis, however, has the advantage of lower detection limits. Detection limits suffer with post-capillary affinity detection because of the high fluorescence background and the need to determine a small change in the background level upon complex formation. Decreasing the concentration of BF in the post-capillary reactor may improve the detection limit by lowering background noise, however, the ultimate limit is also determined by the binding affinity between BF and IgG Fc variants. Therefore, complex formation prior to CZE–LIFD is advantageous in determining the

total amount of IgG. However, post-capillary affinity detection has significant advantages in maintaining resolution and selectively detecting IgG Fc variants in complex sample matrices.

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References

[1] V.R. Anicetti, B.A. Keyt and W.S. Hancock, Trends Biotechnol., 7 (1989) 342.
[2] R.G. Nielsen, E.C. Rickard, P.F. Danta, D.A. Sharknas and G.S. Sittampalam, J. Chromatogr., 539 (1991) 177.
[3] N.M. Schultz and R.T. Kennedy, Anal. Chem., 65 (1993) 3161.
[4] D. Schmalzing, W. Nashabeh, X. Yao, R. Mhatre, F.E. Regnier, N.B. Afeyan and M. Fuchs, Anal. Chem., 67 (1995) 606.
[5] O. Reif, R. Lausch, T. Scheper and R. Freitag, Anal. Chem., 66 (1994) 4027.
[6] L. Shimura and B. Karger, Anal. Chem., 66 (1994) 9.
[7] H.A. Fishman, O. Orwar, N.L. Allbritton, B.P. Modi, J.B. Shear, R.H. Scheller and R.N. Zare, Anal. Chem., 68 (1996) 1181.
[8] D.J. Rose and J.W. Jorgenson, J. Chromatogr., 447 (1988) 117.
[9] W.G. Kuhr, L. Licklider and L. Amankwa, Anal. Chem., 65 (1993) 277.
[10] M. Albin, R. Weinberger, E. Sapp and S. Moring, Anal. Chem., 63 (1991) 417.
[11] R.M. Cassidy, W. Lu and V. Tse, Anal. Chem., 66 (1994) 2578.
[12] C.S. Lee, P.Y. Huang and D.M. Ayres, Anal. Chem., 63 (1991) 464.
[13] P.Y. Huang and C.S. Lee, Anal. Chem., 64 (1992) 977.
[14] P.Y. Huang and C.S. Lee, Biotechnol. Bioeng., 42 (1993) 37.
[15] E.S. Yeung, P. Wang, W. Li and R.W. Giese, J. Chromatogr., 608 (1992) 73.
[16] S.K. Sather and J. King, J. Biol. Chem., 269 (1994) 25268.
[17] L.N. Amankwa and W.G. Kuhr, Anal. Chem., 63 (1991) 1733.
[18] C.L. Colyer, K.B. Oldham and A.V. Sokirko, Anal. Chem., 67 (1995) 3234.