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Design and fabrication of fluorescein-labeled starch-based nanospheres

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ABSTRACT

Fluorescein isothiocyanate (FITC) is covalently attached onto the starch acetate, and the fluorescein-labeled starch acetate can self-assemble to form uniform nanospheres in water. The size of the fluorescent nanospheres can be easily controlled by adjusting the polymer concentration in acetone and the proportions of the water and organic phases. The fluorescent intensity of the nanospheres increases with increasing pH, and its fluorescent intensity has no major effect in the physiologically relevant range from pH 5.7 to pH 8 compared with free FITC. Fluorescence quenching studies prove that the fluorescent nanospheres showed a super fluorescent stability without self-association. The fluorescent nanospheres are degradable and can be potential applied for pH-sensing in biological samples.

1. Introduction

Fluorescent nanoparticles can provide a controlled microenvironment for fluorophore to protect it from unwanted chemical interactions with substrates in the external medium. In addition, the nano-containers composed of fluorescent nanoparticles have considerable advantages, such as high fluorescence intensity and good photo stability. Therefore, the fluorescent nanoparticles have been used as signal magnifiers for a wide range of applications including chemical analysis, biological research and clinical diagnosis. (Fehr, Frommer, & Lalonde, 2002; He, Mortellaro, Leiner, Fraatz, & Tusa, 2003; Stojanovic, Prada, & Landry, 2000).

The fluorescent molecule can be incorporated into nanoparticles using various techniques (Matahwa & Sanderson, 2009; Trau & Battersby, 2001; Wang, Yang, & Tan, 2005). Campbell and Bartlett (2002) developed a fluorescent sphere using physical entrapment of the fluorophores during the polymerization process. However, a drawback of the physical dye immobilization was possible leaching of the fluorophores, which might result in toxicity of the cell and contaminate the biological sample. Additionally, the leaching would cause changes in signal intensity and consequent the incorrect measurements. These problems could be overcome by embedding the dyes covalently. For that purpose the leaching of the fluorophore were avoided, and stable signal intensity can be obtained (Clark, Kopelman, Tjalkens, & Philbert, 1999; Guan & Su, 2008; Mitsuishi, Kikuchi, Miyashita, & Amao, 2003).

Polysaccharide-based nanoparticle has been considered as a fantastic material for biomedical applications because of its biodegradability, biocompatibility and high disease site selectivity (Hornig, Heinze, Hesse, & Liebert, 2005; Jung, Jeong, Kim, & Kim, 2004; Liebert, Hornig, Hesse, & Heinze, 2005). Recently, Heinze and coworker prepared fluorescein and sulforhodamine B marked dextran-based nanoparticles for pH-sensitive nanosensor through a dialysis process (Hornig et al., 2008; Schulz et al., 2009). Incorporation of the dyes in dextran matrix avoids aggregation and compartmentalization. Moreover, the dextran matrix endowed the obtained nanosensor super photostability, and the obtained polysaccharide nanoparticles were non-toxic and easily incorporated into cells. However, the relevant fluorescent stability and biodegradable properties of the dextran-based nanoparticles were not be involved.

We have shown recently that hydrophobically modified starch could form uniform nanoparticles through a nanoprecipitation procedure (Tan et al., 2009; Tan, Xu, Li, Sun, & Wang, 2010). In this work, starch-based nanospheres incorporated with fluorescent dye was prepared by the dropwise addition of water to an acetone solution of fluorescein isothiocyanate (FITC)-labeled starch acetate. The particle size and size distribution could be easily controlled by changing the preparation conditions. Moreover, the fluorescent properties of obtained nanospheres were also investigated in detail.

2. Experimental

2.1. Materials

Waxy maize starch with macromolecular weight (M_W) of 180,000 was obtained according to a previously reported method

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(Chang, Lin, & Lii, 2004). Fluorescein isothiocyanate (FITC), dibutyltin dilaurate (DBTDL) and *N*,*N*-dimethylaminopyridine (DMAP) were obtained from Alfa Co. All the other chemicals were used as received. De-ionized water was used in all reactions.

2.2. Preparation of starch acetate (SA)

The SA was prepared according to the following process. Typically, 2 g of waxy maize starch dispersed in 50 mL of *N*,*N*-dimethylformamide (DMF) was reacted with 3.15 g of acetic anhydride in the presence of 0.02 g DMAP for 3 h at 95 °C. The polymer was precipitated, washed with water three times, and dried at 80 °C under vacuum.

2.3. Preparation of fluorescein labeled SA (FI-SA)

One gram of the SA dissolved in 10 mL of DMSO was reacted with 25 mg of FITC in the presence of 1.5 mL DBTDL for 5 h at 100 °C. The polymer was precipitated, washed repeatedly with water until the filtrate was not fluorescein detected by fluorescence spectrometer. The final product was dried at 60 °C in the oven.

2.4. Preparation of fluorescent nanospheres

The fluorescent nanospheres were prepared by a nanoprecipitation process. Typically, 25 mg of FI-SA was dissolved in 20 mL of acetone. Distilled water (50 mL) was then added dropwise to the polymer solution. The resulting nanoparticle suspensions were stirred at room temperature until acetone was completely vaporized from the aqueous suspension.

2.5. NMR analysis

The NMR spectra were recorded using a Bruker AV400 spectrometer (Ettlingen, Germany) operating at 400 MHz for ^{1}H NMR. The sample was dissolved in d_6-DMSO at 25 °C. The analyses for samples were carried out at 25 °C.

2.6. Nanoparticles analysis

The appearance of nanospheres was characterized by scanning electron microscopy (SEM) using a model XL 30 ESEM microscope (Philips). A droplet of suspension was placed on a silica surface. After the water was evaporated at room temperature, the system was covered by gold before measurement.

The particle size and polydispersity of nanoparticles in aqueous solution were measured by dynamic light scattering using a 90 Plus particle size analyzer (PSA; Brookhaven) at 25 °C. The suspensions were diluted with distilled water to a concentration of about 0.01%. The mean particle size was approximated as the diameter and the combined polydispersity as the polydispersity index (PDI).

2.7. CLSM analysis

The distribution of fluorophores in nanosphere was observed by confocal laser scanning microscopy (CLSM) using a Leica TCS SP2 CLSM. The samples were excited by using a 554 nm He/Ne laser. A droplet of suspension was placed on a glass surface, and visualized directly.

2.8. Fluorescence intensity analysis

The fluorescence intensity was measured at room temperature by a fluorescence spectrometer (Hitachi F-2500) using an emission wavelength of 490 nm. The pH-sensitivity of the nanospheres as well as free FITC was obtained by recording the fluorescence emitted in phosphate buffer solution (PBS) with different pH from 5.7 to 8.0 at fixed wavelength (516 nm).

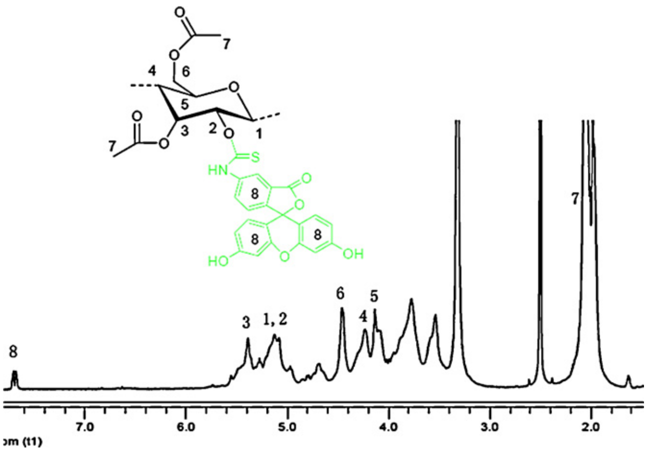

Fig. 1. ^{1}H NMR spectrum of FI-SA in DMSO-d_6.

2.9. Evaluation of fluorescence stabilities

Fluorescence quenching experiment was carried out by addition of small increments of stock solutions of potassium iodide (KI) to 5 mL of 3.26 mg mL^{-1} nanospheres aqueous suspension. The fluorescence emission intensities were recorded at 516 nm by varying the concentration of KI.

Two milliliter of nanospheres aqueous suspension was added in 20 mL PBS (pH 7.4) at 37 °C to complete the *in vitro* degradation test for the fluorescent nanospheres. The fluorescence intensity of the solution was measured by a fluorescence spectrometer (emission at 490 nm) at every designated time.

3. Results and discussion

3.1. Synthesis of FI-SA

FI-SA was synthesized by successively introducing of ester groups and fluorophore into the backbone of starch. In this approach, starch was firstly esterified using acetic anhydride and then labeled with FITC. The acylated process can afford sufficient hydrophobility and consequently avoid the aggregation and collapse of the nanospheres in water. Moreover, it provides a biodegradable polymer-matrix to prevent undesired acid catalyzed hydrolysis of the dyes. The structural feature of FI-SA was characterized by ^{1}H NMR spectroscopy and illustrated in Fig. 1. It can be seen that the aryl protons of FITC appeared at 7.7 ppm, which indicates that the FITC is successfully covalently connected on the starch matrix. The degree of substitute (DS) of acylation is determined to be 2.14 by using Chi's methods (Chi et al., 2008). In addition, fluorescent analysis results show that the DS of FITC is 0.2%.

3.2. Preparation of fluorescent nanospheres

The fluorescent nanospheres were fabricated by a nanoprecipitation method through the dropwise addition of water into an acetone solution of FI-SA, which involves a spontaneous gradient-driven diffusion of amphiphilic organic solvents into the continuous aqueous phase. The energy released in this process leads to rapid dispersion of a polymer-rich organic phase in the form of nanodroplets. Following the initial precipitation of the polymer forming the nanosphere matrix, final solidification of the resultant nanosphere is accomplished by evaporating

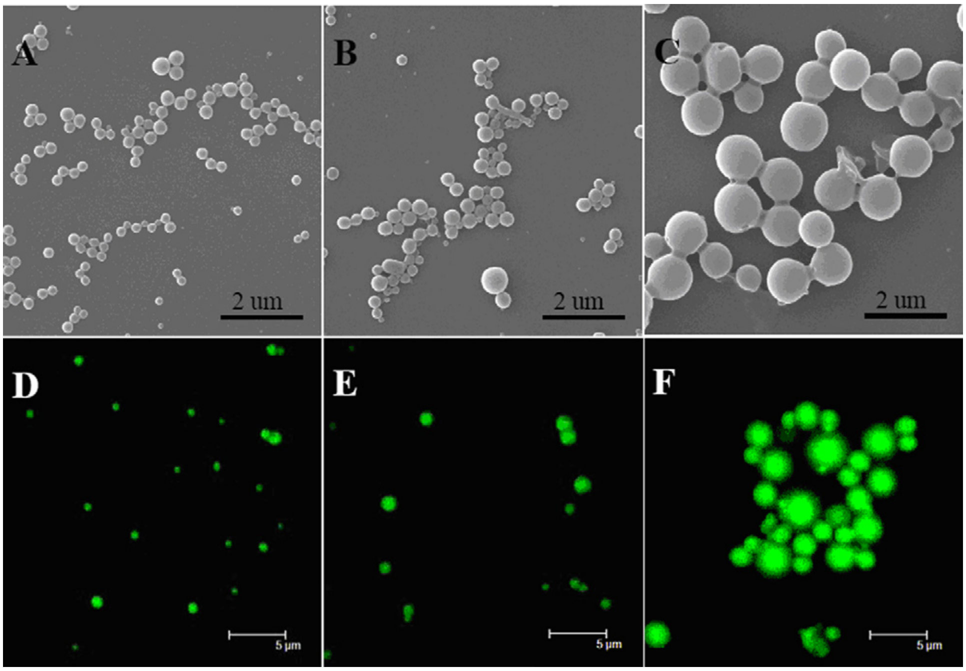

Fig. 2. SEM and fluorescence images of the fluorescent nanospheres prepared by the dropwise addition of water into a polymer acetone solution with concentrations of (A, D) 0.5 mg mL^{-1}, (B, E) 2.0 mg mL^{-1}, and (C, F) 3.5 mg mL^{-1}.

the organic solvents (Niwa, Takeuchi, Nino, Kunou, & Kawashima, 1993; Wehrle, Magenheim, & Benita, 1995). After the complete removal of acetone, sphere-shaped nanoparticles with an even size distribution can be obtained. The SEM and CLSM examination confirmed that the nanoparticles are sphere-shaped with uniform size distribution, as shown in Fig. 2.

Moreover, the size of the starch-based nanospheres can be easily controlled by varying the initial polymer concentration in the organic solvent and the volume proportion between the water and organic phases at the same polymer concentration. The PSA measurement indicates that the diameter increases from 273 to 653 nm as the concentration of FI-SA in acetone increases from 0.35 to 3.75 mg mL^{-1}. (Figs. 2 and 3A) The effect of the polymer concentration on the nanosphere size can be explained by the higher resultant organic phase viscosity, which leads to larger nanodroplets' formation (Chorny, Fishbein, Danenberg, & Golomb, 2002). Similarly, the size of nanosphere increases from 306 to 354 nm as $V_{H/O}$ is decreased from 4 to 1, which resulted from the reinforcement of the interfacial interaction between acetone and water and the compression of the nanoparticles by increasing water content (Sugimoto, 1987). (Fig. 3B) It is noteworthy that all of the nanospheres maintain a lower PDI level range from 0.005 to 0.1.

3.3. Fluorescent properties of nanospheres

For the fluorescent nanospheres, the pH-sensitive dye (FITC) are covalently attached to starch, a question arose whether the polymer matrix change the fluorescent properties of fluorophore (Sun, Scharff-Poulsen, Gu, & Almdal, 2006). A sample with the size of 400 nm (Fig. 2B) is investigated to explore the fluorescent properties of starch-based nanospheres. As shown in Fig. 4A, the fluorescent nanospheres show an emission peaks at 516 nm, which same as the free FITC in aqueous solution. Moreover, the nanospheres suspension was calibrated against sodium phosphate buffer solution of pH 5.7–8.0 to assess its pH-sensitivities. We could conclude that the fluorescence intensity of the nanospheres increases with the increasing pH. (Fig. 4B) This result might be interpreted that, for the dianion form of fluorescein presented in alkaline solution, the fluorescence quantum yield was much

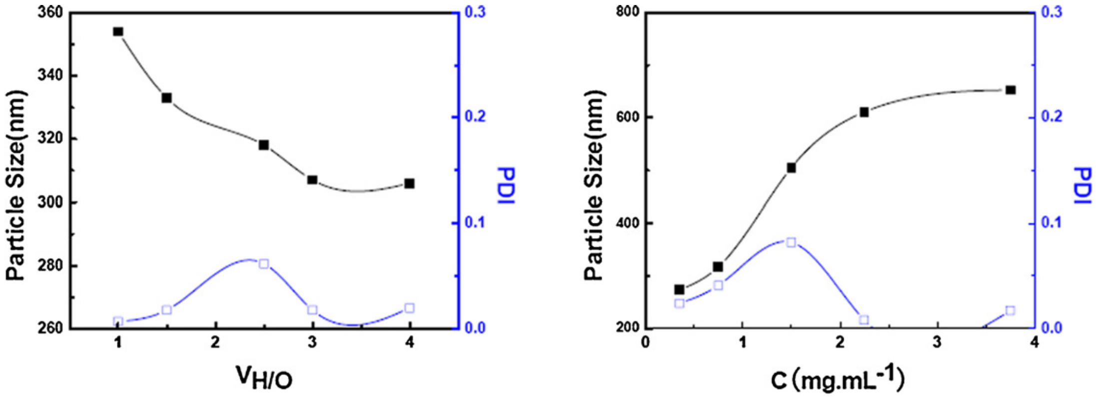

Fig. 3. The effect of FI-SA concentration in acetone and the proportion of water and organic phases ($V_{H/O}$) on the size and size distribution of fluorescent nanospheres.

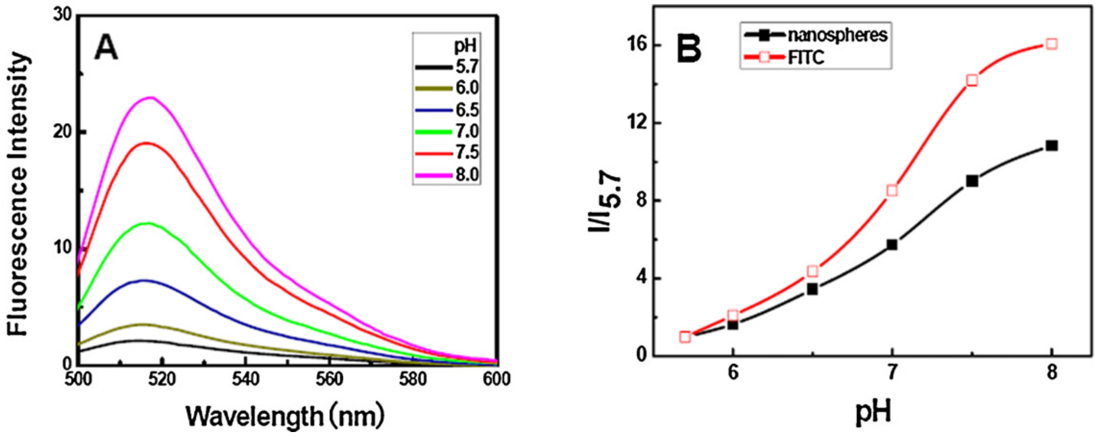

Fig. 4. (A) Fluorescence emission spectra of fluorescent nanospheres suspended in buffer solution with pH rising from 5.7 to 8.0. (B) The fluorescence intensities varying of free FITC and fluorescent nanospheres determined at 516 nm with pH rising from 5.7 to 8.0.

higher than those of its monoanion and neutral molecule presented in neutral and acid solution respectively (Makarova, Ostafin, Miyoshi, Norris, & Meisel, 1999). It should be noted that the fluorescent intensity of the free FITC and fluorescent nanospheres increase about 16-fold and 11-fold, respectively, when the pH of the buffer is increased from 5.0 to 8.0. These facts indicated that incorporating of the indicator dyes in starch matrix has no distinct effect on the fluorescence spectra of the fluorophores. Furthermore, it is noteworthy that the sensitivity of the sensor particles was determined to be in the range between pH 5.7 and pH 8.0, which matches very much for the relevant biological measuring conditions.

To test the quality of encapsulation and sealing of the dye from the surrounding environment, an aqueous quencher of KI was added into both free solution and the nanosphere suspensions to investigate effectiveness of FITC fluorescence quenchers. Fig. 5A shows the effect of KI concentration on the reserved quotient of fluorescent intensity (F/F_0), where F_0 and F are the fluorescence intensities at 516 nm in the absence and presence of varying amounts of KI, respectively. It is clear that the fluorescent nanospheres have a higher reserved quotient than the free dye. This fact implies that FITC is largely encapsulated in the nanospheres and protected by the starch matrix.

The quenching data can also be quantitatively represented as the Stern–Volmer plot, obtained by plotting F_0/F versus [KI] according to the expression (Eftink & Ghiron, 1976; Khan, Ahmad, & Khan, 2010)

$$\frac{F_0}{F} = 1 + K_{SV}[Q]$$

where K_{SV} and [Q] are the Stern–Volmer quenching constant and the concentration of the quencher, respectively.

As shown in Fig. 5B, The K_{SV} plot can be fitted into a straight line intersecting at 1 on the Y-axis, and the apparent K_{SV} values were calculated to be 0.25 and 2.15 for the nanospheres and free FITC, respectively. The result also suggested that fluorophores in nanospheres adopt single conformations without self-association (Patel, Khumsupan, & Narayanaswami, 2010).

3.4. Degradable experiment

In our research, a polysaccharide-based polymer matrix is used to protect the fluorophores. Herein, the effect of the *in vitro* degradation of polysaccharide on the fluorescent properties of nanosphere is also investigated. The nanospheres were cured in a PBS (pH 7.4) at 37 °C to conduct the *in vitro* degradable experiment. As shown in Fig. 6, the release of fluorophores from nanosphere shows two distinct stages according to the variation of its fluorescent intensity. An immediate increase of fluorescent intensity at the initial 7–8 h, this burst stage is predominantly dependent

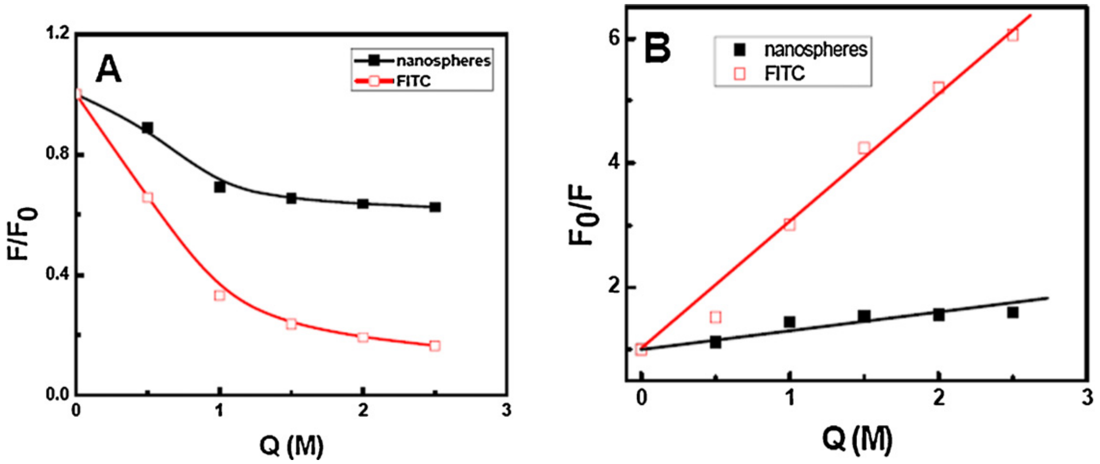

Fig. 5. (A) The fluorescence quenching curve of free FITC and immobilized on the nanospheres by KI. (B) Stern–Volmer plots for fluorescence quenching of FITC by KI in free solution (triangles) and immobilized on the nanospheres.

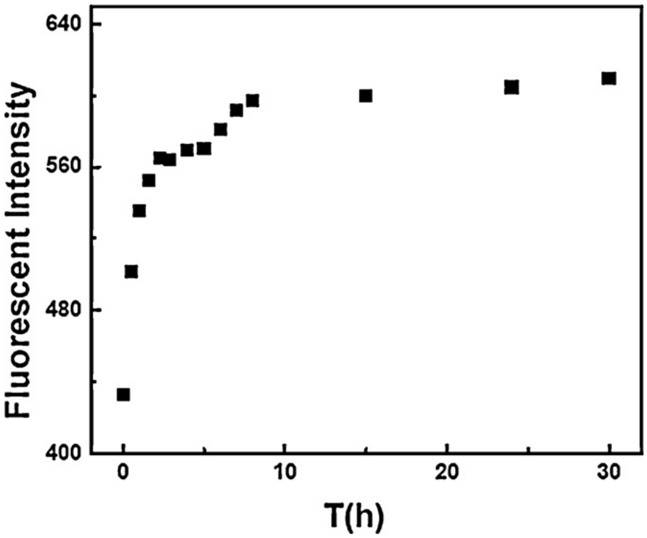

Fig. 6. The fluorescence intensity of the nanospheres in PBS (pH 7.0) at 37 °C at different time.

upon desorption and diffusion of fluorophores from the surface of nanospheres. At the second stage, the fluorescence intensity is slightly increased due to the hydrolyzation of ester bond, resulting to expose the fluorophores attached inside of the nanospheres.

4. Conclusions

In summary, we have successfully fabricated starch-based nanospheres functionalized with an indicator dye of FITC. The nanospheres present a uniform size distribution and the size of the nanoparticles can be tuned by adjusting the initial polymer concentration and the proportions of the water and organic phases. Compared with the free FITC, the fluorescent nanosphere exhibits a higher fluorescent stability due to the covalent linkage between the dye and the starch matrix. Moreover, the incorporation of the indicator dyes in starch matrix has no distinct effect on the fluorescence spectra of the fluorophores, and the fluorescence intensity of the nanospheres increases with the increasing of pH. The fluorescent quenching experiment found that the fluorescent nanospheres showed a super fluorescent stability without self-association. Moreover, the polysaccharide-based fluorescent nanospheres show two distinct release stages at the *in vitro* degradable process. This approach is versatile and can readily be modified to encapsulate other probe molecules inside the starch nanospheres. It has potential applications on the intracellular analysis in the area of biomedical or life-science.

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