

Immunosensing of Trinitrotoluene using Sol-Gel Glasses

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ABSTRACT

Research in recent years has demonstrated that biological molecules such as enzymes, immunoglobulins, and other proteins can be immobilized in sol-gel derived matrices and retain their biological function. Development of glasses with encapsulated biomolecules has opened the possibility of solid-state optical biosensors for the detection and measurement of desired analytes. In this work, we report the successful use of sol-gel silica glasses with encapsulated antibodies for the detection of trinitrotoluene (TNT). Results show that both competitive immunoassay and displacement immunoassay are feasible using antibody-doped glasses, and these materials can detect TNT at <1ppm. Moreover, the sol-gel immobilized antibodies retained the ability to discriminate between TNT and an analog, trinitrobenzene (TNB). Finally, enhanced stability was observed in the sol-gel immobilized antibodies as compared to surface immobilized antibodies.

Keywords: Sol-gel, immunosensing, trinitrotoluene (TNT)

1. INTRODUCTION

We present in this paper a study on the use of sol-gel immobilized antibodies as a sensing element for TNT. A specific, sensitive, and rugged detector is needed to detect TNT in landmines, soil, and groundwater contamination.¹ A variety of biomolecules, including antibodies, can be immobilized successfully in optically transparent, porous, silica matrices by physical entrapment.²⁻¹⁸ 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 sensing elements 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 sensing element 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.²⁻¹⁸ Sol-gel glasses doped with biomolecules have been used as optical and electrochemical sensing elements for a number of analytes.^{7-10, 12, 13, 17} Moreover, some biomolecules such as cytochrome c,¹³ myoglobin,¹⁴ and oxidase enzymes¹⁵ exhibit increased stability when encapsulated in the porous matrix. Antibodies have also been successfully immobilized using the sol-gel approach.¹⁹⁻²⁴ Antigens such as fluorescein, pyrene, and atrazine successfully bind their respective antibodies encapsulated in sol-gel derived glasses. Porous sol-gel glasses with immobilized antibodies, therefore, represent solid-state immunosensors 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.^{25,26} 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.¹ In a similar immunosensor 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.²⁷ In addition to immunosensing methods, another technique to detect TNT based on measuring fluorescence quenching of a conjugated polymer film has also been published.²⁸

We present in this paper an optical immunosensor for TNT with sol-gel encapsulated anti-TNT as the sensing element. The sol-gel approach results in a more robust and rugged sensor 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 sensing elements in both displacement and competitive assays, the fluorescence signal as a function of TNT level exhibits the expected behavior observed in standard immunoassays,^{29,30} indicating that these sol-gel sensors 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 detectors for TNT.

2. EXPERIMENTAL

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 gels with only Ab were prepared with a final Ab concentration from 0.5 μM to 1.0 μM . The gels with TNT Ab pre-bound to TNT-FITC were prepared with a final Ab concentration of 1.35 μM to 2.70 μM , with TNT-FITC concentrations ranging from 4 ppm to 20 ppm.

In the competitive immunoassays, gels containing only Ab was used. Samples were immersed in a buffered solution containing 0.29 ppm TNT-FITC and controlled concentrations of unlabeled TNT. Samples were incubated in the solution containing TNT and TNT-FITC for 3 hours and then washed in water for 2 hours. Fluorescence was measured after washing. In the displacement immunoassays, gels containing Ab-TNT-FITC was used. Unless otherwise stated, the original gels were not washed to remove excess TNT-FITC. Individual samples were incubated in unlabeled TNT for 3 to 4 hours. In the dynamic displacement experiments where percent displacement was determined as a function of time, the aged gel samples and xerogel sample were washed thoroughly with buffer to remove excess TNT-FITC. In all displacement experiments, gels were not washed *after* binding with unlabeled TNT. Fluorescence was measured after incubation in unlabeled TNT.

In the stability experiments, two sets of samples were tested. In one set, Ab was immobilized on polystyrene cuvettes using standard surface adsorption techniques for protein immobilization on microtitre plates.³¹ 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 hours: 1) 0.01 N HCl (pH \approx 2.2), 2) pure MeOH, or 3) 60°C. Both sets of samples were then incubated with TNT-FITC to determine the extent of Ab-TNT (TNT-FITC) binding.

3. RESULTS and DISCUSSION

3.1 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 silica gels exposed to a solution containing TNT-FITC and unlabeled TNT. With the sol-gel encapsulated Ab, increasing concentrations of unlabeled TNT result in decreasing fluorescence signal, as expected. TNT levels <1 ppm can be detected, and signal levels are controlled by tailoring the Ab concentration in the gel.

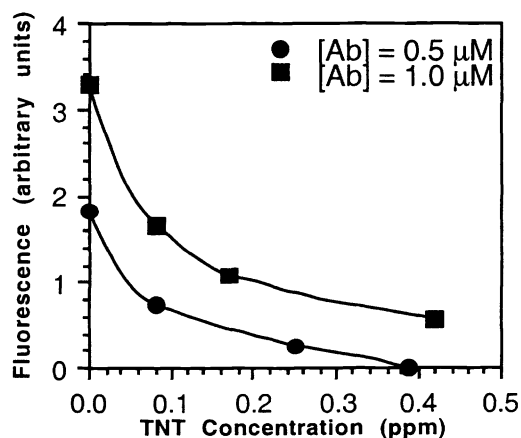


Figure 1. Competitive immunoassay results for sol-gel encapsulated Ab. TNT-FITC concentration was fixed at 0.29 ppm. Fluorescence of blank gels with no Ab was zero.

A standard means of analyzing competitive immunoassays is by plotting B/B_0 vs. unlabeled analyte concentration on a logarithmic scale; the plot is linear for competitive immunoassays.^{26,27} Such a method of expressing the data from competitive binding assays is used routinely to derive a calibration curve for determining the concentration of unknowns. B/B_0 is defined in Eq. (1):

$$\frac{B}{B_0} = \frac{B^* - NSB}{B_0^* - NSB} \quad (1)$$

where B_0^* is the fluorescence signal of sample at 0 unlabeled TNT concentration, B^* is the fluorescence signal of sample, and NSB is the non-specific binding (fluorescence signal of blank sample).

Figure 2 shows a plot of B/B_0 vs. unlabeled TNT concentration on a logarithmic scale for sol-gel encapsulated Ab. The linear correlation confirms that competitive immunoassays can be successfully performed with Ab immobilized in the pores of a silica matrix. A slightly better sensitivity is obtained when using a higher Ab concentration.

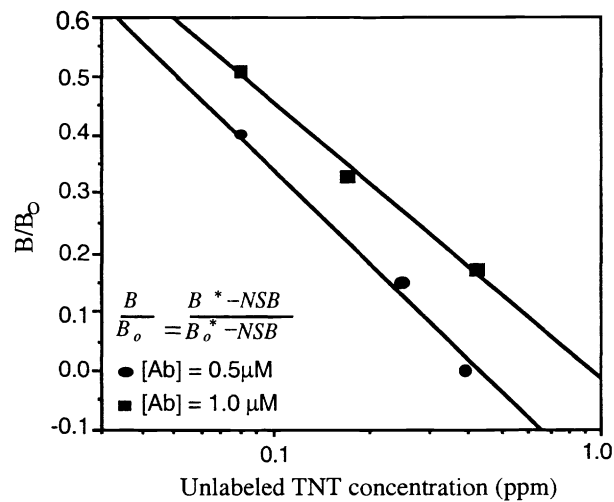


Figure 2. Logarithmic plot of B/B_0 vs. unlabeled TNT concentration for sol-gel encapsulated Anti-TNT in a competitive immunoassay.

3.2 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 pre-bound 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 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.

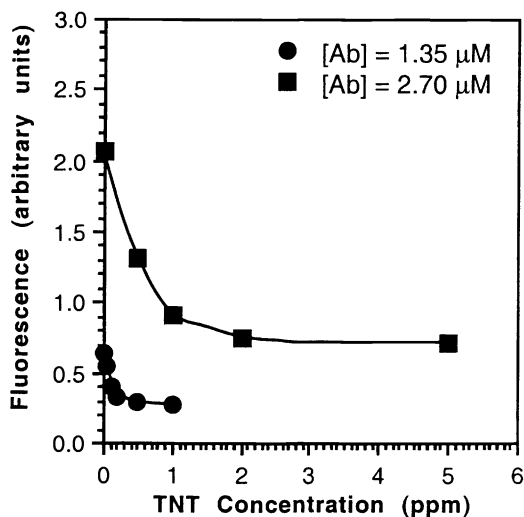


Figure 3. Displacement immunoassay results for sol-gel encapsulated Ab. Original TNT-FITC concentration was 4 ppm for the 1.35 μM Ab samples and 20 ppm for the 2.70 μM Ab samples. Samples were not washed prior to displacement.

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.

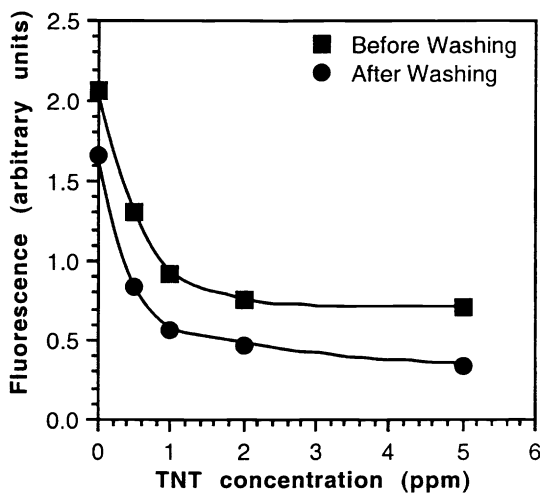


Figure 4. Displacement immunoassays with sol-gel encapsulated Ab showing the effect of washing excess TNT-FITC prior to displacement. $[\text{Ab}] = 2.7 \mu\text{M}$, initial $[\text{TNT-FITC}] = 20 \text{ ppm}$.

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 analog of TNT. The % displaced, as measured by the loss in fluorescence signal in the silica gel, was calculated as a function of time according to equation 2:

$$\%Displaced = \frac{Fluorescence_{t=0} - Fluorescence_{t=t}}{Fluorescence_{t=0} - Fluorescence_{blank\ gel}} \quad (2)$$

As seen in Figure 5, the sol-gel encapsulated Ab retains its ability to distinguish between TNT and TNB. The % displaced by 5.0 ppm TNB approaches the values obtained for 0.5 ppm TNT. 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 minutes for a 1mm thick aged gel suggests that the response time for thin film sensors can be on the order of a few seconds.

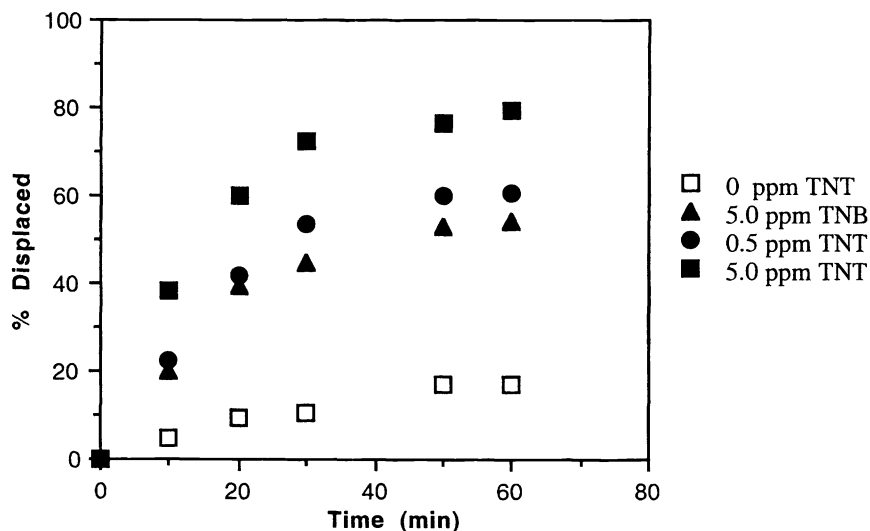


Figure 5. The displacement of TNT-FITC by unlabeled TNT or TNB in aged silica gels. The slight loss in signal in the 0 ppm TNT sample is likely due to incomplete washing of excess TNT-FITC before displacement.

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 % 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 minutes is required to produce 80% displacement whereas in an aged gel, only 60 minutes 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, as pores collapse in the xerogel state, the antibody conformation may be changed leading to decreased binding affinity.

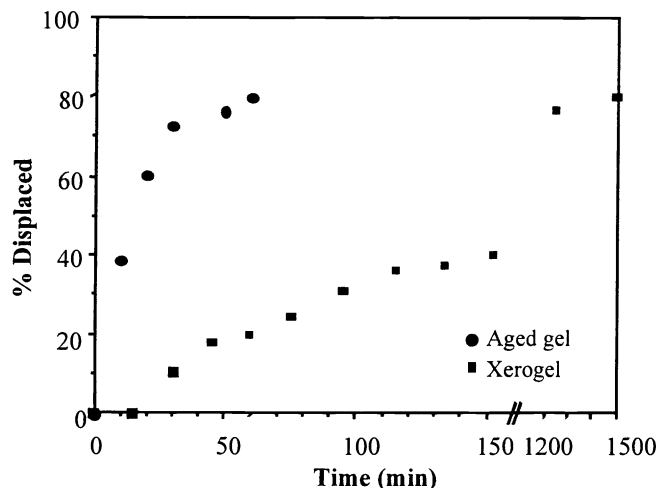


Figure 6. A comparison of the displacement rate of TNT-FITC by unlabeled TNT in an aged gel vs. a xerogel.

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 TNT molecules, being much less than 50Å, still has access to the antibody molecules, although access is slower and binding affinity may be lower.

3.3 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 0.01N HCl, methanol, or 60°C for 24 hours. 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.

Table I. Comparison of the relative stability of sol-gel encapsulated Ab with surface immobilized Ab

Treatment Condition	Relative Signal Surface Immobilized Ab	Relative Signal Sol-Gel Encapsulated Ab
Control	1.00	1.00
HCl	0.68	≈1.00
Methanol	0.87	0.95
60°C	0.75	0.93

The results collectively demonstrate that immunoassays can be successfully performed using sol-gel encapsulated antibodies. Anti-TNT doped silica gels can therefore serve as the detection element in a TNT sensor. By using displacement immunoassays, the signal can be measured directly in the sol-gel sensing element 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 a thin film sensor. 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” immunosensor.

4. SUMMARY

Sol-gel encapsulated anti-TNT antibodies can be used as sensing elements for TNT. Antibodies physically trapped in the silica matrix retain their ability to bind TNT, and both competitive and displacement immunoassays are feasible with the sol-gel based immunosensors. Consequently, these antibody-doped materials can differentiate between different levels of TNT and distinguish between TNT and an analog (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" immunosensor as better stability was observed with the silica-encapsulated Anti-TNT than with the surface-immobilized Anti-TNT.

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