Introduction

Microfluidic chips for use in biochemical analysis have been extensively developed. They offer reduced sample volumes, short operation times, and reduced cost. However, the reduced sample volumes are too small to analyze low-abundant biomolecules, including membrane proteins. Preconcentration of biomolecules prior to analysis can be a solution to the problem. Although many preconcentration techniques for water-soluble biomolecules have been developed, none of these are for membrane proteins.

We have developed alternating current cloud point extraction (ACPE) as a preconcentration technique of membrane proteins on a microfluidic chip. ACPE is based on the principle of cloud point extraction (CPE). In CPE, a test solution containing membrane proteins solubilized in nonionic surfactant micelles is initially heated above its cloud point. The solution is separated into a surfactant-poor phase and a surfactant-rich phase, the latter containing membrane proteins solubilized in the micelles. In ACPE, the test solution is introduced into a microchannel, and AC voltages are applied to microelectrodes integrated in the microchannel. The solution is heated above its cloud point by Joule heating to form a surfactant-rich phase, which is collected around the electrodes by negative dielectrophoresis. Therefore, membrane proteins solubilized in the micelles are concentrated around the electrodes. After turning the AC voltages off, the concentrated membrane proteins will be transferred into a separation channel filled with polymer solution under an external electric field, and size-based separation will be achieved. ACPE has been successfully applied to commercially-available membrane proteins and those extracted from HeLa cells. However, the selectivity of ACPE relies on the hydrophobicity of the analytes. Therefore, the concentration of membrane proteins with lower hydrophobicity (e.g., peripheral proteins, glycoproteins) will be unsatisfactory. Additionally, it is well known that the dynamic range of protein concentrations in analytes span several orders of magnitude. Since the magnitude of concentration enrichment in ACPE is independent of the initial concentration of target molecules, it will be difficult to detect lower-abundant membrane proteins. In other words, it is important to introduce novel interaction into the ACPE technique in an effort to concentrate these analytes efficiently.

In this paper, we report on the use of ferrocenyl surfactants in ACPE. Ferrocenyl surfactants possess ferrocenyl groups at the end of the alkyl chain. Of these, 11-ferrocenyltrimethylundecylammonium bromide (FTMA) possesses a ferrocenyl group at the end of a hydrophobic alkyl chain with a cationic head group. Additionally, the ferrocenyl group becomes cationic if oxidized. Therefore, compared to conventional ACPE, which utilizes only nonionic surfactants (Fig. 1(a)), the hydrophobic region of the micelle becomes cationic, and consequently anionic membrane proteins will be efficiently solubilized (Fig. 1(c)). In this study, we used both reduced FTMA (FTMAred) and oxidized FTMA (FTMAox). We measured the cloud point of a mixed solution comprising Triton X-114 (TX) and FTMA at various concentrations to determine which solution composition is suitable for ACPE. Next, we demonstrated the use of FTMA in ACPE. Adenosine triphosphatase (ATPase), an anionic membrane protein at neutral pH, was utilized as a target molecule. The effect of FTMA on the extraction of ATPase using ACPE was examined.

Experimental

Cloud point measurements

Test solutions comprised 0.5% TX (Wako), 0.1 M lithium sulfate (Kanto Chemical), and 0.5 – 3.5 mM FTMA (Wako). FTMAox was prepared by oxidizing FTMAred with cerium(IV)
sulfate tetrahydrate (Wako) according to the literature. Cloud points of test solutions were determined using an absorption spectrophotometer (V-630BIO, Jasco Corp.) equipped with a water-cooled peltier cell changer (PAC-743, Jasco Corp.). Transmittance of the test solution was measured at 800 nm, and the temperature of the test solution was increased from 10 to 100°C at a rate of 0.5°C/min.

ACPE experiments

Test solutions containing (i) 1.5 mM FTMAred and 0.5% TX, (ii) 3.5 mM FTMAox and 0.5% TX, and (iii) 0.5% TX were utilized in the ACPE experiments. Adenosine triphosphatase (ATPase, SIGMA) was fluorescently labeled with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY® FL, SE, D2184, Life Technologies) as previously described. The isoelectric point of ATPase was calculated to be 5.36 using ExPASy (Swiss Institute of Bioinformatics). The concentration of ATPase was 62.5 mg/L.

A polydimethylsiloxane-glass microfluidic chip with Au electrode (40 μm width, 20 μm gap) was fabricated as previously described. A test solution was introduced into the microchannel using a syringe pump (LEGATO111, KDS) at a flow velocity of 0.33 mm/s. AC voltages (20 Vp-p, 5 MHz) were applied to the microelectrodes using a function generator (WF1963, NF). Fluorescence images were taken using a microscope (BX51, Olympus) equipped with a CCD camera (DP50, Olympus), a 100-W high pressure mercury lamp, a 20× objective lens (NA 0.45), and a dichroic mirror block (U-MWB2, excitation 460 - 490 nm and emission > 510 nm). Images of the ACPE were analyzed using image analysis software (ImageJ, National Institutes of Health). A concentration index (CI) was defined as the ratio of the mean fluorescence intensities above the central electrode (see Fig. 3) at ACPE time t to that at the beginning of the experiment.

Results and Discussion

Cloud point measurements

Figure 2 shows the dependence of the cloud point of test solutions on FTMA concentration. Cloud points of FTMAred solutions increased from 20.7±1.0 to 74.8±3.1°C with increasing FTMAred concentration from 0 to 3.5 mM. Cloud points of FTMAox solutions also increased from 19.9±0.0 to 36.7±0.8°C with increasing FTMAox concentration. These results indicated that both FTMAred and FTMAox form mixed micelles with TX, and that an increase in FTMA concentration leads to an increase in the amount of FTMA incorporated into the mixed micelles. The incorporation of FTMA makes the micelles cationic and induces micelle-micelle repulsion, which leads to an increase in the cloud point of the solutions. Similar results have been reported for mixed solutions comprising TX and the cationic surfactant cetyltrimethylammonium bromide. Therefore, FTMAred solutions exhibited higher cloud points than those comprising FTMAox. These results indicated that if the total concentration of FTMA was the same, the amount of FTMAred incorporated into the mixed micelles is larger than that of FTMAox. The critical micelle concentration of FTMAred and FTMAox was reported as 7×10⁻⁵ and 1×10⁻³ M, respectively. Therefore, FTMAred is incorporated into the micelles more easily compared to FTMAox.

In general, membrane proteins are denatured by heat.
Therefore, it is important to induce clouding at physiological temperatures. When the FTMAred and FTMAox concentrations were 1.5 and 3.5 mM, the cloud points were 40.5 ± 6.1 and 37.0 ± 0.8°C, respectively. Therefore, these solution compositions were subjected to ACPE.

ACPE experiments

Figure 3 shows the results of the ACPE experiments. As shown in Figs. 3(b) - 3(g), fluorescence was observed from all the test solutions by applying AC voltages to the electrodes. Thus, ACPE with FTMA was demonstrated. Figure 3(h) shows the dependence of CI on ACPE time. When the test solution contained 0.5% TX, the CI at 60 s was 6.2 ± 0.7. When the test solution contained 1.5 mM FTMAred in addition to 0.5% TX, the CI at 60 s was 5.9 ± 2.3, which was almost identical to that of the 0.5% TX solution. On the other hand, when the test solution contained 3.5 mM FTMAox in addition to 0.5% TX, the CI at 60 s increased to 10.3 ± 3.7. Therefore, it is expected that the mixed micelles comprising TX and FTMAox possess sufficient positive charge to electrostatically attract ATPase into the micelles.

Conclusions

In this study, we have developed a novel ACPE technique using the ferrocenyl surfactant FTMA. When 3.5 mM FTMAox or 1.5 mM FTMAred were used, the cloud points of the solutions were at physiological temperatures. We applied these solutions to ACPE and demonstrated the extraction of ATPase. ACPE with FTMAred led to almost the same CI observed with a solution comprising only TX. On the other hand, ACPE with FTMAox led to a higher CI compared to the other solutions. The positive charge of FTMAox accounts for the increased CI. We expect the approach outlined in this report to be useful in the preconcentration technique of microchip electrophoresis.

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