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ORIGINAL PAPER

Dimethylditetradecylammonium bromide ($2C_{14}DAB$) as a self-assembled surfactant coating for detection of protein–dye complexes by CE-LIF

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Abstract A self-assembled column coating for capillary electrophoresis in conjunction with laser-induced fluorescence detection (CE-LIF) has been evaluated for the separation and quantitation of protein–dye complexes. This semi-permanent coating, composed of dimethylditetradecylammonium bromide ($2C_{14}DAB$), is inexpensive and easily assembled onto the column and it allows for better peak resolution and greater control over electroosmotic flow. The versatility of long-chained surfactant coatings was determined particularly with respect to their use with fluorescent probes, different pH buffers, and different proteins. Studies were performed to determine the stability of the coating under various pH and buffer conditions. Red-1c, a red luminescent squarylium dye, was used for on-column protein labeling concurrently with the surfactant coating and LIF detection. Protein–Red-1c complexes were excited with a 650-nm diode laser and their emission detected by a photomultiplier tube with a 664-nm filter. A comparison of pre-column labeling and on-column labeling of a two-model protein system (human serum albumin and β-lactoglobulin A) revealed higher efficiencies and greater sensitivities for both proteins using on-column labeling and coated columns. A linear relationship between peak height and protein concentration was obtained by CE-LIF for this on-column labeling method with $2C_{14}DAB$-coated columns and the Red-1c probe.

Dedicated to the 80th birthday of Keith B. Oldham.

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Introduction

Protein separations by capillary electrophoresis in conjunction with laser-induced fluorescence detection (CE-LIF) are complicated not only by the need for fluorescent derivatization but also by protein interactions with the charged surface of the inner capillary wall, arising from the ionization of surface silanol groups. These interactions reduce the separation efficiency and degrade detection limits due to peak broadening [1, 2] and reduced or inconsistent migration times [3]. Often, these factors lead to poor resolution and identification of proteins because of consequent peak overlap and/or reduced peak areas. To avoid protein–wall interactions, a capillary wall coating can be used; but these can be time-consuming to prepare, irreproducible in quality and coverage, degrade rapidly upon use [4], and may be viable only within a very limited pH range [5]. Work by Yassine and Lucy has recently demonstrated the feasibility of using special two-tailed surfactant molecules to create a fast, inexpensive, and effective capillary coating [6].

Yassine and Lucy found that by flushing a capillary with a buffer solution containing a double-chained cationic surfactant they could generate a semi-permanent coating of hydrophobic molecules on the capillary wall, which would repel proteins and thus improve separations [6]. Their coatings generated a positively charged wall coating, resulting in a reversed electroosmotic flow (EOF) and so a reversed polarity (that is, cathode at the inlet end of the capillary) was employed in their studies. Yassine and Lucy employed

surfactants such as dimethylditetradecylammonium bromide ($2C_{14}$DAB), dihexadecylammonium bromide, and dimethyldioctadecylammonium bromide ($2C_{18}$DAB) to create coatings which lasted for 60 trials over 12 days without having to regenerate the coating [6]. The migration times between trials varied by less than 2.3%.

The advantages gained by this coating procedure were demonstrated by Yassine and Lucy for unlabeled proteins detected by UV absorbance. However, in order to achieve greater sensitivity and selectivity, it is sometimes desirable to employ laser-induced fluorescence detection for protein analytes that are themselves natively fluorescent or have been rendered fluorescent by some derivatization or labeling process. Work in our own laboratory has established a number of alternative methods exploiting noncovalent labeling to facilitate protein determination by CE-LIF [7–9]. The potential for interactions between a fluorescent probe molecule and the surfactant capillary coating may be in competition with interactions between the probe and protein analytes, thus reducing the sensitivities that can be achieved by noncovalent labeling methods with surfactant-based coatings. Potential degradation of the coating by the presence of a fluorescent probe molecule in the running buffer, as required by on-column, noncovalent labeling procedures, is also of concern. Hence, this study was conducted to evaluate the utility of double-chained cationic surfactant coatings for CE-LIF assays of noncovalently labeled proteins.

Noncovalent interactions between proteins and dyes have made possible the development of analytical methods that demonstrate high sensitivity when quantifying proteins in complex matrices. Noncovalent labeling of proteins is an attractive alternative to covalent derivatization due to the fact that it entails minimal sample preparation, is fast, and is feasible at biological pH. Pre-column, post-column, and on-column labeling methods can each be employed with noncovalent probes. The last of these methods, by which the dye is incorporated into the separation buffer, thus encountering the analyte throughout the separation as it migrates through the capillary, eliminates the need for sample derivatization prior to injection and separation and does not result in any sample dilution during labeling. A recent comparison of pre-column and on-column labeling of proteins with squarylium dyes [10] revealed higher efficiencies and greater sensitivities for on-column labeling, and so the compatibility of a double-chained cationic surfactant coating with this method is of interest.

The label of interest in this work is a squarylium dye Red-1c, which belongs to the class of 1,3-disubstituted compounds synthesized from squaric acid and two aromatic and/or heterocyclic compounds [11]. This class of dyes has found applications as photocondutors in copying devices, organic solar cells, optical recording media, and as optical sensors for metal determination [12–18]. Squarylium dyes are attracting the attention of analysts for their long excitation and emission wavelengths and resistance to photodegradation. Specifically, the absorbance maximum for Red-1c is 607 nm in the absence of protein (human serum albumin, HSA) and 642 nm in the presence of HSA [10], making the excitation of its protein complexes feasible with an inexpensive and robust diode laser. Because of the inherently low fluorescence of this dye in its free state, Red-1c has been successfully used as an on-column label for proteins in CE-LIF studies [7, 8, 10]. The quantum yield of Red-1c is very low in the absence of HSA but increases significantly, from 0.03 to 0.92, when noncovalently bound to HSA [19]. To improve separation efficiencies for protein mixtures labeled by this (or any related) dye without sacrificing assay sensitivities, we herein describe a comparison of pre- and on-column labeling methods employing $2C_{14}$DAB-coated capillaries.

Materials and methods

Reagents, buffers, and sample solutions

Red-1c was synthesized by Nakazumi and Yagi, as previously described [19, 20]. Proteins (human serum albumin and β-lactoglobulin A, BLG-A), formic acid, ammonium formate, sodium hydroxide, tris–HCl, and boric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formate buffers were prepared to nominal concentrations of 25 or 50 mM by dissolving the appropriate amount of reagent in Milli-Q distilled, deionized water (Millipore, Bedford, MA, USA) and adjusting the pH to 4.5 by the addition of formic acid unless stated otherwise. Buffers were filtered through 0.2-μm nylon syringe filters (Corning, NY, USA) before use. A stock solution of Red-1c dye was prepared in methanol (Burdick and Jackson, MI, USA) to a concentration of 2.1×10^{-5} M and stored in the dark at 4 °C when not in use. Working solutions of the dye were prepared just prior to use by dilution of the stock to the final desired concentration with Milli-Q water or buffer. Stock protein solutions were prepared to a concentration of $0.5–6.0\times10^{-4}$ M in water and stored at 4 °C in the dark. Pre-column mixtures of protein with dye were prepared by adding the proper volume of protein stock solution to diluted dye solution with thorough mixing.

Dimethylditetradecylammonium bromide surfactant was purchased from Sigma-Aldrich. The surfactant was dissolved in filtered running buffer to a concentration of 0.1 mM through a series of sonication cycles consisting of 20 min of sonication at the surfactant's melting point (45 °C) followed by 10 min of stirring. The cycles were repeated twice or until the surfactant was dissolved. Mesityl oxide was used as a neutral EOF marker at a concentration of

0.1 mM in Milli-Q water. Mesityl oxide is sparingly soluble in water and so it was sonicated for an hour and then left to stir overnight to ensure complete dissolution.

Instrumentation

Most capillary electrophoresis experiments discussed herein were carried out on a Beckman P/ACE CE system (Fullerton, CA, USA) with an LIF detector, which was equipped with a 650-nm external diode laser (Oz Optics, Carp, Canada) and a 664-nm DF20 filter (Omega Optical, Brattleboro, VT, USA). Separations were conducted in various lengths of uncoated and surfactant-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with 50 μm ID and 365 μm OD. An Agilent HP3DCE system (Waldbronn, Germany) equipped with a UV absorbance detector was used for unlabelled protein separations on a coated column. A home-built CE system with absorbance detection was also used to determine EOF with the neutral marker, mesityl oxide.

Capillary coating

A modified method similar to Yassine and Lucy's coating procedure [6] was developed and incorporated on the instruments used. A new length of fused silica capillary was flushed with 1 M NaOH for 15 min under high pressure (20 psi) and then with deionized water for 4 min at 20 psi. After the preconditioning steps, the capillary was coated by rinsing with 0.1 mM $2C_{14}$DAB surfactant in 50 mM formate buffer (pH 4.5) for 15 min at 20 psi. Finally, the capillary was rinsed with the formate buffer alone for 6 min at 0.5 psi. The resulting coated capillary was stored filled with the formate buffer between analyses.

Results and discussion

Semi-permanent capillary coatings afford analysts increased flexibility in the realm of method development for large biomolecules such as proteins, which might otherwise experience significant wall adsorption and hence significantly reduced separation efficiencies and sensitivities. However, the coatings themselves must be compatible with other aspects of the analysis, particularly fluorescent derivatization and derivatizing agents in the case of LIF detection. To this end, we have carefully examined the performance of a self-assembled cationic surfactant coating prepared from the two-chained surfactant $2C_{14}$DAB in CE-LIF experiments with on-column and pre-column labeling of model proteins with the noncovalent squarylium probe Red-1c. An assessment of EOF modification, coating stability, and separation efficiencies for uncoated versus coated capillaries will allow us to evaluate the benefits of this procedure.

EOF in surfactant-coated capillaries

The electroosmotic mobility μ_{EOF} of a system depends on many system-specific variables, such as the zeta potential ζ and solution viscosity and permittivity. Furthermore, ζ itself is dependent upon the surface charge density on the capillary wall and the double-layer thickness at the capillary wall, which, in turn, is dependent upon the magnitude of ionic charge and concentration. Hence, EOF or μ_{EOF} can be affected by any of the following parameters: buffer concentration, ionic strength, pH, viscosity, permittivity, and capillary wall composition. This study will focus on effecting a change in the surface charge on the capillary wall (and hence, the EOF) by application of a semi-permanent $2C_{14}$DAB surfactant coating. The positive surface charge imposed by the presence of this bilayer surfactant coating ensures that positively charged analytes will not be adsorbed to the capillary surface and, hence, this should lead to better separations for large, cationic proteins, for example [5, 6, 21].

To determine the stability of the coating during a normal week of use, a 0.1-mM mesityl oxide sample was repeatedly injected (3 s at 0.29 psi), to serve as a neutral marker of EOF. The running buffer in these stability studies was 50 mM formate (pH 4.5), and a separation voltage of −10 kV was applied. In this way, we were able to monitor any change in EOF over the lifetime of the capillary by simply measuring changes in the retention time of the mesityl oxide. Observed changes in EOF would be indicative of a breakdown in the coating because the charge on the wall would change. The average μ_{EOF} found for $2C_{14}$DAB-coated column with 50 mM formate buffer (pH 4.5) was $(7.98\pm0.06)\times10^{-4}$ cm^2 V^{-1} s^{-1} (N=10) over a 1-week period. This value is comparable to that demonstrated by Yassine and Lucy [6]. The less than 1% variability observed in electroosmotic mobility for the coated capillary over the course of a week is excellent relative to the variation in μ_{EOF} observed for a comparable uncoated capillary in use over the course of a week $(8.21\pm0.13)\times10^{-4}$ cm^2 V^{-1} s^{-1} (N=10). It should be noted that the EOF in the case of the coated column was in the direction of (towards) the anode (or positive electrode), while in the case of the uncoated column it was towards the cathode; but in both cases the magnitude of the EOF was comparable. It should also be noted that data from the first ten trials using a newly coated capillary were not included in the average measured μ_{EOF} value, since these were considered "conditioning" runs—to ensure excess surfactant molecules were fully removed and a reproducible surface charge was achieved. Additionally, newly coated capillaries were allowed to sit overnight filled with running buffer prior to their first usage, since this was observed to produce a more stable EOF.

These experiments were conducted under acidic pH conditions, which are not incompatible with normal procedures for the analysis of proteins by CE; but to ensure maximum utility of the surfactant coating, studies at other pH were also conducted. These include the analysis of proteins ribonuclease-A, lysozyme, and α-chymotrypsinogen-A using 50 mM borate buffer (pH 9.5) and 50 mM tris buffer (pH 7.0) with a coated capillary prepared from related surfactant $2C_{18}$DAB (data not shown). The coating was found to be stable under these conditions; however, with 50 mM tris (pH 10) and 50 mM citrate (pH 3.2), the surfactant itself was less soluble and the resulting dynamic coating was less reproducible. As such, the recommended pH range for these coatings with acidic proteins is from 4.5 to 9.5. All subsequent experiments reported in this paper were conducted at pH 4.5.

Resolution of proteins using a $2C_{14}$DAB-coated capillary

Figure 1 depicts electropherograms of individual cationic proteins α-chymotrypsinogen A, ribonuclease A, and lysozyme as well as a mixture of these three proteins.

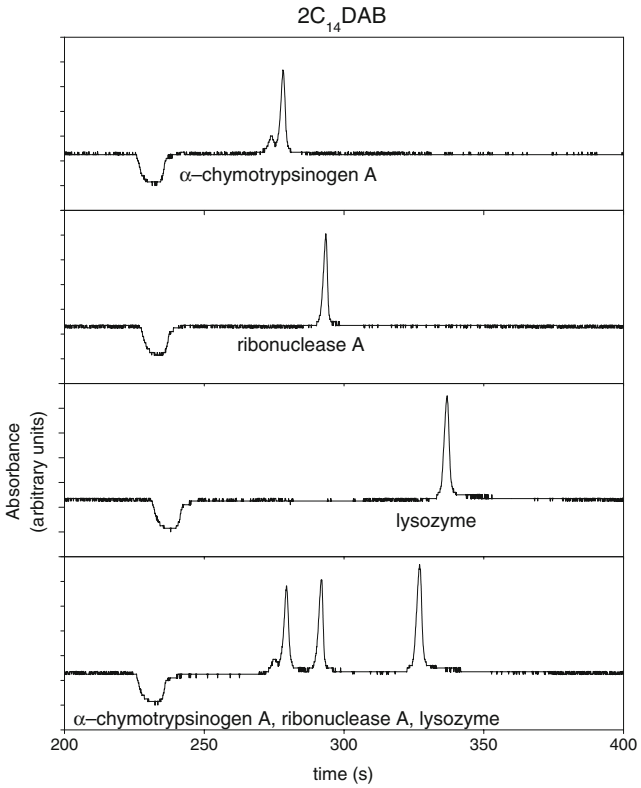

Fig. 1 Electropherograms (from *top* to *bottom*) of 0.1 mg/mL α-chymotrypsinogen A, 0.1 mg/mL ribonuclease A, 0.1 mg/mL lysozyme samples, and a mixture of all three proteins. Separation conditions include: 50 mM formate buffer (pH 4.5), −9 kV separation voltage, 20 s hydrodynamic injection, 50 μm ID×44.7 cm total length (34.7 cm effective length) $2C_{14}$DAB-coated capillary, absorbance detection at 214 nm

Complete resolution of these proteins in less than 6 min serves to demonstrate the effectiveness of the $2C_{14}$DAB coating. In comparison, this same protein mixture separated on an uncoated capillary yielded broader peaks and a significant reduction in average resolution from 2.5 to 1.6 relative units (data not shown). It seems apparent that the double-layer surfactant coating is preventing protein adsorption and hence greatly improving the quality of the separation of free (unlabeled) proteins. However, it still remained to be established that the coating could be equally effective for the separation of fluorescently labeled proteins, as described next.

Effect of dye in running buffer on $2C_{14}$DAB coating

In order to determine if the dynamic surfactant coating would be appropriate for the analysis of noncovalent protein–dye complexes, it was necessary to first assess the effect of the presence of dye in the running buffer (as is characteristic of on-column, noncovalent labeling protocols) on the observed EOF in coated capillaries. To this end, mesityl oxide samples were again employed as markers of EOF, but in these experiments the reproducibility of mesityl oxide transport (or μ_{EOF}) over the course of 5 days using a coated capillary was evaluated with Red-1c present in the running buffer at a concentration of 9.35×10^{-6} M. The average μ_{EOF} found for $2C_{14}$DAB-coated column with 25 mM formate buffer (pH 4.5) containing 9.35×10^{-6} M Red-1c was $(6.16\pm0.05)\times10^{-4}$ cm^2 V^{-1} s^{-1} (N=10) over a 1-week period. The average μ_{EOF} found for a $2C_{14}$DAB-coated column with 25 mM formate buffer (pH 4.5) containing no Red-1c was $(6.67\pm0.12)\times10^{-4}$ cm^2 V^{-1} s^{-1} (N=10) over a 1-week period. The less than 1% variability observed in the electroosmotic mobility for the coated capillary with dye present in the separation buffer compares favorably to the earlier measurement of electroosmotic mobility for coated columns in the absence of dye. Given this indicator of stability, it is reasonable to conclude that the dye does not inadvertently affect the coating and so the use of $2C_{14}$DAB-coated capillaries for dye–protein assays seems warranted. It should be noted that the absolute value of μ_{EOF} (6.67×10^{-4} cm^2 V^{-1} s^{-1}) for the coated column in the absence of Red-1c in this particular study differs from μ_{EOF} reported earlier in this work under similar conditions and this difference may be attributed to such factors as changes in wall surface charge on new pieces of capillary and minor differences in coating protocols. These differences in μ_{EOF} for different coated capillaries mimic the differences seen in μ_{EOF} for different uncoated capillaries, but it should be emphasized that the reproducibility in μ_{EOF} for any one given coated capillary (day-to-day) was high.

The presence of the Red-1c noncovalent protein probe in the running buffer had several potential impacts on the method. First, it contributed to the overall ionic strength of the

buffer solution and so was expected to reduce the rate of EOF. This was observed ((6.16±0.05)×10^{-4} cm^2 V^{-1} s^{-1} with dye present and (6.67±0.12)×10^{-4} cm^2 V^{-1} s^{-1} without dye present). Second, the dye itself is hydrophobic and so may partition into or otherwise associate with the double-layer coating, thus affecting the coating integrity and, in turn, the reproducibility of EOF. Given the high degree of reproducibility observed for μ_{EOF} in the presence of Red-1c, there is no concern about the dye negatively impacting coating integrity. Third, any interactions between the dye and coating may serve to enhance the fluorescence of the dye and in the case of on-column labeling this would increase the background fluorescence signal, thus degrading the sensitivity of the assay. Calibration curves were constructed to evaluate the theoretical limit of detection for this method (see next section). Fourth, given that the dye is insoluble in purely aqueous solution, the presence of 0.4% (*v*/*v*) methanol in the final dye+buffer solution filling the capillary, which was necessary for solubility, may also affect coating integrity. However, recent work by Diress et al. [22] demonstrated the compatibility of double-chain cationic surfactant coatings with up to 60% (*v*/*v*) methanol–buffer mixtures and so, although the presence of some organic solvent in the buffer system would affect the EOF, it is not likely (especially at the low organic levels present in these experiments) to compromise the coating itself.

On-column, noncovalent protein labeling with Red-1c in $2C_{14}$DAB-coated capillaries

Having established the compatibility of the coating with Red-1c dye, it remained to be shown that a mixture of proteins could be separated and labeled on-column in this same system. To this end, we studied HSA and BLG-A samples. Repeated injections of a mixture composed of

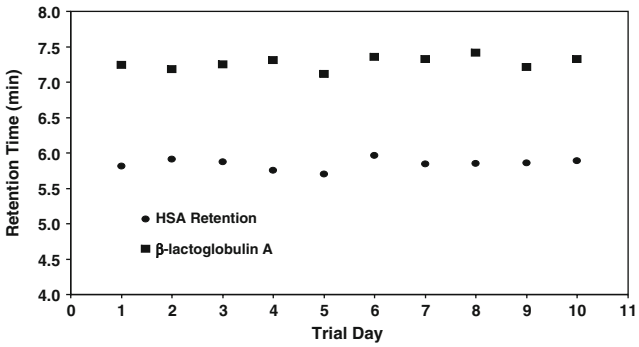

Fig. 2 Day-to-day reproducibility in retention times for on-column, noncovalently labeled HSA and β-lactoglobulin A samples, both at a concentration of 1.0×10^{-5} M, on a surfactant-coated capillary over a 10-day period. Experimental conditions include: −9 kV separation voltage, 3-s pressure injection at 0.5 psi, 50 μm ID×30 cm (24.5 cm effective length) capillary with $2C_{14}$DAB coating, 25 mM formate buffer (pH 4.5), excitation λ=650 nm, emission $\lambda \geq 664$ nm

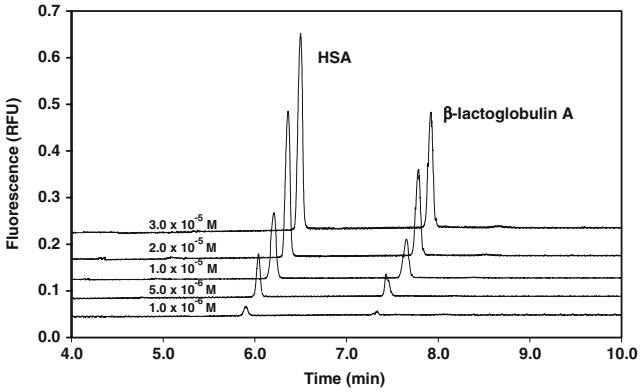

Fig. 3 On-column labeling of mixtures of HSA and β-lactoglobulin A in 25 mM formate buffer (pH 4.5) with 9.35×10^{-6} M Red-1c. Other experimental conditions include: −9 kV separation voltage, 3-s injection at 0.5 psi, 50 μm ID×30 cm (24.5 cm effective length) capillary with $2C_{14}$DAB coating. Protein concentrations range from 1.0×10^{-6} M to 3.0×10^{-5} M for both proteins, as indicated. Electropherograms are offset horizontally and vertically for clarity

10 μM HSA and 10 μM BLG-A onto a surfactant-coated capillary filled with 25 mM formate buffer (pH 4.5) with 9.35×10^{-6} mM Red-1c over a period of 10 days demonstrated excellent reproducibility for the retention times of the noncovalent protein–dye complexes, as seen in Fig. 2. The average retention times for HSA and BLG-A in a mixture over this measurement period translate into net mobilities of (2.35±0.09)×10^{-4} cm^2 V^{-1} s^{-1} and (1.89±0.08)×10^{-4} cm^2 V^{-1} s^{-1}, respectively. The capillary was stored filled with deionized water between days and was flushed at low pressure (0.5 psi) at the beginning of each new day of experiments with 0.1 M NaOH for 3 min, deionized water for 3 min, 0.1 M surfactant solution for 5 min, and finally with deionized water again for 5 min before use. This ensured a consistent coating from day-to-day by regenerating rather than completely stripping and recoating the capillary each day. This procedure proved sufficient to allow ten analyses per day while maintaining retention time and EOF reproducibility from day to day.

Table 1 Figures of merit for CE-LIF separation of on-column, Red-1c-labeled proteins in 30-cm uncoated and surfactant-coated capillaries

	Uncoated	Coated
μ_{EOF} (cm^2 V^{-1} s^{-1})	8.21×10^{-4}± 0.13×10^{-4}	7.98×10^{-4}± 0.06×10^{-4}
R (HSA and BLG-A)	1.5	2.4
N (plates/m)	9.4×10^4	2.0×10^5
Analysis time (min)	15	10

Separation conditions as stated in the text, with an applied separation voltage of +9 kV for the uncoated column and −9 kV for the $2C_{14}$DAB coated column

Quantitation of these model proteins was possible by constructing a calibration curve for each. Figure 3 shows on-column labeling with Red-1c for increasing concentrations of HSA and BLG-A. A subsequent plot of peak area for the protein–dye complex as a function of protein concentration (for a fixed dye concentration of 9.35×10^{-6} M in 25 mM formate buffer, pH 4.5) resulted in regression equations of peak area=9.87×10^{9} (protein concentration, M)+4,005 and peak area=7.89×10^{9} (protein concentration, M)+3,204 with correlation coefficients of 0.984 and 0.979 for HSA and BLG-A, respectively. Based on these standard curves and the standard deviation in the baseline of the corresponding electropherograms, computed limits of detection (3σ) of 60 nM HSA and 90 nM BLG-A were obtained for this method. The mean resolution for these two analytes was R=2.4 and the mean column efficiency for both HSA and BLG-A was 2×10^{5} plates/m. These figures of merit can be compared to corresponding figures obtained for the same protein–dye complexes using an uncoated capillary, as summarized in Table 1.

Conclusions

To be able to employ dynamic surfactant coatings to increase the resolution and separation efficiency of protein separations by CE is an important outcome of this work, and indeed we were able to demonstrate a nearly twofold improvement both in terms of resolution and efficiency in one-third-less analysis time for model protein analysis in a coated versus uncoated column. However, to achieve these improvements without compromising LIF detection sensitivity is an even more important advance in method development. The two-chained surfactant coatings developed by Yassine and Lucy have been shown to be compatible with on-column, noncovalent labeling protocols employing the squarylium dye Red-1c developed in our own lab. As such, the range of protein–dye complexes that can be effectively analyzed by CE-LIF should be extended by the simple incorporation of a $2C_{14}$DAB-coated capillary in the procedure. However, the utility of this coating to facilitate more efficient separations even in the presence of fluorescent probe molecules is likely to be realized for a host of labeled analytes and should not be considered to be limited to proteins only nor to on-column derivatization procedures or noncovalent probe–analyte interactions.

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