Reviews

Enzymes and Other Proteins Entrapped in Sol–Gel Materials

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We review the growing field of entrapment of purified enzymes, whole cells, antibodies, and other proteins in sol–gel matrices prepared from metal alkoxides. Basic aspects of the nature of the trapping and its effects on the dopant activity are discussed, and various applicative uses of these novel bioactive materials are presented, including sensors, biosensors, electrodes, immunoadsorbents, optical components, environment-related materials, and biotechnology-related materials. Outlook of potential applications and future directions of this field are provided throughout the review.

1. Introduction

Bridging the chemistry of inorganic materials with biochemistry carries the unique appeal of creating a link between the inanimate and animate worlds. Implants made of biocompatible materials,[1] the phenomenon of biomineralization,[2] and the use of surfaces as supports for biochemicals[3] are some prominent examples of where such links have been established. The sol–gel process,[4] one of the fastest growing fields in material chemistry,[5] has opened a new route of linking ceramics, glasses, and other inorganic materials on one hand, with bioactive molecules on the other hand, forming a novel, wide-scope family of biochemically reactive materials.

One of the main properties of the sol–gel process which led us to experiment in that direction, was the very fact that it is a room-temperature procedure, such that biomolecules can withstand. The other property which was quite unexpected and actually led to the success of that approach has been that the conditions of the sol–gel process are not harsh enough to denature most of the encapsulated biomolecules. Taking into account that the monomers are highly reactive chemicals (alkoxides of semimetals and metals) capable of altering protein surfaces, taking into account that the sol–gel process involves considerable shrinkage into the xerogel, thus exerting pressure on the proteins and taking into account that the encapsulated enzymes must be oriented so that their active site is easily accessible to the pore network of the matrix, it is quite a nontrivial result that sol–gel-entrapped enzymes should show any reactivity at all. Well, they do,[6] and it is perhaps this unexpected observation that caused a burst of research activity in encapsulating enzymes (Table 1) as well as many other bioactive components. The rapid growth of this young field calls for an interim summary of the significant volume of activity that has been accumulated so far. The purpose of this brief review is to provide this outline.

The matrices described below are basically porous wet gels or xerogels obtained by the hydrolysis and condensation–polymerization of metal and semimetal alkoxides,[4] mostly but not exclusively SiO_2 materials. The detailed description of the procedures for their preparations have been well documented in this journal[7] and elsewhere[8] and will not be repeated here. However, we do list now the advantages and special properties of organically doped sol–gel matrices (for some reviews, see refs 9–13), compared to other prevalent methods of immobilization (such as embedding in plastic matrices or attachment by adsorption or by covalent bond formation to the surfaces of the (porous) materials):

Sol–gel porous matrices in general, and doped matrices in particular (a) have controllable surface area, average pore size, pore size distribution, and fractal dimension; (b) are thermally stable, much beyond the

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Table 1. Sol–Gel Entrapment of Enzymes

dopant	matrix/monomer[a]	comments	ref
	A. Purified Enzymes		
1. acetylated trypsin	SiO_2/TMOS	for comparison with trypsin, see entry 29	22
2. acid phosphatase	SiO_2/TMOS		33, 34
3. alkaline phosphatase	SiO_2/TMOS		6, 12, 33
4. L-amino acid oxidase	cellulose–TiO_2/TiiPr	in fiber form	31
5. L-amino acid oxidase	SiO_2/TMOS		57
6. aspartase	SiO_2/TMOS	highly active	6, 12, 33
7. carbonic anhydrase	SiO_2/TMOS		57
8. catalase		coimmobilized with glucose oxidase	20
9. chitinase	SiO_2/TMOS		6
10. Cu–Zn superoxide dismutase	SiO_2/TMOS	cyanide detector	24, 36
11. cytochrome *c*	ZrO_2–cellulose/$Zr(Bu)_4$	hybrid fiber	54
12. cytochrome *c*	SiO_2/TMOS	redox detector	24, 36
13. cytochrome *c* peroxidase	SiO_2/TMOS	redox detector	37
14. glucose oxidase	SiO_2/TMOS	coimmobilized with peroxidase for glucose detection by dye formation	10, 21–24
15. glucose oxidase	SiO_2/TMOS	reversible optical glucose detector	20
16. glucose oxidase	V_2O_5	electrochemical detection of H_2O_2; film-coated electrode	27
17. glucose oxidase	SiO_2–carbon/TMOS	electrochemical detector (H_2O_2 or ferrocene)	28, 30
18. glucose oxidase	SiO_2/TMOS	electrochemical detection via ferrocene	26
19. glucose oxidase	SiO_2/TEOS	electrochemical detection of O_2	25
20. glucose oxidase	TiO_2–cellulose/TiiPr	electrochemical detection; hybrid fiber	31, 32
21. glucose oxidase	ZrO_2–cellulose/$Zr(Bu)_4$	fiber	54
22. β-glucosidase	SiO_2/TMOS		6
23. hydrogen peroxidase	see entry 14		
24. hydrogen peroxidase	V_2O_5	electrochemical detection	28
25. monoamine oxidase	SiO_2/TMOS		57
26. nitrate reductase	SiO_2/TMOS		57
27. oxalate oxidase	SiO_2/TMOS	coimmobilized with peroxidase	58
28. parathion hydrolase	SiO_2/TMOS		41
29. trypsin	SiO_2/TMOS		12, 22, 33, 34
30. urease	cellulose–TiO_2/TiiPr	stable for several weeks	31, 54
31. urease	ZrO_2–cellulose/$Zr(Bu)_4$	fiber	54
	B. Whole-Cell Extracts and Whole Cells		
32. *Pseudomonas stutzeri* and *Acinetobacter johnsonii*	SiO_2/TMOS	nitrate reductant	42
33. *Thiobacilus thiooxidans*	SiO_2/TMOS	H_2S oxidation	41
34. yeast cells	SiO_2 sol	fermentation, thin films	44
35. yeast cells	SiO_2/TMOS	fermentation	45

[a] TMOS: tetramethoxysilane. TEOS: tetraethoxysilane. TiiPr: titanium triisopropoxide. $Zr(Bu)_4$: zirconium tetra-*n*-butoxide.

range of temperatures which is of relevance for organic molecules; (c) do not photodegrade and, in the case of SiO_2 matrices, are not involved in light-induced reactions with the matrix; (d) do not degrade electrochemically; (e) can be transparent well into the UV (~250 nm for SiO_2), and are highly suitable for optical applications; (f) allow control of conductivity, through the choice of the metal or metal alkoxide; (g) are open to a wide variety of chemical modifications, through the choice of the metals and semimetals, through the use of nonpolymerizable substituents[14a] and through the use of copolymerizations (Ormosils);[14b] (h) enhance the stability of the encapsulated molecules,[15] by virtue of the rigidity of the cage; (i) prevent leaching of proteins due to the effective caging; (j) are easily obtained in a variety of forms: monoliths, optically grade polished monoliths, thin films, fibers, powders, etc., can be miniaturized to microns size,[16] and can be attached to most other materials (plastic, paper, metals, etc.); (k) offer high sensitivities of interactions,[13] which are achieved through the dual action of the doped porous glass: it first acts as a concentrator because of its high capacity of adsorption, followed then by the selected reaction with the dopant; (l) enable the efficient separation of reactant molecules and other reactive species[15,17] which otherwise self-destruct by reaction (enzymatic autodigestion) or quench each other by energy transfer, charge transfer, etc.; (m) have a potential advantage over the use of surfaces in the form of adsorption or covalent bonding, in that adsorbed molecules easily leach out, and covalently attached molecules are prone to chemical degradation of the anchoring bond. The immobilization of enzymes by adsorption or covalent bonding has been extensively reviewed,[18] and the problems of leaching and degradation are well documented (e.g., in Chapter 4 of ref 18a). The protective action of the sol–gel cage solves, as we shall see below, the leaching problem of proteins and enhances significantly their stability.

It is in order to mention also the disadvantages of the sol–gel technology: First, there is the well-known problem of ceramics, namely, their brittleness; second, the narrow-pore network imposes limitations on the rate of chemical interactions, especially in macroscopic monoliths; third, the narrow-average-pore size does not allow yet the interaction with large (bio)polymeric substrates;

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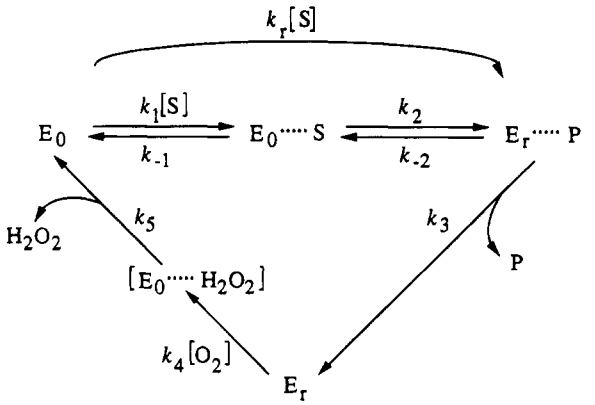


Figure 1. Reaction network of glucose enzymatic oxidation. Here, E_0 is the oxidized enzyme, S is glucose, E_r is the reduced enzyme, P is the product, and the various k's are rate constants. Irreversible chromogenic detectors utilize the regeneration of E_0 from E_r with oxygen, which is reduced to H_2O_2. H_2O_2/peroxidase is then used to form a dye from various nitrogen-containing compounds. Alternatively, H_2O_2 can be detected electrochemically. The first chemical change that occurs in the network is the reversible formation of the complex E_r-P. A new reversible optical sensor base on that step was constructed[20] (see Figures 3–5).

and fourth, although enzymes are firmly encapsulated with zero leachability, for some small encapsulated molecules leachability is yet to be improved.[19] It should be noted, however, that all of these disadvantages are solvable, and intensive work is being carried out by us and in many other labs in these directions.

In the next section, we summarize the results obtained with glucose oxidase, the most studied enzyme in the sol–gel context. We then continue with phosphatases, trypsin, and other purified enzymes (section 3), summarize the entrapments of cell extracts and whole cells in section 4, continue with the sol–gel entrapment of immunoglobulines in section 5, and conclude with various other proteins in section 6.

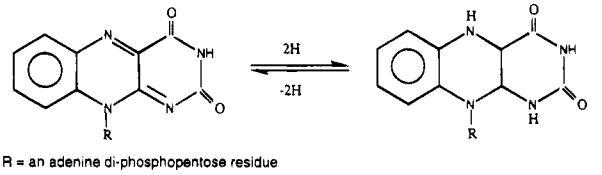
2. Sol–Gel-Entrapped Glucose–Oxidase

The development of disposable glucose sensors is part of an ongoing effort for decentralization of medical diagnostics. This and the high stability, high activity, and low cost of glucose oxidase (GOD) make it a good model for the investigation of photometric and electrochemical biosensors.

GOD is a 154 000-dalton enzyme, capable of catalyzing the oxidation of D-glucose (actually, of β-D-glucopyranose) to D-δ-gluconolactone, for instance with oxygen, which in turn is reduced to hydrogen peroxide. The redox action of that enzyme is through two prosthetic flavin adenine dinucleotide (FAD) groups. This coenzyme is capable of reversible dehydrogenation–hydrogenation which takes place on the isoalloxazine system (see Scheme 1).

On the basis of these reactions and the biotransformations shown in Figure 1, the detection of glucose can be done in a number of ways: Applying the released H_2O_2 in a secondary oxidation reaction which involves the formation of a dye molecule; following oxygen, H_2O_2 or reaction mediators electrochemically; and, following directly the absorption changes of the isoalloxazine. The

Scheme 1



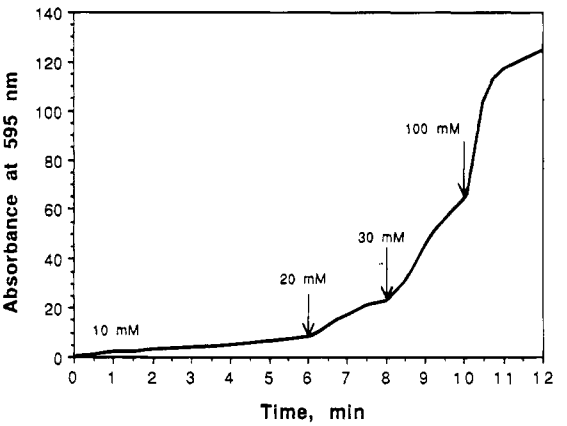


Figure 2. Sensing of glucose by SiO_2 sol–gel-entrapped glucose oxidase. The sensing is indirect and is based on the formation of H_2O_2 (Figure 1). Coimmobilized peroxidase catalyzes an oxidative H_2O_2 coupling reaction of two aza compounds, forming purple indamine, the absorption of which is followed at 595 nm. The response of the sensor to addition of various amounts of glucose, is shown.

latter is a novel approach, developed recently by Braun et al.,[20] and is detailed below.

Several research groups have applied the H_2O_2-based colorimetric method, using coimmobilized peroxidase, the function of which is to catalyze the oxidation of suitable substrates to dyes with the released H_2O_2. A typical procedure for the encapsulation of enzymes which retains their activity is that which was aimed at the construction of a low-cost disposable irreversible glucose sensor:[21,22] GOD and peroxidase were coimmobilized in an SiO_2 sol–gel matrix by dissolving the enzymes in water and shaking it with tetramethoxysilane (TMOS) to which a small amount (0.5%) of poly(dimethylsiloxane) was added. The resulting gel was dried at slightly elevated temperature for 2 weeks. It was then impregnated in a solution of 3.3 mM 3-(dimethylamino)benzoic acid (DMAB) and 2.0 mM 3-methylbenzothiazolinone hydrazone chloride (MBTH) in 0.1 M phosphate buffer at pH 6.5 and then air dried. A purple indamine dye forms upon exposure to O_2 and glucose, which can be followed photometrically by recording the absorbance of the transparent glass at 595 nm. Figure 2 shows the response of a 8 × 2 mm disk to the addition of glucose. Alternative H_2O_2/peroxidase colorations have been used as sol–gel glucose sensors. These include the oxidative dye formation from 3,3′-dimethoxydiphenylhydrazine, aminoantipyrine plus hydroxybenzenesulfonate and *o*-dianisidine.[23,24]

An important aspect of all these systems is the demonstration that **multienzymatic** reactions can be performed successfully in a single sol–gel matrix.

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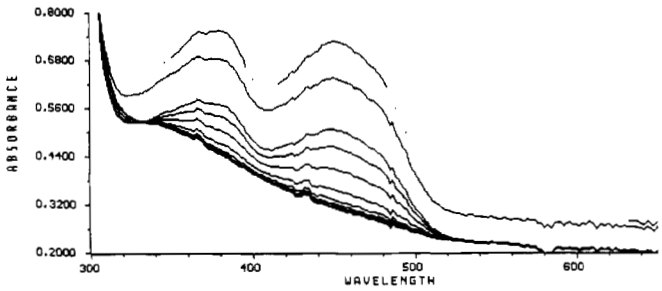

Figure 3. Reduction of FAD (second line from top) to FADH (bottom line) in sol–gel-entrapped glucose oxidase. Shown are spectra recorded at 1 min intervals by immersing the bioactive porous glass in 30 mM glucose. The top line is the absorption in water.

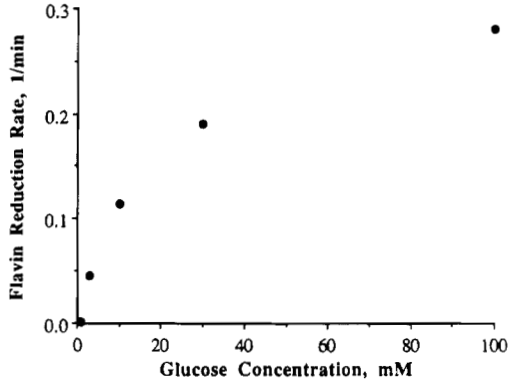

Figure 4. Initial rates of FAD reduction as a function of glucose concentration. The dynamic range of 1–100 mM is of relevance for the detection of human diabetes.

This classical enzymatic system for glucose detection must utilize the complete network of reactions and requires therefore the involvement of O_2 and of detecting reagents for H_2O_2, as summarized in Figure 1. Difficulties arise therefore from the need to saturate with O_2, from the inability to remove readily the colored products by rinsing (due to strong chemisorption), and from the need to regenerate secondary enzymes, such as peroxidase. Consequently, this approach is limited in its use to irreversible, integrating sensors. However, as seen in Figure 1, most of these difficulties can be overcome, if detection of glucose would be limited to the complex E_r–P, without continuing with the oxidation itself. We recall that part of the aromatic character of the isoalloxazine moiety in FAD is destroyed upon reduction, blue-shifting the absorption spectrum of the prosthetic group from the visible (yellow color, 450 nm) to the UV (colorless) (Figure 3). It is on that reversible colorimetric change, which does not require O_2, that Braun et al. based the recyclable and reversible optical sol–gel glucose sensor,[20] which revealed some remarkable properties: the analytical dynamic range is 1–100 mM (as needed by medical requirements); the thermal stability is high (40 °C for 8 h); the chemical stability (such as to added H_2O_2) is high; the leachability of the enzyme is zero; and perhaps most promising, the sensor was stable in daily use for at least half a year. The wide range of glucose concentrations detectability was achieved by calibrating against the initial rate of FAD reduction, as shown in Figure 4. The duration of one cycle, which includes determination of the initial slope of color decay

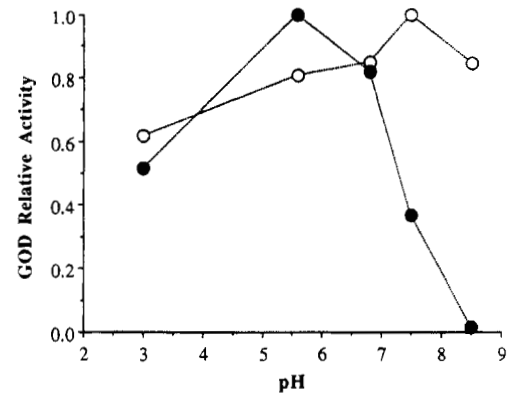

Figure 5. Entrapped glucose oxidase shows higher stability to pH changes (○) compared to solution values (●).[20]

and restoration through flushing with water, is about 2 min. Blood proteins are too large to enter the fine pores of the glass and therefore do not interfere: the response of citrate-stabilized whole blood containing glucose at various concentrations was comparable to that of aqueous solutions. The stabilizing effects of the SiO_2 sol–gel matrix were also apparent in the relatively weak sensitivity of the activity to pH changes (Figure 5), compared to aqueous solutions. Finally, it was found that the specificity of the encapsulated enzyme is reduced and that the reductive complexation occurs with other hexoses such as mannose and galactose. For a full account of the properties of this glucose sensor, see ref 20.

The versatility of the doped sol–gel matrices was revealed also in the construction of electrochemical glucose sensors. Tatsu et al.[25] constructed the first sol–gel glucose flow injection analyzer using an amperometric sensor. The sensor was comprised of TEOS-derived silica–glucose oxidase powder which was attached to an oxygen electrode by a nylon net and a cellulose membrane. Oxygen depletion was used for quantitation of the converted glucose.

Mediated redox electrodes are gaining importance in amperometric biosensing. These sensors utilize an immobilized redox compound (e.g., ferrocene or hexacyanoferrate) which serves as an electron acceptor and affords an oxygen-independent signal. The reduced mediator is regenerated by the anode and the exchanged Faradaic current is proportional to the amount of converted glucose. Audebert and Sanchez constructed a ferrocene-mediated sol–gel biosensor by two successful methods:[26] a two-stage sol–gel preparation procedure using TMOS, and three gels prepared from commercial colloidal silica of various particle sizes. When this gel was kept in wet conditions, it maintained excellent activity and permitted good mobility of the analyte and the chemical mediator. The authors show that more than 80% of the GOD remained active in the gel and that the Faradaic response of the electrode agrees well with theoretical calculations based on this activity.

Motivated by the need to produce stable bioactive silica films on conducting supports Lev et al. used two alternative approaches for the production of sol–gel-

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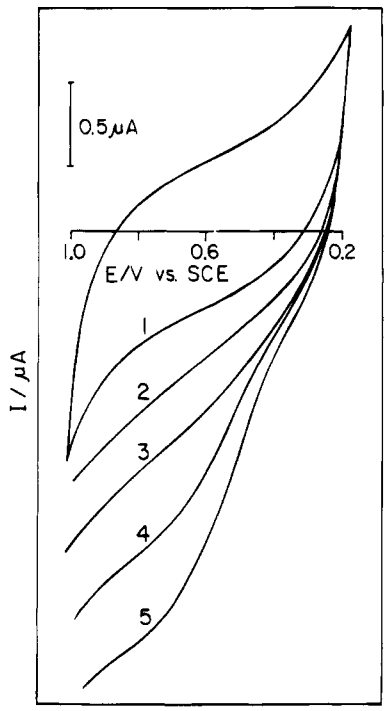

Figure 6. Voltammograms of Pt wire coated by glucose oxidase–vanadium pentoxide thin film. (1) Blank solution (0.1 M, pH = 5.6 phosphate buffer); (2) 1.2 mM; (3) 3.5 mM; (4) 5.5 mM; (5) 8.3 mM glucose solutions. Scan rate: 10 mV/s.

derived amperometric biosensors.[27,28] Thin films of glucose oxidase doped vanadium pentoxide (V_2O_5) was prepared from colloidal suspension of V_2O_5 doped with V^{4+} exhibits good electrical conductivity and adheres well to platinum and other conductive supports. This and the ability to intercalate organic molecules[29] made it suitable as a supporting matrix for active proteins. Typical cyclic voltammograms of this glucose sensors are shown in Figure 6. Sensing in this case is via the produced hydrogen peroxide which is electrooxidized on the metal support. A similar procedure was also used to prepare hydrogen peroxidase vanadium pentoxide biosensor.[28]

Another approach is to improve the stability of bioactive proteins in the hostile environment encountered during the formation of thick bioceramic films is the use of hybrid materials. Lev et al.[28,30] used sol–gel-derived composite silica–carbon electrodes. The composites benefit both from the porosity and rigidity of the silica matrix and from the electrical conductivity of the graphite; a variety of electroanalytical applications has been demonstrated.[30] For the glucose sensor preparation GOD was first adsorbed on the surface of the carbon powder and then used for the preparation of the composite sol–gel film on glassy carbon electrode. Cyclic voltammograms of unmediated and ferrocene-mediated silica–carbon glucose electrode are shown in Figure 7.

A similar approach was reported in an interesting study by Kurokawa et al.,[31,32] who formed glucose

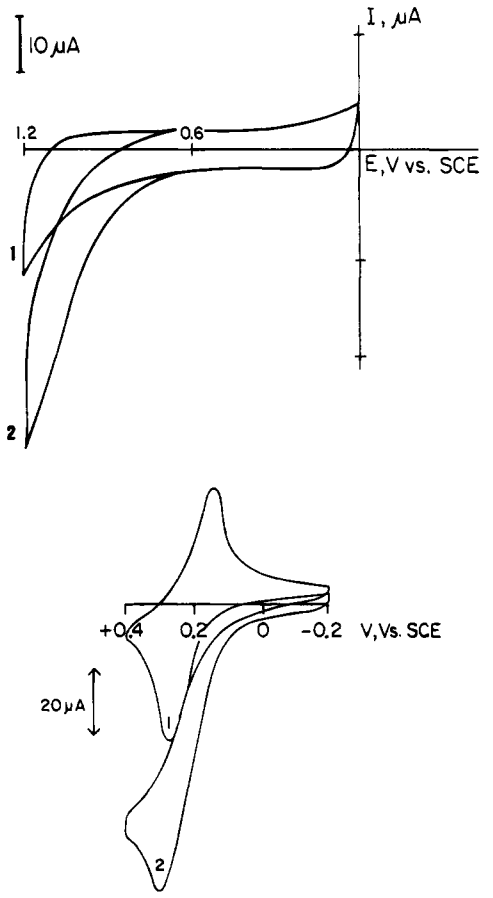

Figure 7. Top: voltammogram (10 mV/s) of glucose oxidase encapsulated in a composite SiO_2 sol–gel/carbon electrode. (1) Blank solution, as in Figure 6; (2) 9.8 mM glucose. Bottom: voltammogram (10 mV/s) of ferrocene-mediated glucose biosensor in phosphate buffer pH 5.6 (1) before and (2) after addition of 10 mM glucose solution.

oxidase doped sol–gel composite fibers made of cellulose and titanium propoxide. They found that the cellulose increases the stability of the hybrid material and that the resulting fiber is stable in various solvents, in high ionic strengths and over a wide pH range (3–10).

3. Sol–Gel Entrapped Phosphatases, Trypsin, and Other Purified Enzymes

Much of our early understanding of protein/sol–gel interactions was gained by studying the phosphatases as model enzymes. We briefly summarize here some of the main observations.[6]

Alkaline phosphatase was trapped in dried SiO_2 xerogel (for details of the procedure, see ref 6) and its dose–response curve determined for the phosphate bond cleavage of *p*-nitrophenyl phosphate (Figure 8a). As seen in Figure 8b, the response curve does not fit a Michaelis–Menten model, which predicts

$$V = -K_m V/[S] + V_{max}$$

where V and V_{max} are the enzyme activity and maximal activity, respectively, K_m is the Michaelis–Menten constant, and [S] is the substrate concentration. (It should be noted, however, that GOD trapped in *wet* gel does obey this equation.[20,23]) Figure 8b is actually a reflection of a distribution of activity efficiencies of the individually trapped molecules. These variations in activity may be the result of different accessibilities of the individual active sites to the pore network in which

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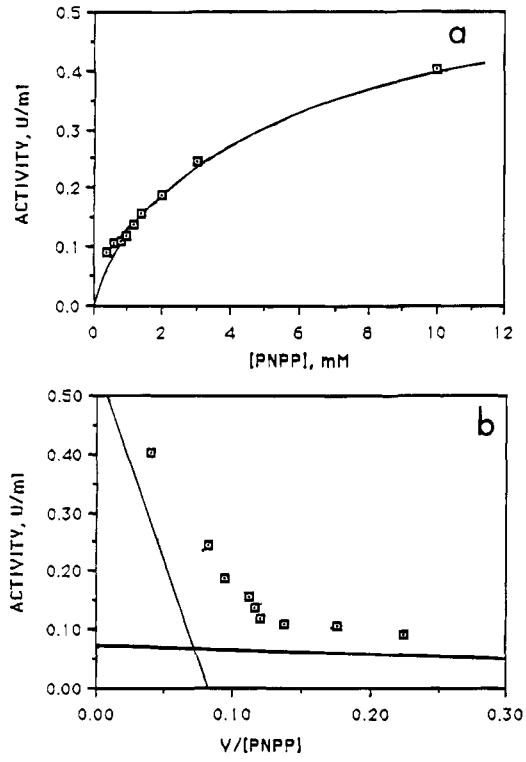

Figure 8. (a) Activity of alkaline phosphatase embedded in dried SiO_2 gel. Formation of *p*-nitrophenol from its phosphate ester is shown. (b) The activity does not fit the Michaelis–Menten equation (solid lines), but approximate domains of high affinity and low affinity (small and high slope angles) are shown.

the substrate resides, or the result of various degrees of denaturation. For convenience of discussion, however, we divide the plot in Figure 8b into two domains, representing the initial (low affinity) and final (high affinity) slopes of the curve. The high-affinity end has a K_m value of 0.8 mM, similar to the K_m value of the phosphate in solution. About 10% of the trapped molecules belong to this unaffected region of activity. The rest have a much higher K_m value (lower affinity) of about 7 mM. However, there is an important tradeoff for this reduction in activity: The trapped enzyme is more stable than the soluble form, both to temperature and to basic environments (the half-life time at 70 °C and pH 9.0 was 2.6 min for the soluble forms but 4.7 min for the trapped form), and it retained its full activity for at least two months. We attribute this enhanced stability to the protective nature of the cage itself and to the rigidity of the SiO_2 matrix, which reduces the freedom of peptide-chain refolding molecular motions which occur in denaturation processes. An even more pronounced protective effect was observed in the case of acid phosphatase:[33] Whereas in solution the half-life time at 70 °C (citrate buffer 0.1 M, pH = 5.6) was less than 0.1 min, in the glass (prepared in the presence of polyethylene glycol 400 and NaF)[34] the half-life time jumped by more than 2 orders of magnitude to 12 min. This is a particularly remarkable observation, as acid phosphatase is known to be much less heat stable than is alkaline phosphatase.

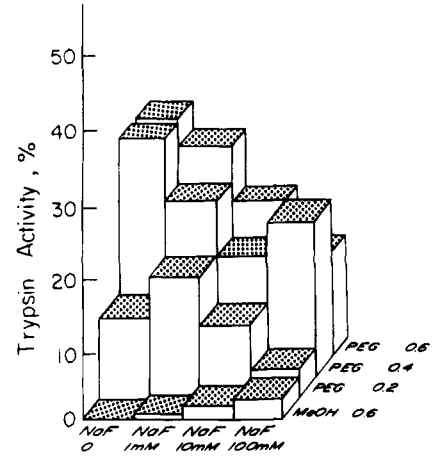

Figure 9. Effects of additives used in the SiO_2 sol–gel matrix preparation on trypsin activity. NaF, methanol, and poly(ethylene glycol) are shown. The porous glasses were incubated overnight at pH 4 and 25 °C, and the activity (in terms of percentage of activity in solution) was determined on the amide *N*-benzoyl-L-arginine-*p*-nitroanilide (0.7 mM, pH 8.0).

Another model enzyme on which we carried out detailed analyses was trypsin.[12,22,33,34] Some of the more interesting observations with that enzyme are as follows:

As perhaps expected, specific conditions of the gel preparation conditions have marked effects on the ensuing activity of the entrapped enzyme. A typical result of the effects of two additives, alcohol and NaF, on trypsin activity (release of *p*-nitroaniline from *N*-benzoyl-L-arginine-4-nitroanilide) is shown in Figure 9. It is seen that while methanol is detrimental to the activity, the replacement of this alcohol, which is used in many sol–gel preparation procedures, with poly(ethylene glycol) 400, solves much of that problem (although the inherent release of methanol during polymerization is still there; only by using alcohol-free silica sol for gel formation, one can overcome this problem). In any event, the maximum activity in Figure 9 is about 45% of the free enzyme, which is quite satisfactory, taking into account the advantages gained by the encapsulation. NaF, an additive used in some sol–gel procedures,[35] is seen in Figure 9 to be of help only in the case of methanol but not in the case of poly(ethylene glycol). Eady–Hofstee plots of the immobilized trypsin still showed concavity, although to a lesser degree, compared to alkaline phosphatase.[34]

The degree of loading of the matrix has a marked effect on the specific activity, as shown in Figure 10. The observed decrease of activity with increase in concentration can be attributed to aggregation of the protein. However, the complexity of the system was evident when nonactive bovine serum albumine was added so that the total protein concentration did not change (Figure 10): an unexpected **increase** in activity was observed, especially significant at the lower enzyme concentrations. In fact, an activity of close to 100% was obtained at a ratio of 20:1 between the nonactive and active proteins.[34] One can speculate that these mixed-protein aggregates have not a homogeneous distribution but an envelope of the trypsin on an albumin core.

Two experiments, which we regard an important, showed that the degree of activity of the trapped enzyme

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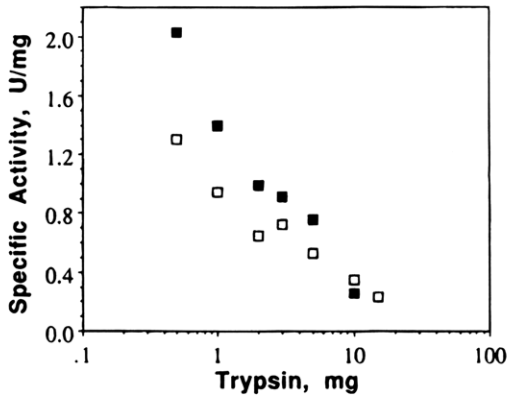


Figure 10. Effect of the loading degree of the matrix with trypsin on its activity. Some glasses (SiO_2 sol–gel with poly(ethylene glycol) 6000 as an additive) contained only the enzyme (□) while in others (■) the total protein load was brought to a constant 10 mg/g glass by adding bovine serine albumine.

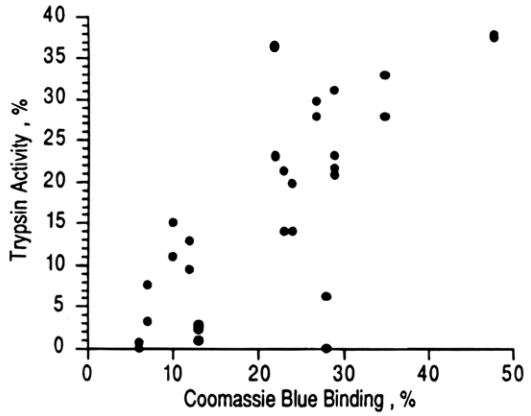


Figure 11. Correlation between trypsin activity (as in Figure 9) and the binding of the entrapped enzyme with Coomassie blue. For experimental details, see ref 33. The correlation is actually between the accessibilities of the enzyme to the amide (Figure 9) and to the blue dye molecule.

is directly correlated to its molecular accessibility. In the first experiment,[33] samples with various degrees of trypsin activity (as obtained, for instance, in Figure 9) underwent enzyme staining with the dye Coomassie blue (which binds to positively charged sites on the surface of the protein). Since the interaction of the dye with the enzyme is nonspecific, the correlation shown in Figure 11 seems to indicate that the prime factor in determining the activity is the molecular accessibility of this enzyme and, to only a lesser degree, denaturation processes. In a second experiment,[34] summarized in Figure 12, the efficiency of a set of inhibitors is seen to be linked to the size of the inhibitor. Indeed, the size of the largest inhibitor (~20 Å) approaches the average pore size of the matrix (30–40 Å, determined by N_2 adsorption) and therefore cannot reach much of the 700 m^2/g surface area which is accessible only through pores smaller than its size.

The absolute entrapment and lack of diffusion of the enzyme (including its nonleachability) was evident by the complete lack of autodigestion of that protein. In contrast to loosely immobilized or soluble trypsin which completely degrades itself, the sol–gel-entrapped enzyme retained its full activity when incubated at pH 7.5 and at ambient temperatures for several months (!).[34] The ability to protect differented encapsulated enzymes which are hostile to each other (of which autodigestion

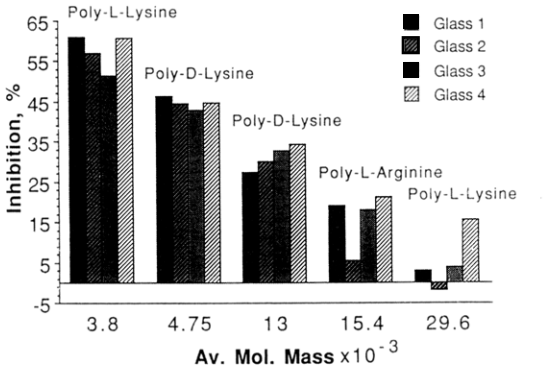
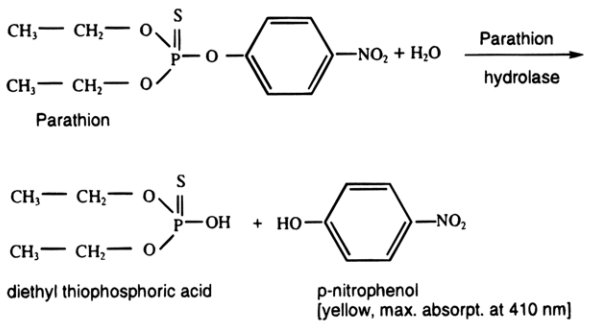


Figure 12. Accessibility of SiO_2 sol–gel-immobilized trypsin to peptide inhibitors (compared to trypsine inhibition in solution). The inhibition efficiency of the benzoyl arginine derivative (Figure 9) is governed by the size (molecular weight) of the inhibitors.[3] Poly-L-lysine (3800), poly-D-lysine (4750), poly-D-lysine (13 000), poly-L-arginine (15 400), poly-L-lysine (29 600). Four different TMOS sol–gel glasses are shown (see ref 33 for details, and Figure 9): (1, 2) methanol as cosolvent; (3, 4) poly(ethylene glycol) as cosolvent; (2, 4) NaF as additive.

Scheme 2



is a special case) is an interesting advantage afforded by the sol–gel approach, yet to be fully explored.

Two enzymatic metalloproteins, CuZn–superoxide dismutase and cytochrome *c* were studied in detail by Ellerby et al.[24,36] These authors demonstrated that the encapsulated cytochrome *c* is capable of undergoing redox processes.[24,36] Likewise, Meuret et al. encapsulated cytochrome *c* peroxidase in TMOS sol–gel matrix[37] and followed up spectroscopically the reversible changes in the oxidation state of iron, upon addition of either H_2O_2 or $Na_2S_2O_4$. Of relevance here are a number of studies on the sol–gel encapsulation of porphines,[38,39] including porphyrines.[40]

Other purified enzymes were successfully entrapped in sol–gel matrices as well. Table 1 lists all enzyme sol–gel entrapments we are aware of. In particular we draw attention to an important development which may open the field to agricultural applications, namely, the entrapment of parathion hydrolase, reported by Armon et al.[41] That enzyme detoxifies and detects that pesticide according to Scheme 2. Typical response curves are shown in Figure 13.

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(40) Fournier, T.; Tran-Thi, T.-H.; Herlet, N.; Sanchez, C. *Chem. Phys. Lett.* **1993**, *208*, 101.
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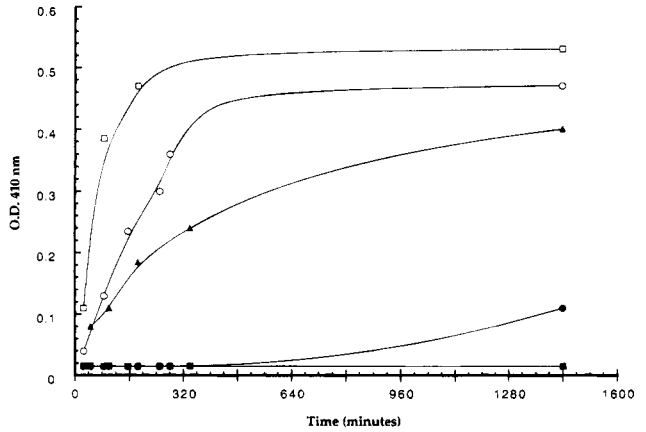

Figure 13. Enzymatic hydrolysis of parathion with SiO_2 sol–gel encapsulated parathion hydrolase. In a typical experiment, to a 10 mg/L parathion in 20 mL of a buffered pH = 8.8 solution, 1 g of the sol–gel powder containing 2.5 U/mL of enzyme was added, □. The activity of other enzyme concentrations are shown as well: (○) 1.0; (▲) 0.5; (●) 0.1 U/mL. Control: sol–gel matrix without enzyme (■).

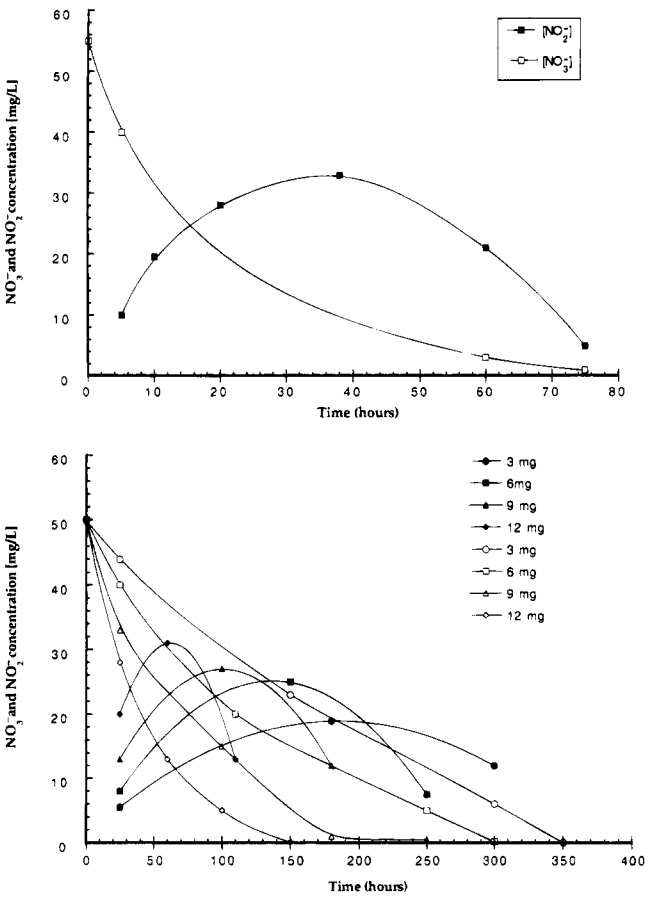

Figure 14. Top: control denitrification reaction of a nitrite solution, using cell extracts of *Pseudomonas stutzeri* and *Acinetobacter johnsonii*. In the example shown, the protein concentration was 4.2 mg/100 mL, and the NO_3^- concentration was 55 mg/L. Bottom: cell extracts of the same bacteria encapsulated in SiO_2 sol–gel at concentrations range of 3–12 mg of protein/100 mL and reacted with a 50 mg/L nitrate solution. The rate of NO_3^- decrease (open symbols) and NO_2^- buildup and decrease (black symbols) are shown.[42]

4. Sol–Gel-Entrapped Cell Extracts and Whole Cells

Another common way of utilizing enzymes is through whole-cell extracts rather than the purified enzyme. Whole-cell extracts were entrapped successfully in sol–gel matrices. An example[42] is the reduction of nitrate to nitrogen by two bacteria, *Pseudomonas stutzeri* and *Acinetobacter johnsonii*, which are the main components in anaerobic denitrification reactor. In a typical experiment, granules of the bacterial biomass were collected from a reactor fed with ethanol and $NaNO_3$. The washed granules were broken to separate cells, washed again with buffered saline, and sonicated at 0 °C, to obtain a suspension of broken cells. This was centrifuged, and the supernatant assayed by the Lowry method. The extracts were trapped in TMOS sol–gel and were used after drying for several days. The activity was monitored spectroscopically, by following the nitrate (275 nm)–nitrite (543 nm) conversion. A typical control experiment, using 4.2 mg protein/100 mL on 50 mg of NO_3^-/L, is shown in Figure 14 (top). Note that the NO_2^- curve passes through a maximum, because it is an intermediate (which is further reduced to N_2). Typical sol–gel results are shown in Figure 15 (bottom): the pattern of behavior is preserved, although it is slower. We already encountered above the slowing of reaction rates with purified enzymes. We believe, though, that this general disadvantage has good chances to be solved by careful parametrization of the matrix properties: thin films[43] and the use of Ormosils[44] are promising future directions.

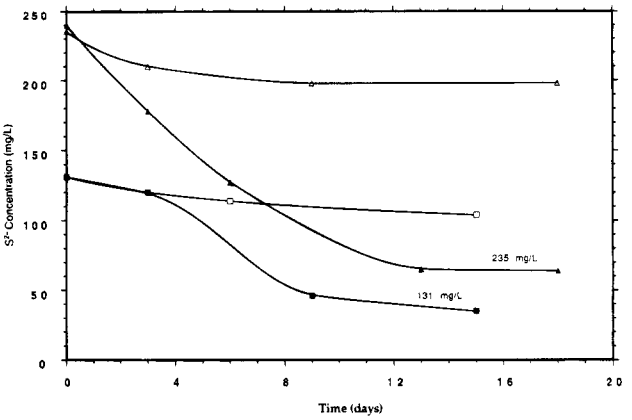

Figure 15. Removal of H_2S from water by *Thiobacillus thiooxidans* cell extracts.[42] The bacteria were grown in Vaksman medium, at 28 °C for 1 month. The cells were collected by centrifugation from large volumes of culture and subjected to sonication in phosphate buffer saline. Protein concentration in the sonicated mixture was 0.56 mg/mL as measured by the Lowry method. The sonicated cells were mixed with tetramethoxysilane (TMOS, 1:1 v/v) and left to gelate for about 1 week. The gelated glass was powdered and added to a reaction volume of 75 mL of water containing H_2S (added as Na_2S) in well-capped vials. Reduction in H_2S concentration was measured periodically against the control without the sol–gel mixture, by the iodometric method. Two experiments are shown: (▲) initial S^{2-} concentration of 235 mg/L (and the control (△) in aqueous solution); and (■) 131 mg/L (and the control, □). The controls show that H_2S itself is somewhat unstable.

In another study from the same research group,[42] the environmentally important process of H_2S removal, by the entrapment of sonicated *Thiobacillus thiooxidanse* was studied. Some typical results are shown in Figure 15 (see figure caption for details).

(42) Armon, R.; Starosvetsky, J.; Green, M., preprint, 1994.
(43) Carturan, G.; Campostrini, R.; Dire, S.; Scardi, V.; de Alteriis, E. *J. Mol. Catal.* **1989**, *57*, L13.
(44) Inama, L.; Dire, S.; Carturan, G.; Carazza, A. *J. Biotechnol.* **1993**, *30*, 197.

The third form of obtaining enzymatic activity is by the use of whole cells. The use of whole cells is quite common in biotechnology but is still not widespread in the standard chemistry laboratory as a routine synthetic method. We hope that the following successful examples in whole-cell sol–gel entrapment may bring this technology closer to the chemistry laboratory, at least from the point of view that the handling of these sol–gel powders resemble, in many aspects, the handling of "standard" laboratory chemicals.

Carturan et al.[44] and Uo et al.[45] studied the sol–gel encapsulation of whole yeast cells, *Saccharomyces cerevisiae* (as spores). Both found that alkoxysilanes cannot be used directly: Uo used prehydrolyzed TMOS,[45] and Carturan used SiO_2 sols.[44] This prehydrolysis approach, which was also used by Ellerby et al.,[36] resembles, in fact, Johnson's silica–sol encapsulation studies,[46] which predated the advent of the sol–gel metal–alkoxide polymerization procedures. Also related to our topic is the study of Carturan et al.,[43] who adsorbed yeast cells on TEOS sol–gel thin films, retaining their activity.

Finally, we mention the trapping of whole *E. coli* cells in TMOS sol–gel,[33] in which we observed that the cell are trapped as aggregates, the size of which, expectedly, increased with cell concentration.

5. Sol–Gel Entrapment of Immunoglobulines

A recent development in bioorganic sol–gel materials is their use for antibody–antigen interactions. Although still in its infancy, several encouraging reports have appeared: Livage et al.[47] have entrapped the parasitic protozoa cells of *Leishmania donovani infantum* which are antigens to specific antibodies in serum samples from infected patients. The antibody–antigen interaction was carried out by the enzyme-linked immunosorbent assay (ELISA), a very popular immunochemical method in which the antibody–antigen interaction is detected by using an enzymatic conjugate which, after attachment to the antibody–antigen complex, allows the detectability, e.g., by performing a chromogenic reaction. In the study of Livage et al.,[47] a peroxidase conjugate was used, oxidizing *o*-phenylenediamine to a yellow dye. As in above-mentioned studies,[36,43,45] it was necessary to carry out the encapsulation in a silica sol (obtained by prehydrolysis of TMOS). The demonstration that ELISA can be carried out in sol–gel system may find many potential applications in medical diagnostics.

Collino et al.[48,49] functionalized sol–gel thin films prepared from (methylamino)propyl-, propylamino-, and propylmercaptotriethoxysilanes, copolymerized with either methyltriethoxysilane or TEOS. Immunoglobulines were then reacted with the free NH or SH groups, and it was found that the activity of the immunoglobulines was retained after anchoring to the surface. In another study Wang et al.[50] encapsulated polyclonal antifluorescein with fluorescein in sol–gel matrix prepared from silica sol, demonstrating that the antibody–antigen complex is retained in the matrix, although with a 2-order-of-magnitude decrease in the affinity constant. It is briefly mentioned in that study that the entrapped antibody is capable of detecting external fluorescein, but no details are given.

In a recent study by Altstein et al.[51] on the encapsulation of antibodies in sol–gel matrices, two important aspects were achieved: It was shown that entrapped polyclonal antibodies can act as sensors to **external** antigens, which can penetrate the porous network and form the antibody–hapten complex; and it was shown that prehydrolysis of TMOS is not necessary—the "classical" direct polymerization can be used. The encapsulation of purified polyclonal immunoglobulins (IgG), binding free antigen in aqueous solutions, was performed using a rabbit anti-2,4-dinitrobenzene (DNB) polyclonal antiserum and 2,4-dinitrophenylhydrazine (DNPH) antigen which exhibits a high degree of cross-reactivity with the antiserum.[52] To reduce to a minimum the amount of irrelevant proteins which may interfere with the encapsulation and the binding properties of the antibodies, DNB IgG was purified from the antiserum using protein A agarose beads. The purified IgG was entrapped in a sol–gel matrix prepared from a mixture of TMOS/an aqueous solution of 30 mM NaCl and 10 mM sodium phosphate buffer, pH 8.2 ($^1/_5$ PBS), = $^1/_{10}$ (by volume), gelled, and dried for 3 days at low temperatures. The final protein concentration in the glass was about 1 mg/g. Binding of DNPH was performed using 1 mL (0.4 g) sol–gel columns pretreated with 1 mg of oxidized phenylhydrazine which was employed as an effective compounds for blocking nonspecific binding to the sol–gel. Binding was monitored employing three sol–gel columns: (A) an experimental column containing 0.4 mg of protein A purified anti DNB IgG; (B) a control column containing 0.4 mg of protein A purified IgG from normal rabbit serum (NRS); (C) a control column with no IgG. All columns were washed prior and post blockage with 20 volumes of 1.0 mL of $^1/_5$ PBS and 20 ng of the antigen (DNPH) were applied to each column. All three columns were washed as above, and the collected fractions from each column were analyzed for the presence of free DNPH using competitive ELISA developed by us for the anti-DNB antiserum.[52] The collected amounts of free antigen, found in the fractions from the two control columns (containing no IgG or IgG from NRS) were 19.6 and 18.7 ng, respectively, whereas the amount found in the fractions of the experimental column containing anti-DNB IgG was only 6.7 ng. The data indicated that sol–gel-entrapped antibodies retain their ability to bind free antigen from an aqueous solution.

The observations summarized in this section, while encouraging, still need to be improved.

6. Other Proteins

Bovine serum albumin (BSA), which was mentioned above in the context of sol–gel trypsin study,[34] was the subject of a detailed study by Saavedra et al.,[53] aimed at shedding further light on the nature of protein–cage

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(52) Altstein, M.; et al., to be published.

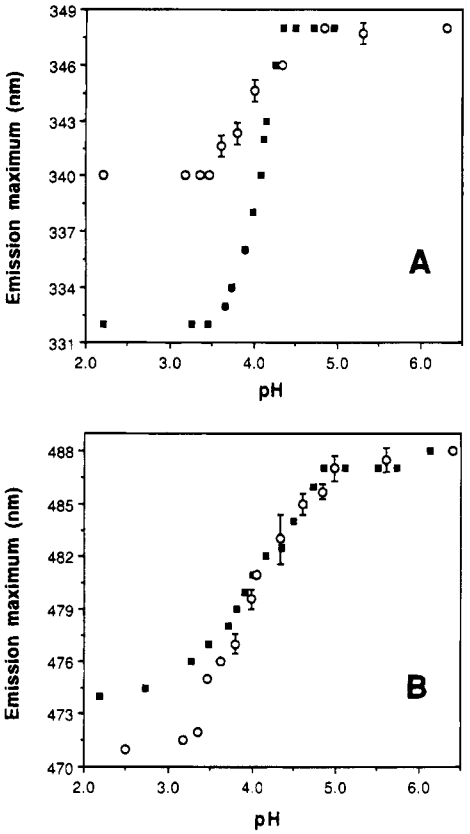

Figure 16. Analysis of structural effects of pH changes on SiO_2 sol–gel entrapped bovine serum albumine (BSA).[53] Shown (A) is the emission maximum as a function of pH for solution (■) and in sol–gel (○). The results for an externally derivatized BSA with 6-acryloyl-2-(dimethylamino)naphthalene are shown in B.

interactions, in sol–gel entrapment. For that purpose BSA was labeled with the fluorescent probe 6-acryloyl-2-(dimethylamino)naphthalene (Ac), yielding Ac-BSA. Both BSA and Ac-BSA were then entrapped in TMOS sol–gel matrices and subjected to pH changes, in order to monitor the possible denaturation of these proteins, using fluorescence spectroscopy. It was found that at neutral pH, the solution emission maxima of BSA (348 nm) and Ac-BSA (489 nm) remained unchanged upon entrapment. The behavior of the emission of these proteins as a function of pH is shown in Figure 16. It is seen that in general, the behavior in sol–gel is similar to that in solution, displaying a sharp blue shift at about the same pH, which signals the transition between two forms (the N and F forms). The solution and sol–gel system do differ in the reversibility of transition between these two forms. Full reversibility is observed in solution but an incomplete one in the gels. In a detailed discussion, Saavedra et al. argue[53] (correctly, we believe) that these observations, along with the results of a denaturating study with guanidine HCl and a fluorescence quenching study with iodide, indicate that BSA is a "hard" protein, which retains its native conformation upon entrapment.

This is contrasted with myoglobin, another protein studied in detail in ref 53, which was found to be "soft", at least in the sense that its native properties are lost upon entrapment. Myoglobin and hemoglobin were also studied by Ellerby et al.[24,36] These authors were able to retain the activity of the encapsulated myoglobin: Met-myoglobin was reduced to deoxymyoglobin, which was then shown spectroscopically to complex with O_2 and CO_2. These observation may lead to the useful development of sensors for O_2 and CO_2. Finally, Kurokawa et al.[54] have reported recently the immobilization of hemoglobin (as well as several enzymes; see Table 1) in fibers made of cellulose acetate and ZrO_2 (from Zr-(n-Bu)$_4$).

The encapsulation of bacteriorhodopsin was reported by two research groups. Weetall et al.[55] entrapped the AbN mutant and found that the light-sensitive properties are retained when immobilized under basic conditions. Wu et al.[56] performed a detailed photophysical characterization of SiO_2-entrapped bacteriorhodopsin and found that the trimeric structure, the structure of the retinal chromophore, and the proton pumping activity were not affected by the encapsulation process. The encapsulation of the photosynthetic membrane protein of bacteriorhodopsin opens the potential of inclusion of this bioactive material as a component in imaging and sensing devices. The sol–gel entrapment of phthalocyanines[40] should also be noted in that context.

7. Conclusion

Although quite young, the field of bioorganically doped sol–gel matrices has already exhibited its diversity and potential applications in many frontiers of modern materials science, including sensors, biosensors, optical materials, biocatalysts, electrochemistry, immunochemistry, and materials for use in environmental sciences. Besides the many advantages of the ceramic sol–gel matrix which led to these many developments, the field is still in its infancy, and a number of problems remain open. These include mainly the diffusional limitations inside the porous network (in the case of large monoliths), the reproducibility of results which are limited by the high sensitivity of the final matrix properties to minute variation of preparation procedures, and the brittleness of the glassy matrix. The outlook is, we believe, that these problems will find their solutions and that many more applications of this new class of materials will be described.

Acknowledgment. We are indebted to our coworkers who shared with us these exciting developments and in particular to N. Aharonson, M. Altstein, A. Bronshtein, S. Druckmann, V. Glezer, G. Gun, S. Rappoport, S. Shtelzer, M. Tsionsky, A. Turniansky, and R. Zusman. Various portions of this interdisciplinary project are funded by the U.S.–Israel Binational Science Foundation, by the Volkswagen Foundation, and by the Israel Ministry of Science and Arts (NCRD). D.A. and M.O. are members of the Farkas Center of Light Energy Conversion. D.A. is also a member of the F. Haber Research Center for Molecular Dynamics. We thank R. Armon[41,42] and J. Livage[47] for permission to report their results prior to acceptance for publication and S. Saavedra[53] for permission to adapt some of his figures.

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