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Capillary sodium dodecyl sulfate-DALT electrophoresis with laser-induced fluorescence detection for size-based analysis of proteins in human colon cancer cells

Capillary sodium dodecyl sulfate (SDS)-DALT electrophoresis (SDS-DALT-CE) refers to CE separation of proteins based on their size; DALT is the abbreviation for Dalton, the unit used to describe molecular weight. In this work, seven proteins from 18 to 116 kDa were denatured by SDS, labeled by 3-(2-furoyl) quinoline-2-carboxaldehyde, separated by SDS-DALT-CE in polyethylene oxide sieving matrix, and detected by laser-induced fluorescence (LIF) in a sheath flow cuvette. This method was combined with detergent differential fractionation, which is a protein fractionation method using a series of detergent-containing buffers to sequentially extract protein fractions from cells, to analyze the proteins in HT29 human colon adenocarcinoma cells. In addition, on-column labeling was demonstrated for protein analysis by SDS-DALT-CE with LIF, and applied to analysis of proteins in a single HT29 cancer cell. Most proteins had molecular masses from 10 to 120 kDa. Similar protein profiles were obtained for single cells and protein extract of a large cell population.

Keywords: Capillary sodium dodecyl sulfate-DALT electrophoresis / Detergent differential fractionation / Human cancer cells / Laser-induced fluorescence / Single cell protein analysis EL 5096

1 Introduction

Capillary SDS-DALT electrophoresis (SDS-DALT-CE), a size-based CE separation method for proteins [1], has several advantages over classical SDS – polyacrylamide gel electrophoresis (SDS-PAGE). These advantages include ease of automation, high analysis speed, small sample volume, as well as accurate quantitation of proteins and determination of their molecular weights. Currently, linear or slightly branched polymers, such as linear polyacrylamide, polyethylene oxide (PEO), polyethylene glycol, dextran, and pullulan, are often used as the sieving matrix for SDS-DALT-CE analysis of proteins [2–8]. Comparing with cross-linked polyacrylamide gel matrix [9, 10], these polymers add great flexibility to SDS-DALT-CE method because they are water-soluble and replaceable between CE experiments.

Although UV absorbance is a common detector in SDS-DALT-CE, it produces low sensitivity for proteins due to the short optical pathlength across the capillary. However, many proteins, especially some regulatory proteins, are

expressed at very low level in cells [11, 12]. Highly sensitive detection technologies such as laser-induced fluorescence (LIF) are needed to characterize such low-abundance proteins. In particular, we are interested in developing a technology to allow the study of protein expression in single somatic cells. Currently, LIF is the most common detector for CE analysis of proteins in single cells [13–20].

Detergent differential fractionation (DDF) is a method used for sequential extraction of proteins from cells or tissues [21–23]. By using a series of detergent-containing buffers including digitonin/EDTA, Triton X-100/EDTA, Tween 40/deoxycholate (DOC), and cytoskeletal solubilization buffer, DDF reproducibly yields four distinct fractions, which contain cytosolic proteins and soluble cytoskeletal elements, membrane and organelle proteins, nuclear membrane and soluble nuclear proteins, and detergent-resistant cytoskeletal filaments with nuclear matrix proteins [23]. DDF is simple, reproducible, and ultracentrifuge-independent. It does not damage the structure or function of the subcellular compartments and can enrich low-abundance species, and the technique has many applications [21, 24].

In this work, a size-based separation method by SDS-DALT-CE with LIF is described for analysis of proteins. This method is combined with DDF to characterize the proteins expressed in HT29 human colon cancer cells. On-column labeling is also demonstrated for protein analysis by SDS-DALT-CE with LIF. Finally, this method is applied to analysis of proteins in a single HT29 cancer cell.

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Abbreviations: **DDF**, detergent differential fractionation; **DOC**, deoxycholate; **FQ**, 3-(2-furoyl)quinoline-2-carboxaldehyde; **PEO**, polyethylene oxide

2 Materials and methods

2.1 CE instrument

The CE instrument was locally constructed as described previously [25]. CE separation was performed in fused-silica capillaries with 140 μm OD, 20 μm or 50 μm ID and 40 cm length (Polymicro Technologies, Phoenix, AZ, USA), which were coated with linear polyacrylamide [26]. Detection was performed with a locally constructed ultra-sensitive LIF detector, based on a sheath-flow cuvette. A 12 mW argon ion laser beam was used for fluorescence excitation at 488 nm. Fluorescence was collected with a 60 \times , 0.7 NA microscope objective at right angles, filtered with a 630DF30 bandpass filter (Omega Optical, Brattleboro, VT, USA), and then detected with a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ, USA), which was biased at 900 V.

2.2 Reagents

Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO, USA). Ammonium persulfate and *N, N, N', N'*-tetramethylethylenediamine were obtained from Bio-Rad (Richmond, CA, USA). 3-(2-Furoyl) quinoline-2-carboxaldehyde (FQ) and KCN were obtained from Molecular Probes (Eugene, OR, USA). The dried FQ was prepared as described previously [20]. Digitonin and PEO ($M_r=100\ 000$) were obtained from Aldrich (Milwaukee, WI, USA). PEO sieving buffer was prepared by dissolving an appropriate amount of PEO in the running buffer. The running buffer contained 0.1 M Tris, 0.1 M CHES and 0.1% SDS (pH 8.6). Before use, the sieving buffer was degassed for 30 min by ultrasonication.

2.3 Cell culture and whole cell lysate of HT29 cells

The HT29 human colon adenocarcinoma cell line (American Type Culture Collection, ATCC No. HTB-38) was grown to 80% confluence in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere. Roughly 10⁶ HT29 cells were washed for four times with phosphate-buffered saline (PBS) to remove the residual substrate from the culture medium. The cells were lysed in 50 μL of 2% SDS and stored at -80°C .

2.4 DDF fractions of HT29 cells

The sequential extraction buffers, including digitonin/EDTA, Triton X-100/EDTA, Tween 40/DOC and detergent-resistant residue extraction buffers, were prepared ac-

cording to Ramsby's protocol [21]. Monolayer-cultured HT29 cells ($\sim 2 \times 10^6$) in a T25 culture flask were washed with PBS for four times. After PBS was completely aspirated, 1 mL of digitonin/EDTA extraction buffer was added. The flask was placed on ice and rocked for 15 min. The extraction solution was then transferred into microcentrifuge tubes and stored at -80°C . To rinse the cells, 2 mL of ice-cold PBS was added into the flask, rocked for 1 min and then aspirated. As above, 1 mL of Triton X-100/EDTA extraction buffer, 500 μL of Tween 40/DOC extraction buffer and 300 μL of detergent-resistant residue extraction buffer were added into the flask and rocked for 30 min, 20 min, and 20 min, respectively. The extraction solutions were collected, aliquoted, and stored at -80°C . Between these sequential fractionation steps, 2 mL of ice-cold PBS was used to wash out the residual solution from the previous fractionation step.

2.5 Precolumn labeling

Typically, 5 μL 2.0% SDS solution was added to 5 μL of protein solution (protein standards or extracts) to denature the proteins at 90°C for 5 min. Then the denatured proteins were derivatized by mixing 5 μL of this solution with 5 μL of 2.0 mM KCN solution in a 500 μL microcentrifuge tube, which contained 100 nmol of previously dried FQ. The mixture was incubated for 5 min at 65°C and then diluted with the running buffer.

2.6 On-column labeling

An 11 kPa negative pressure (siphoning injection) was first applied to the detection end of the capillary for 2 s to inject 10 mM FQ with 1.0% SDS [27]. The protein solution mixed with 10 mM KCN was then injected for 2 s. Finally the FQ-SDS mixture was injected for 2 s again. The injection end of the capillary was then placed in a vial containing the PEO sieving buffer, and the vial was heated at 90°C for 4 min for denaturation and derivatization of the proteins. After reaction, the separation was performed immediately at $-12\ \text{kV}$.

2.7 Single-cell experiment

HT29 cells were washed four times with PBS before single-cell analysis. Cell injection was carried out using a multipurpose capillary holder [27]. Injection was observed under an inverted microscope (model IMT-2; Olympus, Melville, NY, USA). A hydraulic model MX630R micromanipulator (Newport, Nepean, ON, Canada) was used to position the capillary. An 11 kPa negative pressure was applied to the detection end of the capillary for injection

of single cell as well as labeling reagents. KCN was added to the cell suspension immediately before injection to give a final concentration of 5.0 mM. A drop of this cell suspension and a drop of 10.0 mM FQ with 1.0% SDS solution were placed at different locations on a microscope slide. FQ-SDS mixture was first injected for 2 s. The capillary tip was then centered over a cell, and 1 s negative pressure was applied to draw the cell into the capillary. After the cell injection, the FQ-SDS mixture was injected again for 2 s to make a sandwich surrounding the single cell. The SDS lysed the cell inside the capillary very quickly. The capillary tip was then placed in a vial containing the PEO sieving buffer, and the vial was heated at 90°C for 4 min to denature and label the proteins. After reaction, the separation was performed at -12 kV.

3 Results and discussion

PEO (M_r , 100 000) was used as the sieving matrix in this work [4–6]. FQ, a fluorogenic dye [28], was employed to label proteins. A largely excess of FQ was used to improve the labeling efficiency of proteins.

3.1 Sample preparation for SDS-DALT-CE

As in SDS-PAGE, the performance of SDS-DALT-CE is highly dependent on sample preparation. In this study, sample preparation involves both FQ labeling and SDS denaturation. Three different procedures can be used for sample preparation: (1) Proteins can be denatured with SDS first and then labeled using FQ; (2) denaturation and labeling of the proteins can be performed simultaneously; (3) proteins can be labeled with FQ first and then denatured with SDS. Figure 1 shows the electropherograms of five standard proteins using these three procedures. Both procedures (1) and (2) gave good resolution for the proteins (Figs. 1A and B). However, co-eluted peaks were observed for proteins such as carbonic anhydrase, ovalbumin and bovine serum albumin when procedure (3) was used (Figs. 1C). This poorer resolution might be caused if the FQ hindered subsequent SDS binding to proteins, degrading the sieving separation. On the other hand, binding proteins with SDS before FQ labeling (sample procedure 1) ensured that the proteins combined with a large number of SDS molecules. The highly negative charge of the SDS-bound proteins should not be changed dramatically by the subsequently attachment of the FQ labels. Therefore proteins were separated according to their size. Procedure (2) generated similar separation of the proteins as sample procedure (1), which implies that SDS binding to proteins is kinetically faster than FQ reaction with proteins when FQ and SDS are present at the

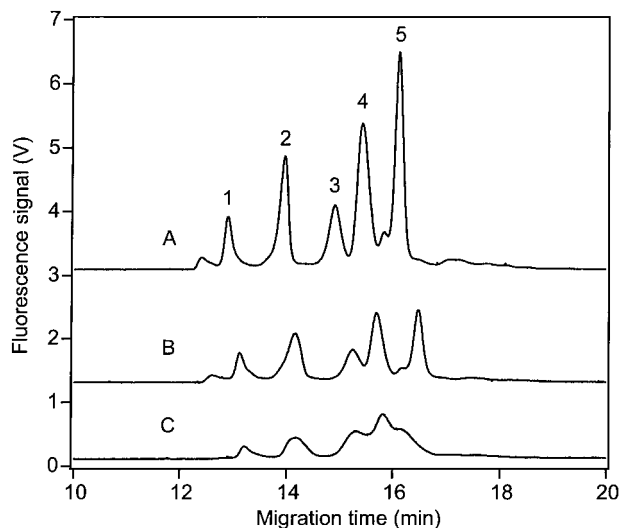


Figure 1. Electropherograms of standard proteins. (A) Proteins were first combined with SDS and then labeled with FQ; (B) proteins were combined with SDS and labeled with FQ simultaneously; (C) proteins were labeled with FQ first and then combined with SDS. Peaks: 1, lactoglobulin; 2, carbonic anhydrase; 3, ovalbumin; 4, bovine serum albumin; 5, conalbumin. Experimental conditions: capillary, 40 cm \times 20 μ m; separation, 300 V/cm; injection, 100 V/cm for 5 s; sieving buffer, 0.1 M Tris-0.1 M CHES with 2% PEO and 0.1% SDS; protein concentration, 200 nM each. Traces offset for clarity.

same time. Data shown in Fig. 1 also indicates that the multiple-labeling problem in SDS-DALT-CE is not as serious as that in CZE [29, 30]. SDS-DALT-CE is a size-based separation method. Attachment of different numbers of labels with low molecular mass to the large protein molecule should not dramatically change the size of protein molecule and therefore its migration velocity [31–33].

3.2 Separation conditions

Figure 2 presents the SDS-DALT-CE electropherograms of seven standard proteins obtained in three different concentrations of PEO. Clearly, 2.0% PEO provided better resolution than 1.5% PEO for the proteins. Compared with 2.0% PEO, 2.5% PEO provided better resolution for ovalbumin and bovine serum albumin but worse resolution for trypsin inhibitor and carbonic anhydrase. Our goal is to use this system for analysis of a single cell, where a low concentration of PEO is desired to reduce the viscosity of the solution to facilitate the pressure-driven injection of the cell into the capillary.

The electropherograms of seven standard proteins obtained under different separation voltages are shown in Fig. 3. The resolution did not change significantly with

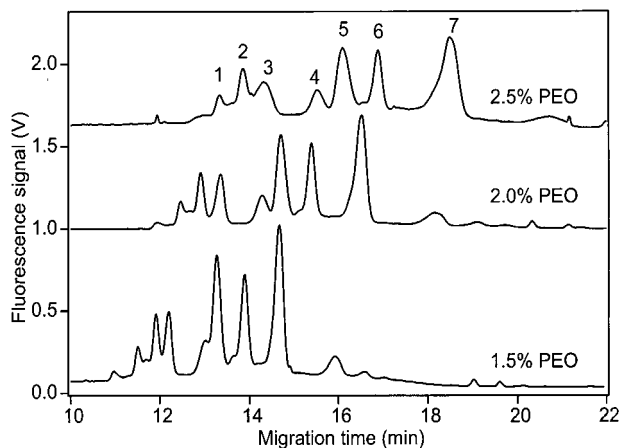


Figure 2. Electropherograms of standard proteins in different concentrations of PEO. Peaks: 1, lactoglobulin; 2, trypsin inhibitor; 3, carbonic anhydrase; 4, ovalbumin; 5, bovine serum albumin; 6, conalbumin; 7, β -galactosidase. Protein concentration, 31.25 nM each. Other conditions as in Fig. 1A. Traces offset for clarity.

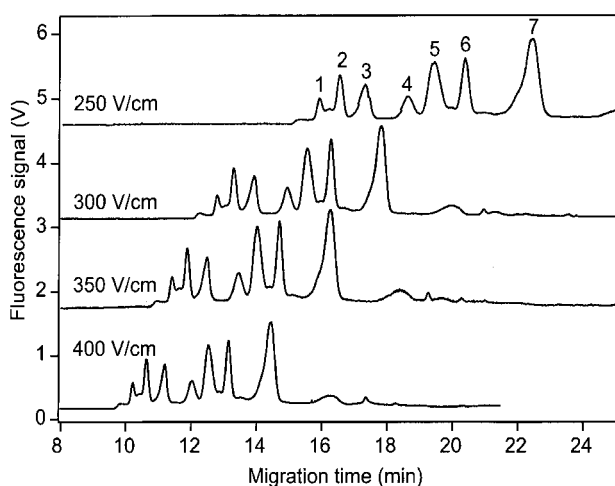


Figure 3. Electropherograms of standard proteins under different separation voltages. PEO concentration, 2.0%; protein concentration, 62.5 nM each. Other conditions as in Fig. 2. Traces offset for clarity.

the separation voltage. As expected, the migration time of the proteins decreased as separation voltage increased. SDS-DALT-CE separation of five standard proteins was also performed in 50 μ m ID coated capillary (see Fig. 4A). The electropherogram was similar to the one shown in Fig. 1A, indicating that capillary radius did not affect the size-based separation. A blank experiment was also performed under the same conditions as those for standard proteins, Fig. 4B. The reagent blank is negligible.

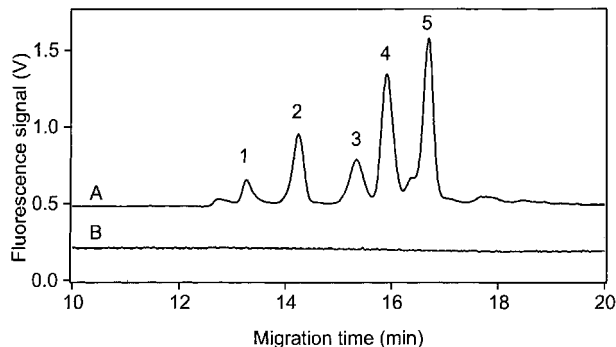


Figure 4. Electropherograms of (A) standard proteins and (B) blank obtained in a 50 μ m capillary. Capillary, 40 cm \times 50 μ m ID; protein concentration, 100 nM each. Peak identification and other conditions as in Fig. 1A. Traces offset for clarity.

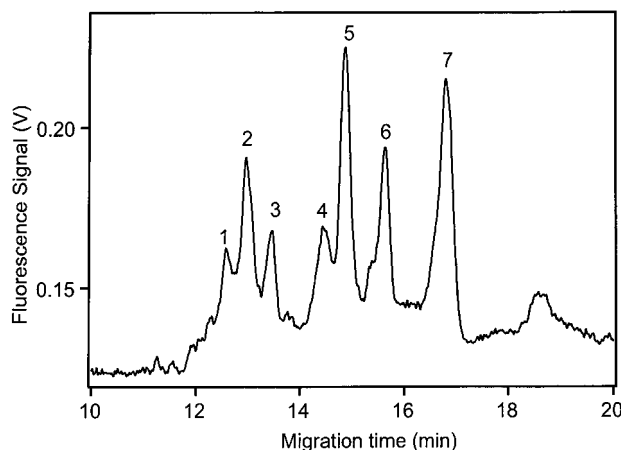


Figure 5. Electropherograms of standard proteins labeled at nanomolar concentrations. Protein labeling concentrations, 7.8 nM each; protein final concentration, 1.56 nM each; injection, 200 V/cm for 5 s. Other conditions as in Fig. 1A.

3.3 Detection limits and reproducibility

The electropherogram of nanomolar concentration proteins (1.6 nM each) is shown in Fig. 5. Note that the proteins were labeled at nanomolar concentrations (7.8 nM each) and diluted by a factor of 5 before injection. The concentration detection limits for all proteins were in the high picomolar range, and the mass detection limits were in the mid- to high-zeptomole range (Table 1). FQ reacts with the ϵ -amine of lysine residues. As expected, sensitivity increased linearly with the number of lysine residues ($\chi^2_n = 2.1$ for six degrees of freedom, $P = 0.05$). Lysine is one of the most common amino acids, and the range in the number of lysine residues observed in these standard proteins is similar to that expected from most eukaryotic

Table 1. Detection limits of standard proteins by SDS-DALT-CE with LIF detection and precolumn labeling

Protein	Molecular mass (Da)	Lysine residues	Conc. LODs (nM)	Mass LODs (zmol)
β -Lactoglobulin	18 400	15	0.39	220
Trypsin inhibitor	20 100	12	0.22	120
Carbonic anhydrase	30 000	18	0.27	140
Ovalbumin	43 000	20	0.26	130
Bovine serum albumin	67 000	59	0.12	60
Conalbumin	78 000	29	0.16	70
β -Galactosidase	116 000	20	0.10	40

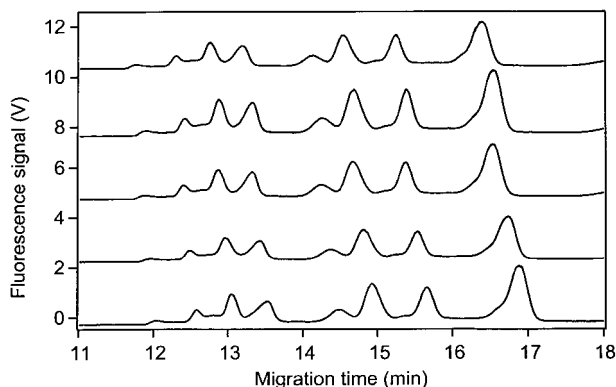
organisms. For example, the average number of lysine residues of proteins in the *Saccharomyces cerevisiae* database is 35 ± 30 .

Figure 6 shows the replicate separation of the standard proteins by SDS-DALT-CE with LIF. A fresh protein solution was labeled for each electropherogram. Related statistical data are summarized in Table 2. Relative standard deviations (RSDs) for migration time were from 0.7% to 0.9% for the proteins. RSDs of peak area were from 5% to 8%. The slow drift in migration time may have been due to either evaporation in the separation buffer or drift in the room temperature during the experiment. A linear relationship, $r = 0.986$, was obtained between the average migration times of the seven proteins and the logarithm of their molecular weights.

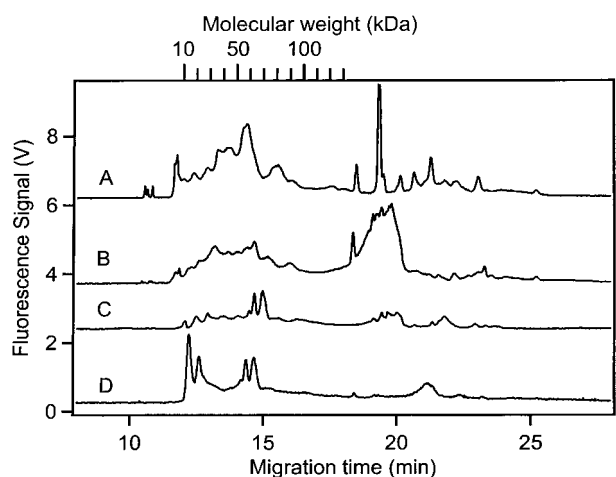
3.4 Analysis of DDF fractions of HT29 cells by SDS-DALT-CE

Similar to standard proteins, DDF fractions of HT29 cells were denatured by SDS, labeled with FQ and then analyzed by SDS-DALT-CE with LIF using 2.5% PEO as the sieving buffer. Figures 7A–D, respectively, show the electropherograms of cytosolic fraction, membrane/organelle fraction, nuclear fraction and cytoskeletal/nuclear matrix fraction of HT29 cancer cells. More than 20 peaks were observed for cytosolic and nuclear fractions. For both fractions, the proteins were distributed across a very wide molecular weight range, with a number of components migrating after the largest of our molecular weight standards. Some of these peaks may be due to very high molecular weight proteins. However, we did not employ a reducing agent to disrupt disulfide bonds, and some of the late-migrating peaks may be due to protein complexes.

The cytoskeletal/nuclear matrix fraction gave the simplest profile. The membrane/organelle fraction had the poorest resolution, which likely was because the membrane con-

**Figure 6.** Replicate separations of standard proteins by SDS-DALT-CE with LIF and precolumn labeling. Protein concentrations, 62.5 nM each. Other conditions as in Fig. 1A. Traces offset for clarity.**Table 2.** Statistical data by SDS-DALT-CE with LIF and precolumn labeling

Peak No.	Protein	Molecular weight	Migration time (average, min)	RSD _T	RSD _A
1	β -Lactoglobulin	18 400	12.42	0.7	5
2	Trypsin inhibitor	20 100	12.88	0.7	7
3	Carbonic anhydrase	30 000	13.34	0.8	8
4	Ovalbumin	43 000	14.28	0.8	6
5	Bovine serum albumin	67 000	14.70	0.9	7
6	Conalbumin	78 000	15.42	0.9	7
7	β -Galactosidase	116 000	16.59	0.9	7

**Figure 7.** Electropherograms of (A) cytosolic fraction, (B) membrane/organelle fraction, (C) nuclear fraction, and (D) cytoskeletal/nuclear matrix fraction, of HT29 human colon cancer cells. Capillary, 40 cm \times 50 μ m; PEO concentration, 2.5%. Other conditions as in Fig. 1A. Traces offset for clarity.

sists of hydrophobic proteins and lipoproteins, which are notoriously difficult to analyze by SDS-PAGE. Overall, the sequential fractions of HT29 cells gave distinct protein profiles, either in the expressed amount or the molecular weight distribution. Taking into account the difference of the final extraction volumes in the four-fractionation steps, as pointed out by Ramsby *et al.* [21], cytosolic and membrane/organelle proteins made up most proteins in these cells.

3.5 On-column labeling of standard proteins

On-column labeling of proteins in SDS-DALT-CE is more complicated than that in CZE because it involves both fluorescent labeling and SDS denaturation. Here a “sandwich” mode was employed for on-column labeling of proteins, which provided efficient contact of the labeling reagents with proteins and improved the labeling efficiency and reproducibility. In previous studies, on-column labeling for CE-LIF was usually performed by injection of the analytes (sample solution or single cell) first and then followed by a plug of labeling reagent [17, 34]. That simpler labeling chemistry required one less injection of reagents, but did not allow all the analytes to efficiently mix with the labeling reagent.

Figure 8 shows replicate separations of seven standard proteins by SDS-DALT-CE with LIF and on-column labeling. The concentration detection limits and RSDs of migration time and peak area are listed in Table 3. The detection limits were observed to be within the nanomolar range. RSDs were from 0.7% to 0.9% (migration time), and from 4% to 8% (peak area). The migration time increased linearly with the logarithm of molecular weight, $r > 0.99$.

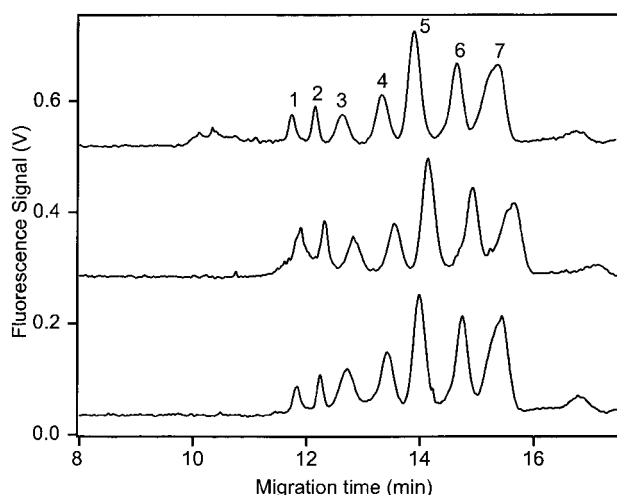


Figure 8. Replicate separations of standard proteins by SDS-DALT-CE with LIF and on-column labeling. Protein concentration, $0.2 \mu\text{M}$ each. Peak identification and other conditions as in Fig. 1A. Traces offset for clarity.

Table 3. Statistical data by SDS-DALT-CE with LIF and on-column labeling

Protein	Migration time (min)	LODs (nM)	RSD _T (%)	RSD _A (%)
β -Lactoglobulin	11.84	10	0.8	4
Trypsin inhibitor	12.26	9	0.7	6
Carbonic anhydrase	12.63	9	0.8	6
Ovalbumin	13.45	6	0.8	6
Bovine serum albumin	14.00	3	0.7	5
Conalbumin	14.74	4	0.8	7
β -Galactosidase	15.47	5	0.9	8

3.6 Analysis of proteins in single human cancer cells

CZE is the most often used CE mode for analysis of proteins in single cells. CZE with native LIF, for instance, has been demonstrated for analysis of carbonic anhydrase and hemoglobin in single erythrocytes as well as green fluorescent protein expressed in single kidney cells [13, 14, 16, 18]. CZE-MS has also been demonstrated for analysis of hemoglobins in single erythrocytes [35, 36]. Recently, we have described CZE-LIF for analysis of the total proteins in single HT29 cancer cell as well as a *Caenorhabditis elegans* zygote [17, 19].

Here, SDS-DALT-CE, a size-based separation mode, was applied to analysis of total proteins in single HT29 human colon cancer cells. On-column labeling was employed. As in the case of on-column labeling of standard proteins, SDS with FQ was first injected. Then a single HT29 cell and KCN was injected. Finally, SDS with FQ was injected again. The cell was lysed by SDS very quickly inside the capillary. The “sandwich” mode ensured the efficient mixing of SDS, KCN and FQ with the cellular proteins. The capillary tip was heated for 4 min to denature and label single-cell proteins. The labeled proteins were then separated in PEO sieving buffer and detected by sheath flow LIF. Figure 9 shows the electropherograms of three individual HT29 cells. Based on calibration from standard proteins, most of the protein components in a single HT29 cell were observed to have molecular masses from 10 to 120 kDa. As with the DDF data of Fig. 7, a number of components had very large molecular weights. For comparison, the whole cell lysate from a large number of HT29 cells was also analyzed using the same method. As shown in Fig. 9, the protein profile of the HT29 extract was similar to the profile from single cells.

4 Concluding remarks

We have demonstrated that SDS-DALT-CE with LIF is a useful technique for protein analysis. This method is fast, reproducible, highly sensitive, and provides separation

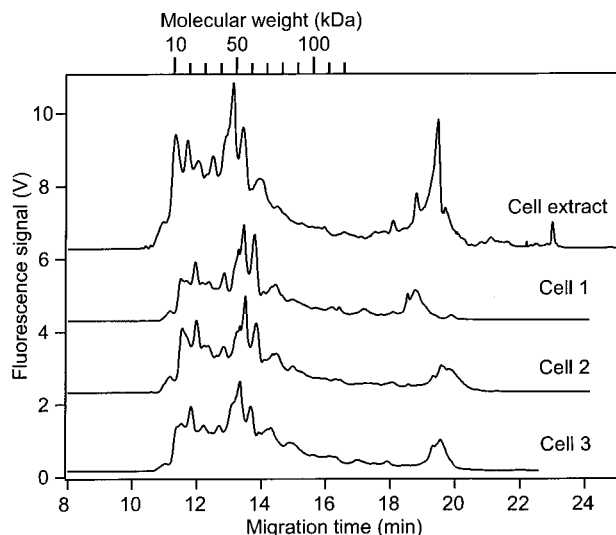


Figure 9. Electropherograms of single HT29 cancer cells and HT29 cell extract by SDS-DALT-CE with LIF and on-column labeling. CE conditions as in Fig. 1. Other conditions see Section 2. Traces offset for clarity.

based on the molecular weight of the protein. It requires a minute sample and is used for analysis of the proteins in single human cancer cells. We have also applied this method to analysis of DDF fractions of HT29 human colon cancer cells. Although distinct protein profiles have been observed for the sequential fractions, further work is needed to enhance resolution. After DDF, the cell fractions are usually subject to analysis by 2-D gel electrophoresis. We have recently developed a 2-D CE system for separation of proteins in a similar way to 2-D gel [37]. This high-resolution method will be combined with DDF to perform proteomic studies of HT29 cancer cells.

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