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Selective capturing and detection of *Salmonella typhi* on polycarbonate membrane using bioconjugated quantum dots

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ABSTRACT

Present work demonstrates the utilization of surface modified polycarbonate (PC) membrane as solid phase and antibody conjugated CdSe/ZnS quantum dots (QDs) as fluorescent label for the sensitive and selective detection of *Salmonella typhi* (*S. typhi*) in water in a period of 2.5 h. PC membrane was surface modified with glycine and activated by EDC/NHS for immobilization of *S. typhi* specific IgG. Antibody immobilized porous PC membrane was incubated with bacteria contaminated water for immunocapturing of *S. typhi*. Antibody conjugated QDs were also prepared by using carbodiimide chemistry. Both modified PC membrane and quantum dots were characterized by using various modern analytical tools. It was estimated that 1.95 molecules of QDs were successfully bio-conjugated per unit of IgG. PC membrane with captured bacteria was incubated with prepared IgG conjugated QDs for the formation of sandwich complex. Analysis of the regions of interest (ROI) in fluorescent micrographs showed that newly developed method based on PC and fluorescent QDs has 100 times higher detection sensitivity (100 cells/mL) as compared with detection using conventional dye (FITC) based methods.

1. Introduction

Various organic fluorophores have been well-established to be used for labeling of biomolecules in various diagnostic applications, but they have limited effectiveness in long-term imaging and multiplexed detection due to their inherent demerits [1,2]. Quantum dots (QDs) are nanoscale crystals of semiconductor material with diameters of the order of 2–10 nm, or roughly 200–10,000 atoms that fluoresce when excited by a light source. Compared with conventional organic fluorophores, QDs exhibit advantageous photophysical properties of broad absorption and narrow emission spectral profile, resistance to photobleaching, larger Stokes shift value, composition- and size-dependent absorption and emission, high molar extinction coefficient value and good quantum yield [3,4]. These properties of QDs enable them to be used in biological systems over extended time periods for single particle detection. In addition, a single wavelength can be used to simultaneously excite QDs of different sizes, and as a result different emission spectra are obtained facilitating multiplexed detection [5,6].

Efforts in the direction of improving the sensitivity of immunodetection by exploring highly fluorescent and photostable QDs as labels have gained considerable momentum over the past decade [7,8]. Bioconjugation of QDs with biomolecules is being reported to have potential prospects in the development of rapid diagnostic systems for detection of pathogenic bacteria [9,10]. Some formats of fluorescence based immunoassays utilizing fluorescent QDs have been reported for detection of *Escherichia coli O159:H7*, *Salmonella typhimurium* and *Listeria monocytogenes* [11–13]. Recently, Goldman et al. reported the development of sandwich fluoroimmunoassay on polystyrene (PS) microtitre plate for the detection of cholera toxin and staphylococcal enterotoxin B up to 60 ng/mL and 250 ng/mL, respectively [14]. However, in general, the immunoassays developed on polystyrene plate have less sensitivity and reproducibility due to immobilization of biomolecules by adsorption on the polystyrene surface [14,15]. Porous polycarbonate (PC) membranes have immense potential as solid phase for the development of fluoroimmunoassays as they have high surface area, ease of generation of desired functionality for stable attachment of numerous biomolecules, sample

Abbreviations: QDs, quantum dots; IgG/Ab, antibody; Ag, antigen; Sty IgG, *Salmonella typhi* specific IgG; Sty Ag, whole-cell *Salmonella typhi* antigen; RAG IgG, rabbit anti goat IgG; GAR IgG-HRP, goat anti rabbit IgG-horse radish peroxidase conjugate.

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enrichment by filtration, quite less non specific adsorption of proteins and are also available in non-autofluorescent forms [16–18]. Surface modification of PC by chemical treatment is a desirable method, out of the various methods reported for immobilization of biomolecules, because of being simple, easy, reproducible and capable of covalent linking of biomolecules to the modified PC surface [19–21]. Bhunia et al. reported the surface modification of PC membrane by its chemical treatment with poly-lysine for immobilization of FITC-IgG for capturing of *L. monocytogenes* [22]. Although method detected the target bacteria selectively but the detection was studied with a fixed bacterial concentration of 7.3×10^8 cells/mL using conventional labeling fluorophore (FITC-IgG).

Present study is focused on surface modification of PC membrane by chemical treatment with glycine and further activation by EDC/NHS for the immobilization of *S. typhi* specific IgG. IgG immobilized PC membrane was directed towards the development of solid phase for sensitive detection of *S. typhi* with the use of bioconjugated CdSe/ZnS QDs as fluorescent label. It was also aimed to characterize the prepared QDs bioconjugates in detail to ensure the good quality of fluorescent label for immunodetection. The challenge was also taken to evaluate the sensitivity and specificity of developed method in water contaminated with mixed bacteria population. Semiquantitative analysis of the samples was also attempted for the detection of *S. typhi* by spectral profiling in lambda scan mode of confocal laser scanning microscope.

2. Experimental

2.1. Materials and reagents

Cadmium oxide (CdO), selenium, sulfur, zinc oxide (ZnO), trioctylphosphine oxide (TOPO), l-octadecene (ODE), thioglycolic acid (TGA), o-dichlorobenzene, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), glycine were purchased from Sigma–Aldrich (St. Louis, USA). Hexane, methanol, acetone, Na_2CO_3, glacial acetic acid, NaOH were purchased from Merck (Mumbai, India). Toluidine blue dye used for estimation of –COOH group was also purchased from Merck (Mumbai, India). All the chemicals were used as received. Deionized water with resistivity of 18.2 MΩ cm^{-1} obtained from Milli-Q water purification system was used throughout the study.

Porous black polycarbonate membrane (cat. no. GTTP02500) with diameter of 0.22 μm was purchased from Millipore, USA. Whole cell *S. typhi* 'O' antigen (Sty Ag) was prepared by heat inactivation at 100 °C for 30 min after culturing on phenol agar plates for the maximum expression of surface "O" antigen on bacterial cell surface as described by Cruickshank et al. [23]. Concentration of bacterial suspension was adjusted to 2×10^8 cells/mL by spectrophotometer at 595 nm and diluted serially to prepare a range of concentrations of bacteria [24]. *S. typhi* specific IgG (Sty IgG) was purified from polyclonal antisera generated in NewZealand white rabbit using whole bacterial cell as an immunogen and purified using affinity chromatography based purification kit (Prosep A®). 10 mM PBS (pH 8) containing BSA (2 g/L), sucrose (90 g/L), ammonium sulphate (100 g/L) and 0.01% thimerosal was used for making various dilutions of *S. typhi* specific IgG and QD-IgG conjugate. FITC-Sty IgG (FITC-IgG) conjugate in the ratio of 56:1 was supplied commercially by Bangalore Genei, India. Goat anti-rabbit antibody-HRP conjugate (GAR IgG-HRP) and substrate (TMB/H_2O_2) were also purchased from Bangalore Genei, India.

2.2. Bioconjugation of CdSe/ZnS quantum dots with antibody

Highly fluorescent TOPO-capped CdSe/ZnS core/shell QDs were synthesized by successive ion layer adsorption and reaction (SILAR) technique as described by Peng et al. with some modifications [25]. Stable surface -ligand exchange of CdSe/ZnS-TOPO with thioglycolic acid at pH 8 resulted in dispersion of QDs in water. Briefly, CdSe/ZnS-TOPO QDs were dissolved in o-dichlorobenzene and reacted with TGA for 24 h at 50–60 °C under stirring. TGA coated QDs were extracted by the addition of ethanol and centrifuged thrice at 8000 rpm for 3 min to wash excess TGA. Pellet of washed TGA capped QDs was collected, resuspended in MQ water of pH 8 and stored at 4 °C.

Carboxy terminated TGA capped QDs were activated using carbodiimide chemistry for conjugation with antibody. Briefly, QDs (2.5 nM) were incubated with EDC (2.2 mM) and NHS (4 mM) in ratio of 1:50:100 at 37 °C for 30 min under gentle stirring at RT. Bioconjugation of activated QDs with Sty IgG was performed by stirring at RT for 1 h. Bioconjugation process was optimized by varying mole ratios of QD and IgG in the range of 100:1, 50:1, 20:1 and 10:1. Bioconjugated QDs, thus obtained, were centrifuged at 8000 rpm to remove excess Ab and ultracentrifuged to remove unbound QDs according to the method described by Carion et al. with some modifications [26]. In brief, bioconjugated QDs were layered on 70% sucrose solution and centrifuged at 40,000 rpm for 5 min. Different layers formed after ultracentrifugation were collected separately under hand-held UV lamp by micropipettes and studied for their fluorescence and biological activity. Upper layer containing bioconjugated QDs was centrifuged at 3000 rpm to remove residual sucrose solution and stored in Ab storage buffer at 4 °C for further use.

2.3. Characterization of prepared QDs

Synthesized hydrophilic QDs and bioconjugated QDs were characterized by various techniques. Optical characterization of synthesized QDs was done by recording the absorption and fluorescence spectra (excitation wavelength: 405 nm) by spectrophotometer (Perkin Elmer, Lambda 55) and spectrofluorometer (Perkin Elmer LS 55), respectively. All the spectra were recorded with water as blank. Size analysis, size distribution and the shape of synthesized QDs were examined by transmission electron microscopy (Phillips, CM12) with an acceleration voltage of 100 kV at magnification of 2,30,000X. 3 μL of diluted solution of QD sample was dropped on a 200-mesh carbon coated copper grid and dried at RT for TEM sample preparation. An average of 10–20 field views of QDs were considered for recording the observations.

Attenuated total reflectance Fourier transform infrared spectrometry (ATR-FTIR) of non-conjugated and IgG conjugated QDs was performed using a Perkin Elmer Spectrum One spectrophotometer. All ATR-FTIR spectra were collected within the range of 600–4000 cm^{-1} with an average of 16 scans per sample. A Bruker AC 300 spectrometer with a frequency of 300 MHz was used for recording ^1H NMR of IgG conjugated QDs in D_2O solvent with tetramethyl silane as an internal standard. All three layers obtained after ultracentrifugation were collected separately under UV lamp and evaluated by ELISA studies for biological activity of bioconjugated QDs. (Detailed procedure and related results are given in Supplementary Material). The number of QDs per IgG was calculated from the ratio of the concentration of QDs and the concentration of IgG in prepared bioconjugates according to the method of Ji et al. [27] using Eq. (1):

$$N = \frac{C_{QD}}{C_{Ab}} = \frac{A_{max}/\varepsilon_{QD}}{(A_{280} - A_{max} \times r_f)/\varepsilon_{Ab}} \quad (1)$$

where, N is the number of QDs per IgG, C_{QD} is the concentration of QDs attached to the IgG, C_{Ab} is the concentration of IgG attached to the QDs in the conjugate. ε_{Ab} is the molar extinction coefficient of IgG, ε_{QD} is the molar extinction coefficient of QDs and r_f is the ratio of absorbance of QDs at 280 nm to the absorbance at λ_{max}. For the calculation, the molar extinction coefficient of IgG was typically

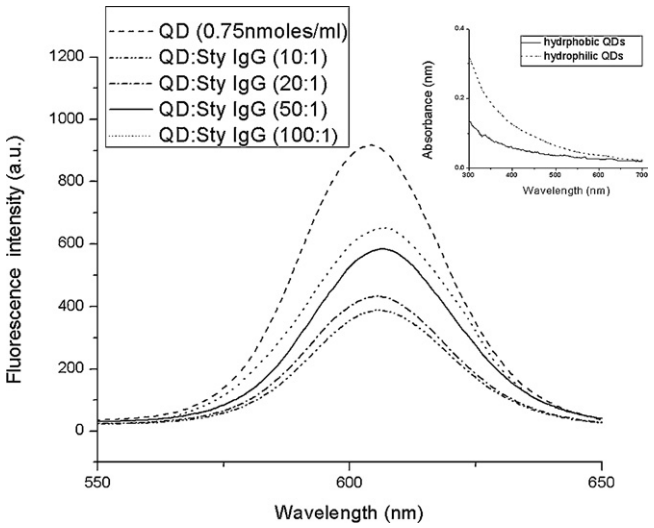


Fig. 1. Fluorescence emission spectra of QDs with absorption spectra of QDs in inset.

taken as $2.1 \times 10^5\ M^{-1}\ cm^{-1}$ as reported in literature [27] and that of water-dispersible QDs was calculated by the calibration curve prepared for absorbance value versus concentration of QDs.

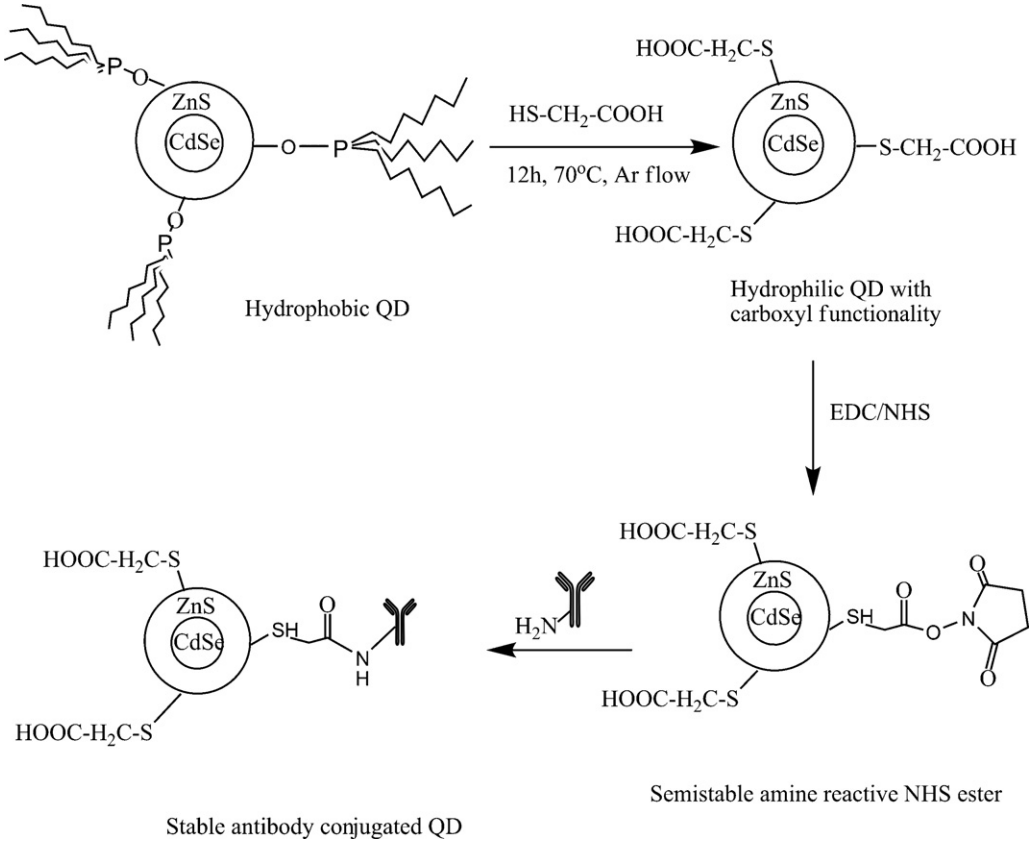
2.4. Surface modification of polycarbonate membrane

For the preparation of solid phase, porous black PC membrane was surface modified by mild chemical treatment. The PC membrane was cut into discs of diameter 0.5 cm using a standard paper punch and used for modification studies. Membrane discs were modified by glycine (100 mg/mL) treatment in the presence of 50 mM Na_2CO_3 under gentle shaking at RT for 6 h. The concentration of surface carboxyl content of the glycine treated PC membrane was determined by calculating the uptake of toluidine blue dye according to the method reported by Drews et al. [28]. This method is based on the assumption that each carboxylic acid group binds to one molecule of toluidine blue. In brief, a membrane disc modified on both sides (with the reactive surface area of 0. 196 cm^2 approximately) was incubated with 5 mL of fresh toluidine blue solution (2×10^{-4} M) in 0.1 mM NaOH for 1 h at 40 °C in a water bath. Afterwards, the solution was removed and the membrane was surface rinsed thrice with fresh 0.1 mM NaOH. The sample discs were then kept in flasks with 5 mL of 50% acetic acid for 30 min at 40 °C to release the bound toluidine blue into the solution. The absorbance of the solutions was measured with a Perkin Elmer Lambda 25 UV spectrophotometer at a wavelength of 630 nm. The number of reactive carboxylic acid groups in the discs was determined from a calibration curve which was obtained by measuring the optical absorbance of different concentration of toluidine blue at 630 nm.

To evaluate the change in hydrophilicity of PC membrane after glycine treatment, contact angle measurements were recorded using goniometer (Rame-Hart, USA Model 100-00-230) after placing a drop of 10 μL of MQ water by microsyringe. All the samples were evaluated in triplicates at ambient temperature (25–30 °C). The experimental values are the average five droplets deposited at different positions throughout two sample surfaces.

2.5. Antibody immobilization on surface modified PC membrane

Glycine treated PC membrane-discs were washed with MQ water and activated with EDC/NHS for 1 h at 37 °C. Activated membrane-discs were incubated with *S. typhi* specific IgG solution (10 μg/mL) at 4 °C for overnight with occasional shaking. After incubation, discs were washed thrice with PBS and blocked with BSA



Scheme 1. Pictorial representation of preparation of antibody immobilized core–shell CdSe/ZnS quantum dots.

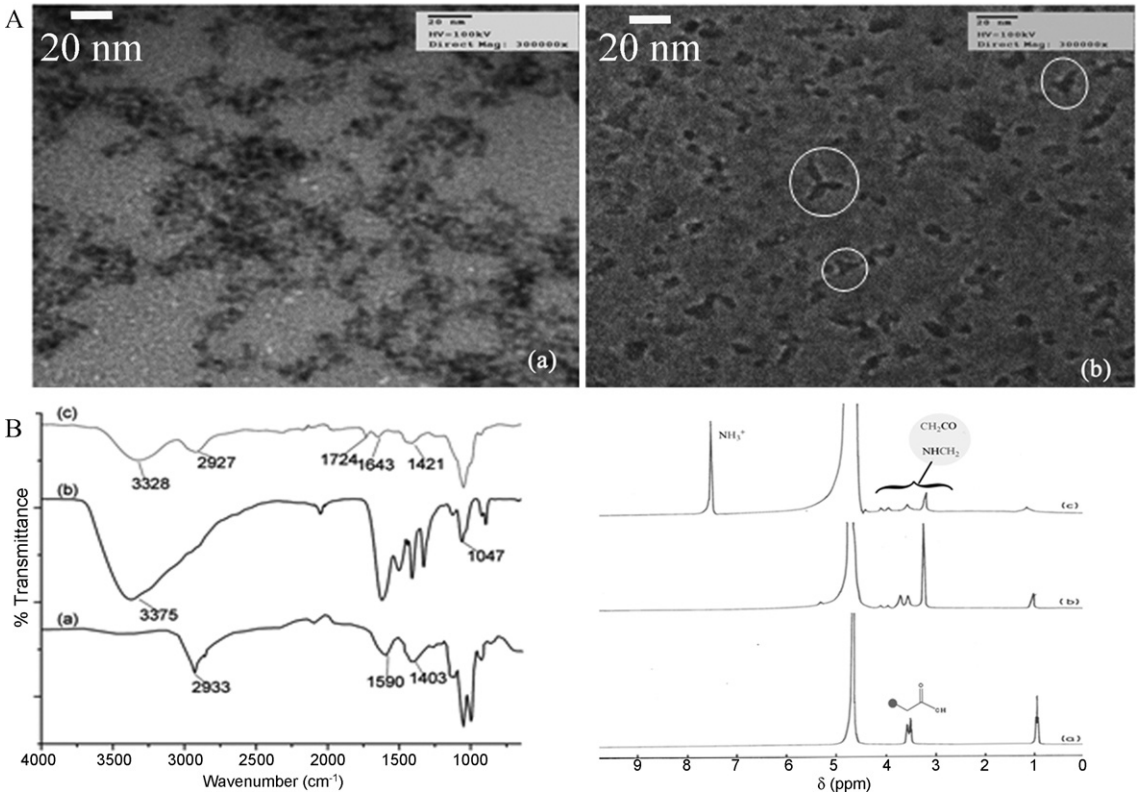


Fig. 2. Characterization of bioconjugated QDs: (A) transmission electron micrographs of (a) hydrophilic QDs and (b) Ab conjugated QDs; (B) FTIR spectra (a) QD, (b) IgG and (c) IgG conjugated QDs; and (C) ^{1}H NMRspectra (a) QD, (b) IgG conjugated QDs and (c) IgG.

(1 mg/mL) at 37 °C for 1 h. After the blocking, discs were washed and stored at 4 °C for further usage.

2.6. Characterization of modified PC membranes

Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy was utilized to characterize the chemical changes in modified and unmodified PC membranes. Surface morphology of modified and unmodified membranes was investigated by scanning electron microscope (Zeiss, EVO 50) by mounting the samples on a metallic stub using double-sided tape. Silver paint was applied on the samples to make them conducting for better resolution. Indirect ELISA studies were conducted using anti rabbit IgG-HRP as secondary antibody for confirming the immobilization of rabbit anti *S. typhi* IgG on modified PC membrane. (Detailed procedure and related results are given in Supplementary material).

2.7. Microscopic studies for detection of S. typhi with semiquantitative analysis

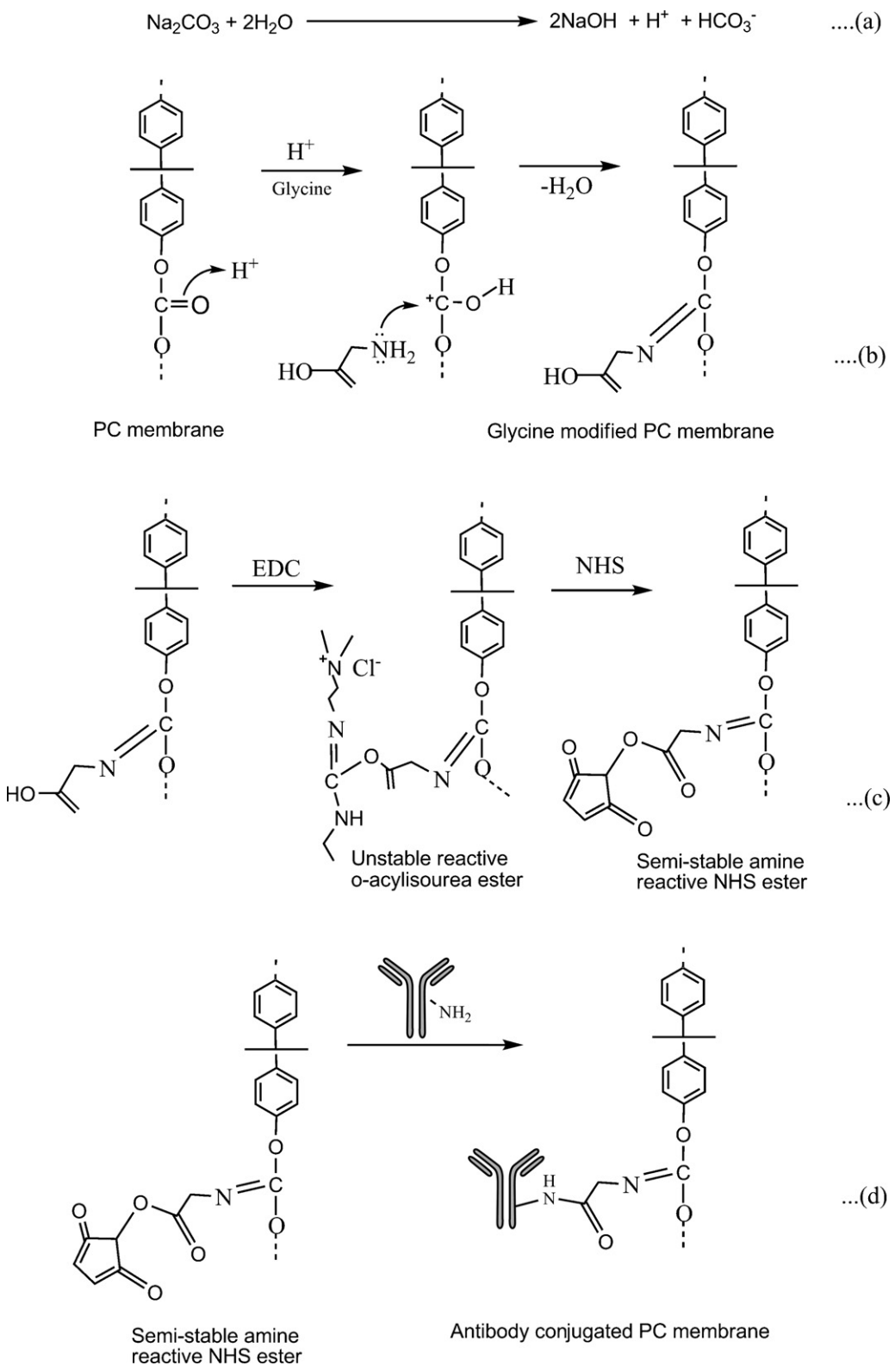
Prepared Sty IgG immobilized PC membrane (PC-Sty IgG) was used as capture matrix for the capturing of targeted bacteria and Sty IgG conjugated fluorescent QDs (Sty IgG-QD) as label in detection method. Ab immobilized membrane discs after blocking with BSA were incubated with 200 μL of Sty Ag (10^8 cells/mL–10^1 cells/mL) for 1 h at 37 °C. After incubation, membrane discs were washed with PBS-T and further incubated with 200 μL of Sty IgG conjugated QDs (1:100 dilution of purified stock of bioconjugated QDs) for 1 h at RT to form a sandwich complex (PC-Ab-Ag-Ab-QDs) on the PC membrane. Incubated membranes were washed vigorously with excess of PBS-T to remove unbound bioconjugated QDs. After washing, membranes were observed for fluorescence and spectral profile by confocal laser scanning microscope (Olympus, FV 1000) with 40X objective. A set of the negative controls without Sty Ag was also kept in triplicates while giving the same treatment as the test discs. In another set of experiments, FITC conjugated Sty IgG was also used as label to compare with QD conjugated IgG based detection on PC membrane. Samples were excited with 405-line and 488-line of multiline Ar ion laser for QDs and FITC based detection, respectively.

Specificity of developed system was also evaluated by cross-reactivity studies. For this, *E. coli* (2×10^8 cells/mL) was incubated in dark for 30 min at RT with FITC (0.2 mg/mL in DMSO) for staining. After incubation, stained *E. coli* suspension was washed by centrifugation for several times to remove the excess FITC dye. FITC-stained *E. coli* suspension was mixed with *S. typhi* (10^6 cells/mL) and used for incubation with Sty IgG immobilized PC membrane (PC-Sty IgG) for immunocapturing. Remaining experimental procedure was performed as described above and finally membranes were examined by CLSM for imaging and spectral profiling.

3. Results and discussion

3.1. Bioconjugation of CdSe/ZnS quantum dots with antibody

The water dispersible CdSe/ZnS QDs were used to prepare Ab conjugated nanoparticle labels for fluoroimmuno-based detection. Schematic representation of the preparation of bioconjugated QDs is shown in Scheme 1. Hydrophobic CdSe/ZnS QDs were successfully prepared by SILAR method as described in literature and converted to carboxy functionalized water dispersible QDs by ligand exchange with thioglycolic acid [29,30]. EDC, a zero-length cross-linker, was selected for bioconjugation of QDs with IgG because it is an established agent with defined coupling chemistry and it is known to yield exclusively QD-protein conjugates in a controlled manner with high bioconjugation efficiencies [31].



Scheme 2. Proposed reaction mechanism for modification of polycarbonate membrane and immobilization of Ab.

3.2. Characterization of non-conjugated and conjugated QDs

Fig. 1 shows the fluorescence emission spectra of Sty IgG conjugated and non-conjugated QDs with absorbance spectra of hydrophobic and hydrophilic QDs in inset. CdSe/ZnS QDs capped with TGA showed characteristic narrow and symmetric emission spectra at 610 nm on excitation at 400 nm. On conjugation with Sty IgG, fluorescence intensity of the QDs decreased and shifted slightly towards higher wavelength. Reason of the decreased fluorescence intensity on bioconjugation is attributed to change in the QD electronic energy caused by chemical interactions or local or electric or stress field which occurred on QD surface during bioconjuga-

Table 1
Number of reactive carboxylic acid group as determined using toluidine blue dye.

Time for treatment with glycine	–COOH (nmoles/cm^2)	Contact angle (deg.)
Unmodified PC membrane	–	68 ± 3
6 h	10.06 ± 0.02	45 ± 3
16 h	10.5 ± 0.11	32 ± 4

tion process [32]. In order to optimize the fluorescence intensity of bioconjugated QDs, amount of QDs was varied for a fixed concentration of Sty IgG. It was observed that although the maximum fluorescence intensity was recorded for QD-Ab ratio of 100:1 but QD:Ab ratio of 50:1 was selected on the basis of good fluorescence intensity along with best biological activity among all the ratios of bioconjugated QDs.

Fig. 2A shows TEM image of non-conjugated and conjugated QDs. The average size of non-conjugated QDs was determined by counting atleast 200 QDs per sample and found to be of about 6 nm in size. It was also recorded that the nanocrystals formed were uniform and well dispersed. Attachment of Y-shaped Ab to QDs was clearly observed after bioconjugation with Sty IgG (Fig. 2Ab). Many such conjugated structures were noticed with varying size which can be attributed to their presence in different focal planes. The size of the conjugated structure was found to be in the range of 25–31 nm (as measured by TEM image analysis software, AMT version 5.4.1.13) which may be explained as the sum of the total sizes of QD (6 × 2 nm) and IgG (14–20 nm). This is the first time report of the clear visualization of Ab conjugated QD structure under TEM. Generally, organic structures (mainly hydrocarbon based) are not well seen under TEM because of less electron density on them. The reason of visualization of prepared QD-IgG bioconjugates clearly and characteristically can be explained by the influence of pH of buffer in which bioconjugates were present. Since, the surface of QD

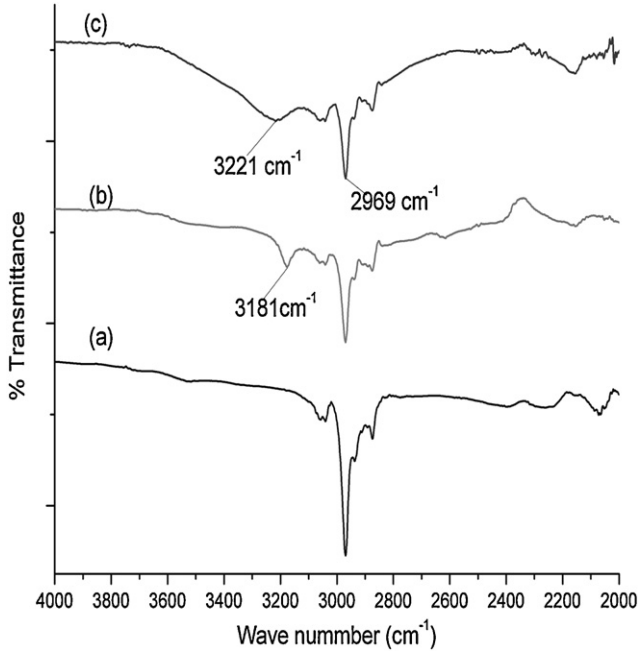


Fig. 3. FTIR spectra showing: (a) unmodified PC membrane, (b) PC membrane modified with glycine treatment and (c) Ab immobilization.

is negatively charged at pH values above the pKa of TGA (3.73) and EDC (5.6) and human IgG is positively charged when pH is below its pI (6.0–7.0), an increase in pH leads to a decrease of positive charge or increase of electron density on IgG which might be enhanced by the interaction with electron rich QDs. Therefore, intensive interactions between the two components of prepared bioconjugated

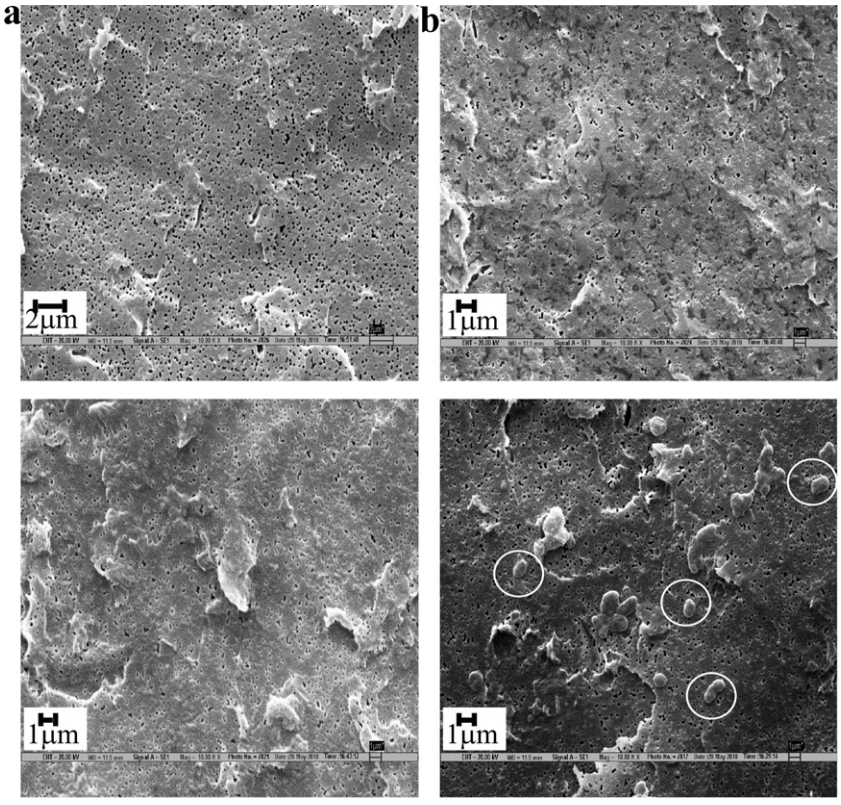


Fig. 4. Scanning electron micrographs: (a) unmodified PC membrane, (b) glycine treated PC membrane, (c) Sty IgG immobilized PC membrane and (d) bacteria captured on antibody immobilized nylon membrane.

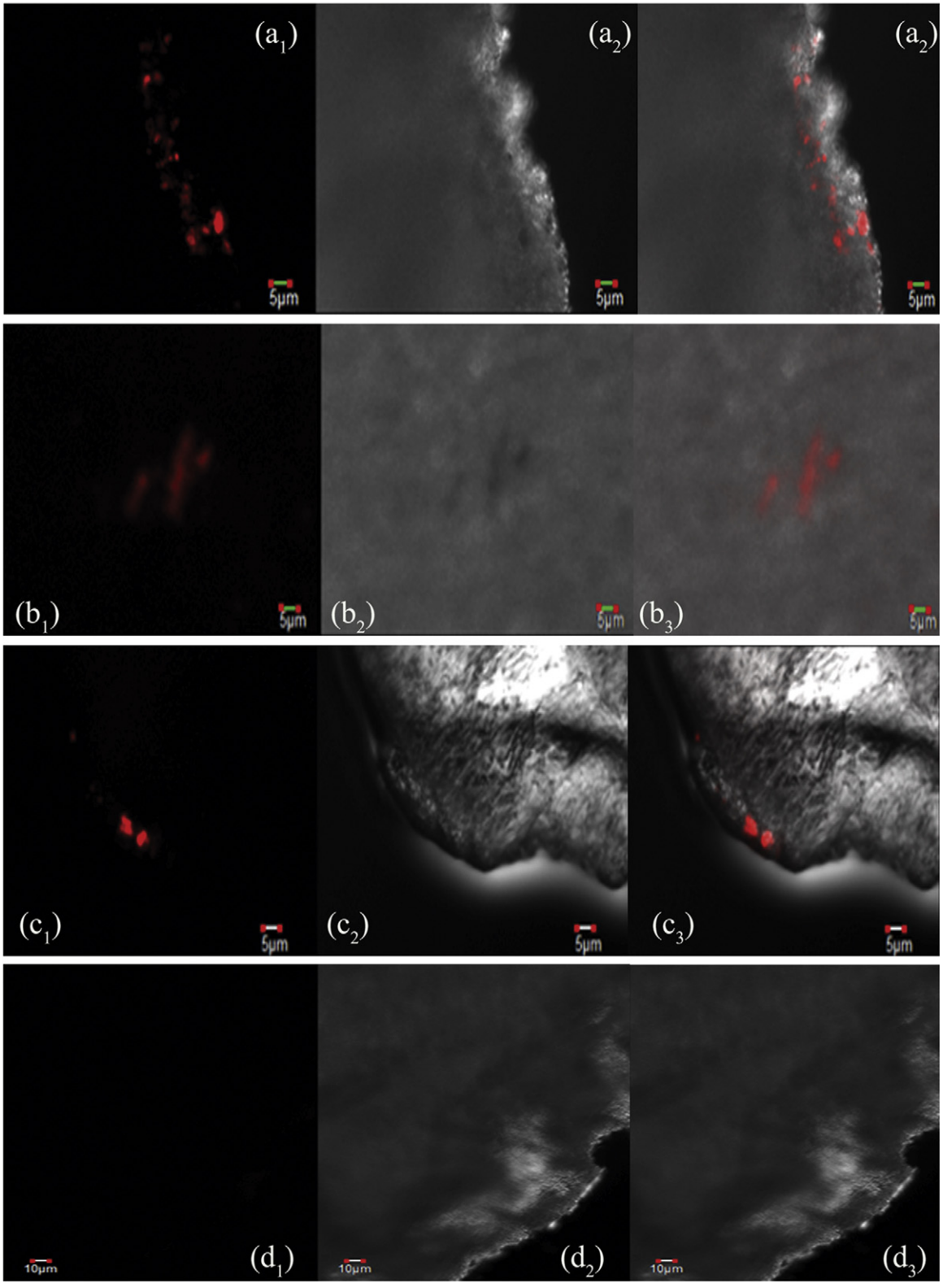
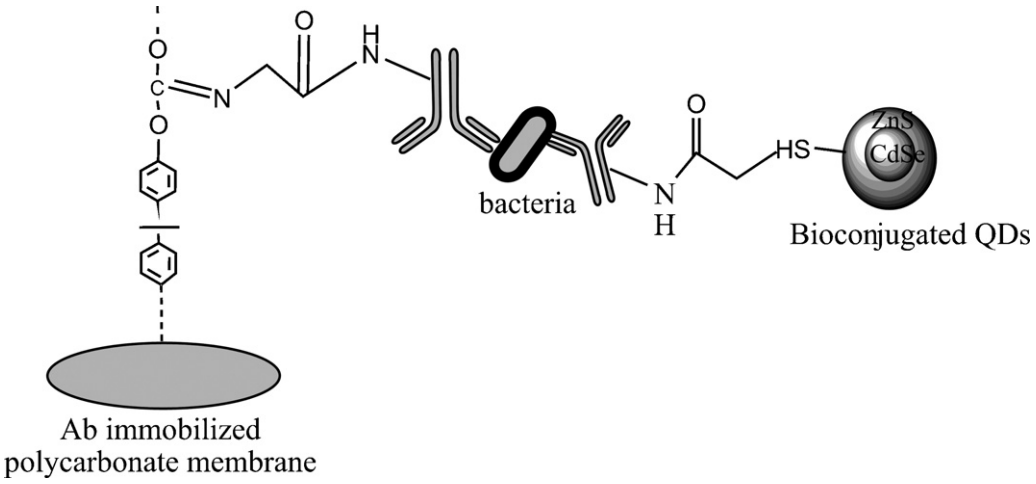


Fig. 5. Confocal laser scanning microscopic images of QD-IgG labeled bacteria on PC membrane incubated with solution of Sty Ag of different concentrations: (a) 10^6 cells/mL, (b) 10^4 cells/mL, (c)10^2 cells/mL and (d) 0 cells/mL. Fluorescent image, DIC image and superimposed image are shown in three different views (1, 2 & 3).

(QD-IgG) in basic pH 8 probably contributed to the visibility of IgG conjugated QDs in transmission electron micrographs.

Fig. 2B shows the FTIR spectrum of TGA capped CdSe/ZnS QD with peak of 2933 cm^{-1} corresponding to $-CH_2$ stretching of $-S-CH_2-$ on the surface of QD. It also showed –COONa peak at 1590 cm^{-1} as QDs were stored in MQ water with pH 8. FTIR spectrum of free antibody is shown in Fig. 2Bb. It showed a peak at 3375 cm^{-1} corresponding to $-NH_2$/–COOH present in the structure of antibody. Spectrum also exhibited a peak at 1617 cm^{-1} attributed to amide bonds in the structure of antibody. Fig. 2Bc shows FTIR



Scheme 3. Schematic representation of sandwich fluoroimmunocomplex formed on modified polycarbonate membrane.

spectrum of Ab- conjugated QD where appearance of new peaks at 3328 cm^{-1} and 1724 cm^{-1} corresponding to free –COOH of the antibody confirmed the conjugation of antibody with QD. Appearance of –CONH peak at 1643 cm^{-1} further confirmed the conjugation of antibody with QD through –CONH bond.

Proton nuclear magnetic resonance spectra of prepared QDs is shown in Fig. 2C. Ligand exchange with TGA on QD was confirmed due to the presence of $–CH_2$ peak of thioglycolic acid at 3.8 ppm as shown in Fig. 2Ca. Pure antibody showed peaks of $–CH_2CO$ and $–NHCH_2$ from 3 ppm to 4.2 ppm region (Fig. 2Cc). Fig. 2Cc also showed a peak of 7.6 ppm corresponding to $+NH_3$ of antibody as the antibody used for NMR studies was from the stock lyophilized after precipitation with ammonium sulphate. Antibody conjugation with water dispersible QDs was confirmed by the appearance of antibody characteristic peak in bioconjugated sample (Fig. 2Cb) between the regions 3–4.2 ppm. Bioactivity and specificity after

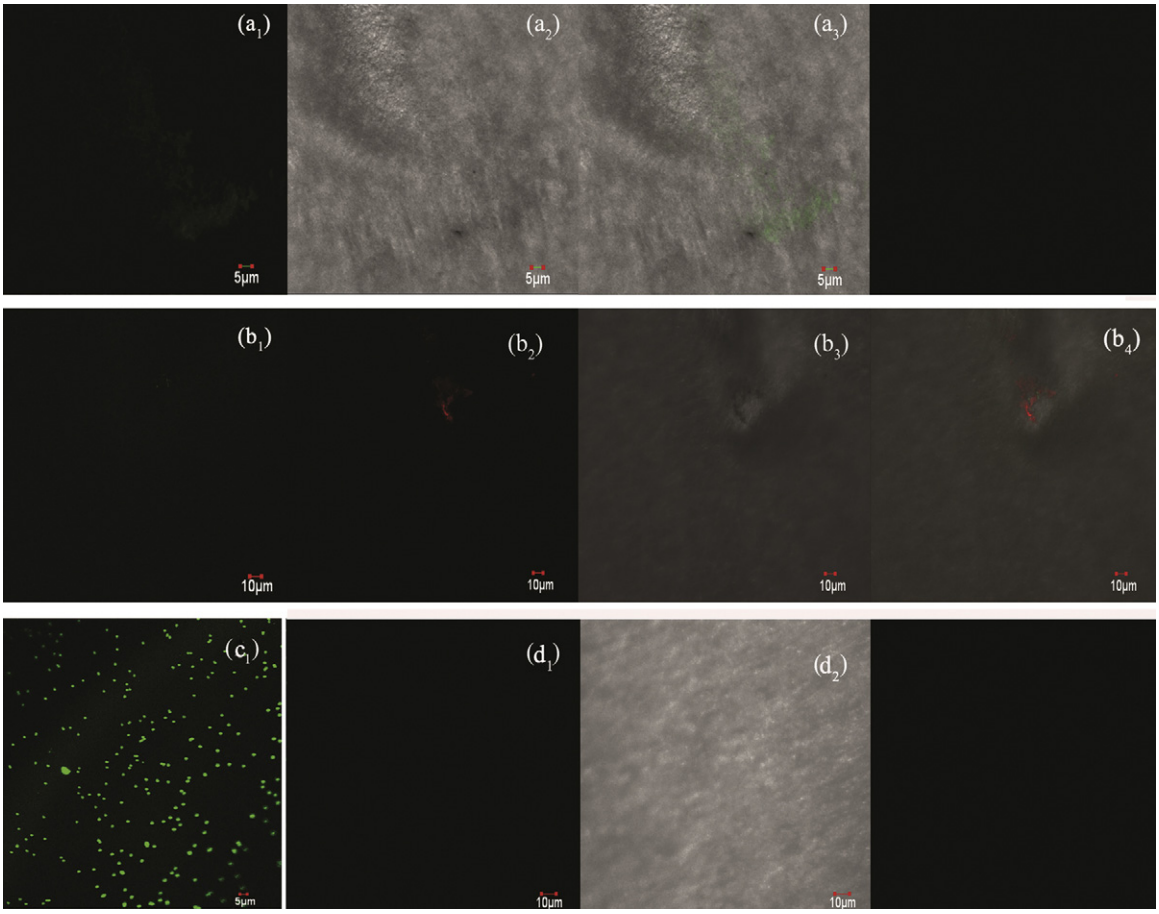


Fig. 6. Confocal laser scanning microscopic images: (a) FITC-IgG labeled bacteria at Sty Ag concentration of 10^4 cells/mL on PC membrane; (b) PC membrane incubated with mixed culture of bacteria containing FITC pre-stained *E. coli* (2×10^8 cells/mL) and Sty Ag (10^8 cells/mL), (b_1) and (b_2) fluorescent images for channel of FITC and channel of QD respectively, (b_3) DIC image and (b_4) superimposed image; (c) FITC pre-stained *E. coli* cells on glass slide and (d) negative control on PC membrane without Sty Ag.

conjugation was confirmed by the good absorbance values recorded for antigen–antibody recognition reaction that occurred between anti rabbit IgG (GAR IgG-HRP) and rabbit Sty IgG conjugated QDs in ELISA studies (Fig. S1, Supplementary Material). An average of 1.95 QDs per IgG were determined to be present in the prepared bioconjugates as estimated using the UV absorbance values (Appendix B Fig. S2, Supplementary Material). The result of quantification of QDs/IgG was found to be in accordance to the TEM analysis studies.

3.3. Surface modification of polycarbonate membrane

Black polycarbonate membrane was selected as solid phase for the development of fluoroimmunoassay because of its non-fluorescent property which allowed the imaging of fluorescent sandwich immunocomplex formed on the membrane. In this study, we have utilized glycine for surface modification and proposed reaction mechanism is shown in Scheme 2. Possible reaction mechanism involves the reaction of carbonyl of polycarbonate membrane in the presence of proton (H^+) with amino group of glycine to form imine bond between PC and glycine. Glycine modified PC membrane forms semi-stable amine reactive NHS ester which further facilitates the formation of stable amide linkage with amino group of antibody. The total number of acid groups on the surface modified PC membrane was also estimated by toluidine blue uptake method and results are shown in Table 1. Unmodified PC membrane disc showed negligible value of carboxyl groups while membrane treated with glycine for 6 h showed the value of 10.06 nmoles/cm^2 of reactive carboxyl groups on the surface of PC membrane. There was no considerable difference in the value of carboxyl groups generated after treatment with glycine for 6 h and 16 h. Therefore, a period of 6 h was selected as treatment time for surface modification of PC membranes for further studies. The water contact angle measurements gave qualitative information about change in surface hydrophilicity after the surface modification of membrane with glycine. (Table 1) Unmodified membrane showed the contact angle value of $(68 \pm 3)^\circ$ which is quite high and corresponds to hydrophobic nature of the virgin membrane [33]. Contact angle value of $(45 \pm 3)^\circ$ and $(32 \pm 4)^\circ$ were recorded for membrane modified for 6 h and 16 h, respectively. Decrease in contact angle value was observed for the modified membrane with the increase in treatment time which was ascribed to the generation of hydrophilic carboxylic group on the surface of PC membrane. Surface modified PC membrane was also evaluated for any autofluorescence property and it was observed that treated membrane remained non- autoflorescent even after the excessive chemical treatment with glycine.

Fig. 3 shows the ATR-FTIR spectra of modified and unmodified PC membranes. IR spectrum of virgin PC membrane is shown in Fig. 3a which showed –CH stretching vibrations of PC at 2969 cm^{-1}. Appearance of –COOH peak at 3181 cm^{-1} in spectrum of glycine treated PC membrane (Fig. 3b) confirmed the covalent attachment of glycine on PC membrane. Fig. 3c showed FTIR spectra of antibody attached modified PC membrane. Appearance of a peak at 3221 cm^{-1} with increased intensity was ascribed to –COOH group of Ab and confirmed the conjugation of Ab on membrane. Possible amino or amide groups of antibody might have also contributed towards the shift of –COOH peak in antibody immobilized membrane.

Scanning electron micrographs of modified and unmodified PC membrane are shown in Fig. 4. Unmodified virgin membrane showed a uniform porous structure (Fig. 5a) which became slightly heterogeneous (Fig. 4b) after surface modification with glycine for 6 h. It was observed that PC surface got somewhat smooth and covered after Ab immobilization (Fig. 4c). Fig. 4d shows the immunocaptured targeted bacteria (*S. typhi*) clearly on antibody immobilized membrane. Similar observations of bacteria bound on membranes have been also recorded by various researchers by SEM studies [34,35]. Results of indirect ELISA studies, performed to confirm the immobilization of Sty IgG on PC membrane, showed increase in absorbance value with the increase in the GAR IgG-HRP conjugate concentration from 1:32,000 to 1:4000 (Fig. S3, Supplementary Material).

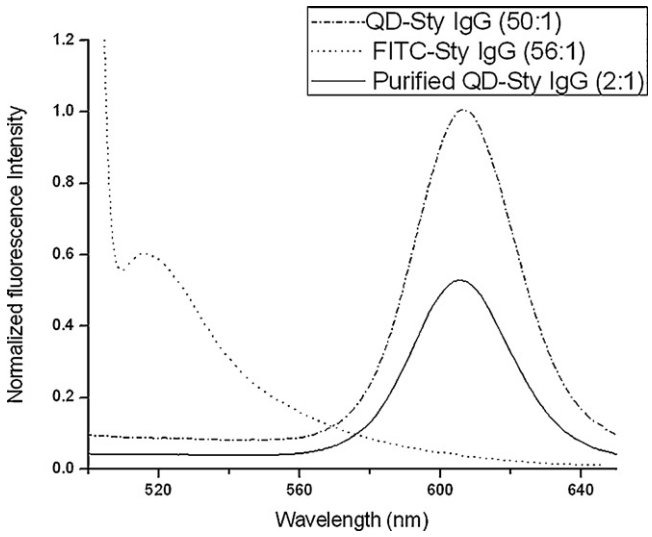


Fig. 7. Fluorescence emission spectra of IgG labeled with FITC and QDs.

3.4. Detection of S. typhi using confocal laser scanning microscopy

A sandwich fluoro-immunodetection method was developed using Sty IgG immobilized membrane and bioconjugated QDs as solid phase and fluorescent label, respectively for the detection of *S. typhi* as shown in Scheme 3. Bright fluorescent red aggregates were observed using CLSM at excitation with 405 nm for the sandwich complex formed on PC membrane (Fig. 5). Intense fluorescence was observed for PC membrane incubated with 10^6 cells/mL–10^2 cells/mL of *S. typhi* antigen. It was also observed that number of fluorescent fields decreased with the decrease in concentration of bacteria used for incubation. No fluorescence was recorded for negative controls which were given the similar treatment except incubation with *S. typhi* antigen (Fig. 5d$_1$). PC membrane without immobilized Sty IgG also did not show any fluorescence under the experimental conditions used for developed detection method. The ROI analysis of the images obtained via lambda scanning mode (band gap: 20, step size: 5 nm) was performed to semiquantitatively study the pattern of fluorescence intensity with change in concentration of bacteria. An average of 20 regions was evaluated for their spectral profiles as recorded by Fluoview software (FV1000-ASW) in 5–10 fields of view for each bacterial concentration. As shown in Fig. 8, the ROI analysis showed maximum fluorescence intensity at 590 nm for 10^6 cells/mL which decreased with decreasing bacteria concentration used for incubation of IgG immobilized membrane. Antibody immobilized PC membrane without incubation with bacteria which was taken as negative control showed negligible intensity under the same conditions of scanning.

In order to verify the selective binding of targeted bacteria, we used *E. coli* cells pre-stained with dye (FITC) having green fluorescent emission to clearly differentiate non-specific binding of *E. coli*. Bright fluorescent green small cocci-rod shaped structures of FITC stained *E. coli* were observed on the glass slide as shown in Fig. 6c$_1$. Negligible green fluorescence was seen on antibody immobilized PC membrane incubated with a mixed culture of bacteria (Fig. 6b$_1$)

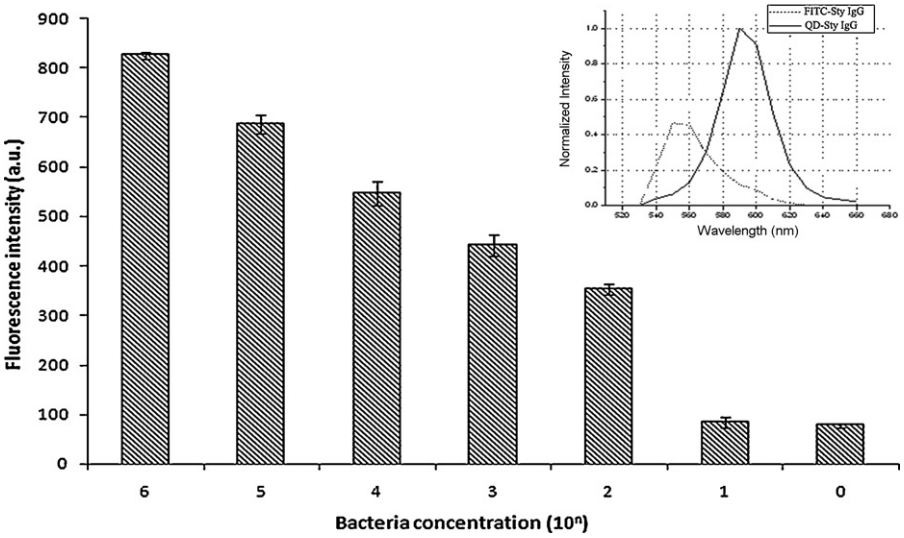


Fig. 8. Semi-quantitative analysis of ROIs on PC membrane using QD-IgG for different concentration of Sty Ag. Inset shows the CLSM spectral profile of ROIs for detection using FITC labeled Sty IgG and QD labeled Sty IgG.

whereas appearance of bright red fluorescence on this membrane confirmed the selective capturing of *S. typhi* from a mixed culture (Fig. 6b$_2$). With regard to detection limit and selectivity of the method for target bacteria in the sample, the detection limit of developed method for *S. typhi* is better than several methods for the bacteria detection based on immunoreactions strategies. Su et al. reported the use of immunomagnetic separation coupled with functionalized QDs for simultaneous detection of multifood borne pathogenic bacteria in food samples upto level of 10^3 cfu/mL [36]. Xue et al. reported the fluorescence detection of *E. coli* using water soluble CdSe QDs [37]. However, their method is highly non-specific since it was based on direct coupling of QDs with bacterial membranes without using any specific binding molecule (antibody). Moreover, no study was conducted for determining the cross-reactivity with bacteria of similar origin, Enterobacteriaceae.

Prepared QDs labeled IgG (QD-IgG) conjugate was also compared with conventional dye (FITC) labeled IgG (FITC-IgG) conjugate and results of emission spectra obtained using spectrofluorometer are presented in Fig. 7. It was observed that after purification, there was decrease in the fluorescence intensity of the bioconjugated QDs which can be explained by the loss of free QDs in pellet obtained in lowermost layer of sucrose gradient obtained after ultracentrifugation as shown in Fig. S1 (see Supplementary Material). However, the fluorescence intensity of purified QDs-IgG conjugates (1:1.95) was almost comparable to that of commercial FITC-IgG conjugates (1:56). Good fluorescence intensity of bioconjugated QDs was attributed to the higher extinction coefficient and good quantum yield of fluorescent CdSe/ZnS QDs and was therefore used for sensitive bacteria detection in this study.

Fig. 6a shows the CLSM micrograph of sandwich immunocomplex formed on Ab immobilized PC membrane using FITC-IgG for the labeling of immunocaptured bacteria. Fluorescent aggregates were observed for the concentration of bacteria up to 10^4 cells/mL. No fluorescence could be recorded for lower bacterial concentration. Whereas, in case of the sandwich complex formed using QD-IgG, fluorescent aggregates were observed for the bacterial concentration of 10^2 cells/mL (Fig. 5c$_1$). Fig. 8 (inset) shows the spectral profile of the brightest ROI of fluorescent sandwich complex formed on PC membrane as recorded by CLSM for both types of labels. A decrease of 50% in fluorescence intensity was observed for detection method using FITC as compared to QD as label. Shift in emission spectra of the immunocomplexes formed on PC membrane was observed for both types of labels (QD-IgG and FITC IgG). Dwarakanatha et al. also reported the shift in emission peak of QD upto 60 nm after binding with *E. coli* O111:154 [38]. In present study, prepared CdSe/ZnS QDs show characteristic narrow and symmetric spectrum with emission maxima at 610 nm which shifted slightly and became broader in the range of 590–610 nm after binding with bacteria. Similarly, FITC-IgG showed shift in emission from its maxima of 520 nm to the range of 540–550 nm after binding with bacteria. The shift in spectral profile confirmed the binding of bacteria with labeled IgG. These results of comparative studies using the two types of labels clearly show that bioconjugated QDs are more sensitive than FITC-IgG conjugate for the specific detection of pathogenic bacteria on PC membrane.

4. Conclusion

Water dispersible CdSe/ZnSe quantum dots were bioconjugated with *S. typhi* specific IgG antibody using carbodiimide chemistry. Spectrophotometrically, 1.95 QDs per IgG were determined to be present in prepared bioconjugates which was confirmed by TEM observations also. In addition, polycarbonate membrane was surface modified by chemical treatment of glycine and 10.06 ± 0.02 nmoles of –COOH group were estimated to be present per cm^2 of membrane treated for 6 h. A sensitive, specific and rapid fluorescence based detection method was demonstrated using prepared IgG conjugated QDs and surface modified PC membrane. Developed immunoassay captured and detected *S. typhi* specifically in water upto the bacterial concentration of 100 cells/mL within 2.5 h. Furthermore, immunoassay developed on PC utilizing bioconjugated QDs was found to be 100 times sensitive than a similar assay using a common organic dye labeled IgG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.02.052.

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