Sol−Gel-Based Immunoaffinity Chromatography: Application to Nitroaromatic Compounds†

Alisa Bronshtein, Nadav Aharonson, Avner Turniansky, and Miriam Altstein*

Institute of Plant Protection, The Volcani Center, Bet Dagan, 50250 Israel

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A sol−gel-based method for immunoaffinity purification using sol−gel-entrapped anti-dinitrophenyl (DNP) antibodies (Abs) was developed. Polyclonal antiserum (whole antiserum) and purified immunoglobulines (IgGs, isolated from the whole antiserum), which recognize nanogram quantities of a variety of di- and trinitroaromatic compounds, were entrapped in SiO_2 sol−gel-derived matrixes, and their binding properties were examined with 2,4-dinitrophenylhydrazine (DNPH) used as an analyte. Binding properties of the entrapped Abs were determined by the evaluation of the optimal sol−gel composition for entrapment and the optimal conditions for binding and elution of the analyte. We found that a hydrophilic, flexible "wet" gel with a tetramethoxysilane:aqueous ratio of 1:8, enriched with 10% PEG exhibited high binding capacities with low nonspecific binding. Under the tested conditions the sol−gel-entrapped Abs bound the analyte in a dose-dependent, highly reproducible manner (antibody- and antigen-wise), and binding was equally effective with either polyclonal whole antiserum or protein A purified IgGs (eliminating the need to purify IgGs from the whole antiserum). The analyte could easily be eluted at high recoveries (90%) and the Abs were well-retained in the sol−gel matrix and did not leach out even at extreme pH conditions or in organic solvents. The sol−gel immunoaffinity columns exhibited binding capacities that were either significantly higher or did not differ significantly from those of protein A−agarose covalently coupled Abs over a wide range of IgG (0.5−15 μL corresponding to 1−30 μg protein) and analyte amounts (20−320 ng).

Introduction

Immunochemical methods such as enzyme immunoassays (EIA) have become increasingly important during recent years for the determination of pesticides and other xenobiotics.[1−4] Commercial kits for about 50 pesticides are available, and more than 200 assays have been described in the literature. The EIAs that have been developed so far, for food analysis and environmental monitoring, employ polyclonal antibodies (Pabs) and monoclonal Abs (Mabs). Recently, genetically engineered Abs (recombinant Abs, Rabs) for a variety of analytes have been generated and integrated into EIAs.

Development of a diagnostic immunoassay technology for detection of pesticide residues and environmental contaminants requires the development of an accurate and sensitive quantitative assay and the establishment of simple extraction, cleanup, and concentration procedures which differ from those currently used for the chemical analytical methods.[4] The requirement for different cleanup and concentration methods emerges from the fact that EIAs are being performed in aqueous solutions and not in organic solvents as in the common methods based on high-pressure liquid chromatography (HPLC), gas chromatography (GC), and GC combined with mass spectrometry (GC−MS) and because organic solvents interfere with the EIA. Thus, the cleanup and concentration methods have to be based on an aqueous basis and have to be able to remove the interfering components from the tested samples. Immunoaffinity purification (IAP) provides a promising approach to sample clean up and concentration and is fully compatibility with the EIA.

Although IAP has been in use for over a decade,[5] its implementation for the analysis of pesticides and environmental contaminant residues is still limited. Successful detection through IAP requires, first and foremost, immobilization of the Abs, which in many cases is a lengthy, multistep process. Consequently, there is a definite need for simplified methods of Ab immobilization and the introduction of new, simple, chemically stable, and nonreactive matrixes, to exploit the full analytical potential of this approach. Sol−gel technology,[6] which enables incorporation of bioactive molecules

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* Corresponding author. Tel: 972-3-968-3710. Fax: 972-3-968-3835. E-mail: vinnie2@netvision.net.il.

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into ceramics, glasses, and other inorganic materials,[6−9] offers promising solutions for all of the above requirements.

The sol−gel process is a method for preparation of inorganic oxide matrixes of metals and semimetals by direct hydrolysis and polycondensation of active monomeric precursors. For silicon, the most studied and used element in this context are alkoxides of silicon, such as tetramethoxysilane (TMOS), which was used in the present study. Upon addition of water, this monomer polymerizes into a porous silica which may take the form of wet gels, xerogels, or organically modified sol−gels (Ormosils). The resulting matrix has high surface area and porosity, inertness and stability to chemical and physical agents, and visible and UV optical clarity. The reactions are performed at room temperature (rt), thus enabling the entrapment of organic and bioorganic molecules within the forming silica network, by their simple addition to the polymerizing mixture. The biomolecules, which are strongly encapsulated within the matrix and cannot diffuse out, generally retain their activity, gain higher stability, and can react with ligands that diffuse into the highly porous matrix. The moderate temperatures and the mild hydrolysis and condensation−polymerization conditions allow proteins to be entrapped without being denatured. The enhanced stability of the entrapped biomolecules and the physical and chemical properties of the matrix are among the reasons for the attractiveness of the sol−gel approach to immobilization in general and that of proteins in particular.

The sol−gel chemical route to materials has been studied quite intensively in recent years, resulting in many biomaterials with diverse applications, including the successful immobilization of Abs, numerous biotechnologically important enzymes, the construction of biosensors, chemical and pH sensors, enzymatic electrodes, construction of bioactive optical components, and the preparation of environment-related biomaterials.[7−22] The entrapping matrixes in all of the above studies were mostly SiO_2 materials or their derivatives.

The successful application of the sol−gel doping methodology to a wide variety of proteins and enzymes, and the above-mentioned long list of potential advantages of the sol−gel technique, prompted us to extend the range of these bioactive materials to include additional enzymes and Abs for agricultural and environmental immunosensing applications. In a previous study we successfully entrapped esterases within sol−gels for monitoring organophosphorus and carbamate insecticides[23,24] and entrapped anti-atrazine Abs for monitoring triazine herbicides.[24−26] Although entrapment of the above molecules was shown to be successful, the exact nature of the entrapment of these proteins is yet to be fully understood and the design of an optimal procedure is yet to be mastered. In the present study, we have extended the scope of the technique by the entrapment of anti-nitroaromatic Abs for further characterization of the sol−gel-entrapped Abs and examination of their properties as an IAP device for clean up and concentration of environmental contaminants.

Nitroaromatic compounds are of great environmental concern in the United States, with many of them having been documented in the EPA's final National Priority List of waste sites in the United States,[27] and also in Europe.[28] The nitroaromatic derivatives found most frequently as environmental contaminants are 2,4-dinitrotoluene and 2,6-dinitrotoluene, which are used in plastics, dyestuffs, and ammunition manufacture; and nitrophenols, which are used as pesticides. Nitroaromatic derivatives are also used in agriculture as insecticides, e.g., Parathion (4,6-dinitro-*o*-cresol); as herbicides, e.g., Ethalfluralin; and as fungicides, e.g., Quintozene. Since these compounds and their metabolites are often found as contaminants of food, soil, and water, there is clearly a need for extensive monitoring of nitroaromatic compounds in the environment.

In the present study, we developed a sol−gel-based IAP method using sol−gel-entrapped anti-dinitrophenyl (DNP) whole antiserum and IgGs (isolated from the whole antiserum) and compared their characteristics with those in solution and those bound to the commonly used IAP resin Protein A−agarose beads.

Experimental Section

Antiserum. Polyclonal anti-DNP antiserum (whole antiserum; total protein concentration ranged from 50 to 85 mg/mL, depending on the batch; Sigma) generated in rabbits against DNP−bovine serum albumin (BSA) conjugate as an immunogen, was used throughout the study.

Immunochemical Methods. *Purification of Anti-DNP IgGs from Whole Antiserum.* Protein A−agarose beads (0.8 mL of suspension, 4% cross-linked agarose, *p*-nitrophenyl chloroformate-activated, Sigma) were washed twice with 0.8 mL of 0.5 M carbonate buffer (CB), pH 9.6, and packed in a Pasteur pipet at rt. The column was washed with 4 mL of 30 mM NaCl in 10 mM sodium phosphate buffer, pH 8.2 (buffer A), followed

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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. DNP antiserum (20 μL) was applied to the column and the wash-through was collected and applied twice more, to ensure maximal binding. The column was washed 10 times with 1 mL of buffer A, and elution of IgGs was performed with four 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 with Centricon-30 tubes (Amicon, Beverly, MA). The IgG fraction was resuspended in 60 μL of CB and tested for protein content (by the Bradford method[29]) and for binding titers by enzyme linked immuno-sorbent assay (ELISA), as described below. Protein content was found to range from 1.4 to 3.4 mg/mL.

Preparation of Dinitrophenyl (DNP)−Ovalbumin (OV) Coating Antigen. The coating antigen was prepared by a modification of the method of Tager.[30] Briefly, 0.2 mg of difluorodinitrobenzene (Sigma) dissolved in ethanol was added dropwise to 20 mg of OV dissolved in 1 mL of 10 mM K_2CO_3, pH 11.2 (2:1 molar ratio of hapten:carrier). The reaction was stirred for 24 h at rt and dialyzed against 5 L of phosphate-buffered saline (0.15 M NaCl in 50 mM sodium phosphate buffer, pH 7.2, PBS). Protein content was determined by the Bradford method[29] and was found to be 9.8 mg/mL. The product was used as the coating antigen for the ELISA described below.

Comparison of Anti-DNP Whole Antiserum and IgG Titers. The assay served to determine the loss of binding capacity in the course of IgG purification from the anti-DNP antiserum. Wells of microtiter plates (NUNC Maxisorp microtiter plates, Roskilde, Denmark) were coated with 200 μL of 0.03 μg/mL DNP−OV conjugate (6 ng) diluted in CB. After an overnight incubation at 4 °C, the wells were washed three times with PBS containing 0.1% (v/v) Tween-20 (Sigma), pH 7.2 (PBST), and 200 μL of protein A purified IgGs or anti-DNP whole antiserum (diluted 1:40 000, 1:80 000, 1:160 000, and 1:320 000 in CB) was added to the wells and incubated overnight at 4 °C. Plates were washed as above with PBST, and 200 μL of goat anti-rabbit horseradish peroxidase (GAR−HRP, Sigma), diluted 1:40 000 in PBST, was added to the plates. The plates were incubated for 2 h at rt, rinsed with PBST, and tested for HRP activity by the addition of 200 μL of substrate solution that contained 96 μg/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 100 μL of 4 M sulfuric acid, and the absorbance was measured with a Labsystems Multiscan Multisoft ELISA reader at 450 nm. The data revealed similar titers for both preparations (whole antiserum and IgGs isolated from the whole antiserum), indicating that no loss of binding capacity had occurred in the course of the protein A purification process.

Determination of Dinitroaromatic Compounds by ELISA. This assay served to determine the extent of the free nitroaromatic compound (dinirophenylhydrazine, DNPH; Fluka) that did not bind to the sol−gel-entrapped, protein A−agarose-bound-, or soluble whole antiserum or IgG. Content of unbound DNPH was determined by the two-step competitive ELISA, in which the compound in solution competed with an antigen−carrier conjugate immobilized on a 96-well microtiter plate for binding an anti-DNP whole antiserum or IgG.

The two-step competitive ELISA was performed essentially as previously described.[24] Briefly, wells of microtiter plates were coated with 200 μL of 0.03 μg/mL DNP−OV conjugate (6 ng) diluted in CB. After an overnight incubation at 4 °C, the wells were washed with PBST as above, and 100 μL of tested sample or standard (12 serial dilutions of DNPH, ranging from 0.005 to 10 ng/well) was added to the wells, together with 100 μL of anti-DNP whole antiserum or IgG (diluted 1:40 000 in PBST). Plates were incubated overnight at 4 °C and washed as above with PBST, and 200 μL of GAR−HRP diluted 1:40,000 in PBST was added. Plates were incubated for 2 h with the secondary Ab, rinsed with PBST, and tested for HRP activity as described above. Under the tested conditions the I_{20} and I_{50} values of the assay were 0.11 $\pm$ 0.02 and 0.86 $\pm$ 0.11 ng/mL, respectively. Contents of DNPH and other dinitroaromatic compounds were determined by comparison with a DNPH calibration curve. Each sample was tested in duplicate, at five dilutions. Only samples that paralleled the calibration curve were considered.

Sol−Gel Entrapment of Whole Antiserum and IgGs. Method I.[31] The entrapment was carried out by a two-step procedure in which hydrolysis was followed by polymerization of tetramethoxysilane (TMOS, ABCR, 99%, Karlsruhe, Germany). An acidic silica sol solution was obtained by mixing TMOS with 2.5 mM HCl in double-distilled water (DDW), at molar ratios of 1:8 and 1:12 in the presence and absence of 10% poly(ethylene 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 1 min, until a clear solution was obtained, and was then sonicated for 30 min 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.

Protein A purified IgGs (0.5−77 μL, corresponding to 0.7−250 μg of protein) or anti-DNP whole antiserum (1−40 μL, corresponding to 0.063−2.02 mg of protein or 1.5−60 μg of IgG) and similar protein amounts of normal rabbit serum (NRS, Sigma) or NRS−IgGs (Sigma) (which served as control proteins intended to determine the extent of nonspecific adsorption) were premixed with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99.99%, Sigma), pH 7.5, to a final volume of 0.5 mL, and added to an equivolume amount of prehydrolyzed TMOS. The solution was quickly mixed for 5 s and gelation occurred within 1−2 min. After 10 min the gels (total volume of 1 mL) were washed four or five times with 2 mL of HEPES buffer or PBS, pH 7.5, at 2 h intervals 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 stored overnight at 4 °C. Gels exhibited high stability and could be used for over two month after preparation. Sol−gels prepared by this procedure are termed "wet gel" throughout the present paper.

Method II.[32] One milliliter of TMOS was mixed with 10 mL of PBS (an approximate PBS:silane molar ratio (r) = 82:1). The proteins to be encapsulated (IgG isolated from anti-DNP whole antiserum or NRS−IgG) were premixed into the above buffer solution. The amount of encapsulated IgG was 250 μg. The mixture was stirred for approximately 3 min, until a clear sol was obtained. Gelation occurred within another 5−10 min. The reaction was usually carried out in vials immersed in an ice−water mixture, and the resulting gels were aged for 24 h at 4 °C and then lyophilized until a xerogel was obtained as a fine white powder. The doped xerogels (0.4 g) were kept at 4 °C until use. Sol−gels prepared by this method are termed "dry gel" throughout the present paper.

Binding of Analytes to Sol−Gel Entrapped with Anti-DNP Whole Antiserum or With IgG. 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 binding, columns were kept under buffer at all times during the experiment. Binding was performed with DNPH, which exhibited the highest cross reactivity with the anti-DNP whole antiserum and with purified IgG.[24] Xerogel powder (1 g) was transferred to similar syringes and processed as above.

Two sets of experiments were performed: one in which a constant amount of analyte (20 ng) was bound to various amounts of sol−gel doped with IgGs or whole antiserum (0.5−77 μL containing 0.7−250 μg of protein and 1−40 μL containing 1.5−60 μg of IgG, respectively) and a second, in which various amounts of analyte (20−640 ng) were bound to a

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constant amount (30 μg of IgG in 20 μL) of anti-DNP whole antiserum. In both sets of experiments the analyte (DNPH) was applied, in a volume of 1 mL PBS, to the doped sol−gel column. The flow-through (unbound analyte) was collected and applied to the column twice more, to ensure maximal binding. The unbound analyte was washed out with 20 mL of DDW (pooled). The pooled sample was concentrated in a Speed Vac system (Savant) in the presence of 0.005% Tween-20, resuspended in 1 mL of DDW, and tested for DNPH content by ELISA, as described above. Binding of DNPH to "dry gels" was performed similarly with 0.4 g of xerogel.

Binding experiments were performed with sets of three sol−gel columns: (A) an experimental column containing anti-DNP whole antiserum or protein A purified IgG; (B) a control column (for the determination of nonspecific binding, namely, adsorption to all sites other than the active site of the Abs) containing NRS or NRS−IgG (NRS was used for the determination of nonspecific binding because the antiserum was generated in rabbits); and (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 below as total binding and that to columns B and C as nonspecific binding. Specific binding (i.e. binding to the active site of the Ab) is defined as the difference between the total binding and the nonspecific binding. Binding in all cases was highly reproducible and the variations between experiments did not exceed 10%.

Elution of DNPH from Sol−Gel-Entrapped Abs. Elution of DNPH was performed with sol−gel columns (1:8 + PEG) doped with 20 μL of anti-DNP whole antiserum (corresponding to 30 μg of IgG) to which 195−213 ng of DNPH were bound (out of 640 ng that were applied to the column). After the regular binding procedure, columns were washed with 20 mL of PBS, and elution was performed with 10 mL of either 0.1 M glycine−HCl buffer, pH 3.5, or absolute ethanol (99.8%). The eluate was passed through a Centricon-30 tube and the DNPH content in the flow-through was determined by ELISA as described above.

Leaching of Abs from the Sol−Gel Matrix. Anti-DNP whole antiserum (20 μL, corresponding to 30 μg of IgG) was entrapped in the sol−gel matrix as described above. Columns were washed with 20 mL of PBS, followed by an additional rinse with 10 mL of eluting solvent (0.1 M glycine, pH 3.5 or absolute ethanol). The sample was concentrated with a Centricon-30 (with glycine as the eluting solvent) or a Speed Vac (with ethanol as the eluting solvent) and resuspended in 1 mL of PBST. Ab titers were determined by ELISA against a calibration curve of anti-DNP whole antiserum that underwent Speed Vac or Centricon-30 concentration as described above.

Binding of Analytes to IgG in Solution. Anti-DNP purified IgGs (0.5−15 μL, corresponding to 1−30 μg of protein) were incubated with 20 ng of DNPH in a total volume of 400 μL, and the mixture was incubated for 15 min at rt on a slow shaker. At the end of the incubation, bound DNPH 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 material was determined by two-step competitive ELISA as described above.

Binding of Analytes to Anti-DNP IgG Immobilized on Protein A Agarose. Protein A−agarose beads (0.8 mL, binding capacity 10 mg of human IgG per mL; Sigma) were washed twice with 0.8 mL of CB. Anti-DNP IgG (0.5−15 μL, corresponding to 1−30 μg of protein) or anti-DNP whole antiserum (20 μL, corresponding to 30 μg of IgG) diluted in CB to a final volume of 200 μL was added to the beads and incubated for 1 h (on a slow shaker) at rt and then overnight at 4 °C. Beads were washed twice with 10 bed volumes (8 mL) of CB, resuspended in 8 mL of CB, and subjected to cross-linkage by means of 20 mM dimethyl pimelimidate (DMP, Sigma) for 1 h. The reaction was stopped by the addition of 8 mL of freshly prepared 0.2 M ethanolamine (Sigma), pH 8.0, for 2 h, after which the protein A−agarose immobilized anti-DNP IgGs were washed three times with 8 mL of PBS, packed into a column in a Pasteur pipet, and subjected to binding. DNPH (20−320 ng in 200 μL of PBS) was applied to the columns, and the wash-through was collected and applied twice more, to ensure maximal binding. Columns were washed with a total volume of 20 mL of DDW and concentrated by Speed Vac in the presence of 0.005% Tween-20. Samples were resuspended in 1 mL of DDW, and the amount of unbound material was determined by two-step competitive ELISA as described above.

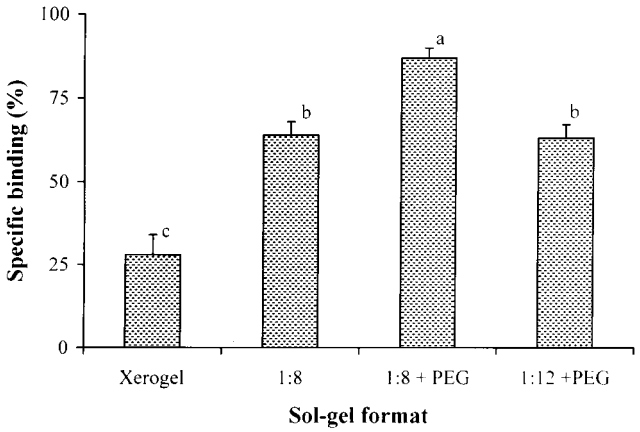

Figure 1. Effect of sol−gel format and composition on binding of DNPH. 1:8 and 1:12 signify the TMOS:HCl molar ratios (2.5 mM in DDW). Binding was performed with 250 μg of anti-DNP IgG. The amount of unbound DNPH in each sol−gel format was determined by ELISA. Samples were tested, in duplicates, at three to five concentrations which paralleled the DNPH standard curve. The amount of bound material was calculated from the difference between the total amount applied on the sol−gel column (20 ng, defined as 100%) and the free material that was found in the flow-through solution. Specific binding represents the difference between the percentage of total binding and the percentage of DNPH that was bound nonspecifically to the sol−gel matrix doped with an equivalent amount (250 μg) of NRS−IgG and could not be recovered from the columns. PEG = poly(ethylene glycol). Each bar represents the mean ± SEM of three experiments. Means with the same letter do not differ significantly at $P < 0.01$.

Statistical Analysis. Statistical analysis was performed by ANOVA. Differences among means were tested for significance by the Newman−Keuls test at $P < 0.01$.

Results and Discussion

The first part in the establishment of an IAP procedure is the determination of optimal conditions for Ab immobilization. Previous studies[6] revealed that the preparation procedure and composition of sol−gel have major effects on the surface area, morphology, average pore size, and pore geometry of the matrix, and affect, therefore, the activity of the entrapped molecules. We found[23,25] that the successful entrapment of dopants requires substantial screening of the sol−gel preparation procedure parameters; therefore, the first set of experiments in the present study included examination of the effects of sol−gel formats and composition on binding. Four different sol−gel formats were tested for their ability to bind analytes. They were comprised of xerogels ("dry gels"), prepared by method II, and three "wet gel" variants, prepared by method I, at different silane:water ratios (1:8 and 1:12) with and without PEG. Sol−gels were doped with IgGs which had been purified by protein A−agarose beads from an anti-DNP polyclonal whole antiserum, and the analyte was DNPH.

The results in Figure 1 show that binding of DNPH to the doped Abs differed according to the sol−gel format

and that the best binding activity of the entrapped anti-DNP IgGs was achieved with composite gels having an *r* ratio of 1:8 + PEG. The gel-encapsulated Abs prepared by this protocol bound 87% of the DNPH applied to these sol−gel columns. IgGs entrapped in the other two "wet" formats (1:8 and 1:12 + PEG) were slightly less effective, and the binding did not exceed 64 and 63%, respectively, of the amount applied to the column. The IgGs entrapped in the dry xerogel exhibited a low binding capacity (28%). The low extent of binding in the xerogel resulted either from damage to the Ab during the lyophilization process or from the drastic pH changes occurring during the hydrolysis of the TMOS (which was performed in the presence of the Ab). It is also possible that the reduced binding was due to increased confinement of the protein caused by the shrinkage of the gel as a result of lyophilization. The greater binding in the two-step "wet gel" procedure may have resulted from the avoidance of drastic pH changes and the constant aqueous environment of the Abs. The addition of PEG improved binding, probably by creating a composite matrix that contains a higher number of pores, resulting in a somewhat more flexible, highly porous, and protective matrix than ordinary silicates. The reason for the lower binding at the1:12 format is not clear at present. Similar results were obtained with anti-atrazine Mab,[25] in which hydrophilic, flexible "wet gels" prepared by the two-step method exhibited higher binding capacity by the entrapped Abs than xerogels, which exhibited very low binding, and among the "wet gels", the 1:8 + PEG composition exhibited the highest binding. Interestingly, this format also resulted in the highest activity of entrapped acetyl-cholinesterase,[23] suggesting that the 1:8 + PEG composition provides favorable conditions for a variety of entrapped biomolecules. In light of the above results, the 1:8 + PEG format was used in all further experiments.

A major requirement in the development of any IAP method is low nonspecific binding to the selected resin. To examine the extent of entrapment or nonspecific adsorption of DNPH to the doped proteins or silica matrix, we determined the recovery of DNPH from nondoped sol−gel columns and from sol−gel columns doped with a NRS−IgG. Recovery of DNPH from these columns (defined as the amount of DNPH that was obtained after extensive washing of the column) was monitored under the same experimental conditions as those used for sol−gels doped with anti-DNP IgGs. In most experiments the extent of nonspecific binding ranged between 2% and 6% and never exceed 10% of the loaded analyte. Nonspecific binding was not affected by the amount of analyte applied on the sol−gel column or on the protein load (see below). In general, binding was highly reproducible, and the coefficient of variance did not exceed 10%.

The second part of the establishment of an IAP procedure is the determination of optimal conditions for the Ab−antigen interaction. Experiments in this part were designed to determine binding capacities and the effects of protein load on binding. First, the extents of binding of a constant amount of DNPH (20 ng) to various amounts of anti-DNP IgGs (1−30 μL, corresponding to 1.5−44 μg of protein) were determined. The results shown in Figure 2 revealed that binding depended on the amount of Ab entrapped in the matrix and that 79% of the applied analyte (corresponding to 15.8 ng DNPH) bound to 30 μL of anti-DNP IgGs. The extent of nonspecific adsorption to sol−gels that contained equivalent amounts of NRS−IgG ranged between 6% and 10%.

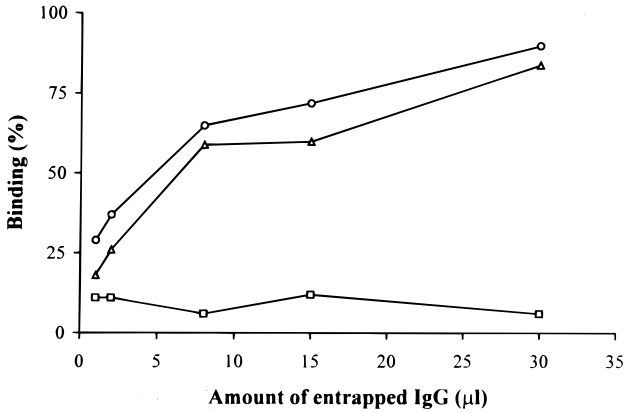

Figure 2. Dose−response curve of DNPH total (○), nonspecific (□) and specific (△) binding to sol−gels columns. A constant amount (20 ng) of DNPH was applied on sol−gel columns (1:8 + PEG) doped either with 1−30 μL (1.5−44 μg of protein) anti-DNP IgG or with equivalent amounts of NRS−IgG.

Another factor that was tested for its effect on binding was the protein load. In a previous study with sol−gel-entrapped acetyl cholinesterase from electric eel (ee-AChE), we found that the amount of the entrapped enzyme had a profound effect on the enzymatic activity and that an increase in the concentration of entrapped enzymes resulted in a marked decrease in activity.[23] In the present study we tested the effect of high nonspecific protein loads on binding by comparing the binding of sol−gel-entrapped anti-DNP whole antiserum (which contains 50.7 mg/mL total protein) with that of sol−gel-entrapped protein-A-purified IgGs (which contains 1.4−3.4 mg of protein/mL). The experiment was carried out with 0.063−2.02 mg of total antiserum protein (corresponding to 1.5−60 μg of IgG) and 1.5−44 μg of purified IgG (i.e., a 45 times smaller amount of total protein). Since the Ab concentrations in both preparations were similar, activities could be compared on a volume basis.

As indicated in Figure 3, the presence of large amounts of nonspecific protein did not impair binding; on the contrary, the high protein content improved binding at all tested doses. For example, 94% binding was obtained with only 10 μL whole antiserum, compared with 30 μL of protein A-purified IgGs needed to bind the same amount of DNPH. The nonspecific binding was not affected by the presence of large amounts of nonspecific proteins. It is possible that the large amounts of nonspecific protein in the whole antiserum protected the IgG from damage caused to protein A-purified IgG during gel formation. This finding reveals a great advantage of the sol−gel technique, as it eliminates the need to purify IgGs from whole antiserum prior to entrapment. All further experiments (unless otherwise indicated) were performed with whole antiserum rather than protein A-purified IgGs.

It is interesting to note that the presence of nonspecific proteins in the doped sol−gels has different effects

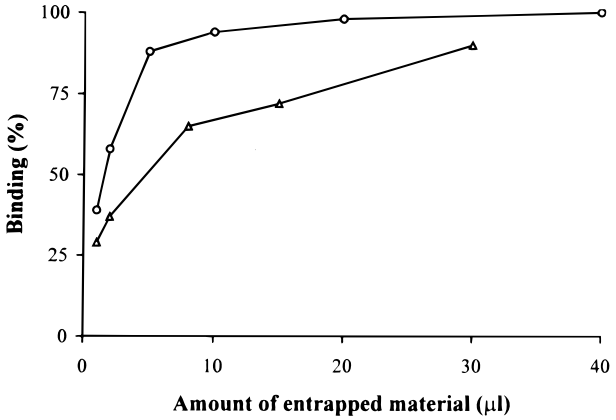

Figure 3. Comparison of the binding of DNPH (20 ng) to sol−gel columns (1:8 + PEG) doped with anti-DNP whole antiserum (1−40 μL, corresponding to 0.063−2.02 mg of total protein or 1.5−60 μg of IgG) (○) or purified IgG (1−30 μL corresponding to 1.5−44 μg) (△). The extent of nonspecific binding did not exceed 10%.

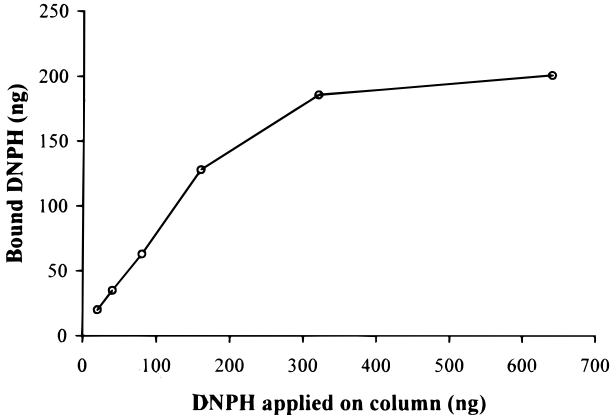

Figure 4. Binding capacity of sol−gel (1:8 + PEG) entrapped anti-DNP antiserum. Binding was determined with a constant amount of whole antiserum (20 μL, corresponding to 30 μg of IgG) and 20−640 ng/mL of DNPH. The extent of nonspecific binding did not exceed 6%.

on different biomolecules. In a previous study with anti-atrazine Mab hybridoma culture fluid and its purified IgGs, we found that specific and nonspecific binding at high (1.3−2.6 mg) and low (in the microgram range) protein contents were identical, i.e., the nonspecific proteins did not impair binding, but did not have any "protective" effect on the Abs.[26] Contrary to these results, an increase in the ee-AChE content (from 1.8 to 36 μg) reduced activity by 90%.[23] It may very well be that the variations in the intrinsic properties of the esterase and Ab molecules (e.g., different overall structural conformations, different structure of the active site, possible variability in polarity under entrapment conditions) cause these molecules to react differently to the gelation process and the matrix properties. Such differences may have a major impact on the overall activity of the entrapped biomolecules and this must be taken into consideration in any entrapment.

Determination of binding capacities with respect to DNPH load on the column (20−640 ng) at a constant whole antiserum amount (20 μL, corresponding to 30 μg of IgG) revealed dose dependency and a maximal binding capacity of 200 ng of analyte (Figure 4). The

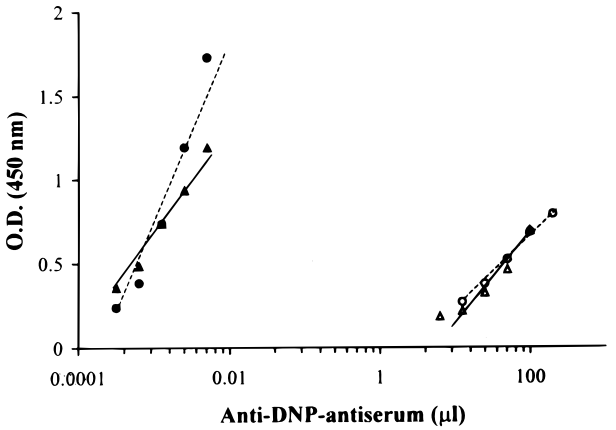

Figure 5. Leaching of anti-DNP antiserum from the sol−gel matrix. Anti-DNP whole antiserum (20 μL) was entrapped in sol−gel and washed with the eluting solution as described in the Experimental Section. Ab titers in the eluate were determined by ELISA. Four serial dilutions of the concentrated ethanol eluate (ranging from 1:2 to 1:16 and representing 100, 50, 25, and 12.5 μL) and five serial dilutions of the concentrated glycine eluate (corresponding to 100, 50, 25, 12.5, and 6.25 μL) were used to determine the amount of leached Ab. The amount of the leached Ab present in the glycine eluate (△) and the ethanol eluate (○) was determined from calibration curves of anti-DNP whole antiserum (▲ and ●), for glycine and ethanol experiments respectively, composed of five serial dilutions (ranging from 1:40 000 to 1:640 000 and representing (5−0.312) × 10^{-3} μL of the anti-DNP whole antiserum.

degree of nonspecific binding (to columns containing an equivalent amount of NRS) did not increase with the increasing amount of applied DNPH and did not exceed 6% of the initial amount.

An important aspect of the development of any IAP method is the ability to elute the analyte. Bound DNPH was eluted from sol−gel-entrapped whole antiserum with either 0.1 M glycine buffer at pH 3.5 or absolute ethanol. Under these conditions, elution with glycine buffer resulted in a recovery of 69% and that with ethanol in 91%. The ability to elute an analyte from sol−gel-entrapped Abs was also proved with anti-atrazine Mabs. Elution of atrazine was performed with high- and low-pH buffers (0.1 M triethylamine buffer at pH 11.5 and 0.1 M glycine buffer at pH 3.5) and resulted in recoveries of 86% and 69%, respectively.[26]

Another important aspect of the development of an IAP method is the ability to maintain the Abs firmly attached to the supporting matrix. Theoretically, leaching can be a much more severe problem in sol−gel than in other methods because of the high porosity of the matrix and the fact that Abs are not covalently bound to it. In a previous experiment we found that extensive washing of anti-atrazine sol−gel-entrapped Mabs with PBS resulted in negligible leaching which, in most cases, was below the detection limit of the assay.[26] In the present study we tested the effects of the eluting buffers and solvents on leaching. The degree of leaching of the entrapped Abs was evaluated with sol−gels doped with 20 μL of whole antiserum (30 μg IgG), and columns were washed with 10 mL of the eluting buffer or solvent. Figure 5 shows that the amount of leached Ab was very low (0.05% and 0.12% of the Ab entrapped in the sol−gel matrix, for washing with 0.1 M glycine buffer, pH 3.5, and absolute ethanol, respectively). To evaluate this

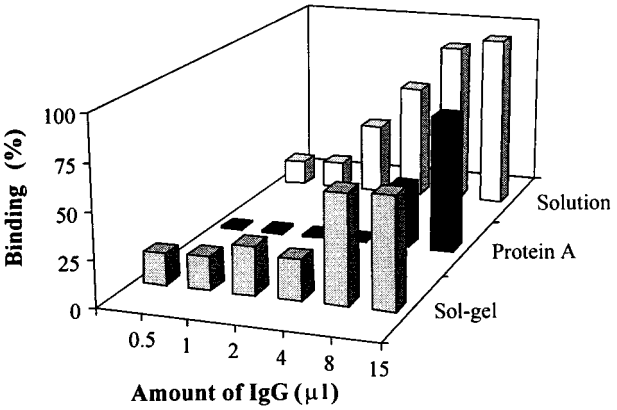

Figure 6. Comparison of DNPH binding by sol−gel-entrapped anti-DNP IgG with that by IgG in solution and IgG coupled to protein A−agarose beads. The same amounts of IgGs (0.5−15 μL or 1−30 μg) were used for entrapment in the sol−gel (1:8 + PEG), binding to protein A−agarose beads, and in solution. DNPH (20 ng) was incubated with the doped sol−gels, protein A−agarose beads, or the IgGs in solution for 15 min at rt. The extent of nonspecific binding to the sol−gel columns did not exceed 10%.

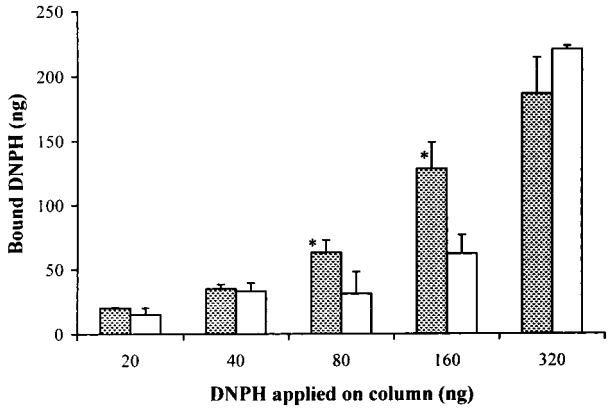

Figure 7. Comparison of the binding capacity of sol−gel (1:8 + PEG) entrapped anti-DNP antiserum (shaded bars) with that of protein A−agarose-coupled IgG (open bars). A constant amount of whole antiserum (20 μL, corresponding to 30 μg of IgG) was used. Binding was monitored with 20−320 ng/mL of DNPH. Values, at each concentration, represent the amount bound (in ng) ± SD of three to six repetitions. The extent of nonspecific binding to the sol−gel columns did not exceed 6%. An asterisk (*) indicates a significant difference in DNPH binding at $P < 0.01$.

limit we found that the amount of the Ab present in 50 μL of the glycine eluate or 25 μL of the ethanol eluate (out of a total volume of 1 mL eluate) corresponded to 0.0005 or 0.0006 μL of whole antiserum, respectively. In a previous study, performed in our laboratory with anti-atrazine Mabs, we found that leaching was very low (0.4%) even when sol−gels were kept at rt for 53 days.[26] This observation agrees with practically all studies on sol−gel entrapment of proteins which have shown that leaching is either negligible or zero.

IAP methods have long been used for the purification of many compounds.[5] The most commonly used approach in IAP uses an Ab covalently linked to protein A−agarose beads.[33] To be able to evaluate whether sol−gel-entrapped Abs may provide an alternative approach to the existing IAP methods, it was necessary to compare the binding properties and capacity of the sol−gel-entrapped Abs with protein A−agarose covalently coupled Abs.

IgGs were covalently coupled to protein A−agarose resin with DMP as a cross-linker. The binding capacity of the bound IgG was compared with that of sol−gel-entrapped IgG for a constant amount of DNPH (20 ng). Figure 6 clearly shows that binding of DNPH to the sol−gel entrapped Abs was higher at almost all tested IgG doses, except at 15 μL, where binding to sol−gel-entrapped IgG was slightly lower than that to the protein A−agarose IgGs (12 compared with 15 ng of applied DNPH, respectively). Comparison of the binding capacity of a constant amount of Abs (30 μg) cross-linked to protein A−agarose beads with that of sol−gel-entrapped Abs at different DNPH concentrations revealed that the binding capacities of the sol−gel Abs are either significantly higher (at 80 and 160 ng of DNPH) or do not differ significantly (at 20, 40, and 320 ng of DNPH) from those of the protein A−agarose-bound Abs (Figure 7).

The binding of immobilized IgGs (in either sol−gel or protein A−agarose) was also compared with that in solution. Analysis was performed with a constant amount of DNPH (20 ng) and various amounts of IgGs (0.5−15 μL, corresponding to 1−30 μg of protein). Figure 6 shows that binding of DNPH to protein A−agarose IgGs was much lower than that in solution: practically no binding was noticed at small Ab amounts (0.5−4 μL), and at 8 μL it was 2.7-fold lower than that in solution (Figure 6). The lower binding of the protein A−agarose Ab may result either from inactivation of binding sites by the cross-linker (used to link the Ab covalently to the protein A) or from inaccessibility of the analyte to the Ab's binding site due to spatial hindrance of the matrix.

Unlike the protein A−agarose bound IgG, sol−gel-entrapped IgGs exhibited only a moderate loss in binding capacity compared with that in solution (by a factor of 1.5−3 at 2 − 8 μL, respectively) (Figure 6). The lower overall binding of the sol−gel-entrapped IgGs indicates that there was a smaller number of active sites of the Ab molecules accessible to the diffusing antigen molecules or that the Ab molecules entrapped in the sol−gel matrix exhibited lower affinities toward the antigen. This may be due to enclosure of the binding site within the cage in an orientation that prevents analyte access to the active site, because of physical constraints imposed on the molecule by the considerable shrinkage of the sol−gel during its formation, and possibly also as a result of denaturation of molecules during the polymerization and gelation processes. The loss in binding capacity, however, was relatively small, and this in itself is of particular interest in view of the fact that Ab molecules are relatively large (MW 150 000) and their binding activity depends heavily on the proper stereochemical conformation. Similar results were obtained with sol−gel-entrapped anti-atrazine Mabs, for which atrazine binding was 1.4−2.3 times lower than that in solution.[26] It should be noted, however, that the current problems of reduced binding may not be inherent and may be avoided by the use of other sol−gel formats or preparation procedures.

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IAP is currently considered one of the most powerful techniques for purification and isolation of many compounds. The advantages gained from this approach have led to the implementation of this method for purification and preconcentration of proteins, hormones, drugs, and other compounds, from animal and plant tissues, for research and biomedical applications. [5,34]

Recently, IAP has also been emerging as a highly convenient and reliable method for purification and concentration of residues from agricultural and environmental samples.[35] A few selected examples of the application of this approach include purification and concentration of aflatoxins from body fluids and food,[36] atrazine from water samples[37] and plants,[38] phenylurea from plants,[39] polycyclic aromatic hydrocarbons from soil,[40] and clenbuterol from liver and meat.[41] The simplicity of the IAP method in terms of sample manipulations, the reduction of the use of organic solvents, and the compatibility of the method with the emerging EIA and with on-line liquid chromatography and mass spectrometry make this method highly convenient for agricultural and environmental applications.

The increasing applications of IAP create the general need to improve and simplify the method and to reduce its cost. Application of the method to agricultural and environmental samples (as opposed to medical applications) also requires better protection of the Abs and development of highly efficient methods for removal of matrix-interfering components. These special needs emerge from the crude, complex, and diverse nature of the tested samples that in most of the cases are heavily pigmented and contain denaturing factors and organic solvents which may damage the Ab, interfere with the formation of the Ab−analyte complex, and increase background and nonspecific binding. Currently, protein A/protein G-coupled resins are among the most commonly used IAP procedures. These resins have long been found to provide the easiest and most useful means for covalently binding Abs to a solid phase. This is mainly due to the good Ab orientation in a matrix that allows maximal interaction with the antigens. Despite the advantages of these resins, coupling of Abs via protein A/G suffers from drawbacks which reside from the many time-consuming steps associated with the Ab coupling procedure, the partial loss of Ab binding capacity (because of cross-linking to protein A), and the high cost of the resin. Other methods which involve direct coupling of the Abs to resins (by means of activated resins or activated beads)[5,33,35] introduce even more problems such as considerable loss of binding, nonspecific interactions with the resin, and leakage.

The sol−gel technology described in the present study overcomes most of these drawbacks, offers many advantages over commonly used immobilization procedures (e.g., adsorption and covalent binding), and fulfills the requirements of an IAP method for agricultural and environmental needs. The method facilitates the simple, low-cost, and quick one-step entrapment of either IgGs or whole antiserum in a manner that does not require any preliminary Ab purification. The entrapped Abs exhibit dose-dependent, highly reproducible binding, offer capacities that are higher than or comparable with those of protein A−agarose-coupled Abs, and allow simple and quick elution of the analyte with almost no leaching. Sol−gel IAP columns can be used repeatedly without a significant loss of binding activity,[26] but the simple and quick preparation process and the use of inexpensive materials render this unimportant. Another useful and important factor is the relatively short response time (a few minutes) of the sol−gel-entrapped Abs that does not differ considerably from that obtained in solution and the commonly used IAP procedures.[26]

Another important characteristic of the sol−gel method is the enhanced stability the matrix confers on the entrapped biomolecules. In a previous study we found that the sol−gel enabled prolonged storage of Abs and enzymes at rt[23,26] and protected against damage caused by organic solvents (unpublished data). Stabilization (e.g., thermal, pH) of proteins in sol−gel matrixes is a well-documented phenomenon which has been previously demonstrated for a variety of biomolecules.[42,43] The enhanced stability is attributed mainly to the protective nature of the matrix, which reduces the freedom of peptide chain refolding and causes the denaturation and inactivation of biomolecules. 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.[44] The stability to organic solvents which the sol−gel confers on the entrapped biomolecules introduces a major advantage, as it enables elution of target analytes from sol−gel IAP columns under harsh conditions (e.g., extreme pH buffers and/or organic solvents) which are needed in cases of strong Ab−antigen interaction. The stability at rt is also of major importance, since it facilitates prolonged storage and ensures long shelf life of ready-to-use prepacked IAP columns.

Successful entrapment of Abs in a sol−gel matrix has been demonstrated by other laboratories[14,18−21,45] and its possible application as an IAP method has been suggested.[18,19,45] The studies were performed with anti-fluorescein and anti-pyrene Pabs and have shown that sol−gel-entrapped Abs retain their binding capacity, are active after prolonged storage (13 weeks), exhibit a low degree of nonspecific binding, and can be regenerated for 10 binding/elution cycles. The successful entrapment of a variety of Abs (Pabs, Mabs and their IgGs) leads to the conclusion that this is a general phenomenon that

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can be applied to the production and use of Ab-doped sol−gels as "generic reagents" with a variety of Abs to different analytes matched to a variety of applications.

The advantages offered by the sol−gel method for the entrapment of Abs and the advantages conferred by the intrinsic properties of the sol−gel matrix (highly stable, inert, and optically clear) render this method suitable and convenient for IAP and open the way for the exploitation of this method in developing highly selective biosensors/immunosensors for applications in immunochemical detection methods and in IAP for agrochemical, environmental, and biomedical fields of research.

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