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# An ultrasensitive immunosensor array for determination of staphylococcal enterotoxin B

Xiaoming Zhang[a,1], Fei Liu[b,1], Ruifang Yan[a], Pan Xue[a], Yongming Li[b], Lili Chen[a], Chaojun Song[b], Cui Liu[a], Boquan Jin[b], Zhujun Zhang[a,*], Kun Yang[b,**]

[a] Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Materials Science, Shaanxi Normal University, Xi'an 710062, China
[b] Department of Immunology, The Fourth Military Medical University, Xi'an 710032, China

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ABSTRACT

Staphylococcal enterotoxin B (SEB) is a potent gastrointestinal toxin and is heat resistant. SEB is also a potential bioterrorism agent. The ability to measure accurately very low amounts of staphylococcal enterotoxin B in food and other samples is very important. A highly sensitive and stable sandwich fluorescence immunoassay based on a pair of monoclonal antibodies against SEB which were produced by us was developed. Classical sandwich immunoassay was adopted and the glass slides were used as the base of the immunologic reaction. The functionalized fluorescent core–shell silica nanoparticles were used as labels. The fluorescence issued from the labels was detected by a laser-induced fluorescence millimeter sensor array detection platform. The fluorescence intensity has a linear relationship with the amount of SEB in the range of 50 pg/mL–5 ng/mL, and the detection limit of SEB was 20 pg/mL (the absolute detection limit was 0.02 pg). The relative standard deviation (RSD) for 5 parallel measurements of SEB (1 ng/mL) was 9.2%.



## 1. Introduction

Staphylococcal enterotoxins (SEs) are an important group of toxins implicated in several illnesses. There are now 19 known types of SEs and SE-like proteins (SEA–SEV, except that there is no SEF). Several SEs have multiple minor variant forms [1]. The classical and well-recognized SEs include SEA, SEB, SEC, SED and SEE. The remaining SEs are newer and less characterized [2–4]. SEs are also known as biological warfare agents [5–7]. An environmental exposure to even a sub-toxic dose of certain biological agents can lead to serious outcomes, due to the amplification of toxicity via in vivo replication in the human host [8]. As one of the major SEs causing food poisoning, staphylococcal enterotoxin B (SEB) is extremely toxic with a half-lethal dose (LD50) of about 20 ng/kg and a half-effective dose (ED50) of about 0.4 ng/kg [9], so it is very important to detect very low amounts of SEB in food and other samples.

During the past decades, many methods were developed for the detection of SEB, such as Enzyme-Linked Immunosorbent Assays (ELISA) [10–12], reversed passive latex agglutination (RPLA) [13], immunofluorescence technique [14], and a new eight-channel lab-on-a-chip (LOC) for SEB detection also has been reported [15]. Recently, several useful antigen/antibody-based biosensors for SEB assay have been proposed, such as surface plasma resonance (SPR) biosensors [16], resonant acoustic profiling (RAP) [17], biological semiconductors (BSCs) [18], fluidic force discrimination (FFD) assay [19], flow-injection capacitive biosensor [20], and piezoelectric-excited millimeter-sized cantilever (PEMC) sensors [21]. However, these sensors are mostly used in scientific research in special professional departments, and even some of them are more expensive and not suitable for high-throughput detection.

Immunofluorescence technique has a relatively long history, but traditional immunofluorescence technique has lower sensitivity. Laser induced fluorescence detection has higher sensitivity than normal fluorescence detecting method [22]. In this study, we produced a pair of monoclonal antibodies against SEB recognizing different epitopes of same antigen for enhancing the specificity, and constructed a new laser induced fluorescence immunoassay which has higher sensitivity compared with traditional Immunofluorescence technique by using laser as the light source and functionalized fluorescent core–shell nanoparticles (FFCSNs) as a fluorescent labels.

The fluorescence detection was achieved on the laser-induced fluorescence millimeter sensor array detection platform to avoid the use of expensive confocal Lasers Scanning Microscope. The

* Corresponding author. Tel.: +86 02985308184.
** Corresponding author. Tel.: +86 02984774531.
*E-mail addresses:* zhangzj@snnu.edu.cn (Z. Zhang), yangkunkun@fmmu.edu.cn (K. Yang).
[1] These authors contributed equally to this work.

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platform constructed by us can also be extended to other applications, such as protein and drug screening, sub-edged analysis of three-dimensional array of cellular samples and evaluation of the existence of special membrane proteins on the cell surface.

## 2. Experimental

### 2.1. Reagents and solutions

Staphylococcal enterotoxin was obtained from the Academy of Military Medical Sciences, (Beijing). Fetal bovine serum (FBS) was purchased from Gibco (Invitrogen Corp., Grand Island, NY). Anti-SEB mAbs of FMU-SEB-2 used as coating antibody, and anti-SEB mAbs of FMU-SEB-1 used as captive antibody both were made by our group. The glass slides were obtained from Taizhou Dongsheng Glass Co. Ltd. (Zhejiang, China). $Ru(bpy)_3Cl_2$ (Rubpy), Triton X-100 (TX-100), glutaraldehyde were obtained from Sigma (USA). (3-Aminopropyl)-triethoxysilane (APS) was obtained from Jinan DuoWeiQiao chemical industry company (Jinan, China). Tetraethyl orthosilicate (TEOS), n-hexanol, cyclohexane were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Other chemicals used in this study were purchased from Xi'an Chemical Reagent Company (Xi'an China). Phosphate buffer solution (PBS, 0.01 mol $L^{-1}$, pH 7.4) was prepared by dissolving 0.2 g KCl, 8.0 g NaCl, 0.2 g $KH_2PO_4$, and 2.9 g $Na_2HPO_4$ into 1000 mL double distilled water. The double distilled water was used for the preparation of all aqueous solutions. Unless otherwise stated, all chemicals and reagents used in this study were analytical grade quality and without purification.

### 2.2. Apparatus

ACQ-600 ultrasonic processor (Xi'an, China) was used for the dispersion of the FFCSNs, and HC-3018R Refrigerated Centrifuge (Anhui, China) was used for the centrifuge of the FFCSNs. JEM-2100 Transmission Electron Microscopy (Hitachi, Japan) was employed to investigate size and morphology of the FFCSNs. Constant temperature water bath (Zhejiang, China) was used as the incubator of the immunologic reaction. F-4600 fluorescent spectroscopy (Hitachi, Japan) was used to measure the fluorescence of the nanoparticles. Laser induced fluorescence measurement device was made by ourselves for the measurement of the fluorescence intensity (Fig. 1).

### 2.3. Synthesis of FFCSNs

Synthesis of FFCSNs was carried out according to methods described by a previous paper [23] with little change. At first 7.5 mL cyclohexane, 1.8 mL TX-100 and 1.8 mL n-hexanol were mixed for 30 min. After 4.8 mL $Ru(bpy)_3$ deionized water solution was added and stirred for 1 h, the W/O microemulsion was formed. Then 100 μL TEOS was added to the mixture. After stirring for 30 min, 60 μL ammonium hydroxide (25–28%) was added to the mixture and the reaction was continued for 12 h. 100 μL of APS was added to the mixture, followed by the addition of 60 μL ammonium hydroxide (25–28%). The reaction continued for another 12 h. To remove the remaining surfactants and the $Ru(bpy)_3$ absorbed on the surface of the nanoparticles, the silica nanoparticles were centrifuged at 8000 rpm and washed with ethanol as well as water several times. Finally, the nanoparticles were dispersed in water for potential usage.

## 3. Manufacturing of protein microchips

### 3.1. Pretreatment of glass slides

The glass slides were cleaned and pretreated according to Ref. [24]. Shortly thereafter, the glass slides were ultrasonically washed with the detergent and soaked in a piranha solution ($H_2SO_4/H_2O_2$, 3:1 [v/v]) for 12 h and then with 10 mol $L^{-1}$ NaOH solution for another 12 h. After washed thoroughly by double distilled water, the glass slides were dried with $N_2$ stream.

### 3.2. Introduction of aldehyde onto glass slides

Gluteraldehyde (GA) covalent coupling strategies have been applied for immobilizing antibody onto glass slide surfaces, and the chemical modified procedure for the glass slide surfaces is demonstrated in details below. Silanization of the glass slide surfaces was initially carried out by immersed in 2% (v/v) APS solution and holding for 30 min reaction at room temperature. After were washed three times with ultrapure water, the glass slides were dried for 3–5 min using $N_2$ stream. After silanization, the glass slides were cured in an oven for 30 min at 105 °C, and then the glass slides were immersed in 2.5% (v/v) GA solution and the reaction was kept for 1 h at 37 °C. Then, the slides were immerged in PBS buffer (pH 7.4) and washed thoroughly with ultrapure water, then was dried with $N_2$ stream.

### 3.3. Immunoreactions

In order to immobilize antibody onto the dot of the chip to make a microarray, we applied methods of manual spotting. First, small circles ($d$ = 1 mm) were drawn on the chip with a hydrophobic pen. Next, 1 μL anti SEB monoclonal antibody FMU-SEB-2 (mAb) with a final concentration of 100 μg/mL diluted with PBS was dotted in each circle and left overnight at 4 °C. The chip was then washed twice with PBST solution (0.01 M PBS with 0.5% Tween 20, pH 7.4) for 10 min at room temperature and dried under a stream of $N_2$ gas. Subsequently, the chip was blocked with 4% FBS diluted in PBS for 1 h at room temperature to block the spot unreacted with antibody. After the chip was washed with PBST and dried with $N_2$ stream as previous described, 1 μL SEB which was diluted in series concentrations with PBS contained 30% glycerol to prevent evaporation was spotted on the chip and incubated in a wet chamber at 37 °C for 1 h. The chip was then washed twice. Then, 1 μL the other anti SEB monoclonal antibody FMU-SEB-1 (mAb) labeled with FFCSNs diluted with PBS was added on each spot and incubated in a constant temperature water bath at 37 °C for 30 min. The chip was then washed

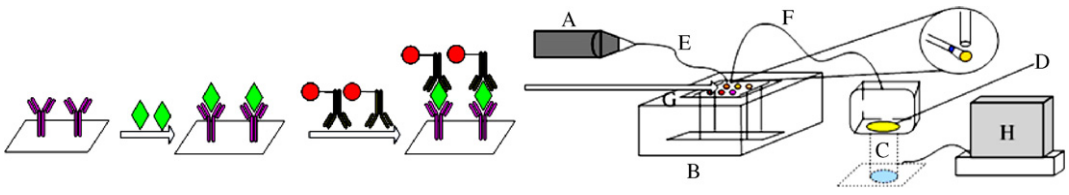


**Fig. 1.** Laser induced fluorescence device. Laser emitted by a semiconductor laser light source (A) (405 nm, 1 mW) was exposed to the millimeter array of spots on the glass slides G which was set in the dark box (B) through optical fiber (E). The fluorescence induced by laser was collected through glass fiber (F) (diameter 3 mm) and detected with a photomultiplier tube (C) after reflected light was filtered by a cut-off filter (D) (450 nm). Then the electronic signal was output by a computer (H).

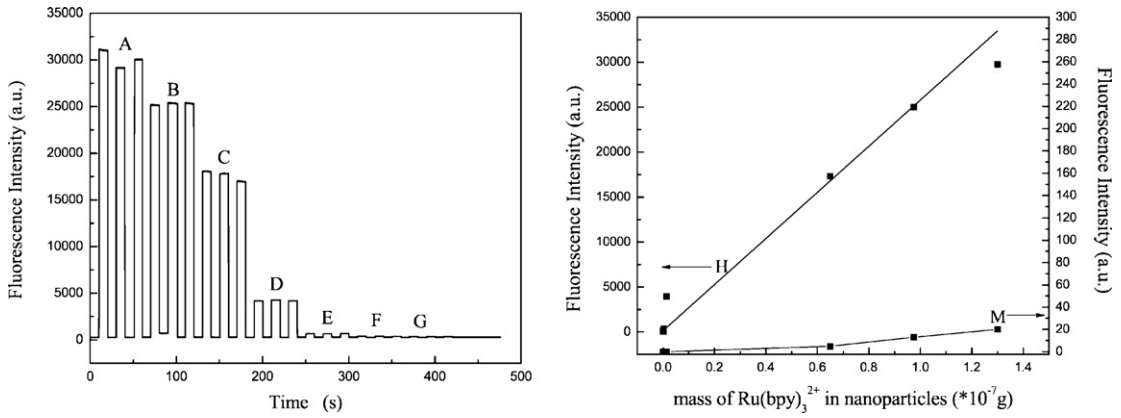


**Fig. 2.** Comparison of the sensitivity of the homemade device. Fluorescence Intensity of nanoparticles induced by laser lamp and Xenon lamp (A)–(G) were intensity of fluorescence of different amount $Ru(bpy)_3{}^{2+}Cl_2$ in Silica nanoparticles induced by laser is shown in left. The linear range is shown in right. H was the response curve of the fluorescent intensity of different amount $Ru(bpy)_3{}^{2+}Cl_2$ in Silica nanoparticles induced by laser, M was the response curve of the fluorescent intensity of different amount $Ru(bpy)_3{}^{2+}Cl_2$ in Silica nanoparticles induced by Xenon lamp.

twice. Blocking and washing processes were performed with the base plate placed in an upside–down position, with gentle shaking on a shaking plate. The fluorescence intensity was measured by the device homemade.

## 4. Results and discussion

### 4.1. The performance of laser induced fluorescence device

During the assembly of the instrument, two kinds of light source (Laser (405 nm) and Xenon lamp (405 nm, Shimadzu, RF-540, 150 W, Slit 20 nm)) were used separately, then the sensitivity of our homemade device using the two kinds of light source were compared. The fluorescent detection results showed that the detection limit of our homemade device using laser as light source was 0.1 ng Rubpy when 1 μL nanoparticle's aqueous solution was used, while it was 50 ng if using Xenon lamp as light source. The sensitivity of the device using laser as light source was 500 higher than that using Xenon lamp as light source. The results demonstrated that the detection limit, sensitivity, and stability of our homemade device using laser as light source can meet the needs of normal immunoassay (Fig. 2).

## 5. Characterization of FFCSNs

### 5.1. Size of the FFCSNs

TEM was used to investigate the morphology of the silica nanoparticles. The image demonstrated that the nanoparticles were $30 \pm 5$ nm in diameter and uniform without aggregation (Fig. 3).

### 5.2. Fluorescence properties of FFCSNs

During the synthesis processing, the concentration of the $Ru(bpy)_3$ doped in the nanoparticles was optimized, and $2.0 \times 10^{-2}$ mol $L^{-1}$ was chosen as the concentration of the $Ru(bpy)_3$s solution lastly in order to avoid the fluorescent quenching.

The fluorescent emission of the nanoparticles is indicated in Fig. 4. $Ru(bpy)_3$ and nanoparticles both have absorbance peaks at 455 nm; the fluorescent emission peaks emerged near 600 nm while the $Ru(bpy)_3$ emission peak is at 595 nm. The emission peaks of thesilica nanoparticles suffer from red shift (595–598 nm) compared to the pure $Ru(bpy)_3$ in aqueous solution.

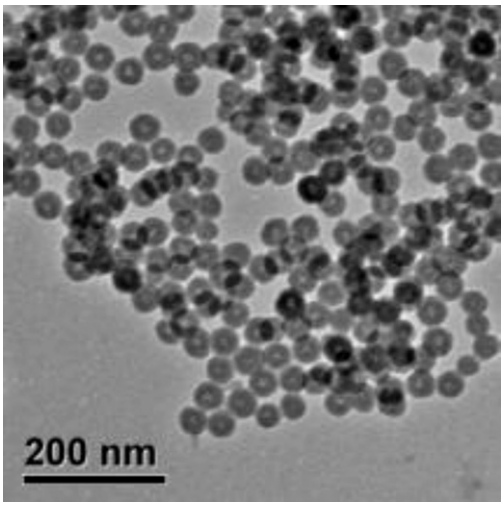


**Fig. 3.** TEM of core–shell fluorescent nanoparticles.

Photo bleaching is a crucial property of fluorophores. In this study, F-4600 fluorescent spectrophotometer was used to observe photo bleaching. We observed an interesting phenomenon that not only our fluorescent nanoparticle's aqueous solution, but also

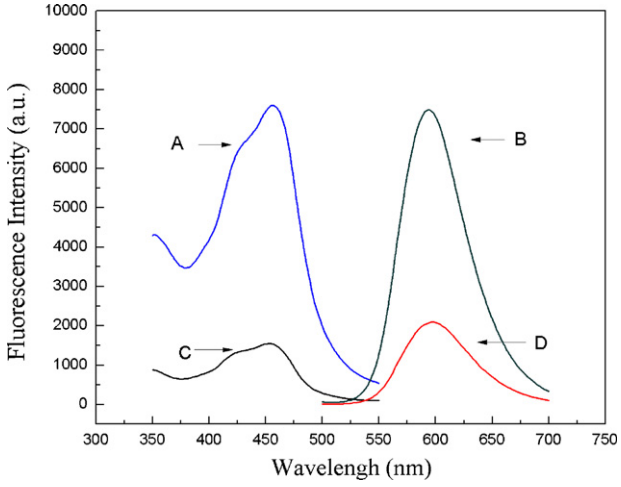


**Fig. 4.** Fluorescent spectrum of the $Ru(bpy)_3$ and our nanoparticles (C) and (D) are the excitation and emission spectrum separately of the $Ru(bpy)_3$, (A) and (B) are the excitation and emission spectrum separately of the nanoparticles.

pure Rubpy aqueous solution both were steady when continuously exposed to light if their concentration is high or volume is large. However, if the concentration and sample volume were lowered down, only pure Rubpy aqueous solution showed obvious photo bleaching. This detail is similar to other researcher's observation [25].

Silica nanoparticles have many pores due to they are synthesized by the hydrolysis of TEOS [26]. In order to study the leakage of the dye, the fluorescent nanoparticles were dispersed in PBS, and the fluorescence was measured. After the nanoparticles were centrifuged and re-dispersed in PBS, the fluorescence was measured once again. This was repeated 4 times in one day. We found that fluorescence intensity decreased only 18% in one day. The results demonstrated that our nanoparticles can be used as Biological fluorescent labels.

### 5.3. Optimization of the FFCSNs-labeled sandwich fluorescent immunoassay (FIA)

A typical "sandwich" FIA was used in our study. We use a pair of monoclonal antibodies of SEB in this study to improve the immunologic specificity. The two monoclonal antibodies recognize different epitopes of same antigen. For optimization of the fluorescent nanoparticle's surface antibody density, we incubated FFSCNs with variable amounts of antibody and examined the effect on the antibody dose response curve. We observed that when the density of the antibody on the nanoparticle's surface was 50 μg antibodies per mg fluorescent nanoparticles, the fluorescent intensity was only about 37.6% at concentration of the SEB 50 pg/mL compared with 200 μg antibodies was used. As the amount of antibody was increased from 50 to 150 μg antibody per mg fluorescent nanoparticles, the assay response increased. Because the assay reached saturation with 200 μg/mg FFCSNs, at this concentration, the assay response was increased slowly at all concentrations of SEB. Thus, there existed an optimum of antibody density at about 150 μg/mg FFCSNs, resulting in the best assay response. Therefore, this ration was chosen for the FFCSNs-labeled sandwich FIA (Fig. 5).

For optimization of the FFCSNs concentration, the fluorescence intensity was monitored at different concentrations of FFCSNs–anti-SEB antibody conjugates. We observed that the fluorescent intensity was enhanced significantly when the FFCSNs concentration was increased from 5 to 50 μg/mL. However, at concentrations >50 μg/mL, the fluorescence intensity did not increase. So we chose 50 μg/mL as the optimal concentration of the FFCSNs (Fig. 6).

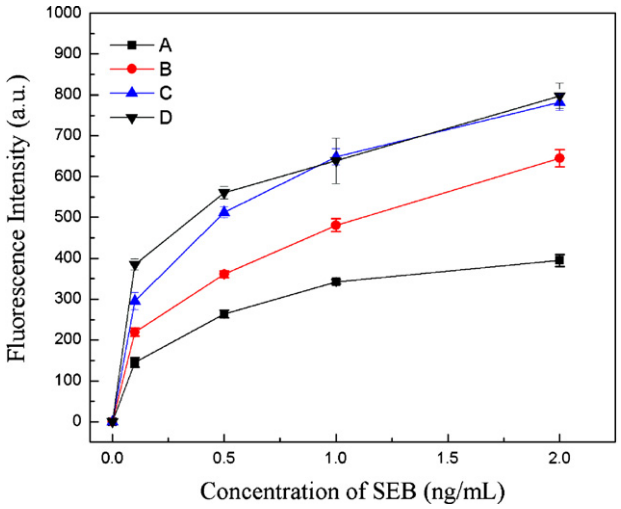


**Fig. 5.** Density of antibody on the surface of the nanoparticles. (A)–(D) were the response curves of different values of antibody microgram per mg fluorescent nanoparticles, separately (A) was 50 μg/mg, (B) was 100 μg/mg, (C) was 150 μg/mg, (D) was 200 μg/mg. The concentration of the antibody–nanoparticles conjugates was 50 μg/mL).

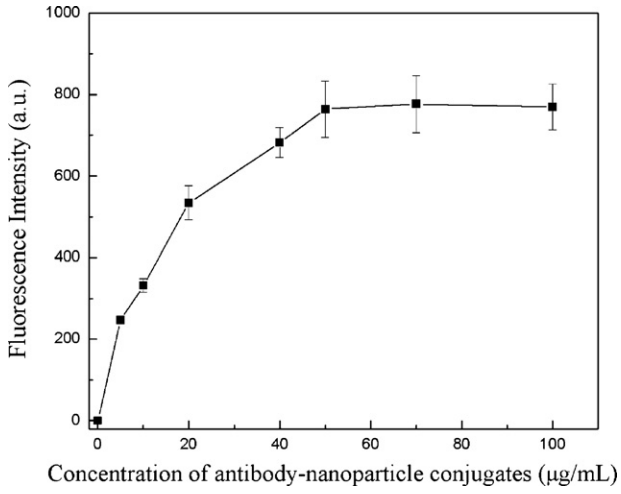


**Fig. 6.** Concentration of the antibody–nanoparticles.

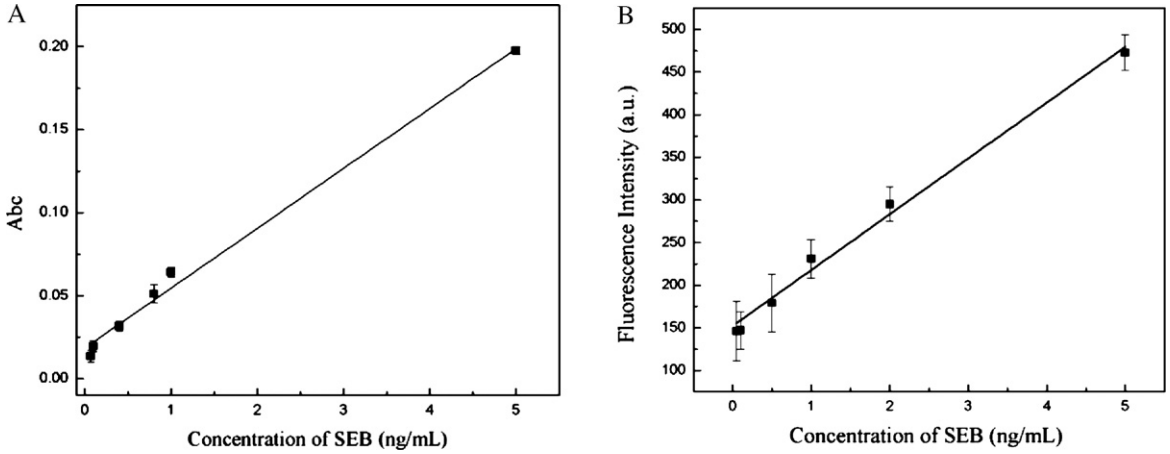


**Fig. 7.** Calibration curve of ELISA and our laser induced fluorescence immunoassay. (A) is the calibration curve of ELISA; (B) is calibration curve of laser induced fluorescence immunoassay.

**Table 1**
Comparison between ELISA and laser induced fluorescence immunoassay.

| | Colorimetric ELISA | Laser induced fluorescence immunoassay |
|---|---|---|
| Regression equation | $\Delta I = 0.03634[SEB] + 0.0177$ ($n = 7$, $R^2 = 0.9940$) | $\Delta I = 66.14[SEB] + 149.8$ ($n = 5$, $R^2 = 0.9904$) |
| Linear range | 0.07–5 ng/mL | 50 pg/mL–5 ng/mL |
| RSD (%) | 6.1% ($n = 9$) | 9.2% ($n = 5$) |
| Detection limit | 0.03 ng/mL | 20 pg/mL |
| Absolute detection limit | 3 pg | 0.02 pg |

**Table 2**
Analytical results of SEB in samples.

| Sample | Colorimetric ELISA (ng/mL) | Laser induced fluorescence immunoassay (ng/mL) | Difference (%) |
|---|---|---|---|
| Lake water 1 | 0.238 | 0.269 | +13.0 |
| Lake water 2 | 0.833 | 0.918 | +10.2 |
| Lake water 3 | 1.55 | 1.63 | +5.16 |
| Milk 1 | 0.661 | 0.737 | +11.5 |
| Milk 2 | 1.12 | 1.22 | +8.93 |
| Milk 3 | 1.53 | 1.61 | +5.20 |
| Serum 1 | 0.561 | 0.638 | +13.7 |
| Serum 2 | 1.03 | 1.14 | +10.7 |
| Serum 3 | 1.47 | 1.54 | +4.80 |

### *5.4. Performance of our new immunologic method*

In this study, a typical "sandwich" method was adopted, and we constructed a new device for the measurement of fluorescence. The calibration curve for the SEB was $I_F = 66.14[SEB] + 149.8$ with $R^2 = 0.9904$ (Fig. 7), the linear range was 50 pg/mL–5 ng/mL. The comparison of the analytical character for SEB between ELISA and laser induced fluorescence immunoassay was shown in Table 1. The result of sample analysis was also compared between ELISA and our laser induced fluorescence immunoassay (Table 2).

## 6. Conclusion

The ability to measure very low amounts of SEB accurately in food and other samples is very important. In this paper, a method employing FFCSNs as fluorescent labels in fluoroimmunoassay of SEB model was established using two mAbs recognizing different epitopes of SEB, and a laser-induced fluorescence millimeter sensor array detection platform. High specificity, sensitivity, very low detection limit were obtained based on this method, our method consisted with ELISA in real sample analysis. Therefore, these merits should make it easily popular and used for various real applications, such as sub-edged analysis of three-dimensional array of cellular samples and evaluation of the existence of special membrane proteins on the cell surface.

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