Preparation and Time-Resolved Fluorometric Application of Luminescent Europium Nanoparticles

Mingqian Tan, Zhiqiang Ye, Guilan Wang, and Jingli Yuan*

Department of Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116012, P. R. China

Received March 26, 2003

Novel silica-coated luminescent europium (SCLE) nanoparticles have been prepared by copolymerization of tetraethyl orthosilicate and 3-aminopropyl(triethoxy)silane with ammonium hydroxide in a water-in-oil microemulsion containing a luminescent Eu^{3+} chelate, Triton X-100, *n*-octanol, and cyclohexane. The nanoparticles were characterized by transmission electron microscopy, X-ray diffraction, and UV−vis and luminescence spectra. The results show that the nanoparticles are spherical and uniform in size, 50 ± 5 nm in diameter, and have high photostability and long luminescence lifetime (770 μs). Because primary amino groups have been directly introduced to the nanoparticle's surface by using 3-aminopropyl-(triethoxyl)silane in nanoparticle preparation, the surface modification and bioconjugation of the nanoparticles are easier and reproducible. The nanoparticles were used for streptavidin (SA) labeling, and the nanoparticle-labeled streptavidin was successfully used in time-resolved fluoroimmunoassay of human α-fetoprotein. The results reveal that the new nanoparticles are favorable for use as a new type of luminescence probe for highly sensitive time-resolved luminescence bioassay.

Introduction

Luminescent nanoparticles have become an attractive area of research in recent years because of their utility in biological detection and biotechnology. It has been known that some luminescent nanoparticles can be used as better luminescence probes for bioassays than the traditional organic dyes. Luminescent semiconductor nanoparticles (quantum dots) used as probes for biological detections have shown great promise because of their unique luminescent properties.[1−4] The main problems of the quantum dots are their poor solubility in water, agglutination, and blinking properties. Gold nanoparticles, as an amplification tag, have been used for the analyses of gene, antibody, and antigen.[5−8] Fluorescent latex nanoparticles, such as polystyrene nanoparticles, have also been developed as probes for biological detections.[9−11] However, the latex nanoparticles have the drawbacks of agglomeration and swelling.[12] Recently, luminophore-doped silica (LDS) nanoparticles containing tris(2,2′-bipyridyl) dichlororuthenium(II) hexahydrate have been developed as a new type of luminescence probe for biological detections.[12−15] Although the LDS nanoparticles are biocompatible, photostable, and easy to be modified to attach to biomolecules, the measurement using this probe suffers the interferences of the strong nonspecific scattering lights, such as Tyndall, Rayleigh, and Raman scatterings.

Time-resolved fluorometry using lanthanide chelate as a luminescence probe has been widely used in highly sensitive fluorescence immunoassay and DNA hybridization assay.[16−23] The most important advantage of this technique is that the method can effectively eliminate

* Corresponding author. Phone: +86-411-3693509. Fax: +86-411-3693509. E-mail: jingliyuan@yahoo.com.cn

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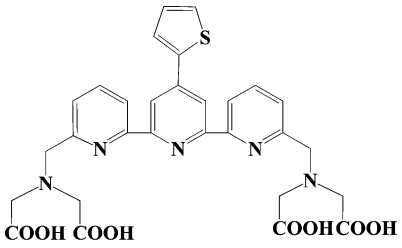
Published on Web 05/18/2004



(TTTA)

Figure 1. Structure of N,N,N^1,N^1-[4′-(2‴-thienyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis(acetic acid) (TTTA).

the short-lived background signals from the biological samples and the optical components, whereas the background noises cannot be removed by the conventional fluorescence method. However, the luminescence of lanthanide chelates is weaker compared with that of organic fluorescence dyes due to lower luminescence quantum yields and smaller molar extinction coefficients of the chelates. Furthermore, photobleaching is still a problem when a lanthanide chelate probe is excited with an intense excitation source for monitoring some real-time biological processes and sensitive detections, such as luminescence bioimaging,[24] even though the photostability of lanthanide chelate is higher than that of the organic dye.

In the present paper, we report a method for preparation of silica-coated luminescent europium (SCLE) nanoparticles. The preparation was carried out in a water-in-oil (W/O) microemulsion containing a luminescent Eu^{3+} chelate, N,N,N^1,N^1-[4′-(2‴-thienyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis-(acetate)−Eu^{3+} (TTTA−Eu^{3+}, Figure 1 shows the structure of TTTA), Triton X-100, *n*-octanol, and cyclohexane by controlling copolymerization of tetraethyl orthosilicate (TEOS) and 3-aminopropyl(triethoxyl)silane (APS) with ammonium hydroxide. The SCLE nanoparticles can emit luminescence of characteristic of TTTA−Eu^{3+} chelate when they are excited by ultraviolet light, and the luminescence is long-lived (luminescence lifetime of 770 μs) and highly stable against photobleaching. Because of the use of APS in nanoparticle preparation, primary amino groups were directly introduced to the nanoparticle's surface, which makes surface modification and bioconjugation of the nanoparticles easier. To evaluate the utility of the new nanoparticles as a luminescent probe for quantitative bioassay, SCLE-nanoparticle-labeled streptavidin (SA) was prepared and used in a sandwich-type time-resolved fluoroimmunoassay (TR−FIA) of human α-fetoprotein (AFP). The result shows that the method is highly sensitive and usable.

Experimental Section

Materials. The ligand TTTA was synthesized according to the synthesis procedure for a structure-similar ligand, N,N,N^1,N^1-[4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis (acetic acid).[25] Anal. Calcd for $C_{29}H_{31}N_5O_9S$(TTTA·$2H_2O$): C, 54.29; H, 4.87; N, 10.91. Found: C, 54.09; H, 4.57; N, 10.41. ^{1}H NMR (DMSO-d_6) δ 8.75 (s, 2H), 8.59 (d, J, 7.8 Hz, 2H), 8.18 (t, J, 7.8 Hz, 2H), 8.10 (d, J, 3.6 Hz, 1H), 7.87 (d, J, 5.1 Hz, 1H), 7.75 (d, J, 7.8 Hz, 2H), 7.34−7.31 (m, 1H), 4.72 (s, 4H), 4.28 (s, 8H). The solid chelate of TTTA−Eu^{3+} was synthesized by reacting TTTA with $EuCl_3$ in aqueous solution using a reported method.[25] SA was purchased from Chemicon International Inc. Goat polyclonal and mouse monoclonal anti-human AFP antibodies were purchased from Nippon Bio-Test Laboratories Inc. and OEM Concepts Co., respectively. The standard solutions of human AFP were prepared by diluting human AFP (Nippon Bio-Test Laboratories, Inc.) 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. Sulfosuccinimidyl-6-(biotin-amido)hexanoate (NHS-LC-biotin) was purchased from Pierce Chemical Co. Double-distilled water was used in the nanoparticle preparation. Unless otherwise stated, all chemical materials were purchased from commercial sources and used without further purification.

Instrumentation. The ^{1}H NMR spectra were recorded on a Bruker DRX 400 spectrometer. The transmission electron microscopy (TEM) was performed using a JEOL JEM-2000EX transmission electron microscope. X-ray diffraction (XRD) spectra were measured on a Rigaku D/Max-γB diffractometer. The X-ray diffraction pattern was taken from 5 to 70° (2θ value) using Cu Kα radiation, and a generator voltage of 40 kV and a current of 70 mA. Luminescence spectra and emission lifetimes were measured on a Perkin-Elmer LS 50B spectrofluorometer. UV−vis absorption spectra were measured on a Tianmei 7500 UV−vis spectrophotometer. Luminescence quantum yield of the nanoparticles was measured by using a reported method[26] and a Eu^{3+} chelate of N,N,N^1,N^1-[4′-phenyl-2,2′:6′,2″-terpyridine-6,6″-diyl]bis(methylenenitrilo)tetrakis-(aceticacid) as the corresponding dye (16% of luminescence quantum yield).[25] The TR−FIA 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 following conditions: excitation wavelength 340 nm, emission wavelength of 615 nm, delay time of 0.2 ms, and window time of 0.4 ms.

Preparation of SCLE Nanoparticles. Typically, cyclohexane, Triton X-100, *n*-octanol (4.4:1.1:1, v/v), and aqueous solution of TTTA−Eu^{3+} chelate were added to a flask with stirring at room temperature to obtain a W/O microemulsion. The molar ratio of water to surfactant (Triton X-100) was controlled to be 8.34 in the preparation. The concentration of the Eu^{3+} chelate in microemulsion was varied from 0.0 M (pure silica nanoparticles) to 1.0 mM. After 200 μL of TEOS and 5 μL of APS were added into 28.1 mL of microemulsion with stirring, the polymerization reaction was initiated by adding 200 μL of NH_4OH (28−30%). The reaction was allowed to continue for 24 h at room temperature. The final silica nanoparticles were isolated by adding acetone, centrifuging, and washing with ethanol and water several times to remove surfactant and unreacted materials.

Preparation of SCLE-Nanoparticle-Labeled SA. After 2.0 mg of SCLE nanoparticles was suspended in 1.0 mL of 0.1 M phosphate buffer of pH 7.0 by ultrasonication for 10 min, 4.0 mg of BSA and 0.3 mL of 1.0% glutaraldehyde were added. The solution was stirred at 4 °C for 24 h, and the nanoparticles were centrifuged and washed with phosphate buffer. The nanoparticles were suspended in 1.0 mL of 0.1 M phosphate buffer of pH 7.0 again, and 1.0 mg of SA and 0.2 mL of 1.0% glutaraldehyde were added. The solution was stirred at 4 °C for 24 h again. Then 1.0 mg of $NaBH_4$ was added, and the solution was incubated for 2 h at room temperature. After being centrifuged and washed with phosphate buffer and water, the SCLE-nanoparticle-labeled SA 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. The fractions containing the SCLE-nanoparticle-labeled SA were collected, diluted with 0.05 M Tris−HCl buffer of pH 7.8 containing 0.2%

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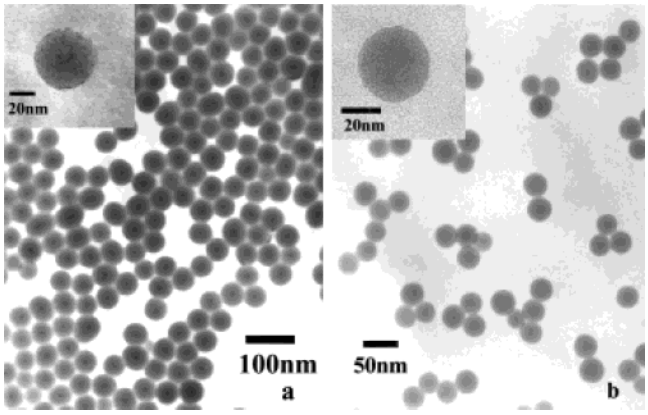

Figure 2. TEM images of SCLE nanoparticles (a) and pure silica nanoparticles (b) at 120 000-fold magnification. The inserts show the higher resolution images of a single SCLE nanoparticle and a pure silica nanoparticle at 300 000-fold magnification.

BSA, 0.1% NaN_3, and 0.9% NaCl, and stored at 4 °C before use.

Preparation of Biotinylated Anti-Human AFP Antibody. After dialyzing (twice) 0.5 mL of goat anti-human AFP antibody solution (0.5 mg/mL) at 4 °C for 24 h against 3 L of saline water, 0.5 mL of water, 8.4 mg of $NaHCO_3$, and 6 mg of NHS-LC-biotin were added with stirring. After stirring for 1 h at room temperature, the solution was further incubated at 4 °C for 24 h. The solution was dialyzed twice each for 24 h at 4 °C against 3 L of 0.1 M $NaHCO_3$ containing 0.25 g of NaN_3, and then 10 mg of BSA was added. The solution was stored at −20 °C before use. When the biotinylated antibody solution was used for immunoassay, it was diluted 500-fold with 0.05 M Tris−HCl buffer of pH 7.8 containing 0.2% BSA, 0.1% NaN3, and 0.9% NaCl.

Immunoassay of Human AFP Using SCLE-Nanoparticle-Labeled SA. After anti-AFP monoclonal antibody was coated on the wells (50 μL per well) of a 96-well microtiter plate by physical absorption method,[27] 40 μL of human AFP standard solution was added to each well. After incubation at 37 °C for 1 h, the wells were 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. Then 40 μL of biotinylated anti-AFP antibody was added to each well and the plate was incubated at 37 °C for 1 h. After the wells were washed, 40 μL of the SCLE-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.

Results and Discussion

By the hydrolysis copolymerization of TEOS and APS with ammonium hydroxide in a W/O microemulsion containing aqueous solution of TTTA−Eu^{3+} chelate, surfactant Triton X-100, cosurfactant *n*-octanol, and oil phase cyclohexane, the well-defined SCLE nanoparticles with spherical shape and a narrow size distribution were obtained. The nanoparticles were characterized by transmission electron microscopy, X-ray diffraction, UV−vis absorption, and luminescence spectroscopic methods.

Figure 2 shows the TEM images of the nanoparticles prepared in the presence (a) (1.0 mM of TTTA−Eu^{3+} in W/O microemulsion was used) or in the absence (b) of TTTA−Eu^{3+} chelate. As shown in Figure 2a, SCLE nanoparticles are spherical and uniform in size, 50 ± 5 nm in diameter. Similar to the 2,2′-bipyridine-Ru^{2+} chelate-doped silica nanoparticles,[12] the dark dots embedded inside the silica network that can be observed on the TEM image of higher solution (the inset TEM image in Figure 2a) imply that the Eu^{3+} chelate molecules in the nanoparticles are existed by physical coating of silica network, whereas no black dots can be observed in the pure silica nanoparticles (Figure 2b). Because the stability of lanthanide(III) chelate with polyacid derivative of terpyridine is very high (higher than that of EDTA chelate)[25,28] in aqueous solution and the pure TTTA−Eu^{3+} chelate (prepared by dissolving solid chelate of TTTA−Eu^{3+} with distilled water) was used for the preparation of SCLE nanoparticles, it can be considered that the dark dots embedded in the nanoparticles are TTTA−Eu^{3+} chelate, not other inorganic europium(III) compounds.

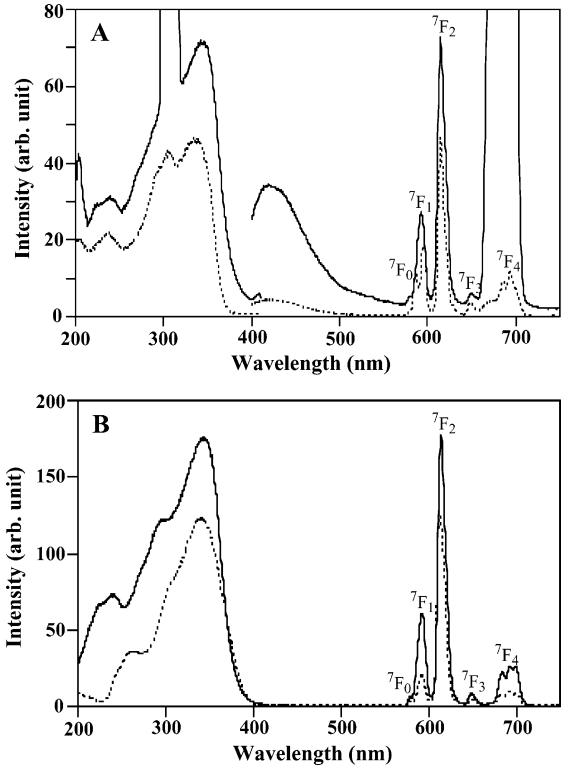

Figure 3. (A) Excitation and emission spectra of SCLE nanoparticles (solid line, 0.2 mg/mL) and free TTTA−Eu^{3+} chelate (dashed, 1.0 μM) in aqueous solution measured by normal luminescence mode. (B) Excitation and emission spectra of SCLE nanoparticles (solid line, 0.2 mg/mL) and SCLE-nanoparticle-labeled SA (dashed line, 0.13 mg/mL) in aqueous solution measured by time-resolved luminescence mode with the following conditions: delay time 0.2 ms, gate time 0.4 ms, and cycle time 20 ms.

In addition, the characterization of the nanoparticles by luminescence spectrometric measurement also indicates that the compound embedded in SCLE nanoparticles is TTTA−Eu^{3+} chelate. The excitation and emission spectra of SCLE nanoparticles and free TTTA−Eu^{3+} chelate in aqueous solution are shown in Figure 3A. Both the nanoparticles and free TTTA−Eu^{3+} chelate show the same excitation and emission maximum wavelengths at 336 and 615 nm, respectively. This

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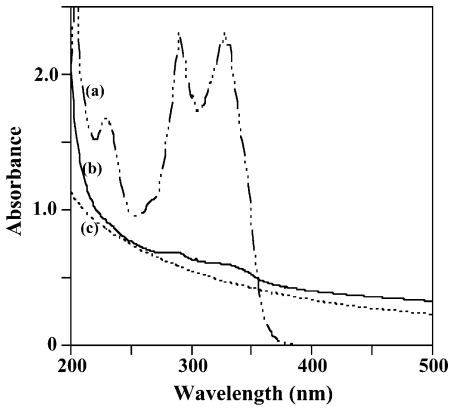

Figure 4. UV−vis spectra of free TTTA−Eu^{3+} chelate (a), SCLE nanoparticles (b), and pure silica nanoparticles (c).

spectrum pattern is typical for a luminescent europium-(III) chelate, while the excitation is caused by the ligand absorption; after the energy is transferred from the ligand to the central Eu^{3+} ion via an intramolecular intersystem crossing mechanism, the energy emission between the resonance energy levels of Eu^{3+} ion occurs.[29] Because the excitation of Eu^{3+} chelate is dependent on the ligand absorption, the same excitation spectrum patterns of SCLE nanoparticles and free TTTA−Eu^{3+} chelate reveal that the Eu^{3+} chelate embedded in SCLE nanoparticles consists of TTTA−Eu^{3+} chelate. In the emission spectra of SCLE nanoparticles and free TTTA−Eu^{3+} chelate, the sharp parks at 587, 596, 615, 648, and 694 nm correspond to the $^5D_0 \rightarrow {}^7F_{0,1,2,3,4}$ transitions, and the broad band between 400 and 500 nm is caused by the ligand emission. The time-resolved luminescence spectra of SCLE nanoparticles and the nanoparticle-labeled SA are also shown in Figure 3B. As expected, the excitation and emission spectra of SCLE nanoparticles and the nanoparticle-labeled SA show the same spectrum patterns. Compared to the normal luminescence spectra, two strong scattering peaks in excitation and emission spectra and the broad band of ligand emission were eliminated completely in the time-resolved luminescence spectra.

The luminescence quantum yields of SCLE nanoparticles, SCLE-nanoparticle-labeled SA, and free TTTA−Eu^{3+} chelate in aqueous solution were measured to be 1.1, 1.1, and 15.0%, respectively. The luminescence quantum yields of SCLE nanoparticles is remarkably lower than that of free TTTA−Eu^{3+} chelate. This phenomenon can be explained as follows. As shown in Figure 4, because pure silica also has relatively strong absorption at excitation wavelength (336 nm), and the amount of TTTA−Eu^{3+} chelate in SCLE nanoparticles is rather small compared to silica, in the absorption of SCLE nanoparticles at excitation wavelength only a few percent of absorption is caused by Eu^{3+} chelate (effective for the luminescence emission), whereas the main absorption is caused by silica (ineffective for the luminescence emission). The luminescence quantum yield was measured with the condition[26] that the optical densities of SCLE nanoparticles and the corresponding dye at the excitation wavelength were set to similar

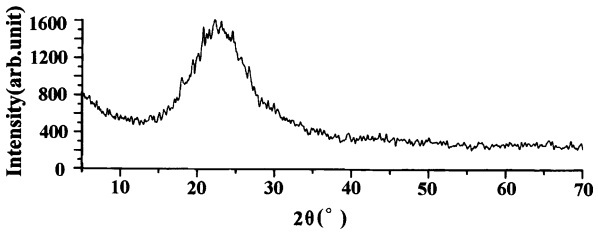

Figure 5. XRD spectrum of SCLE nanoparticles.

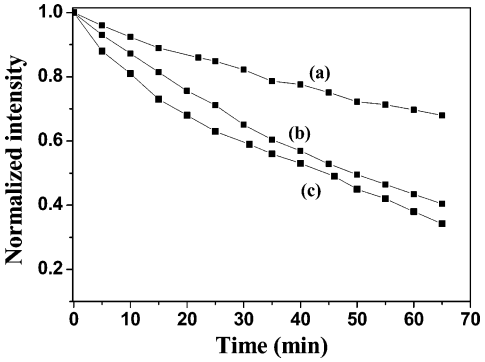

Figure 6. Results of photobleaching experiments: (a) SCLE nanoparticles, (b) pure TTTA−Eu^{3+} chelate, and (c) Rh6G; in aqueous solution phase with a 30-W deuterium lamp excitation source.

values, and was calculated by comparing the integrated luminescence intensities of the nanoparticles and the corresponding dye; therefore, SCLE nanoparticles give lower luminescence quantum yield due to the ineffective absorption of silica in SCLE nanoparticles. The luminescence lifetime of the nanoparticle-labeled SA solution was measured to be 770 μs. This result shows that the nanoparticles have a luminescence lifetime long enough for time-resolved luminescence measurement.

The XRD spectrum of SCLE nanoparticles is shown in Figure 5. The nanoparticles synthesized from the microemulsion show only a broad band in the spectrum, and no sharp diffraction peak corresponding to crystalline structure. This XRD pattern is typical for amorphous materials or ultrasmall crystalline materials where diffraction peaks cannot be well resolved. A similar result was also obtained in a reported work about silica-coated iron oxide nanoparticles,[30] and shows SCLE nanoparticles are noncrystalline materials, and TTTA−Eu^{3+} chelate molecules in the nanoparticles are in a noncrystalline or ultrasmall crystalline state.

To evaluate the photostability of the nanoparticles, the photobleaching experiments of the SCLE nanoparticles, pure TTTA−Eu^{3+} chelate, and rhodamine 6G (Rh6G) were performed in the aqueous solution by using a 30-W deuterium lamp as an excitation source. The emission intensity was recorded at every 5-min interval for a period of 65 min. As shown in Figure 6, the emission intensities of Rh6G and pure TTTA−Eu^{3+} chelate were decreased approximately 65 and 59%, respectively, whereas the emission intensity of the nanoparticles was only decreased 32% in the same period. The high photostability of the nanoparticles is caused by the fact that the Eu^{3+} chelate in the nanoparticles is coated surroundingly by silica, which isolates

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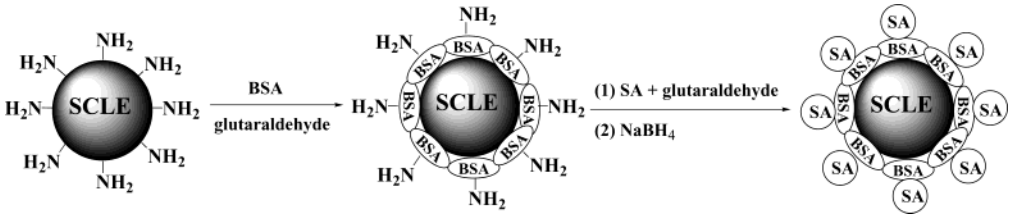

Figure 7. Schematic representation of SA conjugation processes of SCLE nanoparticles.

the chelate from the outside environment (such as solvent molecules and free radicals caused by light exposure) and, therefore, effectively protects the chelate from photodecomposition.

Because the SCLE nanoparticles were prepared by the copolymerization of TEOS and APS, the primary amino groups have been directly introduced to the nanoparticle's surface. These amino groups can be used directly to bind the nanoparticles to biomolecules. To evaluate the utility of SCLE nanoparticles as a new type of luminescence probe for biolabeling and quantitative bioassays, SCLE nanoparticles were used for SA labeling and TR−FIA of human AFP. As shown in Figure 7, the amino groups of SCLE nanoparticles were reacted first with amino group of BSA by glutaraldehyde coupling method[31] to form a layer of BSA on the nanoparticle's surface, and then the BSA-coated nanoparticles were conjugated to SA by coupling the amino groups of SA and BSA (a BSA molecule has 59 amino groups[32]) with glutaraldehyde. The labeling procedure described here is not only simple and reproducible, but the nanoparticle-labeled SA also remains stable for a long time. The calibration curve of TR−FIA using the SCLE-nanoparticle-labeled SA for human AFP is shown in Figure 8. The detection limit, defined as the concentration corresponding to 3SD (standard deviations) of background signal, is 85 pg/mL. This detection limit is low enough for the assay of human AFP in serum.[22]

In summary, SCLE nanoparticles of uniform size distribution and small size have been prepared and characterized, and their utility to time-resolved fluorometry was demonstrated in the present work. The amino groups on the surface of the nanoparticles directly introduced by using a copolymerization technique make bioconjugation of the nanoparticles easier. As a new luminescent reagent, the use of SCLE nanoparticles as a luminescence probe combined the advantages of both luminophore-doped silica nanoparticle probes and lanthanide latex luminescence probes, including smaller size, high hydrophilicity and biocompatibility, ease of modification to attach biomolecules, and ease of elimination of the nonspecific scattering lights by using time-resolved measurement mode. Although the results are preliminary, the properties of long luminescence lifetime, good photostability, and biocompatibility suggest that this new type of luminescent nanoparticles would be useful also for highly sensitive time-resolved luminescence bioimaging and biochip technologies.

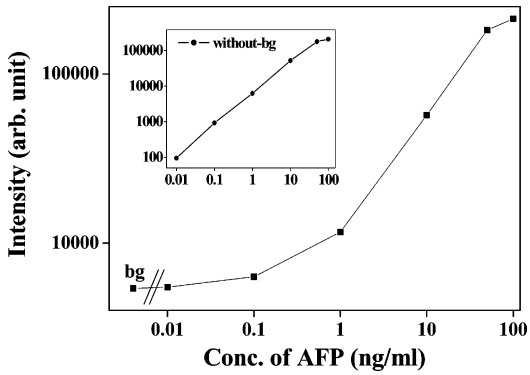

Figure 8. Calibration curve of TR−FIA by using SCLE-nanoparticle-labeled SA for human AFP. The inset curve is plotted for background-subtracted signals vs the concentrations of human AFP.

Acknowledgment. This work was supported by the National Natural Science Foundation of China (20175027) and Scientific Research Innovation Foundation of the Chinese Academy of Sciences (01010406).

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