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Staining Method for Protein Analysis by Capillary Gel Electrophoresis

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Abstract

A novel staining method and the associated fluorescent dye were developed for protein analysis by capillary SDS-PAGE. The method strategy is to synthesize a pseudo-SDS dye and use it to replace some of the SDS in SDS–protein complexes so that the protein can be fluorescently detected. The pseudo-SDS dye consists of a long, straight alkyl chain connected to a negative charged fluorescent head and binds to proteins just as SDS. The number of dye molecules incorporated with a protein depends on the dye concentration relative to SDS in the sample solution, since SDS and dye bind to proteins competitively. In this work, we synthesized a series of pseudo-SDS dyes, and tested their performances for capillary SDS-PAGE. FT-16 (a fluorescein molecule linked with a hexadodecyl group) seemed to be the best among all the dyes tested. Although the numbers of dye molecules bound to proteins (and the fluorescence signals from these protein complexes) were maximized in the absence of SDS, high-quality separations were obtained when co-complexes of SDS–protein–dye were formed. The migration time correlates well with protein size even after some of the SDS in the SDS–protein complexes was replaced by the pseudo-SDS dye. Under optimized experimental conditions and using a laser-induced fluorescence detector, limits of detection of as low as 0.13 ng/mL (bovine serum albumin) and dynamic ranges over 5 orders of magnitude in which fluorescence response is proportional to the square root of analyte concentration were obtained. The method and dye were also tested for separations of real-world samples from *E. coli*.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is one of the most commonly used methods for protein analysis. Usually, a staining and destaining process is involved in order to properly detect the separated protein bands. Capillary electrophoresis (CE), and more recently microchip-based electrophoresis, is a miniaturized electrophoresis technique that has many advantages (e.g., high separation efficiency, short separation time, low mass detection limit, on-line detection, and automated operation) over traditional slab gel techniques. SDS-PAGE has been performed in capillaries^{1–5} and on microchips.^{6–9} Unfortunately, the staining and destaining process used in conventional slab gels cannot be simply adopted in CE. Typically, the separated proteins are detected in-column by either an ultraviolet (UV) absorbance or a laser-induced fluorescence (LIF) detection system.

UV absorption is arguably the most frequently used detection mode in CE. It is also commonly employed in capillary SDS-PAGE,^{1,2} since protein–SDS complexes absorb light around 280 nm due to the aromatic side groups of amino acids and around 200–220 nm due to the peptide bonds between amino acids. LIF detection is preferred when low limit of detection (LOD) and wide linear dynamic range are desired. Native fluorescence of proteins has been explored for direct protein detection,¹⁰ but it is not widely accepted because of the use of expensive UV lasers. Often, proteins are somehow fluorescently labeled and then measured by a LIF detection

system using a commonly available laser such as an air-cooled argon ion or helium–neon laser.^{6,11,12}

Labeling proteins reliably and reproducibly is challenging, although a lot of progress has been made.¹³ Proteins have been covalently linked with fluorescent dyes mainly via the amine groups on the proteins.^{12,14–16} These proteins can then be bound with SDS and separated by capillary gel electrophoresis. Protein concentrations of as low as 3×10^{-10} M (4–10 ng/mL) were successfully analyzed by capillary SDS-PAGE.¹⁷ However, most of these protocols are cumbersome and suffer from incomplete and ambiguous labeling,¹⁶ resulting in complex electropherograms. Alternatively, proteins can react with SDS first and the protein–SDS complexes are then dynamically labeled with fluorescent dyes before electrophoresis.^{8,18–20} Presumably due to the low binding efficiency and high background noise, only moderate low LODs (30–500 ng/mL) were achieved.^{8,20} In this paper, we introduce a novel strategy to stain proteins for capillary SDS-PAGE.

Figure 1 presents a schematic presentation of this novel approach. As SDS reacts with proteins, a given amount of pseudo-SDS dye is added in the solution. The pseudo-SDS dye consists of a negatively charged fluorescent group covalently linked with a long carbon chain. Since the long carbon chain is equivalent to the dodecyl group and the negatively charged fluorescent group resembles the sulfate group of SDS, the pseudo-SDS dye has the same function as SDS when binding to proteins. At the end of the reaction, all protein molecules will be attached with some pseudo-SDS dye, as well as SDS. Increasing the dye concentration relative to SDS in the reaction solution, the number of dye molecules bound to a protein can be enhanced accordingly. As a result, the protein detection sensitivity can be enhanced correspondingly since each protein molecule can be attached with an increased number of fluorophores. The goal of this work is to demonstrate the feasibility of this staining strategy.

EXPERIMENTAL SECTION

Chemicals

Bovine serum albumin (BSA, 66 kDa) and human transferrin (76 kDa) were obtained from Sigma (St. Louis, MO). Protein size marker was purchased from Amersham Biosciences, UK. Acrylamide, *N,N'*-methylene bisacrylamide (Bis), *N,N,N',N'*-tetramethylethylenediamine (TEMED), and ammonium persulfate (APS) were obtained from Bio-Rad Laboratories (Hercules, CA). Fluorescein isothiocyanate isomer I (FITC), amines with various alkyl chains, SDS, and other common chemicals were obtained from Fisher Scientific International Inc. Aqueous solutions were prepared with DI water purified by a NANOpure Infinity ultrapure water system (Barnstead, Newton, WA). All chemicals were used as received.

Preparation of Sieving Matrix

The synthesis procedure has been described previously.²¹ Briefly, 2 g of acrylamide and 32 mg of Bis were dissolved in 100 mL of a buffer solution containing 60 mM Tricine, 21 mM Tris with 0.25% SDS and 1% isopropyl alcohol in a 250-mL glass bottle. After the solution was vacuum degassed, polymerization reaction was initiated by adding 160 μ L of 10% APS and 10 μ L of TEMED in the bottle. The reaction was allowed to proceed overnight at room temperature.

Synthesis of Pseudo-SDS Dyes

A series of pseudo-SDS fluorescence dyes were synthesized by following the process presented in Figure 2. A thiourea group was used as a bridge to link the fluorescence moiety with an alkyl chain. Briefly, 0.05 mmol of FITC and 0.05 mmol of 1-alkylamine were dissolved in dry dichloromethane, and the mixture was stirred at 60 °C for 4 h and cooled down to room

temperature. The product was precipitated out from the solution as a yellow solid. The crude product was collected and recrystallized twice from dichloromethane. The dye products are referred to as FT-*x*, where *x* indicates the number of carbon atoms in the straight alkyl chain. NMR was employed to identify/confirm the dye structures [e.g., For FT-16, ¹H NMR (DMSO, 500 Mz): δ = 10.11(s), 9.84(s), 8.21(s), 8.05(s), 7.71-(m), 7.17–7.16(d), 6.67–6.66(d), 6.60–6.54(m), 3.50(m), 1.57–1.55(m), 1.29–1.23(m), 0.86–0.83(t).] The yields varied from 79 to 88% for the synthesis of FT-2 to FT-18.

Preparation of *Escherichia coli* Sample

The *E. coli* sample was obtained from strain pB0W1/DK8 and prepared following the procedure as described in the literature.²² Briefly, after cells were harvested, they were washed and passed through a French press. The resulting mixture was spun at 18 000 rpm on an Avanti J-26 XPI centrifuge (Beckman Coulter, Fullerton, CA) for 20 min, the supernatant was collected, and its volume was recorded. After the whole solution was diluted by a factor of 4 with 0.02% sodium 1-butanedisulfonate, acetonitrile was gradually added to the solution until a final acetonitrile concentration of 35% (v/v) was reached. The solution was then centrifuged at 13 000 rpm for 10 min to remove the insoluble materials. The supernatant was dried and redissolved in water to the volume recorded.

Preparation of F1 Sample

The F1 sample had the same origin as the *E. coli* sample. After the mixture was spun at 18 000 rpm on the Avanti J-26 XPI centrifuge for 20 min, the supernatant was purified by an anion-exchange chromatograph and gel filtration to remove unwanted proteins.²³

Sample Preparation for Capillary SDS-PAGE

A 10 mg/mL dye stock solution was prepared in 50% (v/v) THF and 50% of an aqueous buffer solution containing 60 mM Tris, 60 mM TAPS, and 5% SDS (pH 8.4). A 1.0 mg/mL dye solution was obtained by diluting the stock solution with a buffer solution containing 60 mM Tris, 60 mM TAPS, and 2.5% SDS (pH 8.4). An aliquot of this solution was then added to a protein sample to achieve a dye/ protein ratio (w/w) of ~1/20. After the solution was cooked at 65 °C for 5 min, it was diluted with water to a proper concentration for capillary SDS-PAGE.

Electrophoresis Apparatus

The experimental setup includes a Glassman high-voltage power supply (Glassman High Voltage Research Inc., Ormond Beach, FL), a confocal laser-induced fluorescence detection system, and a laptop computer for data acquisition. Briefly, a 488-nm beam from an argon ion laser (LaserPhysics, Salt Lake City, UT) was reflected by a dichroic mirror (Chromatech, Canton, MI) toward a 20× objective with a 0.5 numerical aperture (Rolyn Optics, Covina, CA) that focused laser to the bore of the separation capillary. Fluorescence from the capillary was collected and collimated by the same objective and passed through the dichroic mirror and a long-pass filter (cutoff wavelength, 520 nm) to a photosensor module (H5784–01, Hamamatsu) with a spatial filter of diameter of 2.0 mm before the sensor window. The electrophoresis current was monitored by measuring the voltage drop across a 99-k Ω resistor connected between the ground reservoir and grounding electrode. The signal from the photosensor module and the electrophoresis current were acquired by a NI multifunctional card DAQCard-6062E (National Instruments, Austin, TX) and treated with an in-laboratory developed LabVIEW program (National Instruments).

Capillary SDS-PAGE

A 75- μ m-i.d. and 35-cm-long (effective length ~30 cm) capillary was used for the separation. The inner surface of the capillary was coated with a layer of cross-linked polyacrylamide; the

coating process was described previously.²⁴ After the sieving matrix is pressurized into the capillary, a protein sample was electrokinetically injected under a field strength of 290 V/cm for 5 s. The same sieving matrix solution was utilized as the background electrolyte solution in both anode and cathode reservoirs. The separation was also performed at the same field strength of 290 V/cm. The matrix in the capillary was replenished after each run, while the matrix solutions in the reservoirs were used for 5–10 runs. High voltage is utilized for the separation. Caution must be exercised.

RESULTS AND DISCUSSION

In SDS-PAGE, it is generally accepted that SDS binds to proteins, forming SDS–protein complexes at a mass ratio of ~1.4:1 via hydrophobic-hydrophobic interactions.^{25,26} In a separate experiment, we tested the binding strength between different alkyl sulfate surfactants (e.g., decyl, dodecyl, tetradecyl, and hexadecyl sulfate) and proteins and noticed that the longer chain compounds bound to proteins stronger than the shorter ones. These results led us to the idea of this paper: if a fluorescent dye is linked with a long-chain alkyl group, it will bind to proteins just as SDS does. The resulting complex is thus attached with many dye molecules, which should boost the protein detection sensitivity considerably. A fluorescent dye linked with a long alkyl chain is referred to as a pseudo-SDS dye.

To examine the feasibility of this concept, we synthesized a series of pseudo-SDS dyes following the procedure as presented in Figure 2. After the dye products were purified through a chromatographic column, their fluorescence properties were examined. The fluorescence excitation/emission spectra and quantum yields (data not shown) were very similar for all dyes. However, their water solubilities (or hydrophobicities) were quite different. For FT-2–FT-6, 1% (w/v) of the dye solutions could be prepared simply by dissolving the dye in the buffer (60 mM Tris-TAPS buffer, pH 8.4) solution. To prepare solutions of FT-8–FT-18, appropriate quantities of organic solvent or SDS had to be added to the buffer solution to facilitate the dissolution.

Increased hydrophobicity is expected to promote the binding of the dye to proteins. Figure 3 presents the fluorescence response of SDS–BSA–FT-*x* complexes as a function of *x* while all other conditions were maintained the same. The fluorescence response increased with *x*, indicating an increased number of dye molecules associated with BSA. The experimental results showed clearly that the binding strength between pseudo-SDS dyes and protein increased with the lengths of the alkyl chains. FT-18 generated the highest fluorescence signal, but its solubility in buffer was too low (<0.01%, w/v), and preparation of its solution was troublesome. As a compromise between the sensitivity and convenience of operation, FT-16 was selected in this work.

When coexisting in the solution, SDS and dye molecules compete to bind to proteins. Increasing the dye concentration relative to SDS concentration ought to enhance the protein detection sensitivity since more fluorophores can be attached to proteins. To test this hypothesis, we carried out an experiment with varying molar ratios of FT-16 to SDS while their total concentration was maintained to be the same ($[SDS] + [FT-16] = 0.1 \text{ M}$). Figure 4 presents the results. As expected, fluorescence signal increased with the value of $[FT-16]/([SDS] + [FT-16])$, demonstrating that the number of dye molecules bound to a protein did increase with the dye concentration. (Note: The triangular symbol showed a data point when the proteins were reacted with 0.03 M FT-16 only (without SDS). For this data point, $[SDS] + [FT-16] < 0.1 \text{ M}$ due to the limited solubility of FT-16.)

High concentrations of FT-16 were used for protein separations to achieve high detection sensitivities. Unfortunately, separation qualities seemed to have been sacrificed, although

mildly. Often, good resolutions were obtained when $[FT-16]/([SDS] + [FT-16]) < 5\%$. In the sieving matrix, the SDS concentration was kept below the critical micelle concentration ($3\text{--}8\text{ mM}$)²⁷ to minimize dissociation of dye from the SDS–protein–dye complexes, because FT-16 enjoys staying in SDS micelles.

It should be pointed out that the FT-*x* dyes are not particularly stable. In sample solutions, these dyes decompose slowly under ambient temperatures, and the decomposition accelerates at elevated temperatures. Figure 5 presents a few electropherograms of a pure dye solution after being heated at various temperatures for 5 min. The peaks exhibit only the fragments containing the fluorophor. Although the decomposed products were unidentified, they were believed to carry less negative charge than FT-16 since their migration times were longer than that of FT-16. The decomposed fragments interfere with the analysis of proteins with similar migration times.

To reduce the dye degradation, we optimized the heating time and temperature for the formation of dye–protein–SDS complex. Figure 6 presents the effect of heating time on complex formation and dye decomposition at 65 °C. As shown in the bottom electrophoregram, stable saturated dye–protein–SDS complexes did not seem to have been formed if the dye–protein mixture was heated for 3 min or less. On the other hand, too much degradation occurred (see the top electrophoregram) if the dye–protein mixture was heated for more than 10 min. Heating the mixture at 65 °C for 5 min appeared to be a good compromise, and these conditions were chosen for sample preparation in this work.

Another unique property of this dye is that it gets unbound slowly once it binds to proteins. This provides us with the possibility of staining proteins before separation and running the separation without adding any dye in the sieving matrix, which should reduce the background noise and hence increase the detection sensitivity. Currently, fluorescent dyes were added in the separation media to dynamically label proteins.^{8,9} Owing to the high background noise, the LOD was relatively high.⁹ Although a dilution process can be included to reduce such noise and improve the LOD,⁸ the improvement was limited,⁸ and the procedure is not easily practiced in capillary systems.

When some of the SDS in a SDS–protein complex is replaced by FT-16, will migration time correlate with protein size? To answer this question, we performed a capillary SDS-PAGE of a protein size marker that contained proteins of various sizes (14.4–97 kDa), and the results are displayed in Figure 7. As presented in the inset, a good linear relationship ($R^2 = 0.994$) was obtained between molecular weight and migration time.

Under optimized conditions, limits of detection and linear dynamic ranges were measured. Figure 8 presents a calibration curve of BSA. An excellent linear relationship ($R^2 = 0.999$) was obtained between the Log(fluorescence response) and Log[BSA] from 2.0 ng/mL to 0.1 mg/mL. In this test, a concentrated sample was obtained by adding 1 μL of FT-16 solution (1.0 mg/mL in 60 mM Tris–TAPS buffer with 2.5% SDS, pH 8.4) to 10 μL of BSA and human transferrin solution (1.0 mg/mL each in water). Samples at lower concentrations were obtained by diluting the concentrated sample with water. The slope of the relationship was less than 1, indicating that the fluorescence response biased toward the *x*-axis (the BSA concentration). This was likely caused by the dye exhaustion at high BSA concentrations, supported by the fact that the slope increased with increasing dye concentration. Unfortunately, we were unable to utilize high dye concentrations due to the interference of the decomposed compounds.

To obtain the LOD of this method, we prepared a sample by mixing 10 μL of protein solution (2 ng/mL BSA and 2 ng/mL human transferrin) with 1 μL of 1 $\mu\text{g/mL}$ FT-16 in 60 mM Tris–TAPS buffer with 25 $\mu\text{g/mL}$ SDS (pH 8.4). The separation results of this sample are presented in Figure 9. The peak signals of BSA and human transferrin were 43 times and 35 times as

much as the background noise, corresponding to a LOD of 0.13 ng/mL for BSA and 0.15 ng/mL for human transferrin at a signal-to-noise ratio of 3. With the consideration of a top limit of 0.1 mg/mL (see Figure 8), a dynamic range of 5.8 orders of magnitude in which fluorescence signal increases proportionally with the square root of protein concentration was achieved.

To test the applicability of this staining method for real-world samples, we prepared an *E. coli* sample and an F1 sample according to the literature.²³ Figure 10 exhibits the separation results. More than 20 proteins were detected in the *E. coli* sample, and only 5–6 proteins remained in the F1 sample. In the F1 sample, the F1 protein was the major component that remained, indicating the purification process was efficient. Although evaluation of the purification method is beyond the scope of this paper, these results demonstrated the feasibility of our new method to stain proteins for real-world samples.

CONCLUSIONS

We have demonstrated a novel staining strategy and a pseudo-SDS dye for protein analysis by capillary SDS-PAGE. The incorporation of the dye with proteins does not affect the relationship between migration time and protein size. Because many dye molecules can be incorporated with each protein and the separations can be performed without dye in sieving matrixes, protein detection sensitivities can be enhanced considerably. LOD of as low as 0.13 ng/mL (BSA) has been obtained, which represents a more than 100-fold improvement compared to the state-of-the-art dynamic staining methods⁸ for capillary SDS-PAGE. The LODs could be further reduced by employing other pseudo-SDS probes such as a long, straight alkyl group attached with a red fluorescent dye, an energy-transfer dye, and electrochemical tags. Although more dyes can be incorporated into proteins to improve the detection sensitivity when SDS is absent, the best quality separations have been obtained with both SDS and dye bound to proteins. With a LIF system, a dynamic range of 5.8 orders of magnitude in which fluorescence intensity varies proportionally with the square root of protein concentration has been obtained. This can be extremely useful for analyses of real-world samples because protein levels are so diverse in cells. Our preliminary results have also shown that the staining method and dye can also be used for slab gel electrophoresis (results will be published elsewhere).

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Figure 1.
Schematic presentation of pseudo-SDS dye– protein– SDS complex.

Figure 2.
Synthesis of pseudo-SDS dyes.

Figure 3.

Effect of alkyl chain length on binding strength ($n = 3$). The separations were performed using a 35-cm-long (effective length of ~ 30 cm), 75- μm -i.d. and 360- μm -o.d. fused-silica capillary at an electric field of 290 V/cm. Each protein sample was electrokinetically injected under the same field strength for 5 s. The running buffer contained 60 mM Tricine, 21 mM Tris, and 0.25% SDS (pH 7.6). Samples were prepared by mixing 10 μL of BSA and human transferrin solution (1.0 mg/mL each in water) with 1 μL of various dye solutions at a concentration of 1.0 mg/mL in 60 mM Tris–TAPS buffer with 2.5% SDS (pH 8.4) and heating the mixture at 65 $^{\circ}\text{C}$ for 5 min. All solutions were diluted by a factor of 100 with water prior to injection. Peak areas of BSA were used as the fluorescence response in the Y-axis.

Figure 4.

Enhancement of fluorescence intensity with [FT-16] ($n = 5$). In the sample solution, the total molar concentrations of SDS and FT-16 were kept the same (~ 0.1 M), but their relative values were changed. The resulting solutions were diluted by a factor of 10 with water prior to injection. The triangular symbol indicates a data point when the proteins were reacted with 0.03 M FT-16 in the absence of SDS. Peak areas of BSA were used as the fluorescence response. All other conditions were the same as in Figure 3.

Figure 5. Effects of temperature on dye decomposition. The samples contained 0.1 mg/mL FT-16 in 60 mM Tris-TAPS buffer with 2.5% SDS (pH 8.4). After the samples were heated at different temperatures for 5 min, they were diluted by a factor of 20 with water prior to injection. All other conditions were the same as in Figure 3.

Figure 6.

Effects of heating time on dye decomposition and SDS–protein–dye complex formation. The samples were prepared as in Figure 3 with FT-16, heated at 65 °C for various time periods, and diluted by a factor of 100 with water. All the other conditions were the same as in Figure 3. 1, BAS; 2, human transferrin; and *, the decomposed fragments of FT-16.

Figure 7.

Separation trace of protein size marker. The sample contained 1 mg/mL total proteins. After the sample was prepared with FT-16 solution, it was diluted by a factor of 10 prior to injection. All other conditions were the same as in Figure 3. 1, α -lactalbumin (14.4 kDa); 2, trypsin inhibitor (20.1 kDa); 3, carbonic anhydrase (30 kDa); 4, ovalbumin (45 kDa); 5, BSA (66 kDa); 6, phosphorylase b (97 kDa); *, the decomposed fragments of FT-16. The inset is a calibration plot between migration time and protein size.

Figure 8.

Calibration curve of BSA ($n = 5$). Peak areas were used as the fluorescence response. A concentrated (1 mg/mL) BSA sample was prepared using FT-16 as described in Figure 3. All test samples were obtained by diluting this concentrated solution with water before injection. All the other conditions were the same as in Figure 3.

Figure 9.

Separation trace of 2 ng/mL BSA and human transferrin. The sample was prepared by mixing 10 μ L of protein solution (2 ng/ mL BSA and 2 ng/mL human transferrin) with 1 μ L of 1 μ g/mL FT-16 in 60 mM Tris–TAPS buffer with 25 μ g/mL SDS (pH 8.4). The solution was injected directly into the capillary after heating. All the other conditions were the same as in Figure 3.

Figure 10.

Separation of real-world sample. The test sample of *E. coli* was prepared by mixing 10 μL of the *E. coli* sample with 1 μL of FT-16 solution (2 mg/mL in pH 8.4 buffer with 5% SDS). The test sample of F1 was prepared by mixing F1 solution and FT-16 followed the same procedure as in Figure 3. After heated, the resulting samples were injected directly without dilution. All the other conditions were the same as in Figure 3.