

Analysis of Proteins by Capillary SDS-DALT Electrophoresis with Laser-Induced Fluorescence Detection

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Capillary SDS-DALT electrophoresis (SDS-DALT-CE) refers to CE separations of proteins based on their size. DALT (Dalton) is the mass unit used to describe molecular weight. In this article, the highly sensitive analysis of proteins was demonstrated by using SDS-DALT-CE with laser-induced fluorescence (LIF). Proteins were denatured by SDS and labelled by 3-(2-furoyl) quinoline-2-carboxaldehyde (FQ). The FQ-labelled proteins were then separated in pullulan sieving matrix and detected by sheath flow cuvette LIF. This method was used to analyse protein extract of CH27 hybridoma cells, mouse lens protein extract prepared by laser capture microdissection, as well as proteins in single 4355 human breast cancer cells.

Capillary SDS-DALT electrophoresis (SDS-DALT-CE) is a new form of CE method for the size-based separation of proteins. At an early stage, SDS-DALT-CE was performed by using cross-linked polyacrylamide gel formed inside the capillary as the sieving matrix for proteins.^{1,2} However, this capillary gel format suffers from several significant problems, including difficulties in reproducible polymerization of gel inside the capillary, gel breakdown under high electric fields, as well as gel fouling with the residual proteins from the previous separations.²⁻⁴ To solve these problems, polyacrylamide gel has been replaced with linear or slightly branched polymers, including linear polyacrylamide, polyethylene oxide, polyethylene glycol, dextran and pullulan.⁵ Such polymers are water-soluble and replaceable between CE runs, therefore greatly improving the flexibility of SDS-DALT-CE.

UV absorbance is the most widely used detector for SDS-DALT-CE of proteins. However, this detector produces low sensitivity because of the short lightpath in the capillary, limiting the application of SDS-DALT-CE. To improve the sensitivity, laser-induced fluorescence (LIF) has recently been proposed for the detection of proteins in SDS-DALT-CE. Compared with UV detection, the detection sensitivity of LIF is at least two orders of magnitude higher.⁶⁻¹⁰ This enhanced sensitivity is very critical to full-scale characterization of protein expression because many proteins, especially some regulatory proteins, are expressed at very low levels in cells and tissues. Highly sensitive

detection technologies, such as LIF are required to characterize those low-abundance proteins in order to provide meaningful information about cellular and regulatory processes.

Recently we described a highly sensitive method, based on SDS-DALT-CE with LIF, for the analysis of proteins in single human colon cancer cells.¹¹ In this article, this method is further characterized and applied to the analysis of proteins in mouse hybridoma cells, mouse lens, as well as single human breast cancer cells.

Experimental

Apparatus: Separations were performed using a laboratory-built CE instrument with a sheath flow cuvette LIF detector, as described previously.¹² Fluorescence excitation was provided by an argon ion laser operating at 488 nm with a power of 12 mW. Fluorescence was collected with a 60×, 0.7 NA microscope objective at right angles, filtered with a 630DF30 bandpass filter (Omega Optical, Brattleboro, Vermont, USA) and detected with a photomultiplier tube (Hamamatsu, Middlesex, New Jersey, USA), which was biased at 900 V. All separations were performed at -300V/cm. Sample injection was performed at -100 V/cm except for single-cell analysis.

Fused-silica capillaries of 40 cm in length, 140 μm o.d., and 50 μm i.d. (Polymicro Technologies, Phoenix, Arizona, USA) were used in this work. The capillaries were coated with linear polyacrylamide to eliminate electroosmotic flow.¹³

Reagents: Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, Missouri, USA). 3-(2-furoyl) quinoline-2-carboxaldehyde (FQ) and KCN were obtained from Molecular Probes (Eugene, Oregon, USA). The sieving buffer contained 0.1 M Tris, 0.1 M 2-(cyclohexylamino)-ethanesulfonic acid (CHES), 0.1% SDS and 6% pullulan (pH 8.6).

Protein extract of mouse hybridoma cells: A mouse B cell hybridoma line, CH27, was maintained in RPMI (Gibco-BRL) supplemented with 10% fetal calf serum (FCS). Roughly 5×10^5 CH27 cells were washed four times with phosphate-buffered saline (PBS) to remove the residual substrate from the culture medium. The cells were then lysed by 2% SDS and kept at -80°C .

Lens proteins captured by laser capture microdissection (LCM): Sections of 11 μm thick were cut from frozen crystalline lens of 24 to 30 day old mice. The sections on glass slides were stained with Toluidine Blue O and dehydrated in ethanol and xylene. The cells were removed from slide sections using an Arcturus PixCell II LCM system (Arcturus, Mountview, California, USA), solubilized in urea/CHAPS buffer and kept at -80°C .

FQ labelling of protein standard and extract: 5.0 μL protein sample (protein standard or extract) was mixed with 2.5 μL 2.0% SDS and 2.5 μL of 10.0 mM KCN in a 500 μL microcentrifuge tube containing 100 nmol of previously dried FQ.¹¹ No SDS was added to the lysate of hybridoma cells because the cells were lysed by SDS. The mixture was heated at 95°C for 5 min. This procedure was employed to denature the proteins and label proteins with FQ. After labelling, the sample was diluted with the running buffer which contains 0.1 M Tris, 0.1 M CHES and 0.1% SDS.

Single-cell analysis: The MDA-MB-435S human breast cancer cell (ATCC No. HTB-129) was maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FCS. Before a single-cell experiment, the cells were washed four times with PBS to remove the residual substrate from the medium.

Cell injection was performed as described previously.^{11, 14} Right before injection, KCN was added to the cell suspension to give a final concentration of 1.0 mM. Individually, a drop of this cell suspension and a drop of 5.0 mM FQ with 0.5% (w/v) SDS solution were placed at different locations on a microscope slide. First, the capillary tip was moved to the drop of FQ-SDS mixture. A plug of FQ-SDS was injected by applying a negative pressure for 1 s. Then the capillary tip was placed over a cell. The negative pressure was applied again for 1 s, drawing the cell and KCN into the capillary. Finally, the capillary was moved back to inject FQ-SDS again for 1 s. SDS lysed the cell inside the capillary very quickly. The capillary tip

was then heated at 85°C for 5 min to perform on-column labelling of cellular proteins. After labelling, the separation was performed at -300 V/cm .

Results and Discussion

Separation of standard proteins: FQ, a fluorogenic dye, was employed for protein labelling. During the labelling, proteins were also denatured with SDS. This ensured that all proteins have almost identical charge-to-mass ratios so that their charges did not affect the size-based separation. Figure 1 shows the electropherogram of eight standard proteins obtained by SDS-DALT-CE with LIF. Molecular weights and migration times of those proteins are listed in Table 1. A linear plot of the logarithmic molecular weight (Log MW) versus migration time (T_m) is constructed (see insert in Figure 1), and the equation obtained is

$$\text{Log MW} = 2.48 + 0.136 T_m$$

with a linear correlation coefficient (r) of 0.995. Serial dilutions of the protein mixture were analysed and statistical data are also summarized in Table 1. Within the concentration range from 1.0 nM to 50.0 nM, linearities with $r > 0.99$ were obtained for all the proteins, except r of 0.988 for β -galactosidase.

This method can be used to determine the molecular weight of proteins. Figure 2 shows the electropherogram of concanavalin A (Con A), a 52 kDa glycoprotein. The migration time of FQ-labelled Con A is 16.5 min. It corresponds to a molecular

Figure 1: SDS-DALT-CE separation of 8 standard proteins.

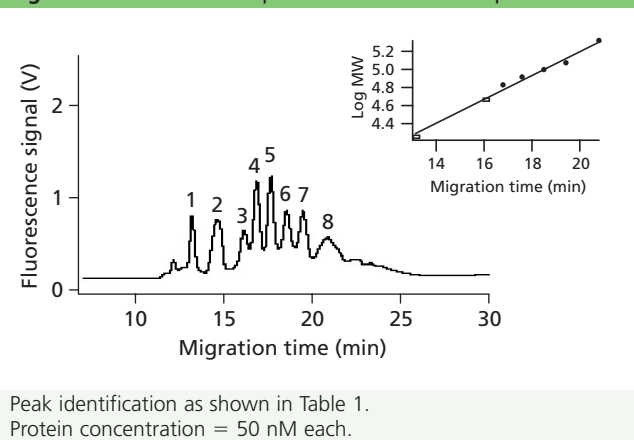


Table 1: Statistical data of standard proteins.

Peak	Protein	Molecular weight (Da)	Migration time (min)	Dynamic range (nM)	r
1	Myoglobin	17600	13.16	1–50	0.997
2	Carbonic anhydrase	29000	14.57	1–50	0.995
3	Ovalbumin	45000	16.08	1–50	0.997
4	Bovine serum albumin	66000	16.79	1–50	0.994
5	Apotransferrin	80000	17.58	1–50	0.993
6	Phosphorylase	97000	18.48	1–50	0.991
7	β -Galactosidase	116000	19.40	1–50	0.988
8	Myosin	205000	20.77	1–50	0.993

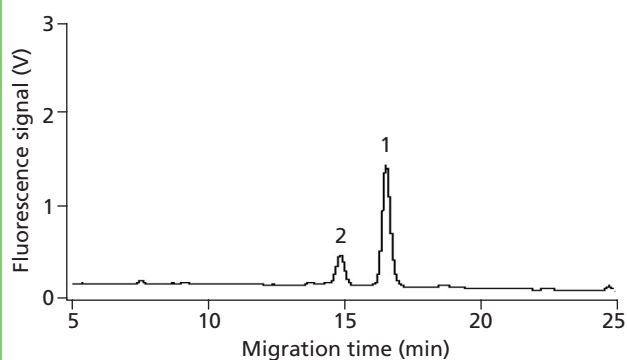
weight of 52 940 Da based on the above equation. This value is 1.8% higher than the accepted molecular weight of 52 kDa, which is much better than the 10% accuracy of SDS-PAGE.

Analysis of protein extract of mouse hybridoma cells: As antigen presenting cells, CH27 B cell hybridoma express class II major histocompatibility complex (MHC) molecule I-E^k on the cell surface. MHC are heterodimeric membrane glycoproteins, which bind diverse peptides of approximately 7–25 amino acids in length and presenting them for recognition to CD4⁺ T cells. Recognition of specific peptide-MHC complexes by T cell is a key step in the generation of an immune response.¹⁵

Compared with UV detection, the detection sensitivity of LIF is at least two orders of magnitude higher.

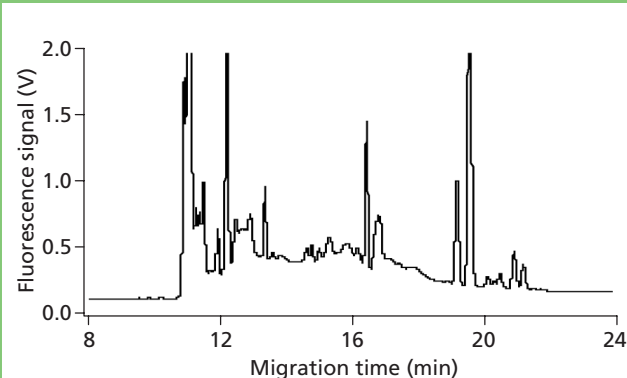
Here SDS-DALT-CE with LIF was used to analyse the protein extract of CH27 cells. A typical protein profile is shown in Figure 3. Around 30 proteins were resolved and those proteins were observed to have molecular weights ranging from 9000 to 215 000 Da, based on the calculation from the standard proteins. The total analysis time, including the sample preparation and separation, was within 30 min, indicating that this method is much faster than SDS-PAGE.

Figure 2: Electropherogram of FQ-labelled concanavalin A.



Peaks: 1 = concanavalin A, 2 = unknown.

Figure 3: SDS-DALT-CE separation of proteins in mouse B cell hybridoma cells.



Analysis of lens proteins captured by LCM: LCM is a rapid, reliable method to procure pure populations of targeted cells from stained heterogeneous tissue sections for subsequent molecular analysis. It is based on a selective adherence of targeted cells to a thermoplastic membrane activated by a low energy infrared laser pulses.^{16,17} Because of the small amount of material captured by LCM, CE should be a useful tool for subsequent molecular analysis of LCM sample since CE only requires a minute amount of sample. Figure 4 shows the electropherogram of a mouse lens protein sample captured by LCM. Roughly 15 peaks were resolved. However, a number of proteins overlapped from 13 to 15 min. Based on their migration times, these proteins are very likely from one particular protein family — the crystallins.¹⁸ Size-based separation of those proteins is fairly difficult because they have very close molecular weights. Future studies on new sieving matrices are needed to improve the resolution of crystallins.

Analysis of proteins in single human breast cancer cells: Single-cell analysis is rapidly expanding our knowledge about biological processes by providing us with an insight into the detailed chemistry of a cell on individual bases. As a micro-separation technique with high resolving power and ultra-small sample volume, CE has been widely used for single-cell analysis, including analysis of single-cell proteins. However, most analyses of single-cell proteins were performed by capillary zone electrophoresis (CZE).^{19–24} Either charge or size information of proteins cannot be directly obtained from the separations.

Figure 4: SDS-DALT-CE separation of mouse lens proteins.

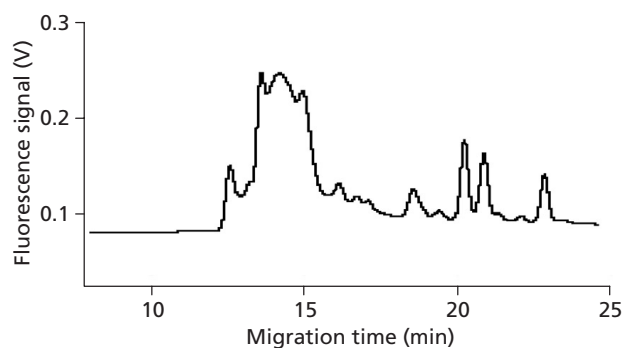
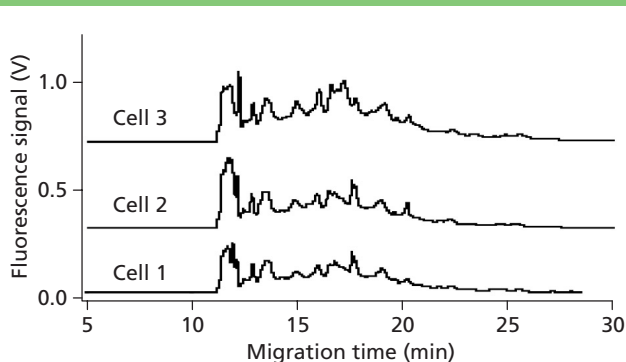


Figure 5: SDS-DALT-CE analysis of proteins in single 435S human breast cancer cells.



We used SDS-DALT-CE, a size-based separation method, for single-cell protein analysis. To ensure efficient contact of labelling reagents with single-cell proteins, a sandwich mode was employed to perform on-column labelling.¹¹ Figure 5 presents the electropherograms of three 435S human breast cancer cells. Around 25 proteins were resolved and most of them were observed to have molecular weights from 10 to 200 kDa. The overall patterns of these cells are remarkably similar. However, a detailed inspection of the electropherograms reveals minor cell-to-cell variation of protein expression. For instance, cell 3 expressed more of the proteins with molecular weights of 50–70 kDa than cell 1 and 2. However, cells 1 and 2 expressed more of an 80 kDa protein than cell 3.

Conclusion

Clearly, SDS-DALT-CE has several advantages over SDS-PAGE. These advantages include high speed, on-line detection, ultra-small amount of sample required and full automation. However, such advantages are attained partly at the expense of the very high resolution characteristic of electrophoresis in polyacrylamide gels.²⁵ Therefore, future studies, such as optimizing sieving matrixes, are needed to improve the resolution of this method. Alternatively, SDS-DALT-CE can be combined with other CE separation modes, such as CZE or capillary isoelectric focusing to perform a two-dimensional separation to achieve high resolution for proteins.²⁶

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