Transactions of the Materials Research Society of Japan 33[3] 795-798 (2008)

Preparation of PEGylated upconversion nanophosphors with high dispersion stability under physiological conditions for near-infrared bioimaging

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Infrared-to-visible upconversion phosphors, erbium ion-doped Y_2O_3 (Y_2O_3:Er) nanoparticles (YNP) were synthesized by homogeneous precipitation method. Since the charge on the YNP surface is positive under physiological conditions, the YNP surface was electrostatically PEGylated using a negatively charged poly(ethylene glycol)-*b*-poly(acrylic acid) (PEG-*b*-PAAc). The surface charge on PEGylated YNP (PEG-YNP) was effectively shielded by the adsorbed PEG-*b*-PAAc. The dispersion stability of the YNP was significantly increased by PEGylation. For example, the PEG-YNP dispersed over 1 week under physiological conditions, which is probably due to the steric repulsion by PEG chains on the YNP surface. Using bovine serum albumin (BSA) as a model antigen, protein-installed YNP was prepared. Both BSA and PEG-*b*-PAAc were added to YNP solution to co-immobilize on the YNP surface (PEG/BSA-YNP). The PEG/BSA-YNP thus prepared specifically recognized anti-BSA antibody and emitted strong upconversion luminescence by near-infrared excitation. The obtained PEG-YNP is promising as a high performance near-infrared bioimaging materials.

Key words: PEG tethered chains, Infrared-excited bioimaging, Upconversion, Bionanosphere

INTRODUCTION

Fluorescence bioimaging is a technique to visualize phenomena in biosystem. Recently, this technology has been widely applied for real time imaging of biological reaction or intracellular kinetics. It is further improving to multicolor imaging visualization simultaneously *in vivo*[1]. The most popular materials for bioimaging are organic dyes and fluorescence protein[2]. However, there are some drawbacks such as easy photobleaching, short stokes shift, and broad absorption and emission bands, which often cause lowering in sensitivity. Recently, quantum dots (QDs) has much attention in this field[3]. QDs solve many problems of organic dyes and fluorescence protein. Several advantages of the QDs as bioimaging materials are tunable fluorescence wavelength by quantum size effect, sharp fluorescent peak, and strong and long time emission[4]. Since these bioimaging materials have to use ultraviolet (UV) light for excitation source, however, UV damages on the biosystem of observation object should be concerned. Therefore, these bioimaging materials can not be used for long time observation. In addition, biomolecules in the observation object generate self-fluorescence under UV excitation and thereby cause an elevated background signal impairing assay sensitivity. It is also serious issue that the UV excitation source is difficult to penetrate in the deep part *in vivo*[5),6)].

In order to solve these problems, infrared-to-visible upconversion luminescence phenomena for bioimaging is intrigued[7)-10)]. It is well known that the low-phonon energy inorganic ceramics NPs containing rare earth ion exhibit the upconversion luminescence (upconversion NP), where the rare earth emits visible light under near-infrared (NIR) excitation by the stepwise excitations. For example, yttrium oxide (Y_2O_3) is a good host matrix of erbium (Er), which is known to show upconversion emission at 550 nm (green) and 660 nm (red) excited by 980 nm NIR excitation source, and other emission lines can be achieved by doping with selected rare-earth ion[11)].

Upconversion NPs possesses several distinct advantages, such as sharp adsorption and emission lines, long lifetimes, and superior photostability. NIR excitation light does not excite the biomolecules. Therefore, the upconversion NPs emission can be measured less subject to self-fluorescence and scattering excitation light, comparing UV excitation, NIR excitation is less

damaging for biosystem.

Although the upconversion NPs proves the principle of bioimaging, they have some drawbacks, such as nonspecific adsorption of the non targeting biomolecules on the upconversion NPs surface, and agglomeration of upconversion NPs under physiological conditions. Particularly, latter is crucial problem. One solution to fulfill the problem of dispersion stability and nonspecific adsorption of the upconversion NP under physiological conditions is the surface modification with biocompatible polymer such as poly(ethylene glycol) (PEG). PEG is well known as biocompatible polymer. The PEG chains have functions both to disperse the particles into water by steric repulsion and to prevent nonspecific adsorption. Our group has already reported several kinds of PEGylated NP for biomaterials[12), 13)]. Preliminary study for PEGylation of the upconversion NP had been done, using electrostatic layer by layer process, where upconversion NP was covered by polyanion, followed by PEG-*b*-polycation to construct PEG layer on the surface[14)].

In this study, PEG monolayer was constructed on the YNP surface by one step treatment using PEG-*b*-PAAc via an electrostatic interaction. The PEGylation conditions of the YNP and the dispersion stability under physiological conditions were evaluated in detail. In order to install the functionality of molecular recognition to the PEG-YNP, bovine serum albumin (BSA), as a model protein, and PEG-*b*-PAAc were co-immobilized on the YNP surface. The ability of specific molecular recognition of the PEG/BSA-YNP was investigated using anti-BSA antibody plate.

EXPERIMENTAL SECTION

Materials: Yttrium nitrate hexahydrate, erbium nitrate pentahydrate, and urea were purchased from Wako Pure Chemical Ind., Japan. PEG-*b*-poly(acrylic acid) (Mn = 5000/3200) was purchased from Polymer Source Inc., Canada. 2-Amino-2-hydroxymethyl-1,3-propanediol, hydrochloric acid and sodium chloride were purchased from Kanto Chemicals, Japan. Bovine Serum Albumin (BSA) was purchased from Aldrich, USA. Anti-BSA antibody was purchased from Rockland, USA. Anti-mouse antibody was purchased from Southern Biotechnology Associates, USA. Microscopical slide glass and cover glass were purchased from Matsunami Glass Ind., Japan. Methyltrichlorosilane was purchased from Acros, Belgium. Micro BCA protein assay reagent kit was purchased from Pierce Biotechnology, USA. All reagents were used as received.

Preparation of PEGylated Y_2O_3:Er nanoparticles: YNP was prepared as described in the previous report[15)]. One mg of YNP was dispersed in 1.0 mL of 10 mM, pH7.0, Tris-HCl buffer and sonicated. PEG-*b*-PAAc solution in 9.0 mL of 10 mM, pH7.0, Tris-HCl buffer (0 – 0.11 g/L) was added into the YNP dispersion. The final concentration of YNP and PEG-*b*-PAAc were 0.1 and 0 - 1.0 mg/mL, respectively. The mixture was stirred for 24 h at 4 °C. Free excess polymers in the solutions were removed by centrifugation (9.0×10^4 g, 15 min, 3 times) and the solvent was substituted to 10 mM, pH 7.4 Tris-HCl buffer, containing 150 mM NaCl.

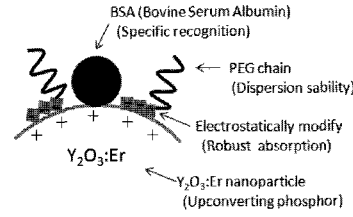

Figure 1. Illustration of PEG/BSA co-immobilized Y_2O_3:Er nanoparticles.

Characterization and evaluation of dispersion stability of the Y_2O_3:Er nanoparticles: The charge on PEGylated YNP surface was measured by ζ-potential analyzer (ZetasizerNano, Malvern, UK). The dispersion stability of the PEGylated YNP under physiological conditions (10 mM, pH7.4, Tris-HCl Buffer, 150 mM NaCl) was evaluated by a dynamic light scattering (DLS) (ZetasizerNano, Malvern, UK).

Preparation and evaluation of PEG/BSA co-immobilized Y_2O_3:Er nanoparticles: PEG/BSA co-immobilized YNP (PEG/BSA-YNP) were prepared by similar method to the PEG-YNP. YNP dissolved in 1.0 mL of 10 mM, pH7.0, Tris-HCl buffer and sonicated. PEG-*b*-PAAc and BSA were dissolved in 9.0 mL of 10 mM, pH7.0, Tris-HCl buffer and the solution was added into the YNP dispersion. The final concentration of YNP, PEG-*b*-PAAc and BSA were 0.1, 0.5 and 0.5 mg/mL, respectively. The mixture was stirred for 24 h at 4 °C. Free excess polymers and BSA in the solutions were removed by centrifugation (9.0×10^4 g, 15 min, 3 times) and the solvent was substituted to pure water. The PEG/BSA-YNP was obtained by freeze drying for 1 day. The purified PEG/BSA-YNP was redispersed in pH7.4, 10 mM Tris-HCl buffer, containing 150 mM NaCl,. The amount of the adsorbed BSA on the YNP was estimated by Micro-BCA protein assay reagent.

Specific molecular recognition of PEG/BSA-YNP was evaluated using anti-BSA antibody plate. The anti-BSA antibody plate was prepared as follows: After the slide glass was cleaned with piranha solution for 1 h, at 25 °C, it was immersed in 2 wt% methyltrichlorosilane for 30 min, followed by drying at 100 °C for 1 day to convert the glass surface to hydrophobic. One mL of the anti-BSA antibody aqueous solution (0 – 50 μg/mL) was dropped on the hydrophobic glass surface and it was dried at 4 °C for 1 day. Anti-mouse antibody plate was also prepared by the

same method (concentration of anti-mouse antibody was 50 μg/mL). Specific interaction of the prepared PEG/BSA-YNP was evaluated as follows: One mL of the PEG/BSA-YNP dispersion was dropped on the antibody plate, and retained for 30 min, followed by rinsing with 1 mL of running water for 10 times. Specific or nonspecific adsorption of the PEG/BSA-YNP was evaluated by a fluorescence microscope (IX71, OLYMPUS, Japan) equipped with a near-infrared excitation source (TCLDM9, THORLABS, USA) observation.

RESULTS AND DISCUSSION

In order to immobilize biocompatible polymer on NP surface firmly, we have to choose specific interaction between the polymer and the surface of NP. In this study, electrostatic interaction between positively charged YNP surface[16] and negatively charged PAAc segment in the PEG-*b*-PAAc was employed. To confirm the adsorption of PEG-*b*-PAAc on the YNP surface, the charge on the PEG-YNP was estimated. Figure 2 shows the ζ-potential of the PEG-YNPs. The ζ-potential of native YNP was about +20 mV at pH7.4. With increasing the [PEG-*b*-PAAc]/[YNP] ratio, the charge was decreased and reached a saturated value, about -7 mV, under the ratio more than 5, which indicates that the charge on the YNP surface was effectively shielded by the adsorbed PEG-*b*-PAAc. However, the charge on the surface of PEGylated YNP under the ratio more than 5 was slightly negative. This result implies that a part of AAc units in the PEG-*b*-PAAc was exposed on the surface to reduce the surface charge, though most of the AAc units were adsorbed on the YNP surface. The result of ζ-potential measurement indicated that [PEG-*b*-PAAc]/[YNP] ratio = 5 was enough to shield the charge on the YNP surface by the PEG-*b*-PAAc. The dispersion stability of the PEG-YNP under physiological conditions was then, investigated as a function of [PEG-*b*-PAAc]/[YNP] ratio by DLS measurement.

Figure 3 shows the relationship between the ratio of [PEG-*b*-PAAc]/[YNP] and the average diameter of PEGylated YNP under physiological conditions. Native YNP was easily agglomerated under physiological conditions, and the apparent particles size was about 1000 nm. With increasing the ratio of [PEG-*b*-PAAc]/[YNP], the particles size of PEGylated YNP was decreased and reached to a saturated value, about 200 nm with the ratio more than 5. The tendency of the average diameter with [PEG-*b*-PAAc]/[YNP] ratio was in good accordance to that of the ζ-potential. These results indicated that PEG-*b*-PAAc was effectively modified on the YNP electrostatically to improve its dispersion stability under physiological conditions.

We have then evaluated long term dispersion stability of the PEG-YNP by DLS measurement. Figure 4 shows variation of the average diameter and its distribution of the PEGylated YNP under physiological conditions as a fraction of storage time. The sample was prepared by the [PEG-*b*-PAAc]/[YNP] ratio of 5. The average

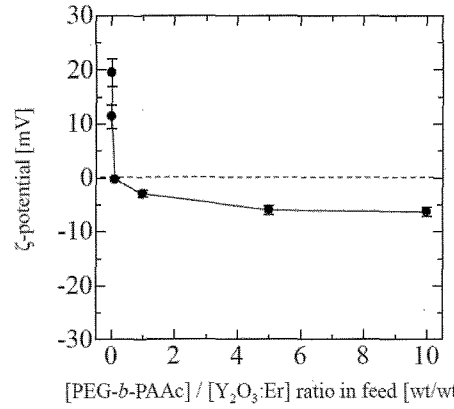

Figure 2. ζ-Potential of PEGylated Y_2O_3:Er nanoparticles depended on [PEG-*b*-PAAc]/[Y_2O_3:Er] under physiological conditions.

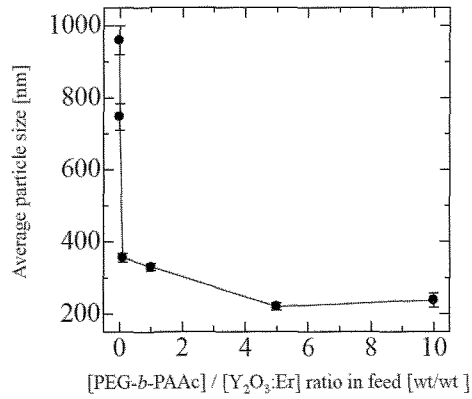

Figure 3. Average particle size of PEGylated Y_2O_3:Er nanoparticles depended on [PEG-*b*-PAAc]/[Y_2O_3:Er] ratio in feed under physiological conditions..

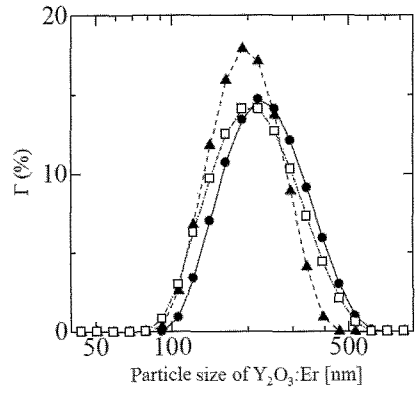

Figure 4. Variation with time of size distribution of PEGylated Y_2O_3:Er nanoparticles under physiological conditions. Closed circle, 0 day; closed triangle, after 3 days; open square, after 7 days.

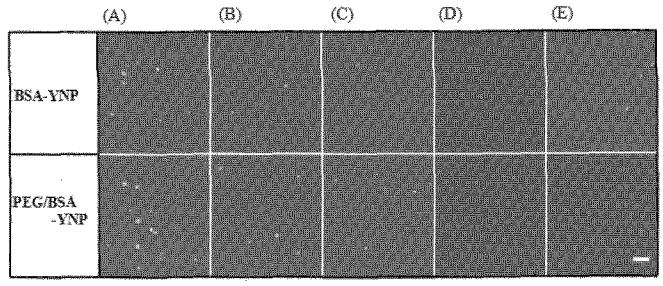

Figure.6 Upconversion luminescence of PEG/BSA-YNP and BSA-YNP on the anti-BSA antibody plate. A, B, C, and D were anti-BSA antibody plate, containing 50, 10, 1, and 0 μg of the antibody, respectively. E was anti-mouse antibody, containing 50 μg of the antibody. Scale bar: 1 μm

diameter and the distribution of the PEGylated YNP have not changed over 1 week under physiological conditions.

In order to improve further functionality to the PEG-YNP thus prepared, protein immobilization on the surface was investigated. We used BSA as a model protein for immobilization. PEG-*b*-PAAc and BSA were co-immobilized on YNP surface, and the ability of specific molecular recognition of the PEG/BSA-YNP was investigated using anti-BSA antibody immobilized plate. The feed ratio of PEG/BSA-YNP ([PEG-*b*-PAAc]/[BSA]/[YNP]) was 5 / 5 / 1 (wt %). As a control, only BSA without PEG-*b*-PAAc was immobilized on the YNP surface (BSA-YNP) under the condition of PEG/BSA/YNP ([BSA]/[YNP]) was 5 / 1 (wt%).

The amount of BSA on the PEG/BSA-YNP surface was 8.5 (molecules/particle), which was determined by micro BCA protein assay. Figure 5 shows the results of anti-BSA antibody plate assay. BSA-YNP was detected anti-BSA antibody and emitted upconversion luminescence. The upconversion emission of BSA-YNP was not observed on the bare hydrophobic treated glass plate. However, it was observed on the anti-mouse antibody immobilized plate, *viz*, the BSA-YNP caused nonspecific adsorption. Furthermore, BSA-YNP was agglomerated under physiological conditions, which the particles size of BSA-YNP was about 800 nm just after preparation (data not shown). These results indicated that the BSA-YNP was not suitable for bioimaging materials. On the other hand, the PEG/BSA-YNP was detected anti-BSA antibody and emitted upconversion luminescence. The upconversion emission intensity of PEG/BSA-YNP was increased with the amount of the anti-BSA antibody on the plate. The upconversion emission of the PEG/BSA-YNP was not observed both on the bare hydrophobic treated glass plate and the anti-mouse antibody plate.

From the result of BSA immobilized on the particle surface interacted with the surface antibody, avoiding the non-specific interaction by the PEG on the YNP. It is thus anticipated that the PEG/BSA-YNP thus prepared was suitable for bioimaging materials.

CONCLUSIONS

YNP was synthesized by homogeneous precipitation method. The surface of YNP was electrostatically modified by PEG-*b*-PAAc. The PEGylated YNP showed high dispersion stability under physiological conditions over 1week. Moreover, PEG-*b*-PAAc and BSA were co-immobilized on the YNP, the PEG/BSA-YNP was recognized anti-BSA antibody and emitted strong upconversion luminescence. Therefore, the PEG/BSA-YNP is promising as a high performance fluorescence bioimaging materials.

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(Recieved December 9, 2007 ; Accepted April 30, 2008)