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Research Article

Size-based protein separations by microchip electrophoresis using an acid-labile surfactant as a replacement for SDS

We demonstrate the use of an acid-labile surfactant (ALS) as a replacement for SDS for size-based protein separations in a microfluidic device. ALS is of interest to the proteomic field as it degrades at low pH and hence can be removed to reduce surfactant interference with down-stream MS. A range of SDS and ALS concentrations were tested as denaturants for microchip electrophoresis to investigate their effects on the separation of proteins from 18 to 116 kDa and to provide a suitable comparison between the two surfactants. The electrophoretic mobilities of the proteins were not significantly affected by the use of ALS instead of SDS. Protein separations with ALS are performed in less than 3 min, which is a significant decrease in the time compared with the previous ALS separations on a slab gel format. We also demonstrate the use of poly-*N*-hydroxyethylacrylamide as a dynamic, hydrophilic chip channel coating that can be applied with a rapid and simple protocol for size-based protein separation. The results reported here could significantly decrease the time and increase the attainable level of automation and integration of the front-end protein fractionation required for "top-down" proteomics.

Keywords:
Acid labile surfactant / Microchip electrophoresis / Protein separation
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1 Introduction

Complete sequencing of the first composite human genome [1, 2] opened the door to a more complete understanding of fundamental biological processes and will uniquely impact emerging methods to understand, identify, and treat diseases [3]. Proteomics is a rapidly expanding field and has achieved a number of successes in deciphering the information encoded within the genome [4]. However, a number of significant technical challenges still face the field to unravel the complexity of the proteome [5–7]. These challenges were laid out by the National Human Genome Research Institute Proteomics Planning Workshop, which identified several key aspects of technology development vital to the field including protein separations and better identification of PTMs [8].

A major obstacle in the proteomics field has been the identification and localization of protein modifications not encoded within the DNA sequence [9, 10]. Over 260 modifications have been identified and can include changes in processing of messenger RNA and PTMs, such as the addition of chemical groups or proteolytic trimming [11, 12]. These modifications can significantly impact the biological interactions of proteins that are critical to normal cell functions [13].

The widely applied method of "bottom-up" proteomics by peptide analysis *via* MS of a tryptic digest does not provide 100% coverage of the protein [14–16]. Although this method is well established and has been an indispensable tool in the proteomics field, it is difficult to determine modifications made to the mature protein when compared with the sequence that is predicted from the open-reading frame [17]. "Top-down" proteomics by whole protein analysis can provide an efficient method to achieve 100% sequence coverage and to identify and localize protein modifications [18, 19]; however, front-end fractionation of the proteome remains a time- and labor-intensive process.

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Abbreviations: ALS, acid-labile surfactant; **GPC,** gel permeation chromatography; **LPA,** linear polyacrylamide; **MALLS,** multi-angle laser light scattering; **βME,** β-mercaptoethanol; **pHEA,** poly-(*N*-hydroxyethylacrylamide)

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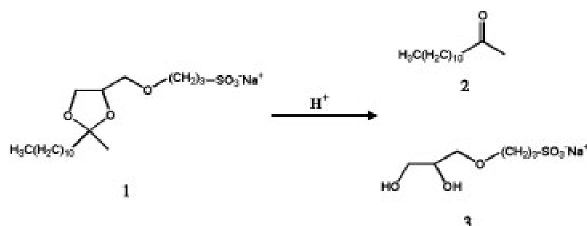
In pioneering work by the Kelleher group, a 2-D protein separation followed by MS/MS was demonstrated to completely characterize whole proteins, including their PTMs [19]. This work shows that it should be possible to lower the barriers to whole protein analysis, so that widespread use of this method can be used to complement other proteomic tools. However, to increase the throughput of any analytical method, the time and labor requirements of all of the processing steps need to be minimized. The front-end separation method employed by the Kelleher group utilizes continuous elution tube gel electrophoresis with an 8 h separation time and off-line connections between the two separation modalities (gel electrophoresis and RPLC) and RPLC with MS [19, 20]. Therefore, the development of an electrophoretic separation method that can be integrated with RPLC, while also being compatible with MS, is necessary to increase the throughput of this emerging technique for proteome analysis.

Size-based protein separations that use SDS to impart the proteins with a constant charge-to-size ratio are an established electrophoresis technique on gel, capillary, and microchip platforms [21, 22]. SDS, however, is incompatible with MS as it suppresses signal from the analyte in MALDI and ESI-MS [23]. To eliminate this problem, an acid-labile surfantant (ALS) has been used to replace SDS in gel electrophoresis because it degrades at low pH (Scheme 1), and therefore does not interfere with down-stream MS analysis [18, 24–26].

Previous reports that compared slab gel electrophoresis using SDS and ALS did not provide consistent results [24, 25]. Ross *et al.* observed that proteins migrated more slowly with ALS in the running buffer, and attributed this to SDS maintaining a higher charge on each protein than ALS [24]. Konig *et al.*, investigated two sets of proteins, and found slower migration with ALS in only one set [25]. Additionally, Konig *et al.*, attribute small changes in electrophoresis conditions (substituting β-mercaptoethanol (βME) for dithiothreitol), buffer drainage, and insufficient protein encapsulation for the differences between SDS and ALS, and for differences between their separations and those performed by Ross *et al.* [25]. In each report, only one SDS and ALS concentration was tested significantly limiting the data set available for comparison.

Here, we demonstrate for the first time the size-based separation of proteins with ALS in a microfluidic chip. These separations are performed in 3 min, compared with the 8 h separations reported for gel electrophoresis [19, 20]. When performing separations in a microchannel instead of a gel, additional considerations such as wall–analyte interactions and protein adsorption must be addressed. Therefore, in addition to using ALS as a replacement for SDS, we demonstrate the use of poly-*N*-hydroxyethylacrylamide (pHEA) as a dynamic wall coating for SDS and ALS protein electrophoresis. This polymer has been previously used as a channel coating for DNA separations and free-solution protein separations and its use is extended here to include protein separations with SDS and ALS in the running buffer [27, 28].



Scheme 1. Decomposition of ALS (1) at low pH into tridecan-2-one (2) and sodium 3-(2, 3-dihydroxypropoxy)propanesulfonate (3) [26].

2 Materials and methods

2.1 Polymer synthesis and characterization

Polymers used in this study were synthesized, purified, and characterized as described previously [27]. Briefly, acrylamide monomer (AMRESCO, Solon, OH, USA) was dissolved in 300 mL of water at 3% w/v with 0.67% v/v isopropanol, which acts as a chain transfer agent to control molecular weight. The solution was stirred and bubbled with nitrogen for 45 min to remove oxygen. The reaction was initiated with 0.03 g of V-50 (Wako Chemical, Richmond, VA, USA), and the reaction was allowed to proceed for 4 h at 50°C in a jacketed reaction vessel. Following the reaction, the solution was placed into 100 000 Da molecular weight cut-off dialysis tubes (Spectrum Laboratories, Rancho Dominguez, CA, USA) for 2 wk with frequent water changes to remove unreacted monomer, reaction reagents, and low molar mass polymers. The purified polymer solution was then frozen, lyophilized, and stored dry until use. pHEA (monomer from Cambrex, East Rutherford, NJ, USA) was synthesized under similar conditions. Monomer was dissolved at 2% w/v with 0.05% v/v isopropanol and initiated with 0.004% w/v 4,4′-azobis(4-cyanovaleric acid) (Sigma-Aldrich, St. Louis, MO, USA).

The molar mass of the polymers was determined by tandem gel permeation chromatography (GPC) multi-angle laser light scattering (MALLS). This method has been previously described in detail [29]. Briefly, the polymer samples were dissolved at 1 mg/mL and fractionated by GPC (Waters, Milford, MA, USA) prior to MALLS and refractive index detection (both Wyatt Technology, Santa Barbara, CA, USA). Each sample was tested three times and the average

Table 1. Summary of polymer properties[a)]

	M_w (MDa)	PDI	R_z (nm)
LPA	2.16	2.6	83
PHEA	3.32	1.5	80

a) M_w, weight-average molar mass; PDI, polydispersity index; and R_z, z-average radius of gyration.

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value of these runs is summarized in Table 1. The light scattering and refractive index data are analyzed using ASTRA software (Wyatt Technology) to determine the molecular weight, polydispersity index and radius of gyration of the samples. The data were fit using the Berry method and the two processing parameters used were a known AUX calibration constant and 100% sample recovery.

2.2 Protein labeling

Proteins were labeled with the amine-reactive Py-1 dye, which is commercially available as Chromeo^{TM} P503 (Active Motif Chromeon, Tegernheim, Germany) [30–32]. This dye is advantageous as it undergoes a large shift in its absorption/emission spectra as well as exhibits a significant increase in quantum yield upon conjugation [30]. The proteins were labeled individually using a method adapted from Craig *et al.* [33], then combined to create a ladder. Proteins used in this study were β-lactoglobulin, carbonic anhydrase, ovalbumin, bovine serum albumin, and β-galactosidase (all proteins from Sigma-Aldrich). Table 2 lists the proteins with their molecular weights, the concentrations at which they were labeled, and the final protein concentration used in the ladder. A stock solution of 1 mg/mL Py-1 was dissolved in DMSO (Fisher Scientific, Pittsburgh, PA, USA). To label the proteins, a solution of 5 mM borate (Sigma-Aldrich), 2 mM SDS (Pierce Biotechnology, Rockford, IL, USA) or ALS (available as RapiGest^{TM} from Waters), and 3% v/v βME (Sigma-Aldrich) with protein at the concentration listed in Table 2 was heated to 99°C for 10 min to denature the proteins. The solutions were then cooled to room temperature, and Py-1 dye solution was added at a final concentration of 0.1 mg/mL. This solution was then heated to 50°C for 10 min to allow the dye to react. Successful conjugation of the dye can immediately be determined visually as the solution changes from blue to pink due the shift in the emission spectrum. The fluorescently labeled proteins were then diluted into a 5 mM borate, 2 mM SDS or ALS, 3% βME solution to create the protein ladder.

2.3 Protein separation

Protein separations were performed in a 75 μm id, 25 cm long fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) on a BioRad BioFocus instrument to identify a suitable microchannel wall coating. The performance of the wall coatings in the capillary is expected to be qualitatively similar to their performance in a microchannel. Prior to coating, the capillary was flushed for 3 min with 1 M NaOH, 3 min with 1 M HCl, then filled with a 0.5% w/v solution of either poly(*N*-hydroxyethylacrylamide) (pHEA), poly(*N,N*-dimethylacrylamide (pDMA), poly(vinylpyrrolidone) (PVP) (Sigma-Aldrich), hydroxypropyl methylcellulose (HPMC) (Sigma-Aldrich), or poly(ethylene oxide) (PEO) (Sigma-Aldrich). The capillary was then filled with 3% w/w linear polyacrylamide (LPA) (2.16 MDa) in 1 × TTE (49 mM Tris (AMRESCO)), 49 mM TAPS (Fisher Scientific), and 2 mM EDTA (Sigma-Aldrich) +0.25% SDS. A protein ladder of β-lactoglobultin (42 μg/mL), carbonic anhydrase (72 μg/mL), ovalbumin (90 μg/mL), and bovine serum albumin (79 μg/mL) was injected for 2 s at 200 V/cm and separated with a field strength of 400 V/cm (Fig. 1).

The proteins were separated on a microfluidic chip using a system custom-built for our laboratory that has been previously reported in detail by Chiesl *et al.* [34]. Briefly, the system consists of a power supply subsystem capable of controlling four electrodes independently with a maximum voltage of 4.5 kV on each electrode. The optical subsystem uses a 488 nm argon ion laser as the excitation source with fluorescence detected by a high-quantum efficiency CCD.

Separations were performed in a glass microchip (Micronit Microfluidics BV, The Netherlands) with a 100 μm "offset T" injector and a 7.5 cm effective separation distance. The chips were dynamically coated using high molar mass poly-(*N*-hydroxyethylacrylamide) polymer [27, 28]. The channels were flushed with 1 M HCl for 10 min. A 0.1% w/v pHEA solution was then flushed through the channels and allowed to contact the surface for 15 min to allow the polymer to dynamically adsorb to create a homogeneous wall coating. LPA (weight-average molar mass is 2.16 MDa) was dissolved at a concentration of 3% w/w in 1 × TTE with varying amounts of SDS or ALS and pressure loaded into the microchannels. The proteins were injected by applying 400 V for 40 s to the waste well while grounding the sample well. Separations were performed at different field strengths between 300 and 500 V/cm, with the pull-back voltages adjusted to achieve currents in the side channels of ~0.2 μA. Electropherograms were analyzed using PeakFit^{TM} software (SPSS, Chicago, IL, USA).

Table 2. Protein labeling and ladder concentrations

Protein	Molecular weight (kDa)	Labeling Concentration (μMol)	Concentration (mg/mL)	Ladder Concentration (nMol)	Concentration (μg/mL)
β-Lactoglobulin	18.4	25	0.46	38	0.7
Carbonic anhydrase	29.5	10	0.30	200	5.9
Ovalbumin	42.7	12	0.50	35	1.5
BSA	66.0	10	0.66	20	1.3
β-Galactosidase	116.3	1.7	0.20	34	4.0

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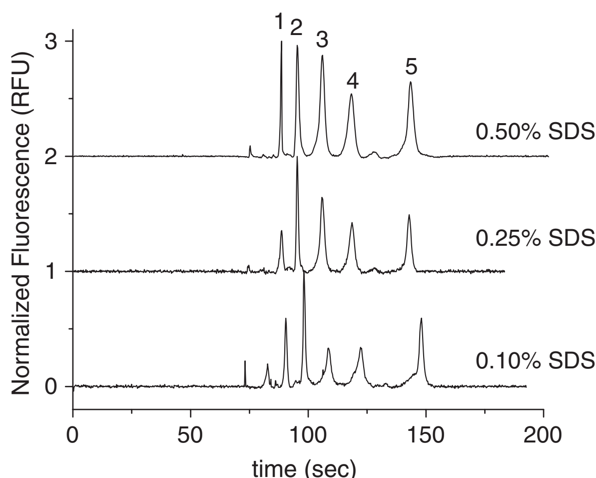

Figure 1. Representative electropherograms of protein separations with three SDS concentrations at 400 V/cm. The fluorescence signal was normalized to the largest peak in the trace. Peak labels correspond to (1) β-lactoglobulin, (2) carbonic anhydrase, (3) ovalbumin, (4) bovine serum albumin, and (5) β-galactosidase.

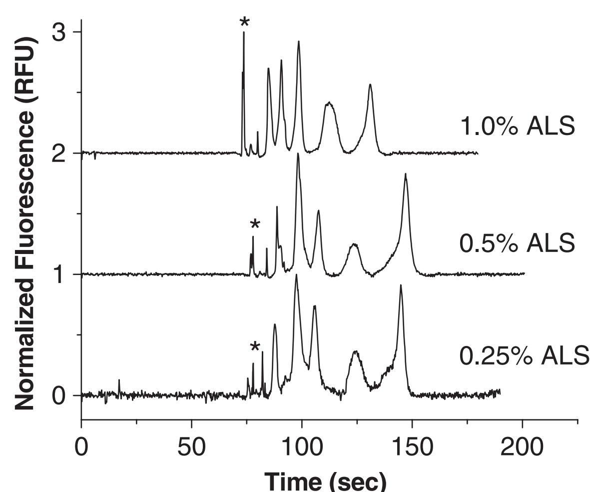

Figure 2. Representative electropherograms of protein separations at three ALS concentrations with a field strength of 400 V/cm. An asterisk represents a peak from unconjugated Py-1 dye. The fluorescence signal was normalized to the largest peak in the trace and peak order is the same as described in Fig. 1.

3 Results and discussion

Proteins can interact with the microchannel wall *via* hydrophobic, hydrogen bonding, or electrostatic interactions. Irreversible interactions can result in a loss of the protein peak, foul the capillary, and engender EOF, leading to non-uniform axial and radial flow profiles that can greatly reduce peak efficiency [28, 35]. Additionally, reversible interactions can result in peak broadening and tailing [28].

An investigation of ''dynamic'' (*i.e.* non-covalently bound) surface coating was performed using fused silica capillaries. Results obtained in a fused silica capillary should be qualitatively similar to the coatings' relative performances in microfluidic chips. Five different polymer coatings were tested: poly(*N*-hydroxyethylacrylamide), pHEA; poly(*N*,*N*-dimethylacrylamide), pDMA; poly (vinylpyrrolidone), PVP; hydroxypropyl methylcellulose (HPMC); and poly(ethylene oxide), PEO. These coating polymers were selected because each has been previously reported as a useful dynamic coating for other biomolecule separations applications [28, 36, 37]. We found that the pHEA coating provided the highest separation efficiency of the coatings tested (Supporting Information Fig. 1). HPMC- and PEO-coated capillaries did not provide resolved protein peaks. Additionally, proteins eluted in the shortest time with the pHEA coating (data not shown), indicating that the other coatings had either a higher degree of wall–analyte interactions or a higher velocity EOF (or both). These results are consistent with previously published reports in which channel coatings that are more hydrophilic have resulted in better separations [36]. It has been previously reported that pHEA performs well as a dynamic coating for free-solution CE separations of proteins. However, this is the first demonstration of PHEA and is a useful adsorptive (''dynamic'') microchannel wall coating for protein size separations by SDS microchip electrophoresis.

The physical properties of the pHEA and LPA polymers as determined by tandem GPC-MALLS are listed in Table 1. Protein separations using the ladder as summarized in Table 2 were first carried out using SDS as the surfactant. Field strength and SDS concentration were varied to investigate their effects on mobility and separation efficiency, as well as to validate the efficacy of pHEA as a dynamic coating for this type of separation.

SDS concentrations of 0.10%, 0.25%, and 0.50% w/v were tested using a separation field strength of 400 V/cm. The SDS concentration can be an important factor in the separation as proteins may not be fully denatured below certain concentrations or may not have a constant charge-to-size ratio. A semi-log plot of the inverse of migration time *versus* the log of protein molecular weight (Fig. 3) showed a linear relationship indicating a constant SDS binding ratio at each concentration [38]. Proteins were separated with 0.25% w/v SDS in the running buffer at field strengths of 300, 400, and 500 V/cm. The separation efficiency was not significantly affected by field strength within the range tested (data not shown); 400 V/cm was chosen to achieve a rapid separation while not operating the electrical power supply near its maximum.

Following successful protein separation using pHEA as a dynamic coating with SDS as the ionic surfactant, separations were performed using ALS at 0.25%, 0.50%, and 1.0% w/v. Representative electropherograms obtained at each ALS concentration are shown in Fig. 2. The mobilities of the proteins were very similar at all ALS concentrations, with 1.0% ALS separations being slightly faster. This also occurred when using SDS; 0.5% SDS-proteins migrate slightly faster than 0.1 and 0.25% SDS and may be due to a

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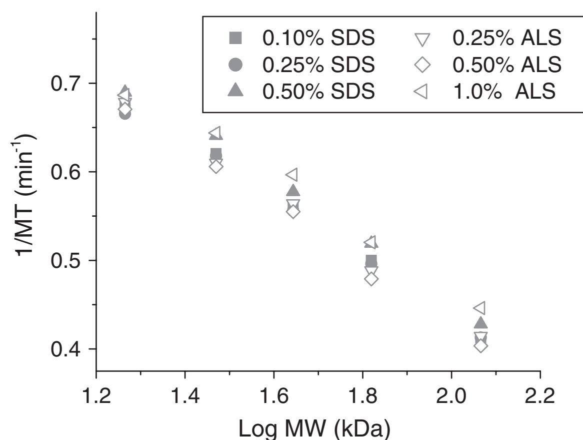

Figure 3. Inverse migration time *versus* log molecular weight for the protein ladder using SDS and ALS at a field strength of 400 V/cm. $n = 3$.

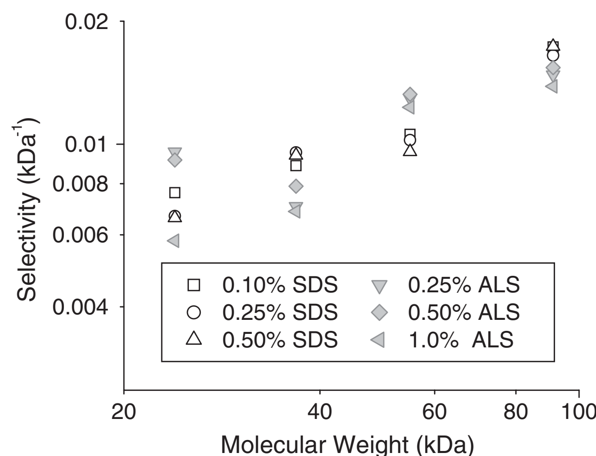

Figure 4. Selectivity *versus* molecular weight with the molecular weight being the average molecular weight of the two proteins used to calculate the selectivity. $n = 3$.

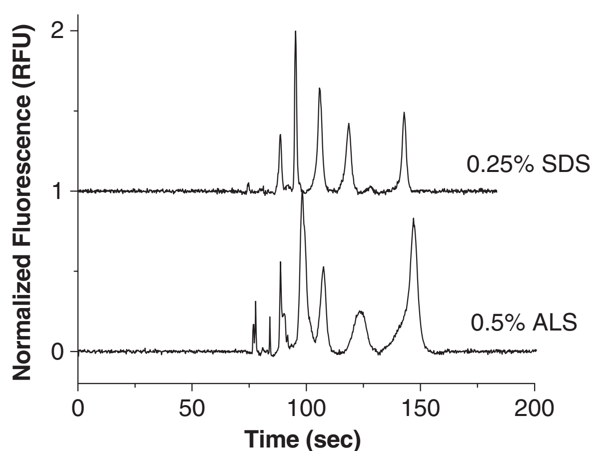

Figure 5. Comparison of protein separations using 0.50% SDS and 0.50% ALS with a field strength of 400 V/cm. The fluorescence signal was normalized to the largest peak in the trace and the peak order is the same as described in Fig. 1.

small degree of Joule heating at the higher surfactant concentrations. Protein mobility with ALS was approximately the same as SDS as shown in Fig. 3, indicating that the protein–surfactant complexes had similar charge-to-size ratios with the two different surfactants. Additionally, this indicates that the selectivity of the separation did not substantially change as a result of using ALS (Fig. 4). Therefore, future studies performing protein separations with SDS can be adjusted to the use of ALS in their separations with the expectation of little or no effect on the separation time or the intervals at which protein fractions eluting from the channel are collected.

Separation efficiency is a calculated measure of the quality of an analysis, that reflects changes that occur in both the electrophoretic mobilities and the peak widths (the latter normalized to the separation distance), allowing a comparison of results among different studies in which different channel lengths were used. For this reason, separation efficiency has been a standard method of evaluating protein separations despite potential protein heterogeneities (*e.g.* glyosylation) represented within a single peak. Therefore, while hypotheses may be made as to source of the slightly broader peaks when using ALS, additional studies are needed to clarify this. However, Supporting Information Figs. 2 and 3 show the separation efficiency of the ALS separations *versus* 0.25% SDS and compared with other recent publications on SDS protein separations on chip to provide additional information on the comparison of ALS with SDS. The electropherograms in Fig. 5 clearly show that similar sieving performance can be achieved with ALS compared with SDS on a microfluidic chip.

4 Concluding remarks

We have successfully demonstrated size-based protein separations using an acid-labile surfactant to replace SDS on a dynamically coated microfluidic chip significantly decreasing separation times compared with slab gel electrophoresis. The migration times of the proteins were not significantly different using ALS *versus* SDS, indicating that the protein–surfactant complexes have similar charge-to-size ratios. Although the protein peaks were slightly broader with ALS than with SDS, similar separation performance can be achieved with ALS. Using ALS instead of SDS can provide significant advantages in terms of integrating protein separation modalities, as well as the front-end separation with MS. This report demonstrates that ALS may be utilized on a microfluidic device for rapid protein separations that provide the opportunity to integrate with a second separation dimension and greatly increase the throughput of top-down proteomics.

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