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Effect of Nanoparticles in Protein Separation by Capillary Electrophoresis

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Identification of proteins and analysis of their function are important for the development of diagnostic and therapeutic markers.[1-3] Since Human Genome Project (HGP) is in imminent completion, researchers has been interested in deciphering the structure and function of the numerous proteins translated from the genetic sequence.[4] Although the human genome may contain about 30,000 genes, more proteins are produced through post-translational modifications such as proteolytic processing,[5] glycosylation,[6] and phosphorylation.[7] Consequently, there is a critical need for analytical techniques that can provide function and identification of numerous and diversified proteins.

Determination of molecular weight is one of the most important aspects of protein characterization. Mass spectrometry (MS) with either matrix-assisted laser desorption and ionization (MALDI)[8] or electrospray ionization (ESI)[9] has been a method of choice for accurate molecular weight determination. However, fragmentation during ionization or inclusion of solvent molecules can lead to incorrect sizing of proteins. Also, variations in ionization efficiency imply that accurate quantitative results are difficult to obtain.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been one of the commonly used methods for the determination of protein molecular weight for over three decades.[10,11] The combination of isoelectric focusing in the two dimensional gel (2D-gel) format has made SDS-PAGE a key tool for protein research. In SDS-PAGE, proteins are separated by size on cross-linked polyacrylamide slab gels with relatively low electric fields (10 ~ 30 V/cm). Electrophoresing SDS-protein complexes through a sieving matrix allows separation of species exclusively on the basis of size since denaturing and reducing proteins in the presence of SDS results in similar charge-to-mass characteristics for most proteins. SDS-PAGE has the advantage of simultaneously analyzing multiple samples on one gel. However, the process is slow and labor intensive due to the requirement of manual preparation of gel, low separation voltage, and post-separation staining of the proteins. In an effort to address these drawbacks, SDS-PAGE has been adapted for use in capillary electrophoresis (CE).[12-14] CE has been proven to have numerous advantages including high speed separation, high separation efficiency, and enhanced resolution over slab-gel electrophoresis (SGE). CE is also feasible for simultaneous multiple operation by using capillary array or parallel channels in a microchip.

The development of separation medium is one of the most important factors for analyte separation by CE since migration behavior and resolution of species are determined by a sieving matrix. A number of different polymers such as cellulose derivatives including methyl cellulose (MC),[15] hydroxyethyl cellulose (HEC),[16] and hydroxypropylmethyl cellulose (HPMC),[17] linear polyacrylamide (LPA),[18] poly-*N*,*N*-dimethylacrylamide (PDMA),[19] polyvinyl pyrrolidone (PVP),[20] polyvinyl alcohol (PVA),[21] and polyethylene oxide (PEO)[22] have been employed. Most of the polymers require the capillary wall-coating, resulting in the problems associated with capillary fouling, coating inhomogeneity, and limited lifetime. To overcome these difficulties, the developments on the separation matrix have focused on the combination of good separation efficiency with dynamic coating and low viscosity for high throughput analysis. For example, mannitol added poly-*N*-isopropylacrylamide (PNIPAM),[23] and gold nanoparticle (GNP)[24,25] showed the potential for this purpose. However, it turned out that mannitol-added PNIPAM had a little dynamic coating ability for the reproducible DNA separation. In the case of GNP, the preparation of gold nanoparticle took long (> 4 h) and the cost was high for nanoparticle production.

Here, we have developed the sieving matrix containing ytterbium oxide nanoparticles (YbNP) mixed with PEO. Since these nanoparticle and PEO are commercially available, the preparation of the matrix was relatively cheap and took less than 30 min. To our knowledge, this is the first approach for the size-dependent separation of proteins using the sieving matrix containing ytterbium oxide nanoparticle.

In general, the conformation of proteins is close to globular shape, which is not feasible for the size-dependent separation. Thus, protein is needed to be unfolded and become linear. It is known that from the intrinsic viscosity studies, SDS induces the conformational change of protein at monomer concentrations higher than 0.1 mM.[26] SDS-protein complex is not globular, but rather a rod-like extended polypeptide chain containing a significant degree of order. The binding was thought to be caused by primarily hydrophobic interaction, and the unit rod length was measured as 7.4 nm.[27] Most proteins bind SDS at the ratio of 1 : 1.4 (mol/mol) and thus have almost identical charge-to-mass ratios.[28] Under this condition, it has been reported that solution behavior of SDS-protein complex is a unique function of the polypeptide chain length. Figure 1(A) shows the electropherogram of six protein standards (α-lactalbumin, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b, and β-galactosidase) without SDS in the buffer. Not even partial separation of protein standards was obtained and this could be attributed to their similar electrophoretic mobilities probably due to their globular shape. From

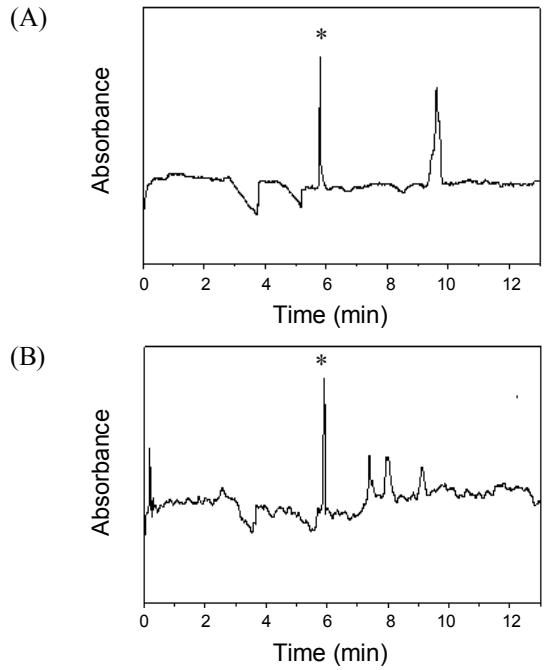

Figure 1. Effect of SDS and DTT on the protein separation. 1.5% PEO (Mw = 600,000) in 100 mM Tris, 100 mM CHES buffer (pH 8.7); electric field strength, 300 V/cm; electrokinetic injection at 6 kV for 20 s; capillary: 75 μm i.d., 40 cm total length, 30 cm effective length; UV detection at 214 nm. (A) Without SDS and DTT, (B) With SDS but, without DTT in protein sample. Peak assignment, * = orange G (reference marker), 1 = α-lactalbumin (14.2 kDa), 2 = carbonic anhydrase (29.0 kDa), 3 = ovalbumin (45.0 kDa), 4 = bovine serum albumin (66.0 kDa), 5 = phosphorylase B (97.0 kDa), and 6 = β-galactosidase (116.0 kDa).

the experiment, it was confirmed that not only SDS but also dithiothretol (DTT) was required for the size-dependent separation of proteins. DTT is known to reduce the disulfide bridge of proteins and produces perfect linear type polypeptide chain.[29] In Figure 1(B), only partial separation of protein mixture was observed when only SDS was added in the buffer.

Figure 2 shows the separation of SDS-protein complex at different YbNP concentrations with the buffer containing 0.5% PEO. Better separation efficiency of protein mixture compared to that in Figure 1 was obtained with the buffer containing SDS, DTT, and low concentration of PEO as shown in Figure 2(A). With this low concentration of PEO (0.5%, M_r = 600,000), it is believed that the separation is caused by the transient entanglement mechanism (a rod-like molecules drag the polymer along as they are encountered during migration).[16] As the concentration of YbNP increased, highest resolution was observed at 0.005% YbNP with 0.5% PEO as shown in Figure 2(C). It is known that the interaction of nanoparticle and polymer causes the limited movement of both nanoparticle and polymer, leading to the formation of immobilized and restricted mobility regions around the nanoparticle (known as the formation of nanoparticle-polymer composite).[30] From the experiment, it is obvious that the effective sieving network was formed for SDS-protein complex separation at these nanoparticle and polymer concentrations.

An interesting fact was that the migration behavior of SDS-

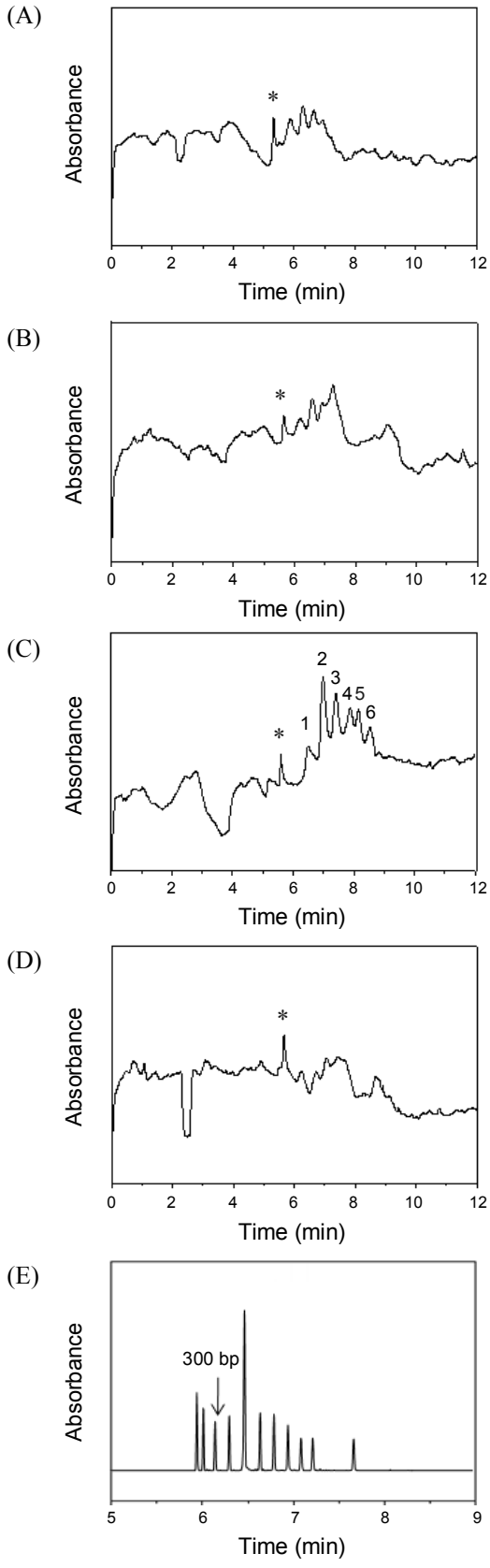

Figure 2. Effect of ytterbium nanoparticle (YbNP) on the SDS-protein separation. 0.5% PEO (Mw = 600,000) in 100 mM Tris, 100 mM CHES buffer (pH 8.7, 0.1% SDS); other conditions were the same as in Fig. 1. (A) No YbNP, (B) 0.0025% YbNP, (C) 0.005% YbNP, (D) 0.01% YbNP, (E) 100 bp dsDNA ladder, 0.02% YbNP in 0.06% PEO.

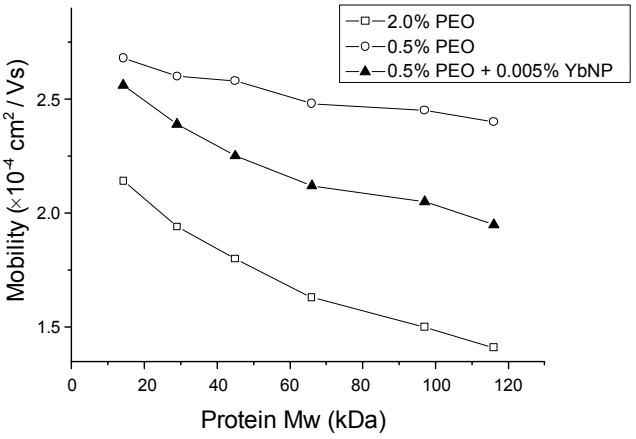

Figure 3. Mobilities of SDS-protein complex for 2.0% PEO, 0.5% PEO, and 0.5% PEO + 0.005% YbNP.

protein complex and double-stranded DNA (dsDNA) was somewhat different. As shown in Figure 2(E), under optimized concentrations of nanoparticle and PEO, better separation efficiency was obtained with dsDNA sample.[31] For example, the numbers of theoretical plate were 4.8×10^5/m and 5.1×10^6/m for SDS-BSA and 300 bp dsDNA, respectively. For calculation, the ratio of 1 : 1.4 for protein and SDS binding was applied, and the resultant molecular weight of each BSA-SDS chain was close to that of 300 bp dsDNA. The different migration behavior could be attributed to the different degree of flexibility between SDS-protein complex and dsDNA. The comparison of flexibility might be possible if the persistence length (the length which the segment of polymer shows rod-like behavior) of each chain is considered. It is known that the persistence lengths are 3 ~ 10 nm and 45 ~ 50 nm for SDS-protein complex and dsDNA, respectively.[32,33] It seems that the interaction between analyte chain and the nanoparticle-polymer composite might be weakened for more flexible SDS-protein complex.

As shown in Figure 3, polymer/nanoparticle composite is the key for improved separation of SDS-protein complex. At 0.5% concentration of PEO only, the separation was deteriorated. Interestingly, at the higher concentration of PEO at 2.0%, the resolution was somewhat improved. However, the viscosity of this solution was higher, and it can cause longer introduction time of the separation buffer into the capillary for each run (10 min *vs*. 1 min for 2.0% and 0.5% PEO, respectively). It means that the higher concentration should not be recommended for high throughput analysis and automation of the system.

YbNP nanoparticle is cheaper and easier to handle for the buffer preparation compared to other types of nanoparticles especially gold. Also, the viscosity of the nanoparticle containing sieving matrix is very low (< 15 cp), which generates the potential for automation and multiplexing for proteomics research. Integration of this sieving matrix to lab-on-a-chip system for protein study is under progress in our laboratory.

Experimental

Chemicals. Bare fused silica capillaries with 75 μm i.d. and 360 μm o.d. were purchased from Polymicro Technologies (Phoenix, AZ, USA). A detection window was prepared by burning the polyimide coating with a hot sulfuric acid. Total length of the capillary was 40 cm with the effective length of 32 cm. The platinum electrode (ϕ 0.5 mm) was obtained from Sigma-Aldrich Co. (MO, USA). Tris(hydroxymethyl)aminomethane (TRIZA base), sodium dodecyl sulfate, CHES, dithiothreitol, 2-mercaptoetheanol, hydrochloric acid, ethylenediamine-tetraacetic acid (EDTA), 100 bp dsDNA ladder and protein standard (1.0 mg/mL, α-lactalbumin, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase b, β-galactosidase) were purchased from Sigma-Aldrich Co. Polyethylene oxide (M_r = 600,000) and ytterbium oxide (Yb_2O_3) nanoparticles were also obtained from Sigma-Aldrich Co. Deionzed water (Mili-Q reagent water system, MA, USA) was used throughout the experiment.

Capillary electrophoresis with nanoparticle. A high-voltage power supply (−30 kV, Spellman, NY, USA) was used for electrophoresis with the electric field strength of 100 V/cm ~ 300 V/cm. A UV detector (Lambda 100, Bischoff, Germany) at 214 nm was employed for protein detection. The control of the high-voltage power supply and data collection at 7 Hz was performed by an in-house LabView program with an IBM compatible computer. The current in the capillary during experiment was monitored with DM-340 digital multimeter (Miltek, CA, USA). A vacuum pump (G-25S, Sinku Kiko Co., Japan) was used for the introduction of the buffer and capillary regeneration.

A sample buffer (120 mM Tris/HCl/1.0% SDS, Ph 6.6) and a running buffer (100 mM TRIZMA base/100 nM CHES/0.1% SDS, pH 8.7) were filtered once with 0.25-μm membrane filter paper (Milipore Co., MA, USA). A 2.0 μL of standard protein sample was added into a 750 μL of the sample buffer, then mixed with 750 μL of water. A 200 μL of this solution was mixed with 100 μL of the sample buffer, 5.0 μL of DTT, 10 μL of orange G (reference marker), and 85 μL of water. This mixture was vortexed for 5 min, stored at 100 °C for 10 min, then cooled at 0 °C in the ice bath for 3 min before injection.

A stock solution of nanoparticle (0.2%, w/v) in the running buffer was prepared with ultrasonication for 30 min. Then, it was diluted for the concentration of 0.005 ~ 0.01% by the buffer containing PEO (M_r 600,000). This solution was homogeneously mixed by vigorous stirring for 4 h, then degassed by vacuum. The buffer containing nanoparticle was pushed into the capillary with the positive pressure for 1 ~ 2 min. Two glass vials for the buffer containing nanoparticle were placed on both ends of the capillary. The capillary was electrokinetically equilibrated by applying the voltage the same as the separation electric field for 10 min before sample injection. The injection for the sample was performed at 4 kV for 4 s. After each run, the capillary was flushed with water for 15 ~ 30 min, and then a new running buffer containing nanoparticle was introduced into the capillary.

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