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ORIGINAL PAPER

Synthesis of Fluorescent Silica Nanoparticles and Their Applications as Fluorescence Probes

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Abstract This paper presents the synthesis of organic dye molecules embedded silica nanoparticles by Stöber method and their applications as fluorescence probes in cell imaging. By modifying the surface of fluorescent silica nanoparticles (FSNs) with amino, biologically functionalized and monodisperse FSNs can be obtained. In this work, FSNs were conjugated with monoclonal anti-Carcinoembryonic Antigen (anti-CEA) antibody via covalent binding. The antibody-conjugated FSNs can be used to label the SPCA-1 cells successfully, demonstrating that the application of FSNs as fluorescence probes in fluorescence imaging and bioassay would be feasible.

Keywords Fluorescent silica nanoparticles · Luminescence · Fluorescent probe · Cellular imaging

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Introduction

With the advancement of nanoparticles technology, engineered fluorescent nanoparticles such as quantum dots (QDs) [1, 2], dye-doped nanoparticles [3, 4], and so on, have attracted a lot of attention in recent years. Despite the excellent brightness and photostability of QDs for in vivo imaging applications, the risk of systemic toxicity remains high, given their incorporation of heavy metals (e.g., Pb, Cd), precluding their widespread use and ultimate clinical translation [5]. Therefore, dye-doped nanoparticles appear very promising. Additionally, it has been suggested that each dye-doped nanoparticle contains a large number of dye molecules, which can result in an increase of the quantum yield of the dyes and produce a strong emission signal when they are properly excited [6]. Silica coating is one of the most popular techniques for dye molecules surface modification. Because of the inert silica matrix, dye-doped silica nanoparticles possess great photostability, chemical stability, biocompatibility and easy modification for bioconjugation [7].

To our knowledge, there are mainly two kinds of routes for preparing silica nanoparticles. One is based on reverse microemulsion system [8, 9], the other is the Stöber method [10, 11]. Water-in-oil microemulsion method has advantages in that it does not require extreme conditions of temperature and pressure and the particle size and shape can be simply controlled by varying microemulsion parameters. However, the technique requires the use of large amounts of surfactants and organic solvents. The Stöber method involves the condensation of tetraethylorthosilicate (TEOS) in ethanol-water mixtures under alkaline condition at room temperature. Compared with reverse microemulsion system, the Stöber method is remarkable in its simplicity and does not require surface modification or

addition of surfactants to achieve excellent control of size and smooth spherical morphology of silica particles [12].

Some previous work [13–17] has reported the development of inorganic dye-doped silica nanoparticles by using reverse microemulsion method. These nanoparticles are hydrophilic and can couple with biomolecules easily based on silica surface chemistry, but the fluorescence intensity is limited due to low quantum yield of inorganic dyes [3]. On the other hand, compared with inorganic dyes, organic dye molecules seem to be better option for biolabeling and bioanalysis because of their relatively high intrinsic quantum yield [18]. Furthermore, the entrapment of the organic dye molecules within the silica matrix should result in an increase of the quantum yield of the fluorophores, thus enhancing the overall brightness of the fluorescent probe [6]. However, it is difficult to prepare organic dye-doped silica nanoparticles because of the hydrophobic nature of organic dyes as compared to the hydrophilic surface of silica and moreover, most dyes are not easily modified without affecting their luminescence properties. Accordingly, to achieve the challenge of the synthesis of organic dye-doped silica nanoparticles synthesized by a simple, economic and environmentally friendly method would be able to develop a new type of fluorescent material for bioimaging and bioanalysis, which is extremely needed.

In this work we describe the synthesis of FSNs which are designed for cellular imaging. The newly designed FSNs synthesis strategy can be used for various dye molecules in the silica matrix. We have tested two dye molecules, Fluorescein isothiocyanate (FITC) and Tetramethyl rhodamine isothiocyanate (TRITC). The experimental results show that both of them are successfully doped inside the silica nanoparticles (FITC-doped silica nanoparticles and TRITC-doped silica nanoparticles) and FSNs are uniform in size and exhibit good dispersivity and high luminescence. Besides, the fluorescence intensity of FSNs does not sharply decrease under continuous illumination with a 100 W mercury lamp. With amino groups on the surface, the FSNs are easily immobilized with anti-CEA antibody via covalent conjugation using glutaraldehyde. In vitro experiments on cellular uptake of the FSNs are carried out by using SPCA-1 cells and MRC-5 cells. SPCA-1 cells are used to test the targeting effect of FSNs due to the overexpression of CEA on the cell surface. CEA-negative MRC-5 cells are used as a negative control. Confocal laser scanning microscopy is conducted to have a qualitative assessment of cellular uptake of antibody-conjugated FSNs. Finally, in vitro cytotoxicity of the FSNs is investigated also by employing SPCA-1 cells through the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is compared with that of the free dye molecules.

Materials and Methods

Materials

FITC, TRITC and N-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS) were purchased from Sigma-Aldrich. TEOS, ammonium hydroxide (25 wt%), ethanol and glutaraldehyde (25 wt%) were supplied from China National Medicine Group. Fetal bovine serum (FBS), RPMI-1640 and DMEM medium were ordered from Sino-American Biotechnology Co. Ltd. Human lung adenocarcinoma SPCA-1 cells and human embryonic lung fibroblasts MRC-5 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Mouse monoclonal anti-CEA antibody and FITC-labeled mouse monoclonal anti-CEA antibody were purchased from Zymed Laboratories, Inc and GeneTex, Inc, respectively. All other reagents were of analytical grade. Double distilled water was used for all the experiments.

Preparation of FSNs

The FSNs were prepared through a two-step process. In the first step, the dye molecules were covalently attached to the silane coupling agent (AEAPS) by reacting the amine groups of AEAPS with the thioisocyanate groups of dyes [19]. In the experiment, 1 mg of dye molecules was dissolved in 1 mL of anhydrous ethanol and dispersed by ultrasonic. To the solution, 5 μL of AEAPS was added. The mixture was sealed in a desiccator and stirred gently for 24 h in the dark. The precursor dye-AEAPS was obtained.

In the second step, 3 mL of TEOS, 2 mL of H_2O, 4.8 mL of ammonium hydroxide and appropriate amount of dye-AEAPS anhydrous ethanol solution were added to 50 mL of ethanol. After stirred for 3 h at 40°C, the core of FSNs formed. To further stabilize the dye molecules in the silica nanoparticles, another silica protective shell was prepared on the core of FSNs. 0.3 mL of TEOS was added to the solution and aged for another 3 h. Finally, the sample was centrifuged at 12,000 rpm for 10 min to collect FSNs. The collected particles were washed with ethanol by centrifugation and decantation several times in order to remove unreacted chemicals.

Surface Functionalization of FSNs

Modification of FSNs with AEAPS was similar to that as reported [20]. Appropriate amount of FSNs was dispersed in a mixture of 25 mL methanol and 15 mL glycerol with 30 min ultrasonication. Then 0.1 mL of AEAPS was added into the mixture and dispersed by vigorous stirring. Through the whole experiment, the temperature was retained at 60°C. The resultant amino-modified FSNs were

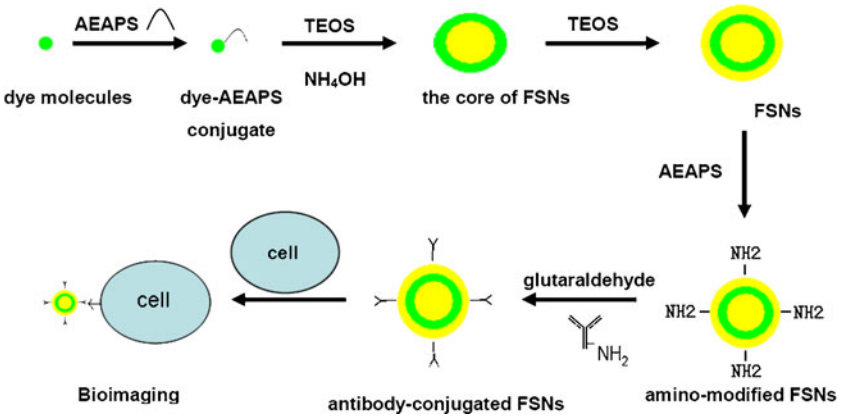

Fig. 1 Schematic illustrations of synthesis of FSNs and their used as fluorescence probes

washed with ethanol and phosphate-buffered saline (PBS, 0.01 M, pH 7.4) for three times, respectively. Finally, the nanoparticles were retained in PBS.

Covalent Immobilization of the Antibody onto FSNs Surface

The anti-CEA antibody was connected with the amino groups on the surface of FSNs by glutaraldehyde [21]. We added 0.1 mL 1% glutaraldehyde to the 0.1 mL amino-modified FSNs solution vibrating for 1 h. Then 0.1 mL anti-CEA antibody was incubated with amine-activated FSNs for 12 h at 4°C. At last, the antibody-conjugated FSNs were centrifuged and washed with PBS several times to remove the excessive antibody, and kept at 4°C in PBS.

Cell Culture and Labeling Experiment

SPCA-1 cells that overexpress CEA (positive control) and MRC-5 cells that have less CEA (negative control) were chosen in the labeling experiments. RPMI-1640 medium was used for SPCA-1 cells and DMEM was used for MRC-5 cells, both mediums were supplemented with 10% FBS. The cells were cultivated in their respective mediums at 37° C in a humidified atmosphere of 5% CO_2. Then the cells were digested and resuspended in the medium. 1×10^5 cells were transferred into a six-well cell-culture plate. After 24 h of incubation, the cells were carefully rinsed with PBS. Then the nanoparticles-dispersed culture medium (100 μg/mL) was added to the plate and incubated for 1 h. The labeled cells were carefully rinsed with PBS to remove the unbound nanoparticles and the fresh serum-free medium (500 μL) was added to the plate. As control, CEA-negative MRC-5 cells were also treated with antibody-conjugated FSNs under the same conditions. The fluorescent imaging of the cells with 488 nm and 543 nm excitation was carried out respectively on a Zeiss LSM 510 META confocal laser scanning microscope.

In Vitro Cytotoxicity

The in vitro cytotoxicity was measured by using MTT assay in SPCA-1 cells. Cells were firstly placed into a 96-well cell-culture plate at a density of 40,000–50,000 cells/mL and

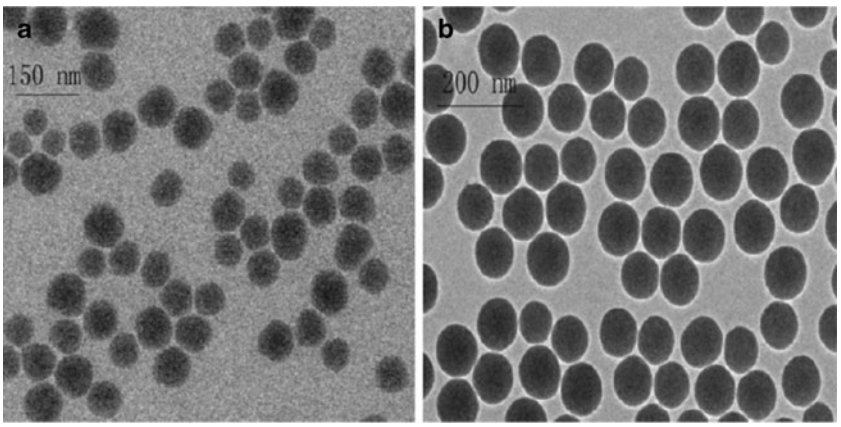

Fig. 2 TEM micrographs of **a** the core of FSNs and **b** FSNs

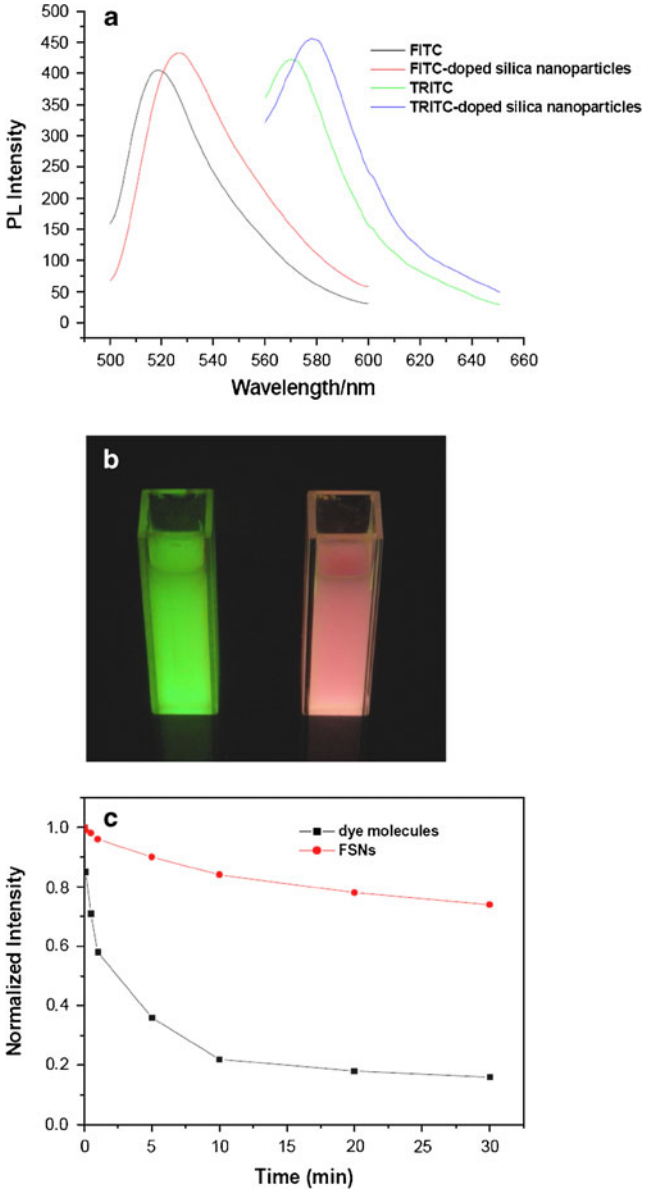

Fig. 3 **a** PL spectra of two kinds of free dye molecules in ethanol and FSNs in water under excitation at 488 nm and 543 nm, respectively; **b** the photograph of both FSNs under UV irradiation at 365 nm. FITC-doped silica nanoparticles (left cuvette) and TRITC-doped silica nanoparticles (right cuvette); and **c** PL-time spectra of organic dye molecules and FSNs with 100 W mercury lamp excitation

maintained in RPMI-1640 supplemented with 10% FBS for 24 h. Then cells were incubated with different concentrations of FSNs (10–100 μg/mL) and organic dye molecules (20 μg/mL), respectively. The plate was incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. Subsequently, 80 μL of 5 mg/mL MTT solution was added into each well and the plate was incubated at 37°C for another 4 h. Then supernatant was removed before DMSO was added to each well to dissolve the formazan. The absorbance at 570 nm was detected with spectrophotometric microplate reader (Thermo Scientific Multiskan Spectrum). Each data point was collected by averaging that of three wells, and the untreated cells were used as controls. The following equation was used to calculate the inhibition of cell viability:

$$\text{Cell Viability}\,(\%) = \mathrm{T}/\mathrm{C} \times 100$$

where T is mean absorption of value treatment group and C is the mean absorption value of control.

Instruments and Measurements

The size and morphology of FSNs were measured on a JEOL model JEM-2010 transmission electron microscope (TEM) operated at 200 kV. The samples for TEM analysis were obtained by placing a drop of the nanoparticles-dispersed aqueous solution onto a copper micro-grid and evaporated in air at room temperature. Fluorescence measurements were performed on a Varian Cary Eclipse Fluorescence Spectrophotometer. To investigate the photostability of FSNs when they were exposed to an aqueous environment for biological applications, a photobleaching experiment for FSNs was performed with mercury lamp for excitation on Olympus HG-100 W. The FTIR spectra of the samples were obtained on a Thermo Nicolet 370 FTIR spectrophotometer. Pressed pellets were prepared by grinding the powder samples with KBr in an agate mortar. The spectra were scanned from 4,000 to 400 cm^{-1}.

Results and Discussion

Synthesis and Characterization of FSNs

This paper describes the use of the Stöber method to prepare highly luminescent monodispersed silica nano-

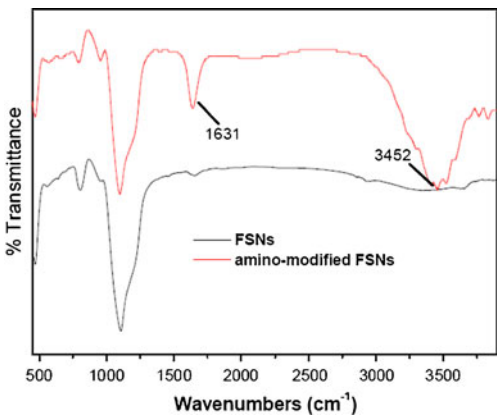

Fig. 4 FTIR spectra of FSNs and amino-modified FSNs

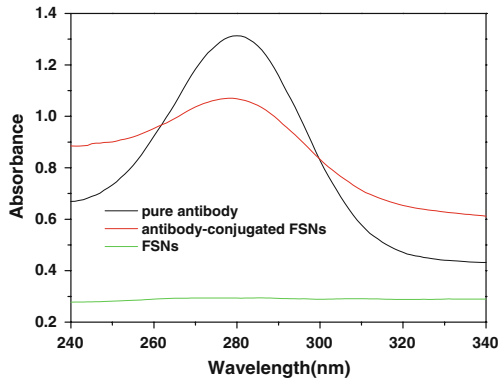

Fig. 5 UV–vis absorption spectra of the anti-CEA antibody, antibody-conjugated FSNs and pure FSNs

particles that encapsulated fluorescent dye molecules. Unlike microemulsion-based method often used to prepare FSNs, this technique completely avoids the use of potentially toxic organic solvents and surfactants. Further conjugation of the nanoparticles to biomolecules is easier because there is no need to wash the nanoparticles off surfactant molecules, which often requires multiple washing steps when microemulsion technique is used to prepare nanoparticles [22].

Figure 1 schematically depicts the series steps involved in the development of FSNs used as fluorescence probes.

Figure 2 shows the typical TEM images of the core of FSNs and FSNs. The core of FSNs are 48±5 nm in size and have a good monodispersity with smooth morphology. After they were coated with another silica shell, the size of the FSNs is increased to 80± 4 nm.

The optical properties of the FSNs are investigated using the PL spectra. The PL spectra of the both FSNs in water solution present a slight red shift as compared with the pure dye molecules in ethanol in Fig. 3a. The red-shifted emission band moves to longer wavelengths as the solvent polarity increases. [23].

The photograph of FSNs under UV illumination (365 nm) is shown in Fig. 3b. The bright green and red fluorescence emitted from FITC-doped silica nanoparticles and TRITC-doped silica nanoparticles, respectively, can be clearly observed from the whole dispersion, suggesting that FSNs can be well dispersed in water solution due to the silica coating.

Although fluorescent dye molecules have a higher quantum yield, they can be rapidly photobleached in the presence of oxygen [24]. This has been one of major problems in their applications as fluorescence probes, especially in real-time imaging. In order to investigate the photostability of FSNs when they are exposed to an aqueous environment for biological applications, the FSNs were taken for the photobleaching experiment in water solution excited with a 100 W mercury lamp. The result is compared with the pure dye solution (Fig. 3c). A 1 mL portion of sample solution was taken in a quartz

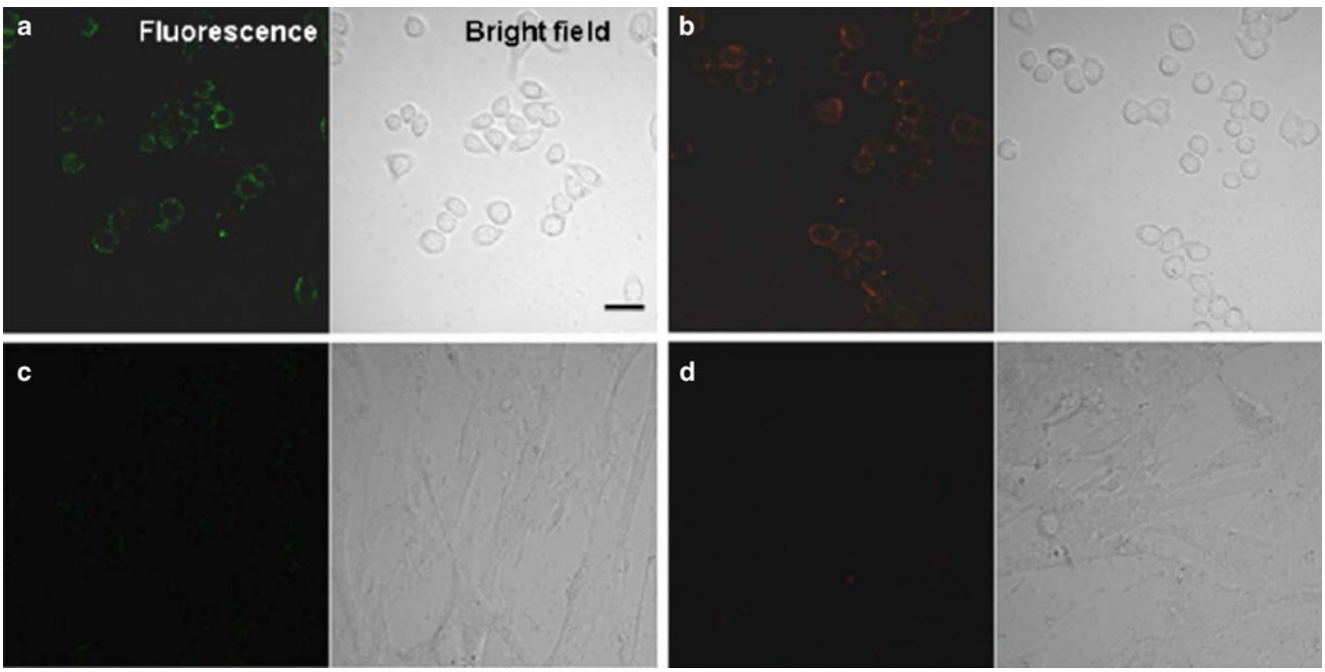

Fig. 6 Confocal microscopy images of SPCA-1 cells treated with **a** antibody-conjugated FSNs (FITC) and **b** antibody-conjugated FSNs (TRITC); and of MRC-5 cells treated with **c** antibody-conjugated FSNs (FITC) and **d** antibody-conjugated FSNs (TRITC) at 37°C after 1 h incubation. The scale bar corresponds to 20 μm

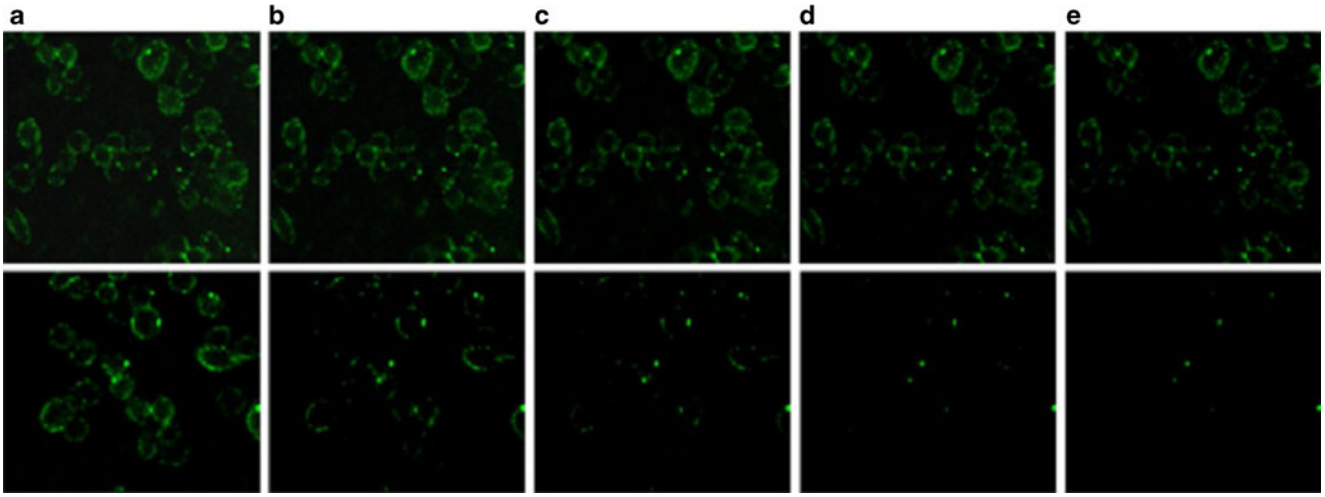

Fig. 7 Photostability comparison of the stained SPCA-1 cells by using antibody-conjugated FSNs (top row) and FITC-labeled anti-CEA antibody (bottom row). Images (**a**), (**b**), (**c**), (**d**), and (**e**) were taken after 1 min, 5 min, 10 min, 15 min, and 20 min of continually intense excitation, respectively

cell, and the experiment was conducted on a spectrofluorometer. After continuous excitation for 0.5 h, the FSNs show a little photobleaching, the emission intensity decreased to 80% of their initial intensity. However, the intensity of pure dye molecules dropps to 17%. These results suggest that the FSNs are more photostable than conventional organic dyes. It is considered that the photostability has been effectively improved for the FSNs as a result of the silica shell acting as a barrier for protecting dyes from the external environment. This improvement would thereby be very crucial for FSNs used in bioanalysis and bioimaging.

Surface Modification of FSNs

Silica is a very versatile matrix particularly because of its inert chemical properties, optical transparency, and the relative ease of its derivatization with functional groups like amino (-NH_2), chloride (-Cl), carboxylic (-COOR) and thiol groups (-SH) [11]. Further surface modification can immobilize biotin, DNA, antibody, enzymes, and other proteins on silica nanoparticles. In this study we modified the surface of FSNs with amino groups through AEAPS with reaction with hydroxyl groups on the silica surface. Figure 4 gives the FTIR spectra of FSNs and amino-modified FSNs. Compared with the spectrum of unmodified nanoparticles, the amino-modified nanoparticles possess the adsorption band in 3,452 cm^{-1} due to the stretching vibration of N–H bond, and in 1,631 cm^{-1} attributed to the bending vibration of the N–H bond [25]. Therefore it can be used to confirm the successful conjugation of AEAPS on the FSNs surface.

Bioapplicable Properties of FSNs

In order to test the targeting capability of the as-prepared amino-modified FSNs, herein, the anti-CEA antibody was immobilized onto nanoparticles to synthesize antibody-conjugated FSNs. Figure 5 shows the absorption spectra of antibody before and after attaching amino-modified FSNs. It is well known that the absorption peak of pure antibody can be observed at around 280 nm. From the Fig. 5, the absorption spectrum of antibody-conjugated FSNs shows a pronounced peak corresponding to pure antibody, indicating that the bioconjugate between amino-modified FSNs and antibody has been successfully formed.

To confirm targeting ability, CEA-positive SPCA-1 cells were incubated with antibody-conjugated FSNs. As shown in Fig. 6a and b, under excitation at 488 nm and 543 nm,

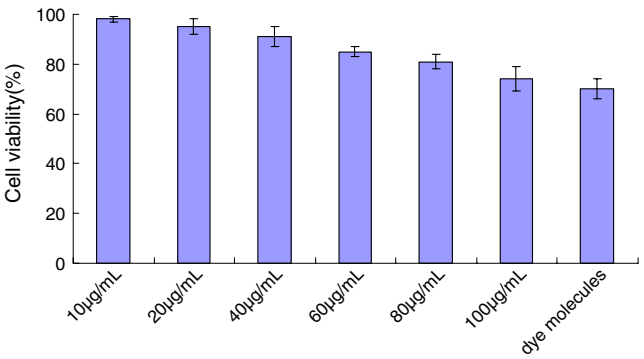

Fig. 8 In vitro viability of SPCA-1 cells treated with free dye molecules and FSNs for 24 h

the two strong luminescence signals (green and orange fluorescence) are respectively observed in the SPCA-1 cells surface after 1 h of incubation at 37°C. Furthermore, almost one-to-one correspondence between SPCA-1 cells shown in both bright field and fluorescent field proves that antigen has been recognized by the antibody on the nanoparticles. The CEA-negative MRC-5 cells were treated with antibody-conjugated FSNs and imaged under similar conditions. The reduced uptake of nanoparticles by MRC-5 cells is clearly shown in Fig. 6c and d, when compares with SPCA-1 cells under same conditions. These results imply that antibody-conjugated FSNs selectively accumulated on the surface of the SPCA-1 cells and can perform as fluorescence probes for bioapplications.

The effect of the photostability of FSNs in bioimaging was further investigated by incubating SPCA-1 cells with antibody-conjugated FSNs and FITC-labeled anti-CEA antibody, respectively. Then the two samples were excited for 20 min by continuous illumination with a 488 nm Ar laser and fluorescent images were acquired every few minutes. As displayed in Fig. 7, the fluorescence on the outer membrane of cells stained by FITC-labeled anti-CEA antibody fades quickly, while the fluorescence intensity of the antibody-conjugated FSNs decreases very slowly. The green signals are still clearly distinguishable to the naked eye after 20 min of continuous intensive irradiation, further indicating that FSNs have good photostability.

It is reported that organic dyes in applications such as biological imaging and dection could be toxic to cells [26] and therefore the use of organic fluorophores as fluorescence markers has some obvious limitations. To some extent, this phenomenon can be effectively prevented through the surface coating. By MTT assay, the cytotoxicity of the FSNs was determined on SPCA-1 cells. The results show that cell viability of nanoparticles was greater than 85% at concentration up to 80 μg/mL after 24 h of incubation (Fig. 8), while the cell viability of organic dye molecules was 70±3.8%. Therefore, these data show that the FSNs can be applied in bioimaging and considered to possess low cytotoxicity.

Conclusions

In this paper, we have developed highly luminescent, extremely photostable and biocompatible FSNs using the Stöber mehod. These FSNs can be used for bioconjugation as they have an easily biomodifiable silica surface. This simple synthesis approach can be applied to various fluorescent dye molecules to prepare FSNs with different emission wavelengths, making it feasible to use the FSNs as fluorescence probes for bioimaging and fluorescent immunoassays.

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