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ORIGINAL PAPER

Preparation and Time-Resolved Luminescence Bioassay Application of Multicolor Luminescent Lanthanide Nanoparticles

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Abstract Because highly luminescent lanthanide compounds are limited to Eu^{3+} and Tb^{3+} compounds with red (Eu, ~615 nm) and green (Tb, ~545 nm) emission colors, the development and application of time-resolved luminescence bioassay technique using lanthanide-based multicolor luminescent biolabels have rarely been investigated. In this work, a series of lanthanide complexes covalently bound silica nanoparticles with an excitation maximum wavelength at 335 nm and red, orange, yellow and green emission colors has been prepared by co-binding different molar ratios of luminescent Eu^{3+}–Tb^{3+} complexes with a ligand N,N,N^1,N^1-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo) tetrakis (acetic acid) inside the silica nanoparticles. The nanoparticles characterized by transmission electron microscopy and luminescence spectroscopy methods were used for streptavidin labeling, and time-resolved fluoroimmunoassay (TR-FIA) of human prostate-specific antigen (PSA) as well as time-resolved luminescence imaging detection of an environmental pathogen, *Giardia lamblia*. The results demonstrated the utility of the new multicolor luminescent lanthanide nanoparticles for time-resolved luminescence bioassays.

Keywords Lanthanide · Multicolor luminescent nanoparticle · Biolabeling · Time-resolved luminescence bioassay

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Introduction

In recent 20 years, time-resolved luminescence bioassay technique using lanthanide (mainly Eu^{3+} and Tb^{3+}) complexes as labels has been widely used for highly sensitive clinical diagnostics and various bioassays [1–3]. Because the luminescence of lanthanide complexes has the properties of very long lifetime, large Stokes shift and sharp emission profile, the microsecond time-resolved luminescence measurement using lanthanide complexes as the labels can effectively eliminate the short-lived background luminescence from the raw biological samples or scatterings from nearby optics, the specific long-lived signal can be selectively measured with a high signal-to-noise ratio.

Multicolor luminescence biolabeling technique entails the deliberate introduction of two or more luminescent biolabels to simultaneously monitor the functions of different biomolecules. This approach has been applied in a number of areas, such as DNA sequencing [4], luminescent microscopy bioimaging [5], and clinical diagnostics [6], with advantages of shorter assay time and lower assay cost, allowing for simplified assay protocols, decreasing sample volume required, and most importantly, making the measurement of the closely related disease markers in a sample feasible, reproducible, and reliable [7]. For luminescent lanthanide complex biolabels, although Sm^{3+}, Eu^{3+}, Tb^{3+} and Dy^{3+} complexes have been used as biolabels for multilabel time-resolved fluoroimmunoassay [8], because Sm^{3+} and Dy^{3+} complexes are weakly luminescent with shorter luminescence lifetime, only Eu^{3+} and Tb^{3+} complexes with emission maximum wavelengths at ~615 nm (Eu^{3+}complexes, red emission) and ~545 nm (Tb^{3+} complexes, green emission) are suitable to be used as

the labels for highly sensitive bioassays. This has limited the effective application of lanthanide biolabels for multiplexing time-resolved luminescence bioassays.

On the other hand, the recent advances in luminescent Eu^{3+} or Tb^{3+} complex-encapsulated nanoparticles, such as polystyrene latex particles [9–13] and silica nanoparticles [14–20], have provided new opportunities for developing luminescent lanthanide nanoparticle-based biolabels. It has been demonstrated that the luminescent lanthanide nanolabel, shielding large numbers of luminescent lanthanide complex molecules inside a coating layer, can provide a highly amplified signal as well as enhanced photostability compared with a lanthanide complex label. The nanoencapsulation technique also provides an opportunity for developing multicolor microsecond-lifetime luminescent nano-biolabels by encapsulating different ratios of highly luminescent Eu^{3+}–Tb^{3+} complexes in the nanoparticles. The bis-lanthanide ensemble consisting of a mixture of Eu^{3+}–Tb^{3+}complexes has been successfully used to generate ratiometric luminescent probes base on the different emission behaviors of Eu^{3+} and Tb^{3+} complexes [21, 22]. The immobilization of some emissive $Eu^{3+}$and Tb^{3+} complexes in a silica matrix was also demonstrated to be useful luminescent sensors for pH and oxygen [23, 24]. Recently, Zhang and coworkers demonstrated that multicolor luminescent lanthanide nanoparticles could be prepared by co-binding different molar ratios of Eu^{3+} and Tb^{3+} complexes in silica nanoparticles, and investigated their application to time-resolved fluoroimmunoassay [25]. However, because these multicolor luminescent nanoparticles require deep UV excitation with peak excitation wavelength of ~280 nm, their practical utility as biolabels for time-resolved luminescence bioassay is unsatisfactory.

In the present work, a series of lanthanide complexes-covalently bound silica nanoparticles with a longer excitation maximum wavelength at 335 nm and different emission colors (red, orange, yellow and green) has been prepared by co-binding different molar ratios of luminescent Eu^{3+}–Tb^{3+} complexes with a ligand N,N,N^1,N^1-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo) tetrakis (acetic acid) (PTTA-Eu^{3+}/Tb^{3+}) inside the silica nanoparticles. Characterizations by transmission electron microscopy (TEM) and fluorometric methods indicate that the nanoparticles are spherical and uniform in size with long luminescence lifetimes (>1.0 ms) and high photostabilities. To confirm the usefulness of the new nanoparticles as biolabels for time-resolved luminescence bioassays, the nanoparticle-labeled streptavidin was prepared and used for time-resolved fluoroimmunoassay (TR-FIA) of human prostate-specific antigen (PSA) as well as time-resolved luminescence imaging detection of an environmental pathogen, *Giardia lamblia*.

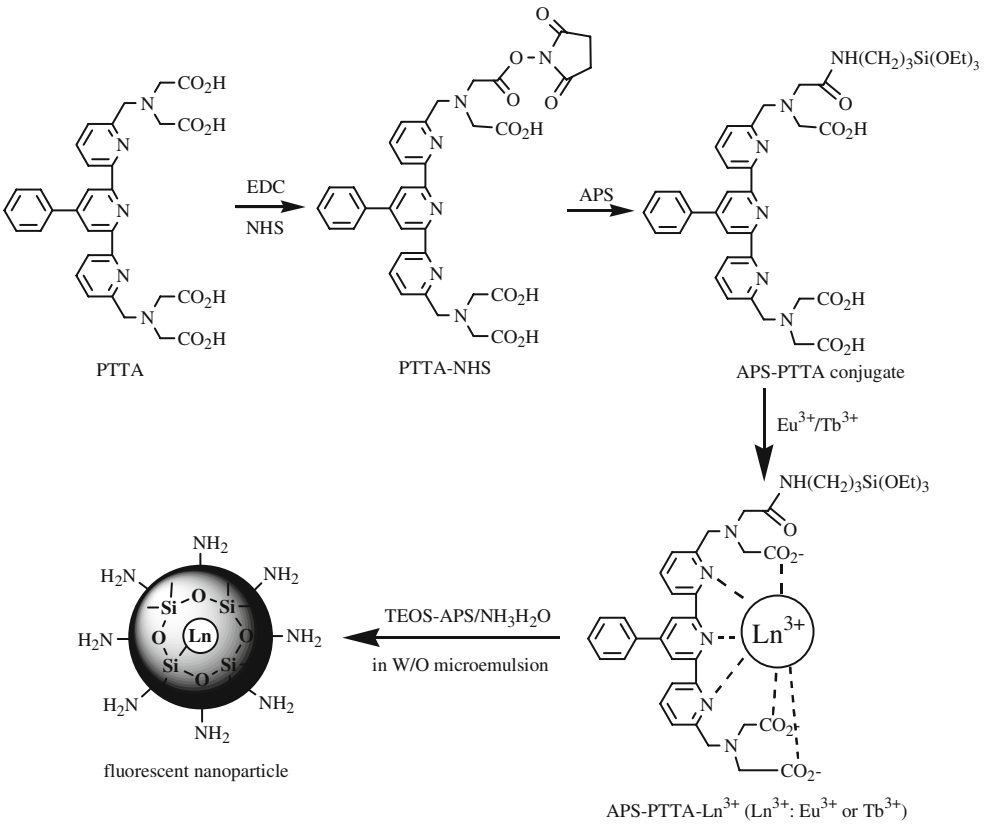
Experimental

Materials and physical measurements

The ligand, N,N,N^1,N^1-(4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl)bis(methylenenitrilo) tetrakis (acetic acid) (PTTA, structure see Scheme 1), was synthesized by using a previous method [26]. Anal. Calcd for $C_{31}H_{29}N_5O_8 \cdot 1.5H_2O$ (PTTA·1.5H_2O): C, 59.37%; H, 5.14%; N, 11.17%. Found: C, 59.49%; H, 4.91%; N,11.40%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.51 (s, 8H), 4.13 (s, 4H), 7.56–7.67 (m, 5H), 7.94 (d, J, 8.0 Hz, 2H), 8.03 (t, J, 8.0 Hz, 2H), 8.56 (d, J, 8.0 Hz, 2H), 8.70 (s, 2H), 12.46 (s, 4H). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHS), 3-aminopropyl(triethoxyl)silane (APS), tetraethyl orthosilicate (TEOS), and Triton X-100 were purchased from Acros Organics. Streptavidin (SA) was purchased from Chemicon International Inc. Mouse monoclonal and goat polyclonal anti-human PSA antibodies and rabbit anti-mouse IgG antibody were purchased from OEM Concepts Co. Biotinylated goat anti-human PSA and rabbit anti-mouse IgG antibodies were prepared and used according to a previous method [14]. *Giardia lamblia* and its mouse monoclonal antibody were purchased from Biotech Frontiers Pty. Ltd. The standard solutions of human PSA were prepared by diluting human PSA antigen (Biogenesis Ltd.) with 0.05 M Tris-HCl buffer of pH 7.8 containing 5% bovine serum albumin (BSA), 0.9% NaCl, and 0.1% NaN_3. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

The shape and size of the nanoparticles were characterized by using a JEOL JEM-2000EX transmission electron microscope. Luminescence spectra and emission lifetimes were measured on a Perkin-Elmer LS 50B spectrofluorometer. The TR-FIA of human PSA was carried out with a FluoroNunc 96-well microtiter plate as solid-phase carrier and measured on a Perkin-Elmer Victor 1420 multilabel counter with the conditions of excitation wavelength, 340 nm; emission wavelength, 615 nm; delay time, 0.2 ms; and window time (counting time), 0.4 ms. All luminescence images and time-resolved luminescence images were obtained with a laboratory-use luminescence microscope [27]. The microscope, equipped with a 100 W mercury lamp, a UV-2A filters (excitation filter, 330–380 nm; dichroic mirror, 400 nm; emission filter, >420 nm) and a color CCD camera system, was used for the normal luminescence imaging measurement with an exposure time of 500 ms. The microscope, equipped with a 30 W xenon flashlamp, UV-2A filters and a time-resolved digital black-and-white CCD camera system, was used for the time-resolved luminescence imaging measurement with the conditions of

Scheme 1 Preparation principle of the multicolor luminescent lanthanide nanoparticles



delay time, 60 μs; gate time (counting time), 1 ms; lamp pulse width, 6 μs; and exposure time, 240 s.

Preparation of the nanoparticles

To a solution of 4.6 mg EDC and 0.8 mg NHS dissolved in 80 μL of anhydrous ethanol was added a solution of 1.88 mg PTTA·1.5H_2O dissolved in 20 μL of 0.05 M sodium carbonate buffer of pH 9.5. The solution was stirred for 1 h at room temperature, and then 1.5 μL of APS was added. The reaction was continued for another 2 h, and then a 100 μL solution containing $EuCl_3$/$TbCl_3$ (total metal ion concentration is 0.03 M, molar ratio of Eu^{3+}/Tb^{3+} was 0:1, 1:5, 1:3, 1:1, 3:1, 5:1 and 1:0, respectively) was added to form the luminescent precursor (APS-PTTA-Ln^{3+} conjugate, Ln = Eu or Tb).

To a water-in-oil (W/O) microemulsion prepared by mixing 1.77 g of Triton X-100, 5.8 g of cyclohexane , 1.32 g of 1-octanol, l.5 μL of free APS and 300 μL of water was added the above precursor with stirring to make a homogenous solution. After the solution was stirred for 30 min at room temperature, 100 μL of TEOS and 60 μL of concentrated aqueous ammonia (25–28%) were added, and the reaction was allowed to continue for 24 h at room temperature. The final nanoparticles were isolated from the microemulsion by adding 20 mL of acetone, centrifuging, and washing with ethanol and water several times to remove the surfactant and unreacted materials.

Preparation of the nanoparticle-labeled SA

The nanoparticle-labeled SA was prepared according to a previous method [17]. To 1.2 mL of 0.1 M phosphate buffer of pH 7.1 containing 6 mg of BSA were added 2.0 mg of the nanoparticles and 0.3 mL of 1% glutaraldehyde. After stirring for 22 h at room temperature, 1.0 mg of $NaBH_4$ was added, and the solution was incubated for 2 h at room temperature. The nanoparticles were centrifuged and washed with the phosphate buffer and then added to 0.9 mL of the phosphate buffer containing 0.4 mg of SA. After 0.2 mL of 1% glutaraldehyde was added and the reaction mixture was stirred for 22 h at room temperature, 1.0 mg of $NaBH_4$ was added and the solution was incubated for 2 h at room temperature. The nanoparticle-labeled SA was centrifuged, washed three times with the phosphate buffer and used for luminescence imaging detection without further purification. When the nanoparticle-labeled SA was used for TR-FIA, it was further purified by gel filtration chromatography on a Sephadex G-50 column with 0.05 M NH_4HCO_3 of pH 8.0 as the eluent (3.5 mL of the nanoparticle-labeled SA solution was obtained). To the solution were added

3.5 mg NaN_3 and 7.0 mg BSA. The nanoparticle-labeled SA solution was stored at 4 °C and diluted 20 times with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl before use for TR-FIA.

TR-FIA of human PSA

The SA labeled by pure PTTA-Eu^{3+}-nanoparticles (Eu:Tb = 1:0) was used for TR-FIA of human PSA. The procedure is as follows. After anti-human PSA monoclonal antibody (diluted to 10 μg/mL with 0.1 M carbonate buffer of pH 9.6) was coated on the wells (50 μL/well) of a 96-well microtiter plate by physical absorption, 50 μL of human PSA standard solution was added to the well. The plate was incubated at 37 °C for 1 h and washed twice with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.05% Tween 20 and once with 0.05 M Tris-HCl buffer of pH 7.8. The biotinylated goat anti-human PSA antibody was added to each well, and the plate was incubated at 37 °C for 1 h. After the wells were washed, the nanoparticle-labeled SA was added to each well, and the plate was incubated at 37 °C for 1 h. The plate was washed four times with 0.05 M Tris-HCl buffer of pH 7.8 containing 0.05% Tween 20, and subjected to solid-phase time-resolved luminescence measurement on the Perkin-Elmer Victor 1420 multilabel counter.

Luminescence imaging of *Giardia lamblia*

Five μL of *Giardia lamblia* solution (10^6 cysts/mL) was mixed with 20 μL of monoclonal anti-*Giardia* antibody (50 μg/mL), 20 μL of biotinylated rabbit anti-mouse IgG antibody (50 μg/mL) and 5 μL of the nanoparticle-labeled SA (~0.5 mg/mL of the labeled SA in 0.05 M Tris-HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN_3, and 0.9% NaCl) in a tube. After incubation for 48 h at room temperature, the cysts were separated by centrifugation at 500 rpm and washed three times with distilled water to remove the unreacted nanoparticle-SA conjugate. The cysts were mixed with ~100 μL of water, and then spotted on a glass slide for luminescence microscopy imaging detection. To confirm the non-specific binding of the nanoparticles on *Giardia* cysts, a control experiment in the absence of anti-*Giardia* antibody was also carried out with the same method.

Results and discussion

The nonadentate ligand PTTA can form highly stable and luminescent complexes with Eu^{3+} and Tb^{3+} ions in aqueous media. The luminescence quantum yields and lifetimes of free PTTA-Eu^{3+} and PTTA-Tb^{3+} complexes in a borate buffer are 16%, 10% and 1.21 ms, 0.45 ms, respectively [26]. Due to the lack of an active group in PTTA, the luminescent PTTA-Ln^{3+} complexes (Ln = Eu and Tb) are difficult to be used for biolabeling and time-resolved luminescence bioassays. In this work, the luminescent PTTA-Ln^{3+} complexes were used for preparing silica-based multicolor luminescent nanoparticles. The principle of the nanoparticle preparation is shown in Scheme 1. After PTTA was covalently coupled to APS in the presence of EDC and NHS to form an APS-PTTA conjugate [25], the conjugate was

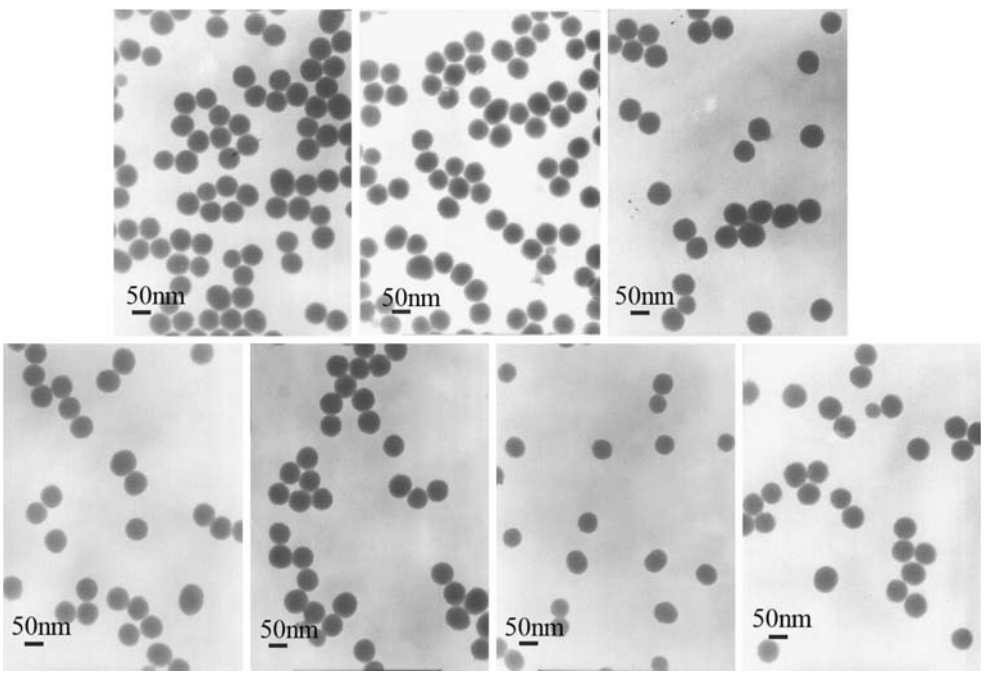


Fig. 1 TEM images of seven kinds of the nanoparticles prepared with PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratios of 0:1, 1:5, 1:3, 1:1, 3:1, 5:1 and 1:0 at 100,000× magnification

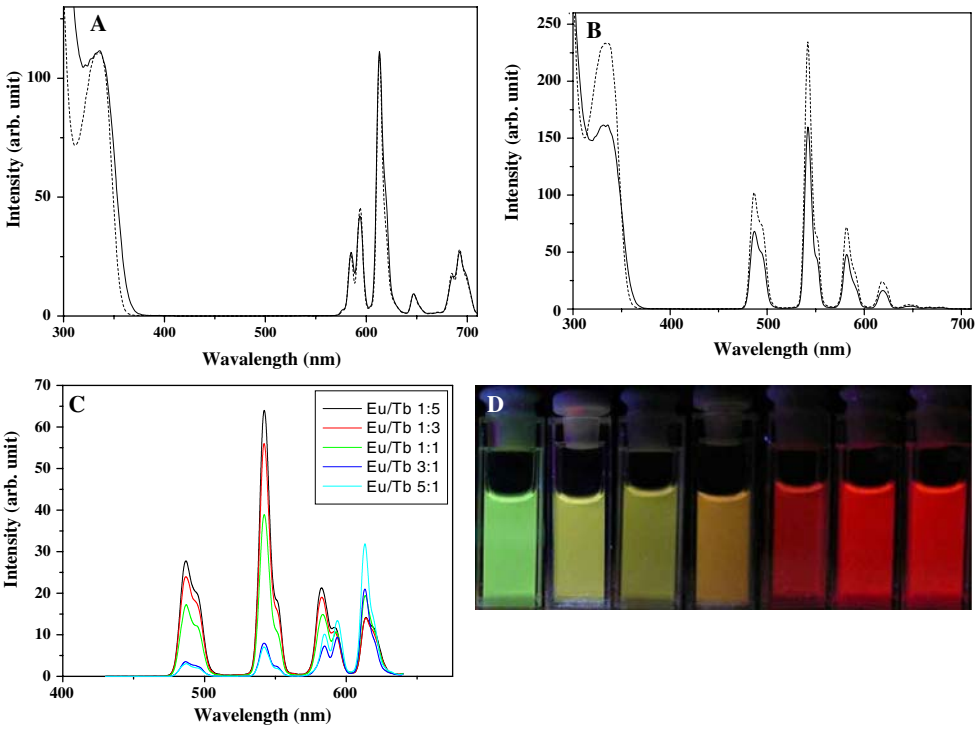


Fig. 2 Time-resolved excitation and emission spectra of **a** the PTTA-Eu^{3+}-nanoparticles (*solid lines*) and free PTTA-Eu^{3+} complex (*dash lines*) and **b** the PTTA-Tb^{3+}-nanoparticles (*solid lines*) and free PTTA-Tb^{3+} complex (*dash lines*) in 0.05 M Tris-HCl buffer of pH 7.8. **c** Emission spectra of the nanoparticles prepared with different molar ratios of PTTA-Eu^{3+}/PTTA-Tb^{3+} complexes. **d** Emission color images of the nanoparticles prepared with different molar ratios of PTTA-Eu^{3+}/PTTA-Tb^{3+} complexes in aqueous solution under irradiation of a 365 nm UV lamp (from left to right, Eu^{3+}:Tb^{3+} = 0:1, 1:5, 1:3, 1:1, 3:1, 5:1, 1:0)

further reacted with different molar ratios of $EuCl_3$/$TbCl_3$ to form the functionalized precursors (APS-PTTA-Ln^{3+}). Then a series of PTTA-Eu^{3+}/PTTA-Tb^{3+} complexes-covalently bound silica nanoparticles having different emission colors was prepared by hydrolysis copolymerization of the precursor with TEOS and APS catalyzed by aqueous ammonia in a W/O microemulsion.

The nanoparticles are characterized with TEM and spectroscopic methods. Figure 1 shows the TEM images of seven kinds of the nanoparticles prepared with PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratios of 0:1, 1:5, 1:3, 1:1, 3:1, 5:1 and 1:0, respectively. All nanoparticles show a small polydispersity, and are spherical and uniform in size, 56 nm (±4 nm of standard deviations) in diameter, without the effect of PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratio. The time-resolved luminescence excitation and emission spectra of free PTTA-Eu^{3+} complex, the PTTA-Eu^{3+}-nanoparticles (PTTA-Eu^{3+}/PTTA-Tb^{3+} = 1:0), and free PTTA-Tb^{3+} complex and the PTTA-Tb^{3+}-nanoparticles (PTTA-Eu^{3+}/PTTA-Tb^{3+} = 0:1) in 0.05 M Tris-HCl buffer of pH 7.8 are shown in Fig. 2a and b, respectively. There is no significant difference on the spectrum patterns between free lanthanide complex and the nanoparticles. The excitation and emission maximum wavelengths are 335 and 612 nm for both free PTTA-Eu^{3+} complex and the PTTA-Eu^{3+}-nanoparticles, and 335 and 542 nm for both free PTTA-Tb^{3+} complex and the PTTA-Tb^{3+}-nanoparticles, respectively. These results indicate that the excitation and emission spectra of the free lanthanide complexes in the nanoparticles are not affected by the silica networks after the complexes are covalently bound into the silica backbones. Figure 2c shows the emission spectra of the nanoparticles co-bound by different molar ratios of PTTA-Eu^{3+}–PTTA-Tb^{3+}complexes under 335 nm excitation. Because the PTTA-Eu^{3+} and PTTA-Tb^{3+} complexes in the nanoparticles have different emission spectra, the spectra of five kinds of the nanoparticles show the mixed spectra of the Eu^{3+} and Tb^{3+} complexes. With the increase of the PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratio in the nanoparticles, the emission

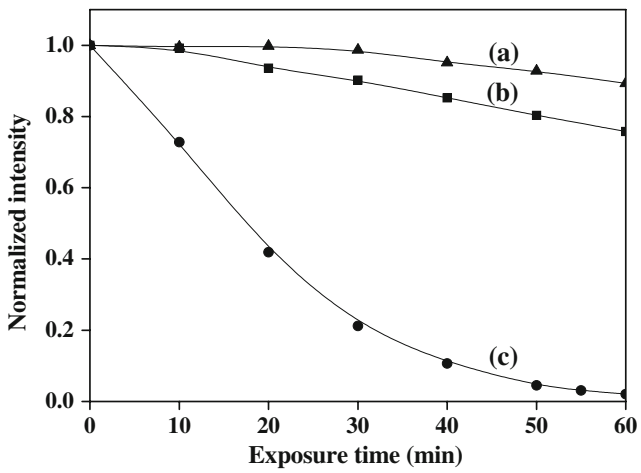


Fig. 3 Photobleaching experiments: **a** the PTTA-Eu^{3+}-nanoparticles, **b** free PTTA-Eu^{3+} complex and **c** rhodamine 6G in 0.05 M Tris-HCl buffer of pH 7.8

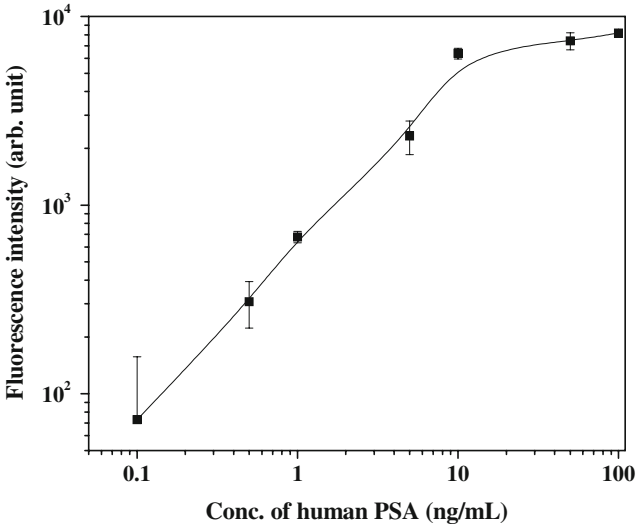


Fig. 4 Calibration curve of TR-FIA for human PSA by using the PTTA-Eu^{3+}-nanoparticle-labeled SA

intensities of the nanoparticles at 612 nm and 542 nm are increased and decreased regularly. Thus, by adjusting the molar ratio of PTTA-Eu^{3+}/PTTA-Tb^{3+} complexes in the nanoparticles, a series of luminescent nanoparticles having different emission spectra (the emission colors of these nanoparticles in aqueous solution are shown in Fig. 2d) was prepared. The emission lifetimes of the PTTA-Eu^{3+}- and PTTA-Tb^{3+}-nanoparticles in 0.05 M borate buffer of pH 9.1 were measured to be1.28 ms and 1.20 ms, respectively, indicating that the nanoparticles have long enough luminescence lifetimes for microsecond time-resolved luminescence measurement.

To evaluate the photostability of the nanoparticles, the photostabilities of free PTTA-Eu^{3+} complex, rhodamine 6G, and the PTTA-Eu^{3+}-nanoparticles in 0.05 M Tris-HCl buffer of pH 7.8 against photobleaching were measured by using a 30 W deuterium lamp as an excitation source. As shown in Fig. 3, after 1 h excitation, the emission intensities of free PTTA-Eu^{3+} complex and rhodamine 6G are 24% and 98% decreased, respectively, whereas that of the nanoparticles is only 11% decreased. This result indicates that the photostability of the luminescent nanoparticles is remarkably higher than those of the free lanthanide complexes and organic dyes, which can be attributed to the coating silica layer that shields the luminescent molecules from the outside environment, such as solvent molecules and free radicals caused by light exposure, to protect the molecules from photodecomposition.

The immunoassay of human PSA was used to confirm the reactivity of the nanoparticle-labeled SA with biotinylated antibody. Figure 4 shows the calibration curve of TR-FIA for human PSA by using the PTTA-Eu^{3+}-nanoparticle-labeled SA. The straight line in the PSA concentration range of 0.1–10 ng/mL can be expressed as log(signal) = 0.943 log[PSA] + 2.796 (r=0.997). The detection limit, defined as the concentration corresponding to 3SD (standard deviations) of back-

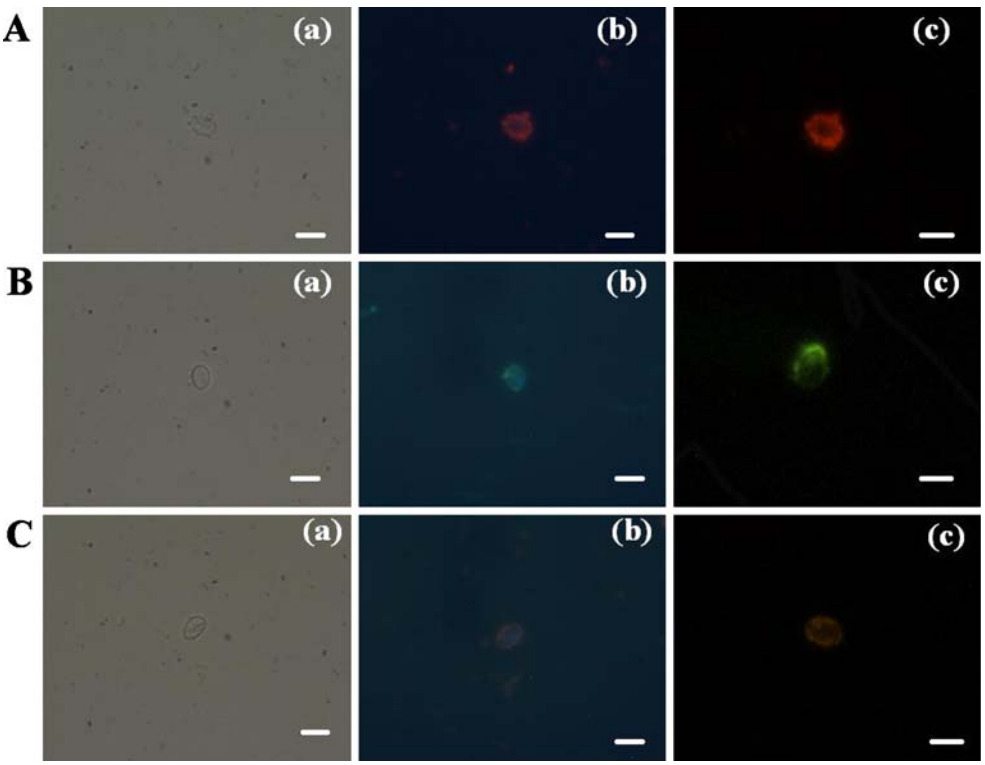


Fig. 5 Bright-field (*a*), luminescence (*b*, under excitation of 330–380 nm with exposure time of 500 ms) and time-resolved luminescence (*c*, under excitation of 330–380 nm with exposure time of 240 s) images of *Giardia lamblia* immunostained by the nanoparticle-labeled SA (PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratio of the nanoparticles: *A*, 1:0; *B*, 0:1; *C*, 1:1) in a water sample. Scale bars, 10 μm. The time-resolved luminescence images are shown in pseudo-color treated by a SimplePCI software [27]

ground signal, is 0.21 ng/mL. This result indicates that the nanoparticle-labeling process has no significant effect on the bio-reactivity of SA, and the nanoparticles-labeled SA prepared in this work can be used for reacting with biotinylated antibody.

The SA, respectively labeled by three kinds of nanoparticles with PTTA-Eu^{3+}/PTTA-Tb^{3+} molar ratios of 1:0, 0:1 and 1:1, was used for immunostaining and luminescence imaging detection of an environmental pathogen, *Giardia lamblia*, in a water sample. Before the detection, a control experiment was carried out using *Giardia* cysts incubated with biotinylated secondary antibody and the nanoparticle-labeled SA. No binding event was found for the nanoparticles onto the *Giardia* cysts surface, indicating that the non-specific absorption of the nanoparticles onto the cysts is negligible. After *Giardia* cysts were incubated with the anti-*Giardia* antibody, biotinylated secondary antibody and the nanoparticle-labeled SA, the bright-field, normal luminescence and time-resolved luminescence imaging detections of *Giardia* cysts were carried out. The results are shown in Fig. 5. It is clear that the *Giardia* cysts stained by the different nanoparticle-labeled SAs display different luminescence colors, corresponding to the luminescence colors of the PTTA-Eu^{3+}-nanoparticles (red), PTTA-Tb^{3+}-nanoparticles (green) and PTTA-Eu^{3+}/PTTA-Tb^{3+}-nanoparticles (yellow). The results of Fig. 5 also indicate that the luminescence imaging of the pathogen can be carried out with time-resolved mode, which has been demonstrated to be a powerful method to eliminate the background luminescence from non-target particles for the complicated environmental samples [20].

Conclusions

By co-binding different molar ratios of luminescent Eu^{3+}/Tb^{3+} complexes inside silica nanoparticles, a series of luminescent lanthanide nanoparticles with multi-luminescence colors has been prepared. These nanoparticles have the advantages of smaller size with uniform size distribution, strong luminescence with a longer excitation wavelength and emission lifetime, high photostability, and direct availability for biolabeling. The availability of the new nanoparticles as biolabels was demonstrated by the TR-FIA and time-resolved luminescence imaging applications. The unique luminescence properties of these nano-biolabels offer an opportunity for developing luminescent multicolor biolabeling-based time-resolved luminescence bioassay techniques, such as multi-label immunological, DNA hybridization and biochip assays.

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