Development of a Diatom-Based Photoluminescent Immunosensor for the Early Detection of Karnal Bunt Disease of Wheat Crop

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ABSTRACT: In India, the major crop is wheat. Its production is severely hampered by seed-borne diseases such as smut and bunt which are responsible for the reduction of crop yield with poor grain quality. In the current study, an attempt was made to prepare a photoluminescence (PL)-based immunosensor for early detection of Karnal bunt (KB) disease. The KB disease-causing pathogen *Tilletia indica* was detected using functionalized diatom frustules as a sensing platform. The teliospore-covered platform, on exposure to light, showed enhanced intensity of PL in comparison to control. This response was directly proportional to the concentration of spores. For the development of a stable frustule-based immunosensor platform, glutaraldehyde was added for the covalent immobilization of the *T. indica* antibody onto amine-functionalized diatom substrates. Frustules of diatom consisting of a nanoporous three-dimensional biogenic silica material exhibit a unique property of emitting strong, visible blue PL under ultraviolet (UV) excitation. PL studies were done to reveal the specificity and binding of the conjugated diatom platform that will distinguish between the *T. indica* (complementary) and *A. niger* (noncomplementary) antigens. Four times better intensity of PL was observed against the complementary one in comparison to a noncomplementary setup (control). The immunocomplex frustule-based platform serves as a suitable sensor platform for early detection of KB.

INTRODUCTION

Karnal bunt (KB) is an important disease of wheat (*Triticum aestivum* L.) and its causative agent is *Tilletia indica* a spore producing fungi, first detected in Karnal (Haryana). It has been frequently reported in regions of Himachal Pradesh, Uttar Pradesh, Punjab, and Uttarakhand. Apart from India, the disease has also been reported in other countries such as Nepal, Pakistan, USA, and so forth. Early detection of plant pathogens is required to manage the infection and prevent its contamination to other healthy plants. Conventional diagnostic methods for the detection and identification of fungal spores usually include identifying unique morphological characteristics and/or DNA-based amplification schemes such as a polymerase chain reaction (PCR), electro chemical enzyme immunoassays, fluorescence, and so forth. However, morphological examinations by electron microscopy are very time consuming and, therefore, routine examinations cannot be carried out on a larger scale. Even though both antibody-based and nucleic acid-based detection have a greatly decreased assay time compared to traditional culture techniques, they still lack the ability to detect microorganisms in “real-time”.

There is a clear need for rapid, reliable, specific, and sensitive analysis systems for detecting a target analyte. The methods that allow real-time monitoring in the field such as biosensors are preferred. Recent advances in biosensors showed that most important characteristics of biosensors are specificity and sensitivity. Specificity strongly depends on the bio-specific interface of biosensors. However, sensitivity depends not only on the (bio) functionalizations but also on the biosensor architecture and transduction elements. Most existing nanofabrication techniques involve only two-dimensional (2D) planar lithography. Thus, new challenges for the biosensor industry are to enhance the signal and fabricate highly sensitive biosensors with a high signal to noise ratio. The simplest way to enhance the signal is to transform the 2D surface into a three-dimensional (3D) surface. Large-scale fabrication of 3D nanostructures on a fine scale is an essential requirement for commercialization. In this context, diatoms have developed elegant solutions producing hierarchical 3D micro- or nanostructures under physiologically compatible and environ-
mentally benign conditions using minimal energy and producing minimal waste.\textsuperscript{15} Diatoms, single-cell eukaryotic microalgae, are present in nearly every water habitat and their silicon dioxide (silica)-based cell walls of 10–100 $\mu$m in size are the most interesting feature to be used in nanotechnology.\textsuperscript{16} Researchers showed that antibody-functionalized diatom 3D biosilica may be successfully utilized as a photoluminescent sensor to identify the goat anti-rabbit IgG molecules.\textsuperscript{17} They also utilized the amine-functionalized diatom frustules for the detection of the bovine serum albumin protein with a detection limit up to $3 \times 10^{-5}$ M. In 2009, Gale and co-researchers also showed the three times enhanced PL intensity after the formation of the immunocomplex on the functionalized diatom biosilica frustules of \textit{Cyclotella} species compared to binding with a noncomplementary antigen.\textsuperscript{18} The present work is showing four times enhancement of PL with the complementary antigen immunocomplex on the functionalized diatom biosilica frustules of \textit{Navicula lundii} for detecting KB in wheat.

The present study is an effort to utilize the efficiency of amine-functionalized diatom (AFD) for the early and specific detection of fungal pathogen \textit{T. indica} in wheat crops using polyclonal antibodies as the specific recognition element.

\section*{RESULTS AND DISCUSSION}

\textbf{Estimation of Teliospores Protein.} The teliospores protein from \textit{T. indica} was isolated and O.D. was taken at 660 nm.

The teliospores protein of samples were in the range of 0.205–0.773 mg/mL.

\textbf{Morphological Analysis of AFD and Cross-linked AFD Glass Substrate.} In order to analyze the ultrastructure of the porous surface in diatom frustules, scanning electron microscopy (SEM) was performed. Morphological analysis of AFD and glutaraldehyde cross-linked AFD substrates is shown in Figures 1 and 2, respectively. The cell wall surface of the AFD substrate, as shown in Figure 1, was coated with APS uniformly all over its peripheral end. The pores of frustules in

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{SEM micrographs of the nonfunctionalized AFD substrate at different magnifications (a) 1500x, (b) 5000x, (c) 10,000x, and (d) 20,000x.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{SEM micrographs of the functionalized cross-linked AFD substrate at different magnifications (a) 1500x, (b) 5000x, (c) 5000x, and (d) 20,000x.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{FTIR spectra of the bare diatom frustules, AFD, and cross-linked AFD glass substrate.}
\end{figure}

AFD were wide (~40 nm) in comparison to cross-linked AFD (<25 nm). This is due to the amine functionalization process which makes the diatom surface more reactive and sensitive.

The binding in between glutaraldehyde and the vacant amine group of AFD leads to the formation of long chains in cross-linked AFD.\textsuperscript{11}

\textbf{Functional Group Analysis of Bare Diatom Frustule, AFD, and Cross-linked AFD.} The infrared spectra of AFD and cross-linked AFD were analyzed in the wavelength range of 500–4000 cm\textsuperscript{-1}, as shown in Figure 3. The vibrations were initiated from 500 cm\textsuperscript{-1} and characteristic peaks of Si–O–Si were observed at 500–700 cm\textsuperscript{-1} with a transmittance of 0.82 and 0.70% for bare diatom frustules and AFD, respectively, whereas for cross-linked AFD sharp peak not observed and lies near 0.77%. The bond Si–O–CH\textsubscript{3} peaks were observed at 1200–1230 cm\textsuperscript{-1} with transmittance of 0.66% for diatom frustules, 0.62 and 0.58% of AFD and cross-linked AFD, respectively. While, cross-linked AFD was affected by glutaraldehyde. The N–H stretching were observed at 1300–1600 cm\textsuperscript{-1} with transmittance of 1.01, 0.92, and 0.63% for diatom frustules with no treatment, AFD and
cross-linked AFD, respectively. The bands with high intensity were observed because of overlapping between the amide group of AFD and cross-linker.\textsuperscript{19} The mechanism behind the covalent binding between the cross-linker and amine-functionalyzed diatom substrates. The CH stretching occur at 2700 cm\(^{-1}\) with a transmittance of 0.90\% for AFD and 0.71\% for cross-linked AFD and negligible peaks were seen in the case of bare diatom frustules. The stretching of OH bonds were seen from 3200 to 3600 cm\(^{-1}\) and transmittance were observed at 0.95, 0.85, and 0.64\% of diatom frustules only, AFD and cross-linked AFD, respectively, as depicted in Figure 3.

Figure 4. Schematic diagram of all step of fabrication of the diatom-based sensor along the sensing mechanism.

Absorption Spectrum Analysis of AFD and Cross-linked AFD. Absorption spectra of AFD and cross-linked AFD were carried out as shown in Figure 5. The absorption intensity showed a higher absorbance from the initial wavelength of 250 nm upto 700 nm in the cross-linked AFD as compared to AFD. The maxima absorbance is seen in the range of 400–450 nm. This is due to the influence of the added cross-linker that has altered the optical characteristics of the diatom frustules.\textsuperscript{17}

PL Emission Spectrum Analysis of AFD and Cross-linked AFD Substrates. Emission spectra of AFD and cross-linked AFD substrates were analyzed using a PL spectrophotometer, as shown in Figure 6. Because of the effect of the gluteraldehyde cross-linker, peak intensity of cross-linked AFD was increased significantly from 350 to 400 nm in contrast to AFD. However, there was no change in \(\lambda_{\text{max}}\) of both substrates. It has also been proven that gluteraldehyde cross-linking in AFD helps in efficient binding of antibody molecules,\textsuperscript{20} as shown in Figure 7. Furthermore, PL spectra of the complex formed between complementary \textit{T. indica} antigen-
antibody-functionalized diatom showed a twofold increase in PL as compared to the other samples (Figure 7).

**Biosensing System.** To confirm the immunospecificity of *T. indica* antibody-functionalized diatom, the sensing platforms treated with *T. indica* (complementary) and *Aspergillus niger* (A. niger) (noncomplementary) antigens in phosphate buffer saline (PBS) was excited at a particular excitation wavelength. We did not find any bonding between *T. indica* antibody and A. niger antigens as they are noncomplementary.

The PL responses were observed with the maximum concentration of *T. indica* ($10^{-1}$) and *A. niger* antigens ($10^{-1}$), where *A. niger* antigen was producing the weak signals as compared to the signals of *T. indica*. Most of the biomolecules, when attached with nanoscale nanoporous diatoms, help to enhance the PL emission.²¹ The PL was enhanced in complementary antigen-binding diatom substrates compared to other treatments. This QC/PL center model proposed the quantum confinement (QC) in the nanoscale range of silicon which is followed by a de-excitation process in a SiO₂-passivated layer system.²²

The frustules of diatom mainly contain porous silica and results in multiple peaks in PL spectra because of the presence of irregular crystallite sizes in its frustules. Different concentrations of the *T. indica* antigen were taken from the range of $10^{-1}$ to $10^{-5}$ and PL intensities were observed (Figure 8). A PL intensity of $10^{-1}$ *T. indica* antigen found to have increased many folds than *T. indica* antibody-functionalized diatoms. These results were also confirmed when the PL activities of *T. indica* and noncomplementary *A. niger* were done. In immunocomplex formation, the complementary antigen *T. indica* showed the linear increase of PL intensity with the spore concentration but after the concentration of spores reached $8 \times 10^4$ spores/mL and it became saturated as the number of antibodies were bound with complementary antigens. When this is compared with noncomplementary antigen *A. niger*, first it increased as the other biomolecules showed the increased intensity and after that it became a constant line as this is noncomplementary and did not show any binding with anti *T. indica* antibody, as shown in Figure 9.

The reason behind the increase was the nucleophilic nature of the *T. indica* antigen. Following its attachment with the antibody, it donates an electron to the nonradiative vacant sites of the *T. indica* antibody-functionalized diatom platform. Therefore, it can be concluded that there is a decrease in the process of nonradiative electron decay and increase in radiative

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![Figure 6](https://example.com/figure6.png)  
**Figure 6.** PL spectra of AFD and cross-linked AFD substrates.

![Figure 7](https://example.com/figure7.png)  
**Figure 7.** PL spectra of different steps involved in biosensor fabrication.

![Figure 8](https://example.com/figure8.png)  
**Figure 8.** PL spectra of different dilutions of antigens involved during biosensor fabrication.

![Figure 9](https://example.com/figure9.png)  
**Figure 9.** PL spectra of complementary and noncomplementary antigens involved in the immunocomplex on the biosensor platform.
emission. This showed the enhancement in PL intensity after the formation of immunocomplex. 18

On the other hand, when we observed the PL intensity of T. indica antibody-functionalized diatoms challenged with a 10⁻¹ A. niger antigen (which also acts as a nucleophile) it was increased only by onefold. This is due to a lack of immunocomplex formation, thus demonstrating that the detection of PL intensity was very selective.

From the above experiments, we can come to the conclusion that this proposed bio silica sensor would be beneficial for the selective detection of T. indica even at a nanomole concentration of the antigen.

CONCLUSIONS

In global food industries, exporting pathogen-free food grains with high nutritional value is one of the primary concerns nowadays. The sensor device must be fabricated in a simple, rapid, sensitive, and reproducible in nature, based on immunoassay techniques, form. From the above results, we can conclude that morphological images of cross-linked diatom formed a smoother outer surface because of the presence of particular functional groups, where the surface was chemically modified with gluteraldehyde. After the addition of complementary antigen of the T. indica spore protein against the antibody-immobilized functional diatom-coated sensing platform, the complementary immunocomplex was formed. There was a significant increase in PL intensity. It also has the ability to detect the antigens in 10 pg values. This ultrasensitive sensing platform can be used for monitoring the fungal pathogens at the field level in an early stage of infection with higher specificity. This will help in disease surveillance and regulation of quarantine conditions.

MATERIALS AND METHODS

Materials. Hydrogen peroxide (30%), hydrochloric acid, 3-amino propyl triethoxy silane (APS) (analytical grade, 98%), absolute ethanol (analytical grade, 99.9%), and gluteraldehyde solution (25%) were purchased from HiMedia. Polyclonal antibody against the spores of T. indica was commercially prepared by Genei Bengalore. For the preparation of solutions, double-distilled water was used.

Characterization of the Fabricating Device. After frustule collection of the freshwater diatom Navicula lundii, morphological analysis of the AFD and gluteraldehyde cross-linked AFD substrate in carboxy methyl cellulose (CMC) were done. SEM was performed and image analysis of both were done. Qualitative imaging of isolated frustules and functionalization were performed by SEM (Superprobe Jeol JXA-8100) done. SEM was performed and image analysis of both were done. Qualitative imaging of isolated frustules and functionalization were performed by SEM (Superprobe Jeol JXA-8100) done. SEM was performed and image analysis of both were done. Qualitative imaging of isolated frustules and functionalization were performed by SEM (Superprobe Jeol JXA-8100) done. SEM was performed and image analysis of both were done. Qualitative imaging of isolated frustules and functionalization were performed by SEM (Superprobe Jeol JXA-8100) done.

Amine Functionalization of Diatoms. Collected frustules of freshwater diatom N. lundii were chemically modified by adding frustules with ethanol in a 1:1 ratio followed by the addition of 3% ammonium persulfate (APS) and after mixing properly heated up to 65 °C for 1 h in a shaking incubator. To remove excess volume of APS, the reactant mixture was centrifuged at 3000 rpm for 10 min. The amine-functionalized diatom frustules were ready for the spin-coating on a clean glass substrate.

Spin-Coating of AFD on a Glass Substrate. For spin-coating of AFD on a glass substrate, prepared the sol gel by using CMC by mixing them in equal proportion. AFD (10 mg) were mixed uniformly with 10 mL of 0.5% CMC on a magnetic stirrer. For CMC coating, we found that 30 μL of 0.5% CMC was suitable to make one film on a 1 × 1 cm glass substrate at 1200 rpm for 40 s by using a spin-coater instrument (spin NXG ME). For preparation of the first layer, we have used 20 μL of 10 mg/10 mL of AFD and spin-coated on a washed clean glass substrate. Likewise, the second layer was deposited and dried in a hot air oven for 10 min which resulted in uniformly coated AFD substrates. The thickness of the film on the glass substrate is around 5 μm. Like this, we have prepared five layers on the glass substrate for CMC coating. After this spin-coating, the CMC with AFD-coated glass slides were kept in a hot air oven at 90 °C for 1 h.

Cross-linking of the AFD Substrate. The 1 × 1 cm spin-coated AFD substrate was transferred to a tissue culture plate containing 1.8 mL of phosphate buffer saline buffer, and 200 μL of gluteraldehyde was added which act as a cross-linker and then the resultant suspension at room temperature was placed.
in a shaking incubator for 20 min. The cross-linked AFD substrates were finally washed with PBS buffer thrice to remove the excess cross-linkers.

**Covalent Immobilization of Specific Antibody on the Cross-linked AFD Substrate.** The covalent immobilization of the specific antibody of *T. indica* was performed by placing the cross-linked AFD substrates into the plate which contain the 1.8 mL of PBS buffer and then added the 200 μL of the antibody. Mixing of substrates was performed at room temperature for 2 h in a shaking incubator. The *T. indica* antibody-conjugated diatom frustule substrates were then rinsed thrice with PBS buffer.

**Immunological Reactions of Antibody-Conjugated Diatom Frustule Substrates with Antigens.** The antibody-conjugated diatom frustule substrates were treated with an equal amount of complementary and noncomplementary antigens. The stocks of *T. indica* antigens were prepared by mixing 200 μL of antigens in 1.8 mL of PBS buffer and all are mixed in an incubator for 2 h in a shaker at 37 °C. The immuno-complex formed by the addition of antibody and complementary antigen. The specific immunocomplexed diatom frustule substrates were again rinsed in PBS buffer.

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**Notes**
The authors declare no competing financial interest.

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