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Plate assay by UC emission from Y_2O_3: Er under near infrared excitation

Y. Saito, K. Shimizu, M. Kamimura*, H. Furusyo*, K. Soga and Y. Nagasaki*
Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510
Fax: 81-04-7124-1526, e-mail: luv-sick1118@sogalabo.jp
*University of Tsukuba, Ten-Noudai 1-1-1, Tsukuba, Ibaraki 305-8573
Fax: 81-029-853-5749, e-mail: nagasaki@nagalabo.jp

Upconversion (UC) phosphors are expected to be used as new photonic probes for biologically-benign systems. To apply the UC phosphors for the bio-photonic devices, chemical durability, dispersion stability in physiological saline and specific interaction with a target substance must be introduced to the UC nanophosphors. We modified the surface of Er-doped Y_2O_3: nanoparticles, one of the UC phosphors, using the bi-layer of poly(acrylic acid) (PAAc) and biotinyl-poly(ethylene glycol)-block-poly[2-(N,N-dimethyl amino)ethyl methacrylate] (biotinyl-PEG-*b*-PAMA), which has a biofunctional block possessing biotinyl group at PEG chain end. The bi-layer modification was confirmed by FT-IR and zeta potential analyses. The amount of the polymers on the surface was estimated by a TGA. The particles modified with the bi-layered polymers did not erode in highly acidic solution, while those without modification completely did at a pH=6.4. The modified particles also could be dispersed in a physiological salt solution for more than 20 h. Due to the specific interaction between biotin and avidin, plate assay experiments on plates with avidin and bovine serum albumin (BSA) resulted in a specific binding of the modified particles only with the plate with avidin.
Key words: upconversion, bioimaging, yittria, plateassay, nanoparticle

1. INTRODUCTION

Fluorescence bioimaging (FBI) implies several problems such as color fading of markers, damage to biological substances and strong scattering in the observation system due to the use of ultraviolet (UV) or visible (VIS) lights as excitation lights. Since the UC phosphors can convert near infrared (NIR) into VIS light, the UC phosphors are expected to be used as a new bioimaging probe for benign biologically system [1].

It is known that low-phonon inorganic materials containing rare earth ions exhibit the UC phenomenon. For example, yttrium oxide (Y_2O_3) is one of good host materials to be doped with several atomic % of erbium (Er), which shows UC emission at 540 nm (green) and 650 nm (red) under 980-nm laser diode excitation [2].

The bio-photonic devices are required to be chemically durable and dispersed in physiological saline. Specific interaction with a target substance is also an important requirement. A solution to fulfill these requirement is surface modification of the UC nanophosphors with biofunctional polymer such as α-acetal-poly(ethylene glycol)-block-[poly(2-(N,N-dimethylamino)ethyl methacrylate)] (acetal-PEG-*b*-PAMA) block copolymer [3, 4]. The acetal group at one end of the PEG chain in the block copolymer can be easily converted into an aldehyde group, where various ligands such as biotinyl groups can be anchored by a reductive amination reaction. The polymer replaced the end to biotin is called biotinyl-PEG-*b*-PAMA (Figure 1). The PAMA segment at the other end of the PEG chain is positively charged polyelectrolyte which gives the copolymer a high ability to be attached on a negatively charged surface of substances by multipoint adsorption. The PEG chain has functions to disperse the particles into water by steric repulsion and to prevent non-specific adsorption to non-targeting substances. The biotinyl group has the ability of specific interaction with avidin in a targeting substance [5, 6]. The biotinyl-PEG-*b*-PAMA can simultaneously achieve to fulfill the all of the above requirements for the bio-photonic devices.

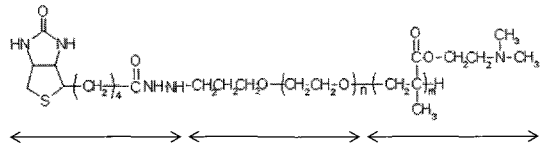

Fig.1. Structure formula of biotinyl-PEG-*b*-PAMA.

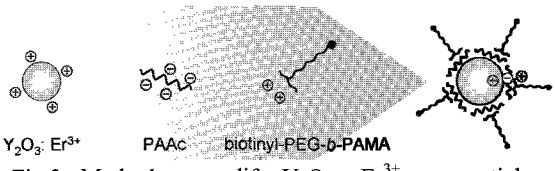

Fig.2. Method to modify Y_2O_3 : Er^{3+} nanoparticles using bi-layer of PAAc and Biotinyl-PEG-*b*-PAMA.

It is reported, however, that the isoelectric point of Y_2O_3 is 8~9 [7, 8]. In other words, the surface of Y_2O_3 particles possesses positive charge under physiological condition (pH=7.4). The positively charged PAMA segment of the block copolymer cannot firmly be electro-statically adsorbed on the Y_2O_3 particles [9]. The authors have solved this problem by inserting an intermediate layer of negatively-charged polymer, poly(acrylic acid) (PAAc) polyelectrolyte as the binder between them (Fig. 2) [10]. The objective of this study is to estimate the effects of the bi-layers modification of the Er-doped Y_2O_3 nanoparticles (Y_2O_3: Er) with the PAAc and acetal-PEG-*b*-PAMA on the above listed requirements for bio-photonic applications. We also

evaluated the specific interaction to the avidin on a plate with the particles modified with the bi-layers of the PAAc and biotinyl-PEG-*b*-PAMA.

2. EXPERIMENTAL

2.1 Sample preparation

The Y_2O_3: Er nanoparticles with 200-nm size were synthesized by a homogeneous precipitation method [11-16]. The yttrium hydroxyl carbonate precursors containing 10 atomic% of Er were precipitated from a starting aqueous solution with $Y(NO_3)_3$ and $Er(NO_3)_3$ using ammonia and carbonate ions as precipitants generated by thermal decomposing of the urea in aqueous solution at 100°C. The precipitated precursors were washed three times with distilled water and calcinated at 900°C for 30 min after drying. The particle size is approximately 200 nm, which was estimated by SEM and dynamic light scattering (DLS) analyses.

The acetal-PEG-*b*-PAMA was synthesized by ring opening anionic polymerization of ethylene oxide followed by the polymerization of 2-(N,N-dimethyl amino) ethyl methacrylate according to the procedure is described in literature [3, 4].

The molecular weight and the structure of the obtained polymer were determined by gel permeation chromatography (GPC) recorded on HLC-8120GPC (Tosoh, Tokyo, Japan), and nuclear magnetic resonance (NMR) using EX-400 (JEOL, Tokyo, Japan). In this study, the molecular weights of the PEG and polyamine segment were 5000 and 3600 respectively. The biotin was adopted to the acetal-PEG-*b*-PAMA refering the method in literature [5, 6].

To give negative charge on the positive surface of Y_2O_3: Er nanoparticles, we modified the surface with PAAc followed by the acetal-PEG-*b*-PAMA which has positively charged polyelectrolyte was adsorbed on the first layer. For the first modification, Y_2O_3 : Er nanoparticles (10 mg) were dispersed into 30 mL of 3 mg/mL PAAcNa solution at pH=7. These particles were purified by 3-fold centrifugations. For the second modification, the acetal-PEG-*b*-PAMA or biotinyl-PEG-*b*-PAMA (15 mg) was attached to the particles by the same procedure as the first modification. The concentration of PAAcNa was 3 mg/mL and that of acetal-PEG-*b*-PAMA was varied to be 0.00050, 0.0050, 0.050, 0.50, 1.25 and 2.5 mg/mL. The particles prepared in this study are abbreviated as follows : Y; unmodified Y_2O_3: Er nanoparticles, YA; Y_2O_3: Er nanoparticles singly modified with PAAc, YAP; Y_2O_3: Er nanoparticles modified with the bi-layer of PAAc and acetal-PEG-*b*-PAMA and sample YAPB; Y_2O_3: Er nanoparticles modified with the bi-layer of PAAc and biotinyl-PEG-*b*-PAMA.

2.2 Characterization

Infrared spectra were recorded on a FTIR-8300 spectrometer (Shimadzu Co., Tokyo, Japan) by a KBr pelletizing method. The zeta potential was monitored using electrophoretic light scattering spectrophotometer with a 10 mW He-Ne laser (OTSUKA ELECTRONICS Co., Tokyo, Japan).

The amount of the polymers on the surface of the particles was estimated by a TGA measurement at a heating rate of 15°C/min in air atmosphere using DTG-60H (Shimadzu Co., Tokyo, Japan). The concentration of PAAcNa was 3 mg/mL and that of acetal-PEG-*b*-PAMA was 0.50 mg/mL in the samples supplied for the analysis.

The Y_2O_3 are known to dissolve into water at a pH=6.4. The chemical disabilities of samples Y, YA and YAP were tested in acidic condition. The 10 mg of samples Y, YA and YAP were dispersed in 30 mmol/L NaOH aqueous solution with pH=11. Subsequently, 0.05 M HCl aq (3 mL) was added to measure the time dependent variation of the pH under acidic condition.

Dispersion stability of the suspensions of the samples Y, YA and YAP was estimated by measuring the time dependent transmittance. We used 10 mmol/L Tris buffer at pH=7 which contains 0.15 M NaCl as a physiological saline for forming the suspension.

Specific interaction of the samples to protein plates were performed for the samples Y, YA and YAPB. The blank plate is a hydrophobic glass slide treated with chlorotrimethylsilane. The bovine serum albumin (BSA) and avidin plates were prepared by soaking the hydrophobic glass slide into solution of BSA (3 mg/mL) and Avidin (0.5, 1, 2, 3 mg/mL), respectively. The samples were dispersed in water (0.2 mg/mL) and weeped on these plates. After 0.5 h, these plates were washed with water. The remained amount of the samples was tested by measuring the 550-nm UC emissions intensity under 980-nm LD excitation in a optical microscope.

3. RESULTS AND DISCUSSION

Using IR spectroscopy, we first confirmed adsorption of polymers on the particle surface.

As shown in Fig. 3, new absorption bands assignable to alkyl groups of PAAc were observed for sample YA, YAP. The absorption around 1000-2000 cm^{-1} shows in sample YAP are assignable to absorption band of amino (C-N) and ether (C-O-C) groups in the PAMA segment and the PEG chain, respectively. These results show that the PAAc and acetal-PEG-*b*-PAMA were firmly adsorbed on the surface of the Y_2O_3: Er nanoparticles even after three-time centrifugal purification processes.

The surface charge of the complex was then evaluated by zeta potential measurements. Figure 4 shows the relation between the concentration of the acetal-PEG-*b*-PAMA vs nanoparticle for the

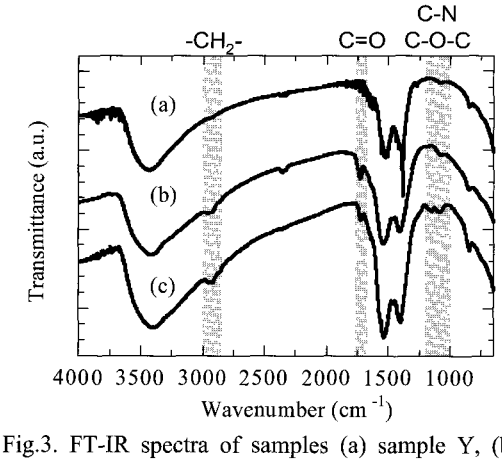

Fig.3. FT-IR spectra of samples (a) sample Y, (b) sample YA and (c) sample YAP.

modification and the zeta potential of the samples Y, YA and YAP. The zeta potential of the sample Y is positive as plotted in the figure with a closed square dot. The zeta potential changed to be negative after the modification of the particles with PAAc (sample YA) due to the existence of the carboxylic acid groups in the PAAc. The zeta potential approached to zero with increasing the acetal-PEG-*b*-PAMA concentration (sample YAP). The change in the zeta potential of the particles by the polymer modification shows that the positively charged surface of the Y_2O_3: Er particles at first covered by the negatively charged PAAc and then the negatively charged surface interacted with the positively charged PAMA end of the acetal-PEG-*b*-PAMA. Finally, the surface was neutralized due to the electrostatic interaction between the negatively charged PAAc and the PAMA end of the acetal-PEG-*b*-PAMA. The reason of the firm modification of the particles with the polymer bi-layers modification discussed above based on the FT-IR observation is the electrostatic interaction among the particle surface and the polymers. The zeta potential saturated at 0.50 mg/mL, which is the minimum required concentration of the acetal-PEG-*b*-PAMA in the treatment solution for the full coverage of the particles.

Quantitative analysis of polymers on the particle surface was carried out by TGA measurement. Figure 5 shows the results of the TGA profiles of the samples Y, YA and YAP. The weight loss of the samples YA and YAP at the temperatures higher than 450 °C were more than that of the sample Y because of the combustion of the PAAc and acetal-PEG-*b*-PAMA. The result also supports the existence of these polymers absorbed on the Y_2O_3: Er particles. The estimated densities of the

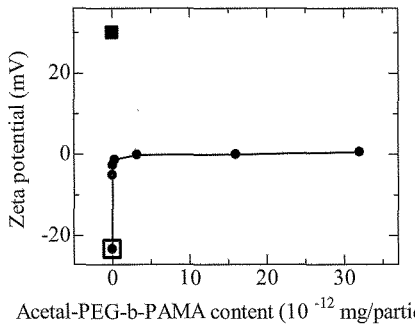

Fig.4. Zeta potential of Y (closed square dot), YA (open square dot) and YAP (closed circle and solid line) in 7.5 mM NaCl solution at 25 °C, pH=7.4.

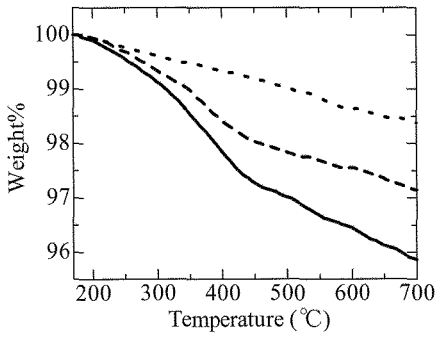

Fig.5. TGA analysis results of the samples Y (dashed line), YA (chain line) and YAP (solid line) under air flow. Programming rate is 15 °C/min.

PAAc and acetal-PEG-*b*-PAMA on the surface were to be 0.01 and 0.006 chains/nm^2, respectively.

Yttrium oxide particles are known to dissolve in an acidic solution under pH less than 6.4. Figure 6 shows the result of the chemical durability test of the unmodified and modified particles.

When Y_2O_3 is eroded under acidic condition, pH of the solution tends to increase up to 6.4. On the contrary, the change in the pH due to the dissolution of the particles delayed by the PAAc modification (sample YA). It is rather interesting to note that the sample YAP didn't dissolve for more than 20 hours under pH less than 3. This result indicates that the bi-layer modification of the Y_2O_3: Er nanoparticles increased eroding resistance under acidic conditions.

To stably disperse the ceramic nanoparticles in a physiological saline, steric repulsion by a surface polymer is inevitable since electrically dispersed nanoparticles are known to coagulate rapidly due to the shielding of electrically repulsive force in the solution with high ionic concentration. Dispersion stability of the samples was tested by measuring the time dependent transmittance of the particle suspension of the physiological saline. When the particles sediment, the transmittance of the suspension increases. As shown in Fig. 7, the sample Y sediments immediately under the physiological condition.

The PAAc can not work as a steric repulsion agent because the single modification by the PAAc does almost not change the sedimentation behavior (sample YA). Only the particles modified with the bi-layer

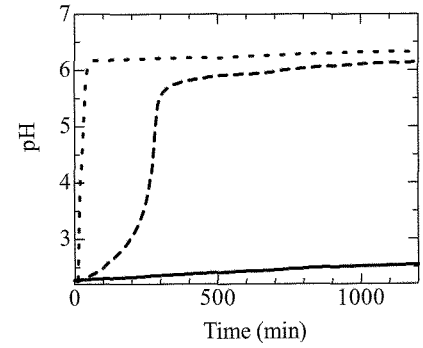

Fig.6. pH changes of the particles suspension after adding 3mL hydrochloric acid (0.05 M). Dashed line shows the sample Y. Chain and solid lines show the results of the samples YA and YAP, respectively.

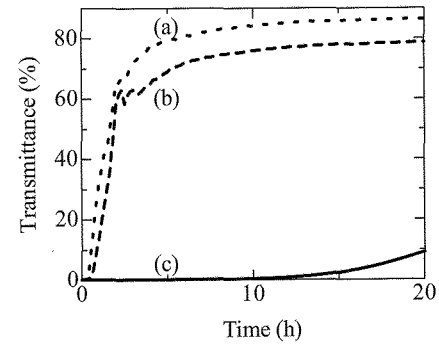

Fig.7. Time dependent transmittance of the physiological saline suspension with samples (a) Y, (b) YA and (c) YAP in physiological saline (10 mM Tris buffer / 0.15 M NaCl, pH=7.4).

polymers, sample YAP, were dispersed stably at least for 20 h, due to the steric repulsion by the acetal-PEG-*b*-PAMA created on the surface of Y_2O_3: Er nanoparticles.

To estimate the specific adsorption of the samples to a particular protein, we observed the UC emissions from the samples remained on the plates; blank, BSA and avidin plates after soaking the plates in the suspension of the samples Y, YA and YAPB. The results are shown in Fig. 8. As shown in Figs. (a) and (b), the samples Y and YA absorbed on the both BSA and avidin plates, though did not on the blank plate, indicating that the samples Y and YA could not recognize the deference of the BSA and avidin. Fig. 8 (c) shows that the sample YAPB could successfully recognize the avidin specifically. The UC emission was only observed from the avidin plate. Fig. 8 (d) shows the relation between the concentration of the avidin on the avidin plate and the UC emission intensity from the remained sample YAPB on the surface of the plate. Clear linear correlation was observed, which confirms the specific interaction between the biotinyl group on the surface of the sample YAPB and the potential use of the probes both for bioimaging and fluorescence immunoassay.

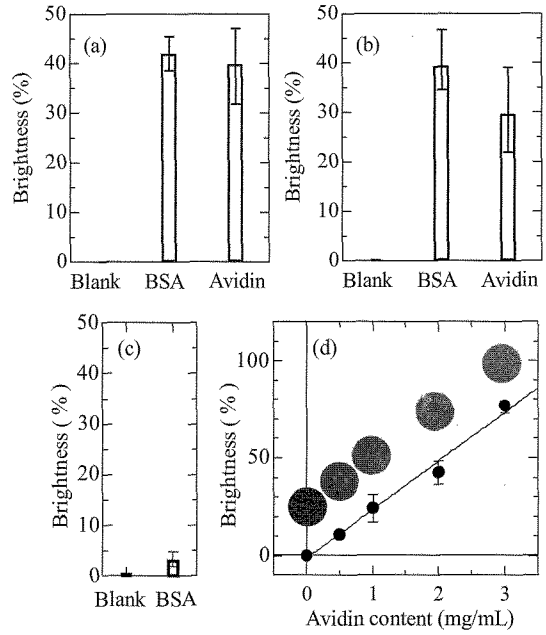

Fig.8. UC emission intensities from the blank, BSA and avidin plates soaked in sample suspension (a) Y, (b) YA and (c) YAPB. Fig. (d) show the relation between the concentration of the avidin for preparing the avidin plate and the UC emission intensity. The UC emission was measured under 980 nm LD laser excitation by monitoring 550-nm UC emission by the fluorescent microscope. The protein plates (a)-(c) are prepared with 3 mg/mL protein solution.

5. CONCLUSION

Y_2O_3: Er nanoparticles were rigidly modified with the polymer bi-layer of the PAAc and acetal-PEG-*b*-PAMA. They are stably attached on the surface of the particles even after three-time centrifugal washing with water. The chemical durability of the particles was improved by the bi-layers modification. The bi-layer coated Y_2O_3: Er was not eroded under a pH=3 for 20 h. The dispersion stability of the particles in physiological saline was also improved by the modification to be suspended for more than 10 h.

In order to install a ligand moiety, the particles were modified with the polymer bi-layers of the PAAc and biotinyl-PEG-*b*-PAMA. Due to the introduction of the biotinyl end to the PEG polymer, the particles were specifically interacted selectively to the avidin plate and did not on the blank or BSA plates. The amount of the adsorbed particles was proportional to the concentration of the avidin on the plate. The Y_2O_3: Er nanoparticles modified with the polymer bi-layers have the specific interaction with avdin and can be used for bio-photonic applications such as fluorescence immunoassay and bioimaging.

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