# Enzymes and Cells Confined in Silica Nanopores

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## ABSTRACT

The sol-gel process opens new possibilities in the field of biotechnologies. Sol-gel glasses are formed at room temperature *via* the polymerization of molecular precursors. Enzymes can be added to the solution of precursors and trapped within the growing silica network. Small substrate molecules can diffuse through the pores allowing reactions to be performed in-situ, within the silica gels. Enzyme are encased by the hydrated silica in a cage tailored to their size, they retain their biocatalytic activity and may even be stabilized within the sol-gel matrix.

Whole cell bacteria have also been immobilized within sol-gel glasses. They behave as a "bag of enzymes" and their membrane protects enzymes against denaturation and leaching. The cellular organization of bacteria cells is preserved upon encapsulation. Experiments performed with *Escherichia coli* induced to β-galactosidase show that they still exhibit noticeable enzymatic activity. Some degradation of the cell walls may even occur increasing the "measured" activity. However silica gels made from aqueous precursors seem to prevent bacteria from natural degradation upon ageing.

Antibody-antigen recognition has been shown to be feasible within sol-gel matrices. Trapped antibodies bind specifically the corresponding haptens and can be used for the detection of traces of chemicals. Even whole cell protozoa have been encapsulated without any alteration of their cellular organization. For medical applications, trapped parasitic protozoa have been used as antigens for blood tests with human sera. Antigen-antibody interactions were followed by the so-called Enzyme Linked ImmunoSorbent Assays (ELISA).

## INTRODUCTION

Entrapment in crosslinked organic polymers is a well known method for the immobilization of enzymes and whole cells. Entrapped biomolecules are physically confined within the polymer matrix and can be reused several times. Organic polymers such as polyacrylamide gels are currently used in biotechnology but silica glasses could offer some advantages such as improved mechanical strength and chemical stability. Moreover they don't swell in aqueous or organic solvents preventing leaching of entrapped biomolecules. However glasses are made at high temperature and, up to now, enzyme immobilization can only be performed *via* adsorption or covalent binding onto the surface of porous glasses [1].

The so-called sol-gel process opens new possibilities in the field of biotechnology [2-4]. Sol-gel glasses are formed at room temperature *via* the polymerization of molecular precursors such

as metal alkoxides. Proteins can be added to the solution of precursors. Hydrolysis and condensation then lead to the formation of an oxide network in which biomolecules remain trapped. Small analytes can diffuse through the pores allowing bioreactions to be performed inside the sol-gel glass. Trapped enzymes still retain their biocatalytic activity and may even be stabilized within the sol-gel cage. A wide range of biological species such as antibodies and whole cells have been trapped within sol-gel matrices. They usually retain their activity but weak interactions with the silica cage actually occur that can change their behavior.

## SOL-GEL CONFINEMENT IN SILICA MATRICES

Sol-gel silica can be synthesized at room temperature via the hydrolysis and condensation of TetraMethyl OrthoSilicate (TMOS), $Si(OCH_3)_4$. Hydrolysis gives reactive silanol groups whereas condensation leads to the formation of bridging oxygen as follows:

$$\equiv Si\text{-}OCH_3 + H_2O \Rightarrow \equiv Si\text{-}OH + CH_3OH \qquad \text{(hydrolysis)}$$

$$\equiv Si\text{-}OH + HO\text{-}Si\equiv \Rightarrow \equiv Si\text{-}O\text{-}Si\equiv + H_2O \qquad \text{(condensation)}$$

The overall reaction is then

$$Si(OCH_3)_4 + 2H_2O \Rightarrow SiO_2 + 4\,CH_3OH$$

Silicon alkoxides are not miscible with water so that a common solvent has to be used. The parent alcohol is currently chosen but a large variety of other organic solvents can also be used [5]. Silicon alkoxides are not very sensitive to hydrolysis. Gelation takes place within several days and even weeks, when pure water is added. Therefore acids and bases have to be added in order to increase the gelation rate. Acid catalysis, below the Point of Zero Charge of silica (pH≈3) mainly increases hydrolysis rates. Chain polymers are formed leading to microporous monolithic gels (average pore diameter < 20 Å). Basic catalysis (pH > 3) enhances condensation, giving dense spherical colloids and mesoporous gels (average pore diameter in the range 50-100 Å) [6].

The encapsulation of proteins within sol-gel matrices is usually performed in two steps in order to avoid denaturation. The first step is the acid hydrolysis of pure TMOS, without alcohol as a co-solvent. Most alkoxy groups are removed giving silisic acid $Si(OH)_4$. A suspension of enzymes in a buffered solution (pH≈7) is then added. Basic condensation takes place rapidly and a mesoporous oxide is formed around the biomolecule. Enzymes are immobilized within the silica gel but their active site may remain accessible and they can react with small chemical analytes through the porous matrix.

The chemical control of the pore size is one of the major challenges of sol-gel encapsulation. Pores have to be small enough to avoid the leaching of entrapped proteins but large enough to allow the diffusion of analytes. The pore size has then to be tailored according to the nature of biospecies. Figure 1 shows the pore size and specific area variation as a function of the amount of water, $h=[H_2O]/[TMOS]$, added during the first hydrolysis step.

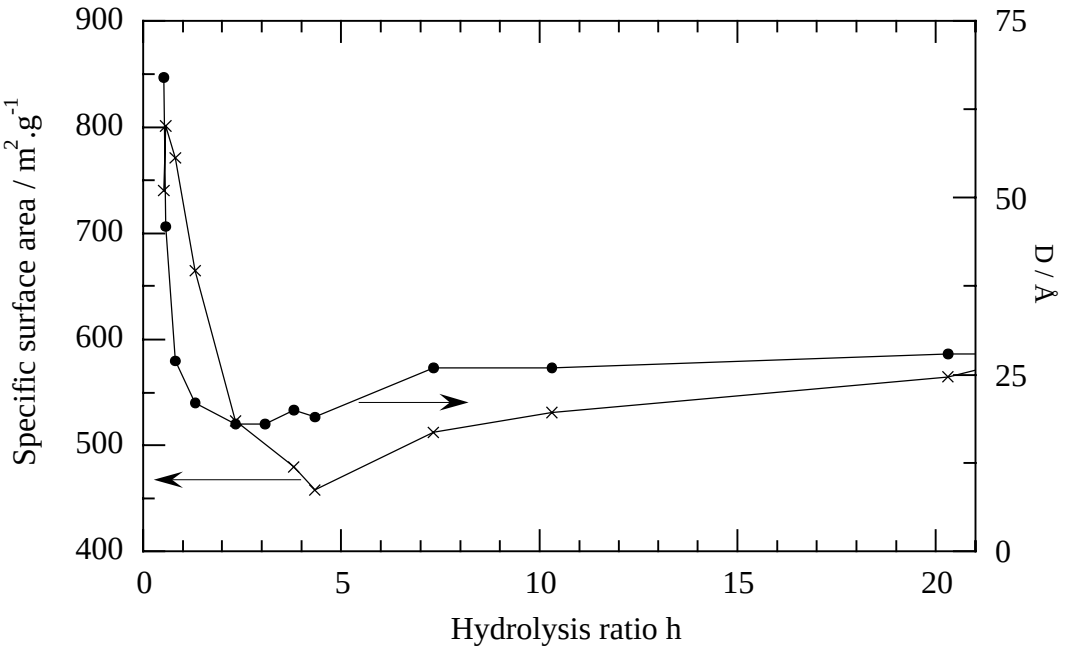


***Figure 1**. Mean pore size as a function of the hydrolysis ratio h=[$H_2O$]/[TMOS]. The pore size is measured on xerogels dried at room temperature.*

They go through a minimum around h≈3. Below this value, an emulsion is formed that disappears just before gelation when the buffered aqueous solution is added in order to adjust the pH around 7.

## CATALYTIC ACTIVITY OF ENZYMES

Enzymes are biological catalysts which are responsible for the chemical reactions of living organisms. Chemically these proteins are made of amino acids linked together *via* covalent peptide bonds. Their high specificity and huge catalytic power is due to the fact that the geometry of the active site can fit exactly that of the substrate. Therefore even small changes in the enzyme conformation can reduce drastically their catalytic activity.

A large number of enzymes have been trapped within sol-gel glasses since the pioneering work of D. Avnir in Jerusalem [7]. Most publications study glucose oxidase (GOD) as a model for enzyme encapsulation. GOD catalyzes the oxidation of D-glucose by molecular oxygen into D-gluconolactone and D-gluconic acid as follow:

$$C_6H_{12}O_6 + O_2 \Rightarrow C_6H_{10}O_6 + H_2O_2$$

The enzymatic activity can be followed in a number of ways. The formation of hydrogen peroxide can be detected by optical measurements using a peroxidase to oxidize an organic dye [8]. The redox reaction at the active site of GOD can be followed with an electrochemical mediator [9]. In our group oxygen consumption was measured directly with an oxygen sensitive electrode. In these experiments the sol-gel solution containing GOD was deposited onto the Pt cathode of a Clark electrode. Oxygen

concentration was measured by amperometric titration at imposed potential. The enzymatic activity is determined *via* the decrease in oxygen concentration after the injection of glucose into the aqueous solution.

Figure 2 shows that the catalytic activity of GOD in silica gels is about the same as when the enzyme is just immobilized at the surface of the electrode

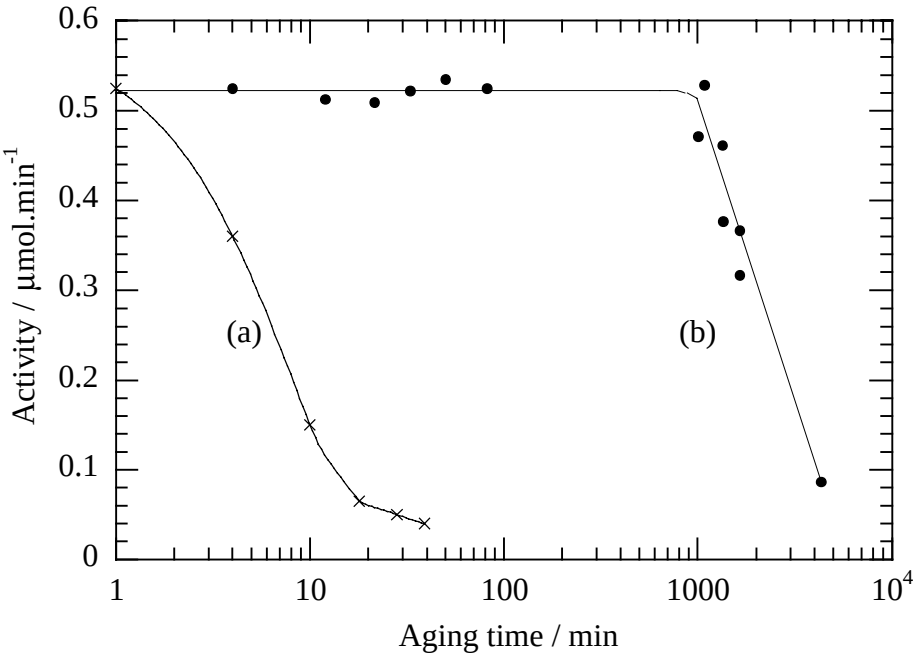


***Figure 2****. GOD activity as a function of time measured onto a Clark electrode :(a) enzyme immobilized at the electrode surface, (b) encapsulated enzyme*

Therefore sol-gel encapsulation does not lead to denaturation during the formation of the silica network. Even after drying at room temperature, silica xerogels still contain enough water to provide a mainly aqueous environment to avoid denaturation of the enzymes. Figure 2 also shows that the catalytic activity of the trapped enzyme is constant during *ca.* 500 min whereas it progressively decreases in the case of enzymes immobilized at the electrode surface. The half-time $t_{1/2}$ (corresponding to a 50% decrease of catalytic activity) is close to 300 min for encapsulated GOD instead of 10 min when the enzyme is only immobilized. Its thermal behavior is also improved. Entrapped GOD retains 60% of its activity at 63°C after 20 h, whereas the half-life of free GOD is only 6.5 min. [10].

These experiments suggest that the silica matrix protects encapsulated enzymes against leaching and denaturation. Leaching is prevented by the small size of silica pores whereas denaturation could be prevented by some restriction of molecular motions. Upon encapsulation the enzyme is encased by the hydrated silica in a cage tailored to its size. The silica matrix constrains the motions of the encapsulated protein molecules and may prevent irreversible structural deformations [11]. Electrostatic interactions may also occur between anionic silicate sites and specific positively charged residues on the protein surface. If these residues are essential for either preserving the protein structure or catalysis, such interactions will decrease the enzymatic activity. Silica surfaces are negatively charged above the Point of Zero Charge (pH≈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 GOD retain its activity upon encapsulation [10]. The detrimental electrostatic interactions can be overcome by complexing the enzyme with a polyelectrolyte that shield the critical charged sites and lactate oxidase can be stabilized by complexing with the weak base PVI (poly(N-vinylimidazole) [12].

Confinement within silica gels does not only protect enzymes against denaturation. It can also provide a chemical surrounding that favors the enzymatic activity. Lipases provide a nice example of such interactions between enzymes and the sol-gel matrix. These enzymes act on ester bonds. In aqueous media they are able to hydrolyze fats and oils into fatty acids and glycerol. In organic media esterification or transesterification reactions occur. However lipases are not soluble in organic solvents and freeze-dried powders are currently used. A least one hydration layer is required for enzymes to retain their bio-activity. Therefore the activity of lipases in organic media is not very good. Actually 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 [13].

Transesterification reactions performed with lipases trapped within silica gels show that their activity strongly depend on the water content of the gels. Aged gels that contain a large amount of water (> 60% in weight) exhibit a good enzymatic activity. They provide a mainly aqueous environment similar to that observed in biological media. However the enzymatic activity decreases drastically upon drying. This mainly depends on the chemical nature of the silica matrix. Gels hydrolyzed with a very small amount of water ($h=[H_2O]/[TMOS] \approx 0.6$) still exhibit a good activity even after freeze-drying, whereas the activity of fully hydrolyzed ($h \approx 20$) silica xerogels is very low. This might be due to the fact that the methyl groups of the alkoxide precursor, $Si(OMe)_4$, have not been completely removed during the formation of the silica gel. Lipases are then encapsulated in pores that contain both hydrophilic Si-OH and hydrophobic Si-OMe surface groups. The amphiphilic peptidic loop may then be in the "open" position. The gel shrinks upon drying preventing further motion of the loop and the active site remains open when the gel is freeze-dried. This is no longer the case with fully hydrolyzed silica gels. Nanopores are then covered only with hydrophilic Si-OH groups and the peptidic loop is in the "close" position. In wet gels there is enough space to allow the motion of the loop, but not in dry gels.

Very nice experiments have been published by M. Reetz who showed that lipases can be almost 100 time more active when trapped within a hybrid silica matrix [14]. The co-hydrolysis of $Si(OMe)_4$ and $RSi(OMe)_3$ precursors provides alkyl groups that offer a lipophilic environment that could interact with the active site of lipases and increase their catalytic activity. Such entrapped lipases are now commercially available and offer new possibilities for organic chemistry, food industry and oil processing.

## BACTERIA IN SILICA GELS

Many intracellular microbial enzymes are produced in large enough quantities to be used in industrial processes. However the cost for their isolation and purification may be quite high. Therefore it might be interesting to be able to directly immobilize micro-organisms containing these enzymes. Moreover, retaining the enzyme within its natural surrounding preserves its stability. Whole-cell immobilization with organic polymers has been extensively studied. It is a

powerful tool for a precise control of the micro-environment of the cell in a wide variety of reactor configurations and external conditions [15].

The first sol-gel experiments were published more than ten years ago by G. Carturan et al. with "*Saccharomyces cerevisiae* " cells [16]. These yeast are involved in the conversion of sugar and carbohydrates into ethyl alcohol and $CO_2$ and are currently employed in the fermentation of beers and the raising of bread. Encapsulated yeast cells follow the well-known Michaelis-Menten law and exhibit almost the same activity as in a solution for more than a year. They even retain their viability and the budding of yeast cells can still be observed after immobilization at the surface of sol-gel films [17].

The sol-gel encapsulation of whole cell bacteria *Escherichia coli* was reported much later [18]. These bacteria were trapped within two different sol-gel matrices, synthesized from alkoxide or aqueous precursors, in order to study the role of the micro-environment.
Sol-gel entrapment with alkoxide precursors was performed via the same two-step procedure as for enzymes. The pH of the acid solution of hydrolyzed TMOS is first increased with a phosphate buffer in order to prevent the denaturation of bacteria. *E. coli* cells, extracted by centrifugation from their culture medium, are suspended in a phosphate buffer solution ($10^9$ cells/ml) and then mixed with the sol-gel solution. A silica gel is formed within a few minutes and left for aging several days at room temperature.
A two steps procedure was also followed for the aqueous route using a solution of sodium silicate (0.4 M, 2ml) and colloidal silica (Ludox HS 40, 1 ml). The pH of such solutions is close to 12. It is first acidified by adding HCl (4 M, 180 µl) in order to decrease the pH down to 7. The bacteria suspension is then added with a phosphate buffer. Gelation takes places within a few minutes, encapsulating the bacteria cells.

Ultra structural observations by transmission electron microscopy of thin slices of wet gels show that the integrity of *Escherichia coli* cells is preserved after sol-gel encapsulation (Figure 3). The capsule, cell walls and plasma membrane are not destroyed and there is a clear separation between the cell and the porous silica matrix.

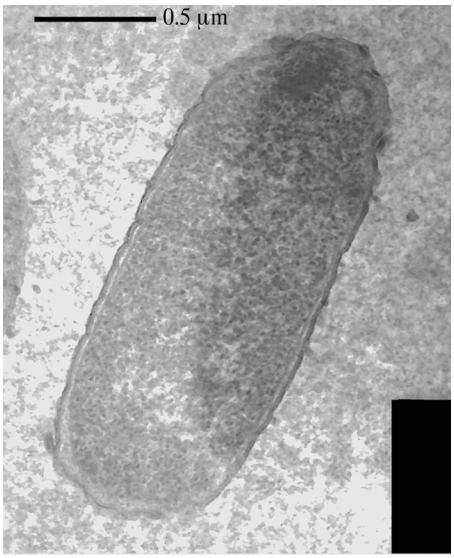


***Figure 3.*** *Transmission electron microscopy of an Escherichia coli cell confined within a wet silica gel made from TMOS (bar = 0.5 µm)*

*E. coli* bacteria were induced, in their culture medium, with IPTG (IsoPropylThioGalactoside) in order to express β-galactosidase enzyme. The β-galactosidase activity of trapped bacteria was measured using p-NPG (p-nitrophenyl-β-D-galactopyranoside) as a substrate. p-NPG can enter the cell without a specific permease and once inside the cell it is cleaved by β-galactosidase into galactose and nitrophenol :

$$\text{p-NPG} + H_2O \Rightarrow \text{p-nitrophenol} + \beta\text{-D-galactose}$$

The saturating concentration of the substrate [p-NPG] has been chosen in order to be much larger than the Michaelis constant of β-galactosidase ($K_M \approx 0.1$ mM for free bacteria in solution) [18]. The rate of the reaction ($V_{max}$) is then nearly independent of [p-NPG] and proportional to the enzyme concentration. The formation of the yellow p-nitrophenol is followed by optical absorption of the solution at $\lambda = 400$ nm. The enzymatic activity was deduced from the initial velocity of the reaction and compared with the activity of free bacteria suspended in a phosphate buffer solution (Table I).

**Table I**. Evolution with time of β-galactosidase activity of free and encapsulated *E. coli*

| | β-galactosidase activity (μmol/min) of *E. coli* | | |
|---|---|---|---|
| | free bacteria | in TMOS silica | in aqueous silica |
| ageing 1 day | 0.030 | 0.045 | 0.035 |
| ageing 7 days | 0.060 | 0.071 | 0.050 |

These experiments show that after one day, the β-galactosidase activity of bacteria in silica gels is slightly higher thant that of free bacteria. This might be due to some denaturation of the cell walls that become more porous allowing easier diffusion of the substrate within the cell. Silica gels prepared from TMOS give the best activity, presumably because of the presence of $CH_3OH$ in the hydrolyzed solution. Results obtained after one week ageing in the buffered solution without nutrients are rather interesting. They show that the activity of bacteria trapped in aqueous gels is now smaller than that of free bacteria suggesting that the silica matrix partially prevents the natural degradation of the cell walls.

It might be interesting to point out that the enzymatic activity of *E. coli* trapped in aqueous gels containing a nutrient such as gelatin does not change significantly after one week of ageing suggesting that these bacteria still exhibit some metabolic activity.

## IMMUNOASSAYS IN SILICA GELS

Antigen-antibody reactions have been performed within sol-gel matrices extending the field of sol-gel chemistry toward immunosensors [19]. However antibodies are large biomolecules. IgG the major immunoglobulin in normal human serum is a monomeric protein with a molecular weight close to 150 kDa and dimensions of about 19 x 56 x 240 $Å^3$. Therefore most immunoassays have been performed with antibodies either bonded to the surface [20] or trapped

within the sol-gel matrix. Specific haptens are then used, they are much smaller and can diffuse easily through the pores of the sol-gel matrix. These reactions have been developed for the biodetection of chemicals such as atrazine, a widely used herbicide. Anti-atrazine antibodies are trapped within the sol-gel matrix and nanograms of atrazine are applied on $SiO_2$ sol-gel columns doped with this antibody. Titration of eluted solutions shows that high amounts of atrazine remain bound to anti-atrazine antibodies inside the silica gel [21]. TNT titration using anti-TNT antibodies has also been reported recently [22].

For medical applications, whole cell parasitic protozoa (*Leishmania donovani infantum* ) have been trapped within sol-gel matrices and used as antigens for blood tests with human or dog sera [23]. As for bacteria, transmission electron microscopy shows that the cellular organization of the parasites is well preserved and that the plasma membrane is unaltered. This is very important as antigenic determinants are situated at the outside surface of the membrane.

The recognition of trapped *Leishmania* cells by antibodies in a serum was followed by the so-called Enzyme Linked ImmunoSorbent Assays (ELISA), a widely used test in parasitology [24]. Specific antibodies bound to Leishmania cells in the silica gel are detected via an enzyme conjugate, horseradish peroxidase (HRP) which, after binding to the antibody-antigen complex will catalyze the oxidation of an organic dye by $H_2O_2$. Optical density measurements show a clear difference between negative and positive sera (Figure 4).

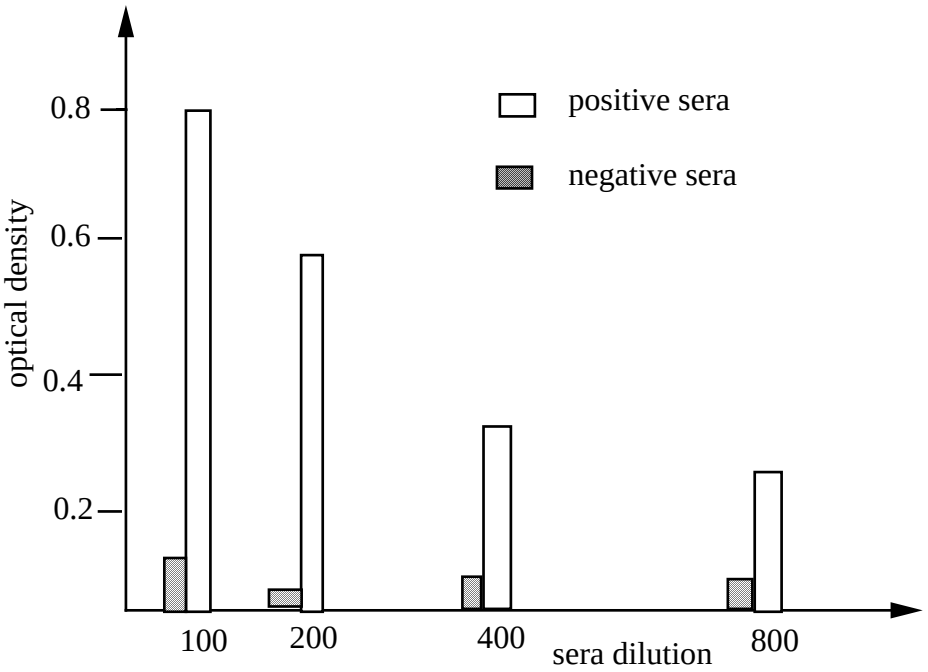


***Figure 4****. Optical detection of antigen-antibody association via ELISA*

The optical density of positive tests decreases regularly with dilution showing that mainly specific antigen-antibody interactions are involved. Only non specifically bound immunoglobulins are washed out giving a very low residual coloration.

These experiments show that large antibodies can diffuse through the pores of the silica matrix and bind specifically to epitopes on the surface of the cell. This of course is no longer possible when pores are not large enough to allow such a diffusion. Assays performed using different silica matrices obtained with different hydrolysis ratios show that pore size is actually a critical parameter (Table II).

**Table II.** ELISA tests with gels of different porosity

| | | | |
|---|---|---|---|
| Hydrolysis ration h = $[H_2O]/[Si]$ | 0.57 | 3 | 20 |
| Average pore diameter (Å) | 45 | 18 | 24 |
| OD+/OD- | 5.6 | 1.8 | 3.4 |

## CONCLUSION

The biological applications of sol-gel chemistry appear to be very promising. Immobilization within silica gels offer several advantages compared to organic polymeric matrices which are nowadays widely used in biotechnology. Biomolecules are trapped inside hard porous glasses that do not swell in water. They are confined within hydrated silica pores and surrounded by an aqueous medium close to that observed in biological media. Motions are restricted by the silica cage avoiding irreversible denaturation. The silica matrix protects biomaterials against external aggression (pH, temperature, solvents...). It may even enhance their activity by providing a controlled micro-environment. Sol-gel encapsulation does not destroy the cellular organization of micro-organisms. This might be one of the major advance of sol-gel chemistry and some promising examples show that living cells can be immobilized within sol-gel matrices. Mammalian tissue such as pancreatic islets have been recently been encapsulated in porous silica gels and transplanted into a diabetic mouse where they retain their activity [25, 26]. The fine porosity of silica gels protects transplanted cells against antibody aggression but permits nutrients to reach the cell and byproducts to escape. Such transplants, if viable for extended lengths of time, could emerge as a new treatment for diabetes.

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