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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].

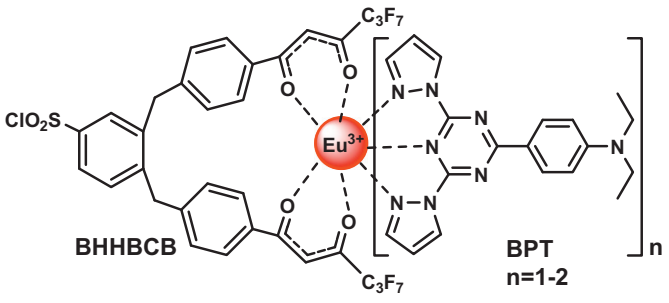

Fig. 1. Structure of the ternary Eu^{3+} complex BHHBCB-Eu^{3+}-BPT.

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