Encapsulation of enzymes and cells in sol-gel matrices for biosensor applications

Anup K Singh*[a], Alok Gupta[a], Ashok Mulchandani[b], Wilfred Chen[b], Rimple B. Bhatia[c], Joseph S. Schoeniger[a], Carol S. Ashley[d], C. Jeffrey Brinker[c,d]

[a]Sandia National Laboratory, Livermore, CA 94551-0969
[b]Dept. of Chemical and Environmental Engineering, Univ. of California, Riverside, CA
[c]Univ. of New Mexico/Center for Microengineered Materials, Albuquerque, NM
[d]Sandia National Laboratory, Albuquerque, NM 87185

ABSTRACT

Porous silicate materials made by low temperature sol-gel process are promising host matrices for encapsulation of biomolecules. Their mechanical strength, chemical inertness, hydrophilic nature, and above all, their optical transparency makes them an exciting platform for development of biosensors. To date, researchers have focussed on sol-gel routes using alkoxides for encapsulation of biomolecules. However, formation of alcohol as a byproduct is an undesired complication as it can have detrimental effect on the activity of entrapped biomolecules. We have developed a novel sol-gel process to encapsulate biological molecules (such as enzymes, antibodies and cells) that uses neutral pH, room temperature, and does not generate alcohol as a byproduct. The process uses sodium silicate as precursor and is carried out in two steps- preparation of a low pH silicate sol followed by gelation at neutral pH in a buffer containing biomolecules. We developed a novel homogeneous immunoassay for 2,4,6-trinitrotoluene (TNT), and have encapsulated the immunoassay reagents in sol-gel matrices to produce dispersible biosensors for the detection of TNT. Using the sol-gel doped with immunoassay reagents, we can detect TNT at low ppm levels. We also report encapsulation of *E. Coli* cells expressing the enzyme organophosphorous hydrolase (OPH) on the cell surface in sol-gel matrices. The cell-doped sol-gel material can be used to develop biosensors for detection of organophosphates.

Keywords: biosensor, immunoassay, TNT, organophosphate, sol-gel, organophosphorous hydrolase

1. INTRODUCTION

Biosensors using immobilized receptors are finding ever-increasing application in a wide variety of fields such as clinical diagnostics, environmental monitoring, food and drinking water safety, and illicit drug monitoring. One of the most challenging aspects in development of these sensors is immobilization and integration of biological molecules in the sensor platform. Numerous techniques-- physical adsorption, covalent attachment, entrapment in polymer and inorganic matrices, have been explored over the years to achieve a high yield, reproducible, robust immobilization technique that preserves the biological activity of recognition molecule without adversely affecting the performance of the transduction component. No single method has emerged as the universal method of choice for each and every application and ongoing efforts strive to optimize these methods to render them adequate for specific applications. Silica host matrices, made by sol-gel process, have emerged as a promising platform for encapsulation of biomolecules such as enzymes[1-3], antibodies[4], and cells[5]. These sol-gel matrices are chemically inert, hydrophilic, biocompatible, and inexpensive to produce. They also exhibit superior mechanical strength, enhanced thermal stability, and negligible swelling in solvents compared to organic polymers. The sol-gel matrix can also be tailored to act as a reservoir for water thereby significantly enhancing the ability to maintain the biological activity of entrapped enzymes, antibodies or cells.

* *Correspondence: Email: aksingh@sandia.gov; Telephone: 925-294-1260

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Sol-gel matrices offer advantages over organic polymers from another viewpoint as well- ease of integration into sensor and transduction platform. Their optical transparency makes it ideal for transduction platforms that rely on transmission of light for detection, such as absorbance or fluorescence measurements[6]. Sol-gel films can be made relatively fast and cast as thin layers on sensor surfaces. For silicon oxide/nitride based sensors, such as field-effect transistors or optical fibers, formation of sol-gel films results in minimum alteration in optical, chemical and physical properties of base material. The hydrophilic nature of silica also allows uninterrupted transport of water and other molecules such as substrates and products of an enzymatic reaction.

In most of the reported applications, sol-gel matrices containing encapsulated proteins or cells have been prepared by hydrolysis and condensation of an orthosilicate such as tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) [1]. First, TMOS is partially hydrolyzed in an acidic medium by addition of controlled amount of water. Next, the biological species is introduced in a suitable buffer to facilitate gelation. The buffer pH is chosen so as to allow the final solution to be close to neutrality in order to avoid denaturation of proteins. However, use of TMOS (or TEOS) as starting material leads to generation of methanol (or ethanol), presence of which in large quantities can be deleterious to proteins and cells. In low temperature aging typically used with encapsulation of biological species, the generation of alcohol proceeds for an extended period of time allowing the encapsulated species to denature over time.

We have developed a new aqueous route for synthesis of sol-gel structures with encapsulated biological entities that uses sodium silicate as a precursor[7]. This approach completely avoids generation of alcohol and at the same time, allows encapsulation to be carried out at neutral pH to preserve biological activity. We used this novel sol-gel process to encapsulate enzymes, antibodies and cells, all of which retained their biological activity after encapsulation. We also report development of dispersible biosensors for 2,4,6-trinitrotoluene (TNT) using enzyme- and antibody-doped sol-gel matrices. The sensing is based on a homogeneous immunoassay where the analyte (TNT) in a sample competes with TNT-enzyme conjugate to bind to a limited number of antibodies present in the solution. The TNT-enzyme conjugate is fully active in the unbound state but when it binds to a TNT antibody, the activity is inhibited. But, the inhibition can be reversed by addition of free TNT. The complex of antibody bound to TNT-enzyme conjugate is encapsulated in a sol-gel matrix together with the substrates for the enzyme. This is the "off" state of biosensor where there is no enzyme activity remaining as all the TNT-enzyme conjugates are bound by antibodies. In the presence of TNT, TNT-enzyme conjugates dissociate from the antibody and become active, catalyzing the conversion of colorless or nonfluorescent substrate into a colored or fluorescent product.

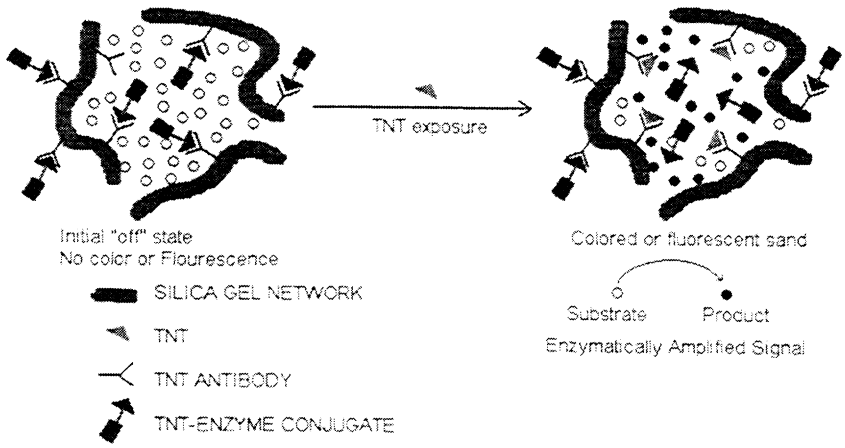

Figure 1: A schematic of dispersible sol-gel biosensor for detection of TNT

We have also successfully encapsulated *E.Coli* cells expressing the enzyme organophosphorous hydrolase (OPH) on the cell surface by sol-gel process using sodium silicate. The cells remain intact and functional

after encapsulation and can be used as biosensors for detection of organophosphates. Organophosphates are toxic compounds that have found extensive use as pesticides, insecticides and potential chemical warfare reagents. Recently, because of environmental, health and national security concerns, significant efforts have been directed towards developing sensitive and portable sensors for these compounds. OPH, a novel recombinant enzyme, has been shown to effectively hydrolyze a variety of organophosphates and has been used in developing direct sensing schemes for them[8, 9].

2. EXPERIMENTAL

2.1 Preparation of Silicate sols

Silicate sols were prepared from sodium silicate [(3.25 SiO_2:Na_2O), H_2O] solutions. The ratio of 3.25 was selected to minimize the volume of acid required for neutralization of alkali. 11.5 g of sodium silicate solution was combined with 34 ml of DI water. To this aqueous solution, 15.4 g of strongly acidic cation exchange resin was added while stirring to bring the pH of the solution to a value of 4. The resin was then removed by vacuum filtration. 2.0 M HCl was added to the filtrate to adjust the pH to 2.0. The low pH value was necessary to enhance aging. The resin was regenerated as needed with 500 ml of 4% acid per 100 g of used resin. The low pH sol solution can be stored at room temperature for up to 48 hours before it gels. A phosphate buffer (1 M, pH 7) containing proteins or cells at the desired concentration was added to the sol solution in 1:5 (vol.) ratio. After mixing, the solution was quickly transferred to cuvettes or microtiter plates. Gelation occurred in approximately 5 minutes. Gels were aged at room temperature for 24 hours prior to use.

To obtain the pore size distribution of the silica gels prepared by the aqueous route, aerogels were obtained by supercritically drying the wet gels[7]. 8 ml of pH 7 buffer was added to 40 ml of the low pH sol and the solution was quickly transferred to cylindrical vials. The gels were allowed to age at 4°C for 3 days. The cylindrical wet gels were washed with pH 7 buffer for two hours to desorb any enzyme molecules not bound in the pores of the gel. The solvent in the pores of the gels was then replaced with amyl acetate. The gels were dried in a Polaron critical point dryer. The nitrogen sorption technique was employed to characterize the aerogels using the Micromeritics ASAP (Accelerated Surface Area and Porosimetry) 2100 Instrument. Prior to analysis, the aerogels were degassed overnight at 1 mm Hg pressure. The BJH models were used to obtain the pore size distribution and pore volume of the gels.

2.2 Development of a sol-gel encapsulated immunoassay system for TNT

The enzyme chosen to develop homogeneous immunoassay for TNT was Glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides*. G6PDH catalyzes conversion of glucose-6-phosphate to 6-gluconolactone while reducing NAD^+ to NADH. The reaction kinetics was followed by measuring absorbance or fluorescence of NADH.

The G6PDH-TNT conjugate was prepared as follows. Trinitrobenzenesulfonic acid (TNBS) was linked to the following spacers - Glycine, 3-aminopropionic acid, γ-amino butyric acid and 6-aminohexanoic acid by reacting 0.76 mmoles of TNBS with 1.52 mmoles of the various linkers. Reaction was carried out at pH 9.5 for 24 hours at room temperature. The product was precipitated by lowering the pH using 2 N HCl. The precipitate was washed extensively with pH 2.0 water and then recrystallized twice in 1:1 Ethanol:water. Dried product (TNP-linker conjugate) was then conjugated to G6PDH by reacting 0.5 mg of G6PDH with TNP-linker conjugate in the presence of EDC and sulfo-NHS for two hours at room temp in dark. After reaction, each conjugate was purified by using a size-exclusion column with 10 mM phospahte buffer saline (pH 7.4) as the eluent. Protein concentration and degree of conjugation was estimated by measuring absorbance at 280 nm and 420 nm respectively.

G6PDH-TNT conjugate and TNT antibody were allowed to bind in solution for 15 minutes and then encapsulated in sol-gel matrix as described above together with the substrate glucose-6-phosphate. The gels were cast in cuvettes or wells of a microtiter plate. After aging for a day, sol-gel was exposed to

different concentrations of TNT for 2 hours. NAD+ was added and the plate (or the cuvette) was read in an absorbance platereader (or a spectrophotometer).

2.3 Encapsulation of cells in sol-gel matrices

E. coli strains XL1-Blue was used as the host cell for expression of OPH enzyme[8]. Plasmid pOPK132 was used for expressing Lpp-OmpA-OPH on the cell surface. Cells bearing the plasmid were grown in 50 ml of LB media buffered to pH 7.0 with 0.017 M KH_2PO_4 and 0.072 M $K_2H_2PO_4$, supplemented with 100 mg/ml ampicillin at 370C. Once the OD600 of cell suspension reached 0.5, expression of OPH on the cell surface was induced with 1mM IPTG. After 48 hours of growth, cells were harvested by centrifugation and were resuspended in 1M phosphate buffer. Cells were then encapsulated in sol-gel matrix as described earlier. Care was taken to ensure that osmolarity of the final buffer was close to physiological level, approximately 150 mM. Gels containing cells were aged for 24 hours at room temperature in presence of excess buffer.

To ascertain the integrity of cell structure, OPH on the cell surface was labeled with fluorescein and cells were imaged using a fluorescence microscope and a cooled CCD, before and after encapsulation. Concentration of cells entrapped in sol-gel was estimated by performing an absorbance scan. Activity of OPH expressed on the cell surface was measured by using paraoxon, an organophosphate surrogate, as a substrate.

3. RESULTS AND DISCUSSION

Figure 2 shows the pore size distribution in aerogels prepared from the wet gels as discussed in the experimental section. The volume-averaged mean pore size is approximately 50 nm, with a very wide distribution to the left of the mean value. The bigger pores allow minimum resistance to diffusion of small molecules such as analytes, substrate and product. Although, the pores are big enough to allow passage of protein molecules, we did not observe significant leaching of the entrapped macromolecules. In fact, the rate of leakage of even small encapsulated molecules such as substrates was quite small. The enzymes and antibodies encapsulated in sol-gel matrices were tested for their activity by performing kinetic and binding assays, respectively and were found to retain as much as 50% of their activity.

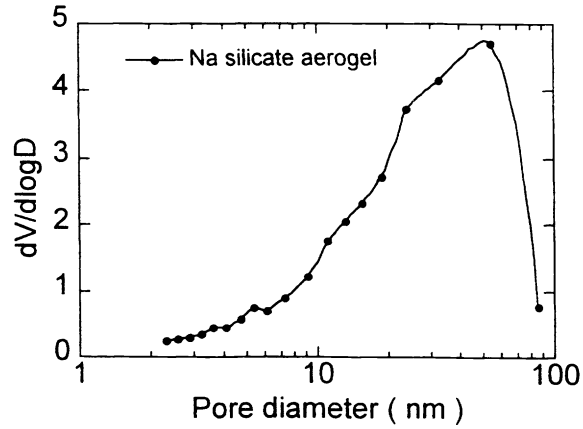

Figure 2: Pore size distribution of aerogels synthesized by sol-gel process using sodium silicate as the precursor.

Figure 3 shows the results of an immunoassay performed using sol-gel encapsulated reagents for detection of TNT. All the components of the assay namely, the TNT-G6PDH conjugate, the antibody, and glucose-6-phosphate were encapsulated in the sol-gel matrix. Only sample containing TNT and the cofactor NAD+ was added later and the change in absorbance (or fluorescence) of NADH was monitored. The preliminary data indicates that we can detect approximately 10 micromolar (2 ppm) of TNT in solution. The same immunoassay when performed in solution leads to a detection limit of 0.3 ppb TNT, over an order of

magnitude better than the sol-gel encapsulated system. We suspect the steric hindrance to the dissociation of antibody-enzyme complex because of the presence of the sol-gel matrix leads to the decreased sensitivity of the assay in sol-gel. Efforts are underway to improve the sensitivity and also to cast the sol-gel composites in spherical particles that can be used for field-testing.

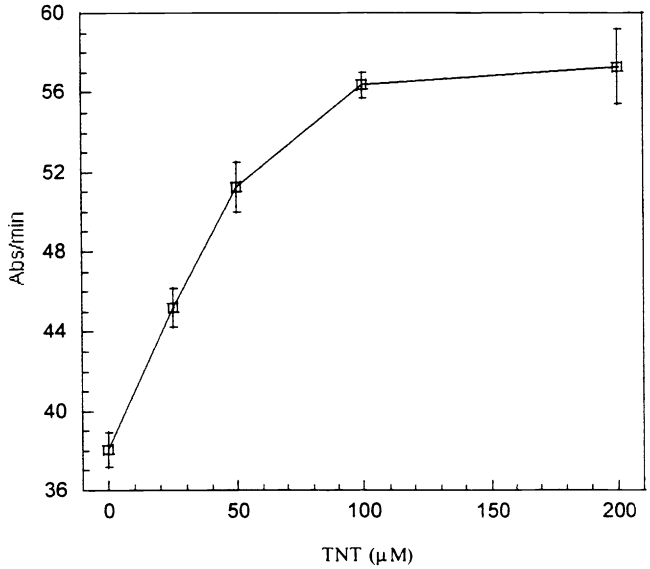

Figure 3: Detection of TNT using immunoassay reagents encapsulated in sol-gel matrix

Figure 4 shows fluorescence micrographs of fluorescein-labeled *E. Coli* cells in solution and in sol-gel matrix, respectively. We observe that there is no significant change in the size or shape of the entrapped cells implying that cells are physically intact after immobilization. Sol-gel process has proven to be a remarkably gentle method for entrapping macromolecules. There are hardly any mechanical or surface stress exerted on the material being encapsulated and this feature has been used advantageously to template molecules[10]. Figure 4 also indicates that the enzyme OPH is distributed uniformly over the cell surface as the fluorescence is uniformly intense over the imaged cell.

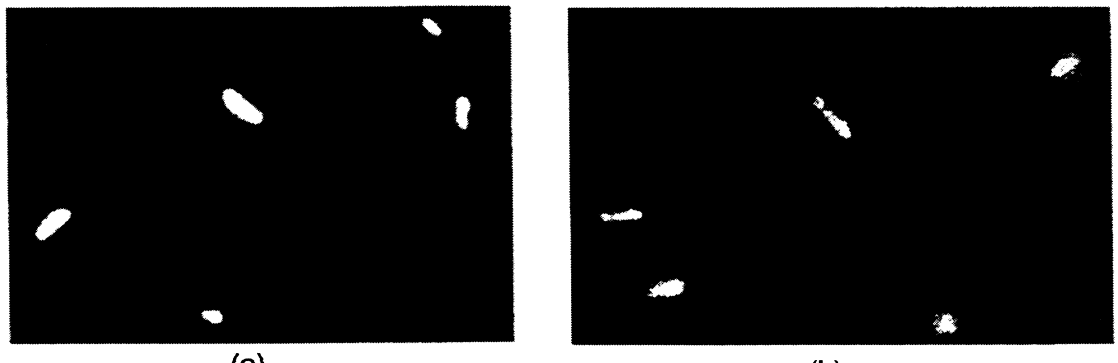

Figure 4: Fluorescence Microscope images of (a) *E. Coli* cells in solution (b) *E. Coli* cells encapsulated in sol-gel matrix. OPH expressed on the cell surface were labeled with fluorescein isothiocyanate. Images were taken with a cooled CCD camera (Photometrics) mounted on an Olympus IX-70 inverted fluorescence microscope using an oil-immersed 100x objective.

It is also worth noting that presence of sol-gel matrix does not give any background fluorescence and transparent nature of the matrix makes it quite easy to image the cells. These features are especially critical for developing absorbance or fluorescence-based biosensors using the immobilized cells.

The OPH molecules on the surface of immobilized cells retained their activity as depicted in figure 5. Paraoxon, an insecticide, was used to determine the activity of immobilized enzyme. Hydrolysis of paraoxon results in production of p-nitrophenol that can be detected by measuring absorbance at 405 nm. Efforts are underway to develop fiber-optic biosensors for detection of paraoxon and other organophosphates using immobilized cells.

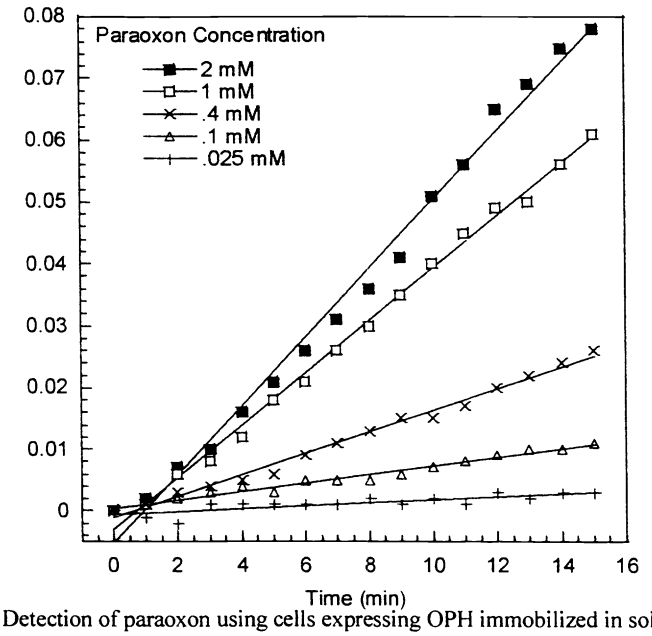

Figure 5: Detection of paraoxon using cells expressing OPH immobilized in sol-gel matrix

4. CONCLUSIONS

A new approach to synthesis of bio-inorganic composites has been demonstrated. Employing sodium silicate as the starting material, generation of alcohol as a byproduct is completely avoided. We successfully used this method to encapsulate enzymes, antibodies and cells in silica gels, and encapsulated biomolecules retained their biological activity. Sol-gel matrices offer a number of advantages over conventional organic polymers as platforms for biosensors owing to their mechanical strength, chemical inertness, hydrophilic nature, and optical transparency. We are evaluating the long-term stability of biomolecules entrapped in sol-gel matrices, a key issue in the development of biosensors.

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