Sol–Gel Encapsulated Anti-Trinitrotoluene Antibodies in Immunoassays for TNT

E. H. Lan,† B. Dunn,*† and J. I. Zink*‡

Department of Materials Science and Engineering, University of California at Los Angeles, Los Angeles, California 90095-1595, and Department of Chemistry and Biochemistry, University of California at Los Angeles, Los Angeles, California 90095-1595

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Antibodies to trinitrotoluene (TNT) were encapsulated in optically transparent sol–gel silica glasses and retained their ability to bind TNT. Both competitive and displacement immunoassays were successfully performed using sol–gel immobilized antibodies. TNT concentrations on the order of ppm were detected. In competitive immunoassays using the sol–gel immobilized antibodies, a logarithmic decrease in fluorescence signal as a function of TNT concentration was observed, similar to competitive immunoassays performed in solution. When encapsulated in the sol–gel silica matrix, the antibodies retained their ability to differentiate between TNT and trinitrobenzene (TNB), an analogue. In displacement immunoassays, the rate of displacement was dependent upon pore morphology, with aged gels exhibiting faster rates than that of xerogels. The relative stability of antibodies was better for sol–gel encapsulated antibodies than for antibodies immobilized using surface attachment. After exposure to HCl, methanol, or 60 °C, the sol–gel immobilized antibodies experienced essentially no loss in ability to bind TNT whereas the surface immobilized antibodies showed as much as 30% loss in ability to bind TNT.

Introduction

The need for reliable, specific, and sensitive detectors for trinitrotoluene (TNT), a commonly used explosive, is escalating due to the need to detect landmines, soil, and groundwater contamination. In addition to sensitivity and specificity, ruggedness is also an important requirement in order for the detector to be used in the field. We present in this paper an exploratory study on the feasibility of using sol–gel immobilized antibodies as the detection element for TNT. A variety of biomolecules, including antibodies, can be immobilized successfully in optically transparent, porous, silica matrices by physical entrapment. One of the benefits of immobilizing biomolecules using the sol–gel encapsulation approach is that the silica network protects the biomolecule. Therefore, these solid-state probes can be highly specific due to the biomolecule and yet rugged because of the surrounding silica matrix. Sol–gel encapsulated antibodies for TNT, as presented here, represent a viable detector for TNT.

Sol–gel encapsulation is an attractive method of immobilizing biomolecules, as proteins and enzymes trapped in the pores of a glass retain their spectroscopic properties and biological activity. Moreover, some biomolecules such as cytochrome c, myoglobin, and oxidase enzymes exhibit increased stability when encapsulated in the porous matrix. Antibodies have also been successfully immobilized using the sol–gel approach. Antigens

1 Department of Materials Science and Engineering.
2 Department of Chemistry and Biochemistry.
* To whom correspondence should be addressed.
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such as pyrene, atrazine, and 2,4-dinitrophenylhydrazine successfully bind their respective antibodies encapsulated in sol–gel derived glasses. Porous sol–gel glasses with immobilized antibodies, therefore, represent solid-state probes for analytes of interest.

The use of antibody–antigen reactions to detect TNT has been demonstrated. An optical fiber approach in which immobilization was achieved by covalent attachment of antibodies to optical fibers was reported to reach ppb detection limits. The portability of such a method is a considerable advantage over instrumental detection methods such as HPLC, GC, and MS whose field usage is limited. In a similar approach, anti-TNT antibodies were covalently attached to the inner walls of a capillary. In this study, a TNT detection limit on the order of ppt was reported. In addition to immunochemistry methods, another technique to detect TNT based on measuring fluorescence quenching of a conjugated polymer film has also been published.

We present in this paper an optical detector for TNT with sol–gel encapsulated anti-TNT as the detection element. The sol–gel approach is more robust and rugged since the antibodies are immobilized in the interior of a porous silica material as opposed to being attached to its exterior surface. Using the sol–gel probes in both displacement and competitive assays, the fluorescence signal as a function of TNT level exhibits the expected behavior observed in standard immunoassays, indicating that these sol–gel probes can successfully discriminate between different TNT concentrations. The results from these proof-of-concept experiments demonstrate that sol–gel immobilized anti-TNT can be used as solid-state optical probes for TNT.

Experimental Section

Reagents. Tetramethyl orthosilicate (TMOS) was obtained from Aldrich Chemical Co. and used as received. Anti-TNT antibody (10.9 mg/mL) and TNT standard solution (102 ppm in water) were obtained from TSD Bioservices (Newark, DE). TNT–fluorescein isothiocyanate (TNT-FITC), 10 mg/mL or 10,000 ppm based on TNT only, in N,N-dimethylformamide (DMF) was custom synthesized by TSD Bioservices. Trinitrobenzene (TNB) standard solution (100 ppm in methanol) was obtained from AccuStandard Inc. (New Haven, CT).

Sample Preparation. Two sets of sol–gel encapsulated antibody (Ab) samples were prepared: one containing only anti-TNT Ab and the other containing anti-TNT Ab that was prebound to TNT-FITC (Ab-TNT-FITC). The Ab-only samples were used in the competitive assay experiments, whereas the Ab-TNT-FITC samples were used in the displacement assay experiments. The silica gel was prepared by mixing 7.60 g of Ab-TNT-FITC samples were used in the displacement assay where percent displacement was determined as a function of time, the aged gel samples (4.0 M Ab prebound to TNT-FITC) were

When preparing silica gels with only Ab, an aliquot of Ab (10.9 mg/mL) was diluted in 0.02 M Tris-HCl, 0.02 M NaCl, pH 8.0 buffer, and 1.5 mL of this buffered Ab solution was mixed with 1.0 mL of silica sol. The total volume (2.5 mL) was then placed into a 1.0 mm thick plastic cassette (Novex, San Diego, CA). The final Ab concentration in the aged gel ranged from 0.5 to 1.0 μM. Ab concentration in all gel samples was calculated on the basis of the volume of the anti-TNT Ab solution (10.9 mg/mL) used in the gel and an Ab molecular weight of 160,000 g/mol.

When preparing silica gels with TNT Ab prebound to TNT-FITC, an aliquot of TNT-FITC (10,000 ppm based on TNT) was diluted with methanol. A 0.10 mL aliquot of this diluted TNT-FITC solution was added to 0.90 mL of 0.02 M Tris-HCl, 0.02 M NaCl, pH 8.0 buffered Ab solution and incubated overnight at 4 °C and 3 h at room temperature. A 1.0 mL aliquot of this Ab-TNT-FITC solution was then added to another 0.50 mL of 0.02 M Tris-HCl/NaCl, pH 8.0 buffer and 1.0 mL of silica sol. The total volume (2.5 mL) was then placed into a 1.0 mm thick plastic cassette. The final concentration of Ab ranged from 1.35 to 2.70 μM, with TNT-FITC concentrations (based on TNT only) ranging from 4 to 20 ppm.

All silica gels with encapsulated Ab or Ab-TNT-FITC were aged for 1–2 days before starting competitive and displacement immunoassays. Prior to the experiments, the aged gel was removed from the cassette and sliced into individual samples of ∼15 mm height, ∼7 mm width, and 1.0 mm thickness. A total of 10–12 samples was obtained from one original aged gel. The one exception was the displacement immunoassay experiment carried out with a xerogel. The xerogel (final dimensions of ∼11 mm height, ∼6 mm width, and ∼2.5 mm thickness), prebound to TNT-FITC, was dried at 4 °C for over 80 days and was ∼25% of the original volume of the aged gel. The xerogel had an Ab concentration of ∼3.5 μM (after correcting for gel shrinkage).

Fluorescence was measured using a SPEX fluorolog (model F12A2) in front-face configuration at room temperature. Samples were excited at 485 nm, and the emission signal was monitored at 525 nm. In measuring the gel samples, fluorescence was taken after removing the 1 mm thick sample from its test solution and placing it in a plastic cuvette (4 mm thickness) filled with water.

Procedures. In the competitive immunoassays, the sol–gel experiment was conducted with samples containing only encapsulated Ab, while the solution experiment was conducted with Ab attached to polystyrene cuvettes using standard surface adsorption techniques for protein immobilization on microtiter plates. In the sol–gel experiment, the gel (Ab) was sliced into individual samples and placed in buffered solution (0.02 M Na phosphate, 0.02 M NaCl, pH 7.0) containing TNT-FITC of 0.5 ppm (based on TNT) and the specified concentration of unlabeled TNT. Samples were incubated in a 1.0 mL solution containing TNT and TNT-FITC for 3 h and then washed in 2 mL of water for 2 h. During incubation and washing, the samples were gently rotated on a rocking plate. In the solution experiment, a buffered solution containing 5.0 ppm TNT-FITC and the specified concentration of unlabeled TNT was placed in the cuvette and incubated for 3 h. The cuvettes were then thoroughly washed with water. Fluorescence for all samples was measured after washing.

In the displacement immunoassays, a gel containing Ab-TNT-FITC was used. Unless otherwise stated, the original gel was not washed to remove excess TNT-FITC. Individual samples were incubated in 1.5 mL of buffered solution (0.02 M Na phosphate, 0.02 M NaCl, pH 7.0) containing only unlabeled TNT for 3–4 days. Samples were gently rotated during incubation. Fluorescence was measured at the end of the incubation period. In the dynamic displacement experiments where percent displacement was determined as a function of time, the aged gel samples (4.0 M Ab prebound to TNT-FITC) were

In one set, Ab was immobilized on polystyrene cuvettes. In the solution experiment, [Ab] = 1.35 µM and [TNT-FITC] = 0.5 ppm. In the solution experiment, Ab was adsorbed on the surface of polystyrene with [TNT-FITC] = 5.0 ppm.

washed thoroughly with buffer to remove excess TNT-FITC. Individual aged gel samples were incubated in 1.5 mL of TNT (0, 0.5, or 5.0 ppm) or TNB (5.0 ppm) with gentle rotation and measured periodically for fluorescence. The xerogel sample was incubated with 5.0 ppm TNT for the displacement experiment. In all displacement experiments, gels were not washed after binding with unlabeled TNT.

In the stability experiments, two sets of samples were tested. In one set, Ab was immobilized on polystyrene cuvettes. In the other set, Ab was immobilized in an aged silica gel as described above. Samples of both the surface-immobilized and sol–gel encapsulated Ab were exposed to three different sets of conditions for 24 h: (1) 0.01 N HCl (pH ≈ 2.2), (2) pure MeOH, or (3) 60 °C in 0.02 M pH 7.0 phosphate buffer. Both sets of samples were then washed and incubated with TNT-FITC (10 ppm based on TNT only) in 0.02 M Na phosphate, 0.02 M NaCl, pH 7.0 buffer for 3 h and then washed thoroughly to remove unbound TNT-FITC. The test samples were compared to the control samples, which were washed prior to TNT-FITC incubation and then washed again, but did not experience any other treatment. All samples were then measured for fluorescence to determine the extent of Ab-TNT (TNT-FITC) binding.

### Results and Discussion

**Competitive Immunoassays.** Sol–gel immobilized Ab retains its ability to bind TNT, and immunoassays using Ab encapsulated in a silica matrix behave similarly to immunoassays using Ab in solution. In competitive immunoassays, labeled and unlabeled antigens compete for a fixed number of antibody sites, and the signal decreases with increasing unlabeled analyte concentration. Figure 1 shows results from competitive immunoassay experiments with Ab encapsulated in aged silicas and Ab in solution exposed to a mixture of TNT–FITC and unlabeled TNT. With both sol–gel immobilized Ab and Ab in solution, increasing concentrations of unlabeled TNT result in decreasing fluorescence signal, as expected. TNT levels <1 ppm were readily detected.

A standard means of analyzing competitive immunoassays is by plotting B/B₀ vs unlabeled analyte concentration on a logarithmic scale; the plot is linear for competitive immunoassays. Such a method of expression the data from competitive binding assays is used routinely to derive a calibration curve for determining the concentration of unknowns. B/B₀ is defined by eq 1:

\[
\frac{B}{B_0} = \frac{B^* - NSB}{B_0^* - NSB}
\]

where B₀ is the fluorescence signal of sample at 0 unlabeled TNT concentration, B* is the fluorescence signal of sample, and NSB is the nonspecific binding (fluorescence signal of a blank sample).

Figure 2 shows a plot of B/B₀ vs unlabeled TNT concentration on a logarithmic scale for Ab in sol–gel silica and Ab in solution (Ab was adsorbed on the surface of polystyrene). The linear correlation confirms that competitive immunoassays can be successfully performed with Ab immobilized in the pores of a silica matrix. As seen in Figure 2, unlabeled TNT concentrations that span almost 2 orders of magnitude (concentrations ranging from 0.2 to 10 ppm) were successfully detected.

The apparent association constant (Kₐ) was determined to be 1.2 × 10⁴ M⁻¹ for the sol–gel encapsulated Ab and 1.7 × 10⁵ M⁻¹ for the Ab in solution using the data from Figures 1 and 2 and the method of Singh et al. This method involves fitting the fluorescence vs antigen (TNT) concentration to a four-parameter model. One of the parameters is the predicted concentration at the response halfway between two asymptotes, its reciprocal being the apparent association constant (Kₐ).

**Displacement Immunoassays.** One of the disadvantages of competitive immunoassays is that washing is required after competitive binding to remove unbound labeled analyte. This washing step can be eliminated by using a displacement immunoassay. In displacement immunoassays, the antibody is prebound to labeled analyte. When exposed to unlabeled analyte, the labeled analyte is displaced, resulting in a signal decrease. Figure 3 shows data from displacement immunoassay experiments with Ab-TNT-FITC encapsulated in aged

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Silica gels and subsequently immersed in a solution with unlabeled TNT. The signal was measured after exposure to the unlabeled TNT solution without any washing steps. As expected, the characteristic decrease in signal with increasing unlabeled TNT concentration is observed. Moreover, the range of TNT detected can be changed by tailoring the antibody concentration in the original silica gel. Washing the Ab-TNT-FITC prior to displacement to remove unbound TNT-FITC has little effect on the response shape of the signal as a function of unlabeled TNT concentration. As seen in Figure 4, removal of excess TNT-FITC essentially lowers the baseline without significantly affecting the “calibration curve” of TNT concentrations.

Kinetic studies were performed to determine the rate of displacement. Aged silica gels with encapsulated Ab-TNT-FITC were incubated with 0 ppm TNT, 0.5 ppm TNT, 5.0 ppm TNT, or 5.0 ppm trinitrobenzene (TNB), an analogue of TNT. The percent displaced, as measured by the loss in fluorescence signal in the silica gel, was calculated as a function of time according to eq 2:

\[
\% \text{ displaced} = \frac{\text{fluorescence}_{t=0} - \text{fluorescence}_{t=t}}{\text{fluorescence}_{t=0} - \text{fluorescence}_{\text{blank gel}}} 
\]

(2)

As seen in Figure 5, the sol–gel encapsulated Ab retains its ability to distinguish between TNT and TNB. The percent displaced by 5.0 ppm TNB approaches the values obtained for 0.5 ppm TNT, i.e., a factor of 10 lower than the actual TNB concentration. Although there is some cross-reactivity between TNB and the Ab, it is evident that the Ab is much more selective for TNT. The fact that most of the displacement is complete after 60 min for a 1 mm thick aged gel suggests that the response time for thin films can be on the order of a few seconds.

The data presented thus far have been for silica gels in the aged state. As the aged gel is allowed to dry, liquid is expelled from the pores and the gel shrinks as the pores collapse, forming a dried gel, termed a xerogel. Kinetic studies were carried out on a xerogel which was dried until the volume was 25% of its original aged gel volume. A displacement immunoassay with a silica xerogel encapsulated Ab-TNT-FITC shows the percent displaced in a xerogel as compared to an aged silica gel (Figure 6). Displacement occurs in the xerogel, although the rate is dramatically slower. As seen in Figure 6, in a dried gel, about 1500 min is required to produce 80% displacement whereas in an aged gel, only 60 min is required. These results indicate that when antibodies are encapsulated in the pores of a silica network, pore size has a profound effect on the rate of antibody–antigen binding. BET analysis of the aged gel indicated an average pore diameter of 200 Å. At these pore dimensions, antigens such as TNT have sufficient access to bind antibodies trapped in the pores of the silica matrix. In the xerogel state where average pore dimensions are <50 Å, access becomes restricted. Moreover, pore collapse upon drying may cause changes in Ab...
conformation, resulting in lower Ab binding affinity for the analyte.

Although displacement rates in the xerogel are much slower, the data show that antibody–antigen reactions continue to take place in the xerogel despite the smaller pore dimensions. Since the estimated pore diameter in the xerogel is smaller than the size of the antibody, the antibodies may have a pore shaping effect in the silica matrix. We speculate that the antibody defines its own pore in the silica network, and the pores in which antibody molecules are located are larger than the average pore diameter in the xerogel. The Ab conformation, however, is probably still altered due to constriction in the smaller pores. The rate of antigen binding may be reduced by changes in the Ab tertiary structure as well as by slower diffusion rates from pore shrinkage.

**Stability Studies.** One of the advantages of sol–gel encapsulation as a method of immobilization is that biomolecules may be stabilized when trapped in the pores of the silica network. Enhanced stability was observed in some proteins\(^{13,14}\) and enzymes,\(^ {15}\) and the experiments reported here represent initial investigations for antibodies. The relative stability for Ab immobilized by surface attachment is compared to Ab immobilized in the interior of aged silica gels via sol–gel encapsulation. Both sets of immobilized Ab samples were subjected to 0.01 N HCl, methanol, or 60 °C. The results show that sol–gel encapsulated Ab has better stability than surface immobilized Ab (Table 1). The relative signal was consistently higher for the sol–gel immobilized samples. Aged gels with encapsulated Ab subjected to HCl, methanol, or 60 °C experienced essentially no loss in its ability to bind TNT-FITC. In contrast, the surface immobilized Ab showed as much as a 30% loss in its ability to bind TNT-FITC.

**Figure 6.** Displacement of TNT-FITC (using 5.0 ppm TNT) in a xerogel as compared to an aged gel. For the xerogel, a zero baseline was established to ensure that changes in signal were due only to displacement by unlabeled TNT.

**Table 1. Comparison of the Relative Stability of Sol–Gel Encapsulated Ab with Surface Immobilized Ab**

<table>
<thead>
<tr>
<th>treatment condition</th>
<th>relative signal surface immobilized Ab</th>
<th>relative signal sol–gel encapsulated Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>HCl</td>
<td>0.68</td>
<td>≈1.00</td>
</tr>
<tr>
<td>methanol</td>
<td>0.87</td>
<td>0.95</td>
</tr>
<tr>
<td>60 °C</td>
<td>0.75</td>
<td>0.93</td>
</tr>
</tbody>
</table>

The results collectively demonstrate that immunooassays can be successfully performed using sol–gel encapsulated antibodies. Anti-TNT doped silica gels can therefore serve as detection elements for TNT. By using displacement immunooassays, the signal can be measured directly in the sol–gel probes, and no washing is required (i.e., a homogeneous assay). From a device standpoint, sol–gel immobilized Ab must be prepared as thin films rather than as monoliths in order to reduce detection time. Moreover, pore size may be a limiting factor for response time. Detection times on the order of seconds are certainly plausible with thin films. At this time, it is uncertain whether sol–gel immobilized Ab can approach the ppb to ppt detection limits previously reported.\(^ {25–27}\) Sensitivity can be improved and optimized by choosing an appropriate fluorescent label as well as tailoring the antibody concentration. The present results demonstrate that one benefit of sol–gel encapsulation is that the silica matrix stabilizes the Ab, leading to a more “rugged” TNT detector.

**Summary**

Sol–gel encapsulated anti-TNT antibodies can be used as detectors for TNT. Antibodies physically trapped in the silica matrix retain their ability to bind TNT, and both competitive and displacement immunooassays are feasible using sol–gel encapsulated antibodies. Consequently, these antibody-doped materials can differentiate between different levels of TNT and distinguish between TNT and an analogue (TNB). Aged gels exhibit a significantly faster response time than xerogels because of the larger pore diameter. Finally, immobilization via the sol–gel process makes possible a more “rugged” detector as better stability was observed with the silica encapsulated anti-TNT than with the surface immobilized anti-TNT.

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