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Preparation of visible-light-excited europium biolabels for time-resolved luminescence cell imaging application

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ABSTRACT

By using a visible-light-excited ternary Eu^{3+} complex, BHHBCB-Eu^{3+}-BPT (BHHBCB: 1,2-bis[4′-(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)-benzyl]-4-chlorosulfobenzene; BPT: 2-(*N*,*N*-diethylanilin-4-yl)-4,6-bis(pyrazol-1-yl)-1,3,5-triazine), as a luminophore, two kinds of novel visible-light-excited europium materials, the silica-encapsulated BHHBCB-Eu^{3+}-BPT (Eu@SiO_2) nanoparticles and BHHBCB-Eu^{3+}-BPT-conjugated bovine serum albumin (BSA–BHHBCB-Eu^{3+}-BPT), were prepared for biolabeling and time-resolved luminescence cell imaging applications. The Eu@SiO_2 nanoparticles, prepared by the copolymerization of 3-aminopropyl(triethoxy)silane–BHHBCB-Eu^{3+}-BPT conjugate, free 3-aminopropyl(triethoxy) silane and tetraethyl orthosilicate in a water-in-oil reverse microemulsion, are monodispersed, spherical and uniform in size, and strongly luminescent with an excitation peak at ~400 nm and a long luminescence lifetime of 346 μs. The BSA–BHHBCB-Eu^{3+}-BPT, prepared by covalent binding of BHHBCB-Eu^{3+}-BPT to BSA, shows also strong visible-light-excited luminescence with a excitation peak at ~400 nm and a long luminescence lifetime of 402 μs. The two materials were used for labeling transferrin and folic acid. Using the time-resolved luminescence imaging of living HeLa cells, the cell-surface receptors of transferrin and folic acid were successfully visualized by the prepared biolabels based on the ligand–receptor affinity binding interaction. The results demonstrated the feasibility of the new materials as visible-light-excited biolabels for the time-resolved luminescence cell imaging.

1. Introduction

A time-resolved (or time-gated) luminescence bioassay technique using luminescent lanthanide complexes as labels has been widely used for highly sensitive clinical diagnostics and biomedical detections [1–3]. Based on the unique luminescence properties of lanthanide biolabels, such as long luminescence lifetime, large Stokes shift and sharp emission profile, the time-resolved luminescence detection technique enables the short-lived autofluorescence and scattering light to be effectively eliminated, allowing the detection sensitivity to be increased remarkably. In recent years, the time-resolved luminescence bioimaging technique that combined time-resolved luminescence measurement with the microscopy imaging technique has achieved great success, to provide a useful tool for the visualization of biofunctional molecules and pathogens in complicated biological and environmental samples [4–11].

For applying time-resolved luminescence imaging technique to living cells, a main problem of commonly used lanthanide labels is their UV excitation wavelength, since the photo-toxicity of UV excitation light can lead to the damage to the living cell samples. To solve this problem, the most efficient method is to extend the excitation wavelength of lanthanide labels toward the visible region. However, to develop a good lanthanide biolabel that can be excited by visible light is difficult due to the requirements of high stability and solubility in aqueous media, strong luminescence with a long luminescence lifetime, and an appropriate biomolecule-coupling group for labeling biomolecules. Recently, we found that encapsulating a visible-light-excited Eu^{3+} complex into silica nanoparticles [12,13] or to conjugate the complex to bovine serum albumin (BSA) [14] was a useful approach for the preparation of visible-light-excited europium biolabels. The key requirement of this approach is that the visible-light-excited Eu^{3+} complex should be covalently bound to silane derivatives or BSA molecules.

On the other hand, for synthesizing visible-light-excited Eu^{3+} complexes, a simple and effective method is to react a β-diketonate-Eu^{3+} complex with one of 4,6-bis(pyrazol-1-yl)-1,3,5-triazine derivatives, including 2-(*N*,*N*-diethylanilin-4-yl)-4,6-bis(3,5-dimethylpyrazol-1-yl)-1,3,5-triazine (DPBT), 2-(*N*,

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N-diethylanilin-4-yl)-4,6-bis(3-methylpyrazol-1-yl)-1,3,5-triazine (MPBT) and 2-(*N*,*N*-diethylanilin-4-yl)-4,6-bis(pyrazol-1-yl)-1,3,5-triazine (BPT), to form the β-diketonate-Eu^{3+}-4,6-bis(pyrazol-1-yl)-1,3,5-triazine ternary Eu^{3+} complex [15–18], since this kind of Eu^{3+} complexes displays a unique energy transfer process from ligand to central Eu^{3+} ion with a singlet pathway mechanism to remarkably red-shift the excitation wavelength of the β-diketonate-Eu^{3+} complex [19]. Among three 4,6-bis(pyrazol-1-yl)-1,3,5-triazine derivatives, BPT is the most effective to extend the excitation wavelength of the Eu^{3+} complex when it is coordinated with a β-diketonate-Eu^{3+} complex in aqueous media [20].

In this work, a recently developed UV-excited chlorosulfonylated tetradentate β-diketonate-Eu^{3+} complex, BHHBCB-Eu^{3+} [21], was combined with BPT for the preparation of visible-light-excited europium biolabels. After binding the ternary complex BHHBCB-Eu^{3+}-BPT to 3-aminopropyl(triethoxy)silane (APS) to form a functional precursor APS–BHHBCB-Eu^{3+}-BPT, the silica-encapsulated BHHBCB-Eu^{3+}-BPT (Eu@SiO_2) nanoparticles with strong visible-light-excited luminescence were prepared by the copolymerization of APS–BHHBCB-Eu^{3+}-BPT, free APS and tetraethyl orthosilicate (TEOS) in a water-in-oil (W/O) reverse microemulsion. Furthermore, by directly coupling BHHBCB-Eu^{3+}-BPT to BSA, the water-soluble, visible-light-excited and strongly luminescent BSA–BHHBCB-Eu^{3+}-BPT conjugate was obtained. Two biomarkers, transferrin and folic acid, were labeled by Eu@SiO_2 and BSA–BHHBCB-Eu^{3+}-BPT, respectively, and their utilities for the visible-light-excited time-resolved luminescence cell imaging application were investigated. Fig. 1 shows the structure of the ternary Eu^{3+}complex BHHBCB-Eu^{3+}-BPT.

2. Experimental

2.1. Materials and methods

The Eu^{3+} ligands BHHBCB [21] and BPT [18] were synthesized according to the previously reported methods. 3-Aminopropyl-(triethoxy)silane (APS), tetraethyl orthosilicate (TEOS), and Triton X-100 were purchased from Acros Organics. Transferrin, folic acid and bovine serum albumin (BSA) were purchased from Sigma-Aldrich. Hoechst 33342 was purchased from Aladdin. HeLa cells and Chang liver cells were obtained from Dalian Medical University. D-Hanks solution [22] consisting of 4.5 g NaCl, 0.2 g KCl, 0.076 g $Na_2HPO_4 \cdot 12H_2O$, 0.03 g KH_2PO_4, 0.175 g $NaHCO_3$ and 0.5 g glucose in 500 mL water at pH 7.4 was prepared in our laboratory, and stored at 4 °C before use. Otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

The luminescence spectra were measured on a Perkin-Elmer LS 50B spectrofluorometer. The shape and size of the nanoparticles were characterized by using a JEOL JEM-2000EX transmission electron microscope (TEM). All bright-field imaging, steady-state luminescence imaging and time-resolved luminescence imaging measurements were carried out on a laboratory-use luminescence microscope [12,23]. A luminescence microscope (TE2000-E; Nikon), equipped with a 100 W Hg lamp, V-2A filters (Nikon, excitation filter, 380–420 nm; dichroic mirror, 430 nm; emission filter, >450 nm) and a cooled color CCD camera system (RET-2000R-F-CLR-12-C, Qimaging Ltd.), was used for the steady-state luminescence imaging measurement with an exposure time of 6 s. The microscope, equipped with a 30 W xenon flashlamp, V-2A filters, and a time-resolved digital black-and-white CCD camera system (Imagex-TGi, Photonic Research Systems Ltd.), was used for the time-resolved luminescence imaging measurements with the conditions of delay time, 100 μs; gate time, 1 ms; lamp pulse width, 6 μs; and exposure time, 120 s. The time-resolved luminescence images are shown in pseudo-color treated by a SimplePCI software [23].

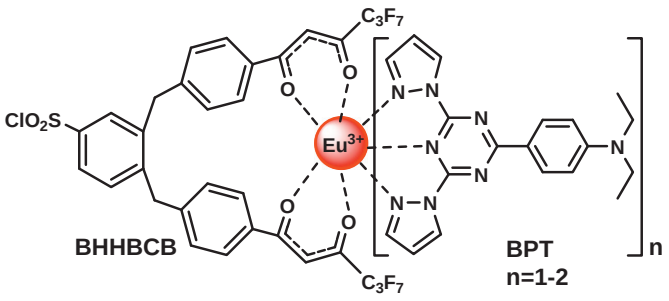

Fig. 1. Structure of the ternary Eu^{3+} complex BHHBCB-Eu^{3+}-BPT.

2.2. Preparation of Eu@SiO_2 nanoparticles

Before the nanoparticle preparation, the APS–BHHBCB-Eu^{3+}-BPT precursor was synthesized by covalent conjugating BHHBCB-Eu^{3+}-BPT to the amino group of APS with the following procedure. To a solution of 22 mg BHHBCB (25 μmol) in 4 mL of dry tetrahydrofuran (THF) was added 14.6 μL (62.5 μmol) of APS with stirring. After the solution was stirred for 3 h at room temperature, 15 mL of THF containing 17.9 mg BPT (50 μmol) and 9.6 mg $EuCl_3 \cdot 6H_2O$ (25 μmol) was added. The solution was further stirred at room temperature for 24 h. After the solvent was evaporated, 30 mL of n-hexane was added to afford a yellow powder of APS–BHHBCB-Eu^{3+}-BPT precursor having an approximate composition $[(APS–NH–SO_2–BHHBCB)Eu(BPT \cdot HCl)_2]Cl \cdot 5H_2O$. Anal. calcd (%): C, 42.25; H, 10.60; N, 4.22. Found (%): C, 42.26; H, 10.70; N, 4.30. In the composition, the two HCl molecules formed during the reactions of APS with BHHBCB and APS–NH–SO_2–BHHBCB with $EuCl_3$ are considered to bind with the diethylamino groups of the two BPT ligands. Because the (triethoxy)silane group in this precursor is not stable, further structure confirmation of the precursor was not carried out.

The Eu@SiO_2 nanoparticles were prepared by the copolymerization of APS–BHHBCB-Eu^{3+}-BPT, free APS and TEOS in a water-in-oil (W/O) reverse microemulsion consisting of 2.37 g of Triton X-100, 1.82 g of n-octanol, 7.25 g cyclohexane and 0.55 mL of water. After different amounts of APS–BHHBCB-Eu^{3+}-BPT (4.0, 6.0, 8.0 and 10.0 mg) dissolved in 1.5 mL of toluene were added into the reverse microemulsion, the solution was stirred at room temperature for 15 min, and then 100 μL of TEOS and 5 μL of APS were added. The copolymerization reaction was initiated by adding 100 μL of concentrated aqueous ammonia, and then the reaction was allowed to continue for 24 h at room temperature. The yellow solid nanoparticles were isolated from the microemulsion by adding 4 mL of ethanol, centrifuging, and washing twice with the 1:2 mixture of ethanol and water, once with the 1:4 mixture of ethanol and water, and twice with distilled water to completely remove surfactant and un-reacted materials.

2.3. Preparation of Eu@SiO_2-labeled transferrin

To 1.0 mL of 0.1 M phosphate buffer of pH 7.1 containing 5 mg of BSA were added 1.0 mg of Eu@SiO_2 nanoparticles and 0.1 mL of 1% glutaraldehyde. After stirring at room temperature for 24 h, the nanoparticles were centrifuged and washed with the phosphate buffer. The nanoparticles were re-suspended in 1.0 mL of the phosphate buffer containing 0.4 mg of transferrin, and then 0.1 mL of 1% glutaraldehyde was added. The solution was further stirred for 22 h at room temperature, and then 0.2 mg of $NaBH_4$ was added. After the solution was incubated at room temperature for 2 h, the Eu@SiO_2-labeled transferrin was centrifuged, washed

three times with the phosphate buffer, and then re-suspended in 0.5 mL of 0.1 M phosphate buffer of pH 7.1 containing 0.5 mg of NaN_3 and 1.0 mg of BSA. The Eu@SiO_2-labeled transferrin was stored at 4 °C before use.

2.4. Preparation of the BHHBCB-Eu^{3+}-BPT conjugated BSA

To 1.3 mL of 0.05 M carbonate buffer of pH 9.3 containing 7.5 mg of BSA ($\sim 1.2\times10^{-4}$ mmol) 200 μL of dimethyl sulfoxide (DMSO) containing 6.6 mg of BHHBCB (7.5×10^{-3} mmol) was added dropwise with stirring. After stirring at room temperature for 1 h, the BHHBCB-conjugated BSA was separated by gel filtration chromatography on a Sephadex G-50 column using 0.05 M $NaHCO_3$ as the eluent. The fractions containing BSA–BHHBCB conjugate were collected, and then the binding ratio of BHHBCB to BSA (the binding number of BHHBCB to a BSA molecule) was determined by the previous method [21] to be ~34.

To 2.0 mL of the above BSA–BHHBCB solution (containing $\sim 1.5\times10^{-3}$ mmol of BHHBCB) 1.8 mL of DMSO containing 1.1 mg of BPT (3.0×10^{-3} mmol) and 1.2 mg of $EuCl_3\cdot6H_2O$ (3.0×10^{-3} mmol) was added with stirring. After incubating at 52 °C for 2 h, the BSA–BHHBCB-Eu^{3+}-BPT conjugate was obtained, and stored at 4 °C before use.

2.5. Preparation of BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid

To 1.0 mL of anhydrous DMSO containing 10 mg folic acid and 200 μL triethylamine were added 5.6 mg N-hydroxysuccinimide (NHS) and 5.6 mg dicyclohexylcarbodiimide (DCC). After the solution was stirred for 4 h in darkness, a portion of 200 μL solution was added to 200 μL of the BSA–BHHBCB-Eu^{3+}-BPT conjugate solution. The solution was stirred for 10 h at room temperature, and then separated by gel filtration chromatography on a Sephadex G-75 column to remove the unreacted folic acid using 0.1 M phosphate buffer of pH 7.1 as the eluent. After adding NaN_3 (0.1%), the solution of the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid was stored at 4 °C before use.

2.6. Luminescence cell imaging applications of the labeled transferrin and folic acid

For the cell imaging application of the Eu@SiO_2-labeled transferrin, HeLa cells were cultured in a 25 cm^2 glass culture vial in RPMI-1640 medium (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Corning Inc.), 1% penicillin and 1% streptomycin (Invitrogen Co.) at 37 °C in a 5% CO_2–95% air incubator. After the culture medium was removed, a mixture of 0.2 mL of the Eu@SiO_2-labeled transferrin in phosphate buffer (2 mg/mL) and 2.0 mL of RPMI-1640 medium was added, and the cells were further incubated for 20 h at 37 °C in the incubator. The cells were washed three times with the phosphate buffer, and then subjected to the luminescence imaging measurement.

For the cell imaging application of the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid, HeLa cells were incubated in 2.0 mL of the D-Hanks solution containing 0.1 mL of the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid for 4 h at 37 °C in the 5% CO_2–95% air incubator. After the nuclei were further stained with 2.5 μM of Hoechst 33342 for 1 h, the cells were washed three times with the phosphate buffer, and then subjected to the luminescence imaging measurement. To confirm the specific binding between the labeled folic acid and the folic acid receptor of the cells, two control experiments were carried out under the same conditions without the nucleus staining step. In the first experiment, the folic acid receptor-free Chang liver cells were used instead of HeLa cells for the incubation with the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid. And in the second experiment, HeLa cells were stained by the free BSA–BHHBCB-Eu^{3+}-BPT conjugate instead of the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid.

3. Results and discussion

3.1. Preparation and characterization of Eu@SiO_2 nanoparticles and BHHBCB-Eu^{3+}-BPT conjugated BSA

The europium complex BHHBCB-Eu^{3+} is a recently developed tetradentate β-diketonate-Eu^{3+} complex [21]. Owing to the presence of chlorosulfonyl group, this complex can be easily bound to the amino-containing molecules by the formation of sulfonamide (–NH–SO_2–) for labeling these molecules. Compared to the classic biolabel of tetradentate β-diketonate-Eu^{3+} complex 4,4′-bis(1″,1″,1″,2″,2″,3″,3″-heptafluoro-4″,6″-hexanedion-6″-yl)chlorosulfo-*o*-terphenyl-Eu^{3+} (BHHCT-Eu^{3+}) [24], because two freely rotatable methylene groups are introduced into the three benzene rings of the ligand to allow the two bidentate β-diketone groups to simultaneously coordinate a Eu^{3+} ion easily, BHHBCB-Eu^{3+} exhibits higher stability, larger luminescence quantum yield and longer luminescence lifetime than those of BHHCT-Eu^{3+} [21]. However, similar to BHHCT-Eu^{3+} and other β-diketonate-Eu^{3+} complexes, BHHBCB-Eu^{3+} is also a UV-excited complex with a maximum excitation peak at 327 nm, which restricts its applicability for the UV light-sensitive living bio-samples. Because BHHBCB is only a tetradentate chelator to coordinate with a Eu^{3+} ion, the coordinative unsaturated central Eu^{3+} ion can be further coordinated by a secondary ligand, BPT, to remarkably shift the excitation wavelength of the Eu^{3+} complex to the visible light region. Thus, for preparing the visible-light-excited europium materials that can be used as biolabels, the BHHBCB-Eu^{3+}-BPT covalently doped silica nanoparticles and conjugated BSA were prepared.

Fig. 2 shows the preparation principles of the Eu@SiO_2 nanoparticles and BSA–BHHBCB-Eu^{3+}-BPT conjugate. For covalent doping BHHBCB-Eu^{3+}-BPT into silica nanoparticles, BHHBCB was preferentially bound to APS, and further reacted with BPT and Eu^{3+} in THF to form the APS–BHHBCB-Eu^{3+}-BPT precursor. In this experiment, the luminescent precursor was synthesized by reacting one APS–BHHBCB with one $EuCl_3$ and two BPT, since the coordination number of a Eu(III) ion is generally 8–10; while one BHHBCB ligand and one BPT ligand have only 7 coordination atoms, the second BPT ligand enables the Eu(III) coordination sites to be saturated. After the hydrolysis copolymerization of APS–BHHBCB-Eu^{3+}-BPT, TEOS and APS in a W/O microemulsion initiated by aqueous ammonia [12], the Eu@SiO_2 nanoparticles were obtained. In this copolymerization, the free APS was used for directly introducing primary amino groups onto the nanoparticle's surface to make bioconjugation of the nanoparticles easier. For conjugating BHHBCB-Eu^{3+}-BPT to BSA, the BHHBCB conjugated BSA was first prepared by the sulfonamide formation reaction between the chlorosulfonyl group of BHHBCB and amino groups of BSA. After reacting with Eu^{3+} and BPT in an aqueous-DMSO medium, the BSA–BHHCT-Eu^{3+}-BPT conjugate was obtained. Although this method allows some primary amino groups of BSA to be bound to the Eu^{3+} complex (~34 primary amino groups per BSA molecule were conjugated to the Eu^{3+} complex), because a BSA molecule has 59 primary amino groups [25], the residual primary amino groups in the BSA–BHHBCB-Eu^{3+}-DPBT conjugate are still available for the labeling of biomolecules [14].

Fig. 3 shows the TEM images of the Eu@SiO_2 nanoparticles prepared with different amounts of the APS–BHHBCB-Eu^{3+}-BPT precursor in the copolymerization system. When the added amount of the APS–BHHBCB-Eu^{3+}-BPT precursor was in the range

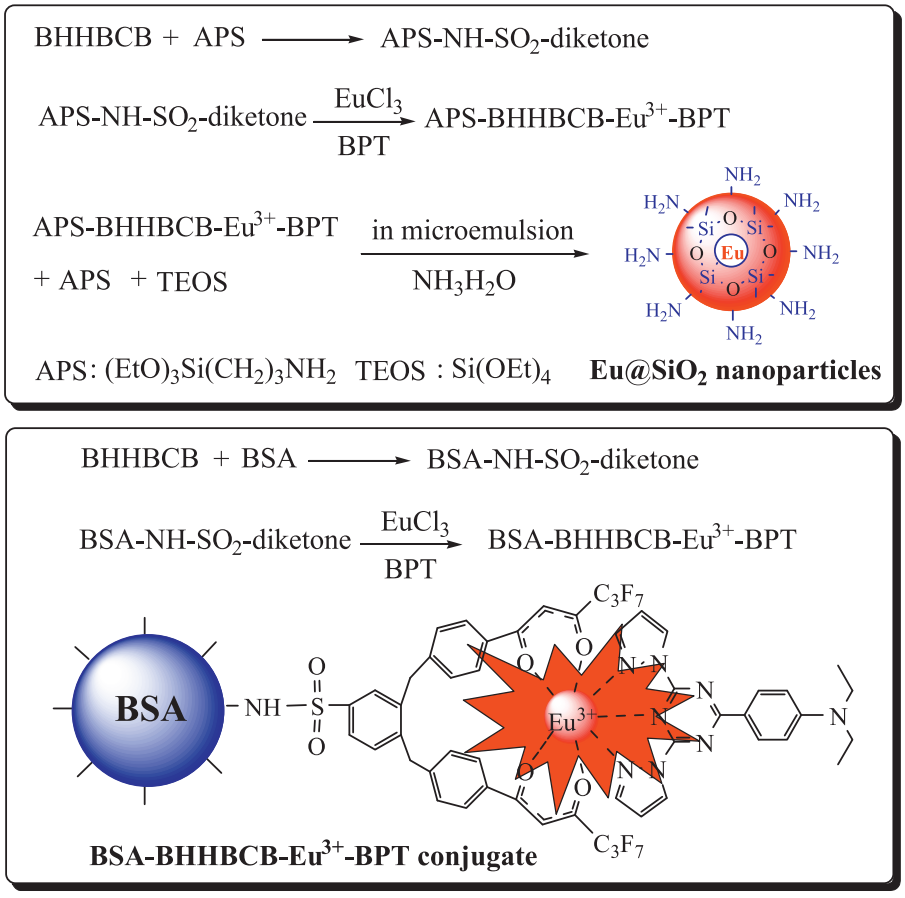

Fig. 2. Schematic diagram of the preparation principles of the Eu@SiO_2 nanoparticles (top) and the BSA–BHHBCB-Eu^{3+}-BPT conjugate (bottom).

of 4.0–8.0 mg, the nanoparticles were monodispersed, spherical and uniform, and ~40 nm in diameter. However, when the precursor amount was further increased to 10.0 mg, the nanoparticles became irregular, less smooth and conglomerate. This phenomenon might be due to the effect of bigger molecular size of the APS–BHHBCB-Eu^{3+}-BPT precursor, since the large amount of bigger APS–BHHBCB-Eu^{3+}-BPT precursor could distort the network of O–Si–O framework during the formation of nanoparticles undergoing the hydrolysis copolymerization of APS–BHHBCB-Eu^{3+}-BPT, TEOS and APS in the aqueous nanodroplets of the W/O microemulsion. Because the nanoparticles prepared with 6.0 mg of the precursor have stronger luminescence than those prepared with 4.0 mg of the precursor and better shape than those prepared with 8.0 mg of the precursor, they were selected for further characterization and bioassay application.

The time-resolved excitation and emission spectra of the Eu@SiO_2 nanoparticles and the BSA–BHHBCB-Eu^{3+}-BPT conjugate in aqueous buffer were measured to examine their visible-light-excitation and emission properties. As shown in Fig. 4, both the Eu@SiO_2 nanoparticles and the BSA–BHHBCB-Eu^{3+}-BPT conjugate showed wide excitation bands ranging from UV to visible light extending to ~475 nm with two excitation peaks at ~335 nm and ~400 nm, and a typical Eu^{3+} emission spectrum pattern with a main emission peak at 613 nm ($^5D_0 \rightarrow {}^7F_2$) and several weak side emission peaks at 574 nm ($^5D_0 \rightarrow {}^7F_0$), 586 nm ($^5D_0 \rightarrow {}^7F_1$), 647 nm ($^5D_0 \rightarrow {}^7F_3$) and 691 nm ($^5D_0 \rightarrow {}^7F_4$). There were no remarkable differences on excitation and emission spectra between the two materials. These results indicate that the ternary complex BHHBCB-Eu^{3+}-BPT can be excited by visible light ($\lambda > 400$ nm) both in silica nanoparticles and in aqueous solution, and silica matrix and water molecules do not affect the excitation and emission wavelengths of the complex. The luminescence lifetimes and quantum yields measured in 0.05 M carbonate buffer of pH 8.5 are 346 μs and ~31% for the Eu@SiO_2 nanoparticles and 402 μs and ~22% for the BSA–BHHBCB-Eu^{3+}-BPT conjugate, respectively, which demonstrate that the two kinds of materials are all highly luminescent with long enough luminescence lifetimes for the 100-μs-level time-resolved luminescence measurement application.

3.2. *Transferrin labeling and cell imaging application of the Eu@SiO_2 nanoparticles*

To evaluate the applicability of the Eu@SiO_2 nanoparticles as a visible-light-excited biolabel for time-resolved luminescence cell imaging application, the Eu@SiO_2-labeled transferrin was prepared by a BSA-coating and the glutaraldehyde-bridging method established by our group [12,26]. Describing briefly, the BSA-coated nanoparticles were firstly prepared by cross-linking the primary amino groups of the nanoparticles and BSA molecules using glutaraldehyde as a bridging reagent. After that, the BSA-coated nanoparticles were further conjugated to transferrin by cross-linking the primary amino groups of BSA and transferrin with glutaraldehyde, and the formed unstable –N=C– bonds (Schiff base bond) were reduced with $NaBH_4$, the Eu@SiO_2-labeled transferrin was obtained. This method allows a flexible BSA 'bridge' to exist between the nanoparticle and transferrin to reduce the effect of the nanoparticles on the bio-reactivity of transferrin [26].

Based on the affinity interaction of transferrin with transferrin receptor on the cell surface [27], the Eu@SiO_2-labeled transferrin stained HeLa cells were prepared by co-incubating the

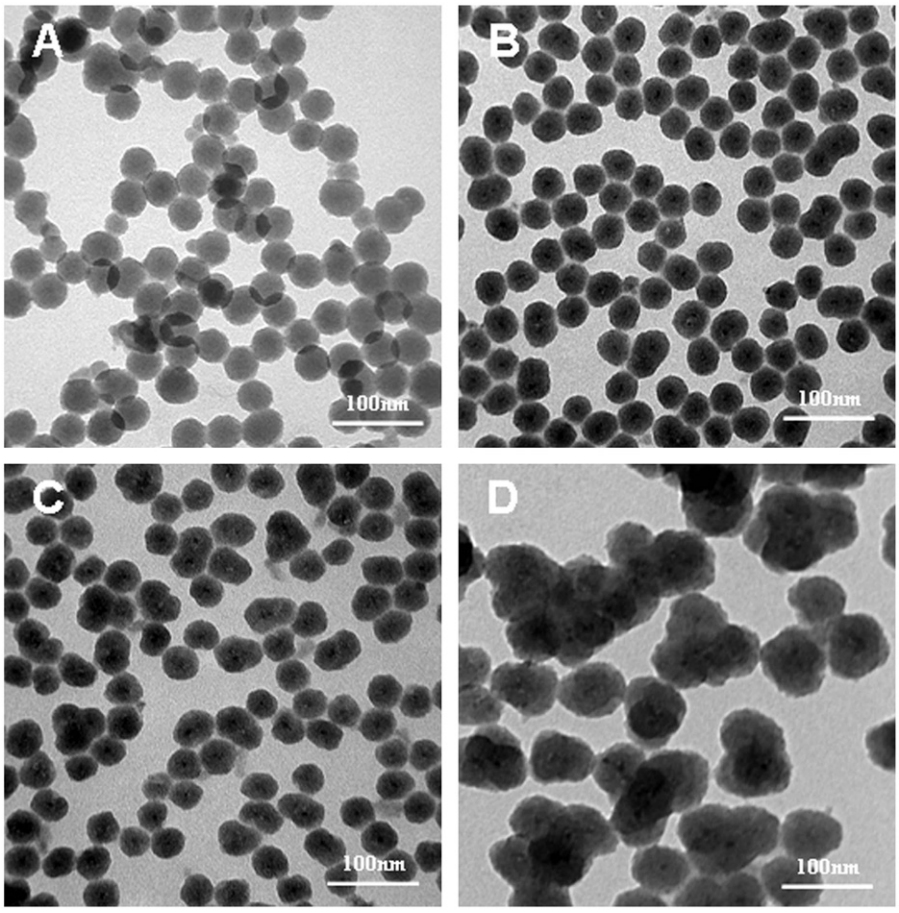

Fig. 3. TEM images of the Eu@SiO_2 nanoparticles prepared with different amounts of APS–BHHBCB-Eu^{3+}-BPT precursor (A, 4.0 mg, 40 ± 2 nm; B, 6.0 mg, 37 ± 3 nm; C, 8.0 mg, 39 ± 5 nm; and D, 10.0 mg, irregular) in the copolymerization system.

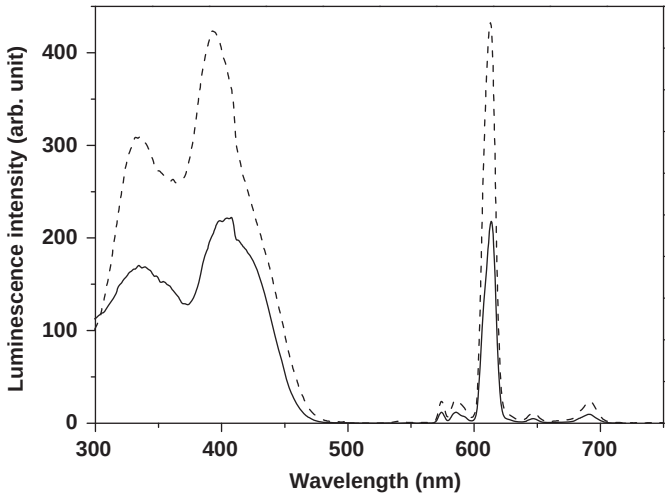

Fig. 4. Time-resolved excitation (300–500 nm) and emission (500–750 nm) spectra of the Eu@SiO_2 nanoparticles (solid lines) and the BSA–BHHBCB-Eu^{3+}-BPT conjugate (dash lines) in 0.05 M carbonate buffer of pH 8.5.

nanoparticles with the cells, and then the HeLa cells were used for the visible-light-excited luminescence imaging both in steady-state and time-resolved modes. Fig. 5 shows the bright-field, steady-state and time-resolved luminescence images of the Eu@SiO_2-labeled transferrin-stained HeLa cells with light of 380–420 nm as excitation source. In contrast to the steady-state luminescence imaging, the time-resolved mode offered highly specific and background-free luminescence cell images, because the short-lived background fluorescence from the components of cells and nanoparticles was completely suppressed by the time-resolved mode. These imaging results demonstrate that the new nanoparticles can be a useful biolabel for the visible-light-excited time-resolved luminescence cell imaging application.

3.3. Folic acid labeling and cell imaging application of the BSA–BHHBCB-Eu^{3+}-BPT conjugate

Folic acid, a vitamin required for one-carbon transfer reactions along several metabolic pathways, is essential for the biosynthesis of nucleotide bases [28], while the folic acid receptor, a glycoprotein that can strongly bind with folic acid with a dissociation constant of 10^{-9}–10^{-10} M [29], is vastly over-expressed in a wide variety of human tumors, especially in epithelial cancer cells [30]. In this work, to evaluate the applicability of the BSA–BHHBCB-Eu^{3+}-BPT conjugate as a visible-light-excited biolabel for time-resolved luminescence cell imaging application, the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid was prepared by the NHS–DCC activation method [31]. In this method, the succinimidyl ester of folic acid that can easily react with the primary amino groups of proteins to form the folic acid–protein conjugate (conjugated by amide bond) was synthesized at first by the esterification reaction between the carboxylic acid group of folic acid and *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC). After the BSA–BHHBCB-Eu^{3+}-BPT conjugate was reacted with succinimidyl ester of folic acid, the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid was obtained.

HeLa cells cultured in 25 cm^2 glass culture flask were incubated with the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid and nuclei dye, Hoechst 33342. After washing 3 times, they were used for the luminescence imaging measurements with light of 380–420 nm as excitation source. As shown in Fig. 6, the steady-state luminescence images of the cells showed only strong blue fluorescence of Hoechst 33342, while a clearly red Eu^{3+} luminescence was observed from the time-resolved luminescence images of the cells without the interference of blue fluorescence. These results not only demonstrate the powerful ability of the time-resolved luminescence imaging technique to eliminate the strong short-lived fluorescence, but also indicate that the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid is practically available for the ligand–receptor binding-based cell imaging application.

To further confirm that the red luminescence signals from the cells of Fig. 6C were attributed to the specific binding between BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid and the folic acid receptor of HeLa cells, two control experiments were conducted by using the folic acid receptor-free Chang liver cells instead of HeLa cells for incubating with the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid

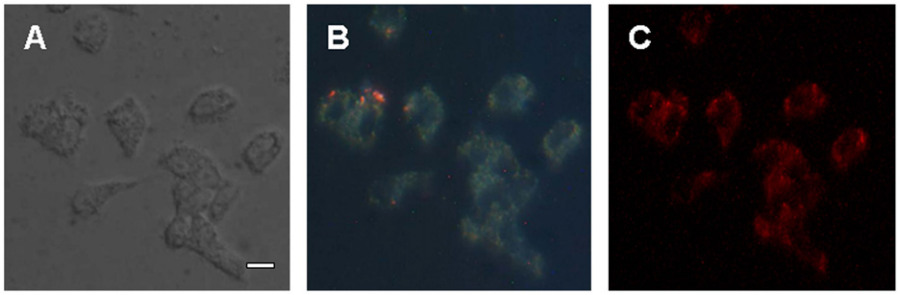

Fig. 5. Bright-field (A), steady-state (B) and time-resolved (C) luminescence images of the HeLa cells that have been incubated with the Eu@SiO_2-labeled transferrin. Scale bar: 10 μm.

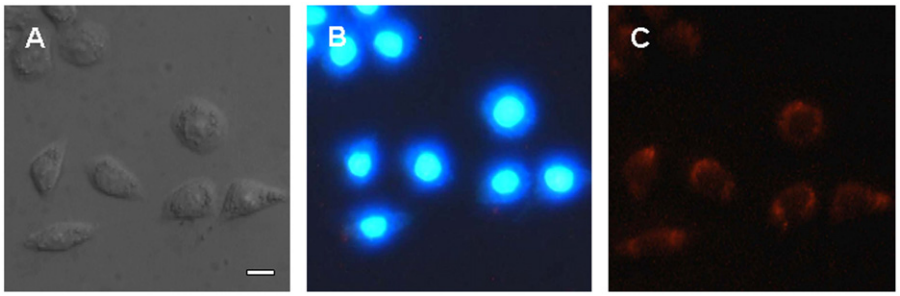

Fig. 6. Bright-field (A), steady-state (B) and time-resolved (C) luminescence images of the HeLa cells that have been incubated with the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid and Hoechst 33342. Scale bar: 10 μm.

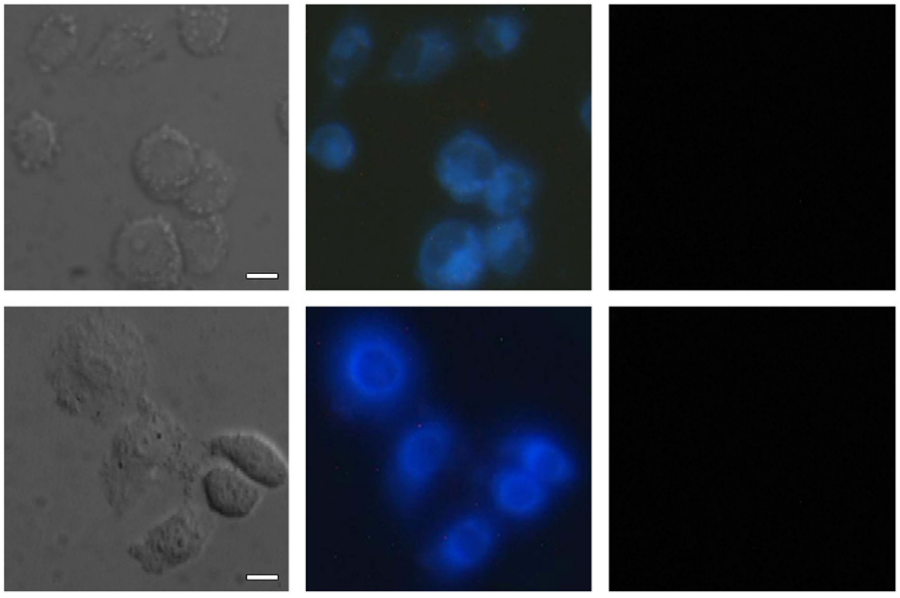

Fig. 7. Bright-field (left), steady-state (middle) and time-resolved (right) luminescence images of the Chang liver cells (top) and HeLa cells (bottom) that have been incubated with the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid and the free BSA–BHHBCB-Eu^{3+}-BPT conjugate. Scale bar: 10 μm.

and the free BSA–BHHBCB-Eu^{3+}-BPT conjugate instead of BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid for incubating with HeLa cells. As shown in Fig. 7, no time-resolved luminescence signals could be observed from the treated Chang liver cells and HeLa cells, even though blue fluorescence was observed from the two kinds of cells. These results demonstrate that the binding of the BSA–BHHBCB-Eu^{3+}-BPT-labeled folic acid with folic acid receptor is highly specific, and the red luminescence signals from the HeLa cells in Fig. 6C are truly emitted from the specific binding product of the labeled folic acid with folic acid receptor, but not from the non-specific adsorption product.

4. Conclusions

In this work, by covalent doping a visible-light-excited Eu^{3+} complex, BHHBCB-Eu^{3+}-BPT, into silica nanoparticles or binding the complex to BSA, two kinds of visible-light-excited europium biolabels, the Eu@SiO_2 nanoparticles and the BSA–BHHBCB-Eu^{3+}-BPT conjugate, were successfully developed. The results of transferrin and folic acid labelings and the application of the labeled transferrin and folic acid to the time-resolved luminescence imaging of HeLa cells demonstrated the feasibility of the new labels for the cell imaging application. Compared to the UV-light-excited lanthanide biolabels, the features of new biolabels, such as strong luminescence, long luminescence lifetime and visible-light-excitation wavelength, enable them to be used for the highly sensitive imaging detection of living cells, which provides a useful tool for the application of the time-resolved luminescence bioassay technique to the UV-light-sensitive bio-samples.

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