



Relative efficiency of zinc sulfide (ZnS) quantum dots (QDs) based electrochemical and fluorescence immunoassay for the detection of *Staphylococcal enterotoxin B* (SEB)[☆]

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ABSTRACT

In this paper an attempt was made to detect *Staphylococcal enterotoxin B* (SEB) both by electrochemical and fluorescence immunoassay methods using zinc sulphide (ZnS) QDs. Wet-chemical method was adopted for the preparation of fluorescent ZnS QDs (diameter ~ 5–10 nm). These QDs were bioconjugated with monoclonal antibodies and then characterized by various method. A detection limit of 0.02 ng mL⁻¹ by fluorescence assay and 1.0 ng mL⁻¹ by electrochemical assay for SEB was achieved. While by sandwich ELISA it is possible to detect 0.24 ng mL⁻¹ only. The sensitivity of all techniques is very good, since the LD₅₀ of SEB is 20 ng kg⁻¹. Electrochemical assay is faster, need low-cost instrument, independent to the size of QDs and found to be one of the best alternative methods as compared to the other existing methods studied herein. The presented method could be expanded to the development of electrochemical and fluorescence biosensors for various agents for field and laboratory use.

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1. Introduction

Staphylococcal enterotoxin B (SEB) is super antigenic in nature causing acute food poisoning known as staphylococcal food poisoning (SFP). SEB is categorized as serious biological warfare agent. The ED₅₀ and LD₅₀ for SEB were found to be 0.4 ng kg⁻¹ and 20 ng kg⁻¹, respectively [1]. Because of its high toxicity it is necessary to detect this toxin in field conditions. Previously, SEB was detected by various methods such as microslide method [2,3] photonic crystal lab-on-a-chip method [4], ELISA [5], liquid chromatography mass spectrometry [6], cantilever sensor [7], piezoelectric crystal immunosensor [8] and surface-enhanced Raman scattering probe [9] etc. (Table 1).

But all these methods are very cumbersome, time consuming as well as expensive. At present, electrochemical immunosensors are the centre of attraction for the researchers because of their simplicity, reproducibility and compatibility with advanced

technology [10]. Enzymes were used as labels for the electrochemical immunosensing of SEB [11,12]. Some reports are also available using various nanomaterials such as carbon nanotubes [13], gold nanoparticles [14], silica nanoparticles [15] and semiconductor nanocrystals [16] which were used earlier for the detection of SEB. Fluorescent based detection of SEB was achieved by various quantum dots [17,18]. It is known that fluorescence immunoassay of QDs depend upon the size. Hence, great control needs to be exercised in their preparation which will increase the cost of detection method. Also it requires costly instrument and is not field usable. The standard ELISA method also needs costly instrument and use enzymes which may deteriorate over a period of time and also it is not field useful. On the other hand electrochemical immunoassay attracted due to their simplicity, low cost and portable field usable instrumentation. Attempts were made by several authors to use metal nanoparticles [26,14,15], quantum dots [27], carbon nanotubes [13] and redox labels [24,25].

Recently QDs get attention due to their good electrochemical as well as fluorescent properties. In the literature the QDs were used to develop immunosensors either by electrochemical or fluorescence methods. And it is not possible to compare relative efficiency of these methods because different types of antigen–antibodies were used by the authors. This is because the sensitivity will also

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Table 1

Methods reported in the literature for detection of SEB.

S. No.	Method for detection of SEB	Detection limit	Authors	References
1	SDS-PAGE technique	50 ng/ μ L	Mary Margaret; Wade et al.; Harold et al.	Int. J. Microbiol. (2011) 1–5; Appl. Microbiol. (1971) (Aug) 214–219
2	Zone electrophoresis	Not mentioned	Ornstein et al.; Davis et al.; Karine Trudeau	Ann. NY Acad. Sci. 121 (1964) 321; Ann. NY Acad. Sci. 121 (1964) 404; Plos One 7 (2012) 1–9
3	Western blotting test	50 ng/mL	Gaithersburg MD 20878	Gaithersburg MD 20878
4	Lymphocyte proliferation assays (flow cytometric assay)	Not mentioned	Betts et al.	J. Immunol. Methods 281 (2003) 65–78
5	T-cell proliferation assay (live-cell assay)	Not mentioned	Chattopadhyay et al.	Nat. Protocols 1 (1) (2006) 1–6
6	Radioimmunoassay (RIA)	1 ng/mL	Howard et al.	Appl. Microbiol. 22 (1971) 837–841
7	Latex agglutination assay	0.5 ng/mL	Hitroshi. et al.	Appl. Environ. Microbiol. 54 (1988) 2345–2348
8	Microslide method	100 ng/mL	Agarwal et al.; McLandsborough et al.	Indian J. Microbiol. 52 (2) (2012) 191–196; Lett. Appl. Microbiol. 12 (1991) 81–84
9	Laser nephelometric assay	0.3 μ g/mL	Hosotsubo et al.	J. Clin. Microbiology, 27 (1989) 2794–2798
10	Enzyme-linked immunosorbent assay (ELISA)	10 pg/mL	Cook et al.	Clin. Vaccine Immunol. 14 (2007) 1094–1101
11	Lateral flow immunodiagnostic	0.25 ng/mL	Boyle et al.	J. AOAC Int. 93 (2010) 569–575
12	Optimal sensitivity plate assay	1.4 ng/mL	Pereira et al.	Ciênc. Tecnol. Aliment 21 (2001) 171–175
13	Immunofluorescent assay	1 ng/mL	Rowe et al.; Genigeorgis et al.	Anal. Chem. 71 (1999) 433–439; J. Food Sci. 31 (2006) 441–449
14	Piezoelectric crystal immunosensor	2.5 μ g/mL	Lin et al.	Biosens. Bioelectron. 18 (12) (2003) 1479–1483
15	Liquid chromatography mass spectrometry	80 ng	Callahan et al.	Anal. Chem. 78 (2006) 1789–1800
16	Colorimetric detector	3.9 ng/mL	Sapsford et al.	Anal. Bioanal. Chem. 394 (2009) 499–505
17	Surface plasmon resonance	10 ng/mL	Slavi'k et al.	Biosens. Bioelectron. 17 (2002) 591–595
18	Magnetoelastic sensor	0.5 ng/mL	Ruana et al.	Biosens. Bioelectron. 20 (2004) 585–591
19	Cantilever sensor	12.5–50 pg/mL	Campbell et al.	Sens. Actuators B 126 (2007) 354–360
20	Carbon nanotubes (optical immunodetection)	0.1 ng/mL	Yang et al.	Int. J. Food Microbiol. 127 (2008) 78–83
21	Gold nanoparticles [Enhanced chemiluminescence (ECL) immunosensor]	0.01 ng/mL	Yang et al.	Int. J. Food Microbiol. 133 (2009) 265–271
22	Chemiluminescence immunoassay (silica nanoparticles)	4 pg/mL	Chen et al.	Food Chem. 135 (2012) 208–212
23	Semiconductor nanocrystals	7.8 ng/mL	Sapsford et al.	Sensors 11 (2011) 7879–7891
24	Micro fluidics system	0.5 ng/mL	Dong et al.	Lab Chip 6 (5) (2006) 675–681
25	Magnetic beads based assay	100 pg	Alefantis et al.	Mol. Cell. Probes 18 (2004) 379–382
26	Matrix-assisted laser desorption ionization time of flight mass spectrometry	70 fmol	Schlosser	Appl. Environ. Microbiol. (2007) (Nov) 6945–6952
27	Electric-field-driven assay	1.8 nM	Ewalt et al.	Anal. Biochem. 289 (2001) 162–172
28	Immunodiffusion assay	0.1 μ g/mL	Meyer et al.	Appl. Environ. Microbiol. 40 (6) (1980) 1080–1085
29	LSPR-based nano-biosensor (Au–Ag NPs)	0.1 ng/mL	Zhu et al.	Opt. Mater. 31 (2009) 1608–1613
30	Array biosensor	ng/mL	Rowe-Taitt et al.	Biosens. Bioelectron. 14 (10–11) (2000) 785–94
31	Magnetic xMAP- technology	3 ng/L	Pauly et al.	Supplementary Material (ESI) for Analyst (2009)
32	NRL-biosensor	100 ng/L	Ligler et al.	Anal. Sci. 23 (2007) 5–10
33	Semi-homogeneous fluidic force discrimination assay	0.001 ng/L	Mulvaney et al.	Biosens. Bioelectron. 24 (2009) 1109–1115
34	Conventional xMAP-technology	200 ng/L	Wang et al.	Immunopharmacol. Immunotoxicol. (2009)
35	RAPTOR	10000 ng/L	Anderson et al.	Biosens. Bioelectron. 14 (2000) 771–777
36	Array tube-system	200 ng/L	Huelseweh et al.	Proteomics 6 (2006) 2972–2981
37	Immunomagnetic, fluorogenic detection	100 ng/L	Yu et al.	Biosens. Bioelectron. 14 (2000) 829–840
38	Microfluidic electrophoretic chipbased immunoassay	8400 ng/L	Meagher et al.	Lab Chip 8 (2008) 2046–2053.
39	Hydrogel-based protein-microarray	1000 ng/L	Rubina et al.	Anal. Biochem. 340 (2005) 317–329
40	Bidiffractive grating (BDG) biosensor	<1000 ng/L	O'Brien et al.	Biosens. Bioelectron. 14 (2000) 815–828
41	QTL-biosensor 2200R	<1000 ng/L	Gooding et al.	Anal. Chim. Acta 559 (2006) 137–151
42	Multiplexed sandwich ELISA with quantum dots	30000 ng/L	Goldman et al.	Anal. Chem. 76 (2004) 684–688
43	Conventional xMAP-technology	14 ng/L	Anderson et al.	Biosens. Bioelectron. 24 (2008) 324–328
44	Fluorescence-based immunoassay	100 pg/well	Khan et al.	Mol. Cell. Probes 17 (2003) 125
45	Lanthanide chelate label (time-resolved fluorescence assay)	10 pg/mL	Peruski et al.	J. Immunol. Methods 263 (2002) 35
46	Silicon-based light-addressable potentiometric sensor (LAPS)	3 pg/mL	Lee et al.	Biosens. Bioelectron. 14 (2000) 795
47	Planar array immunosensor (charge-coupled device (CCD))	5 ng/mL	Wadkins et al.	Biosens. Bioelectron. 13 (3) (1998) 407–15
48	Flow-based microarray platform	4 ng/mL	James et al.	Anal. Chem. 74 (21) (2002) 5681–5687

Table 1 (Continued)

S. No.	Method for detection of SEB	Detection limit	Authors	References
49	Receptor-based immunoassay	1 ng/mL	Mukhin et al.	Anal Biochem. 245 (2) (1997) 213–7
50	Immuno-chromatographic-based hand-held assay	50 pg/mL	Schotte et al.	Clin Lab 48 (7–8) (2002) 395–400
51	Laser-induced fluorescence millimeter sensor array	20 pg/mL	Zhang et al.	Talanta 85 (2) (2011) 1070–1074
52	Multiplexed electrochemical detection (using antibody microarray)	5 pg/mL	Wojciechowski et al.	Sensors (Basel) 10 (4) (2010) 3351–3362
53	Fluorescent latex micro particle immunoassay	0.125–1.0 ng/mL	Medina et al.	J. Agric. Food Chem. 54 (14) (2006) 4937–4942
54	Immuno-chromatographic testing (ICT) using fluorescent immunoliposomes	20 pg/mL	Khreich et al.	Anal. Biochem. 377 (2) (2008) 182–188
55	Flow-injection capacitive biosensor	0.3 pg/mL	Labib et al.	Anal. Bioanal. Chem. 393 (5) (2009) 1539–44
56	Microplate chemiluminescent enzyme immunoassay (CLEIA)	0.01 ng/mL	Liu et al.	Anal. Chem. 82 (18) (2010) 7758–7765
57	Fluorescence-based cytometric bead array (using magnetic microspheres)	Picomolar range	Tallent et al.	Appl. Environ. Microbiol. 79 (5) (2013) 1422–1427
58	Direct skin test	Picogram range	Scheuber et al.	Appl. Environ. Microbiol. 46 (6) (1983) 1351–1356
59	Enzyme-linked immuno filtration assay (ELIFA)	Not mentioned	Dupont et al.	J. Immunol. Methods 128 (2) (1990) 287–291
60	Biosensor detection	0.1 ng/mL	Sapsford et al.	Appl. Environ Microbiol. 71 (9) (2005) 5590–5592
61	Electrochemical immunosensor (based on bio-magnetosomes)	0.017 ng/mL	Wu et al.	Talanta 15 (2013) 360–366
62	Immunomagnetic PCR signal amplification assay	7.5 fg/mL	Panneerseelan et al.	J. Food Prot. 72 (12) (2009) 2538–2546
63	Multiplex PCR-assays	Not mentioned	Mojtaba et al.	J. Paramed. Sci. 2 (2) (2011) 34–40
64	Electrical percolation-based biosensor	5 ng/mL	Yang et al.	Biosens. Bioelectron. 25 (2010) 2573–2578
65	Automated point-of-care system	0.1 ng/mL	Yang et al.	Anal. Biochem. 416 (2011) 74–81
66	Surface-enhanced Raman scattering probe	224 aM	Temur et al.	Anal. Chem. 84 (2012) 10600–10606
67	Photonic crystal lab-on-a-chip method	35 aM	Han et al.	Anal. Chem. 85 (2013) 3104–3109
68	Immunoquantitative real-time PCR	<10 pg/mL	Rajkovic et al.	Appl. Environ. Microbiol. 72(10) (2006) 6593–6599

depend on the stability constant of the antigen–antibody which widely varies. In this paper, we have used same QDs and same antigen–antibody system and compared the results with respect to sensitivity, ease of operation, cost of the equipment etc. Highest sensitivity found by the fluorescence method [0.02 ng mL^{-1}], ELISA [0.24 ng mL^{-1}] and then followed by electrochemical method [1 ng mL^{-1}]. However, all are giving sensitivity at the nanogram levels which is much lower than the LD_{50} value. Hence, all are acceptable and we recommend electrochemical method because of low-cost, no use of enzyme, and independent on QDs size. So, for the utility point of view electrochemical method is more preferred.

In this paper, fluorescent ZnS QDs are used for the electrochemical immunosensing as well as fluorescent detection of SEB. These QDs are biocompatible, non-toxic and can be used as suitable label for the identification of SEB. EDC/NHS chemistry is followed for the bioconjugation of mouse anti-SEB monoclonal antibodies with ZnS QDs (i.e., revealing antibodies) are used for signal production. Direct sandwich ELISA procedure is implemented for the sensing of SEB. For this purpose, capturing antibodies were physically adsorbed on to the screen-printed electrode (SPE). After blocking with bovine serum albumin (BSA), different concentrations of SEB were added. These electrodes were further incubated with revealing antibodies. Finally, 1 N HCl is added on SPE to dissolve the ZnS QDs which were bound in the sandwiched immunoassay and the dissolved zinc ions were analyzed by electrochemical square wave voltammetric (SWV) method because of well reported oxidation potentials of Zn^{2+} -1.0 V vs. Ag/AgCl electrode [19]. The voltammetric current response was increased with increasing the concentration of SEB. Under optimized conditions, we can easily detect SEB at its various concentrations ranging from 10 ng mL^{-1} to $1 \mu\text{g mL}^{-1}$, with a readily achievable detection limit of 1 ng mL^{-1} within 1.5 h. Till date, there is no report for the electrochemical immunosensing of SEB using ZnS QDs.

SEB is also detected by ELISA and QDs-FLISA using the same batch of antigen–antibody and the obtained results were compared in terms of sensitivity, ease of experimentation and

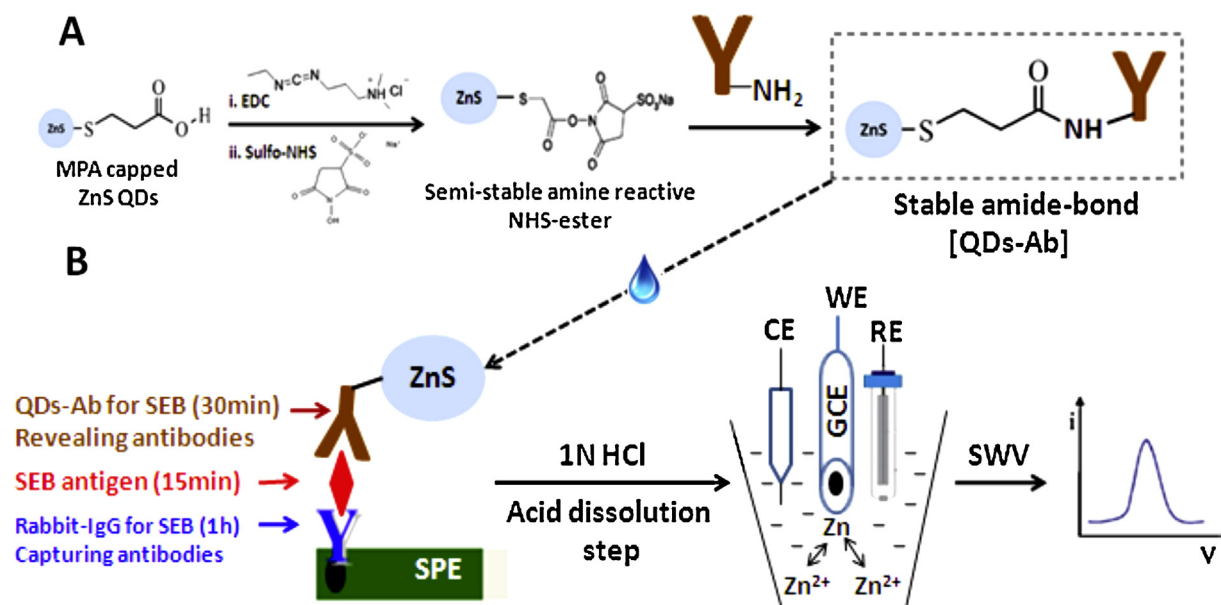
analysis time. In ELISA (detection limit 0.24 ng mL^{-1} , analysis time 3–4 h) and QDs-FLISA (detection limit 0.02 ng mL^{-1} , analysis time 2–3 h) was obtained. As compared to above methods the sensitivity of electrochemical detection was low (i.e., 1 ng mL^{-1} , analysis time 90 min) but it fulfills the condition required for the minimum desired concentration of SEB which can generate illness to human and can be applicable for field diagnosis. As well as electrochemical approach is simple and faster as compared to ELISA and QDs-FLISA which are usually laboratory based techniques that require longer incubation steps and large volume of biomolecules. Also the handling with sophisticated 96-well Maxisorp microtiter ELISA plate was very tedious and required well trained personnel. We are sure that still there is no report in the literature on the immunosensing of SEB based on ZnS QDs using electrochemical method.

2. Materials and method

2.1. Reagents and apparatus

Rabbit anti-SEB polyclonal antibodies (used as capturing antibodies), SEB, and mice anti-SEB monoclonal antibodies (used as revealing antibodies) were purified, validated and estimated in Biotechnology division of our laboratory. Zinc nitrate tetrahydrate $\text{Zn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, sodium sulfide ($\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$), 3-mercaptopropionic acid (MPA), BSA, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), *N*-hydroxysuccinimide ester (NHS) and Tris-HCl were purchased from Sigma–Aldrich. Tris-HCl (0.1 M, pH 7.2) is used for immunoreactions and washing. Chemicals which are used in the analysis but not listed above were of analytical reagent (AR) grade. Aqueous solutions were prepared in ultrapure triple distilled Millipore water for experimentation.

Three-electrode cell system i.e., a platinum electrode, Ag/AgCl/saturated-KCl and glassy-carbon electrode (GCE) was used as counter, reference and working electrode, respectively. All the three electrodes were connected with an Autolab PGSTAT potentiostat/galvanostat (Eco Chemie, The Netherlands). In house



Scheme 1. Bioconjugation mechanism for the tagging of mice anti-SEB antibodies with mercaptopropionic acid-capped ZnS QDs using EDC/NHS chemistry (A) and procedure for the QDs-based electrochemical immunosensing of SEB is shown in (B).

prepared screen-printed electrodes (3 mm in diameter) were used for physical immobilization of biomolecules. Quanta 400 ESEM (The Netherlands) was used for SEM/EDAX measurement. Implen nanophotometer (serial no. 1257) was used for UV–vis spectrum analysis. Raman spectra of unfunctionalized and carboxylic (–COOH) group functionalized ZnS quantum dots were taken with the help of Renishaw inVia Raman microscopy. ELISA plate reader (Bio-Tek Instruments Inc., USA) was used to perform ELISA. Multimode micro plate reader (model 54MLFPTA, Bio-Tek Instruments Inc., USA) was used for quantum dot based fluorescence-linked immunosorbent assay (QD-FLISA). Triple distilled deionized water was obtained from water purification system (Millipore, USA). All electrochemical measurements were performed at room temperature.

2.2. Synthesis of mercaptopropionic acid-capped zinc sulfide quantum dots

Mercaptopropionic acid (MPA) functionalized zinc sulfide quantum dots were synthesized via the following procedure. First, zinc nitrate (0.04 M, 20 mL) solution A and sodium sulfide (0.02 M, 20 mL) solution B was prepared separately. Mercaptopropionic acid (MPA) (0.64 mmol, 36 mL) was prepared and stirred

for 5 min solution C. In the next step, 2 mL of solution A was dropped slowly in the solution C with constant stirring for 10 min. Then this mixture was titrated with tetrabutylammonium hydroxide (TBAH). Adjusted pH was 7.2 and stirred for 10 min followed by rapid addition of 4 mL of solution B. Then, wait for 5 min before adding another 6 mL of solution A. pH 7.2 was maintained by adding TBAH with constant stirring for 5 min. The obtained MPA-capped zinc sulfide quantum dots were clear and colorless. The final volume of the quantum dots solution was 50 mL and the concentration was about 1.6 mM as reported in the literature [20]. Uncapped zinc sulfide QDs were also synthesized for comparison (Supplementary information).

2.3. Preparation of zinc sulfide QDs tagged mouse anti-SEB monoclonal antibodies (revealing antibodies)

EDC/NHS-chemistry was used in this paper for the preparation of ZnS QDs mouse anti-SEB monoclonal antibody conjugates as shown in Scheme 1A. This method was available in the literature [21]. Before conjugation the carboxylic groups present on the surface of ZnS QDs were first activated by adding 10 mM EDC and NHS. After stirring for 30 min, this mixture was centrifuged for 1 min at 5000 rpm. The solution was decanted and the pellet was

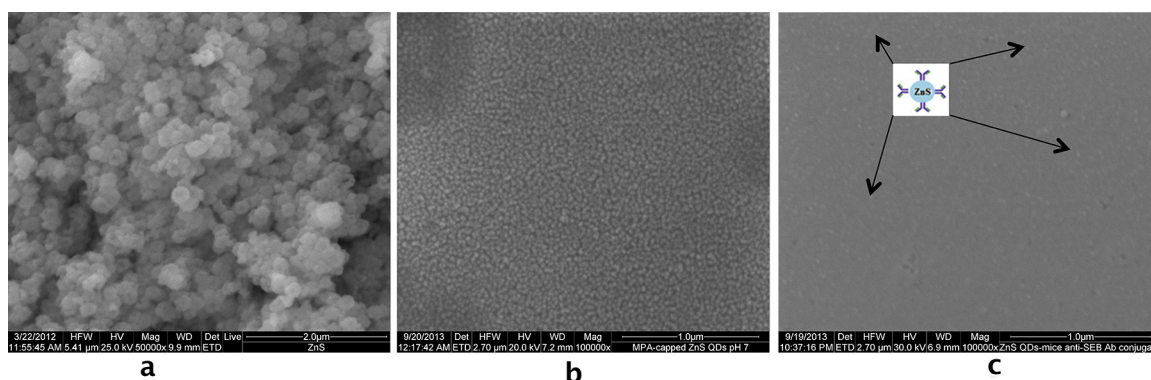


Fig. 1. (a) SEM-characterization of ZnS QDs, (b) MPA capped-ZnS QDs and (c) zinc sulfide QDs tagged mouse anti-SEB monoclonal antibodies conjugates.

dissolved in 250 μL Tris-HCl. In the next step, the activated QDs solution was mixed with 160 μL of monoclonal mouse anti-SEB antibodies (1 mg mL^{-1}) for 1 h. After that, this mixture was centrifuged for 10 min at 14,000 rpm. The supernatant solution was decanted and the collected revealing antibodies were resuspended in 500 μL Tris-HCl containing 0.04% BSA. These revealing antibodies were used for sensing of SEB.

2.4. Fabrication of electrochemical immunosensor

Electrochemical immunosensing of SEB was performed using the principle of direct sandwich ELISA but here ZnS QDs linked antibodies were used as revealing antibodies in place of enzyme linked antibodies. In the standard protocol, physical adsorption of 5 μL of rabbit anti-SEB polyclonal antibodies (concentration $125\text{ }\mu\text{g mL}^{-1}$) optimized in our previous studies was coated on screen-printed electrode (SPE) herein used as an immobilization platform as shown in Scheme.1B. Then, the disposable SPE is left for the incubation for 1 h at 37°C . To reduce the nonspecific adsorption effect, solution of 3% BSA in 0.1 M Tris-HCl pH 7.2 was used for blocking. In the next step, 5 μL of different concentrations of SEB in buffer solution was added and further incubated at 37°C for 15 min. SEB antigen was captured due to the highly specific and selective antigen–antibody interaction and immunocomplex was formed. Subsequently, 5 μL of mouse anti-SEB monoclonal antibodies tagged with ZnS QDs (revealing antibodies) were added and incubated at 37°C for 30 min (optimized time). 0.1 M Tris buffer pH 7.2 was used to rinse the electrode surface after each incubation step. Before going to the electrochemical measurement, the acid dissolution step was carefully performed. In this step, 20 μL of 1 N HCl solution was added on SPE for 30 min to dissolve the captured ZnS QDs bound in the immunosensing layer and to release the zinc ions. Then, this SPE was carefully transferred in the cell containing 2 mL of acetate buffer (0.2 M, optimized pH 5.0) and SWV-experiment was performed using GCE as a working electrode as described in the next coming section.

2.5. Electrochemical detection

In order to get well-defined, sharp and highly reproducible voltammetric signal following parameters such as conditioning potential of +0.6 V for 60 s, deposition potential of -1.4 V for 160 s, equilibration time of 10 s, modulation amplitude of 30 mV, step potential of 5 mV, and square wave frequency of 35 Hz (optimized in this study) were used as it is for all the electrochemical

measurements. GPES 4.9 software was used for the baseline correction of the obtained voltammogram.

3. Results and discussion

3.1. Scanning electron microscopy (SEM)-characterization of ZnS QDs, MPA-capped ZnS quantum dots and zinc sulfide QDs tagged mouse anti-SEB monoclonal antibodies conjugates

Size and morphology of the capped and uncapped ZnS QDs was studied by scanning electron microscopy (SEM). SEM image shows that the prepared quantum dots were regular in shape and uniformly distributed. The diameter of the MPA-capped ZnS QDs was less than 20 nm that was much smaller than the uncapped ZnS QDs. The capping agent covalently bound to the surface of the quantum dot via disulfide bond and reduced the surface tension of the dot. SEM image of ZnS QDs tagged mouse anti-SEB monoclonal antibodies conjugation is shown in Fig. 1c. The carboxylic group functionalized ZnS QDs surface can easily bound to the mice anti-SEB IgG with high density which are called “bionanoconjugates or revealing antibodies”. It was clearly seen in the SEM-image that 10–20 nm sized quantum dots were present in the prepared bionanoconjugates. In addition, Raman, EDX and UV–vis characterization data of QDs are given in the Supplementary information in Fig. S2–S4, respectively.

3.2. SDS-PAGE analysis of purified rSEB, mouse anti-SEB IgG, rabbit anti-SEB IgG and zinc sulfide QDs tagged mouse anti-SEB monoclonal antibodies conjugates

SDS-PAGE was performed to check the purity of SEB, rabbit anti-SEB IgG and mouse anti-SEB IgG. Two bands are observed that correspond to heavy and light chain of the purified rabbit and mice IgG raised against SEB (lane 3 and 4, respectively in Fig. 2a). A sharp band around 28.4 kDa (lane 2 in Fig. 2a) clearly indicates the presence of SEB toxin. Conjugation of mice anti-SEB antibodies with QDs was also confirmed by SDS-PAGE. Mice anti-SEB monoclonal antibodies were covalently bound with MPA-capped ZnS QDs using EDC/NHS chemistry. Due to large size, revealing antibodies (QD–Ab) did not run and remained in the loading wells of the gel (lane 1 in Fig. 2(b)). Therefore, after staining with coomassie blue intense band is visualized in the loading well in lane 1 of Fig. 2b which corresponds to the covalently bounded QD–Ab conjugates. The two weak bands at 27 kDa and 52 kDa were also observed in lane 1 of Fig. 2b. This may be possible that two weak bands in the gel are appearing from the residues of antibodies

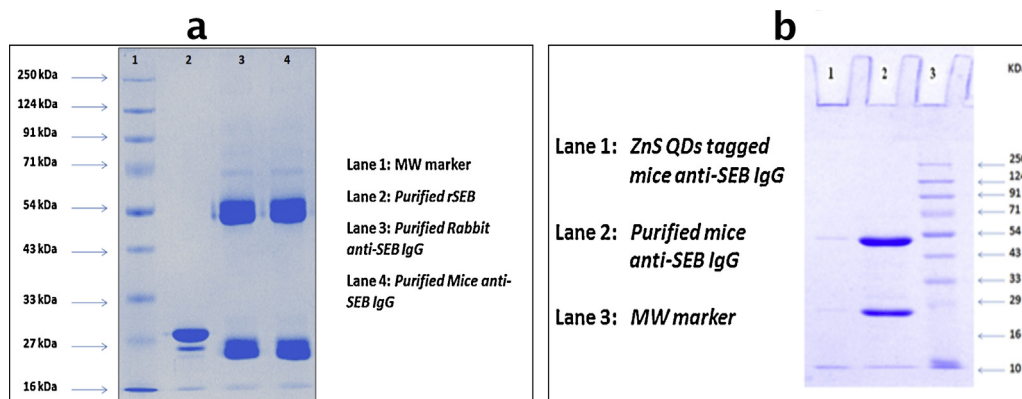


Fig. 2. (a) 12% SDS-PAGE gel image of molecular weight marker (lane-1), purified rSEB (lane 2), purified rabbit anti-SEB IgG (lane 3) and purified mice anti-SEB IgG (lane 4). (b) SDS-PAGE of mice anti-SEB monoclonal antibodies tagged with ZnS QDs (lane 1), mice anti-SEB IgG (lane 2) and molecular weight marker (kDa) lane-3.

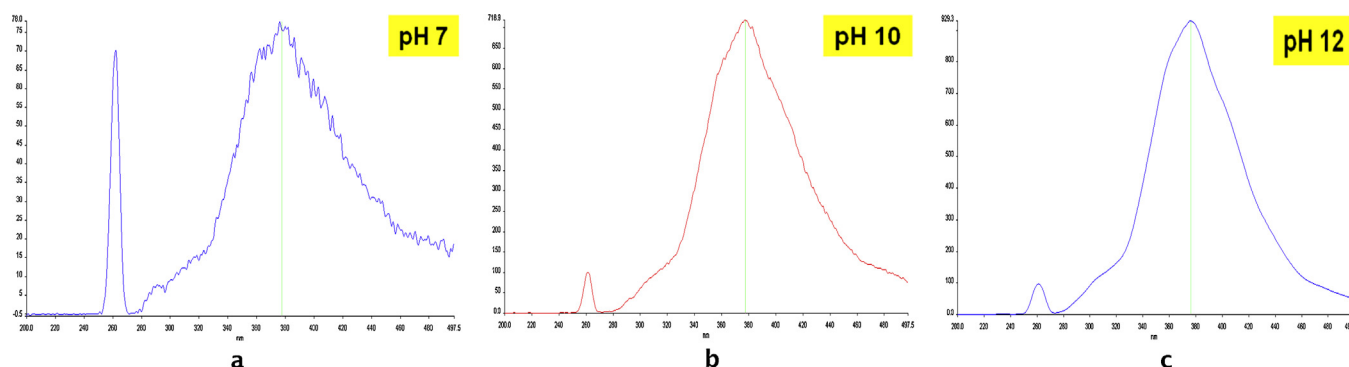


Fig. 3. PL-excitation-emission-spectra of MPA-capped ZnS QDs at different pH conditions (a) at pH 7, (b) at pH 10 and (c) at pH 12.

during purification after conjugation. Another possibility is that due to the presence of small amount of reducing agents such as 2-mercaptoethanol, dithiothreitol, etc., during SDS-PAGE, sample preparation might partially cleave the loosely bound antibody fragments attached with QDs. In the present method, antibodies were covalently bound with ZnS QDs and zinc also have excellent biocompatibility. Since the chances of bond breaking are very less after conjugation, SDS-PAGE method for characterization of QDs-antibody confirmatory binding with different linkers is reported in the literature [23]. Western blot for the validation of purified antibodies is given in Fig. S1. Electrochemical SWV, BCA and AFM-characterization data of revealing antibodies are provided in the Supplementary information in Fig. S8–S10, respectively.

3.3. Effect of pH on photoluminescence (PL)-spectra of MPA-capped ZnS QDs

PL-study was performed to check the fluorescence properties of MPA-capped ZnS QDs. Luminescent properties of ZnS are very well known [30,28]. When ZnS crystal was excited with an external light source, a pair of electron-hole was generated. After excitation, these electron-hole pairs recombined to give emission wavelength. To show the PL-characteristics, ZnS QDs absorb high energy photons which excite the electrons to reach in the conduction band (CB) from the valence band (VB) of Zn^{2+} levels. These excited electrons decay non-radiatively to the surface states and then decay radiatively to the valence band and a lower energy photon was emitted [29]. The energy of excitation and emission was recorded in terms of wavelength and the number of electron-hole pairs generated was determined in terms of intensity with the help of spectrophotometer. The results indicated that highest intensity was obtained for the ZnS QDs at pH 12 condition when

were excited at 260 nm and the emission wavelength was observed at around 378 nm (Fig.3c). The photoluminescence emission was observed due to presence of the band edge and stoichiometric vacancies [32]. The obtained results were compared with those bulk ZnS material reported. The PL-emission was observed at 450 nm for bulk ZnS by Chen et al. [31]. In case of ZnS nanoparticles, the PL-emission wavelength was decreased and reached at around 400 nm [29]. At pH 12 conditions, high density of carboxylate ($-\text{COO}^-$) ions were present on the QDs surface which repel them from each other. Hence, good dispersity was observed in their dispersions. Further, increasing the pH from 12 of the ZnS QDs dispersion the luminescence intensity was very poor which could not be observed. This was due to the formation of precipitate of zinc hydroxide and there were no more QDs. In more acidic medium (less than pH 6) the luminescence emission intensity was also very small and could not be recorded. This was due to the protonation of MPA attached to the ZnS QDs and consequent removal of capping from the ZnS surface. This will make the QDs unstable and they will tend to agglomerate. The PL-spectra of MPA-capped ZnS QDs at pH 7 and pH 10 are given in Fig. 3(a) and (b), respectively. Similar effect of lower and higher pH on fluorescence intensity was reported in the literature for the mercaptoacetic acid capped CdS quantum dots [33].

3.4. Sensitivity of ELISA and QDs-FLISA for SEB

The sensitivity for detection of SEB was checked using ELISA and QDs-FLISA. For this purpose, 96-well Maxisorp microtiter ELISA plate was first coated with 100 ng mL^{-1} (optimized from checker board) of rabbit anti-SEB IgG ($100 \mu\text{L/well}$). After blocking with BSA, different concentrations of SEB antigen were added in respective wells. For ELISA, 100 ng mL^{-1} (optimized from checker board) of mouse anti-SEB IgG was added and after that $100 \mu\text{L}$ of

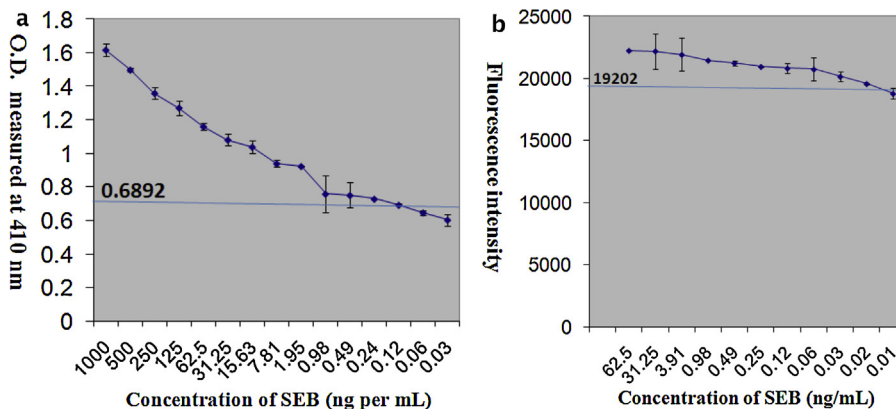


Fig. 4. (a) ELISA and (b) is QDs-FLISA of SEB at various concentrations.

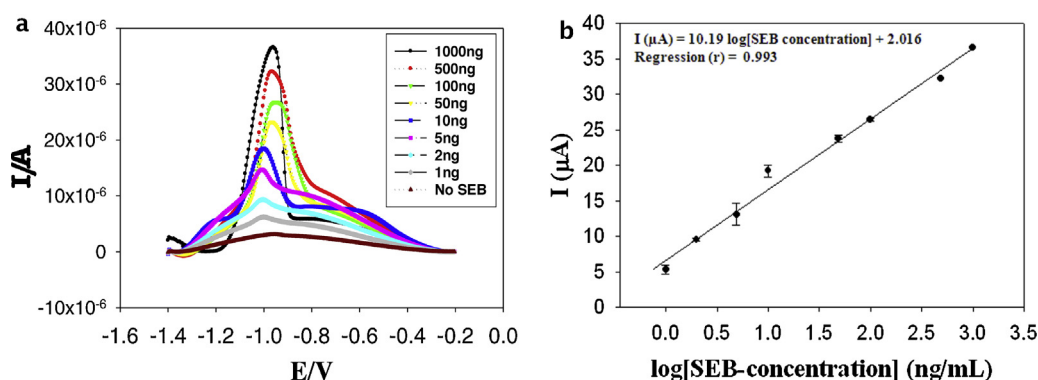


Fig. 5. (a) Square wave voltammetric detection of SEB at various concentrations i.e., 1 ng mL⁻¹, 2 ng mL⁻¹, 5 ng mL⁻¹, 10 ng mL⁻¹, 50 ng mL⁻¹, 100 ng mL⁻¹, 500 ng mL⁻¹ and 1000 ng mL⁻¹. In control experiment, SEB is not taken. Deposition for 160 s at -1.4 V; conditioning for 60 s at +0.6 V; SW-frequency (35 Hz); step potential (5 mV); modulation amplitude (30 mV); voltammetric stripping scan from -0.2 V to -1.4 V; and equilibrate for 10 s. (b) Calibration curve for the immunosensing of SEB.

HRP-conjugated anti-mouse immunoglobulin (1:1000) were added in each well. Plate was washed three times with PBST and padded dry after each step of incubation. In the next step, fixed amount (100 μ L/well) of substrate (ABTS + H₂O₂) was added to each well. Then the plate was incubated for 30 min in dark at 37 °C for the color development. After this, plate was read using microplate reader at 410 nm wavelength. For QDs-FLISA, after addition of SEB antigen then optimized dilution (1:800) of ZnS QDs tagged mouse anti-SEB IgG from checker board was added and the fluorescence intensities were recorded at 380 nm wavelength when the plate was excited at 260 nm. Fig. 4(a) and (b) is obtained for detection of SEB using indirect sandwich ELISA and QDs-FLISA, respectively. The cut off value was calculated using the standard formula given by Snyder et al. [22]: cut-off value = (3 \times standard deviation) + mean value of blank i.e., for ELISA, 3 \times 0.02640 + 0.61 = 0.689 was obtained and for QDs-FLISA, 3 \times 565.5 + 17505.6 = 19202 was obtained. The detection limit was found to be 0.24 ng mL⁻¹ and 0.02 ng mL⁻¹ of SEB using ELISA and QDs-FLISA, respectively.

3.5. Optimization of various parameters

Before going for electrochemical detection of SEB various parameters were need to be optimized to achieve the high sensitivity. These parameters including: square wave frequency, modulation amplitude, deposition time, incubation time and pH of buffer medium. In brief, 35 Hz frequency, 30 mV amplitude, 160 s deposition time, 30 min incubation time and pH 5 of the buffer was optimized and used as such throughout the experiments for the electrochemical detection of SEB. Optimization details and results are mentioned in Fig. S5(a–c), S6 and S7 in the Supplementary information.

3.6. Square wave voltammetric (SWV) detection of SEB

Square wave voltammetry is a useful electrochemical technique studied for the analysis of various metal ions in their trace quantity. In this approach, electrochemical detection of SEB was performed using monoclonal mouse anti-SEB IgG tagged with ZnS QDs conjugates which were used as revealing antibodies. 1 N HCl solution was used to dissolve the bound ZnS QDs which were captured in the sandwiched immunosensing layer. The glassy carbon electrode was used as working electrode during the SWV scan and the aqueous soluble Zn²⁺ ions were detected. The current response was increased with increasing the concentration of SEB antigen. The square-wave voltammograms obtained at different

concentrations of SEB antigen are shown in Fig. 5a. The calibration curve was plotted as shown in Fig. 5b. Under optimized conditions, the peak current was increased with increasing the concentration of SEB ranging from 10 ng mL⁻¹ to 1 μ g mL⁻¹ and 1 ng mL⁻¹ detection limit was achieved at room temperature. BSA-blocked primary antibody was directly incubated with revealing antibodies without addition of SEB was considered as blank. Response was not observed in the blank experiment. Cut-off value was calculated by the standard formula i.e., cut-off value = blank response + 3 \times standard deviation. We can state that square-wave voltammetric response greater than 4.62 μ A (blank response + 3 \times standard deviation; 3.51 + 3 \times 0.37 = 4.62 μ A) was SEB positive. The detection limit i.e., 1 ng mL⁻¹ was easily achieved under optimized conditions. In this approach, the sensitivity obtained for the SEB was comparatively low from ELISA and QDs-FLISA but this technique satisfies the condition of minimum requirement for the detection of SEB. The electrochemical signal was obtained due to highly specific monoclonal SEB antibodies which were covalently bound to the capped-ZnS QDs and hence contain high density of ZnS QDs for each SEB antibody which was mainly responsible for the binding to SEB antigen. 14.28% Zn was present in 85.72% of revealing antibodies by weight. The voltammetric signal was achieved by dissolved ZnS QDs which were captured in the sandwiched immunocomplex and the trace amount of zinc ions was determined using SWV-technique. This methodology was obviously very simple and reproducible for the detection of SEB antigen.

4. Conclusions

In this paper, a novel method for electrochemical and fluorescence based immunosensing of SEB was explored, based on non toxic and biocompatible ZnS QDs was described and the results were compared with conventional ELISA method. Our results obtained by fluorescence method are close to the reported values in the literature. However, electrochemical technique is preferable due to the following reasons: (1) sufficient sensitivity, (2) does not suffer from problem with particle size and agglomeration of quantum dots, (3) faster analysis, (4) cheaper instrument, and (5) can be done even onsite. The sensitivity obtained by electrochemical SWV-method and QDs-FLISA was 1 ng mL⁻¹ and 0.02 ng mL⁻¹, respectively. In the conventional ELISA, HRP-enzyme linked antibodies were used and 0.24 ng mL⁻¹ detection limit was achieved for SEB. Hence, electrochemical detection method is found to be more suitable but for laboratory fluorescence method can be used because of its high sensitivity.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.btre.2015.02.004>.

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