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A novel sensitive staphylococcal enterotoxin C_1 fluoroimmunoassay based on functionalized fluorescent core-shell nanoparticle labels

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Abstract

A highly sensitive fluoroimmunoassay for the determination of staphylococcal enterotoxin C_1 (SEC_1) is proposed. It is based on the functionalized fluorescent core-shell nanoparticles as the labels coated with anti-SEC_1 monoclonal antibodies in "sandwich" fluoroimmunoassay. With the simple, inverse microemulsion polymerisation method, the functionalized fluorescent core-shell nanoparticles were prepared easily. The preparation process produces a silica shell on the surface of the $Ru(bpy)_3Cl_2$ (Rubpy) dye with one step cohydrolysis of tetraethylorthosilicate (TEOS), and the coupling agent (3-aminopropy1)triethoxysilane (APS) provided the amine groups that can be used for biological conjugation. The nanoparticles were then labeled with the anti-SEC_1 monoclonal antibodies and the antibody-labeled nanoparticles were successfully used for the determination of SEC_1. The calibration graph for SEC_1 was linear over the range 1.0–75.0 ng ml^{-1} with a detection limit of 0.3 ng ml^{-1}. The regression equation of the working curve was $I_F = 24.583 + 0.6426[SEC_1]$ (ng ml^{-1}) ($r = 0.9991$). The relative standard deviation (RSD) for five parallel measurements of 25.0 ng ml^{-1} SEC_1 was 2.5%. Furthermore, the application of fluorescence microscopy imaging in the study of the antibody labeling and sandwich fluoroimmunoassay with the functionalized fluorescent core-shell silica nanoparticles was also explored. The results demonstrate that the method offers potential advantages of easily labeling to the antibody, sensitivity, simplicity and reproducibility for the determination of SEC_1 and is applicable to the determination of SEC_1 in real samples and enables fluorescence microscopy imaging for the determination of SEC_1.

Keywords: SEC_1; Functionalized; Fluorescent core-shell nanoparticle; Rubpy; Fluoroimmunoassay; Fluorescence microscopy imaging

1. Introduction

Toxins in environmental samples can be produced by many routes such as industrial, agricultural, or military activity. Toxic compounds may also be found in environmental samples as a result of terrorist activity. Pollutants, such as pesticides and explosives from munitions storage facilities, also contaminate soil, ground water and food and are known to be toxic to humans at ng ml^{-1} levels. Toxins also occur naturally in the food supply. Mycotoxin contamination is a particular problem due to fungal infection of grains and peanuts and can still be present after food processing. Foodborne microbial diseases affect a large number of people each year. One of the most frequent diseases is gastroenteritis, which is caused by the ingestion of food contaminated with staphylococcal enterotoxins (SEs) (Balaban & Rasooly, 2000). Therefore, sensitive detection and monitoring of toxins in clinical fluids, environmental samples, food, and drinking water require new approaches in order to expedite appropriate countermeasures (Ligler et al., 2003). SEs belong to a family of major serological types of heat stable enterotoxins (SEA through SEE and SEG through SEJ). These toxins cause toxic shock-like syndromes and have been implicated in food poisoning and several allergic and autoimmune diseases.

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SECs are a group of highly conserved proteins with significant immunological cross-reactivity. The three antigenically distinct SEC subtypes are SEC_1, SEC_2, and SEC_3. As low as 100 ng SEs are sufficient to cause symptoms of intoxication in humans (Balaban & Rasooly, 2001, 2000). These biological effects and their toxicity make the detection of these toxins very important from the standpoint of public health concerns. Traditional methods for the detection of these toxins have employed many methods such as high pressure liquid chromatography, capillary electrophoresis and mass spectrometry, preceded by significant sample preparation steps to eliminate interference from other components of the sample matrix.

Some methods have been developed for the determination of SEs in different matrices. The analytical techniques have been put forward in the issued papers, including liquid chromatography and mass spectrometry (Kientz, Hulst, & Wils, 1997; Ligler et al., 2003; Rasooly & Ito, 1999), radioimmunoassays (RIAs) (Lam, Wan, Boulet, & Le, 1999), enzyme-linked immunosorbent assay (ELISA) (Giletto & Fyffe, 1998), piezoelectric crystal (PZ) sensor (Lin & Tsai, 2003), fluoroimmunoassays (Goldman et al., 2002), polymerase chain reaction (PCR) (Mantynen, Niemela, Kaijalainen, Pirhonen, & Lindstrom, 1997), electrochemical method (Dong, Luo, Feng, Li, & Gao, 2001) and fluorescence-based biosensor (Rowe-Taitt et al., 2000). Recently chemiluminescence imaging has been used for the determination of SEC_1 in our research group (Luo, Zhang, Chen, & Ma, 2006). However, these methods are often either less sensitive or have their intrinsic disadvantages such as technically complex, time consuming or demanding restricted available reagents for signal amplification. Immunoassay among the methods has been growing in popularity and acceptance because of the reduction in the requirement for extensive sample preparation and high selectivity (Ligler et al., 2003; Schneider, 2003), but it often requires the use of radioactive tracers or carcinogenic reagents.

In this paper, a novel SEC_1 fluoroimmunoassay method was established, where amino-modified fluorescent core-shell nanoparticles as the labels were conjugated with anti-SEC_1 monoclonal antibody using glutaraldehyde as a crosslinker. As most of the available immunoassays, SEC_1 was measured upon specific interaction between captured SEC_1 antigen and functionalized fluorescent core-shell nanoparticles-labeled anti-SEC_1 monoclonal antibody. In comparison to conventional organic dye labels, the functionalized fluorescent core-shell nanoparticles labels offers the advantages of both higher photostability to the excitation of light and stronger luminescence resulting in an amplification factor similar to that of enzyme labels because thousands of fluorescent dye molecules are encapsulated in the matrix that also serves as a shield to protect the dye from photobleaching (Bagwe, Yang, Hilliard, & Tan, 2004). Moreover, the functionalized fluorescent core-shell nanoparticles can be prepared easily under mild condition with the inexpensive reagents and simple inverse microemulsion polymerisation procedures, they have uniform sizes and high functionality and can be easily labeled with anti-SEC_1 monoclonal antibody (Bourgeat-Lami, 2002; Huhtinen, Soukka, Lovgren, & Harma, 2004; Liu et al., 2005; Santra, Zhang, Wang, Tapec, & Tan, 2001; Tan, Wang, Ye, & Yuan, 2006; Wang et al., 2004; Xu, Ayloott, Kopelman, Miller, & Philbert, 2001; Yang et al., 2003; Yang, Zhang, Qu, Yang, & Xu, 2004). The results show that the method offers potential advantages of simplicity, sensitivity and reproducibility to fluoroimmunoassay for SEC_1, and is applicable to the determination of SEC_1 in complicated samples such as water and milk. In addition, we also explore the application of fluorescence microscopy imaging in the determination of the SEC_1 with the fluorescent nanoparticles.

2. Materials and methods

2.1. Reagents and solutions

SEC_1, two monoclonal antibodies against SEC_1 with the protocol, were kindly donated by Prof. B.Q. Jin (Department of immunology, The Fourth Military Medical University, Xi'an, China). $Ru(bpy)_3Cl_2$ (Rubpy) and Bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). APS was obtained from Acros (Geel, Belgium). The compounds Na_2CO_3, $NaHCO_3$, KH_2PO_4, Na_2HPO_4, NaCl, KCl, Tween-20, NaH_2PO_4 and $NH_3 \cdot H_2O$ (28–30 wt%) were purchased from Xi'an Chemical Reagent Company (Xi'an, China). Tetraethylorthosilicate (TEOS), *n*-hexanol, Triton X-100 (TX-100) and cyclohexane were obtained from Shanghai Chemical Plant (Shanghai, China). Distilled, deionized water was used for the preparation of all aqueous solutions. Unless otherwise stated all chemicals and reagents used in this study were of analytical grade quality. Low fluorescent 96 well transparent microtiter plates used for the assay were obtained from Corning Incorporated (Troy, Michigan, USA).

The coating solution was 0.05 mol l^{-1} carbonate buffer, pH 9.6, containing 1.59 g Na_2CO_3, and 2.93 g $NaHCO_3$ per liter. PBS buffer (0.15 mol l^{-1}), pH 7.4, was prepared by dissolving 0.2 g KH_2PO_4, 2.9 g Na_2HPO_4, 8.0 g NaCl, and 0.2 g KCl in one liter of water. Ninety six-well plates (96-well plates) were rinsed with PBST solution (PBST: PBS solution containing 0.05% (v/v) Tween 20). SEC_1 antigen and antibody were diluted with PBSTB solution (PBSTB: PBST containing 0.1% (w/v) BSA).

2.2. Synthesis of functionalized fluorescent core-shell nanoparticles

Synthesis of functionalized fluorescent core-shell nanoparticles was carried out according to the methods described elsewhere (Santra et al., 2001; Wang, Yang, & Tan, 2005) with little modification. The water in oil (W/O) microemulsion was prepared at room temperature first by mixing 1.77 ml surfactant TX-100, 7.5 ml oil phase

cyclohexane and 1.8 ml cosurfactant *n*-hexanol. An aqueous Rubpy dye solution, 0.48 ml, was then added. The resulting mixtures were subsequently homogenized using a magnetic stirrer in order to form water-in-oil microemulsion. In the presence of 100 μl of TEOS, hydrolysis reaction was initiated by adding 60 μl of $NH_3 \cdot H_2O$ under stirring condition. The reaction was allowed to stir for 12 h. Then 100 μl of APS and 60 μl of $NH_3 \cdot H_2O$ were added to the mixtures under stirring. The reaction was allowed to stir for another 12 h. After the reaction was completed, acetone was added to break the microemulsion and recover the particles followed by centrifugation (about 12000 g) and washing with ethanol and water several times to remove surfactant molecules and physically adsorbed Rubpy dye from the particle's surface. The particles were air dried. The synthesized, functionalized fluorescent core-shell nanoparticles were characterized by spectrofluorophotometery for fluorescence intensity and by transmission electron microscopy (TEM; Hitachi H700, Hitachi, Tokyo, Japan) for the size and morphology.

2.3. Covalent immobilization of the antibody onto functionalized fluorescent core-shell nanoparticles surface

The anti-SEC_1 monoclonal antibody was directly immobilized onto the functionalized fluorescent core-shell nanoparticles with well-established glutaraldehyde method (Kiselev et al., 1999) as shown below. (1) Fluorescent core-shell nanoparticles 2 mg was dispersed into the PBS buffer containing 5% glutaraldehyde for about 2 h; (2) the nanoparticles were separated by centrifugation and washed with PBS three times. After the nanoparticles were re-dispersed in PBS, they were further incubated with anti-SEC_1 monoclonal antibody for 12 h at 4 °C with shaking; (3) The antibody-coupled fluorescent core-shell nanoparticles were washed with PBS several times to remove excess antibody and kept at 4 °C in PBS.

2.4. Sandwich fluoroimmunoassay of SEC_1 using the nanoparticle-labeled anti-SEC_1 monoclonal antibody

After anti-SEC_1 monoclonal antibody (100 μl per well) diluted 100-fold in 0.05 mol l^{-1} carbonate buffer, pH 9.6, was coated on the wells (100 μl per well) of a 96-well plate by physical absorption overnight at 4 °C, these wells were washed three times with PBST solution. The SEC_1 standard solution or sample (100 μl per well) were added to each well. After incubation at 37 °C for 1 h, these wells were washed with PBST buffer. Then the antibody-coupled fluorescent core-shell nanoparticles (100 μl per well) were added to each well and the plate was incubated at 37 °C for 1 h. These wells were washed three times with PBST buffer, and subjected to fluorometric measurement with the instrumentation system consisting of a spectrofluorophotometer with Xenon discharge excitation source (Shimadzu RF-540, Shimadzu, Kyoto, Japan) and a bifurcated glass optical bundle with 4.0 mm diameter at the common end (Oriel Co., Stratford, CT, USA). A new framework was made to hold one end of the bifurcated optical bundle toward the excitation beam and its other end toward the photodetector. The common end inserted perpendicularly in the well was also fixed with the framework.

Sandwich fluoroimmunoassay was also performed in Olympus inverted microscope system (Olympus, Model IX70, Toyko, Japan) with a 100 W high pressure mercury lamp (Olympus, Model BH2-RFL-T3, Tokyo, Japan) used as the light source. The 96-well plates used above was mounted over the microscope stage. The excitation light which comes from high pressure mercury was introduced through the inverted microscope objective from underneath the chamber. A CCD camera (Pixera, model PVC100C, Los Gatos, CA, USA) interfaced with a Pentium computer was employed for the acquisition of imaging of the SEC_1. The software of 1.2 Million Pixel Digital Camera v2.5 was used to deal with the imaging. In order to investigate the dispersivity and the fluorescence image of the functionalized fluorescent core-shell nanoparticles before and after conjugation with the antibody by using the microscope system, the solution of nanoparticles was dropped onto a slide for fluorescence microscopy observation.

3. Results and discussion

3.1. Preparation of functionalized fluorescent core-shell nanoparticles

The principle of the inverse microemulsion polymerization method to prepare the fluorescent nanoparticles was described earlier (Santra et al., 2001). TEOS and APS were used for nanoparticle preparation. Generally, TEOS and APS undergo hydrolysis reactions simultaneously to form the monodisperse spherical nanoparticles with the amino groups on the surface of the nanoparticles. However, the speed of hydrolysis of TEOS was faster than that of APS (Willner & Katz, 2000). The layer of amino groups formed with the hydrolysis of APS on the surface of nanoparticles could be obstructed by further formation of Si–O–Si bond with the hydrolysis of TEOS on the nanoparticle's surface and the remainder APS which has not been hydrolyzed can be embedded in the core of the silica nanoparticle. In order to solve this problem, we delayed the addition time of APS after 12 h hydrolysis of TEOS in W/O microemulsion. Using this method, more free amino groups were directly introduced on the nanoparticle's surface by using APS in the nanoparticle preparation, making the surface modification and bioconjugation of the nanoparticles easier.

3.2. Characterization of functionalized fluorescent core-shell nanoparticles

The inverse microemulsion method yielded uniform functionalized fluorescent core-shell nanoparticles. These

nanoparticles were characterized using microscopic methods. The results showed that the particle sizes of nanoparticles were about 47 ± 5 nm (Fig. 1). In addition, spectrofluorometric measurements were used to characterize the nanoparticles. Free Rubpy dye exhibited an emission at 595 nm when excited at 435 nm in aqueous solution. However, the emission maximum of the nanoparticles shifted 5 nm to the longer wavelength compared with the pure Rubpy dye (data not shown), indicating that the spectral characterization of the Rubpy dye did not change to any great extent when it was doped inside the nanoparticles.

3.3. Preparation of the nanoparticle-labeled antibody and sandwich fluoroimmunoassay of SEC_1

After the hydrolysis reaction of TEOS and APS, some amino groups were introduced on the nanoparticle's surface. These amino groups made the modification and the bioconjugation of the nanoparticles easier. As shown in Fig. 2a, the nanoparticles were first coupled with glutaraldehyde, and then the glutaraldehyde-coated nanoparticles were conjugated to the antibody by reacting the aldehyde groups of the glutaraldehyde-coated nanoparticle and the amino groups of the antibody. The typical "sandwich" type fluoroimmunoassay SEC_1 was used as described in Fig. 2b.

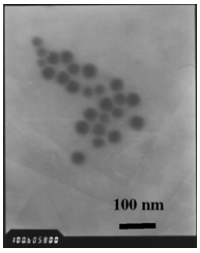

Fig. 1. TEM micrographs of functionalized fluorescent core-shell nanoparticles at 100,000× magnification.

A pair of antibodies that recognize different epitopes of the same antigen was used to capture and detect a certain antigen. In this study, we use the fluorescent nanoparticles-labeled anti-SEC_1 monoclonal antibody to capture the SEC_1 antigen and detect the fluorescence intensity for the determination of SEC_1. It is very easy to separate the nanoparticles-labeled anti-SEC_1 monoclonal antibody from the unreacted antibody by centrifugation and it is also a commonly used method to separate the silica nanoparticles from the biomolecules (Santra et al., 2001; Tan et al., 2006). There would be no fluorescence response signal if the nanoparticles were not labeled with anti-SEC_1 monoclonal antibody. The results of detection of the fluorescence intensity show that SEC_1 monoclonal antibody has been successfully conjugated to the nanoparticles.

3.4. Optimization of fluorescent nanoparticles surface antibody density

To investigate the effect of antibody density on fluorescence intensity, we incubated fluorescent nanoparticles with variable amounts of antibody and examined the effect on the antibody dose–response curve. In Fig. 3, one can observe that for 5 μg antibody per mg fluorescent nanoparticles, there was only about 43% dose response at a concentration of 20 ng ml^{-1} SEC_1 compared with that when 100 μg antibody was used. As the amount of antibody was increased from 5 to 50 to 100 μg antibody per mg fluorescent nanoparticles, the assay response increased. However, at 200 μg antibody per mg fluorescent nanoparticles, the assay response was increased slowly. Thus, there existed an optimum antibody density at about 100 μg per 1 mg

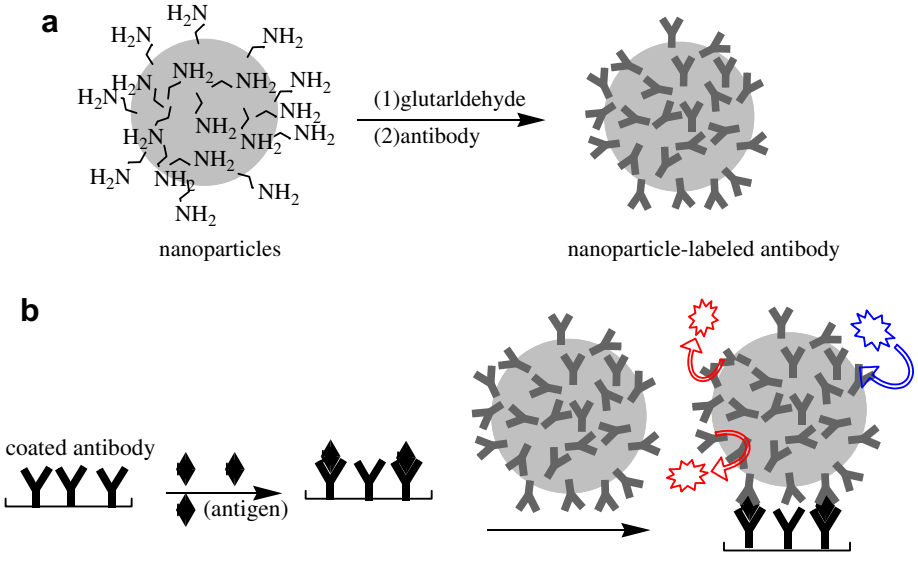

Fig. 2. Schematic representation of antibody immobilization process onto functionalized fluorescent core-shell nanoparticles (a). Principle of the sandwich fluoroimmunoassay (b).

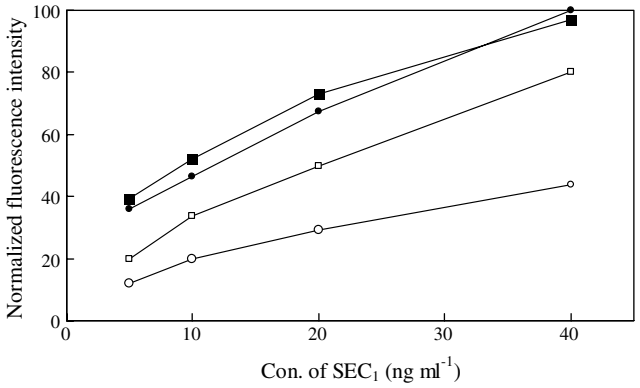

Fig. 3. Effect of fluorescent microsphere surface antibody density on assay response. Antibody density was increased by increasing amounts of antibody incubated per milligram of nanoparticles: 5 (open circles), 50 (open squares), 100 (closed circles) and 200 (closed squares) μg.

fluorescent nanoparticles, resulting in the best assay response. Therefore, incubation of 100 μg anti-SEC_1 monoclonal antibody with 1 mg fluorescent nanoparticles was recommended for further studies.

3.5. Optimization of fluorescent nanoparticles concentration

The effect of fluorescent nanoparticles concentration on assay response was determined with the results shown in Fig. 4. It can be seen that the fluorescence intensity increased greatly with increasing fluorescent nanoparticles concentration from 0.1 to 10 μg ml^{-1}. This enhancement abated at high reporter concentrations because of increased nonspecific binding and the approach of specific antigen-labeling saturation. It can also be noted that the noise increased but slowly with increasing fluorescent nanoparticles, 0.1–10 μg ml^{-1}, and increased greatly with increasing fluorescent nanoparticles, 10–100 μg ml^{-1}. Thus, the maximum signal/noise ratio was obtained at an intermediate concentration of fluorescent nanoparticles. Hence 10 μg ml^{-1} was chosen for the analytical procedure (see Fig. 5).

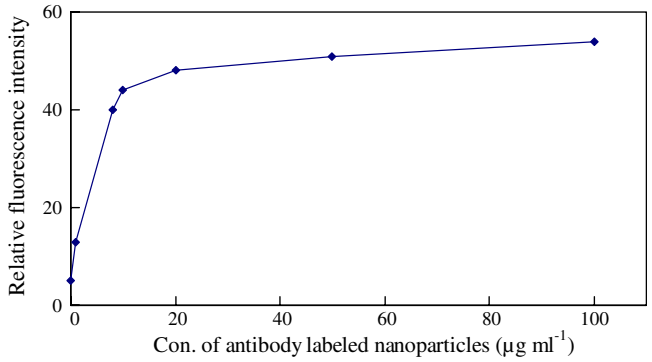

Fig. 4. Effect of nanoparticles labeled with antibody concentration on assay response at fixed 100 μg antibody per mg nanoparticles in the presence of 30.0 ng ml^{-1} SEC_1.

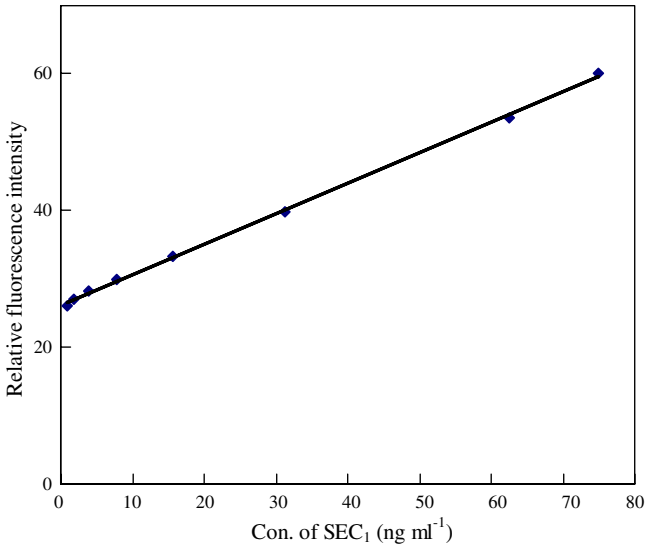

Fig. 5. Calibration curve of sandwich fluoroimmunoassay using the functionalized fluorescent core-shell nanoparticles-labeled antibody for SEC_1.

3.6. Working curve, detection limit, and precision

Under the optimum experimental conditions, the calibration graph of emission intensity (I_F) versus SEC_1 concentration was linear in the range of 1.0–75.0 ng ml^{-1}, and the detection limit was 0.3 ng ml^{-1} (3σ). The regression equation of working curves was $I_F = 24.583 + 0.6426$ [SEC_1] (ng ml^{-1}) with a correlation coefficient of 0.9991. The RSD was 2.5% for samples of 25.0 ng ml^{-1} of SEC_1 for 11 replications. Although there was fluorescence response signal from a wide range of concentrations of SEC_1 in this study, linearity range for the SEC_1 was 1.0–75.0 ng ml^{-1} and there was no linear correlation below 1.0 ng ml^{-1}. There would be a non-zero intercept in fluorescence intensity vs. concentration plot. This result maybe due to the existing physical adsorption between the nanoparticles and the antigen at low concentrations of SEC_1, which leads to no linear correlation below 1.0 ng ml^{-1}. In this study, SEC_1 was measured based on the interaction between antigen and monoclonal antibody. This method has the advantage of specificity in immunoassay, because the interaction between antigen and monoclonal antibody is specific intrinsically. Although there would be cross reactivities in immunoassay, in previous studies (Luo et al.,

Table 1
Results of recovery test

Sample	Original (ng ml^{-1})	Added (ng ml^{-1})	Found (ng ml^{-1})[a]	RSD (%)	Recovery (%)
Water1	0	10.0	9.8	2.1	98.0
Water2	0	10.0	10.3	3.2	103.0
Water3	0	20.0	20.2	1.9	101.0
Milk1	0	50.0	52.4	3.4	104.8
Milk2	0	50.0	48.9	2.5	97.8

[a] Average of three determinations.

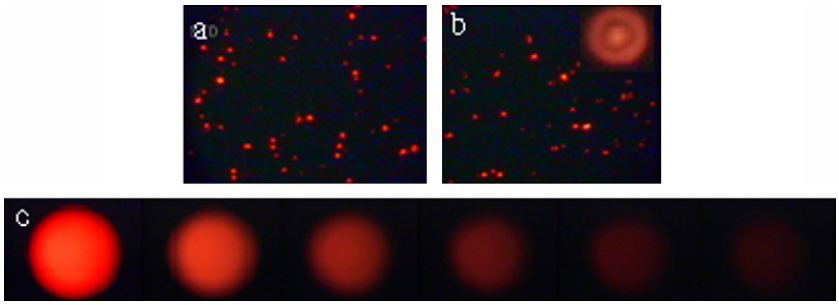

Fig. 6. Fluorescence image of functionalized fluorescent core-shell nanoparticles before (a) and after (b) labeled with antibody. The solution of nanoparticles was dropped onto a slide. Magnification 100 was used for the images in (a) and (b). Inset in (b): magnification 400. Fluorescence image of fluoroimmunoassay for SEC_1 with functionalized fluorescent core-shell nanoparticles-labeled anti-SEC_1 monoclonal antibody with 96-well plates (below). The concentration of the SEC_1 was 40.0, 30.0, 10.0, 5.0, 2.0, 0.5 ng ml^{-1} from left to right respectively.

2006; Dong et al., 2001) it has been proven that the interaction between SEC_1 antigen and anti-SEC_1 monoclonal antibody is also specific.

3.7. Recovery test of SEC_1 in water and milk samples

The feasibility of applying the proposed sandwich fluoroimmunoassay to measure toxin levels in a complex matrix was studied. This was conducted by adding various levels of SEC_1 into milk and water samples. The results of recovery test are shown in Table 1.

3.8. Fluorescence microscopy imaging

The dispersivity of the fluorescent nanoparticles or quantum dots (QDs) can often be investigated with TEM or SEM. The fluorescence images are usually observed using a fluorescent microscope system (Xie et al., 2005). The single and multiple nanoparticle imaging was also investigated by using an Olympus inverted microscope system. The functionalized fluorescent core-shell nanoparticles and the antibody-labeled functionalized fluorescent core-shell nanoparticles were found to be of good dispersivity as shown in Fig. 6a and b. To investigate the feasibility of using functionalized fluorescent core-shell nanoparticles in fluorescence microscopy imaging for the fluoroimmunoassay, the fluorescence microscopy imaging was used for determination of various concentrations of the SEC_1. Fig. 6c shows the fluorescence microscopy images of the fluoroimmunoassay for SEC_1 with functionalized fluorescent core-shell nanoparticles. It can be seen from the image that the fluorescence intensity gradually increased with increasing concentration of the SEC_1.

4. Conclusions

The ability to measure accurately and precisely very low amounts of toxins in clinical fluids, environmental samples, food, and drinking water is very important. In this contribution, a novel method for SEC_1 detection was developed with fluoroimmunoassay and nanoparticle labels. The dynamic range and precision of the assay met the requirements for a quantitative method. The proposed method is sensitive, simple and can reliably measure SEC_1 in food and water samples. Therefore, these merits should make it easily popular and should be used for various real applications.

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