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Cholesterol Biosensors Based on Oxygen Sensing Alginate-Silica Microspheres

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ABSTRACT: Cholesterol determination in body is important in diagnosis of diseases like coronary heart disease, arteriosclerosis, diabetes, and obstructive jaundice. This research aims at developing fluorimetric cholesterol biosensors based on self-assembled mesoporous alginate-silica (Algilica) microspheres. For preparing the biosensor, Pt-(II)-octaethylporphine (PtOEP; oxygen sensitive metalloporphyrin) dye has been loaded in the Algilica microspheres using the solvent-mediated precipitation method. Cholesterol oxidase (ChOx) was then covalently conjugated to PtOEP/Algilica microspheres using EDC and NHS reagents. PtOEP dye and enzyme encapsulation, activity and stability were then analyzed. Layer-by-layer self-assembly was finally performed using PAH and PSS polyelectrolytes to minimize leaching of the biosensor components. The prepared biosensor exhibited linearity over a range of 0.77–2.5 mM O_2 (K_{SV}: 0.097/mM of O_2) obtained using from Stern–Volmer plots. The biosensor response to standard cholesterol displayed a linear analytical range from 1.25 to 10 mM of cholesterol with regression coefficient of 0.996 (1.25–3.75 mM), 0.976 (1.25–6 mM), and 0.959 (1.25–10 mM) and response time of 10 min. Thus, the prepared cholesterol biosensor shows great potential in the diagnosis of hypercholesterolemia.

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KEYWORDS: alginate–silica; microspheres; cholesterol biosensor; PtOEP dye

Introduction

Monitoring the derangement in the cholesterol metabolism is of paramount importance to the modern society due to its profound implication in various diseases. National Cholesterol Education Program (NCEP) guidelines correlates a human total serum cholesterol level of 6.21 mM or above [4.88 mM for low density lipoprotein (LDL) cholesterol level] to a strong indication of hypercholesterolemia (Ahmad et al., 2010). This condition if persisting can lead to the development of coronary heart disease (CHD), arteriosclerosis, Niemann–Pick disease, myxoedema, nephrosis, diabetes mellitus, and obstructive jaundice in patients (Steven and Quarfordt, 1977; Suman and Pundir, 2003). Unfortunately, patient's reliability on cholesterol lowering drugs has been questioned by most researchers. Researchers claim that the drug therapy, in many cases, has led to symptoms of amnesia, cognitive loss, and personality disorders in patients (Brown et al., 2005; http://www.newswithviews.com/Ellison/shane14.htm). Diagnostics have therefore gained tremendous attention in healthcare for the management of cholesterol level in patients.

Enzyme based methods are by and large the most widely used procedure nowadays for the diagnosis of hypercholesterolemia. They are better suited for this purpose than methods like ultracentrifugation, HPLC, and electrophoresis owing to their superior characteristics such as selectivity, sensitivity, and rapid response (Krauss and Burke, 1982). The methods are typically based on co-immobilization of cholesterol oxidase (ChOx), cholesterol esterase, and peroxidase in a porous matrix (Arya et al., 2008; Basu et al., 2007; Li et al., 2003; Pierre, 2004; Singhal and Malhotra, 2007; Suman and Pundir, 2003; Wu and Choi, 2003). Mesoporous materials are excellent candidates for enzyme encapsulation as earmarked by their highly porous nature and low absorption and emission in the visible spectrum (Ouyang et al., 2006; Scott et al., 2001). Several researchers have explored mesoporous substances for enzyme-based sensors that include Graphite/Ruthenium (II) complex silicon (Marazuelaa et al., 1997), TEOS-derived sol–gel (Kumar et al., 2000), octadecylsilica/TEOS sol–gel (Wu and Choi, 2003), and alginate–silica (Stein et al., 2007). Mesoporous alginate-silica (Algilica) microspheres have the advantages of having both hydrophilic and hydrophobic microenvironments. The hydrophilic part is contributed by alginate whose COOH groups are centers for the chemical coupling of oxidases to the matrix by reagents like

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EDC–NHS (Carbodi-imide coupling; Ghanem and Ghaly, 2004; Stein et al., 2007), While the hydrophobic part is contributed by silica, it has capability to hold hydrophobic substances such as cholesterol and sensing dyes.

The sensing chemistry of enzymatic methods typically involves the catalysis of cholesterol by ChOx enzyme in the presence of oxygen thereby leading to the production of Δ-cholestenone and hydrogen peroxide at the same time. Peroxidase consumes hydrogen peroxide (H_2O_2) resulting in the conversion of *o*-dianisidine to quinoneimine in the color reaction. One shortcoming of this method is the coloration of the reaction mixture which restricts the reusability of these enzymes. The second being the presence of bilirubin in serum samples which chemically interferes with the peroxidase for H_2O_2 in the color reactions (MacLachlan et al., 2000). This competition produces a false negative result proportional to bilirubin concentration in serum. A significant reduction in this chemical interference can be achieved by replacing the peroxidase with oxygen sensitive fluorophores for end-point estimation (Fig. 1). Extraordinary sensitivity of flourophores ruthenium and metalloporphyrins (like Platinum and Palladium) in extremely low oxygen circumstances makes them highly efficient optical oxygen sensors and this has been reported by many researchers (Marazuelaa et al., 1997; Papkovsky et al., 1995; Stein et al., 2007; Wu and Choi, 2003; Yeha et al., 2006). Platinum(II) octa ethyl porphyrin (PtOEP) is a more viable luminescent oxygen indicator owing to its high chemical and photochemical stability, large Stokes' shifts of about 200 nm, high quantum yields and extinction coefficient, long excited-state lifetime, absorbance in visible range (Bansal et al., 2006; Borisov and Wolfbeis, 2008) and longer radiative lifetime of 100–200 μs suggesting its efficient phosphorescence quantum efficiency even at room temperature (Zhou et al., 2007). Depletion in the oxygen level in cholesterol reaction system can be traced spectrophotometrically monitoring the changes in fluorescence intensity of metalloporphyrins. This modified sensing approach offers low cost and reusability in addition to other attractive features of enzyme-based procedures.

This research aims at developing a cholesterol biosensor with immobilized ChOx in Algilica microspheres. Solvent-mediated precipitation technique was used to adsorb PtOEP dye into the Algilica microspheres (Ghanem and Ghaly, 2004). ChOx from *Streptomyces* sp. was chemically coupled by Carbodi-imide within the spheres and layer-by-layer (LBL) self-assembly was used for enzyme leaching control (Brown et al., 2005; Li et al., 2008; Lolekha et al., 2004; Pollegioni et al., 1999; Srivastava et al., 2005; Zhu et al., 2005b). Co-entrapment of cholesterol esterase was not performed out as it leads to a very slow reaction and a slow response; therefore, any cholesterol present in esterified form needs to be pretreated with free cholesterol esterase to derive the free cholesterol for sensing application.

Materials

Sodium alginate (viscosity 3,500 cps, 12–80 kDa), (3-glycidyloxypropyl)trimethoxysilane (GPTS), PtOEP, polyethylene glycol dodecyl ether (Thesit), tetrahydrofuran (THF), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodi-imide hydrochloride (EDC), *N*-hydroxysuccinimide sodium salt (NHS), fluorescein isothiocyanate-dextran (FITC-Dextran, 70 kDa), Bradford reagent, cholesterol (389 Da), Poly(allylamine hydrochloride) (PAH), poly(sodium 4-styrene sulfonate) (PSS), ChOx (EC 1.1.3.6, 31.8 U/mg *Streptomyces hygroscopicus*, 55 kDa), and glucose oxidase (GOX; G2133 from *Aspergillus niger*, Type VII, 144 kDa) and sodium chloride were purchased from Sigma–Aldrich (Mumbai, India). Ammonia solution (30% AR) was obtained from Merck (Mumbai, India), and bovine serum

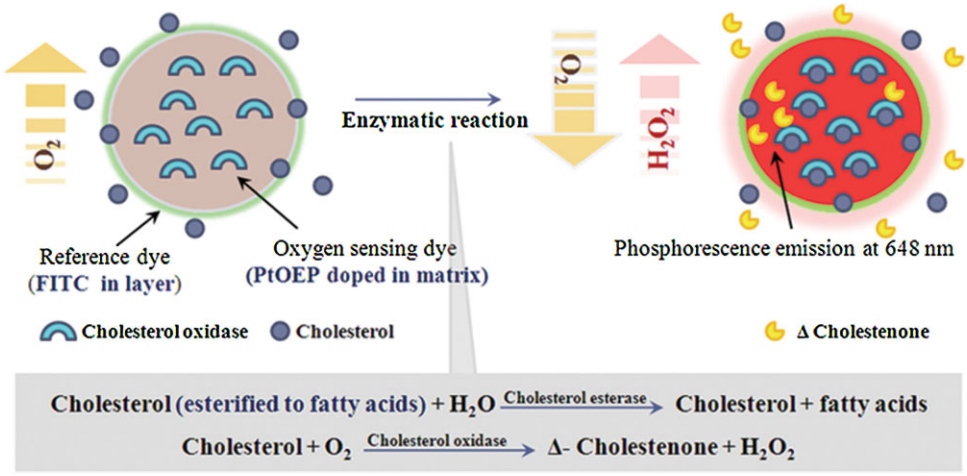

Figure 1. Schematic of working principle of alginate silica/PtOEP/ChOx biosensor. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

albumin (BSA, 66 kDa) was purchased from SRL Pvt. Ltd. (Mumbai, India). All chemicals were used as received.

Preparation of PtOEP Dye-Doped Alginate-Silica Microspheres

Alginate-silica microspheres were prepared by a modified protocol developed by Stein et al. (2007). Briefly, 1.5 (wt.%) aqueous alginate solution and GPTS were mixed in a 1:1 volumetric ratio and stirred at high speed for 26 h, resulting in an alginate-modified silanol. Two milliliters of the precursor silanol was taken and added to 3 mL of water under stirring condition. After 5 min, 1.25 mL of 30% ammonia solution was added to the above mixture and stirred for 20–30 min, and was followed by the addition of 10 mL of water and an additional stirring time of 40 min. An extra 40 mL of DI water was added and the suspension was stirred for another 4 h. The resulting microparticle suspension was washed with DI water using four centrifugation cycles. The morphology, chemical nature, and size distribution of microspheres were characterized using SEM (JEOL 35C, Japan), FTIR (Nicolet Instruments Corporation, Magna 550), and DLS (Beckman Coulter MS4 Particle Counter/Size Analyzer). The mass transfer of hydrophobic and hydrophilic entities in and out was verified using fluorescence microscopy (Leica DMIL LED; Leica Microsystems, Germany). Approximately 250 μL of stock particle suspension was placed in a microcentrifuge tube and dried under vacuum, followed by adding 250 μL of a 500 μM PtOEP solution prepared in THF. The container was sealed to prevent volatilization of THF and stirred for 30 min, after which 30 μL of water was added and the suspension stirred for an additional 30 min. One hundred microliters of DI water added to the suspension initiated a solvent-mediated controlled precipitation of PtOEP into the Algilica particles (Stein et al., 2007). The suspension was subsequently rinsed four times with DI water. PtOEP loaded microspheres were characterized by CLSM (IX 81; Olympus, Tokyo, Japan) and fluorescence spectroscopy (F-2500; Hitachi, Tokyo, Japan).

Encapsulation of Macromolecules

Macromolecular encapsulation of BSA (model protein) and GOx (model enzyme) was performed using Carbodi-imide coupling method (Ghanem and Ghaly 2004; Stein et al., 2007). Approximately 500 mg of Algilica microspheres were suspended in 1 mL of BSA solution (4 mg/mL) prepared in sodium acetate buffer (0.05 M, pH 3.9). The suspension was incubated at 100 rpm for 2 h for adsorption of free protein in mesoporous particles. Following adsorption, acetate buffer (pH 3.9) rinsed particles were placed in 500 μL of 25 mM EDC and 15 mM NHS in 0.05 M sodium acetate buffer (pH 5.0) to couple the amine and carboxylic acid functionalities on BSA and alginate, respectively. The suspension was stirred for 4 h at 250 rpm and then rinsed with DI water. BSA loading efficiency and leaching were studied following encapsulation. Similarly, GOx was encapsulated and evaluated for encapsulation and leaching profiles. SDS–PAGE was carried out to study leaching profile with a miniVE system (GE Healthcare Bioscience Corp., Mumbai, India) using gel of 12% polyacrylamide (1.0 mm, 10 wells). The running condition was 100 mA for 210 min.

Stability of Encapsulation

GOx enzyme was used as a model enzyme to study the effect of matrix on the enzyme activity within microspheres. GOx activity was monitored through a colorimetric assay, based on the oxidation of *o*-dianisidine through a peroxidase-coupled system (Zhu et al., 2005a).

$$\beta\text{-}d\text{-Glucose} + O_2 + H_2O \xrightarrow{\text{GOx}} d\text{-Gluconic acid} + H_2O_2$$

$$H_2O_2 + o\text{-dianisidine(reduced)} \xrightarrow{\text{Perosidase}} o\text{-dianisidine(oxidized)} + H_2O$$

The activity was measured at regular intervals of 7 days for 3 weeks using the following relation

$$\text{Activity(U/ml)} = \frac{\{\Delta A_{500}/\text{min of particles(max.)} - \Delta A_{500}/\text{min of particles(min.)}\} * 3.1 * \text{dilution factor}}{7.5 * \text{sample of volume}}$$

where 3.1 is the volume of solution taken for UV measurement, 7.5 is the millimolar extinction coefficient of *o*-dianisidine, and $\triangle A$ is the change in absorbance at 500 nm.

Preparation of ChOx/PtOEP-Doped Algilica Microspheres

ChOx loading was performed using Carbodi-imide coupling method mentioned above (Kim et al., 2007; Stein et al., 2007). Briefly, 500 mg of Algilica/PtOEP microspheres were suspended in 1 mL of ChOx solution (3.14 mg/mL) prepared in sodium acetate buffer (0.05 M, pH 4.0). EDC and NHS in the ratio of 1.0:0.6 were used for coupling the enzyme to the matrix. The particles were washed thrice with DI water to remove unbound enzyme, and then the particles were used for further studies.

Layer-by-Layer (LBL) Self-Assembly

For deposition of each coating, solutions of PSS, PAH, and (FITC-conjugated PAH) PAH-FITC were prepared in 0.25 M NaCl at a concentration of 2 mg/mL (Hau et al.,

2003). The supernatant of the particles was removed and the 100 μL of particles resuspended in 1 mL of PAH-FITC solution. Adsorption was allowed to proceed for 30 min, after which the suspension was centrifuged at 3,000 rpm for 10 min to separate the spheres from remaining unadsorbed polyelectrolyte following the adsorption period, during which the particles were continuously vortexed in micro-centrifuge tubes, the particles were rinsed three times with DI water and subsequently resuspended in 1 mL of PSS solution. The process was repeated for the oppositely charged polyelectrolyte and then alternated until a total of three bilayers of PAH/PSS films were formed.

Study of Interference of Surfactant and Cholesterol

Thesit solutions of 0.3% (v/v) and 1% (v/v) were prepared in isopropanol (10% v/v) and DI water. Five hundred microliters of each solution was incubated with 100 mg of PtOEP doped Algilica microspheres at room temperature. The supernatant was collected at specific time intervals and replaced with equal quantity of thesit solutions. Cumulative peak emission intensities of the supernatants were plotted against time intervals to monitor the dye leaching in thesit solutions. Similarly, effect of cholesterol in different concentrations was studied for the effect on the PtOEP fluorescence emission spectra.

Oxygen Sensitivity Testing and Cholesterol Biosensing

Oxygen sensing was carried out using a spectrofluorimeter with a 150 W Xenon lamp as a visible excitation light source. Different oxygen standard concentrations (0.77–16.0 mM) in a gas stream were produced by controlling the flow rates of oxygen. The flow rate was maintained to control the oxygen concentration. Oxygen sensing properties of PtOEP doped in algilica microspheres were characterized by I_0/I value and Stern–Volmer quenching constant, K_{SV}, obtained from following equation

$$\frac{I_0}{I} = 1 + K_{sv}[O_2]$$

where I_0, I, and $[O_2]$ are phosphorescence intensities in the absence, in the presence of oxygen and oxygen concentration, respectively. K_{SV} was obtained from a linear plot. Reversibility experiments were determined by bubbling oxygen and nitrogen consecutively into the sensing particles, at the same time measuring oxygen concentration, and fluorescence emission intensity. The emission scans were obtained at 30 s intervals. All the experiments were performed at room temperature. Cholesterol concentrations of 1.25, 2.5, 3.75, 5.0, 6.0, 7.5, and 10 mM were prepared in 10% (v/v) of iso-propanol and 1% (v/v) thesit. One hundred milligrams of ChOx/PtOEP/Algilica microspheres were suspended in 500 μl of standard cholesterol solutions. The emission spectra of the suspension were also collected at intervals of 1 to 10 min to calculate the response time. The reduction in dissolved oxygen was monitored fluorimetrically for each of the aliquots of the cholesterol at 535 nm excitation wavelength. The emission intensities for each aliquot were taken at 648 nm and plotted against the cholesterol concentrations.

Results and Discussion

Preparation of PtOEP Dye-Doped Alginate-Silica Microspheres

Alginate-silica microspheres were synthesized using the modified protocol described earlier. The particles were characterized by SEM and fluorescence microscopy. SEM images indicate that the microspheres have a smooth surface and spherical morphology (Fig. 2Ia–d). Additionally, a highly porous network was observed in the internal structure of the microsphere. Fluorescence microscopic imaging shows that these pores are large enough to allow internalization of macromolecules (Fig. 2IIa–c). The microspheres exhibited high internalization of both hydrophilic (FITC-Dextran, 70 kDa) and hydrophobic (FITC) dye molecules confirming the hydrophilic–hydrophobic nature of the microspheres. Particle size distribution (in terms of volume %) were acquired by DLS as shown in Figure 2IV. The data suggest polydispersity in the particle size. Almost 60% of the as-synthesized particles were in the size ranging between 40 ± 10 μm. Particle size distribution was found to be proportional to the amount of aqueous ammonia added during synthesis process. Lower concentration of ammonia leads to formation of smaller sized particles with low silica content (Stein et al., 2007). PtOEP dye was doped into the vacuum dried microspheres by solvent-mediated precipitation technique. CLSM image indicates dye loading in the particles (Fig. 2IIIa–c). The dye distribution within the microspheres was verified by line scan (Fig. 2IIIc). FT-IR spectra are represented in Figure 3c. Absorbance band at 1,730 cm^{-1} confirms the formation of ester bond between carboxylic acid group on alginate and alcoholic group on GPTS. The presence of a very strong Si–O bond absorbance band between 1,100 and 1,000 cm^{-1}, confirms the condensation of the silanol by ammonia leading to formation of the silicate structure.

Encapsulation of Macromolecules

Bovine serum albumin (66 kDa), a protein with a molecular weight close to ChOx, was used to study the encapsulation efficiency of Algilica microspheres. Immobilization of BSA was carried out using the Carbodi-imide coupling method. Leaching profile (Fig. 4a) was calculated using Bradford reagent for determination of protein content. The supernatant was collected at each step during loading and were tested for protein content using Bradford test. The concentration of protein leached out was determined using

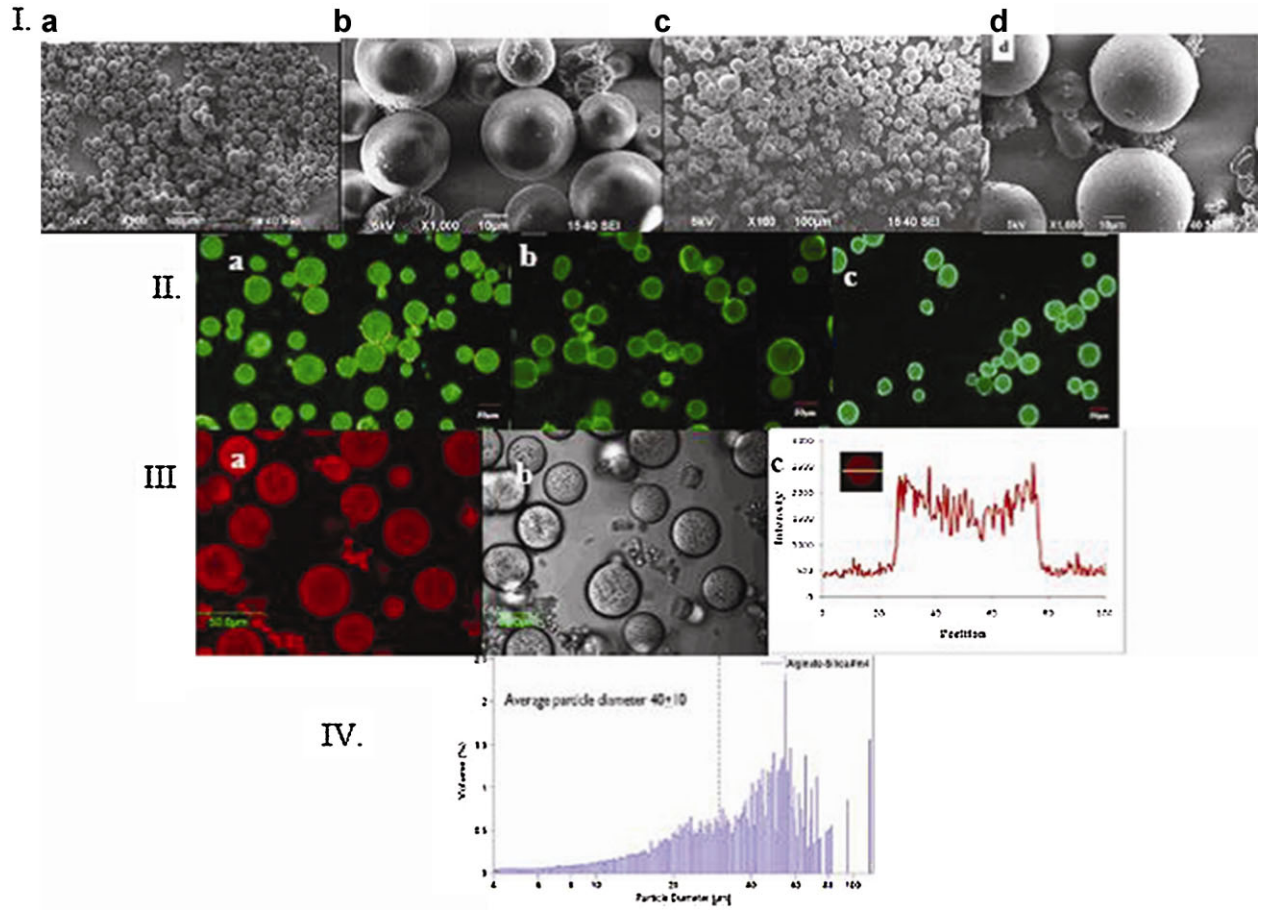

Figure 2. Characterization of Algilica particles: **I**. Scanning electron micrographs of (**a**,**b**) plain alginate-silica microspheres and (**c**,**d**) LBL-coated alginate-silica microspheres. **II**. Fluorescence microscopic imaging (**a**) FITC (MW = 389.28 Da), (**b**) FITC-Dextran (MW = 70 kDa), (**c**) FITC-Gox-loaded microspheres. **III**. Confocal imaging of (**a**) PtOEP-loaded microspheres, (**b**) DIC image of PtOEP-loaded microspheres, (**c**) CLSM Line scan for PtOEP-doped alginate-silica microspheres. **IV**. Particle size distribution analysis. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

a calibration curve for BSA (50–1,400 μg/mL). Percent leaching from the microspheres was evaluated by comparing with theoretical loading. A phenomenal reduction in leaching was observed after EDC–NHS coupling as indicated in Figure 4a. The encapsulation efficiency was calculated for 500 mg of microspheres using the relation

$$\%\mathrm{EE} = \frac{\text{Initial amount loaded-Amount detected in sample}}{\text{Initial amount loaded}} \times 100$$

The encapsulation efficiency was found out to be 61.32% for the immobilization of 2.48 mg/mL of BSA in 500 mg of spheres. The microspheres were stored in deionized water for 4 months to determine the leaching occurring post-encapsulation (Fig. 4b). It was observed that only 0.114% of BSA leached into the solution from microspheres during 26 h of wet storage. The result from Bradford method was further corroborated by SDS–PAGE technique which again showed negligible leaching after coupling (Fig. 4c).

Stability of Encapsulation

Stability studies were performed using a model enzyme. Figure 2IIc shows the fluorescence microscopy image of (FITC-conjugated GOx) FITC-Gox encapsulated within microspheres which confirms successful encapsulation of GOx in Algilica microspheres. Enzyme activity was evaluated as a parameter of enzyme stability (Fig. 4d). Activity (U/mL) was monitored by colorimetric method after every 7 days using the relation.

$$\mathrm{Activity(U/ml)} = \frac{\{\Delta A_{500}/\text{min of particles(max.)} - \Delta A_{500}/\text{min of particles(min.)}\} * 3.1 * \text{dilution factor}}{7.5 * \text{sample volume}}$$

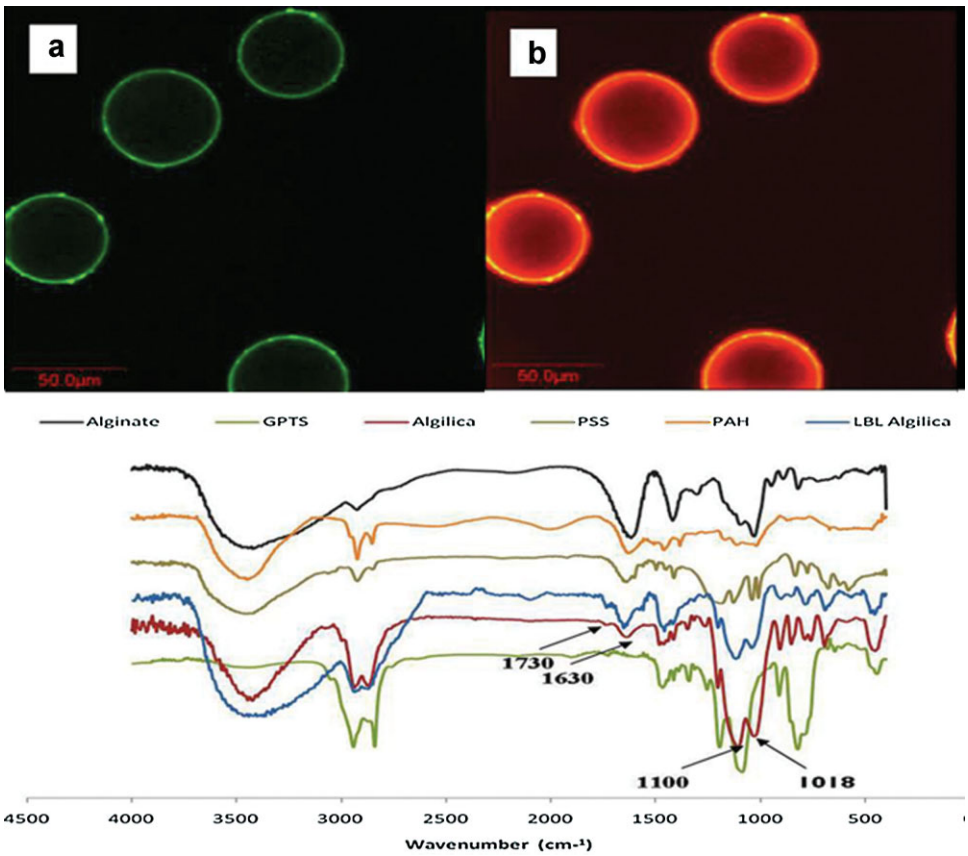

Figure 3. Confocal imaging of (**a**) (PAH-FITC/PSS)$_2$-coated PtOEP/alginate-silica microspheres at excitation with single wavelength (**b**) at excitation with two wavelengths, and (**c**) FTIR overlay scan of alginate, 3-glycidoxypropyltrimethoxysilane (GPTS), alginate-silica microspheres, PSS, PAH, and (PAH/PSS)$_2$-coated alginate-silica microspheres. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

The millimolar extinction coefficient of quinoneimine dye was taken to be 7.5 and the sample volume which is the volume containing GOx loaded microspheres, was taken to be 100 μL. The activity of 145.95 U/mL was recorded at T_0. The activity at the end of 3 weeks incubation for GOx loaded particles under wet storage was found to be 133.8 U/mL. Thus, a difference in activity of 8.3 U/mL was observed over 3 weeks of study. A reduction of 8.29% U/mL in activity can be partly attributed to the leaching of enzyme from the matrix and to the effect of Carbodi-imide crosslinker. However, cross-linking has a major role in preventing the macromolecules from getting leached out. Therefore, use of EDC–NHS has both positive and negative function for enzyme stability and activity.

Layer-by-Layer Self-Assembly on ChOx/PtOEP Algilica Microspheres

LbL assembly was performed on microspheres for maintaining stability of sensing assay within the matrix. LbL self-assembly of PAH and PSS nanofilms has been traditionally used for reducing leaching or controlling rate of release from a matrix. Two bilayer nanofilms were formulated in separate sets of Algilica samples which were confirmed using CLSM. CLSM image of LBL-coated microspheres illustrates an overlay between the excitation wavelength of PtOEP and the emission of FITC, thereby producing an orange color (Fig. 3a,b). Such an LbL assembled system can be used for ratiometric analysis of sensor using two fluorescent dyes and main advantage of such a system is the capability of exciting FITC and PtOEP in a single step due to their close excitation maxima.

Study of Interference of Surfactant and Cholesterol

The leaching of dye is negligible in DI water due to hydrophobic nature of dye. PtOEP-loaded microspheres were then incubated in a solution of thesit (*n*-dodecyl polyethylene glycol ether, a nonionic surfactant) and isopropanol (cholesterol solubilizer) in order to study the effect of surfactant on emission spectra of PtOEP/Algilica particles. It was found that PtOEP leaching increases

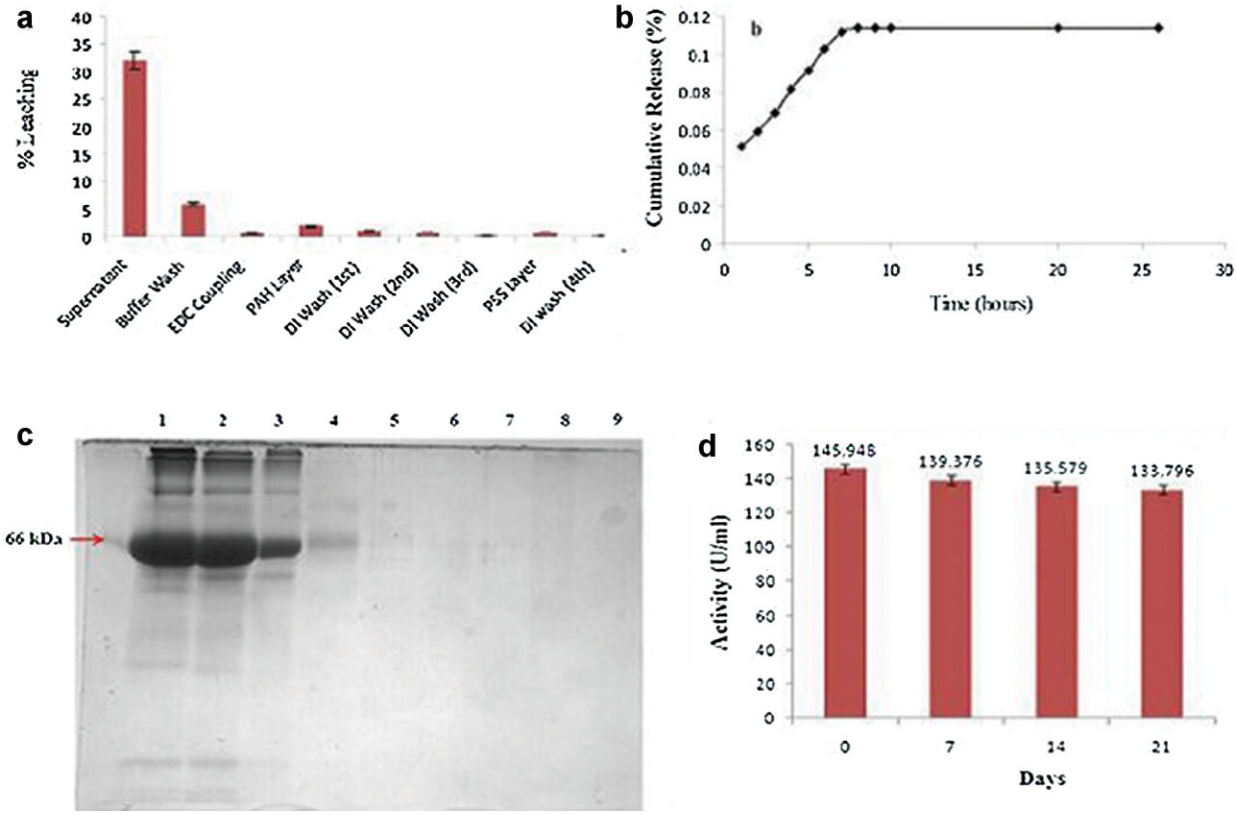

Figure 4. Leaching profile of BSA (**a**) during loading in alginate-silica microspheres (**b**) post-loading. **c**: Electrophoretic patterns of leached BSA are also included. The lanes represent: Native BSA (standard), BSA released in supernatant after loading, BSA released after buffer wash, BSA released after Carbodi-imide coupling steps (5,6,7), BSA leaching after PAH deposition and subsequent washes (8,9), BSA leaching after PSS deposition and subsequent washes. **d**: Activity profile of Gox in plain alginate-silica microspheres determined at pH 7.4 after EDC crosslinking (25 mM). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

with increasing surfactant concentration. An increase in thesit from 0.3% to 1% leads to corresponding increase in leaching by 0.38%. A maximum cumulative intensity of 1.78% of PtOEP-doped particle intensity was observed after 17 days of incubation in 1% (v/v) thesit and 10% (v/v) isopropanol. The incubation of PtOEP-doped microspheres with thesit causes a solubilization of encapsulated PtOEP from the microspheres. This limits the usage of sensing assay for certain number of repetitions. Similarly, the effect of cholesterol on the excitation spectra of PtOEP was studied as described in Figure 5a and b. It indicates no interferences for absorption maxima of cholesterol and PtOEP at $\lambda_{max} = 286$ and 535 nm, respectively. The graph also shows that there are no effects of increasing cholesterol concentrations on the emission spectra of the PtOEP (Fig. 5c). The use of thesit for dissolving cholesterol limits the use of sensors because the PtOEP dissolved in particles leaches out in presence of thesit and the leaching increases with increase in thesit percentage (Fig. 5d). The interference studies confirm the suitability of individual components for developing biosensor without optical interference.

Oxygen Sensitivity Testing and Cholesterol Biosensing

Response of PtOEP doped in the Algilica microspheres under various oxygen concentrations is shown in Figure 6a. This test was repeated and fluorescence intensity changes were monitored by increasing and decreasing oxygen concentrations. It is evident from Figure 6b that the intensity changes are fully reversible and no measurement hysteresis is observed. I_0/I ratio was used to calculate the oxygen sensitivity of the microspheres (Fig. 6c). For PtOEP doped in the Algilica matrix, the plot exhibited linearity in the low oxygen concentration up to 2.5 mM $[O_2]$. Furthermore, nonlinearity (logarithmic response) of the sensor response on higher oxygen concentration was observed. The correlation factor of the plot, R^2, was estimated to be 0.986 for $[O_2]$ concentrations up to 15.89 mM. However, it was observed that the Stern–Volmer plot (Fig. 6d) measures concentration at least for oxygen concentrations up to about 2.5 mM ($R^2 = 0.991$). This proves that the sensing system can be very useful in determining the dissolved oxygen in aqueous fluids. K_{SV} in the low oxygen concentration range was found to be 0.097 U/mM, which indicates that oxygen is

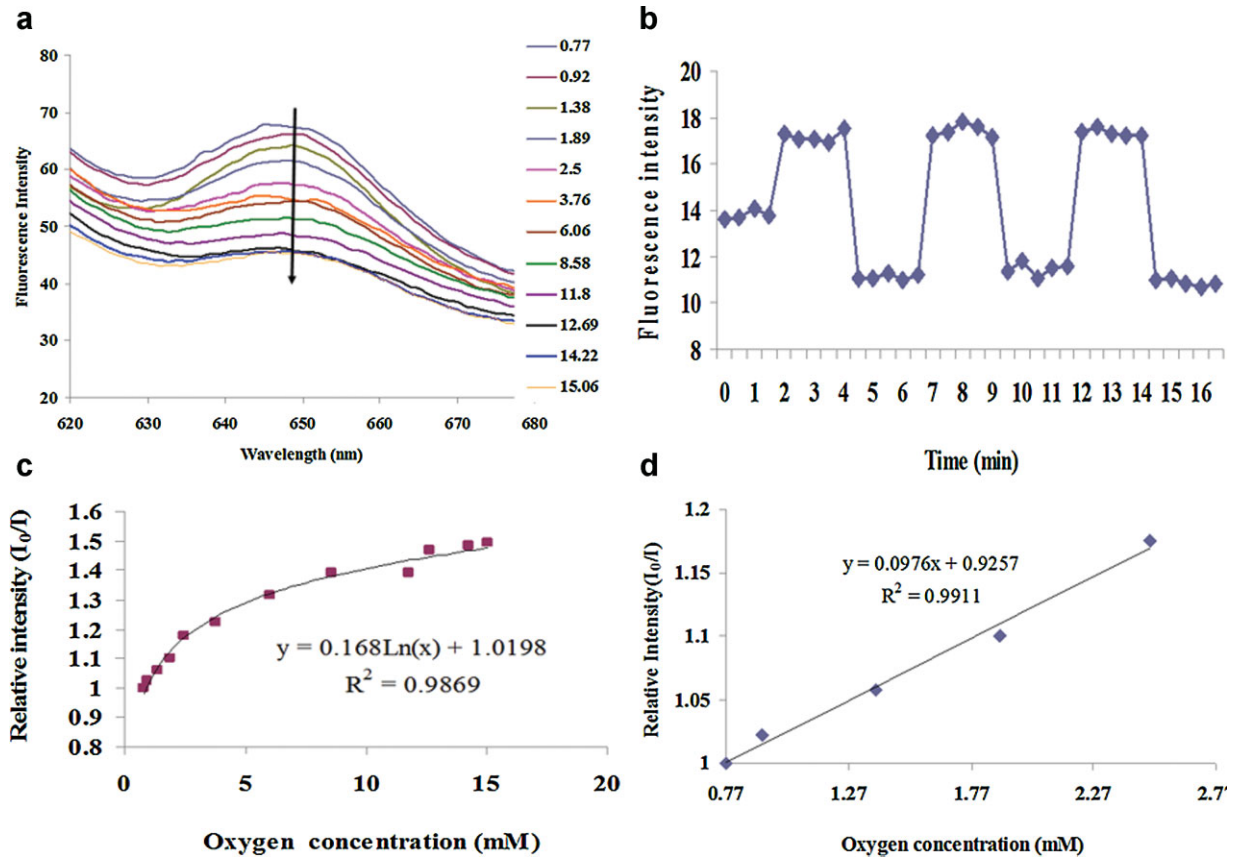

Figure 5. Oxygen sensitivity testing. **a**: Oxygen-dependent quenching of PtOEP within algilica microspheres. **b**: Reversibility of biosensor. **c**: Relative intensities of microspheres at different [O_2]. **d**: Stern–Volmer plot for oxygen sensing. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

able to diffuse inside the matrix and interact with encapsulated PtOEP molecules.

Cholesterol-dependent reduction in oxygen concentration of the reaction mixture is shown in Table I. Subtle changes in emission intensity were observed on moving from lower to higher concentrations because of the slower diffusion rate of cholesterol. Upon increasing the concentration the intensity also increases in steps 2 and 4. The emission intensity was used to calculate the ratiometric intensities I_0/I values at different cholesterol concentrations. The response time of the sensor was determined for the cholesterol concentration using 3.75 mM. Figure 7a illustrates that saturation in enzyme response was observe in about 10 min. The ratiometric Stern–Volmer plot is shown in Figure 7b. The plot shows a linear range up to 4 mM cholesterol concentrations. The determination of concentrations up to 4 mM confirms the usage of sensor for hypocholesterolemia with a very high accuracy in this range ($R^2 = 0.996$). The sensor works on increasing the concentrations up to 10 mM with less but acceptable efficiency. The sensor capability lies in the accurate determination of LDL cholesterol level (where $R^2 = 0.976$ for cholesterol concentrations up to 6 mM). The sensor however offers adequate accuracy for the estimation of hypercholesterolemia in cases where total serum cholesterol is used as analyte as illustrated by the correlation values ($R^2 = 0.959$ for cholesterol concentrations above 6 mM).

Conclusions

A novel sensing system based on Algilica microspheres and ChOx has been demonstrated for the in vitro monitoring of cholesterol levels. In this novel work the oxygen sensitive dye, PtOEP, has been explored for the first time as the sensing element for cholesterol sensing. The enzyme-based sensor has been used to detect cholesterol in isopropanol/thesit/DI water. The resulting Algilica/Chox/PtOEP sensor shows several fold better detection performance than the existing optical-based biosensors for cholesterol when operated at the optimum pH value of 7.4 and temperature 25°C. The linear response of this sensor lies in the range of 1.25–10 mM of cholesterol concentration. A detection limit of less than 1.25 mM of cholesterol, a high sensitivity

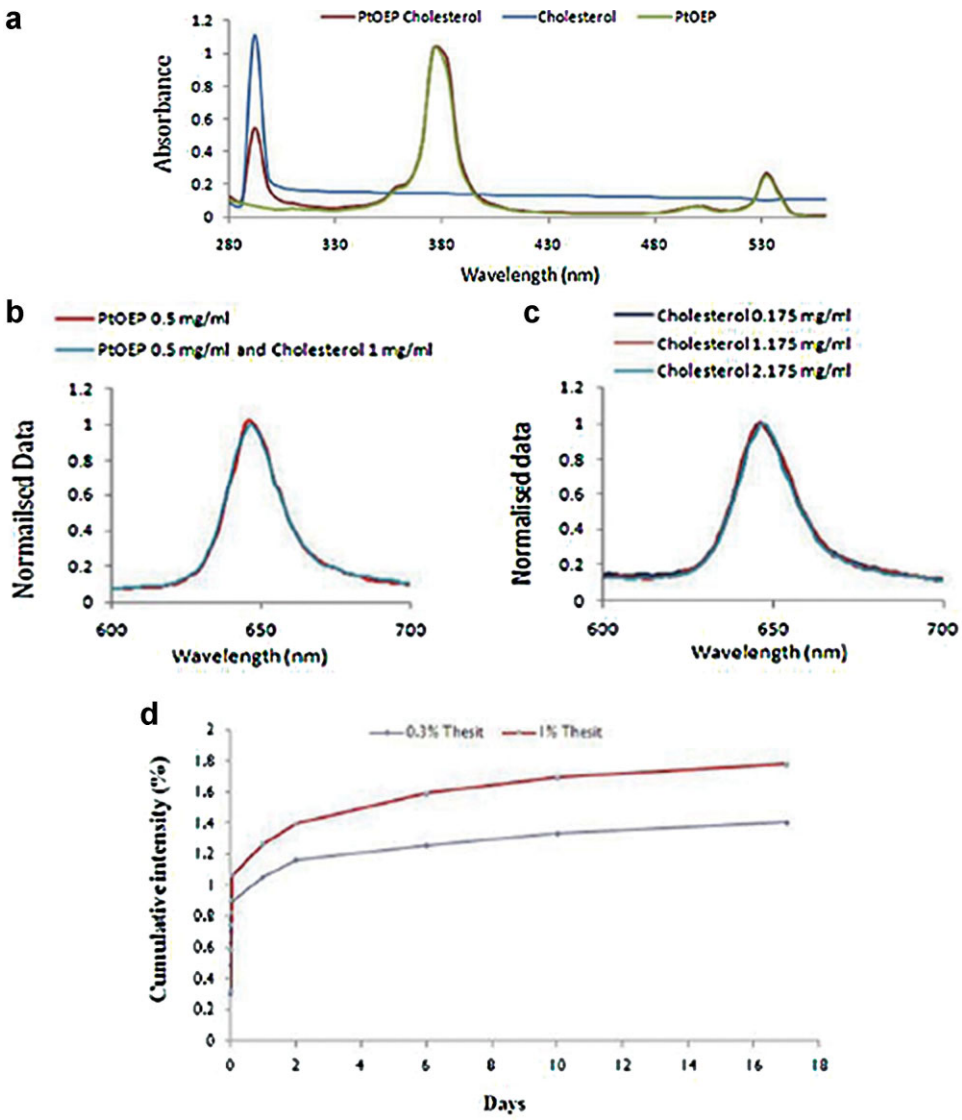

Figure 6. **a**: Absorption spectra for study of interference of cholesterol and PtOEP. **b**,**c**: Emission spectra of PtOEP for studying effect of concentration of cholesterol. **d**: Effect of thesit on leaching of PtOEP from microspheres. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Table I. Intensity profile showing peak emission values observed for the sensor for standard cholesterol concentrations.

Cholesterol concentration (mM)	Emission intensity (648 nm)
0	60.01
1.25	64.43
2.5	68.82
3.75	72.18
5.0	74.27
6.0	76.31
7.5	78.65
10	82.11

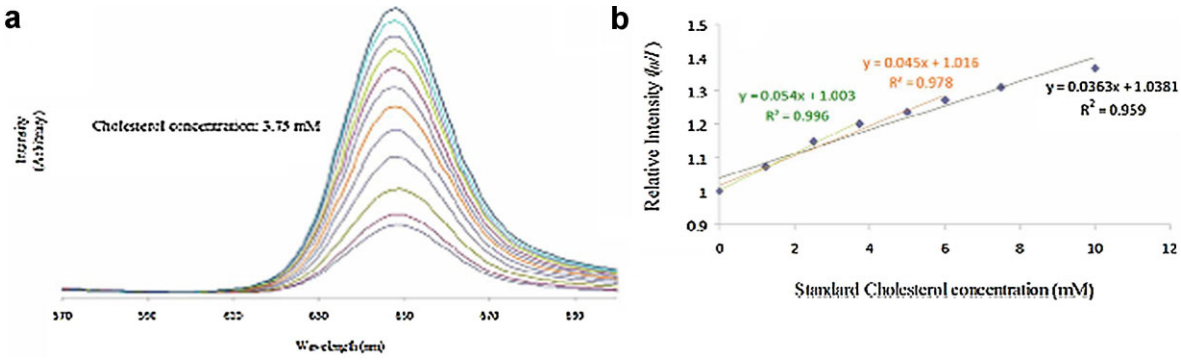

Figure 7. **a**: Emission spectra at intervals of 5 s. **b**: Relative intensities of microspheres with different standard cholesterol concentrations. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

and selectivity, and a response time of 10 min has been found from the sensor. The Algilica/PtOEP/ChOx-based cholesterol sensor was satisfactorily employed for the estimation of cholesterol concentrations that are representative of hypercholesterolemia state with high accuracy.

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