Encapsulation of Enzymes, Antibodies, and Bacteria

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Abstract

The development of biotechnology requires the immobilization of active biospecies such as enzymes or cells on solid substrates. Immobilization is mainly restricted to polymers, but inorganic substrates such as silica could offer some advantages: improved mechanical strength, chemical and thermal stability, and no swelling in aqueous or organic solvents. Sol-gel glasses can be formed at room temperature, and biomolecules can be added to the solution of precursors. Hydrolysis and condensation then lead to the formation of a porous silica network in which biomolecules remain trapped. In this chapter, the sol-gel routes to bioencapsulation are reviewed, including the encapsulation via the alkoxide route and the aqueous route. Doped silica sol-gel matrices have been also evaluated in order to improve the host properties as stabilization of biomolecules, reduction of electrostatic interactions, biocompatibility, and mechanical

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properties. Some examples of enzymes and antibodies immobilization in silica gel for biosensing and biocatalysis are described. Finally, miscellaneous applications as controlled drug delivery and encapsulating bacteria using silica gel are demonstrated.

Introduction

The industrial development of biotechnology requires the immobilization of active biospecies such as enzymes or cells on solid substrates. Numerous techniques, physical adsorption, covalent grafting, entrapment, or encapsulation have been explored. But no single method or material has emerged as a generic and universal technology. Nowadays, immobilization is mainly restricted to polymers, but inorganic substrates such as silica could offer some advantages: improved mechanical strength, chemical and thermal stability, and no swelling in aqueous or organic solvents. Moreover hydrophilic silica is not toxic and biologically inert. Therefore combining fragile biomolecules with tough materials becomes a highly innovative research field, and physical encapsulation within sol-gel glasses could offer new opportunities for biotechnologies.

Sol-gel glasses are formed at room temperature, and biomolecules can be added to the solution of precursors. Hydrolysis and condensation then lead to the formation of a porous silica network in which biomolecules remain trapped. Small analytes can diffuse through the pores allowing bioreactions to be performed inside the sol-gel glass.

The first experiments showing that enzymes could be trapped within silica gels were reported by F.H. Dickey in the mid-1990s (Dickey 1955), and the immobilization of trypsin in silica gels was published about 30 years ago (Johnson and Whateley 1971). However, the interest of these papers was not realized at this time, and most work on sol-gel bioencapsulation really began almost 20 years later during the last decade of the twentieth century (Carturan et al. 1989; Braun et al. 1990; Ellerby et al. 1992). A wide range of biological species, proteins, antibodies, and even whole cells have been trapped within sol-gel matrices during the past decade. Several good review papers report on the main achievements of sol-gel encapsulation (Avnir et al. 1994; Dave et al. 1994; Lin and Brown 1997; Wang 1999; Gill and Ballesteros 2000; Livage et al. 2001; Jin and Brennan 2002).

Sol-Gel Routes to Bioencapsulation

Encapsulation Via the Alkoxide Route

The Usual Two-Step Process

The ability to form hybrid organic–inorganic materials under aqueous, room-temperature conditions (at which proteins and cells are active) opens up the possibility to extend sol-gel processing to the encapsulation of biologicals. Like organic

dyes, biomolecules could be added to the solution of alkoxide precursors before hydrolysis and condensation. However, the gentle conditions associated with sol-gel chemistry are still too harsh for most proteins, and the sol-gel process has to be slightly modified in order to fit the requirements of biology.

Water and alkoxides are not miscible so that a cosolvent, usually the parent alcohol, is currently added in order to mix both reagents and get a clear solution. However, alcohol denatures proteins so that such a solvent should be avoided when mixing water and alkoxides. Actually, alcohol has been shown to be an unnecessary additive in the silicon sol-gel chemistry (Avnir and Kaufman 1987). Hydrolysis occurs at the water/silane interface giving alcohol as a reaction product. The mixture then becomes rapidly homogeneous even if unstirred. Better mixing and faster hydrolysis are obtained when the preparation is sonicated before adding proteins (Ellerby et al. 1992). Hydrolysis is usually performed under acid conditions (HCl) in order to speed up the reaction and get fully hydrolyzed $Si(OH)_4$ species. The pH of hydrolyzed solutions then lies typically around pH $\approx$ 2. However, proteins are usually not stable outside a narrow pH range around pH 7, and a buffer has to be added to the hydrolyzed solution in order to increase pH above 5 before mixing with the aqueous suspension of proteins. The pH buffer being close to 7, basic conditions are obtained, condensation is quite fast, and biomolecules become rapidly trapped within the growing oxide network (Fig. 1). A porous oxide is formed, with pore diameters of the order of few manometers.

Entrapped biomolecules usually retain more than 60% of their native activity in fresh gels. However, such gels are very soft, and they have to be aged in the wet state to allow the completion of condensation reactions. Some shrinkage then occurs, and biomolecules may lose some 20% of their activity during this aging process. Gels can be kept in the wet state or dried, but drying has to be performed very carefully in order

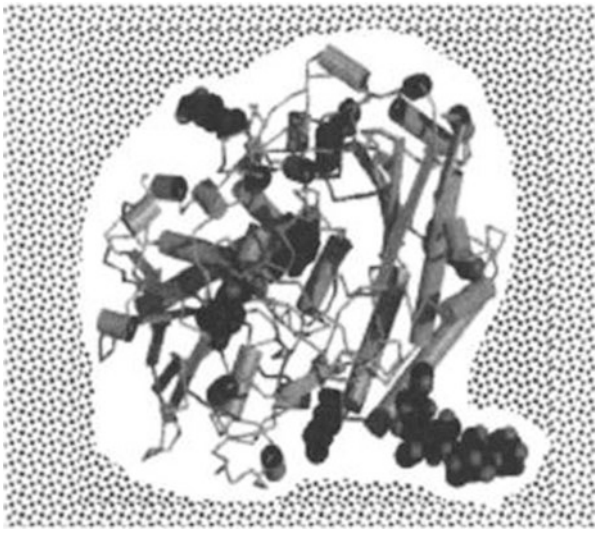

Fig. 1 Upon encapsulation, enzymes are encased in a silica cage tailored to their size

to prevent the denaturation of biomolecules. Actually most proteins require an aqueous environment to preserve their native structure, and even when dried, silica xerogels have to contain at least 30 wt% of water in their porous structure.

Overcoming the Alcohol Release Problem

TMOS, $Si(OMe)_4$, is currently used as a precursor rather than TEOS, $Si(OEt)_4$. Methanol is then released that has a polarity closer to water and is less harmful than ethanol. However, even when the neat alkoxide is used as a precursor, concentrations of methanol up to 8 M could be reached via the full hydrolysis of tetramethoxysilane $Si(OCH_3)_4$. Such an amount of alcohol can be harmful for enzymes and cells. The enzymatic activity of bovine liver catalase in a TMOS-based silica matrix actually shows a 70% reduction in the presence of 5 vol% methanol (Miller et al. 1996). It has also been shown that concentrations of methanol larger than 0.5 M lead to the lysis of *Escherichia coli* bacteria (Coiffier et al. 2001). It must therefore be eliminated before adding biomolecules.

Some authors perform hydrolysis in the presence of a large excess of water, using hydrolysis ratios $h = [water]/[alkoxide]$ up to $h = 50$. Released alcohol is then highly diluted, gelation occurs almost entirely in water, and xerogels with surface areas in excess of 1000 $cm^2\ g^{-1}$ are obtained (Conroy et al. 2000).

Evaporation or distillation of the alcohol produced during hydrolysis has been suggested as an efficient solution (Ferrer et al. 2002). However, silicon alkoxide precursors are usually not fully hydrolyzed during the first acid-catalyzed step; alcohol can still be released during condensation and aging in the presence of the immobilized species. In the so-called Biosil process, hydrolysis is performed in the gas phase so that alcohol elimination is much easier (Cappelletti et al. 1999; Carturan et al. 2001).

Silicon alkoxide precursors can also be chemically modified in order to release nontoxic by-products such as glycerol upon hydrolysis. A new class of precursors, named polyglyceryl silicates (PGS) based on polyol esters of silicates and siloxanes, have been suggested (Gill 1998). They exhibit several advantages: high water solubility, auto-hydrolysis in water without catalyst, and bioprotective alcohols such as glycerol are produced upon hydrolysis. The main drawback of this process is that PGS precursors are not commercially available and need to be synthesized first via the transesterification of partially hydrolyzed TMOS with glycerol. A solid product is formed that can be rapidly hydrolyzed and gelled in water at neutral pH. Stable transparent xerogels are obtained after washing and drying.

Improving the Host Properties: Hybrid Silica Matrices

Steric Hindrance. The use of additives during sol-gel encapsulation has been widely explored in order to stabilize biomolecules against the denaturing stresses encountered upon entrapment. Interactions between additives and proteins can be used advantageously to maximize their stability and function. Stabilization may arise from improved conformational stability or alteration of their hydration (Eggers and Valentine 2001a). Entrapment in the presence of osmolytes such as sugar or amino acids increases significantly the thermal stability and bioactivity of enzymes

(Brennan et al. 2003). Osmolytes can actually be added directly to the protein solution before mixing with hydrolyzed silica precursors. They are then involved in the modification of hydration effects, even in aqueous solutions (Eggers and Valentine 2001b). It is also possible to optimize the stability of proteins in the precursor solution, before encapsulation. This was obtained with the calcium-binding protein oncomodulin, by adjusting the level of Ca^{2+} present during entrapment. Fluorescence experiments show that the protein, entrapped in a fully folded state in the presence of excess Ca^{2+}, is partially unfolded when the level of Ca^{2+} decreases. Calcium maintains the structure and stability of the protein during entrapment that is then protected against denaturation by chemical reagents such as alcohol produced during the hydrolysis of alkoxides (Zheng et al. 1998).

Surface Interactions. Steric hindrance is not the only cause for the limited mobility of entrapped enzymes. Electrostatic interactions may also occur between silicate sites and specific residues on the protein surface. Silica surfaces are negatively charged above the point of zero charge (pH $\approx$ 3), and electrostatic interactions mainly depend on the isoelectric point (IEP) of the protein. Experiments performed with three different oxidases, glucose oxidase (IEP = 3.8), glycolate oxidase (IEP = 4.6), and lactate oxidase (IEP = 9.6), show that only glucose oxidase retains its activity upon encapsulation. Electrostatic interactions decrease the catalytic activity of the two other positively charged oxidases. However these detrimental electrostatic interactions can be reduced by complexing the enzyme with a polyelectrolyte that shields the critical charged sites. Thus, lactate oxidase can be stabilized by complexing with the weak base PVI (poly(*N*-vinylimidazole) (Chen et al. 1998; Heller and Heller 1998), whereas polyvinyl alcohol (PVA) was shown to improve glucose oxidase activity on long term (Niu and Lee 2002).

The hydrophilic/hydrophobic media provided by the host surface can also be of primary importance toward entrapped species reactivity and stability. Since pristine silica surface is of hydrophilic nature, it is possible to enhance its hydrophobic character using silicon alkoxide bearing alkyl chain (C_nH_{2n+1} $Si(OR)_3$). This approach revealed to be particularly successful for the immobilization of lipases (Reetz 1997) (see section "Entrapped Lipases as Biocatalysts").

Biocompatibility. Adding biopolymers to the mineral matrix appears as a good procedure to improve the biocompatibility of the host network. Mainly three common polymers were investigated as silica additives for bioencapsulation.

1. *Chitosan:* This polysaccharide, obtained by deacetylation of natural chitin, is polycationic at pH 7 and should be able to bind silica through electrostatic interactions. TEOS/chitosan membranes were obtained by mixing the pre-hydrolyzed alkoxide with a biopolymer solution. These hybrid membranes are pH sensitive and were shown to be suitable for selective drug permeation (Park et al. 2001). Sol-gel/chitosan composites have been used to immobilize horseradish peroxidase on a carbon electrode in order to make amperometric hydrogen peroxide biosensors (Miao and Tan 2001). Chitosan can be used as an enzyme-stabilizing host and a dispersant in order to homogeneously mix silica

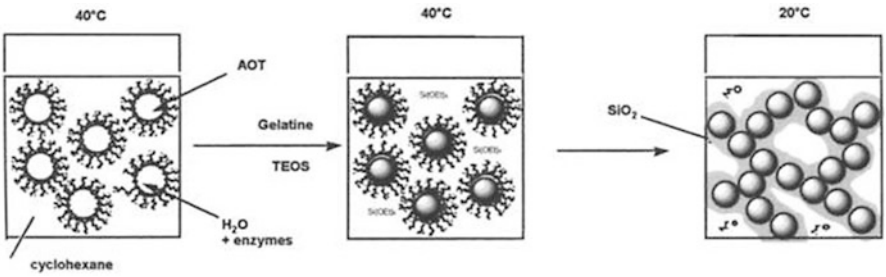

Fig. 2 Design of biocompatible gelatine/silica hybrid materials (see text for details) (Adapted from Schuleit and Luisi 2001)

with an organic polymer (Cho et al. 1997). Alternatively, amine-containing alkoxide precursors can be used to make covalent bonds with the free deacetylated amino group of chitosan through a glutaraldehyde coupling agent. When compared to the biopolymer alone, better stability in organic solvent and larger capacity for enzyme immobilization are observed (Airoldi and Monteiro 2000).

2. *Gelatine:* Depending on the way it is prepared from collagen, this protein can be positively or negatively charged at pH 7. In the first case, it can form hybrid materials when mixed with TEOS. This was nicely achieved using reverse micelles. In the first step, enzymes are immobilized within the aqueous environment of these micelles dispersed in cyclohexane. Gelatine, heated above its gel temperature, is added and also dissolves in the water droplets, whereas TEOS, diluted in cyclohexane, slowly condenses at their surface. Cooling the mixture down to room temperature leads to the formation of a gelatine network, which is further reinforced by polymerizing alkoxides, leading to a hybrid gel (Fig. 2) (Watzke and Dieschbourg 1994; Schuleit and Luisi 2001). As with chitosan, it is also possible to create covalent bonding between the biopolymer and the silica network. In this context, the 3-(glycidoxypropyl) trimethoxysilane (GPSM) precursor has been used allowing to cross-link the gelatine network via electrophilic addition on amino or carboxylic acid groups of the peptide chain (Brasack et al. 2000; Ren et al. 2001, 2002).
3. *Alginate:* The alginic acid polysaccharide bears a global negative charge at pH 7 that is usually compensated by sodium ions. Adding divalent cations such as Ca^{2+} induces the cross-linking of the polymer and therefore gel formation. Alginate gels can be obtained as microcapsules by a dropping process and can be used as immobilization matrices for cell transplantation. In a first approach, pre-formed wet calcium alginate beads were suspended in a solution of TMOS in hexane. Partial hydrolysis of the alkoxide leads to the formation of water-soluble silicon species that can permeate the alginate gel and polymerize within the capsule (Heichal-Segal et al. 1995). The gas-phase Biosil process was also applied to the encapsulation of animal cells in silica-alginate beads. In a typical microcapsule elaboration procedure, the size of alginate droplets can be controlled by flowing air at the tip of the extrusion nozzle. It was possible to

saturate this airflow with a mixture of silicon alkoxide vapors to impregnate the droplet with silica precursors. Partial hydrolysis and condensation occur before the droplet reaches the calcium chloride solution, reducing the surface tension of the solution and therefore resulting in small microspheres (200 μm) (Boninsegna et al. 2003). Alternatively, it was possible to use a mixture of TMOS and 3-aminopropyl-trimethoxysilane to form a silica layer on Ca^{2+}/alginate beads, whereas the methoxy groups of TMOS molecules can be hydrolyzed to form a silica gel. Positively charged amino groups can interact with the alginate surface to anchor the mineral deposit while allowing the addition of another alginate layer to provide a biocompatible outer surface (Sakai et al. 2001, 2002).

Mechanical Properties. Apart from the improved biocompatibility, added polymers may modify the structure and mechanical properties of silica gels. For instance, adding polyethylene glycol (PEG) to a TEOS-based silica gel leads to hybrid materials with improved optical transmittance, better resistance to cracking, and reduced pore surface polarity that enhance the enzymatic activity of entrapped lipases (Keeling-Tucker et al. 2000). Moreover, even though PEG is not a biopolymer, hybrid composites were shown to be cytocompatible (Kros et al. 2001). Similar improvements were obtained with PVA (Pierre and Buisson 2001). Silica-Nafion composite films have been recently used for making amperometric phenol biosensors (Kim and Lee 2003). It appears that adding Nafion not only overcomes the brittleness of the film but also increases the long-term stability of the sensor. Moreover, the pore size increases allowing faster response times and lower detection limit.

The Aqueous Route

As mentioned earlier, even if alcohol is not added to the precursor solution, the hydrolysis and condensation of silicon alkoxides lead to the formation of alcohol as a by-product. The aqueous route does not produce such harmful by-products and should therefore be preferred to the alkoxide route. However, the formation of aqueous silica gels is more difficult to control, and very few papers actually report on sol-gel encapsulation using aqueous precursors (Liu and Chen 1999; Bhatia et al. 2000; Finnie et al. 2000; Coiffier et al. 2001).

Aqueous sol-gel chemistry is based on the condensation of solute precursors via pH modifications (Jolivet 2000). Ionic dissociation and hydrolysis occur when a silicate salt is dissolved in water leading to more or less protonated silicate species $[H_nSiO_4]^{(4-n)-}$. In the case of sodium silicate Na_2SiO_3, a convenient commercial precursor for silica, a basic aqueous solution is obtained that contains Na^+ and $[SiO(OH)_3]^-$ species at pH $\approx$ 12.

As for the alkoxide route, a two-step procedure is currently followed (Bhatia et al. 2000). $[Si(OH)_4]$ neutral precursors are first formed via the acidification of a sodium

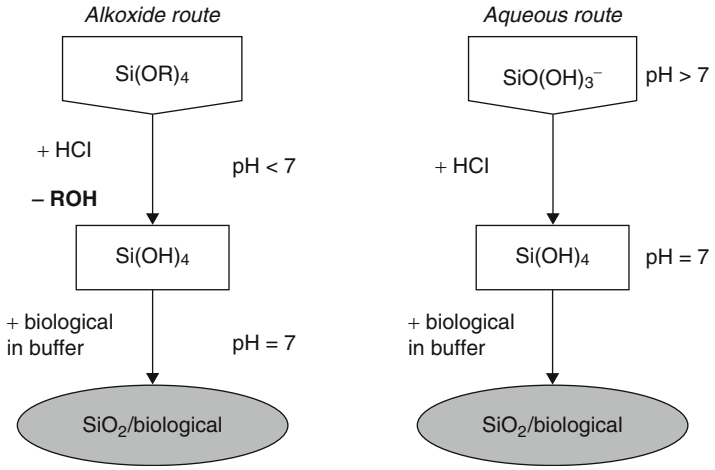

Fig. 3 Comparison of alkoxide and aqueous encapsulation pathways

silicate solution, and condensation then occurs by mixing this solution with a suspension of enzymes in a buffer solution.

Acidification can be performed by adding an acid, such as HCl. However, large amounts of Na^+ and Cl^- ions are released in the solution. They could be harmful for cells and must be removed before encapsulation. This can be done using a proton exchange resin, but concentrated silica solutions lead to fast gelation inside the resin itself. Dilute sodium silicate solutions have then to be used for bioencapsulation at neutral pH giving brittle silica gels. A way to overcome this drawback is to use two different kinds of silica precursors: sodium silicate for gelation and colloidal silica particles for mechanical reinforcement (Finnie et al. 2000; Coiffier et al. 2001). This strategy affords a better control of reactant concentration and allows the design of an encapsulation process very similar to the traditional alkoxide pathway, thus providing a basis to compare both routes (Fig. 3).

Hybrid silica matrices for bioencapsulation involving the aqueous pathway are hardly found in the literature. Alginate/silica composites have been recently obtained by deposition of a silica layer from a sodium silicate solution on Ca^{2+}-alginate beads previously coated with polylysine (Coradin et al. 2001). This polymer was shown to favor silica condensation, allowing the use of low concentrations of silicate and, thus, limiting its gelation to the capsule surface rather than in the whole solution. This approach was successful in obtaining hybrid membranes with a low concentration of silicate. The beads showed improved mechanical stability when compared to alginate coatings. Moreover, the silica layer did not noticeably modify membrane diffusion properties. Finally, these beads were found to be suitable for enzyme encapsulation. Glycerol, PVA, and gelatine were also added to sodium silicate/silica nanoparticles mixtures for the encapsulation of bacteria (Nassif et al. 2003). All these additives provided enhanced viability of entrapped cells.

Non-Silica Sol-Gel Matrices

The sol-gel chemistry of silica is much more versatile than that of other oxides, and about 99% of sol-gel encapsulation experiments have been made with silica matrices.

Some experiments report on the realization of amperometric biosensors based on alumina sol-gel matrices (Liu et al. 1999; Liu et al. 2000a, b). Alumina is formed via the hydrolysis of aluminum alkoxides $Al(OPr^i)_3$ and heated around 90 °C to give a boehmite sol (γ-AlOOH). This sol can be mixed with an aqueous suspension of enzyme and deposited onto an electrode (Liu et al. 1999). The pore size can be easily controlled by changing the Al/H_2O ratio allowing the encapsulation of molecules of different sizes. Amperometric tyrosinase biosensors, for instance, were made in which the enzyme (polyphenol oxidase) is trapped in a matrix with large pores, whereas smaller pores are formed to trap the $[Fe(CN)_6]^{4-}$ mediator (Liu et al. 2000a).

Silica and alumina are nonconducting oxides. Therefore they have to be mixed with conducting graphite powders to make electrodes. Semiconducting oxides such as V_2O_5 or TiO_2 would offer obvious advantages for the realization of amperometric biosensors. Glucose oxidase has been trapped within vanadium oxide or titanium oxide gels for making glucose biosensors (Glezer and Lev 1993; Kurokawa and Ohta 1993). Actually titania also exhibits photocatalytic properties that can be used for the selective reduction of H_2O_2 in the presence of O_2. Hence TiO_2 has been suggested as a catalytic electrode material for oxidase biosensors (Cosnier et al. 1999). Titania is currently formed via the hydrolysis of titanium alkoxides, but TiO_2 gels are very brittle. Therefore, composite materials in which TiO_2 is mixed with cellulose or a poly(vinyl alcohol) copolymer have been used (Kurokawa et al. 1993; Chen et al. 2001).

Titanium is one of the most biocompatible materials and is widely used as dental and orthopedic implants. An oxide layer is formed at the surface of the titanium metal onto which cells should be able to grow. Therefore, sol-gel-derived titania coatings are being developed for biomedical applications. Osteoblast-like and bone marrow stromal cells have been shown to attach well to these sol-gel coating and spread normally at their surface (Haddow et al. 2000). Such properties could open new opportunities for the encapsulation of living cells within titania gels!

Biomolecules in Silica Gels

Enzymes for Biosensing and Biocatalysis

Enzyme Immobilization in Silica Gels

A wide range of proteins has been trapped within silica gels. They have been shown to retain most of their activity and could be used for a large variety of applications including chemical sensing, optics, or local probes (Jin and Brennan 2002). The light transduction of bacteriorhodopsin and phycoerythrin has been exploited for the

realization of photonic devices (Weetall et al. 1993, 1996; Wu et al. 1993; Chen et al. 1995; McCraith et al. 1997).

The properties of entrapped proteins have already been extensively reported in many review papers (Avnir et al. 1994; Lin and Brown 1997; Dave et al. 1994; Livage et al. 2001). Therefore we shall focus on the sol-gel entrapment of enzymes. These biocatalysts are responsible for the chemical reactions of living organisms. Their high specificity and huge catalytic power are now widely used in biotechnology for making biosensors and bioreactors. However, they have to be immobilized in order to be reusable. Natural and synthetic polymers such as polysaccharides, polyacrylamides, or alginates are currently used for enzyme immobilization via covalent binding or entrapment. Enzymes could be grafted at the surface of silica gels, but this does not bring any real improvement as such a process can also be performed with porous glasses. Therefore, most works deal with the encapsulation of enzymes within a silica gel.

The specific properties of enzymes are due to the fact that the geometry of their active site can fit exactly that of the substrate, according to the so-called lock and key model. With sol-gel encapsulation, enzymes are only physically trapped, without covalent bonding, within the hydrophilic aqueous environment provided by the silica matrix. Therefore, most enzymes are not denatured by sol-gel encapsulation, and their catalytic properties are closed to that of their water-soluble counterparts. Moreover, enzymes can even be stabilized in the gel. The silica matrix prevents their leaching and protects them against external reagents. In some cases, during encapsulation, the silica network grows around enzymes that remain trapped within pores tailored to their size. Their mobility is then somehow restricted preventing the deformation of their active site. Moreover the chemical nature of the nanopores in which enzymes are trapped can be chemically controlled in order to provide a favorable environment.

A large number of enzymes have been trapped within silica gels as already reported in several well-documented review papers (Avnir et al. 1994; Gill and Ballesteros 2000; Gill 2001; Livage et al. 2001; Jin and Brennan 2002). Therefore only two examples will be described here showing how sol-gel encapsulation can be used for making biosensors and bioreactors.

Entrapped Glucose Oxidase for Glucose Monitoring

In biology, the oxidation of glucose by molecular oxygen is catalyzed by an enzyme called glucose oxidase (GOD). D-Glucose is oxidized to gluconolactone by the reduction of a flavin group, which is then deoxidized by the conversion of oxygen into hydrogen peroxide.

$$\text{D-Glucose} + O_2 - (\text{GOD}) \rightarrow \text{gluconicacid} + H_2O_2$$

Such a reaction has been extensively used for the titration of glucose in clinical analyses for the diagnosis of diabetes (Wilson and Turner 1992). Glucose detection can be made via the consumption of O_2, the redox reaction at the active site of GOD or the formation of H_2O_2, leading to different sensor configurations.

The formation of hydrogen peroxide can be detected via the enzymatic oxidation of an organic dye by a horseradish peroxidase (HRP) (Dave et al. 1994). The peroxidase enzyme is then trapped within the silica gel together with the glucose oxidase. H_2O_2 molecules produced at the active site of GOD diffuse through the porous gel and react with the organic dye at the active site of HRP. The whole glass becomes colored when dipped into a glucose solution, and a fair correlation is observed between the optical density of the gel and the amount of glucose in the solution (Braun et al. 1992; Yamanaka et al. 1992; Shtelzer and Braun 1994).

The electrochemical detection of redox reactions at the active site of GOD can be followed via electrochemical detection. However, due to the large size of the enzyme, electrons cannot tunnel directly from the active site to the electrode. Molecular mediators such as ferrocene and its derivatives (FcD) have to be co-immobilized in the gel in order to transfer electrons. Ferrocene is then deoxidized at the electrode, and the faradaic current is proportional to the amount of glucose (Audebert et al. 1993; Niu and Lee 2002).

Oxygen concentration can be measured directly with an oxygen-sensitive Clark electrode. The doped sol-gel film is deposited onto the Pt cathode, and oxygen concentration is measured by amperometric titration at imposed potential. Oxygen depletion can then be used to quantify glucose concentration (Fig. 4) (Bergogne et al. 1999).

Amperometric detection has been widely used for sol-gel biosensing, and many devices have been described (Lev et al. 1997; Wang 1999; Collinson and Howells 2000; Przybyt and Bialkowka 2002). These methods are very convenient, but silica is not electronically conductive (Willner and Katz 2000). Therefore carbon–ceramic composite electrodes (CCE) in which an enzyme-loaded carbon powder is mixed to the sol-gel solution have been developed (Gun and Lev 1996; Sampath and Lev 1996; Wang et al. 1997).

Hybrid organic-silica matrices can easily be made by sol-gel chemistry. Hydrophobic hybrid electrodes limit water penetration to a very thin layer at the surface of the electrode that can be renewed by a simple polishing step (Kuselman et al. 1992; Li et al. 1999). Sol-gel matrices containing additives such as chitosan, Nafion,

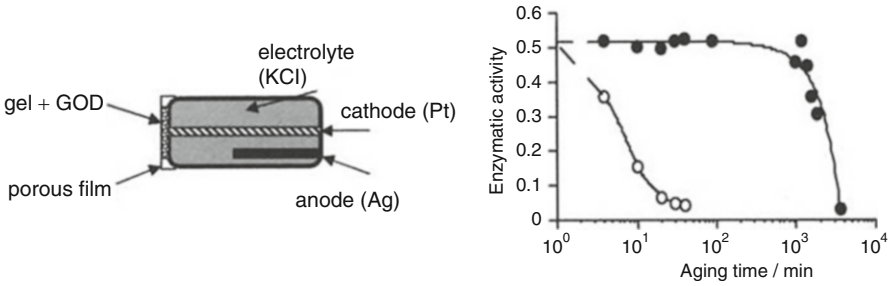

Fig. 4 Monitoring glucose oxidase (GOD) activity via O_2 depletion: (left) oxygen-sensitive Clark electrode description; (right) evolution of enzymatic activity of free (open circle) and entrapped (full circle) GOD (adapted from Bergogne et al. 1999)

polyvinyl alcohol, PDMS, dextran, etc. have been described in order to improve the properties of sol-gel biosensors (Wang et al. 1998; Wang and Dong 2000; Miao and Tan 2001; Mizutani et al. 2001; Gulcev et al. 2002; Kim and Lee 2003). Silica inks, using polyvinylpyrrolidone (PVP) as a binder, can be deposited by screen printing (Nagata et al. 1995).

Another advantage of sol-gel encapsulation is that electrodes can be prepared in virtually any shapes: thick films, micro-tips (≈10 mm) (Gun et al. 1994), disk, rods (Sampath et al. 1996), or optical fiber coatings (Doong and Tsai 2001). Microarrays are now widely used for the analysis of biocomponents. Several methods such as covalent binding or affinity capture have been developed to produce protein microarrays, but they all have potential limitations. The entrapment of proteins in sol-gel-derived microspots could therefore offer new possibilities. Recent works report on the preparation of pin-printed biosensor arrays (PPBSA) based on protein-doped xerogels (Cho et al. 2002). Spots about 100 μm in diameter are deposited onto glass plates or light-emitting diodes. Xerogels are doped with $[Ru(dpp)_3]Cl_2\ 5H_2O$ and glucose oxidase in order to detect simultaneously O_2 and glucose. Reliable titration can be performed in the range 0.1–10 mM for glucose and 0.1–100% for O_2. Antibody microarrays have also been described showing that sol-gel processing and pin-printing techniques can be combined to make biosensing microarrays (Rupcich et al. 2003).

Entrapped Lipases as Biocatalysts

Confinement within silica gels does not only protect enzymes against denaturation. It can also provide a chemical surrounding that favors the enzymatic activity. The ability to tailor the matrix properties, by modifying sol-gel chemistry, enables optimization of the bioactivity of encapsulated enzymes. Hybrid materials can be used to control the polarity of the internal environment within the nanopores. Hydrophobic hybrid organic–inorganic materials can then be produced. They are more suitable for the encapsulation of lipophilic enzymes that would not remain functional in polar matrices (Gill 1998; Brennan et al. 1999).

Lipases provide a nice example showing how a chemical control of the sol-gel matrix can be used to improve enzymatic activity. Lipases catalyze hydrolysis and esterification reactions. In aqueous media, they hydrolyze fats and oils into fatty acids and glycerol, whereas esterification reactions occur in organic media. Actually most lipases are interfacial activated enzymes. In an aqueous solution, an amphiphilic peptidic loop covers the active site just like a lid. At a lipid/water interface, this lid undergoes a conformational rearrangement which renders the active site accessible to the substrate (Schmid and Verger 1998). First attempts to immobilize lipase in silica gels obtained from tetramethoxysilane (TMOS) $Si(OCH_3)_4$ by Reetz et al. gave very poor activities (Reetz et al. 1995). Methyltrimethoxysilane (MTMS) $CH_3–Si(OCH_3)_3$ was then co-condensed with TMOS, creating an hydrophilic/hydrophobic Si–OH/Si–CH_3 interface at the gel surface, favorable to lipase activity. Thus, increasing the MTMS:TMOS ratio greatly improved the catalytic activity, up to 1300% in a pure MTMS gel when compared to enzymes in solution (Reetz et al. 1996a). Nevertheless, MTMS only exhibits three hydrolyzable functions, and the

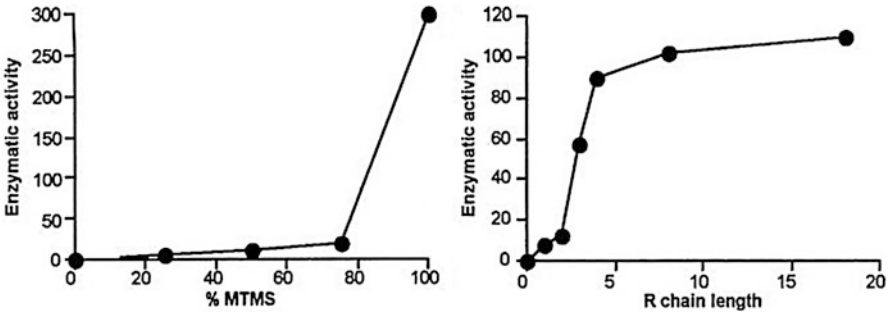

Fig. 5 Optimizing hybrid silica gels for lipase encapsulation: influence of MTMS amount (left) and R chain length in R–Si$(OCH_3)_3$ added alkoxide (right) on entrapped lipase enzymatic activity (adapted from Reetz 1997)

gels thus formed are poorly condensed. Following studies therefore focus on the effect of the chain length of the R group of the R–Si$(OCH_3)_3$ additional alkoxide. It was shown that the enzymatic activity increases for chain lengths up to five carbon atoms, no improvement being observed for longer, more hydrophobic derivatives (Fig. 5). Even though similar behavior was observed for different lipases, the optimum TMOS: R–Si$(OCH_3)_3$ ratio and R chain length appear to depend on the enzyme (Reetz 1997). Due to the excellent properties of these materials, they were patented and are now commercially available (Fluka).

Since then, several improvements were proposed. The first one is dedicated to the effect of drying on the activity of entrapped lipases. Room temperature and freeze drying, leading to xerogels, and supercritical drying, leading to aerogels, were compared. The latter appears as the most efficient in decreasing the capillary stress induced by the evaporation process. As a consequence, gel shrinkage is decreased, allowing an easier diffusion of substrates and products therefore limiting enzyme denaturation. It was also showed that these materials could be further improved by adding PVA to the precursor solution (Pierre and Buisson 2001). In parallel, several studies were devoted to the design of easy-recovered catalysts. Silica gels containing lipases were deposited on millimeter-sized glass beads, allowing their recovery by filtration (Reetz et al. 1996b). Alternatively, it was proposed to incorporate iron oxide nanoparticles with the gel for magnetic separation (Kuncova and Sivel 1997; Reetz et al. 1998).

Antibodies for Immunoaffinity-Based Biosensing

The highly specific and sensitive recognition of antigens by antibodies can be used for immunoaffinity purification. Antibodies, trapped within a silica gel, bind a specific molecule from a mixture of compounds. Small molecules (haptens) have to be bound to a macromolecular carrier such as bovine serum albumin (BSA) to induce an immune response and stimulate the production of antibodies. These

antibodies are then able to recognize specifically the target analyte (hapten) and have been used for the detection of various chemicals such as fluorescein, dinitrophenyl (DNP), dinitrobenzene (DNB), and even TNT (Wang et al. 1993; Aharonson et al. 1994; Jordan et al. 1996; Bronshtein et al. 2000; Lan et al. 2000; Vazquez-Lira et al. 2003).

The extensive use of chemicals in agriculture and their persistency in the geosphere cause environmental problems, mainly for drinking water resources. Highly sensitive detection methods are then required to detect traces, and immunochemical reactions are good candidates for such analyses. Specific antibodies trapped in silica gels have been used for the detection of pesticides (Hock et al. 1995) and herbicides (atrazine, isoproturon) (Turniansky et al. 1996; Bronshtein et al. 1997; Pulido-Tofino et al. 2001).

For medical applications, whole cell parasites (*Leishmania donovani infantum*) have been used as antigens. Sol-gel encapsulation was performed directly inside the microwells of a standard polystyrene microtiter plate currently used for immunoassays. Gelation occurs inside the wells within about 5 min. Human or dog sera are then poured into these wells, and the antigen–antibody association is detected via the so-called enzyme-linked immunosorbent assays (ELISA) which provide a very sensitive method for the optical detection of antibodies (Fig. 6) (Barreau et al. 1994; Livage et al. 1996; Correia de Costa et al. 1996).

Miscellaneous Applications

Controlled Drug Delivery

The controlled release of drugs is becoming increasingly important for oral, transdermal, or implantable therapy. Some reports show that porous silica matrices could offer new possibilities as drug carriers (Sieminska and Zerda 1996; Carturan et al. 1997; Böttcher et al. 1997). In vitro experiments show that the liberation rate of nifedipine, a well-known drug against hypertension, can be controlled by changing the pore size or the hydrophilic–hydrophobic balance of the silica matrix (Böttcher et al. 1998).

In vivo experiments have been performed with toremifene citrate, a nonsteroidal antiestrogenic compound that exhibits antitumor activity in breast and endometrial

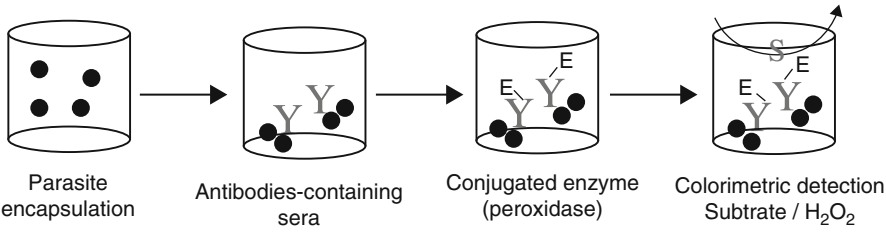

Fig. 6 Parasite detection via ELISA assays

cancer. Toremifene-containing xerogels were subcutaneously implanted in mice. The drug was labeled with tritium in order to follow the amount of toremifene remaining in the silica implant (Kortesuo et al. 2000). About 16% of the drug was still trapped in silica implants after 6 weeks. Moreover, silica xerogels do not lead to any tissue irritation, and a fibrotic capsule forms around the implant.

Biocompatibility is actually a major problem for implants, and bioactive sol-gel glasses have been developed. They are typically made of a mixture of silica and calcium phosphate in order to show some affinity for the in vitro and in vivo nucleation of apatite (Hench et al. 1998). Such sol-gel glasses can also be used as implantable drug carriers. Experiments performed with ibuprofen, an anti-inflammatory drug, show that both processes, formation of an apatite layer and drug release, occur simultaneously. The release kinetics is mainly influenced by the solubility of the drug and the pore size of the bioglass (Ramila et al. 2003; Hall et al. 2003).

Microdevices

The sol-gel technique allows the reproducible preparation of capillary electro-chromatography (CEC) microcolumns that could be used for high-performance separation in microfabricated devices (microchips). Fluid-hydrolyzed silica sols can be injected into the microchip channels. Condensation occurs inside the device micro-HPLC column made of silica gel (Constantin et al. 2001).

Protein can be added to the solution of precursor in order to add some biocomponent to the CEC column. Chiral compounds, bovine serum albumin (BSA), and ovomucoid (OVM) have been trapped within such sol-gel CEC in order to check their chiral selectivity. These capillary columns showed good enantioselectivity toward selected enantiomers such as tryptophan, benzoin, eperisome, or chlorpheniramine (Kato et al. 2002).

Encapsulating Bacteria

One of the major advances in the field of sol-gel materials would be the possibility to trap whole cells within porous silica matrices, and a whole chapter of this book is devoted to the encapsulation of eukaryotic cells (fungi, mammalian cells, etc.). Therefore, we shall focus our discussion on prokaryotic cells (bacteria). Their metabolic activity can be used for a large variety of processes in food industry, waste treatment, and the production of chemicals or drugs.

Bacteria are known to be able to bind selectively large amounts of metals. They can therefore be used in remediation technologies for the removal of heavy metals from polluted waters (Al-Saraj et al. 1999). Cells, spores, and surface-layer proteins of *Bacillus sphaericus* bacteria have been trapped within sol-gel ceramics (biocers) for the in situ bioremediation of uranium mining waste pile waters (Raff et al. 2003). Biocers were made by dispersing vegetative cells, spores, and surface-layer proteins (S-layer) in aqueous silica sols. Entrapment does not influence the metal-binding properties of cells and S-layers that have been shown to exhibit high-binding

capacity toward uranium and copper. However, spores lose most of their activity upon encapsulation. Uranium and copper can be easily removed from the bioceramic by using citric acid so that biocers appear to be suitable for the realization of reversible filters.

Experiments performed with *Escherichia coli* showed that these bacteria retain their enzymatic activity when trapped in a silica gel (Fennouh et al. 1999). They still exhibit a β-galactosidase activity and are able to hydrolyze *p*-NPG (*p*-nitrophenyl-β-D-galactopyranoside) into galactose and nitrophenol. The enzymatic activity of entrapped *E. coli* follows a typical Michaelis behavior. Their catalytic activity is even slightly better than for free bacteria suspended in an aqueous solution. This was assigned to some lysis of the membrane of bacteria cells during encapsulation, allowing a better diffusion of the reactants (Fennouh et al. 2000; Coiffier et al. 2001).

Actually, the viability of whole cells in a silica matrix is a crucial point for sol-gel to compete with usual encapsulation processes in polymer matrices. However, very few experiments have been performed to check the long-term viability of trapped cells after encapsulation. Experiments performed with *Pseudomonas* sp. bacteria showed that they retain their ability to metabolize atrazine, a widely used herbicide. Entrapped cells lose much of their activity upon immobilization, but partial activity could be restored by adding nutrients suggesting that bacteria may remain alive, at least for some time (Rietti-Shati et al. 1996).

The formation of acetate was observed when lactate was added to a gel containing anaerobic sulfate-reducing bacteria providing evidence of the metabolic conversion of lactate to acetate. Encapsulated bacteria survive the gelation procedure and are able to continue normal metabolic activity within the gel matrix. This activity decreases with time but can be regenerated by immersion in nutrient solution, even after several weeks (Finnie et al. 2000).

Lev et al. in Jerusalem recently reported on the behavior of genetically engineered luminescent *Escherichia* obtained by coupling a gene promoter sensitive to chemical or physical stress to a reporter gene coding for luminescent proteins (GFP, RFP). These recombinant cells were trapped in sol-gel silica films deposited on glass plates. They appear to maintain their ability to synthesize luminescent proteins in the presence of chemical inducers over months, either through repeated uses or under continuous flow (Premkumar et al. 2001, 2002a). The stress-dependent luminescence properties of these cells provide information about their state during the sol-gel process and within the silica gels. It was then used to optimize the sol-gel procedure (pH, water/TMOS ratio, drying time, thickness of the films). Moreover, luminescent cells can be observed by confocal microscopy showing that bacteria are homogeneously distributed within the film with limited aggregation. Neither cell proliferation nor leaching could be observed after several days. Finally, the possibility to entrap simultaneously cells from two different strains within the same gel opens new possibility for dual or multiple sensing (Premkumar et al. 2002b; Sagi et al. 2003).

Generic tests such as culturability and glycolysis have been reported recently to follow the viability of *Escherichia coli* in silica gels. They show that the usual two-step sol-gel procedure, using alkoxides as precursors, is still very detrimental to

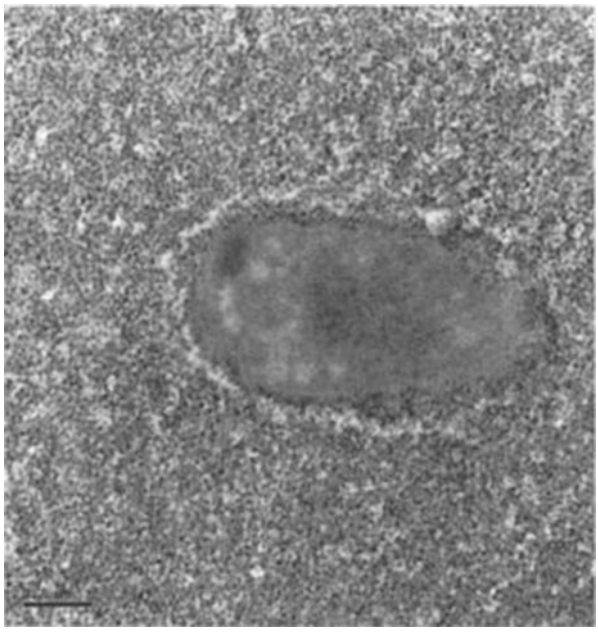

Fig. 7 *E. coli* bacteria entrapped in an aqueous silica gel

bacteria. Much better results are obtained when aqueous precursors are used and when sol-gel encapsulation is performed in the presence of additives such as glycerol (Nassif et al. 2002). Bacteria then remain culturable for several weeks after entrapment. They are still able to form colonies when dispersed on a culture medium. Their metabolic activity toward glycolysis was followed by ^{14}C titration and ^{13}C NMR experiments using marked glucose molecules. They show that in aqueous silica gels, bacteria are able to maintain a constant internal environment when trapped and remain viable for several weeks (Nassif et al. 2003) (Fig. 7).

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