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Polystyrene latex particles containing europium complexes prepared by miniemulsion polymerization using bovine serum albumin as a surfactant for biochemical diagnosis

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ABSTRACT

Luminescent particles have been attracting significant attention because they can be used in biochemical applications, such as detecting and imaging biomolecules. In this study, luminescent polystyrene latex particles were prepared through miniemulsion polymerization of styrene with dissolved europium complexes in the presence of bovine serum albumin (BSA) and poly(ethylene glycol) monomethoxy methacrylate as surfactants. The solubility of the europium complex in styrene has a strong effect on the yield of the particle. Europium tris(2-thenoyl trifluoroacetonate) di(tri-*n*-octyl phosphine oxide), which has a high solubility in styrene, was sufficiently incorporated into the polystyrene particles compared to europium tris(2-thenoyl trifluoroacetonate), which has a low solubility in styrene. The luminescence property of the europium complex could remain intact even after its incorporation through the miniemulsion polymerization. In the aqueous dispersion, the resulting particles could emit strong luminescence, which is a characteristic of the europium complex. The antibody fragments were covalently attached to BSA-covered particles after a reaction with a bifunctional linker, *N*-(6-maleimidocaproyloxy)succinimide. The time-resolved fluoroimmunoassay technique showed that 3.3 pg/mL of human α-fetoproteins (AFP) can be detected by using the resulting luminescent particles. An immunochromatographic assay using the resulting particles was also performed as a convenient method to qualitatively detect biomolecules. The detection limit of AFP measured by the immunochromatographic assay was determined to be 2000 pg/mL. These results revealed that the luminescent particles obtained in this study can be utilized for the highly sensitive detection of biomolecules and in vitro biochemical diagnosis.

1. Introduction

Luminescent nanoparticles have been widely used in biomedical fields for the highly sensitive detection and imaging of biomolecules (e.g., DNA and proteins) and cells due to their favorable emission properties and the facility of surface functionalization [1–9]. However, the weak photo-stability of genetically induced green fluorescent proteins (GFP) or the conventionally used organic dyes interferes with their biological applications [10]. For example, the photo-bleaching of dyes limits the spatial tracing of proteins or organelles in cells during their life span (normally from several hours to one day). Therefore, the europium β-diketonate complex is one of the most preferred luminescent probes for biological applications [10–14]. Its luminescence properties have been well described by Binnemans [15]. The europium complexes emit luminescence at around 615 nm with a narrow band width and a large Stokes shift (~270–290 nm), which can spectrally distinguish their luminescence from that emitted from coexisting molecules in biological samples. Notably, the luminescence lifetime of the europium complexes is generally up to several hundreds of nanoseconds, which is significantly longer than that of typical organic fluorescent molecules [15]. To date, Niu et al. reported highly sensitive diagnostic methods of time-resolved detection of biomolecules, low-density lipoproteins [16], and thrombin [17] by making full use of the long luminescence lifetime of the europium complexes.

Generally, most biochemical assays are performed in aqueous solutions. In aqueous environments, the luminescent emission of

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the europium complexes is strongly suppressed due to energy loss through the non-radiative activation of the $^5D_0$ state to the O—H vibration of water molecules [15]. One effective method for protecting the europium complexes from exposure to the aqueous environments and retaining their intact luminescence properties is to incorporate the europium complexes in hydrophobic polymer matrices [18]. Several useful methods for incorporating the europium complexes in polymer particles have been reported in recent years [19–21]. Among them, miniemulsion polymerization is the most effective technique for incorporating the europium complexes within the polymer particles [22,23].

In this study, polystyrene latex particles containing europium β-diketonate complexes were prepared by miniemulsion polymerization. The chemical structures of the europium complexes and the schematic structure of the polystyrene particles are illustrated in Fig. 1a and b, respectively. One of the most significant features of this study is the use of bovine serum albumin (BSA) as a surfactant for miniemulsion polymerization. Because the amphiphilic protein BSA is known to adsorb onto hydrophobic surfaces non-specifically, the addition of BSA to the miniemulsion polymerization system is expected to stabilize the monomer droplets, leading to the formation of BSA-coated polystyrene latex particles. In addition, functional groups (e.g., amino, carboxyl, or thiol groups) included in the peptide sequence of BSA can be utilized for further surface functionalization of the resulting particles. These functional groups in BSA can form a covalent bond with commercially available multifunctional reagents containing maleimide, succinimide, thiols, aldehydes, etc. In the current study, a bifunctional reagent was used as a linker between the antibody fragments and BSA adsorbed on the luminescent polystyrene particles (Fig. 1b). We investigated the practical utility of the luminescent polystyrene particles modified with antibody fragments using a time-resolved fluoroimmunoassay (TRFIA) and an immunochromatography assay (ICA).

2. Materials and methods

2.1. Materials

Styrene (St. Kanto Chemical) was distilled under reduced pressure prior to use. The polystyrene hydrophobe was prepared by conventional radical polymerization using α,α'-azobisisobutyronitrile in THF. Bovine serum albumin (BSA; Mitsubishi Chemical Medience, Corp.) and poly(ethylene glycol) monomethoxy methacrylate ($M(PEG_{23})$); Shin-Nakamura Chemical Industry were used as received. L-Ascorbic acid (AscA; Kanto Chemical) and 34.5% hydrogen peroxide (H_2O_2; Kanto Chemical), which was used as a redox initiator, were used without purification. Europium tris(2-thenoyl trifluoroacetonate) [$Eu(TTA)_3$] and europium tris(2-thenoyl trifluoroacetonate) di(tri-*n*-octyl phosphine oxide) [$Eu(TTA)_3(TOPO)_2$] were prepared according to previously reported procedures [8,9]. The rabbit polyclonal anti-human α-fetoprotein (AFP) IgG antibody (Nippon Bio-Test Laboratories (NBLi)), rabbit monoclonal anti-human AFP IgG antibody (NBLi), anti-(human AFP rabbit IgG) polyclonal IgG antibody (NBLi), *N*-(6-maleimidocaproyloxy)succinimide (EMCS; Dojindo Laboratories), and 2-aminoethanethiol hydrochloride (AET; Tokyo Chemical Industry) were used as received. The compositions of the buffer solutions used in the immunoassays are listed in Table S1. A Pierce Micro BCA assay kit (Micro BCA Reagent A(MA), Micro BCA Reagent B(MB), and Micro BCA Reagent C(MC)) was purchased from Thermo Fisher Scientific and was used as received.

2.2. Methods

2.2.1. Preparation of the luminescent particles

The desired amount of europium complex ($Eu(TTA)_3$ or $Eu(TTA)_3(TOPO)_2$) and 0.104 g of PSt were dissolved in 1.04 g of St in a 12-mL glass vial. In another glass vial, 0.27 g of $M(PEG_{23})$, 0.061 g of BSA, and 0.017 g of AscA were dissolved in 5 mL of deionized water. The monomer solution and the aqueous solution were mixed with a magnetic stirrer for 20 min at room temperature. The miniemulsions were prepared with an ultrasonic homogenizer (UH-300, SMT) for 15 min in an ice bath. Argon gas was bubbled for 15 min to remove the oxygen from the miniemulsions. Polymerization was initiated by the addition of 0.12 g of H_2O_2, and then the emulsion was stirred at room temperature for 6 h. The conversion of St was gravimetrically determined. Briefly, a certain amount of the latices was lyophilized to remove the unreacted reagents, and the conversions were calculated from the weight ratio of samples before and after lyophilization. The obtained latices were purified by dialysis through a hollow fiber filter (SPECTRUM P/N M10S260-01P, pore-size = 50 nm, Spectrum Laboratories) for more than 1 h. The amount of BSA adsorbed on the particles was measured using the micro BCA assay. The purified latices (0.05 wt%, 2.0 mL) were mixed with the micro BCA reagent (5.0 mL) and incubated at 60 °C for 1 h. The absorbance (at 560 nm) of the filtrate, which was obtained by passing the solution through a membrane filter, was measured by a Tecan Sunrise microplate reader.

2.2.2. Preparation of the antibody fragments

The anti-human AFP rabbit monoclonal IgG was treated with pepsin to cleave the crystallizable fragment (Fc) of IgG into the small subfragment $F(ab')_2$ consisting of two antigen-binding sites connected with a disulfide bond. One milliliter of acetate buffer (0.1 M, pH 4.5) was added to 1.5 mL of the anti-human AFP rabbit monoclonal IgG solution. The resulting IgG solution was desalted by chromatography using a small column filled with 8.3 mL of Sephadex G-25 (GE Healthcare), and acetate buffer (0.1 M, pH 4.5) was used as an eluent. Pepsin dissolved in acetate buffer (90.6 μL, 3.2 mg/mL) was added to 0.9 mL of the IgG solution (11.7 mg/mL) and was shaken for 20 h at 37 °C. The pepsin-treated mixture was purified by chromatography with 106 mL of Sephacryl S-200 (GE Healthcare) using acetate buffer (50 mM, pH 5.0) as an eluent. The fractions containing $(Fab')_2$ were determined from the molecular weight of the lysate using gel electrophoresis. The concentration of $(Fab')_2$ in the solution was calculated by measuring the absorbance at 280 nm. F(ab') was obtained by a reductive reaction of $F(ab')_2$. An AET aqueous solution (20 mg/mL, 71 μL) was added to 1.0 mL of the $F(ab')_2$ solution and stirred at 37 °C for 90 min. The mixture was purified by chromatography with 8.5 mL of Sephadex G-25 using 50 mM phosphate buffer containing *N,N,N',N'*-ethylenediamine tetraacetic acid (pH 7.0) as an eluent. The resulting F(ab') was used immediately after purification because the thiol groups of F(ab') easily retune themselves to $F(ab')_2$ by forming a disulfide bond.

2.2.3. Surface modification of the luminescent particles with the antibody fragments

A schematic of the procedure for the surface modification of the particles with F(ab') is illustrated in Fig. 1b. ET(8.8) was chosen as a proper sample because it displayed good dispersion stability and high luminescence efficiency. The dispersion medium of ET(8.8) was exchanged with phosphate buffer (0.1 M, pH 7.0) by dialysis. To introduce maleimide groups on ET(8.8), 75.4 μL of an EMCS/DMF solution (15 mM) was added to 1.6 mL of ET(8.8) (5.2 mg/mL) and stirred for 1 h at room temperature. After dialysis of the resulting mixture against 2 L of phosphate buffer (0.1 M, pH 7.0) through a cellulose tube (MWCO: 12–14 kDa), 955 μL of the