Fluorescent Silica Nano-Particles and Magnetic beads Immunoassay: an Optical Tool for Detection of Staphylococcal enterotoxin B (SEB) in Milk

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ABSTRACT

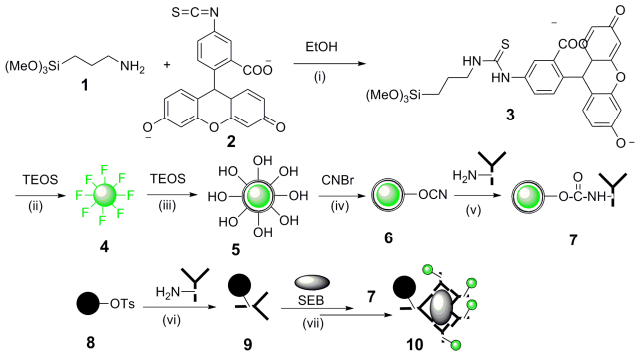
This paper describes the use of fluorescein-dye-doped silica nanoparticles (F-SiNPs) as fluorescent signal amplifiers in a magnetic bead based immunoassay to detect toxins. The reverse microemulsion (W/O) method was used to synthesize F-SiNPs (~ 100 nm of diameter) using a co condensation of tetraethyl orthosilicate (TEOS) with trimethoxysilylpropyl-fluorescein dye and further encapsulation within a silica layer. The F-SiNPs were characterized using SEM, TEM imaging, and FTIR spectroscopy. To determine usefulness of these particles as a diagnostic signaling reagent, milk was spiked with Staphylococcal enterotoxin B (SEB) and the SEB toxin was captured by using magnetic beads with attached SEB specific antibodies. F-SiNPs covalently linked with secondary SEB specific antibody were then used to label captured SEB toxin. The relative fluorocount method was used to generate a signal from the F-SiNPs and magnetic bead immunoassay. The lowest amount of SEB detected was100 pg/ml in the spiked milk samples.

Keywords: Fluorescein dye, Silica nanoparticles, SEB toxin, magnetic beads, and chemiluminescence.

1 INTRODUCTION

Fluorescin organic dye doped silica particles (SiPs) are very versatile materials that have been applied in various bio-analytical applications [1]. This dye has some deficiencies such as, photo-degradation [2] and decreased fluorescent intensity upon bio-conjugation [3]. The dye doped fluorescent particles offer significant advantages over the fluorescent dye alone [4]. Encapsulation of fluorescent molecules into SiPs often increases their photostability and emission quantum yield due to their isolation from possible quenchers like oxygen and water [5]. Further, it facilitates the functionalization of the surface for covalent conjugation to enzymes and antibodies, which used as a fluorescent probes [6].

The reliable colloidal stability, biocompatibility and reactivity with various coupling agents of SiPs allow the design and synthesis of dye-doped SiPs with desired structures and properties [7]. Some groups have synthesized fluorescein-doped SiPs [8]; however, the fluorescent intensity of such dye-doped SiPs decreases relatively fast.



Scheme 1: Synthetic steps for F-SiNPs and its application in SEB detection assay. (i) anhyd. EtOH, rt, 16h., (ii) W/O reverse microemulsion method, (iii) encapsulation of F-SiNPs with silica layer, (iv) Na_2CO_3, acetonitrile, rt., 24 h., (v) *anti*-SEB polyclonal antibody, PBST (ph 7.2), 37 °C, 24 h., (vi) covalent immobilization of *anti*-SEB polyclonal antibody with magnetic beads, (vii) magnetic bead and F-SiNPs based SEB detection assay.

In this work, we demonstrate that uniform fluorescein-dye-doped silica nanoparticles (F-SiNPs) **5** can be synthesized using a reverse microemulsion (water in oil, W/O) method (as shown in scheme 1) and after the further deposition of a silica layer, the fluorescence properties of F-SiNPs become almost independent of pH values and other photobleaching factors. The F-SiNPs were used to label *anti*-SEB polyclonal antibodies and these were applied for SEB detection using a magnetic bead (MB) based immunoassay. The SEB detection signals were generated through relative fluorocounting.

Staphylococcal enterotoxin B (SEB) is one of the many toxin produced by the Gram-positive bacterium *Staphylococcus aureus*. While SEB is known as the causative agent of certain food poisoning it is also considered a biological select agent. Thus a rapid and sensitive identification of SEB is critical to the mitigation of the suspect agent. Here, we describe a method to capture and detect SEB that is rapid (2 h), sensitive (100 pg/ml) and can easily be scaled for high-throughput methods. Due to the extraordinary fluorescence properties of F-SiNPs **5**, the proposed method can be performed within 2 h including setup and reading time.

2 EXPERIMENTAL

2.1 Reagents and Instrumentation

All the chemical reagents used in this study were purchased from Sigma Aldrich, St. Louis, MO, USA, Fisher Scientific, New Jersey, USA), or Invitrogen molecular probes (Euegne, Oregon, USA). Antibodies were purchased from Immunology Consultant Laboratory, Newberg, OR USA. SEB toxin was provided by Dr. Gregory Bohach (Univ. of Idaho, Moscow). Supra Gemini 35 VP FE-SEM (Zeiss) coupled with Thermo Electron (Zeiss) model was used for electronic imaging of F-SiNPs. FLx800 plate reader (from BioTek instrument Inc., Winooski, VT, USA) instrument was used for fluorocounting. Fluoro Max-3 (Horiba Jobinyvon) model was used for emission fluorescence spectra and with fluorescence version 2.0.5.0 software.

2.2 Synthesis and Surface Modification of F-SiNPs 5

2.2.1. Synthesis of monomer fluorophore 3.

First, the monomer fluorophore precursor was prepared according to the method described by A. V. Blaadern et al. [9]. The fluorescein isothiocynate (FTC; 2.57 x 10^{-3}) was reacted to the 3-aminopropyltriethoxysilane (APS; 0.0427 M) in anhydrous ethanol for at least 12 h under magnetic stirring in a dried flask. Water was excluded to prevent hydrolysis of the APS molecules.

2.2.2. Synthesis of encapsulated F-SiNPs 5.

The F-SiNPs were synthesized from tetraethoxy silane (TEOS) and monomer precursors **3** in water-in-oil microemulsion [5, 10]. The microemulsion was prepared by mixing 1.77 ml of triton X-100 (surfactant), 1.8 mL of *n*-hexane (co-surfactant), 7.5 ml of cyclohexane (oil), 0.5 ml of DI water and 0.5 ml monomer flurophore **3**. The silica precursor (100 µl of TEOS) was added to the microemulsion solution and stirred for 30 min. The silica polymerization reaction was completed by adding 60 µl NH_4OH and then stirring for 24 h. The microemulsion was broken by the addition of 20 ml of acetone to the microemulsion, followed by sonicating, vortexing, and centrifuging. The dye-doped silica NPs were thus separated from the microemulsion. Finally the resulting NPs were washed four times with 95% ethanol and one time with acetone. The samples were then left dry overnight. To encapsulate these NPs, the above procedure was repeated once again for 4 h, with 50 µl of TEOS, 20 µL of NH_4OH and without monomer fluorophore **3**. The surface functionalization of SiNPs at each step was characterized using FT-IR spectroscopy. The size and morphology of F-SiNPs **5** were characterized using a scanning electron microscopy.

2.2.3. Surface modification of F-SiNPs with *anti*-SEB polyclonal antibody.

Dried F-SiNPs **5** were suspended in 5 ml of 2 M sodium carbonate solution (activation buffer) by ultrasonication. A solution of cyanogen bromide (CNBr) in acetonitrile (0.25 g of CNBr dissolved in 0.5 ml of acetonitrile) was then added dropwise to the particle suspension under stirring for 10 min at room temperature (rt). Activated particles **6** were washed twice with ice-cold water. Antibody immobilization onto the silanized surface was achieved using the cyanogens bromide pretreated fluorescent nanoparticles **6**, as shown in scheme 1 [11]. A 0.5 µl portion of *anti*-SEB polyclonal antibody diluted in PBS buffer (pH 7.2) was added to the surface-modified nanoparticles **6** with the final concentration of 1 µg/ml, and suspension was stirred for 24 h at 4 ºC. Antibody immobilized nanoparticles were then treated with 1.5 ml of 0.03 M glycine solution for 30 min. The final product 7 was washed, resuspended in PBS (pH 7.2) buffer, and stored at 4 ºC for future use.

2.2.3. Fluorescence and Photostability Test.

A solution of 0.1 µM of FTC and a suspension of 0.1 µg/ml of F-SiNPs **6** were prepared in 0.1N NaOH and used for emission fluorescence characterization. Each solution (2 ml) was placed into a quartz cuvet and placed in fluorospectrometer. The emission spectrum was recorded after applying excitation wavelength 466 nm with entrance and exit slit of 5 nm. To check photostability of F-SiNPs, the two samples were prepared (0.1 µM of FTC and 0.1 µg of F-SiNPs **6**) in PBS buffer (pH = 7.2) and placed (5 mL each) into a glass tube. The tubes were placed into a beaker having water at room temperature and set up in front of an ARC lamp. Fluorocounting of each sample was done at two hr. intervels, keeping sample temperature constant at rt. Further F-SiNPs were washed before each reading.

2.3 SEB Toxin Detection Assay

Coupling of tosylated magnetic beads (Dynabeads® M-280 Tosylactived) with *anti*-SEB polyclonal antibodies was performed as described in Invitrogen protocol [12] and the modified beads were stored in 2 ml PBSTB (PBS + 0.05% Tween-20 surfactant + 3% bovine serum albumin) buffer (~ 3 mg magnetic beads/ml) at 4 °C prior to use. *Anti*-SEB antibody magnetic beads (4 µl) were mixed with commercially available 1% fat milk (100 µl) samples, pre-spiked with different concentrations of SEB toxin (1, 10, 25, 50, 100, 500, 1000, and 10,000 pg/ml). The milk alone was used as a negative control. The magnetic beads were mixed in each sample by gently pipeting the mixture up and down and then incubating at 37 °C on rotator for 30 min. The tubes were then placed into the magnetic rack, and beads were allowed to separate for 2 min before the milk was removed. The beads were then washed one time with 150 µl PBST buffer and suspended in PBS buffer (90 µl). The PBS suspension containing F-SiNPs-*anti*-SEB antibody conjugate 7 (10 ng/ml) was then added (10 µl) to each tube and placed on rotator for 30 min at 37 °C after which MBs of each tube were washed three times with 150 µl of PBST and finally one time with PBS. The MB were then resuspended in PBS (pH = 7.2) with a final volume of 200 µL and transferred into a 96 well plate. The plate was inserted into fluorocount reader. Fluorescence was determined by exposing to 485±20 nm and relative fluorescent emission units (RFU) recorded at 528±20 nm.

3 RESULTS AND DISCUSSION

SEM, TEM and FTIR Characterization of F-SiNPs:

In this paper, biomolecules were immobilized onto the surface of cyano-functionalized silica nanoparticles doped with organic dye. The careful synthesis of nanoparticles is very important and thus the concentration of each reactant, reaction time, and temperature is critical for the morphology of nanoparticles. The synthetic method (W/O reverse microemuslion) as shown in scheme 1 is very good for synthesis of uniform organic dye doped and silica encapsulated nanoparticles. The SEM and TEM were employed to characterize the silica encapsulated F-SiNPs **5**. As shown in fig. 2a, and b, the core-shell structural particles were spherical and uniform and observed in the range of 100 nm of diameter.

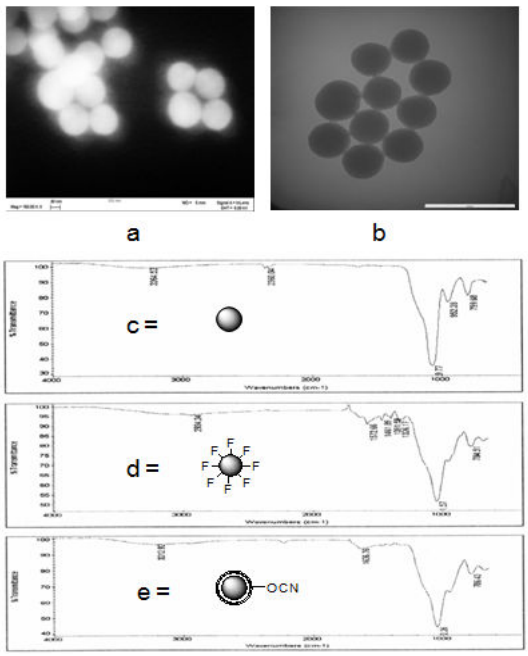


Figure 1: (a) SEM (scale bar = 100nm), and (b) TEM (scale bar = 0.25 µm) images of (F-SiNPs). FTIR analysis of (c) plain silica nano particles, (d) F-SiNPs **4**, and F-SiNPs **6** after cyanogens bromide reaction.

Fig. 2c-e shows the Fourier Transform Infrared (FTIR) spectra of pure and modified silica nanoparticles. A sharp and strong Si-O-Si stretching peak (~ 1100 cm^{-1}) was observed in all surface modified-samples indicating that the main structure was not changed by the modification reaction. The peak at ~ 960 cm^{-1} corresponding to a silanol group is very prominent in plain silica compared to the modified one, indicating that functionalization has occurred. The FTIR spectrum of F-SiNPs **4** showed absorption at ~ 2934 cm^{-1} which indicates the presence of $-CH_2$ groups. Two additional peaks were observed for sample **4** compared to plain silica, at ~ 1572 cm^{-1} (amide CONH stretching) and 1326 - 1461 cm^{-1} (aromatic stretching). This indicates that the fluorescein molecule is present in the SiNPs. Finally, a medium peak observed at 2220 cm^{-1} in fig. 2e corresponds to nitrile (CN group), clearly indicating the cyanogen modification of F-SiNPs **5**.

Fluorescence and Photostability Test of F-SiNPs 6.

The newly designed fluorescein dye doped silica NPs **6** emit high fluorescence signal as shown in fig. 2a, and are comparable to the starting material (fluorescein dye), which was used as a positive standard.

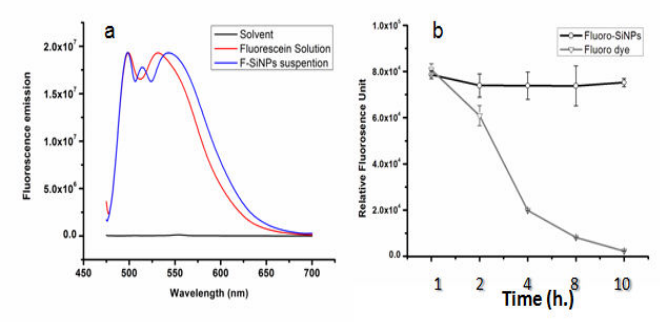


Figure 2: (a) Comparisons of emission fluorescence spectra of F-SiNPs **6** (0.1 µg/ml) and fluorescein dye (0.1 µM) in PBS (pH= 7.2) buffer, (b) comparative photostability measurement of F-SiNPs **5** (0.1 µg/ml) and fluorescein dye (0.1 µM) in PBS buffer. The samples were excited with a HBO 50 W mercury arc lamp, keeping sample temperature constant (at room temperature).

The fluorescence emission intensity of (0.1 µg/ml) F-SiNPs was observed similar to the emission properties of free fluorescence dye (0.1 µM) at λexc. = 466nm and λem. = 520nm. The photostability measurements of F-SiNPs were carried out by continuously illuminating particle samples with a 50W mercury lamp. Fig. 2b shows the photostability of F-SiNPs that were prepared by the W/O reverse microemulsion method (top curve) as compared to the 0.1 µM aqueous solution of the dye in PBS (pH = 7.2) buffer. As expected the dye doped SiNPs were highly photostable in comparison with the free dye. This indicates that the silica particles are loaded with a high amount of fluorescein dye, and that they are encapsulated with silica layer, which protects them from photobleaching factors and did not allow leakage of the dye when immersed in aqueous solution. The high fluorescence emission properties of F-SiPNs make them potentially useful for bioanalysis and biomedical imaging.

SEB Toxin Detection Assay

In this experiment we demonstrated that the target SEB toxin protein was captured from the milk sample by using the MB conjugated with polyclonal *anti*-SEB antibody. We also showed that the F-SiNPs labeled with secondary anti-SEB antibody reacted with the MB isolated SEB and resulted in an optical signal.

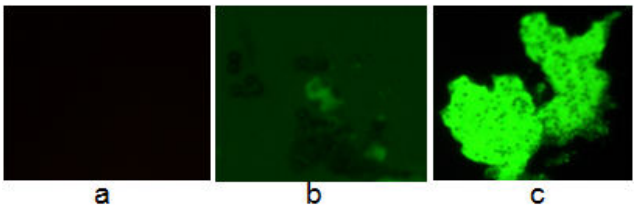


Figure 3a: Fluorescent microscopic images of (a) MB-*anti*-SEB antibody (showing no fluorescence), (b) MB- *anti* SEB antibody and F-SiNPs without SEB toxin (showing negligible nonspecific binding), (c) MB-*anti*-SEB antibody-SEB toxin-F-SiNPs **7** (showing dense binding of F-SiNPs **7** with SEB protein in sandwich model).

In fig. 3, the images show the fluorescent signals of (c) MB-SEB-F-SiNPs with only (a) MBs and (b) MB-F-SiNPs without SEB. An intense fluorescent signal is noted in (c) from the sandwich assay of MB-SEB-F-SiNPs, while MB alone gave no fluorescent emission and the fluorescent signal of MB-F-SiNPs is almost equal to the blank. This indicates that the F-SiNPs labeled secondary antibody is binding with target and giving the desired optical signal.

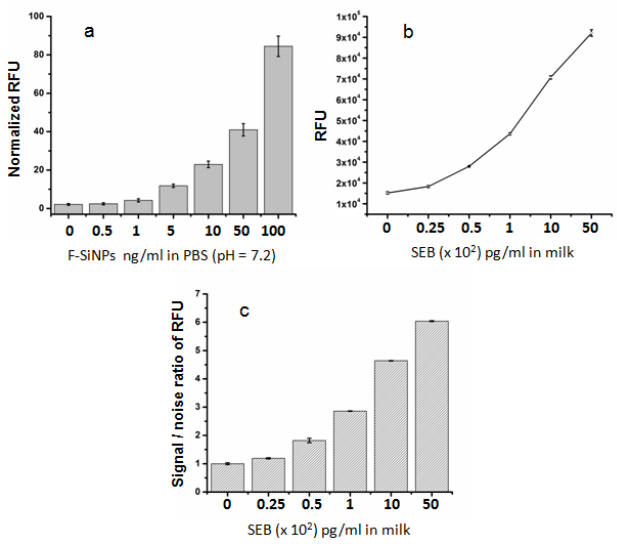


Figure 4: (a) Calibration graph of F-SiNPs **6,** (b) RFU for detection of SEB through sandwich model of MB-SEB-F-SiNPs **7** from SEB spiked samples. (c) RFU of signal and noise ratio for SEB detection method. For reading RFU, F-SiNPs and MBs were resuspended in 200 µl of PBS (pH = 7.2).

A fluorocounting calibration curve was obtained from F-SiNPs **6** at different concentrations (fig. 4a), which shows that 10 ng/ml F-SiNPs gives 25 times more fluorescence than the blank. For the MB-SEB assay in milk, 10 ng/ml F-SiNPs **6**-*anti*-SEB antibody were incubated with the different concentration of SEB in milk (fig. 4b), where MB (without SEB in milk sample) was used as a negative control. The ratio of RFU between signal and noise were calculated and showed in fig. 4c. The minimum statistically significant fluorescence signal was measured at a concentration of 100 pg/ml of SEB ($p < 0.05$) in the sample. Dose dependent behavior was observed for the detection of SEB between 25 pg/ml and 50 pg/ml. Each experiment was performed in triplicate set and the values of RFU are the average with standard deviations.

4 CONCLUSION

This research showed that the synthesis of fluorescein dye-doped-silica nanoparticles (F-SiNPs) and its encapsulation with a silica layer using W/O reverse micro emulsion method, yield uniform (~100 nm of diameter), highly fluorescent and photostable nanopaticles. The preliminary studies of the application of F-SiNPs for biological detection showed the possibility for the SEB toxin detection in milk samples. The lowest detection limit was observed from 100 pg/ml of SEB solution. The SEB detection assay system presented here can be used as high-throughput assay format, because this assay was performed in the total volume of 100 µl and the total time of this assay is less than 2h. In summary, the F-SiNPs could easily be utilized for development of a variety of detection systems for biological materials.

5 ACKNOWLEDGEMENTS

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