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RESEARCH ARTICLE

A functional approach toward xerogel immobilization for encapsulation biocompatibility of *Rhizobium* toward biosensor

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Abstract **Sol-gel derived silica has tremendous applications as a biocompatible scaffold for the immobilization of cells. The use of xerogel as a matrix in the blueprint of biosensors is an appealing proposition due to several inimitable characteristics of xerogels, primarily because of their high porous nature, amendable pore size, and exceptionally large internal surface area. Morphological (X-Ray Diffraction and Thermogravimmetric Analysis) and optical (Fourier Transform Infrared and UV-Vis absorption) studies of the silica matrices with entrapped *Rhizobial* (Rz) structure of the biomaterial has been made. Temporal and concentration dependent studies were conducted for impregnated samples; it showed that the response time for the new biosensor for determining the concentration of Rz is less than 20 min. In this work, first time a novel avenue to create a generic approach for the fabrication of biosensor has been created.**

Keywords biosensor, Fourier Transform Infrared spectroscopy (FTIR), *Rhizobium*, Thermo Gravimmetric Analysis (TGA), sol-gel, xerogel, X-Ray Diffraction (XRD)

Introduction

Detection and identification of bacteria is an important aspect of our world today. Outbreaks of pathogenic bacteria, occurring either naturally in food or possibly being used as weapons by bioterrorists for contamination of food, air, and water, are constantly in the news. Therefore, identifying the specific bacteria responsible for these outbreaks and their potential source is of great importance (Zourob, 2008; Kishen et al., 2003). The biocompatibility for biosensor depicts at this juncture a simple and commercially inexpensive screening of immobilized cell samples, which may be expanded to encompass the detection of a wide variety of pathogenic, infectious, and malignant cells without the need for a fluorescent or a radiolabeling process and other optical microscopic methods.

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The low processing temperature of sol-gel technology combined with the intrinsic biocompatibility and environmental friendliness makes it an ideal technology for the fabrication of bioactive materials. The sol-gel-derived materials bequeath excellent matrices for entrapping a variety of organic, inorganic compounds and biologically imperative molecules (Tsai et al., 2003; Barbe et al., 2008; Desimone et al., 2009). The gel-derived materials are excellent model systems for studying and controlling biochemical interactions within constrained matrices with enhanced bioactivity because of their residual hydroxyl ions, micro-pores and large specific surface. The sol-gel derived film or layer not only bestows a good degree of biocompatibility, but also a high specific surface area (which can be targeted as a carrier of adsorbed drugs) and as an external surface, whose rich chemistry allows easy functionalization by suitable biomolecules (Gupta and Kumar, 2008). Concerted efforts have been made in merging the bacterial preservation schemes with the construction of sensor cell arrays on platforms such as biochips or optical fibers, hopefully leading to effective miniaturized whole-cell biosensor system. These approaches embrace immense vow for fabrication of a workable biosensor in the future panorama. Nevertheless, their eventual implementation in practical devices calls for significant

enhancement of current knowledge on formulation of reporter microorganisms (Niki et al., 2005; Shaomin et al., 2007; MacDonald et al., 2008). Soil-inhabiting bacterium *Rhizobia* subsists as free-living soil saprophyte or as nitrogen-fixing endo-symbiont of legume host plants. It reveals symbiotic liaison with plant-legume species in root nodules. The bacteria fix atmospheric nitrogen to form ammonia, which is ancillary assimilated by the plants. This relationship is acknowledged to augment escalation of legume crop by agriculture without the addition of nitrogen-containing fertilizers. Convicting this rationale of ecological interaction, the research has focused on *Rhizobia* infecting herbaceous exotic and endemic legumes of agricultural implication. The natural *Rhizobia* of wild legumes sprouting in arid zones reveals higher tolerance to prevailing ominous conditions akin to salt stress, elevated temperature and drought, since, the symbiosis necessitates successful fortitude of bacteria even under stressed environmental conditions. In the current effort, bacterial culture of *Rhizobia* has been encapsulated *in silica* matrices at assorted concentrations by low temperature sol-gel method. Metal-alkoxide, Tetraethylorthosilicate [$Si(OC_2H_5)_4$] is used as the precursor and RhodamineB (RhB) is used for tagging the bacteria because of its high quantum efficiency, strong fluorescence yield and short relaxation time ~ 4 ns (Niki et al., 2005; Sharma et al., 2008a, 2008b). Various optical and photophysical (XRD, TGA, FTIR and nonlinear) assets of these samples are studied by using second harmonic of Nd:YAG laser at 532 nm. UV-visible absorption spectra of samples have been recorded and it is concluded that the absorption drops off with increase in the concentration of bacteria. Transmission of the laser light after fleeting through the samples is documented.

Materials and methods

Solitude of *Rhizobia*

Investigational site

Haryana state lies between 27° 37′ and 30°35′ North Latitudes and 74° 28′ and 77° 36′ East Longitudes. The average maximum temperature ranges barely from 40 to 48°C. The normal annual rainfall is 450 mm, showing a semi-arid climate. Hisar region occupies 29°5′5” North Latitude and 75°45′55″ East Longitude.

Anthology of root nodules and segregation of Rhizobia

Root nodules of the commonly growing wild legume *Acacia nilotica* along with seedlings were collected from fields of Hisar, subsequently crammed in plastic bags and carried to the laboratory, where bacterial strains were secluded. In the progression, nodules were alienated from the roots and washed once in running tap water and then thrice in sterilized distilled water for surface sterilization, afterwards, bacterial seclusion is carried out using serial dilution agar plate technique as reported by Somasegaran and Hoben, (1985) using YEMA (Yeast Extract Mannitol Agar) medium containing 0.0025% Congo red dye. Plates were incubated at 28±1°C and observed every day for the growth. Bacterial colonies appeared after 2–3 days were chosen and streaked on YEMA plates. Pure culture was attained with one or more re-streaks of single picked colonies. *Rhizobial* isolates obtained were auxiliary subjected to their morphological and cultural characterization. Besides this, for confirmation test, pure cultures were grown in YEM broth having bromothymol blue (BTB) (Vincent, 1970) and then inveterate by Gram’s staining technique (Rao, 1999). The purified cultures were justified by observing their contagion when inoculated to their host-plants grown in vermiculite and sterile sandy soil under greenhouse conditions containing N-free Hoagland nutrient solution as the sole nutritional source (Arnon and Hoagland, 1940).

Sample preparation

The silica matrices were assembled by the archetypal procedure of hydrolysis and polycondensation reactions of Tetraethyl orthosilicate (TEOS) (Sharma et al., 2008a, 2008b). All the solvents were of research grade and used without further purification. It is a two step protocol of silica xero-gels; in the first step, silica sample was prepared and in the second step, the aging of the sample is done in a controlled manner to get homogeneous dye doped silica matrices. At first, ethanolic dye (5 mL) was added to the metal alkoxide precursor TEOS (5 mL) and is stirred using magnetic stirrer for 15 min for gel formation. The metrology *in silica* gels is customized as similar to the step for forming gel network. The present study is limited to the alkoxide sol-gel method, in which the inorganic polymerization occurs in two steps: hydrolysis and condensation reactions. During the sol-gel transformation, the viscosity of the solution gradually increases as the sol becomes interconnected to form a rigid, porous network of gel. To keep Rz active no catalyst is used and then the gel is kept for aging for a week at room temperature (30°C). The resulting material produced was known as a xerogel. Post-doping of *Rhizobia* (tagged with Rhodamine B) was done in blank silica samples. The concentration of RhB was taken constant at 0.5 mM and the amount of Rz was varied from 0 to 0.4 mL at a step of 0.1mL. The silica samples typically have 1 mm thickness and visually appeared to have a good surface finish.

Experimental setup

The experimental set up used for recording the temporal response and concentration dependent studies of the samples under test is shown in Fig. 1. The experiment was performed using nanosecond laser pulses from a Q-switched, frequency doubled Nd:YAG laser (Minilite Continum, Inc.). The laser

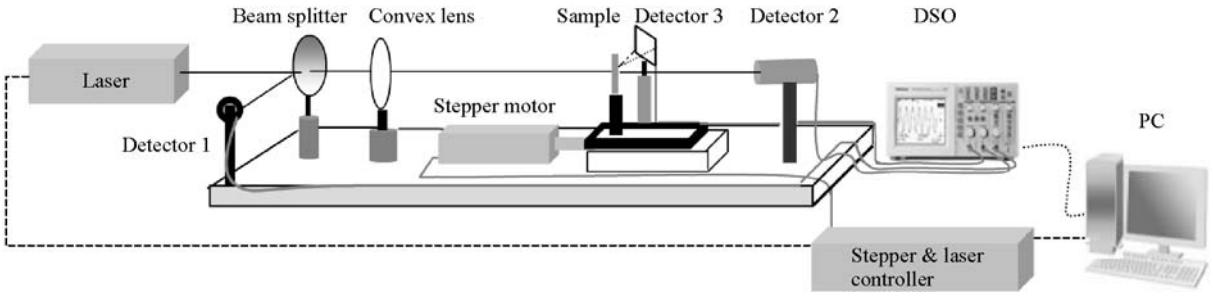

Figure 1 Schematic diagram of z-scan set up.

beam was focused using a lens and the sample was placed at the focal point. The optical transmittance through the test sample was recorded at different time ranging from 0 to 60 min and at different concentration of Rz.

As bacteriorhodopsin film has shown the broadband optical limiting property at 532 nm of Nd:YAG laser (Huang et al., 2004). Hence, for curiosity, the optical limiting property of Rz sample was recorded by translating the sample across the focal region along the axial direction that was similar to the direction of the propagation of laser beam. In z-scan set up, the far-field aperture transmittance is measured for a constant laser input as the sample is scanned along the z-direction through the focus of the lens. As the sample is scanned along the beam length, its effective focal length will change, since the incident intensity is changing. This change will be reflected in the intensity distribution at the aperture in the far field. The amount of energy transmitted by the aperture will depend on the sample location on the z-axis. The transmission of the beam through an aperture placed in the far field is measured using an energy probe placed after the sample. Pump intensity is changed by using neutral density filters. Laser pulses are fired at low repetition rate (1 Hz) to avoid accumulative thermal effects in the samples and the data acquisition is automated.

Results and discussion

Seclusion of *Rhizobia*

To begin with, a good number of isolates were obtained in 3 days from the root nodules of *Acacia nilotica* seedlings, which showed their fast growth. Quite similar acid producing aptitude, of Shah et al. (1995) findings were recorded, as it has turned the yeast mannitol agar containing bromothymol blue to yellow color. Isolates were confirmed as *Rhizobia* (Rz-25) after the confirmatory test in pot experiments using sterile sandy soil and vermiculite under controlled environmental conditions in three replicas (Data not shown). The gummy-circular colonies with convex-smooth edges, glistening-translucent or pinkish-white color due to the non-absorbance of congo red were appeared in all the gram-negative isolates of *Rhizobia* (Anand and Dogra, 1991).

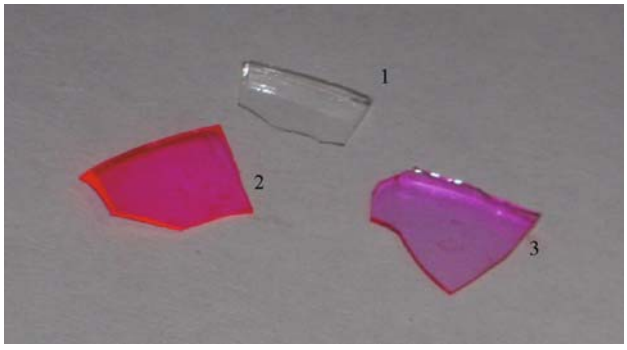

Figure 2 Visual characteristic of the grown samples (Blank (1), RhB co-doped Rz (2 = pre-doping, 3 = post-doping)).

Morphological and optical characteristics

The as prepared blank, RhB doped and RhB codoped Rz samples are shown in Fig. 2. The samples seem to have good surface finish. Phase identification of the samples was performed by a Rigaku (Miniflex-II) X-ray diffractometer in the 2 θ range of 0–80° with scanning speed of 0.01° min^{-1}. For this study, the sample was powdered by crushing in the crucible. It is an ideal experimental approach to investigate the formation of amorphous material and the nature of the X-ray diffraction response. It depicts the amorphous nature of the material which is expected in glass as only one peak (d-value is 3.84) corresponding to 23° is observed in the recorded pattern.

To depict the nature of sample the TGA thermogram (Fig. 3) of the blank sample was recorded using SDT Q600 from TA instruments. The powdered sample was put as it in the metal pan of the instrument. The spectra show glass to glass transition at 70°C. Glass transitions occur as the temperature of an amorphous solid is increased. This is due to the sample undergoing a change in heat capacity; no formal phase change occurs. A closer look at the Figure shows that ~17% weight loss occurs in the sample in the temperature range 0-550°C. In this temperature range the host material had not undergone any phase transition and hence is suitable for temperature, pressure and bio-sensing* up to 550°C (*Some bionucleo-proteins viz., an isolate of *Thermus aquaticus*; *Taq* polymerase, a well known polymerase chain reaction enzyme

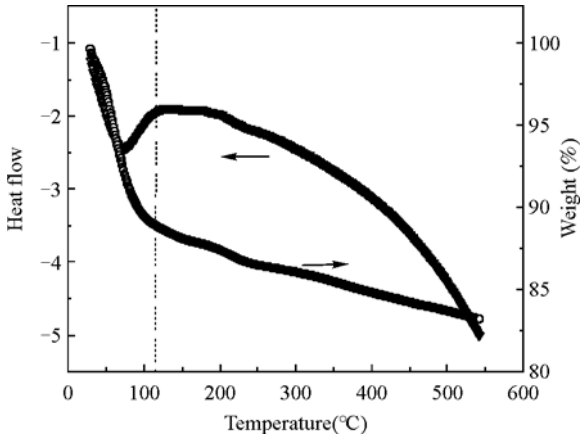

Figure 3 TGA thermogram of blank sample.

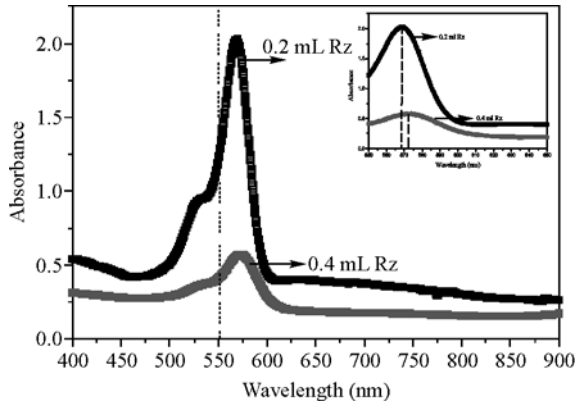

Figure 4 Concentration dependent UV-Vis absorption spectra of Rz doped silica matrices.

used in amplification of nucleic acids can withstand temperature up to 100°C). UV-VIS absorption spectra of the doped silica samples at various concentrations have been recorded using a Perkin Elmer lambda 35 spectrophotometer. Typical absorption spectra of 0.2 ml and 0.4 ml bacteria are shown in Fig. 4. Though the absorption curve does not mention the nonlinear effects, as the light source in the spectrophotometer is insufficient to cause these effects, yet the absorption measurement is used to determine the suitable wavelength of the excitation source. It is found that the absorption decreases with increase in the concentration of bacterial culture of *Rhizobia*. This result of concentration quenching is in contrary to Beer's law and the reason for such observation is not clearly understood. We propose for the first time that, at low concentration due to more exposed surface area of Rz for the absorption is more because the effective path length is higher that causes more absorption. In addition, the role of unsaturated double bonds, absorption by delocalized electron cloud, scattering event, local fields, quantum effects, non-Arrhenius diffusion mechanism and other nonlinear effects could affect the absorption that needs further attention. However, at higher concentration of Rz they may form aggregates giving rise to effectively shorter path length and the increase of scattering events is perhaps the reason for subsequent decrement in absorption. With the increase of concentration, the ligaments become bigger and saturated that has less number of unsaturated conjugated bonds and localized electron clouds. Consequently, the nature of their energetically stable configuration and has less surface effects causes lower absorption. Moreover, the electronic and ionic polarization responsible for the absorption in this case is relatively lower. Furthermore, a red shift of ~8nm that we observed with the increase in concentration of *Rhizobia* confirms the entrapment of *Rhizobia* in silica matrices due to aggregate formation.

Fourier Transform Infrared (FTIR) spectra of dye doped samples (powdered sample with KBr) (with (+ Rz) and without (-Rz)) are recorded and shown in Fig. 5. The observed band in the range 3000–3700 cm^{-1} is assigned to

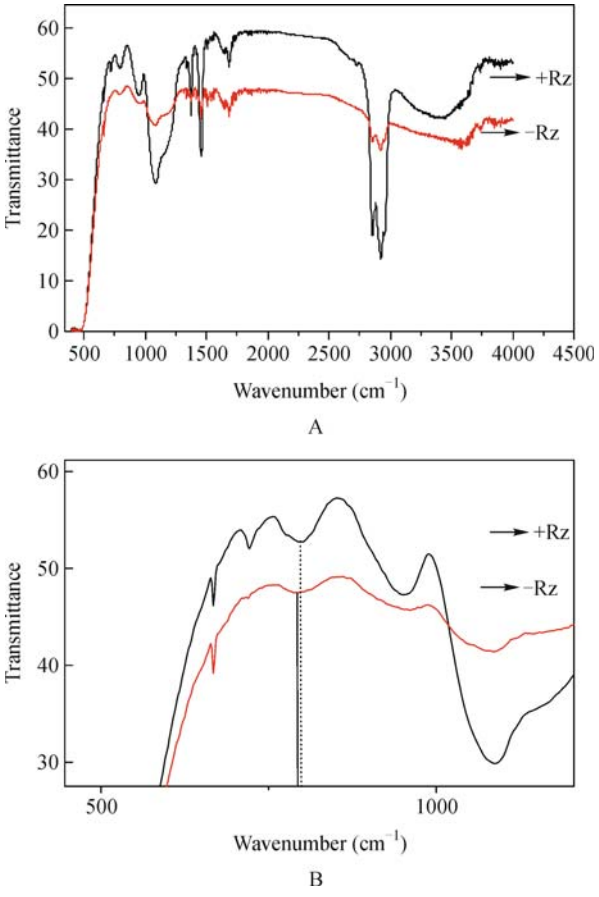

Figure 5 FTIR spectra of dye doped samples (with and without Rz) (A) and the inset of a portion of it (B).

OH stretching vibrations of the Si-OH group and strong Si-O bands are associated with the peak ~ 928 cm^{-1}. Some portion of the wide band is due to aromatic stretch. The peak located at 798 cm^{-1} is associated with symmetric Si-O-Si stretching. It confirms the formation of silica matrices. All the peaks are blue shifted in the sample with Rz as depicted by absorption spectra too. It confirms that Rz has binded with RhB molecules. The glassy matrices at 532 nm show in Fig. 6

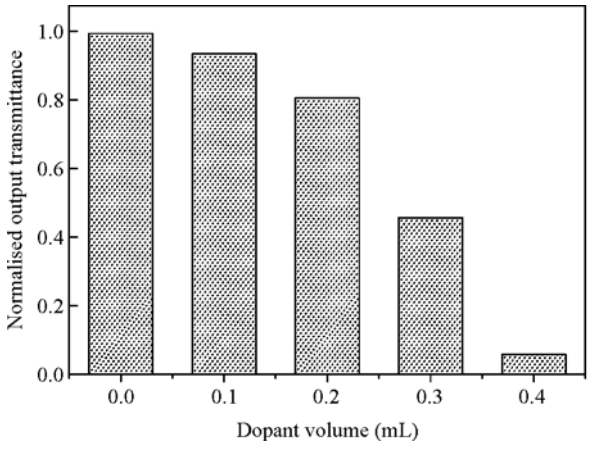

Figure 6 Variation of output intensity with dopant volume.

that there is a decrease in output intensity with the increase in concentration of the *Rhizobia*. This curve is crucial to ensure accuracy during testing with fiber optic sensor. Further, the dopant concentration should be within the dynamic range of the assay else, the photodetector should be reasonable sensitive. The optical limiting study of the sample is made (data not shown) and it is observed that it does not show optical limiting behavior. Figure 7 represents the temporal response of sensor when tested at 0.2 mL of Rz at 532 nm of second harmonics of Nd:YAG laser. Output intensity decreased significantly with time up to about 20 min. For the first 10–12 min, there is a significant reduction in output intensity. From 10 to 20 min the gradient of output intensity reduction decreases. After 20 min the output intensity reading began to stabilize. Using this sensor method, the total bacterial concentration could be measured in ~20 min. This is significantly faster than current techniques, such as conventional calorimetric method, which takes about 1 h. The transmittance is less in post-doped samples than in pre-doped samples.

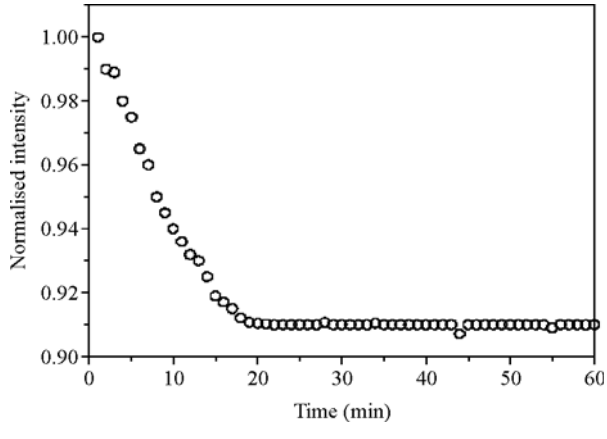

Figure 7 Temporal response of the sample at 0.2 mL of Rz.

Conclusions

A new method for rapid, reproducible and a sensitive detection of *Rhizobia* has been reported for the first time. Various concentrations of Rz are entrapped *in silica* matrices by sol-gel method in the form of layers. Its morphological and optical studies support the successful incorporation of Rz within the matrices. This method enables single-step detection and quantification of microgram levels of *Rhizobia* directly without destroying the sample. The response time for the new biosensor for determining the concentration of *Rhizobia* in a sample is ~20 min.

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Compliance with ethics guidelines

This article does not contain any studies with human or animal subjects performed by any of the authors.

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