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Diatom-Based Label-Free Optical Biosensor for Biomolecules

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Abstract Diatoms are unicellular algae, which fabricates ornate biosilica shells called frustules that possess a surface rich in reactive silanol (Si–OH) groups. The intrinsic patterned porous structure of diatom frustules at nanoscale can be exploited in the effective detection of biomolecules. In this study, the frustules of a specific diatom *Amphora* sp. has been functionalized to detect bovine serum albumin (BSA). The functionalization of the diatom frustule substrate is achieved by using 3-aminopropyltriethoxysilane (APES). The field emission scanning electron microscopy (FESEM) results showed an ornately patterned surface of the frustule valve ordered at nanoscale. The Fourier transform infrared (FTIR) spectra confirmed the N–H bending and stretching of the amine group after amine functionalization. The emission peaks in the photoluminescence (PL) spectra of the amine-functionalized diatom biosilica selectively enhanced the intensity by a factor of ten when compared to that of a bare diatom biosilica. The result showed a significant quenching of PL intensity of BSA at around 445 nm due to the interaction of amine-functionalized diatom–BSA protein complex. The detection limit was found to be 3×10^{-5} M of BSA protein. Hence, the study proves that the functionalized frustule of *Amphora* sp. is an effective quantitative analytical tool for optical label-free biosensing applications.

Keywords Diatom · *Amphora* sp · Biosensing · Photoluminescence · Bovine serum albumin

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Introduction

In the last few decades, people have shown great interest toward the submicron and the nanoscale world [1]. The increasing global demand for ever smaller structures in electronic, optical, chemical, and biomedical device has forced the development of synthetic nanotechnology to a greater extent. As a result, researchers undergo a bottom-up approach to engineer systems by looking at natural nanostructures. This often produces biologically inspired devices and materials that are superior to current devices [2, 3].

Microalgae are a good example of unicellular organisms in which complex nanostructures are found [4-7]. Among them, diatoms are particularly interesting due to their morphologies and material properties [8, 9]. Their protoplasts are enclosed in an amorphous silica cell wall called a frustule, consisting of two valves joined together by a series of silica bands linked together along the margins. The valves of diatom exhibit species-specific ornamentations consisting of regular arrays of chambers. The chambers range in diameter from a few hundred nanometers up to a few microns, and they are circular, polygonal, or elongate through the whole silica skeleton, as tube-like structures [10].

The diatom frustule structure has special optical effects, such as photoluminescence (PL) [11, 12] and light focusing [13]. The frustules are composed of hydrated silica ($SiO2 \cdot nH2O$), which has a free silanol group; thus, they can be chemically modified to bind molecules, such as proteins [14] and mercury ions [15].

Most of the biosensors were designed to detect only lower concentration of single biomolecules since the signal of PL quenching is coincided with the increase of amide content in the surface [16]. In our study, we chose the bovine serum albumin (BSA) as the target biomolecule, because serum albumins are the most abundant protein in plasma [17]. As the major soluble protein constituents of the circulatory system, they have many physiological functions [18]. Among them, BSA has a wide range of function such as binding, transport, and delivery of fatty acids, porphyrins, bilirubin, steroids, etc. It acts as a home to specific binding sites for metals, pharmaceuticals, and dyes [19]. BSA has two tryptophan moieties at positions 134 and 212 as well as tyrosine and phenylalanine [20]; the protein intrinsic emission is due to aromatic amino acid residues. The binding properties of BSA and drugs were investigated by many researchers [21-25], but there are only limited works found using diatom as a transducer.

In this study, we explored the potential of diatom frustule as a transducer in optical detection of BSA protein by PL quenching technique especially from *Amphora* sp.

Materials and Methods

Preparation of Diatom Frustules

The marine algal samples were collected as per APHA standard methods [26] from Muttukadu backwaters, Tamilnadu, India (lat. 9.8° N and long. 79.1° E). After sample filtration, a stock culture of the diatom *Amphora* sp. was isolated in the laboratory. The diatoms were cultured at 25 °C, under a regime of 12 h light and 12 h darkness. Samples were grown in sterilized filtered sea water supplemented with f/2 growth medium [27].

After growth using selective medium, the diatoms were centrifuged and rinsed with distilled water to remove salt contents. Samples were treated with 50:50 (*v/v*) water, followed by 30 % hydrogen peroxide and incubated at 90 °C for 3–4 h, followed by the addition of hydrochloric

acid to remove the organic matter and clean the frustules. Samples were collected by sieving to prevent damage to the frustules, rinsed copiously with distilled water, and stored in 70 % ethanol [28].

Surface Modification of Diatom Frustules

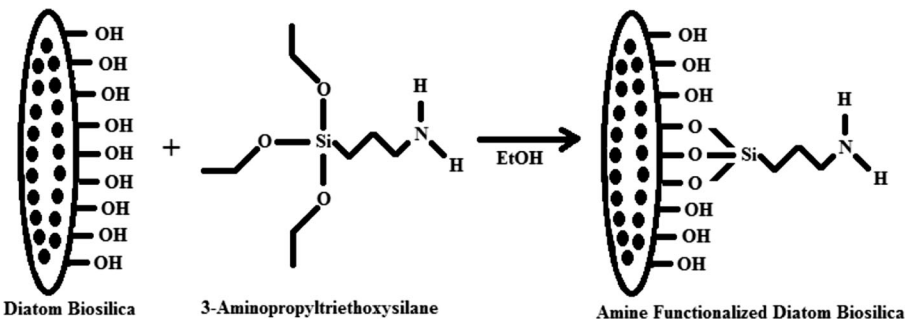
The diatom frustule surface of *Amphora* sp. was chemically modified as illustrated in Scheme 1. Of the diatom frustule, 1.0 mL was dispensed in 30 mL of toluene in a single-necked 250-mL round-bottomed flask. Of the sterile distilled water, 0.3 mL was then added to the mixture and stirred for 2 h at ambient temperature to allow the water to disperse through the diatom frustules which made a fully hydroxylated silica surface. Then, 1.7 mL of APES was added to the mixture which was taken to reflux for 6 h at 60 °C. After that, the mixture was cooled and washed with toluene, 2-propanol, and distilled water thrice. And the final product was stored in 70 % ethanol.

UV–Visible and Photoluminescence Studies

Aqueous solution of 1.0 mL (10^{-3} M) of amine-functionalized diatom was titrated with 20 μL of successive additions of BSA protein ($1–5\times10^{-5}$ M), then the absorption and emission spectra for all the solutions were recorded.

Characterization

The frustules of *Amphora* sp. were centrifuged and resuspended in methanol to view the morphological features by field emission scanning electron microscopy (FESEM; FEI Quanta 250 FEG) [29]. Fourier transform infrared (FTIR) spectroscopic analysis (FTIR Spectrophotometer TENSOR, Bruker) was performed on bare diatom frustule and amine-functionalized diatom frustules to obtain the information about chemical modification. A drop of both the samples were mounted onto the FTIR sample holder separately [30]. The absorption spectra of the amine-functionalized diatom with successive addition of protein were characterized using UV–visible spectroscopy (JASCO V-650 Spectrophotometer) with a wavelength range from 200 to 900 nm. The optical emission properties of functionalized diatom frustules were characterized using photoluminescence (PL) spectroscopy. The PL spectra of the frustule samples before, after amine functionalization, and with successive addition of protein were scanned to determine any modification in the optical intensity of the frustules. The samples were centrifuged and 1.0 mL of sample was loaded into a PL sample holder. These samples



Scheme 1 Surface modification of diatom frustules

were excited with a 370-nm light source from a 150-W Ozone-free Xenon arc lamp (HORIBA JOBIN YVON FluoroMax-4) equipped with a Czerny-Turner monochromator (1,200 grooves per millimeter) with an accuracy of 0.5 nm [31]. All PL measurements were carried out at room temperature and corrected for the baseline spectra.

Results and Discussion

The FESEM images showed two adjoined halves; each halve has an oval-shaped structure (~17 μm) resting on top of a ring-shaped girdle band which confirms that the frustules were of pennate diatom *Amphora* sp. The frustule valve has an ornately patterned porous structure ordered at the submicron and nanoscale. The pore structure consists of ~200-nm pores that ring the perimeter of the valve (Fig. 1). Isolation of frustule from the cell wall of diatom *Amphora* sp. was done by acid digestion and it effectively separates the valve and girdle band structures [32].

The FTIR spectra clearly shows the characteristic peaks of diatom frustule, which includes Si–O–Si bending at 689 and 810 cm^{-1}, [33] Si–O–Si stretching at 947 cm^{-1}, and [33] O–H stretching of surface-bound hydroxyl groups at 3,361 cm^{-1}, which would include bound water and Si–O–H. It also represents the characteristic peaks for carboxyl C–H stretching at 2,885 cm^{-1} (CH_2) and 2,972 cm^{-1} (CH_3) [34]. This is due to the residual organic materials still adhered to the frustules even after acid digestion [34]. After amine functionalization, the FTIR spectra had a strong peak at 1,119 cm^{-1}, which is characteristic of C–H bending of the Si–O–CH_3 moiety [35]. Furthermore, the C–H stretching peaks at 2,929 and 2,972 cm^{-1} were considerably enhanced, which was attributed to the propyl group on the amine-functionalized diatom frustules. The weaker peaks at 1,463 and 1,643 cm^{-1} were assigned to the N–H bending and stretching of the amine group [34, 35], and the peak near 3,361 cm^{-1} was enhanced, which was also attributed to N–H stretching [35]. So far as the chemical modification of *Amphora* sp. has not been attempted, this study revealed it through the FTIR spectra of bare diatom and amine-functionalized diatom (Fig. 2).

The optical properties of bare diatom frustules and amine-functionalized diatom frustules were evaluated using PL. The PL measurements of frustules were performed at room temperature. The PL intensity was measured at different excitation wavelengths [36]. It was observed

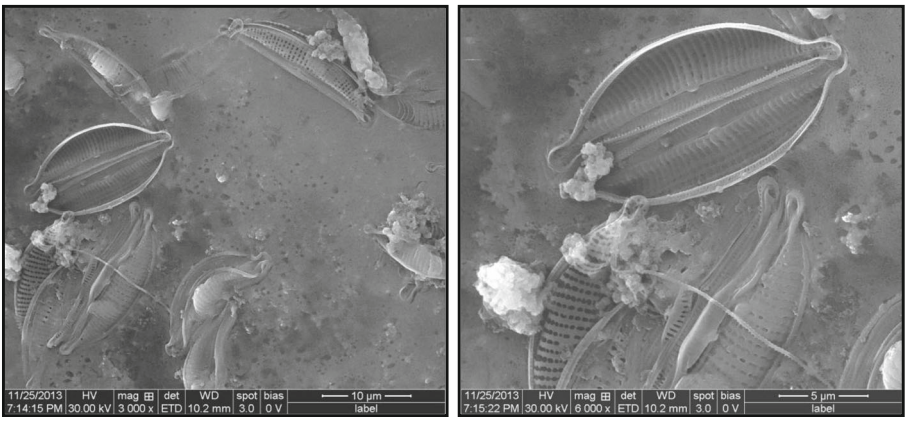


Fig. 1 FESEM micrographs of the frustules valve isolated from the pennate diatom *Amphora* sp.

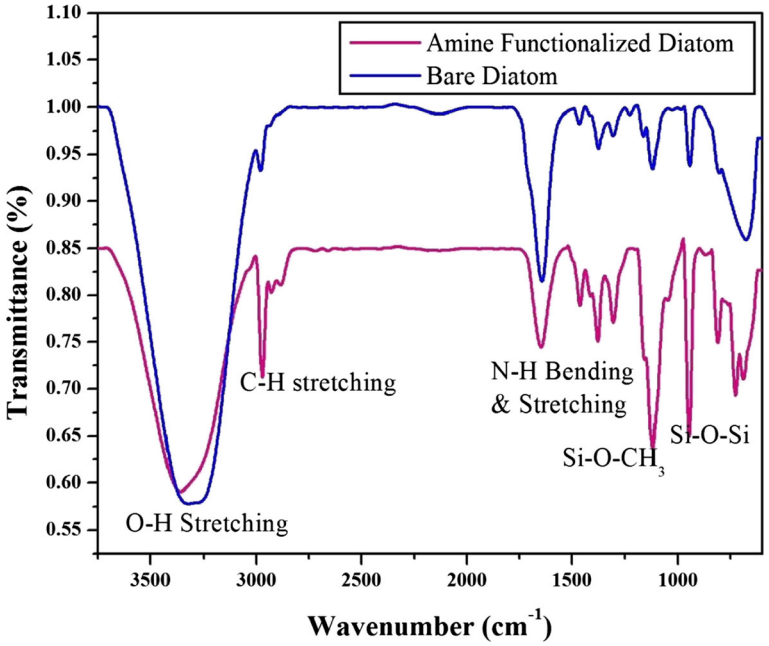


Fig. 2 FTIR spectra of diatom frustules before and after amine functionalization

that the sample frustules emitted strong blue photoluminescence centered at 440 nm when samples were excited at 370 nm. After the amine functionalization of the diatom frustules, the photoluminescence was slowly quenched, since the passivation process affects the surface recombination centers, which were the radiative points responsible of the diatoms PL [37]. It shows that the PL intensity of bare diatom was increased over a factor of 10 after the diatom frustules were covalently functionalized with APES (Fig. 3).

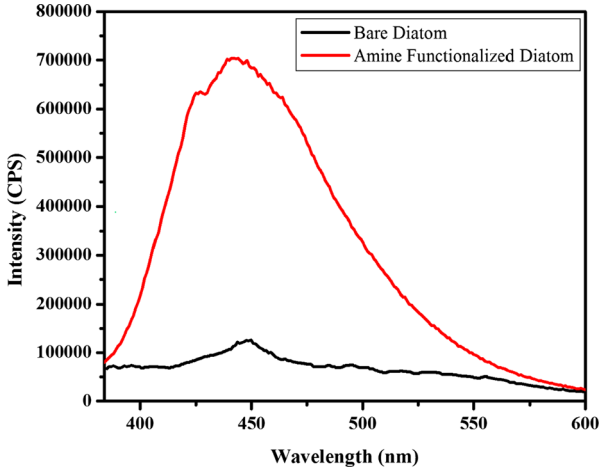


Fig. 3 PL spectra of bare and amine-functionalized diatom frustules

Figure 4 showed the absorption spectrum of amine-functionalized diatom with different concentrations of BSA. In the presence of BSA, the absorbance of amine-functionalized diatom was increased markedly, without any peak shift. This inference was due to the BSA molecules getting adsorbed on the surface of amine-functionalized diatom and involved in the formation of ground state amine-functionalized diatom–BSA complex [38].

The equilibrium for the formation of a complex between amine-functionalized diatom and BSA is given in Eq. 1:

amine-functionalized diatom+nBSA $\Leftrightarrow^{Kapp}$ amine-functionalized diatom−BSA, (n=1)

$$K_{app} = \frac{[amine-functionalized\ diatom - \mathrm{BSA}]}{[amine-functionalized\ diatom\ ] \cdot [\mathrm{BSA}]} \quad (1)$$

where K_{app} represents the apparent association constant.

The effect of different concentrations of BSA on the PL emission spectrum of amine-functionalized diatom was shown in Fig. 5. The PL intensity quenched by successive addition of BSA to the amine-functionalized diatom was due to the adsorption of excitation and emission photon flux and the presence of amino acid residues such as tyrosine, tryptophan, and phenylalanine [39, 40]. The quenching happened till 3×10^{-5} M, then it reaches the saturation level and there was no more quenching taken place. It may be due to the limitation of binding sites in the amine-functionalized diatom surface.

The origin of strong blue PL is attributed to the tiny nanostructures on the frustules of diatom species. Nucleophilic molecules that attach to nanoscale diatom frustules surface with nanoscale topology increases the PL emission by donating electrons to non-radiative defect sites, this results in higher quantum efficiency of the amine-functionalized frustules surface [41, 42].

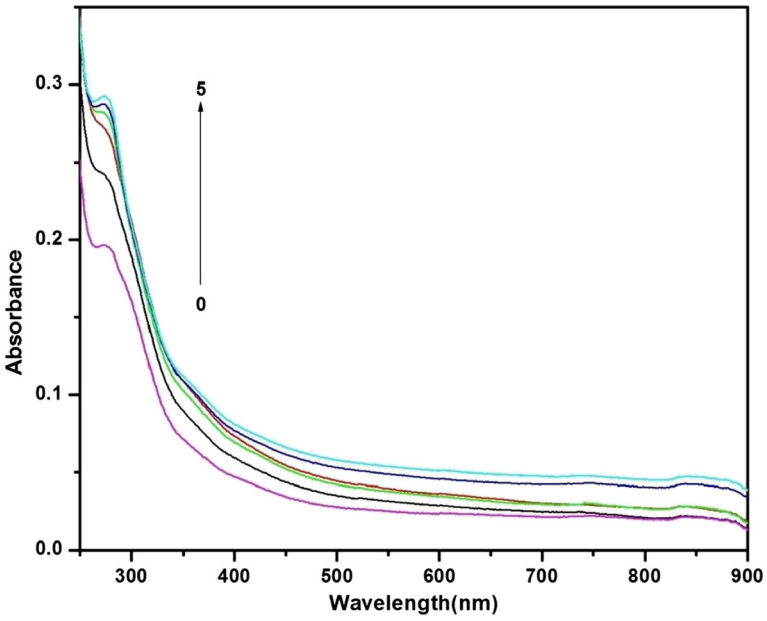


Fig. 4 Absorption spectra of amine-functionalized diatom in the presence of BSA in the concentration range of $0–5\times10^{-5}$ M

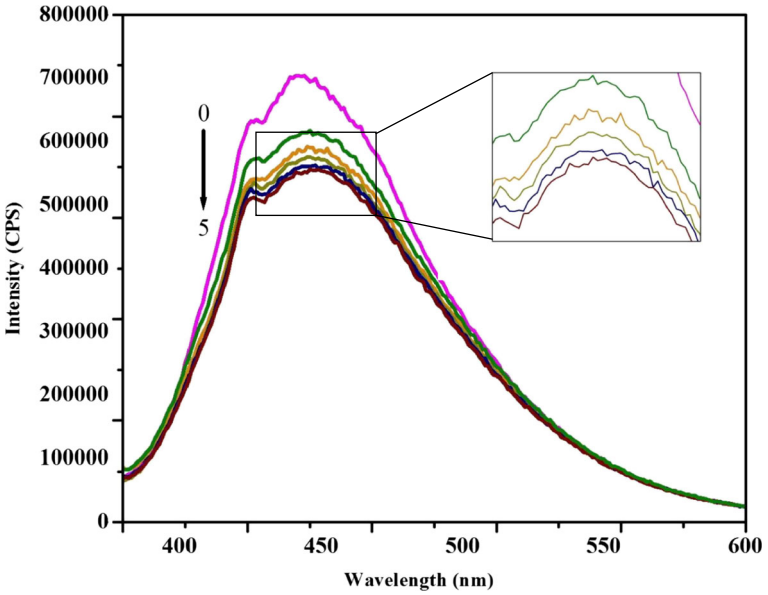


Fig. 5 PL spectra of amine-functionalized diatom in the presence of BSA in the concentration range of 0–5× 10^{-5} M

Conclusion

The diatom frustules isolated from the cell wall of the pennate diatom *Amphora* sp. were functionalized with amine group and the protein was detected. The UV–visible and PL spectra detects the BSA concentration at 3×10^{-5} M and it clearly indicates that BSA quenches the PL intensity of amine-functionalized diatom through complex formation. The results concluded that the ornately patterned porous structure with large flat surface area of amine-functionalized diatom *Amphora* sp. serves as a novel microscale transducer platform for the selective and label-free PL-based detection of biomolecules.

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