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Sol–Gel Matrixes Doped with Atrazine Antibodies: Atrazine Binding Properties†

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Sol–gel materials with antibody properties are described. These were constructed by the entrapment of monoclonal anti-atrazine antibodies (Mabs) in SiO_2 sol–gel derived matrixes, which successfully recognized and bound atrazine, a widely used herbicide. The binding properties for atrazine were evaluated by comparing sol–gel entrapped anti-atrazine hybridoma culture fluids with entrapped purified immunoglobulins (IgGs). In the study, ^{14}C-labeled and unlabeled atrazine, which are stable to hydrolysis or to other chemical decay processes, were used as analytes. Leaching of the antibodies was found to be zero. Stability was tested under various storage conditions and was found to be 100% for at least 2 months at room temperature, compared with a drop of 40% in solution. The response time was found not to differ considerably from that obtained in solution. Other factors tested included reproducibility of binding, dose response, nonspecific physisorption of atrazine to the ceramic matrix, and elution recoveries of atrazine from the doped sol–gel columns. An advantage of the sol–gel methodology is the elimination of the need to purify the IgGs from the Mab hybridoma culture fluids.

Introduction

The sol–gel chemical route to materials is the basis of a general methodology for the incorporation of bioactive molecules into ceramics, glasses, and other inorganic materials.[1–4] The activity in this field has been quite intensive in recent years, resulting in many biomaterials with diverse applications,[2–5] including the successful immobilization of numerous biotechnologically important enzymes, the construction of biosensors and of enzymatic electrodes, construction of bioactive optical components, and the preparation of environment-related biomaterials.[6–11] The entrapping matrixes have been mostly SiO_2 materials or their derivatives and composites, but other oxides have been used as well.[12] The moderate temperatures and the mild hydrolysis and condensation–polymerization conditions of the monomeric metal and semimetal alkoxides allow the entrapment of proteins without their denaturing. Enhanced stability of the entrapped biomolecule, the stability, inertness and high surface area and porosity of the matrix, the optical transparency, and the ease of the direct heterogenization procedure are among the reasons for the attractiveness of the sol–gel approach to immobilization in general and that of proteins in particular.

A family of sol–gel entrapped proteins which is of special interest comprises the immobilized antibodies, which are of potential use in biomedical and environmental immunochromatography and immunosensing. Some earlier reports in this area include the study of Wang et al. on the successful entrapment of the complex of fluorescein with polyclonal fluorescein antibody;[13] our own successful demonstration that sol-gel-entrapped purified immunoglobulins (IgGs) from an anti-dinitrobenzene polyclonal antiserum antibody retain the capacity to bind an external antigen, namely, 2,4-dinitrophenylhydrazine, from solution;[14] the covalent binding of antibodies to functionalized sol-gel films;[15] the development of a sol–gel enzyme-linked immunosorbent assay (ELISA) test for antigenic parasitic protozoa;[6] the immunochromatography of nitropyrene;[16]

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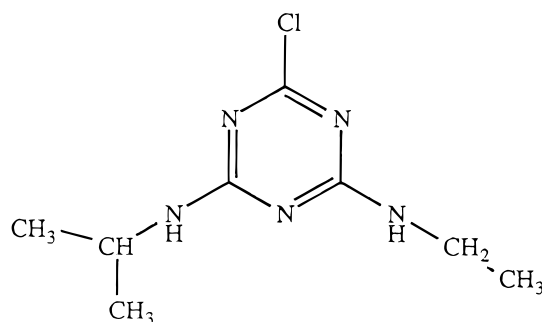
and the incorporation of antifluorescein in sol–gel thin films.[17] From these studies with entrapped antibodies it emerges that although the entrapment procedure is easy and convenient, the exact nature of the trapping of these proteins is yet to be fully understood and the design of an optimal procedure yet to be mastered.

Atrazine (**1**), which is at the focus of this study, is one of the *s*-triazine herbicides. In our previous preliminary study on the entrapment of anti-atrazine monoclonal antibodies (Mabs) in an SiO_2 sol–gel matrix,[18] we found that the formation conditions and composition of the sol–gel play a crucial role in determining the ability of the antibodies to bind the analyte from aqueous solutions. The present investigation used



Atrazine (1)

the optimal sol–gel preparation procedure and composition that were established in the previous study.[18] We have further characterized the binding properties of entrapped anti-atrazine Mabs, compared the binding capacities of sol–gel-entrapped anti-atrazine hybridoma culture fluids with those of purified IgGs, analyzed the stability of the entrapped Mabs under a variety of storage conditions, tested the leaching of the Mabs from the sol–gel matrix, examined the extent of nonspecific adhesion of atrazine to the ceramic matrix, evaluated the elution recoveries of atrazine from doped sol–gel columns, and compared the binding properties of the entrapped antibodies with those obtained in buffer solutions. The results of the present study add to the knowledge of the properties of sol–gel-entrapped antibodies and set the basis for further development of the sol–gel-based technology into an immunochemical analytical methodology for purification, concentration, and monitoring of compounds of high biomedical, environmental, and agricultural concern.

Experimental Details

Sol–Gel Entrapment of the Antibody. The entrapment was carried out by a two-step procedure, in which hydrolysis is followed by polymerization of tetramethoxysilane (TMOS, ABCR, 99%, Karlsruhe, Germany).[19] An acidic silica sol solution was obtained by mixing TMOS with 2.5 mM HCl in triple-distilled water (TDW), at a molar ratio of 1:8, containing 10% polyethylene–glycol (PEG-400, Merck, Germany; average molecular weight of 400 g/mol, corresponding to approximately seven methylene units in the chain). The mixture was stirred for a minute until a clear solution was obtained and sonicated for 30 min in a Branson (Energieweg, The Netherlands) Model R-3, 55 W 0.5 L sonicator or in an ELMA (Singen-Hohentwiel, Germany) ultrasonicator bath (Model T-460/H, 285 W, 2.75 L). The reaction was carried out in a well-ventilated fume hood.

Hybridoma culture fluids (200 or 400 μL, corresponding to 1.3 and 2.6 mg of protein, respectively, unless otherwise indicated) from monoclonal mouse anti-atrazine antibodies[20] (AM7B2, kindly provided by Dr. A. Karu, UCLA, Berkeley, CA) to be encapsulated in the sol–gel were concentrated in Centricon-30 tubes (Amicon), diluted in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99.99%, Sigma), pH 7.5, to a final volume of 0.5 mL, and mixed with an equivolume amount of prehydrolyzed TMOS. All other proteins to be encapsulated in the sol–gel (IgGs purified from the hybridoma culture fluid, or fetal calf serum, FCS, Biological Industries, Israel) were dissolved in 0.5 mL of 50 mM HEPES buffer, pH 7.5, and mixed with TMOS as above. The solution was quickly mixed for 5 s, and gelation occurred within 1–2 min. After 10 min the gels were washed four or five times with 2 mL of HEPES buffer or PBS, pH 7.5, at 2 h intervals (unless otherwise indicated) and were kept wet (with 2 mL of HEPES buffer or PBS, pH, 7.5, on top) at 4 °C until use. Best results were obtained with gels that were kept at 4 °C and were used on the second day after preparation. Protein concentration was 1.3–2.6 mg in 1 mL total gel volume (unless otherwise indicated).

Binding of Atrazine to Sol–Gel Entrapped Antibodies. Wet gels were thoroughly crushed, transferred into inverted 5 mL plastic syringes, and packed in 1 mL columns. Sol–gel columns were washed, prior to sample application, with 50 mL of PBS. For optimal activity columns were kept under buffer at all times during the experiment. Forty nanograms (unless otherwise indicated) of unlabeled atrazine (analytical grade, 98–99%, kindly provided by Agan Chemicals, Israel) or ^{14}C-labeled atrazine (atrazine-ring-UL ^{14}C; 20 000 cpm, s.a. 5 mCi/mmol; radiochemical purity 95%; Sigma) were applied, in a volume of 1 mL PBS, onto the sol–gel column. The eluent was collected and applied onto the column two more times, to ensure maximal binding. When radiolabeled atrazine was used, columns were washed with PBS and 20 × 1 mL fractions were collected. Four milliliters of Ultima Gold XR scintillation liquid (Packard) were added to each fraction, and the radioactivity (representing the amount of unbound atrazine) was counted in a Beckman Model LS 1701 scintillation counter. When unlabeled atrazine was used, unbound atrazine was washed out with 20 mL of TDW, the sample was concentrated in a Speed Vac system (Savant), resuspended in 1 mL of TDW, and tested for atrazine content by the two-step competitive ELISA as described below.

Binding experiments were performed using sets of three sol–gel columns: (A) an experimental column containing anti-atrazine Mabs or protein A purified IgGs

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(see below); (B) a control column (for the determination of nonspecific binding; namely, adsorption to all sites that are not the active site of the antibodies) containing FCS (the rational for using FCS for the determination of nonspecific binding is based on the fact that the hybridoma cultures of the anti-atrazine Mabs were grown in the presence of FCS); (C) an empty control column with no protein. The protein concentration in all columns was the same regardless of the nature of the entrapped protein. The extent of binding to column A is referred to in the text as total binding, and that to columns B and C as nonspecific binding. Specific binding (namely, binding to the active site of the antibody) is defined as the difference between the total binding and the nonspecific binding.

Elution of Atrazine from Sol–Gel Entrapped Antibodies. Elution of atrazine was performed using sol–gel columns with radiolabeled bound atrazine. After the regular binding procedure, columns were washed once again with 10 × 1 mL of PBS, and elution was performed with 10 × 1 mL of either 0.1 M glycine–HCl buffer, pH 3.5, or 0.1 M triethylamine buffer, pH 11.5. Elution was followed by 10 × 1 mL washes of PBS and all 30 fractions were monitored for radioactivity as described above.

Leaching of Antibodies from the Sol–Gel Matrix. Anti-atrazine Mabs were entrapped in the sol–gel matrix as described above. Ten minutes after gelation, the gels were washed four times with 2 mL of HEPES buffer or PBS (at room temperature) at 1 h intervals and transferred (with 2 mL of HEPES buffer or PBS on top) to 4 °C for an additional period of 18 h. The pooled supernatants (total volume of 10 mL) were concentrated by Centricon-30, dissolved in 200 μL of 0.5 M sodium carbonate buffer, pH 9.6 (Carbonate buffer), and tested for antibody titers, by ELISA (using a calibration curve of hybridoma culture fluid Mabs) as described below.

Binding of Atrazine to Antibodies in Solution. Anti-atrazine monoclonal hybridoma culture fluid (500 μL) was concentrated prior to binding in a Centricon-30 and diluted in PBS to a final volume of 200 μL. Unlabeled atrazine (64, 128, and 256 ng) was added to the Mabs in an equivolume ratio, and the mixture was incubated for 15 min at room temperature on a slow shaker. At the end of the incubation, the bound atrazine was separated from the unbound compound by means of Centricon-30 tubes. The bound complex was washed twice with 400 μL of PBS, and the amount of unbound atrazine was determined in the flow-through by the two-step competitive ELISA as described below.

Immunochemical Methods. *A. Purification of Anti-atrazine IgGs from Hybridoma Culture Fluid.* Protein A agarose beads (0.8 mL of suspension, 4% cross-linked agarose, *p*-nitrophenyl chloroformate-activated, Sigma) were packed in a Pasteur pipet at room temperature. The column was washed with 4 mL of 30 mM NaCl in 10 mM sodium phosphate buffer, pH 8.2 (buffer A), followed by an additional wash with 0.4 mL of 0.1 M citrate buffer, pH 3.5, and a third wash with 4 mL of buffer A. Monoclonal mouse anti-atrazine hybridoma culture fluid (200 μL) was applied onto the column, and the eluent was collected and applied two more times to ensure maximal binding. The column was washed 10 × 1 mL with buffer A, and elution of IgGs was performed by 4 × 1 mL washes with citrate buffer, pH 3.5. The citrate buffer fractions were collected into tubes containing 0.2 mL of 1 M Tris, pH 9.4 (final pH of mixture was about 6.5), and the collected fractions, containing the purified IgGs, were concentrated using Centricon-30 tubes. The IgG fraction was resuspended in 100 μL of Carbonate buffer, tested for protein content (by the Bradford method[21]), and for binding titers (by ELISA), as described below.

B. Determination of Antibody or IgG Titers by ELISA. Wells of microtiter plates (NUNC Maxisorp microtiter plates, Roskilde, Denmark) were coated with 100 μL of (1 μg) protein A (Sigma) diluted in Carbonate buffer. After an overnight incubation at 4 °C, the wells were washed five times with PBS containing 0.1% (v/v) Tween-20, pH 7.25 (PBST), and 100 μL of anti-atrazine Mabs (diluted 1:200, 1:400, 1:800, and 1:1600 in Carbonate buffer) or any other tested samples: protein A purified IgGs (diluted 1:200, 1:400, 1:800, and 1:1600 in Carbonate buffer), Mabs which had been stored at room temperature for various lengths of time (diluted 1:200, 1:400, 1:800, and 1:1600 in Carbonate buffer), or samples from the leaching experiments (concentrated 25-fold and diluted 1:2, 1:4, 1:8, and 1:16) were added to the wells and incubated overnight at 4 °C. Plates were washed as above with PBST, and 100 μL of atrazine–horseradish peroxidase (HRP) tracer (prepared by the modified active ester method as previously described),[22] diluted 1:2000 in PBST, were added to the plates. The plates were incubated for 1 h at room temperature, rinsed with PBST, and tested for HRP activity by the addition of 100 μL of substrate solution that contained 96 mg/mL tetramethylbenzidine (TMB) and 0.004% H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5. The reaction was stopped after 10–20 min by the addition of 50 μL of 4 M sulfuric acid, and the absorbence was measured with a Labsystems Multiscan Multisoft ELISA reader at 450 nm. Protein A purified IgGs, Mab titers in samples from leaching experiments, or Mabs that had been stored in solution at room temperature for various lengths of time, were compared with the titers of "untreated" hybridoma culture fluid Mabs.

C. Determination of Atrazine Content by ELISA. Atrazine content was determined by the two-step competitive ELISA. Assays were carried out using a "haptenated enzyme" format in which the Mab was immobilized on microtiter plates via protein A, and atrazine in solution competed with atrazine–HRP conjugate for binding to the Mabs.

The two-step competitive ELISA was performed essentially as previously described.[22] Wells of microtiter plates were coated with 100 μL (1 μg) of protein A diluted in Carbonate buffer. After an overnight incubation at 4 °C, the wells were washed five times with PBST, and 100 μL of anti-atrazine Mabs (diluted 1:200 or 1:400 in Carbonate buffer) were added to the wells for an overnight incubation at 4 °C. Plates were washed as above with PBST, and 50 μL of tested sample or standard atrazine (12 serial dilutions, ranging from 0.001 to 2 ng/well), in duplicate, were added to the wells together with 50 μL of atrazine–HRP tracer, diluted

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Table 1. Binding of Atrazine to Sol−Gel-Entrapped Anti-atrazine Mabs and IgGs[a]

	total binding (%)		specific binding (%)	
atrazine Mab	radiolabeled atrazine	unlabeled atrazine	radiolabeled atrazine	unlabeled atrazine
culture fluid				
200 μL	30 ± 6 (n = 5)	37 ± 7 (n = 3)	27 ± 5 (n = 5)	24 ± 4 (n = 3)
400 μL	50 ± 4 (n = 6)	58 ± 5 (n = 3)	47 ± 3 (n = 6)	45 ± 8 (n = 3)
none	3 ± 2 (n = 5)	13 ± 10 (n = 3)		
purified IgG				
300 μL	41 ± 0.3 (n = 3).	n.t.	38 ± 0.3 (n = 3)	n.t.
600 μL	63 ± 2 (n = 3)	n.t.	60 ± 2 (n = 3)	n.t.
none	3 ± 0.1 (n = 3)	n.t.		

[a] 40 ng of ^{14}C-radiolabeled or unlabeled atrazine was applied on sol−gel columns containing 200 or 400 μL (1.5 and 3.0 mg of protein, respectively) hybridoma culture fluid from mouse monoclonal anti-atrazine antibodies, or IgGs purified from 300 and 600 μL hybridoma culture fluid. The amount of unbound atrazine was determined either by direct counting of radioactivity (labeled atrazine) or by ELISA (unlabeled atrazine). Total binding represents the difference (as %) between the total amount of atrazine (defined as 100%) applied on columns containing anti-atrazine Mab or purified IgGs, and the amount of free atrazine that was found in the flow-through solution. Specific binding represents the difference between the percentage of total binding and the percentage of atrazine that was bound nonspecifically to the sol−gel matrix (in the absence of any protein) and could not be recovered from the columns. Values are presented as the mean ± SEM of n = 3−6 experiments. IgG, immunoglobulin; Mab, monoclonal antibody; n.t., not tested.

1:1000 in PBST. Plates were incubated as above, the reaction was stopped after 10−20 min by the addition of 50 μL of 4 M sulfuric acid, and the absorbence was measured with a Labsystems Multiscan Multisoft ELISA reader at 450 nm. Atrazine content was determined by comparison with an atrazine calibration curve. Each sample was tested, in duplicate, at three dilutions, which paralleled the atrazine calibration curve.

Results

Binding Properties of a Fixed Dose of Atrazine to Sol−Gel-Entrapped Anti-atrazine Mabs. The first set of experiments was designed to determine the binding extent of sol−gel-encapsulated anti-atrazine antibodies, to compare the binding of nonpurified hybridoma culture fluids with that of purified IgGs and to determine the extent of nonspecific binding of atrazine to the sol−gel matrix. The data in Table 1 indicate that the mouse monoclonal anti-atrazine antibodies bind free atrazine and that the nonspecific binding of atrazine to the sol−gel matrix is low, ranging from 3 to 13%. Binding experiments were performed using radiolabeled atrazine as well as unlabeled atrazine, in order to compare a direct quantification method (determination of radioactivity) with a method (ELISA) that itself introduces variability. The data indicate that both methods are very similar and the values with the two methods were very close.

Hybridoma culture fluids contain, in addition to anti-atrazine antibodies, a high concentration of proteins that originate from the fetal calf serum (FCS) that is added as a nutritional supplement to the hybridoma cell culture. The concentration of antibodies in a typical fluid culture is usually less than 0.7% (<0.05 mg/mL of Mab in a total protein concentration of 6−7 mg/mL). Utilization of hybridoma culture fluids in sol−gel matrixes results, therefore, in entrapment of a large quantity of nonspecific proteins. To determine whether such proteins cause undesired nonspecific adsorption, we purified the IgG fraction from the hybridoma fluid culture, using protein A agarose beads, and compared the binding capacity of sol−gels that contained aliquots of hybridoma culture fluids with that of sol−gels that contained purified IgGs.

Since antibody titers tend to decrease in the course of the IgG purification process, we first determined the exact titer of the purified fraction. Comparison of the titers of the purified IgGs with those of the Mabs present in hybridoma culture fluids, by ELISA, revealed a loss of 50%. Therefore, the amount of purified IgG that was entrapped in the sol−gel was derived from an amount of culture fluid that was 1.5 times greater than the amount that was used with the nonpurified Mabs (namely, the amount of IgG that was entrapped in 1 mL of sol−gel originated from 300 and 600 μL of hybridoma culture fluid, compared with 200 and 400 μL, respectively, that were entrapped when unpurified hybridoma culture fluid Mabs were employed). Because of the small amount of protein present in the purified IgG fraction, we were unable to determine the exact protein content that was entrapped in the sol−gel. As can be seen from Table 1, the binding capacity of the sol−gel columns that contained purified IgGs was only slightly higher than that obtained using sol−gels with hybridoma culture fluids. Despite the high protein content in sol−gel columns that contained hybridoma culture fluids, the nonspecific binding of atrazine was low, similar to that found in sol−gels containing purified IgGs.

Dose Response. Binding of atrazine to the sol−gel-entrapped antibodies was found to exhibit dose dependency with respect to the amount of antibody that was entrapped in the matrix. Examination of the extent of binding (of a constant amount of atrazine, 40 ng) to Mabs present in various amounts of hybridoma culture fluid (100−700 μL; 0.73−5.11 mg of protein) revealed a linear increase in binding with increasing amounts of entrapped Mabs (Figure 1). Total binding ranged from a minimum of 14% (at 100 μL) to a maximum of 65% (at 700 μL). The extent of non-specific adsorption to sol−gels, that contained equivalent amounts of FCS, was low and ranged between 1 and 7% (Figure 1). The binding to nondoped sol−gel columns that contained no protein was 3% (data not shown).

Binding of atrazine to sol−gel-entrapped Mabs also showed dose dependency with respect to the amount of loaded analyte. Examination of the specific binding as a function of the atrazine load (4−128 ng) revealed increased binding as the amount of atrazine applied to the column increased (Figure 2). The degree of nonspecific binding (to columns containing an equivalent amount of protein contributed by FCS) also increased with the increasing amount of applied atrazine, but in no case did it exceed 20% of the initial amount. The

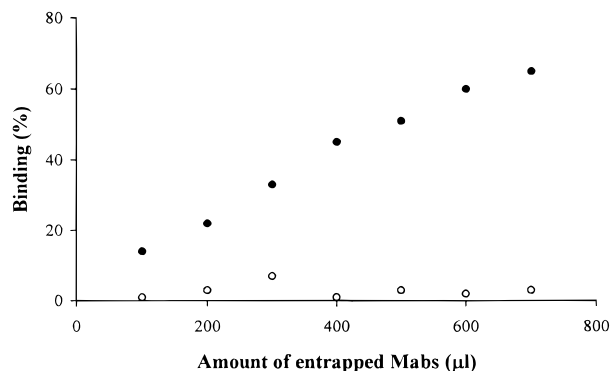

Figure 1. Dose response curve of atrazine binding to sol–gels doped with various amounts of Mabs (●) and fetal calf serum (FCS) (○). A constant amount (40 ng) of ^{14}C-radiolabeled atrazine was applied on sol–gel columns doped either with 100–700 μL (0.65–4.55 mg of protein) or with equivalent amounts of FCS. All other details are as described in the legend to Table 1.

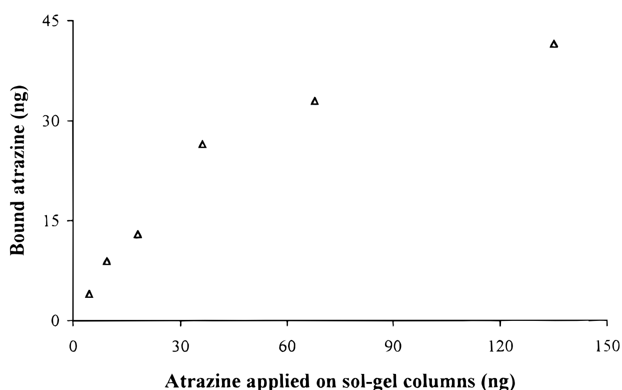

Figure 2. Dose response curve of atrazine binding to sol–gel columns doped with anti-atrazine Mabs. A constant amount (500 μL) of hybridoma culture fluid Mabs (3.25 mg of protein) or an equivalent amount of FCS was entrapped in the sol–gel matrix. Binding was monitored with 4–128 ng/mL of unlabeled atrazine. The amount of unbound atrazine was determined by ELISA. The data depict specific (△) binding which was calculated from the difference between total binding (in the presence of anti-atrazine Mabs) and nonspecific binding (in the presence of FCS).

degree of nonspecific adsorption that was obtained with sol–gel that did not contain any proteins, was similar. Scatchard kinetic analysis[23] of the binding data revealed that, under the tested conditions, the binding capacity of the sol–gel-entrapped Mabs was 45 ng.

Comparison with Solution Activity. Although entrapment of antibodies in the sol–gel matrix was successful, it is reasonable to assume that only part of the entrapped molecules are biologically active. This assumption is based on the fact that some of the entrapped molecules undergo denaturation during the gelation proces as a result of the high reactivity of the monomers that form the polymer, or because of the considerable shrinking that exerts pressure on the proteins in the course of the gelation process. Molecules may also be inactive because of their spatial orientation that hinders accessibility of the hapten to the active site. To address this issue, we compared the binding capacity of sol–gel-entrapped anti-atrazine Mabs with that of those in solution. Analysis was performed with different amounts of atrazine (64, 128, 256 ng) and a constant amount of anti-atrazine hybridoma culture fluid (500 μL). The data reveal that the extent of binding of the entrapped Mabs was moderately (1.4–2.3-fold) lower than that in solution (Figure 3).

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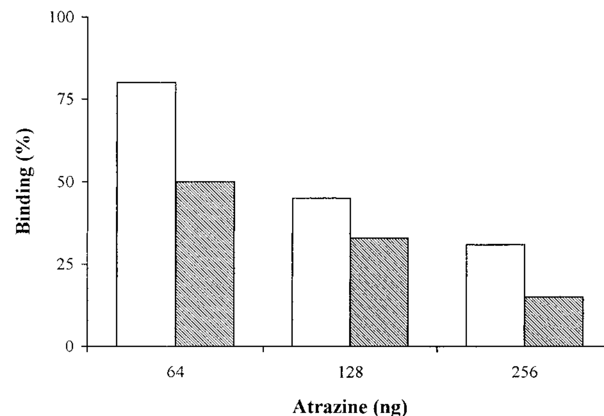

Figure 3. Comparison of the binding capacity of sol–gel-entrapped anti-atrazine Mab (cross-hatched bars) with that of Mabs in solution (open bars). The same amounts of anti-atrazine Mabs (500 μL, 3.25 mg) were used for entrapment and binding in the sol–gel and for binding in solution. Atrazine (64, 128, and 256 ng) was incubated with the doped sol–gels or the Mabs in solution for 15 min at room temperature. The amount of unbound atrazine was determined by ELISA. Data represent specific binding (defined as the difference, in percent, between the total and nonspecific binding). Nonspecific binding was determined in the presence of an equivalent amount of FCS (sol–gel experiments) and IgG from normal mouse serum (solution experiments).

Table 2. Effect of Incubation Time on the Binding of Atrazine to Sol–Gel Entrapped Anti-atrazine Mabs[a]

experimental conditions	binding (%)	
	15 min	18–24 h
sol–gel		
total binding	70	77
nonspecific binding	4	9
specific binding	66	68
solution		
total binding	62	67
nonspecific binding	3	7
specific binding	59	60

[a] Atrazine (40 ng, ^{14}C radiolabeled) was incubated with doped sol–gels containing 500 μL (3.25 mg of protein) hybridoma culture fluid from mouse monoclonal anti-atrazine antibodies, or with 100 μL of Mabs in solution. Incubation was carried out for 15 min (at room temperature) and overnight (18–24 h, at 4 °C). Nonspecific binding was determined in the presence of an equivalent amount of FCS in both experiments.

The results of testing for possible effects of incubation time and of comparison between sol–gel and solution behavior are collected in Table 2. Remarkably, the binding values obtained in sol–gel after 15 min incubation are quite similar to those obtained overnight, and to those obtained in solution.

Stability. The ability of the sol–gel-entrapped Mabs to bind free atrazine from solutions was retained for at least 53 days, and the amount of atrazine that was bound to the sol–gel columns did not change with storage time (Table 3). The unchanged binding capacity of the entrapped Mabs was retained regardless of the conditions of storage, and sol–gels that were kept at room temperature retained their binding capacity similarly to those that were kept at 4 °C. The extent of nonspecific binding to sol–gels doped with FCS or to

Table 3. Binding Capacity of Atrazine Mabs Entrapped in Sol–Gel as a Function of Storage Time and Temperature[a]

storage time, temp	total binding (%)		
	sol–gel + Mab	sol–gel + FCS	nondoped sol–gel
1 day, 4 °C	74	4	0
3 days, RT	72	2	2
17 days, 4 °C	74	n.t.	n.t.
17 days, RT	76	8	7
31 days, 4 °C	74	0	0
31 days, RT	76	0	0
53 days, 4 °C	76	3	4
53 days, RT	73	4	5

[a] 40 ng of ^{14}C-radiolabeled atrazine was applied on undoped sol–gel columns, and sol–gel columns doped with 500 μL of hybridoma culture fluid from mouse anti-atrazine Mabs, or FCS. Protein content in all sol–gels was 3.25 mg. The amount of unbound atrazine was determined by direct counting of radioactivity in the flow-through solution. Total binding was calculated as indicated in the legend to Table 1. Values obtained with sol–gel columns containing FCS or no protein indicate nonspecific binding. Mab monoclonal antibodies; FCS, fetal calf serum; n.t., not tested.

Table 4. Comparison of the Activity of Sol–Gel-Entrapped Anti-atrazine Mabs with That in Solution as a Function of Storage Time and Temperature[a]

storage time, temp	activity (%)	
	sol–gel	solution
1 day, 4 °C	100	100
3 days, RT	97	n.t.
16–17 days, RT	103	100
31–36 days, RT	103	62
53–55 days, RT	99	39

[a] Activity of sol–gel-entrapped Mabs was determined as described in the legend to Table 3. Activity of Mabs in solution was determined by ELISA as described in Experimental Details. Values express the ratio (as percent) between the activity of the Mabs at various storage times and the activity at 1 day of storage at 4 °C (defined as 100%). n.t., not tested.

the matrix alone did not change significantly with storage time or temperature and in all cases did not exceed 8%.

The ability of the entrapped Mabs, which had been kept for different storage times at room temperature, to bind free antigen was compared with that of antibodies in solution that had been kept under the same conditions. As indicated in Table 4, the sol–gel antibodies retained their full activity throughout the entire period of the experiment, unlike the antibodies that were kept in solution, which lost about 40–60% of their activity in a period of 36–55 days of storage at room temperature.

Leaching. Another important aspect of stability is the leaching of biomolecules from the entrapping matrix, which can severely affect any immunochemical method. The known high porosity of the sol–gel matrix that was used in our study led us to examine the degree of antibody leaching from the matrix. Evaluation of the degree of leaching of the entrapped Mabs was performed in sol–gels that contained several different amounts of entrapped Mabs (200, 400, and 600 μL, corresponding to 1.3–3.9 mg of protein/1 mL of sol–gel). As can be seen from Figure 4, the amount of leached Mabs was very low, in fact negligible, and in most cases was below the detection limit of the assay. To evaluate this limit, we note that the binding capacity of the antibody that was present in 50 μL of the concentrated supernatant (representing one-fourth of the total sample) was lower

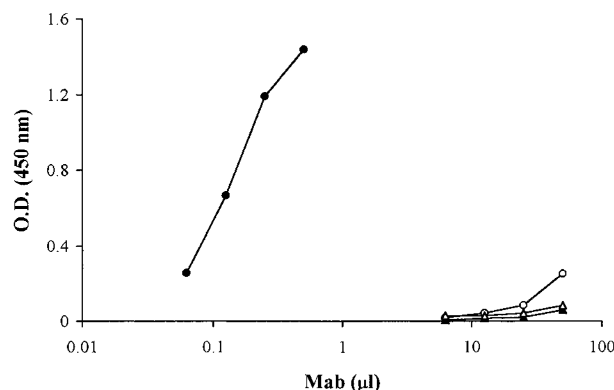

Figure 4. Leaching of anti-atrazine Mabs from sol–gels. Anti-atrazine Mabs [200 μL (○), 400 μL (▲), and 600 μL (△)] were entrapped in sol–gels as described in the Experimental Details. Supernatant solutions were collected, concentrated (25-fold) and redissolved in 200 μL of Carbonate buffer. Four serial dilutions of the supernatant (ranging from 1:2 to 1:16 and representing 50, 25, 12.5, and 6.125 μL of the concentrated supernatant) were used to determine the amount of leached antibody. Analysis was performed by ELISA, and the amount of antibody in the supernatant was determined from calibration curves of anti-atrazine Mabs (●), composed of four serial dilutions (ranging from 1:200 to 1:1600 and representing 0.5, 0.25, 0.0125, and 0.06125 μL of the Mab hybridoma culture fluid).

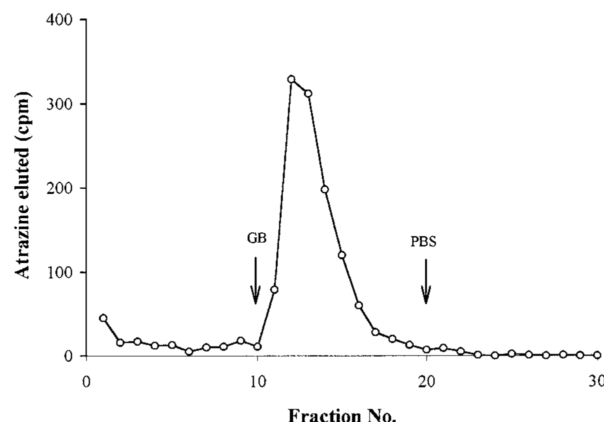

Figure 5. Typical elution profile of atrazine from sol–gel-entrapped anti-atrazine Mabs. 40 ng of ^{14}C-radiolabeled atrazine were applied on sol–gel columns doped with 400 μL (3.25 mg of protein) hybridoma culture fluid from mouse anti-atrazine Mabs. The total amount of atrazine that was bound to the entrapped Mabs was 62%. The amount of eluted atrazine was determined by direct counting of radioactivity in each of the collected fractions. Left and right arrows indicate application of 0.1 M glycine buffer (GB), pH 3.5, and PBS, respectively.

than that obtained with 0.0625 μL of the Mab. Thus, less than 0.12% of the total amount of Mabs encapsulated in the sol–gel leached. Leaching was also determined in sol–gels that were kept at room temperature or at 4 °C for 53 days. The activity that was found in the sol–gel supernatant after 53 days was low, representing 0.4 and 0.3% of the entrapped Mab, respectively.

Elution of the Bound Analyte. Elution of bound atrazine from sol–gel-entrapped Mabs was performed using 0.1 M glycine buffer at pH 3.5 (Figure 5) or 0.1 M triethylamine buffer at pH 11.5 (data not shown). The elution with glycine buffer resulted in a recovery of 69%, whereas that with triethylamine buffer was 86% of the atrazine that was bound to the column. The data shown in Figure 5 represent an experiment that was performed

with sol–gel columns that contained 400 μL entrapped Mabs. Similar recoveries (66 and 73%, respectively) were obtained with glycine buffer when elution was performed with columns that contained 600 and 700 μL of Mabs.

Discussion

In the present study, we entrapped anti-atrazine mouse Mabs in an SiO_2 sol–gel matrix and characterized their binding properties with respect to dose dependency and stability under various storage conditions. Aspects tested included the reproducibility of binding, the leaching of the Mabs from the sol–gel matrix under various conditions, the nonspecific adhesion of atrazine to the ceramic matrix and the elution recoveries of atrazine from doped sol–gel columns. The study also compared the binding capacities of sol–gel-entrapped anti-atrazine hybridoma culture fluids, with those of purified IgGs, and with those in solution. The study was performed with ^{14}C labeled and unlabeled atrazine (as analytes), which is a stable compound, and its monitoring is not affected by external physical or chemical factors such as hydrolysis or decay. The sol–gel composition and formation conditions (which are known to have a major effect on the binding properties of the entrapped antibodies) were based on our previous findings[18] that indicated that hydrophilic wet SiO_2 sol–gel matrixes, in which the TMOS:aqueous ratio is 1:8, enriched with 10% (v/v) PEG, exhibit high binding capacities with low nonspecific adhesion.

The results of the present study indicate that anti-atrazine Mabs entrapped in the sol–gel matrixes retained their ability to bind nanogram amounts of free antigen from solution in a dose-dependent manner (antibody- and antigen-wise). The binding capacity of 500 μL Mab (corresponding to 3.25 mg of protein/1 mL of sol–gel) entrapped in sol–gel and incubated with atrazine for 15 min at room temperature was found to be 45 ng. In addition, the study indicates that specific and nonspecific binding are not affected by the presence of large amounts of nonrelevant proteins (Table 1). This finding reveals a great advantage of the sol–gel technique: the need to purify IgGs from Mab hybridoma culture fluids prior to the entrapment process is eliminated. The presence of relatively large amounts of protein in the sol–gel may, however, have an impact on the reproducibility of the system. This emerges from the comparison of the coefficients of variation in the two respective sets of experiments. The comparison revealed relatively high variabilities, ranging from 16 to 45% in sol–gel experiments performed with Mabs originating from unpurified hybridoma culture fluids, as oppose to a variation of 2–6% in experiments performed with IgGs. It is important to note that our experimental system has not yet been fully optimized, and it may very well be that under optimal conditions even the use of unpurified hybridoma culture fluids would result in highly reproducible results.

Sol–gel entrapment of antibodies exhibited a very high stability. Storage of the sol–gel-entrapped antibody for 53 days at room temperature did not cause a decrease in its binding capacity, and the amount of atrazine that was bound did not differ from that obtained with entrapped antibodies that had been kept for 1 day at 4 °C (Table 3). In contrast, antibodies that were kept in solution (with 0.01% thimerosal) at room temperature in a sealed tube, for 36 and 55 days, lost 40 and 60% of their activity, respectively, compared with those that were kept in solution at 4 °C (Table 4). Stabilization of proteins in sol–gel matrixes is a well-documented phenomenon that has been demonstrated for a variety of enzymes. Early examples include a 100-fold increase in the thermal stability of silica sol–gel acid phosphatase[24] and a marked decrease in the sensitivity of glucose oxidase to a wide range of pH changes;[25] and a recent example is the remarkable increase in the thermal stability of lipase entrapped in alkylated sol–gel silicas, as observed by several research groups.[7,26] The enhanced stability has been attributed mainly to the intimate nature and rigidity of the ceramic cage, as compared with organic polymer caging, exposed adsorption or exposed covalent attachment.[24] The ability of the cage silanols to bind the protein at several of its sites, is another factor contributing to the enhanced stability of the entrapped biomolecule.[24] An important aspect in the development of any immunoaffinity purification method is the ability to elute the analyte and regenerate the sensing molecule. The elution and regenerability were investigated using buffers with low (3.5) and high (11.5) pH values which dissociate the antigen–antibody interaction. Under these conditions 70–85% of the bound atrazine was dissociated from the antibody and was eluted from the sol–gel column (Figure 5). Although we were able to elute over 80% of the bound atrazine from the column, the results indicate that only 33–44% of the initial response could be recovered in one cycle. It has to be determined whether the elution conditions damaged the antibody, the sol–gel matrix, or both.

Another important factor that plays a major role in immunochemical assays is the leaching of the biological sensor (antibody in our case) from the matrix in which it is entrapped, adsorbed, or covalently bound. Our data indicate that sol–gel-entrapped Mabs do not leach from the matrix (Figure 4) even if the gels are kept for long periods (53 days) at room temperature. This observation agrees with practically all studies on sol–gel entrapment of proteins that have shown that leaching is either negligible or zero. As noted in a recent review by Dave et al.,[7] this makes the application of proteins for sol–gel sensing purposes, more attractive than the use of small molecules.

Although entrapment of antibodies in the sol–gel matrix offers many advantages, part of the biological activity of the molecule is lost because of its complete enclosure within the cage or because of an orientation within the cage which obstructs the active site, and possibly also because of denaturation during the polymerization and gelation processes. Of these factors, steric obstruction is probably more important than denaturation for the case of globular rigid proteins. This is evident from the fact that Michaelis–Menten behavior is maintained in many instances,[25] which is possible only if there is a homogeneous population of entrapped

(24) Braun, S.; Rappoport, S.; Zusman, R.; Shtelzer, S.; Druckman, S.; Avnir, D.; Ottolenghi, M. In *Biotechnology: Bridging Research and Applications*; Kluwer: Boston, MA, 1991.
(25) Shtelzer, S.; Braun, S. *Biotechnol. Appl. Biochem.* **1994**, *19*, 293.
(26) Reetz, M. T.; Zonta, A.; Simpelkamp, J. *Biotechnol. Bioeng.* **1996**, *49*, 527.

protein molecules. Denaturation due to entrapment would yield a whole spectrum of activities, among which Michaelis–Menten behavior would not usually be preserved. Nevertheless, as shown by Edmiston et al.,[27] soft proteins are indeed affected by the gel shrinkage. Our results indicate a reduction in the bioactivity of the entrapped Mab, but the loss is relatively low (1.4–2.3-fold; Figure 3). This result is of particular interest in view of the fact that antibody molecules are relatively large (MW 150 000) and their binding activity depends heavily on the proper stereoconformation.

Of particular usefulness is the relatively short (15 min) response time of the sol–gel-entrapped antibodies, which does not differ considerably from that obtained under similar incubation conditions after an overnight incubation (Table 4). The results indicate that under the tested conditions accessibility of the analyte to the biosensing entrapped molecule is not hindered.

(27) Edmiston, P. L.; Wambolt, C. L.; Smith, M. K.; Saavedra, S. S. *J. Colloid Interface Sci.* **1994**, *163*, 395.

(28) Note added in proof: Cichna et al. described recently the successful sol–gel entrapment of antibodies to polycyclic aromatic hydrocarbons (Cichna, M.; Knopp, D.; Niessner, R. *Anal. Chem. Acta* **1997**, *339*, 241).

Conclusions

It was shown that the method for entrapment of antibodies in a sol–gel matrix is a simple one-step procedure that maintains the antibodies' binding capacity, enhances stability, enables dissociation of the analyte from the antibody at high recoveries, and shows no leaching, thus offering many advantages over commonly used immobilization procedures such as adsorption and covalent binding. The ability to immobilize a biorecognition element in a sol–gel matrix that is highly stable, inert, and optically clear opens the way for the development of highly selective biosensors/immunosensors for applications in immunochemical detection methods and in immunoaffinity purification.

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