Supplementary information

Sensitive detection of Staphylococcal Enterotoxin B (SEB) using Quantum dots by various methods with special emphasis on an Electrochemical immunoassay approach

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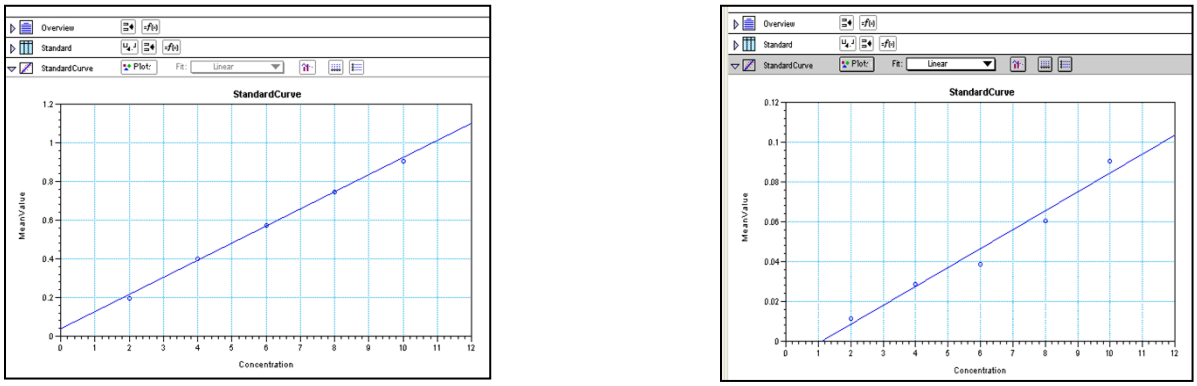
1. Affinity chromatography based purification, estimation and SDS-PAGE analysis of SEB and IgG samples

Unpurified rabbit anti-SEB polyclonal IgG and mice anti-SEB monoclonal IgG was purified by affinity chromatography based method as per details given by the manufacturer. In this method, unpurified serum sample was first mixed properly with the binding buffer. After that the "*Protein A Cartridge"* was washed with regeneration buffer. For this purpose, regeneration buffer was passed through the cartridge at the approx flow rate of 1mL/min. Then the cartridge was equilibrated by binding buffer by passing the binding buffer through the cartridge at the same flow rate. Then, we had loaded the sample-binding buffer mixture by passing it to the "*Protein A Cartridge"* at the approx flow rate of 0.5mL/min. Binding buffer was passed through the cartridge after the sample loading at the flow rate of about 1mL/min. Desalting cartridge was washed with [N-(2-hydroxyethyl)peiperazine-N'-(2-ethanesulfonic acid)] i.e. (HEPES) buffer by passing it through the cartridge at an approximate flow rate of

1mL/min. Then we had attached the one end of the "*Protein A Cartridge"* to another end of the desalting cartridge. Then, we had eluted the cartridges with elution buffer by passing it through the cartridges at an approximate flow rate of 0.5mL/minute. Elute was contained the purified IgG at physiological pH. After it, we had detached both the cartridges and regenerate them. "*Protein A Cartridge"* was regenerated by regeneration buffer by passing it through the cartridge. HEPES buffer was passed through the desalting cartridge and was regenerated. These cartridges are ready and can be used for another affinity chromatographic purification.



(a) **(b)**

Fig.1. (a) Shows the standard graph obtained for the estimation of rabbit anti-SEB IgG (b) shows for estimation of mice anti-SEB IgG using BCA-protein estimation method

Bicinchoninic acid protein assay kit and spectrophotometric method was utilized for the estimation of purified rabbit anti-SEB polyclonal IgG and mice anti-SEB monoclonal IgG concentration. For this purpose, bicinchoninic acid (BCA) working reagent was prepared by mixed with 50 parts of reagent A (containing bicinchonic acid, sodium carbonate, sodium tartrate, and sodium bicarbonate in 0.1N NaOH, pH 11 with 1 part of reagent B (containing 4% (w/v) copper(II) sulfate pentahydrate). In the standard procedure, BCA working reagent was

taken in 200µL are mixed with 0, 2, 4, 6, 8, 10µL part of a standard protein (BSA) solution of known concentration. 10µL of water without protein was used as blank. Unknown purified IgG sample was assayed with the known concentration of BSA protein standard. After that, the plate was incubated at 37°C for 30min. In the next step, absorbance was recorded at 562nm and the unknown protein concentration was determined by comparison to the standard curve. It was estimated that 2.4mg/mL IgG is present in rabbit-IgG of SEB and 1.0mg/mL IgG was present in mice-IgG of SEB.

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protocol was followed as per details given by the manufacturer. In this experiment, separating gel mixture (12% final concentration in 5mL) was prepared by mixing the stock solution of 30% acryl amide (2mL), 1.5M Tris-buffer, pH 8.8 (1.25mL), 10% SDS (50µL) and distilled water (1.65mL) and then N,N,N′,N′-tetramethylethylenediamine (TEMED) (5µL) and 10% ammonium persulphate (50µL) was added. This mixture was transferred into the gel mould and overlay it with 50µL of butanol and then kept for 40min for polymerization. In the next step, 2.5mL of 5% stacking gel was prepared by mixing 415µL of 30% acrylamide, 315µL of 1M Tris-buffer, pH 6.8, 25µL of 10% SDS and 1.7mL distilled water and then 5µL TEMED and 25µL ammonium persulphate was added. After that, stacking gel was transferred into the gel mould that contains the already polymerized separating gel. At the same time, suitable comb was carefully inserted without any air bubbles to make slots or loading wells for sample application. After complete polymerization, PAGE apparatus was appropriately filled with 1x running buffer (composition of 10x running buffer in 500mL is 93.75gm glycine, 5gm SDS and 15.125gm Tris). Then, the comb was carefully removed and the samples such as QDs-antibody conjugates and pure IgG samples were loaded in separate wells. Standard protein molecular weight marker was loaded in separate slot

for the accurate molecular weight determination of protein samples. After that 10mA fixed current was applied till the samples crossed the stacking gel and then the electric current was increased to 20mA. It was performed until the molecular weight marker reaches at the bottom of the gel.

Electrophoretic transfer of protein from gel to membrane:

After that, cassette was dissembled and the membrane was carefully removed. Then, staining was performed for 2hrs to visualize the protein bands using coomassie brilliant blue dye. In the next step, destaining was performed for 2hrs using destaining solution containing distilled water, methanol and glacial acetic acid (in 5:4:1 ratio). After that, SDS-PAGE image of the sample was taken.

2. Synthesis of lead sulfide (PbS) quantum dots

The lead sulfide quantum dots were synthesized as per reported procedure [1]. For the synthesis of PbS QDs, 10 mL of 0.1M $Pb(NO_3)_2$ solution was stirred at 500rpm for 30min. Then, appropriate quantity of NaOH was added to adjust the pH 7 of this solution. Nitrogen gas was passed in this solution for 30min and 10mL of 0.1M Na_2S was added drop-wisely. Under controlled conditions, the black precipitate of lead sulfide QDs was formed in the solution due to the combination of S^{2-} ions with Pb^{2+} ions and stirred this solution at 1000rpm for 1h and then kept for 24h. After that, this solution was filtered. Thus obtained QDs were three times washed with absolute ethanol and finally three times washed with distilled water. Thus obtained PbS QDs was dried for 24h at 60°C and was obtained. These QDs do not have any capping agent.

3. Synthesis of mercaptoacetic acid-capped lead sulfide quantum dots

We adopted the procedure for the synthesis of mercaptoacetic acid-capped cadmium sulfide quantum dots [2] and used this procedure in this work for the synthesis of mercaptoacetic acid (MAA) functionalized lead sulfide quantum dots. In the typical experiment, 0.5mmol MAA and 0.5mmol $Pb(NO_3)_2$ were added in a round bottom flask and dissolved in 250mL water. Then, 0.1 M NaOH solution was added dropwise to adjust the pH 6 in the constantly stirred solution. After that, 20mL of 0.5mmol $Na_2S{\cdot}9H_2O$ was added drop-wise under nitrogen atmosphere in the vigorously stirred solution. MAA capped PbS QDs were precipitated with absolute ethanol. To remove the impurities in the prepared QDs they were washed with water and again precipitated by ethanol and finally redispersed in water. Then, 0.05M Tris–HCl buffer solution was added to adjust the pH 7.4. These QDs capped with mercaptoacetic acid were used for further work.

4. Preparation of lead sulfide QDs tagged mice anti-SEB monoclonal antibodies bionanoconjugates

There are various methods for the conjugation of antibodies with quantum dots [3-5]. Herein, PbS QDs mice anti-SEB monoclonal antibody conjugates were prepared by EDC/NHS chemistry as per scheme 1(A). This procedure was reported in the literature [6]. Carboxylic group present on the surface of PbS QDs were not able to directly link with the amino group of antibodies. First of all, activation of carboxylic groups was required for the attachment of antibodies on the surface of carboxylic group functionalized PbS QDs. Hence, for the activation of carboxylic groups present on the surface of PbS QDs, were first dispersed in Tris-HCl then 10mM EDC and 10mM NHS were added. Then, stirred this mixture for 30min and after that centrifuged at 5000rpm for 1min. Supernatant solution was removed and redispersed the pellet in

250µL Tris-HCl. After that, mice anti-SEB monoclonal antibodies (1mg/mL, 160µL) were added in the activated QDs solution and mixed for 1hr and then centrifuged for 10min at 14,000rpm. Supernatant solution was removed and the purified QDs-antibody conjugates were collected. Thus obtained bionanoconjugates were resuspended in 500µL Tris-HCl containing 0.04% BSA. These bionanoconjugates were used for further experimentation.

5. Development of sandwich ELISA for the detection of SEB

For the detection of SEB in buffer first we have to optimize the concentration of rabbit anti-SEB IgG and mice anti-SEB IgG. For this purpose, checkerboard titrations were done using following procedure: Different concentrations of rabbit anti-SEB IgG were taken from 0ng to 500ng and mice anti-SEB IgG were taken from 0ng to 600ng coated on 96-well Maxisorb microtiter ELISA plate in fixed amount (100µl/well). 3% BSA in Phosphate buffer saline (PBS)-buffer was used for the blocking of unabsorbed sites of wells and incubated for 2 hours at 37ºC. After that wells were washed three times with PBS containing Tween 20 (PBST)-washing buffer (i.e. 0.05% Tween20 in PBS, pH 7.4) using ELISA-plate washer. In the next step, 500ng SEB-antigen fixed amount (100µl/well) was added in wells and incubated for 1hr at 37ºC. Again wells were washed thrice with PBST. 100µl of HRP-conjugated anti-rabbit immunoglobulins (1:1000) were added to each well and incubated for 1hr at 37ºC. Color was developed using [2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid)] i.e [ABTS] and H_2O_2 as substrate fixed amount (100µl/well) was added and plate was incubated at 37ºC for 30minutes in dark and plate was read at 410nm in an ELISA plate reader (Bio-Tek Instruments Inc., USA). Results were noted and interpreted for the optimized concentration of rabbit anti-SEB IgG and mice anti-SEB IgG.

6. Development of QDs-FLISA for the detection of SEB

For the detection of SEB in buffer first we have to optimize the appropriate dilution of bionanoconjugates i.e. PbS QDs tagged mice anti-SEB IgG. For this purpose, checkerboard titrations were done using following procedure: Different concentrations of 50ng, 100ng, 150ng, 200ng, 250ng, 300ng and 400ng of SEB-antigen (100µl/well) was suspended in carbonate-bicarbonate buffer (pH 9.6) and adsorbed on 96-well Maxisorb microtiter plates overnight at 4ºC in 100µl volume. Phosphate buffer containing no antigen was taken as control. Unadsorbed sites of wells were blocked with 3% BSA for 2 hours at 37ºC. After that, different dilutions of mice anti-SEB antibodies tagged with PbS QDs such as 1:100, 1:200, 1:400, 1:800, 1:1000, 1:1500, 1:2000, 1:3000, 1:4000 and 1:5000 were added in respective wells, and plate was incubated for 1 hour at 37ºC. After each step, wells were washed thrice with PBST using ELISA plate washer (5 minutes for each wash). Finally, plate was read at 438nm using microplate reader (spectrophotometer). (Excitation wavelength 320nm).

7. SDS-PAGE of purified SEB toxin, mice anti-SEB IgG and rabbit anti-SEB IgG

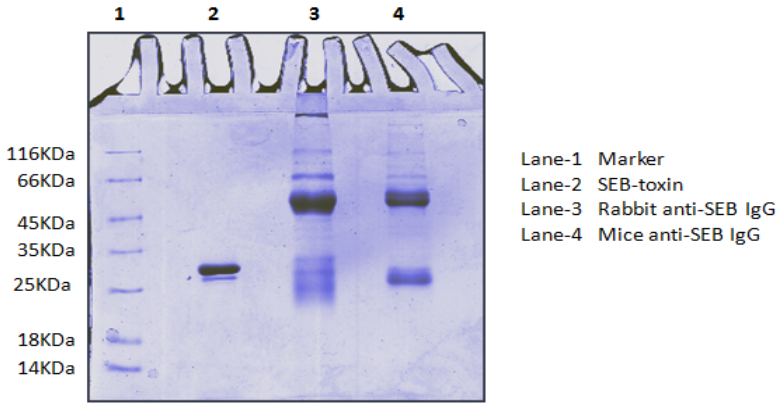


Fig.2. SDS-PAGE of SEB toxin, mice anti-SEB IgG and rabbit anti-SEB IgG

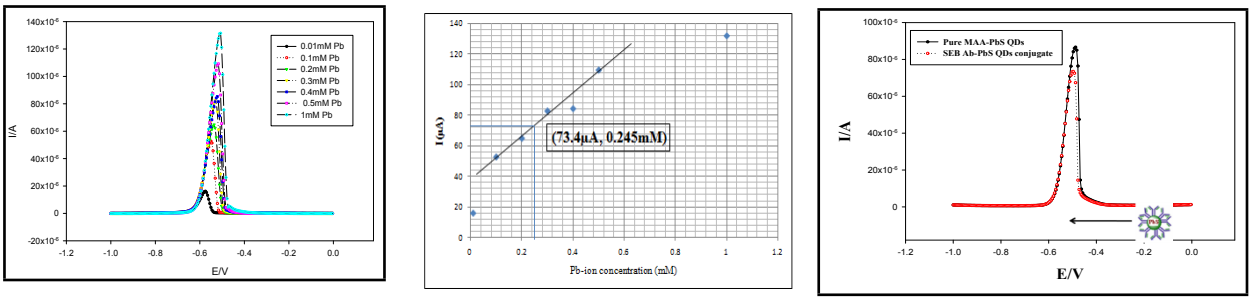
Each antibody molecule has two kinds of amino acid chains, the heavy chains and the light chains. SDS-PAGE analysis of antibodies generated in mice and rabbit against SEB was

performed to determine the presence of light and heavy chains which must be required for the specific binding of the target toxin i.e. SEB toxin. It also demonstrated that both the antibodies are present in working condition and can be used for the immunoassay of SEB. Working condition of antibody means that the presence of Fab fragments in antibodies, which is associated for binding with the epitope of the target analyte. SDS-PAGE of SEB toxin was also performed in the different lane (i.e. lane 2). A sharp band was coming around 27-29KDa clearly indicated that SEB toxin is a protein molecule and position of the band is reported for SEB in the literature. SDS-PAGE analysis of rabbit anti-SEB IgG and mice anti-SEB IgG was shown in lane 3 and lane 4 respectively.

8. Quantitative determination of the [Pb:Ab] ratio present in the 5µL of lead sulfide QDs tagged mice anti-SEB monoclonal antibodies conjugates

For the quantitative determination of [Pb:Ab] ratio present in the 5µL of lead sulfide QDs tagged mice anti-SEB monoclonal antibodies conjugates that is the final volume of the bionanoconjugates which was used for the electrochemical immunoassay of SEB. Simplest two step procedure was utilized for determination. In the first step, amount of Pb present in QDs-antibody conjugates was determined using standard square wave voltammetric method. Amount of IgG present in QDs-antibody conjugates was determined in the second step using standard BCA-protein estimation method. Finally, it was calculated that Pb:Ab ratio in the 5µL of bionanoconjugates was 1:4.8 by weight. We can state that about 82.76% antibodies were conjugated with 17.24% Pb by weight. So, there is large amount of specific mice anti-SEB monoclonal antibodies on PbS quantum dot nanocrystal and high sensitivity for the electrochemical detection of SEB was achieved. These steps are described below:

Step1. Determination of amount of Pb present in QDs-antibody conjugates



(a) **(b)** **(c)**

Fig.3(a). Shows the SWV obtained for the different concentrations of Pb-ions, (b) shows the standard curve for the determination of Pb present in QDs-antibody conjugates and (c) the SWV obtained for the PbS QDs and its bionanoconjugates

For the determination of quantity of lead present in QDs-antibody conjugate were prepared in standard solution of different concentrations of Pb-ions such as 0.01mM, 0.1mM, 0.2mM, 0.3mM, 0.4mM, 0.5mM and 1mM. After that 5µL of each concentration was mixed with 2mL of acetate buffer and then square wave voltammetric analysis was performed Fig.3(a) and the standard curve was plotted fig.3(b). In the next step, 5µL mice anti-SEB monoclonal antibodies tagged with PbS QDs was put on the surface of the SPE and then 20µL of 1N HCl was added and kept for 30min. After that, this SPE was dipped in 2mL of acetate buffer (0.2M, pH 5.2). In the next step, GC-electrode was dipped in this solution and SWV was performed using same experimental conditions fig. 3(c). For the mice anti-SEB monoclonal antibodies tagged with PbS QDs, 73.4µA peak current was observed and it was corresponding to 0.245mM Pb concentration. Finally, 253.8ng Pb was present in 5µL mice anti-SEB monoclonal antibodies tagged with PbS QDs.

Step2. Determination of amount of IgG present in QDs-antibody conjugates

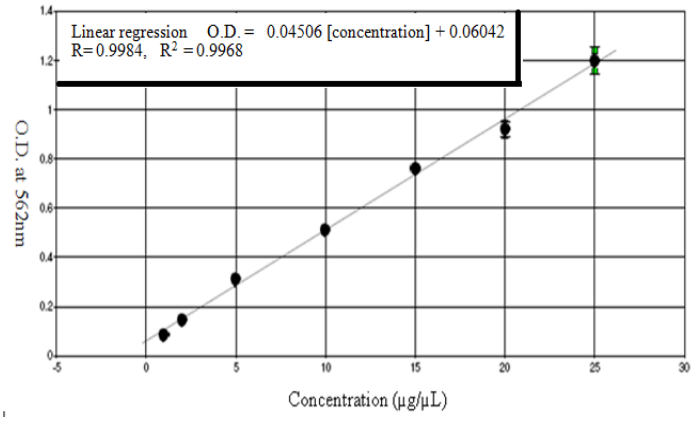


Fig.4. Standard graph obtained for increasing concentration of BSA using BCA-protein estimation method.

For the determination of amount of IgG bound with lead sulfide quantum dots standard solution of BSA (1µg/µL) was prepared and standard curve was plotted using BCA-protein estimation method. From the standard curve as shown in fig.4. we had obtained 0.24µg/µL concentration of protein i.e. mice anti-SEB antibody present in the antibody-quantum dots bionanoconjugates. Since, 1.2µg of mice anti-SEB monoclonal antibody was present in 5µL of the bionanoconjugates which was the final amount used in the electrochemical immunoassay of SEB for the experiments. It was calculated that Pb:Ab ratio in the 5µL of bionanoconjugates was 1:4.8. It means about 82.76% antibodies were conjugated with 17.24% Pb by weight. So, there is high loading of monoclonal antibodies on each quantum dot nanocrystal and high sensitivity for the electrochemical detection of SEB was achieved.

9. SEM-characterization of PbS QDs

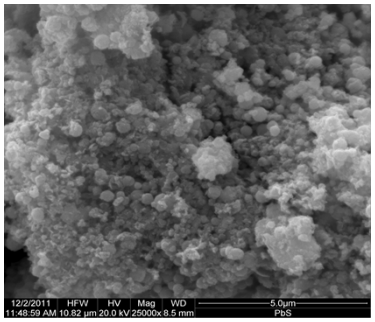


Fig.5. SEM characterization image of the PbS QDs

Morphology of the capped and uncapped PbS QDs was determined by scanning electron microscopy (SEM). The SEM image of uncapped PbS QDs showed that the prepared quantum dots are uniform and regular but particles are agglomerated due to the attractive force between the nanocrystals which leads to become the particle size larger (Fig.5).

10. FT-IR characterization of PbS and MAA-capped PbS quantum dots

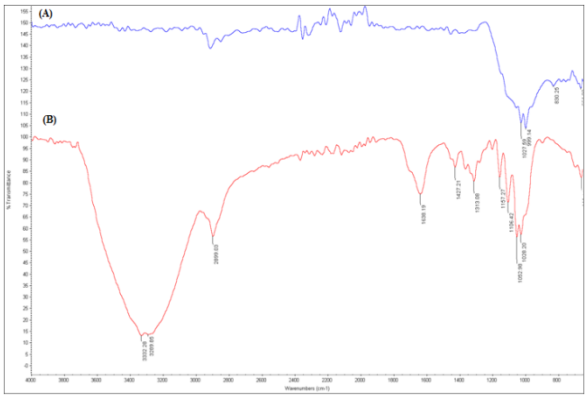


Fig.6. (a) shows the FT-IR spectra of uncapped PbS QDs and (b) is the FT-IR spectra of MAA capped-PbS QDs.

Fourier-transformation infra-red spectroscopy (FT-IR) spectrum of uncapped PbS QDs is shown in Fig.6a. In this spectrum only two weak characteristic peaks for the Pb-S band were observed at 830cm^{-1} and 1027cm^{-1}. Whereas in the case of MAA-capped PbS QDs the most pronounced IR absorption bands occurred at 3332-3289cm^{-1} (υ OH, COOH), 2899cm^{-1} (υ_s C-H),

1638cm^{-1} (υ C=O), 1427cm^{-1} (υ_s COOH), 1313cm^{-1} (υ C-O). This indicates carboxyl and carbonyl groups coexistence on the surface of the MAA-capped PbS QDs (Fig.6b). While the peaks for S–H vibration were absent in MAA-capped PbS QDs due to the disappearance of S–H vibration in FT-IR spectra of MAA capped PbS QDs was the result of the covalent bonding between thiols and Pb atoms on the QDs surface. Some reports are available for the FT-IR spectrum of lead sulfide nanomaterials capped with various kinds of acids like aminocaproic acid capped PbS nanoparticles (size around 9.9nm) shows bands at 1380 cm^{-1}, 1630 cm^{-1}, 1530cm^{-1}, 1545 cm^{-1}, 2850cm^{-1} - 2950cm^{-1} and 3300cm^{-1} to 3600cm^{-1} [7]. FT-IR of oleic acid capped PbS nanocrystals (size~7.3nm) shows vibrational bands at 2917cm^{-1} (CH_2 asymmetric stretching), 2850cm^{-1} (CH_2 symmetric stretching), 2975 cm^{-1} (C-H stretching), 1276cm^{-1} (C–O stretching), 1535cm^{-1} ($\upsilon_{asymmetric}$ COO^- stretching) and 1410cm^{-1} ($\upsilon_{symmetric}$ COO^- stretching) [8]. Till now, there is no report for the FT-IR spectrum of MAA-capped PbS QDs.

11. UV-VIS characterization of MAA-capped PbS quantum dots

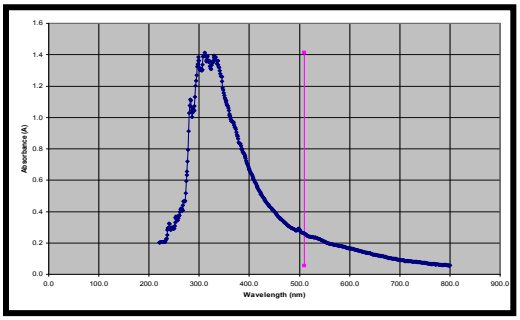


Fig.7. UV-visible absorption spectrum of the MAA capped-PbS QDs.

To monitor the optical properties of the nanomaterials, UV–VIS spectroscopy technique was used. The optical properties of MAA- capped PbS QDs were characterized by UV–visible absorption spectrometry at room temperature. The characteristic absorption peak of the MAA

capped PbS QDs is located at 320nm as shown in Fig.7. The broad absorption spectra was observed & peaks appear in the range of 300-330nm. There are various reports in the literature for the UV-VIS absorption spectrum of lead sulfide nanomaterials. UV-VIS spectra of mercaptoacetic acid capped PbS-nanoparticles (diameter~3nm) were labeled with oligonucleotides DNA probes and peak around 254nm was observed [9]. Different values for UV-VIS absorption of capped PbS QDs were widely reported by various authors. They were found in the range between 200nm to 450nm [10-14].

12. Photoluminescence (PL) emission spectra of MAA-capped PbS quantum dots

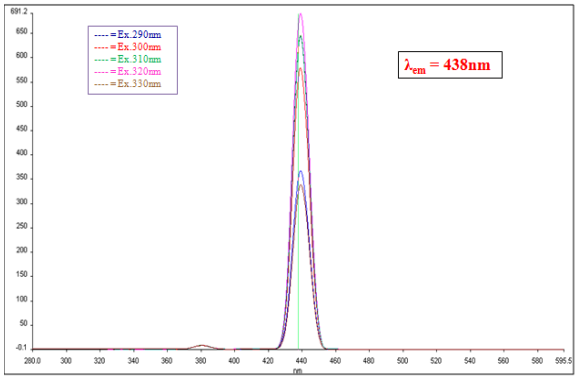


Fig.8. Photoluminescence spectra of the MAA capped-PbS QDs at different excitation wavelengths

Photoluminescence emission (PL) spectra of the prepared MAA-capped PbS QDs are shown in Fig.8. The light of different wavelengths such as 290, 300, 310, 320 and 330nm was given to excite the QDs sample at room temperature. At any of these excitation wavelengths the PL-emission spectral peak of MAA-capped PbS QDs was found at around 438nm. It was concluded that maximum fluorescence emission intensity of the functionalized PbS QDs was obtained when excited at 320nm. It was reported, that Tetrapod like PbS microcrystals

synthesized by hydrothermal method showed photoluminescence emission spectrum due to the radiative recombination of the surface states and the emission peak was observed at 440nm when excited at 275-325nm [15]. Our report was close to the values as reported in the literature.

13. Effect of pH on the fluorescence intensity

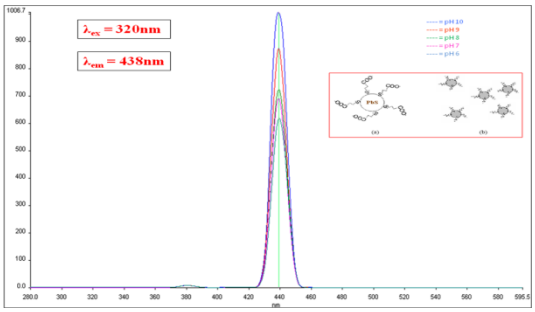
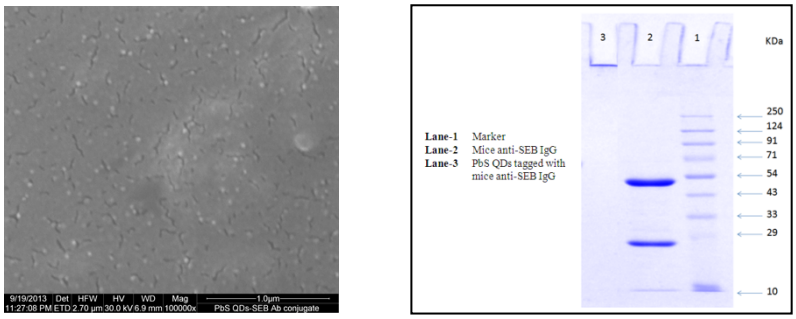


Fig.9. Photoluminescence spectra of the MAA capped-PbS QDs at different pH in aqueous medium.

pH is a very important parameter which could effect the fluorescence intensity of the MAA-capped PbS QDs which was soluble in the aqueous medium. To study the effect of pH, the pH of the medium in which the QDs were dissolved was changed from 6 to 10 and the fluorescence emission spectrum was recorded as shown in fig.9. It was observed that the intensity of the fluorescence emission spectrum of the QDs was increased with increasing the pH of the QDs dispersion and the intensity was maximum at pH 10 as shown in (Fig.6). Hence, fluorescence intensity increased with increasing pH due to the deprotonation of the –COOH groups and the formation of $–COO^-$ ions on to the QDs surface which will make the quantum dots to repel them each other which will provide them good dispersity in their dispersions. Another reason is that in basic medium due to the deagglomeration of QDs particle size was reduced and more number of single QDs was present in their dispersion. Further, increasing the

pH of the QDs dispersion the fluorescence intensity was very poor which could not be observed. This was due to the formation of hydrated product of the lead (Pb) at pH more than 10 and the lead ions will form the hydroxide in the basic medium and the precipitate of lead hydroxide was formed. In more acidic medium (less than pH 6) the fluorescence emission intensity was also very small and could not be recorded. This was due to the protonation of the disulfide bond attached to the PbS QDs and thus removal of the mercaptoacetic acid capping from the PbS QDs surface. This will make the QDs unstable and they will tend to agglomerate. A similar report for the effect of pH on the fluorescence intensity of CdS quantum dots was available in the literature [2].

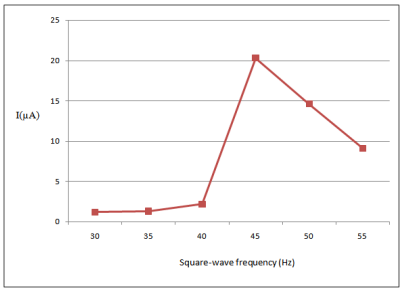
14. SEM and SDS-PAGE characterization of lead sulfide QDs tagged mice anti-SEB monoclonal antibodies conjugates

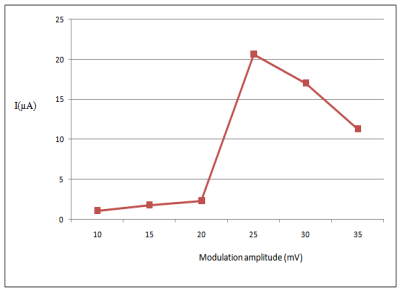


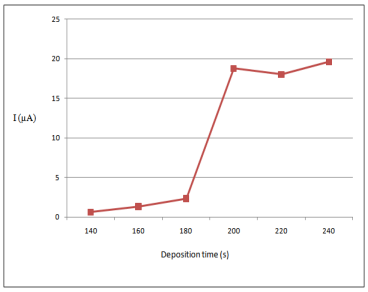
(a) **(b)**

Fig.10. (a) SEM characterization image of the mice anti-SEB monoclonal antibodies tagged with PbS QDs and (b) shows the SDS- PAGE characterization.

SEM image of the lead sulfide QDs tagged mice anti-SEB monoclonal antibodies conjugates is shown in Fig.10(a). It is interesting to find that the quantum dots were not agglomerated while bound to the antibodies. These bionanoconjugates may contain several PbS







QDs per each SEB-antibody. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique was followed for the confirmatory binding of PbS QDs with specific monoclonal antibodies against SEB. Polyacrylamide gel can easily separate the proteins in the form of intense band coming according to their molecular size and weight. In the functional structure of an antibody both light chains and heavy chains should be present which can be used for the binding with toxin. Lane 2 represented that both light chains and heavy chains were present in the mice anti-SEB monoclonal antibodies. These antibodies were covalently bound with carboxylic group functionalized PbS QDs using EDC/NHS chemistry. Since, quantum dots are not protein molecules and once they bind with antibodies they are not able to transfer on to the membrane and remain in the loading wells due to the formation of larger size of the bionanoconjugates as compared to single quantum dot. Therefore, visible intense blue band appeared at the loading wells for lane 3 of the SDS-PAGE in fig.10(b) that corresponded to the PbS QDs tagged antibody conjugates. Lane 1 represented the magic marker used for the monitoring of protein separation and determining approximate size of proteins during SDS-PAGE. This method was used for previously the characterization of quantum dots tagged antibody conjugates and reported in the literature [3].

15. Optimization of square-wave frequency, modulation amplitude and deposition time

(a) **(b)** **(c)**

Fig.11(a) is the calibration curve for the optimization of square-wave frequency, (b) modulation amplitude and (c) deposition time.

Various SWV parameters were optimized so as to obtain the high response of the voltammetric signal for the SEB detection. These optimization results were obtained for 10ng/mL SEB antigen concentration. For the optimization of square wave frequency, we had increased it from 30Hz to 55Hz. Normally, in voltammetric immunosensor the useful range of frequency is 8-250Hz [16]. The square wave frequency is optimized at 45Hz because at this frequency the maximum voltammetric response of the immunosensor was achieved as shown fig.11(a). At higher frequency than 45Hz, the voltammetric response was decreased due to the influence of the large capacitive current. The modulation amplitude will also influence the response of the immunosensor generally it is suitable in the range of 5-100mV. Therefore, it was expected to optimize it to achieve the high sensitivity. The modulation amplitude was optimized at 25mV. Further, increasing and decreasing the modulation amplitude the response was decreased (Fig.11b). This is due to the fact that at large modulation amplitude the voltammetric peak will become broad because of non-linearity effect the voltammetric peaks was distorted and hence the potential resolution was low. For the optimization of deposition time, maximum response was obtained at 200s deposition for the dissolved PbS QDs onto the electrode surface. It was observed that while changing the deposition time from 140s to 200s the voltammetric peak current is increased and become saturated after 200s (Fig.11c). Further increasing the deposition time the response was not increased and almost saturated. The increase of deposition time better supports the QDs detection due to the increase of the quantity of the reduced lead coming from the lead ions in equilibrium with PbS QDs at optimized conditions. Optimizations of these parameters were reported in the literature for the cadmium sulfide quantum dots [17].

The effect of very low and very high square wave frequency and modulation amplitude was mentioned in the literature [16].

16. Optimization of rabbit anti-SEB antibody (capturing antibody) concentration

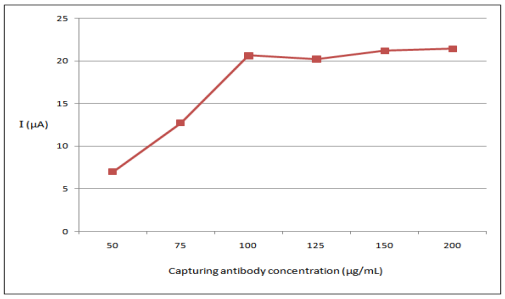


Fig.12. Calibration curve for the optimization of rabbit anti-SEB antibody (capturing antibody) concentration.

Rabbit anti-SEB antibody (capturing antibody) concentration is one of the most important parameter which would affect the response of the immunosensor. Thus, there is a need for the optimization of CAb concentration to get the maximum sensitivity. The condition was optimized for 10ng/mL of SEB-antigen. For this purpose, the screen-printed electrodes were incubated with various amounts of capturing antibody for 1h and followed by blocking with 3% BSA for 15 min. Then, 10ng/mL SEB antigen is added on each electrode. After it, 5µL of PbS QDs conjugated with mouse anti-SEB antibody (revealing antibody; fixed amount of 5µL) were incubated for 15min at 37^0C. The voltammetric peak current was increased when the concentration is increased from 50µg/ml to 100µg/ml. Further, increasing the concentration from 100µg/ml to 200µg/ml the response was almost constant (Fig.12). It indicates that at 125µg/ml CAb concentration was sufficient and optimized

for capturing of all the antigens. Hence, 125µg/ml concentration of the CAb was used in further experimentation.

17. Optimization of incubation time for mice anti-SEB antibody conjugated with MAA-capped PbS QDs (revealing antibody)

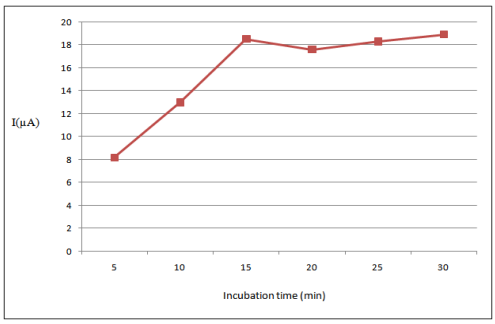
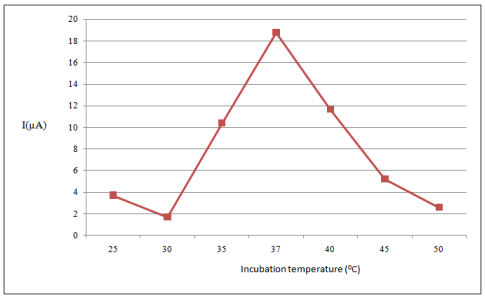


Fig.13. Calibration curve for the optimization of incubation time for mice anti-SEB antibody conjugated with MAA-capped PbS QDs (revealing antibody)

The voltammetric response mainly depended on the incubation time for MAA-capped PbS QDs-mice anti-SEB antibody conjugates (revealing antibody) in the sandwiched assay. The effect of incubation time of these QDs-antibody conjugates on the SWV peak was studied. With increasing incubation time the voltammetric peak current increased since more and more QDs-antibody conjugates has combined to SEB antigen immobilized on the electrode surface. The voltammetric current reached a maximum at 15min and longer incubation time did not improve the response (Fig.13). A longer incubation time could result in a large nonspecific signal. Thus, the optimal incubation time was 15min. Incubation time studies were previously done in the literature for the detection of neutravidin using cadmium telluride (CdTe) quantum dots [6].

18. Optimization of incubation temperature for SEB-antigen

Fig.14. Calibration curve 

for the optimization of incubation temperature for SEB-antigen

For the optimization of incubation temperature, SPE were incubated with 125µg/mL concentration of capturing antibody for 1h and followed by blocking with 3% BSA for 15min at 37^0C. Then, 10ng SEB antigen is added on each electrode and incubated for 15min at different incubation temperatures. After it, PbS QDs conjugated with mice anti-SEB antibody (revealing antibody; fixed amount of 5µl) were incubated for 15min at 37^0C. It was observed that at 37^0C the voltammetric peak current is highest which indicates the formation of stable immunocomplex at the physiological temperature (37^0C). Further, increasing and decreasing the incubation temperature the response is also decreased which indicates that at another temperature the biological activity or efficiency of antibody may be reduced for antigen binding to its active site (Fig.14). Similar observations are reported in the literature [18].

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