Lessons in Nanotechnology from
Traditional and Advanced Ceramics
J.-F. Baumard (Editor)

BIOENCAPSULATION IN SILICA

Jacques LIVAGE, Thibaud CORADIN
Chimie de la Matière Condensée, College de France, Paris, France

Biomineralization offers many examples of nanostructured materials. These biomaterials are currently synthesized from aqueous solutions at room temperature. They show that it is possible to make glasses and ceramics via soft solution chemistry. The mild conditions associated with the so-called sol-gel process lead to the formation of hybrid organic-inorganic nanocomposites. They can even be optimized in order to trap biomolecules such as enzymes within silica glasses opening new possibilities in the field of biotechnology. Even whole cells have been entrapped within silica gels where they remain viable and can be used for the production of metabolites, immunoassays and even for cell transplantation.

1. INTRODUCTION

Nanostructured materials are becoming very popular.[1] They open new possibilities in the field of materials science and extensive researches are nowadays devoted to the controlled synthesis of such materials. However, nanostructured glasses and ceramics are not new. They have been made since the early Cambrian, almost six hundred billions years ago by micro-organisms in order to protect themselves against predators.[2] The example of diatoms is specially interesting. These single cell algae's built an exoskeleton, called frustule, made of silica. Such glass walls have to be strong for protection, transparent for photosynthesis and porous to allow chemical exchanges between the cell and the outside medium.[3] Moreover, they exhibit a wide variety of amazing shapes that are genetically controlled, each species building its own characteristic silica frustule (Fig.1).[4]

Biomineralization offers a large variety of such examples and bio-inspired materials are a real challenge for solid-state chemists. Would they be able to make nanostructured glasses and ceramics in such mild conditions ?

2. SOFT SOLUTION SYNTHESIS OF SILICA GLASSES

For thousands years glasses and ceramics have been made *via* the high temperature processing of solid raw materials such as clays or sand. For the

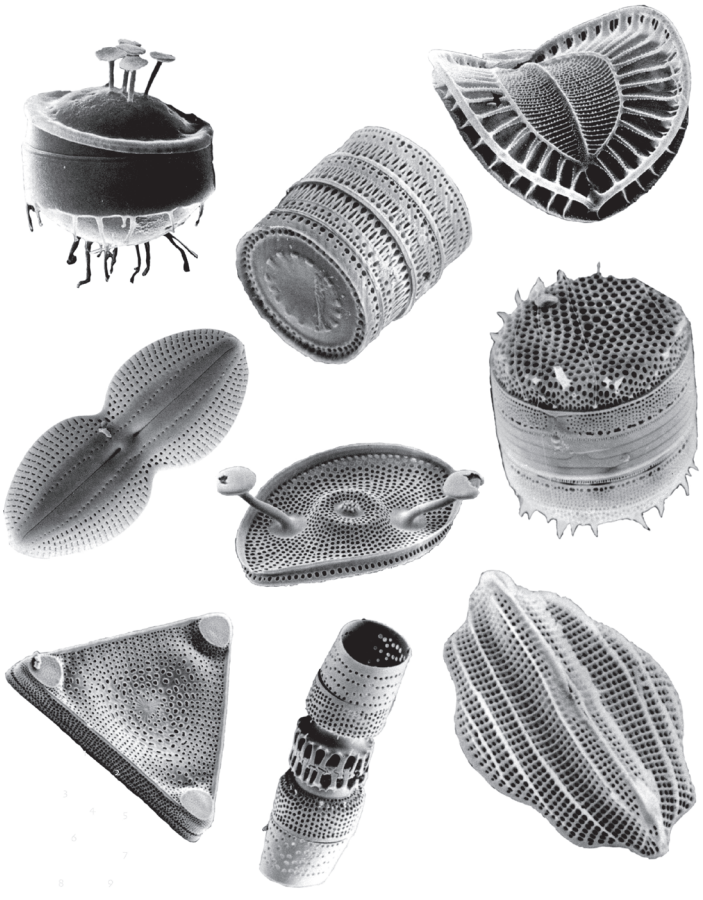

FIGURE 1 - Some examples of diatoms (from Ref. 5).

reaction to proceed at a reasonable rate, powders are crushed together and heated at high temperatures *via* the so-called called "shake and bake" chemistry. In contrast, diatoms are able to make silica glasses at room temperature using the very small amount of silicic acid $Si(OH)_4$ arising from the dissolution of silica by rain water.

Silicic acid is transported through the membrane of diatoms, stored in the cell and transformed into silica in the so-called "Silica Deposition Vesicles" (SDV), and then deposited outside the cell.[6,7] The chemical reaction involved in the transformation of silicic acid into silica is called condensation. It occurs via the formation of one water molecule between two silanol groups to give a bridging oxygen as follows:

$$Si - OH + HO - Si \rightarrow Si - O - Si + H_2O \quad (1)$$

Actually, such a reaction is quite easy to perform *via* the acidification of a sodium silicate solution (water glass). But it is rather difficult to control and usually leads to the precipitation of silica.[8] Therefore chemists are working with other molecular precursors such alkoxides $Si(OR)_4$ where "R" is typically an alkyl group such as $-CH_3$ or $-C_2H_5$. The corresponding alkoxides are called TetraMethylOrthoSilicate (TMOS) and TetraEthylOrthoSilicate (TEOS) respectively. They are commercially available and rather easy to handle.

Two chemical reactions are involved in the formation of silica from silicon alkoxides. The first one, called hydrolysis, leads to the transformation of the alkoxide into silicic acid :

$$Si(OR)_4 + 2H_2O \rightarrow Si(OH)_4 + 4ROH \quad (2)$$

It is followed by the condensation of silicic acid into silica as previously described (eq. 1).

This is the so-called "sol-gel process".[9] Silica is produced from molecular precursors following an "inorganic polymerization reaction". Oligomers are first formed and then colloidal particles that give a sol or a gel of hydrated silica $SiO_2.nH_2O$. Drying and densification can be performed upon heating in order to get bulk silica pieces.

Actually silicon alkoxides and water are not miscible so that a common solvent, usually the parent alcohol ROH, has to be used. Moreover, the chemical reactivity of silicon alkoxides is very low and gelation could take several days. Therefore acid or base catalyses are currently performed, by changing the pH of the water used for hydrolysis. This does not only speed up the kinetics of the reaction, it also changes the morphology of the silica particles. Acid catalysis favors hydrolysis and leads to the formation of chain polymers, whereas base catalysis favors condensation and leads to highly branched species and spherical particles. This is the well-known "Stöber process" currently used for the production of monodispersed silica nanospheres.[10]

3. HYBRID ORGANIC-INORGANIC GLASSES

With the sol-gel process shaped materials can be obtained directly from the solution, allowing the powderless processing of glasses and ceramics. Patents were taken by Schott more than 50 years ago for the sol-gel deposition of coatings on glasses and the industrial production started in the early sixties. The sol-gel process is now very well known and many applications have been developed for the production of thin films, fibers or nanoparticles.[11]

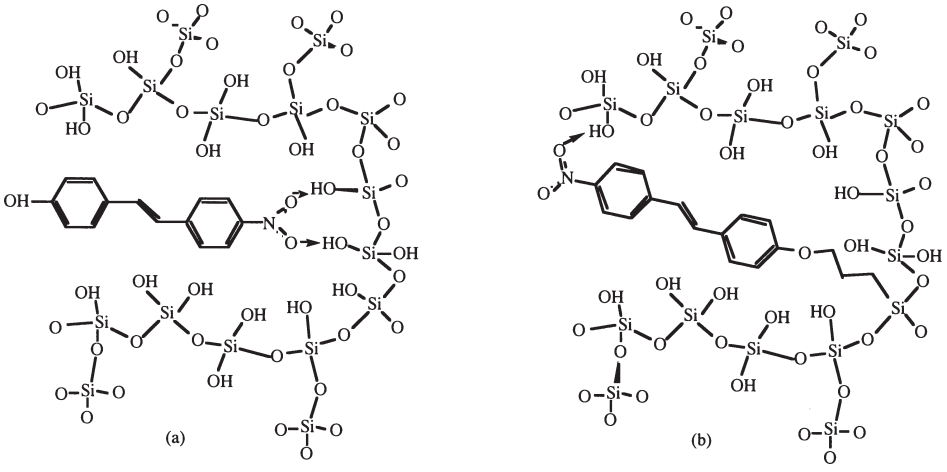

FIGURE 2 - Hybrid organic-silica nanocomposites for optics: the dye can weakly bind the surface of the silica matrix (a) or be covalently linked via functionalized alkoxides (b).

One of main advances of the sol-gel process during the past decades is undoubtedly the synthesis of hybrid organic-inorganic materials.[12] The mild conditions involved in the sol-gel synthesis of metal oxides provide a versatile access to hybrid compounds. The intimate mixing of molecular precursors in organic solvents allows organic and inorganic components to be associated at the molecular level (Fig. 2). These nanocomposites usually exhibit better properties than a simple mixture of both components and are now extensively studied by both polymer chemists and ceramists.[13]

Organic groups bring new properties to the oxide materials. One of the main applications of hybrid materials is for sol-gel optics.[14] The mean size of organic and inorganic phases can be of the order of few nanometers. Therefore they are transparent and can be used for optical applications. Moreover, due to their improved mechanical properties, hybrid sol-gel matrices can be polished down to one nanometer in surface roughness. Sol-gel optics takes advantage of the optical properties of organic dyes together with the hardness and optical transparency of silica. A large number of organic dyes have been entrapped within sol-gel silica matrices. They provide optical properties such as fluorescence, laser emission, photochromism, non linear optics or photochemical hole burning.[15]

Optical devices require dense matrices that can be perfectly polished whereas chemical sensors can be obtained when organic molecules are embedded within a porous sol-gel matrix. Small analytes diffuse in and out of the silica matrix and react with entrapped organic dyes.[16]

4. BIOENCAPSULATION

4.1. Biocompatible sol-gel route

The sol-gel encapsulation of biomolecules is becoming a very popular method.[17-20] Inorganic matrices offer several advantages compared to polymers currently used for bioencapsulation. They exhibit improved mechanical strength and chemical stability. They do not swell in water, preventing the leaching of trapped molecules.

However, sol-gel chemistry is not mild enough for fragile biomolecules such as enzymes. Proteins are denatured by alcohol and have to be kept at a pH close to pH~7. The sol-gel process has then to be modified to fit with the requirements of biomolecules and encapsulation is currently performed in two steps:[21]

i) Acid hydrolysis: Because of its high dielectric constant, methanol is less harmful than ethanol, therefore TMOS is taken as a precursor rather than TEOS and water is added directly without alcohol as a co-solvent. An emulsion is then formed that has to be vigorously shaken (often via sonication) for hydrolysis to take place. Some acid (HCl) is usually added to the water in order to increase hydrolysis rates and the alcohol released during this reaction is sufficient to form a homogeneous solution after few minutes.

ii) Basic condensation: Proteins are kept in a buffered medium around pH~7 and mixed with the aqueous solution of hydrolyzed precursors $Si(OH)_4$. Basic catalysis favors condensation and gelation occurs within few minutes. A porous silica network is formed and biomolecules remain trapped within the growing oxide network. The pore size depends on the sol-gel procedure (hydrolysis ratio, pH, aging, sonication...). It currently ranges between one and ten nanometers.

4.2. Encapsulation of enzymes

A large number of enzymes have been trapped within sol-gel glasses showing that they retain their catalytic activity and can even be protected against degradation by the silica matrix.[22,23] Encapsulated enzymes are trapped in a silica cage tailored to their size. Mobility within this confined space is restricted avoiding the denaturation of the active site that retains its geometrical configuration. 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 or charge of the internal environment within the nanopores.

Lipases provide a nice example showing how a chemical control of the sol-gel matrix can be used to improve enzymatic activity. They are involved

in 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.[24] Their activity in hydrophilic silica matrices is rather poor but they can be almost 100 time more active when trapped within a hybrid silica matrix. Using hybrid precursors such as $RSi(OMe)_3$ or adding polymer additives such as polyethylene glycol (PEG) or polyvinyl alcohol (PVA) provides organic groups that offer a lipophilic environment that could interact with the active site of lipases and increase their catalytic activity.[25-27] Such entrapped lipases are now commercially available and offer new possibilities for organic chemistry, food industry and oil processing.

4.3. Whole cell entrapment

The catalytic activity of enzymes in silica gels has been already extensively studied. However, the example of diatoms suggests that living cells could also be kept inside a silica cage. This is actually one of the major challenge for sol-gel materials. Would it be possible to trap living cells within a porous silica matrix ?

Sol-gel encapsulation could offer a simple and generic method for whole cell immobilization. It does not destroy the cellular organization of micro-organisms. The high porosity of silica gels favors water retention and nutrient diffusion allowing biochemical exchanges between trapped cells and the surrounding medium.

The first paper was published by G. Carturan *et al* showing that encapsulated yeast spores were able to retain their bioactivity.[28] These experiments were performed with *Saccharomyces cerevisiae* that are currently employed for the fermentation of beer and the raising of bread, but other yeast cells have been immobilized for environmental protection or metal recovery.[29,30] The fine porosity of the gel permits substrates to reach the cells and by-products to escape, but prevents cells from leaching out. Silica gels can be washed with water in order to remove fermentation by-products and then used again for several weeks.[31,32]

More recently *Escherichia coli* bacteria have also been trapped within silica gels. Transmission electron microscopy shows that their cellular integrity is preserved by the encapsulation process (Fig. 3a). *E. coli* induced for b-galactosidase still exhibit enzymatic activity showing that substrate molecules can diffuse through both, the pores of the gel and the cell membrane.[33] However the enzymatic activity of trapped bacteria does not mean that they

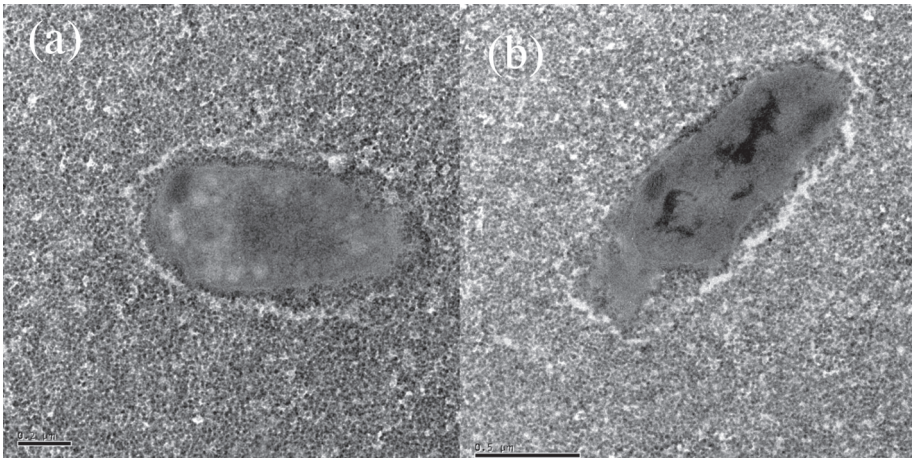

FIGURE 3 - Transmission electron micrograph of E. coli cells in silica gels. a) one day after encapsulation b) one month after encapsulation

remain alive. Bacteria that are damaged or even dead may still maintain some enzymatic activity and then behave as a "bag of enzymes". TEM experiments actually show that some cells may be lyzed after few days of encapsulation (Fig. 3b).

As a matter of fact, the production of metabolites involves more complex pathways that require whole-cell integrity. Maintaining trapped cells alive is therefore a real challenge for sol-gel immobilization and viability tests have to be to performed in order to check the viability of bacteria in a silica matrix.

Genetically engineered *Escherichia coli* have recently been trapped in alkoxide-based silica films. They appear to maintain their ability to synthesize luminescent proteins in the presence of chemical inducers over months.[34] The stress-dependent luminescence properties of these cells provide information about their state during the sol-gel process and within the silica gels, and can be used to optimize the sol-gel procedure.[35]

However, investigating the effect of alcohol release during alkoxide hydrolysis revealed that this process can be harmful for encapsulated cells.[36] In contrast, using aqueous precursors, such as sodium silicate solutions and colloidal silica, which are very similar to the naturally-occurring silicon species used by diatoms, appears more suitable for cell encapsulation.[37]

Despite a number of advantages when compared to polymer systems, sol-gel silica matrices are not yet considered as suitable hosts for cell-based bioreactors. In fact, when entrapped within polymer hosts, cells are still able to divide and the micro-organism population is continuously renewed. In the

case of sol-gel matrices, such division is no longer possible so that efforts should be made to maintain cell viability over a long period of time. This is only possible if the micro-organisms adapt their metabolism to their new confined environment.

In this context, recent experiments have shown that the sol-gel process could be improved to preserve the viability of trapped *Escherichia coli*. About half of these bacteria remain viable after one month when sol-gel encapsulation is performed with aqueous precursors, in the presence of glycerol, a well-known cryo-protective agent currently used for bacteria conservation (Fig 4).[38] Because of space limitation, trapped bacteria cannot divide any longer. Thus, during these experiments, nutrients were not provided to encapsulated cells in order to limit their growth propensity. However, they adapt their metabolism to these new conditions and remain culturable, forming colonies again when the gel is redispersed in a culture media. Moreover, they still exhibit glucose uptake and glycolysis activity, that could be studied *in situ* through radioactivity and NMR measurements.[39] This suggests that trapped bacteria are still able to maintain their cellular homeostasis, *i.e*. to maintain an almost constant internal environment despite changes in the surrounding external medium.

Serratia marcescens bacteria produce a red pigment, called prodigiosin, that exhibits some promising therapeutic properties. In order to improve the viability of bacteria and their metabolic activity within sol-gel silica matrices, the effect of acylated homoserine lactones as "quorum sensing" (QS) molecules was investigated. In Gram-negative bacteria, these molecules are involved in the expression of genes as a function of cell population density.[40] They are

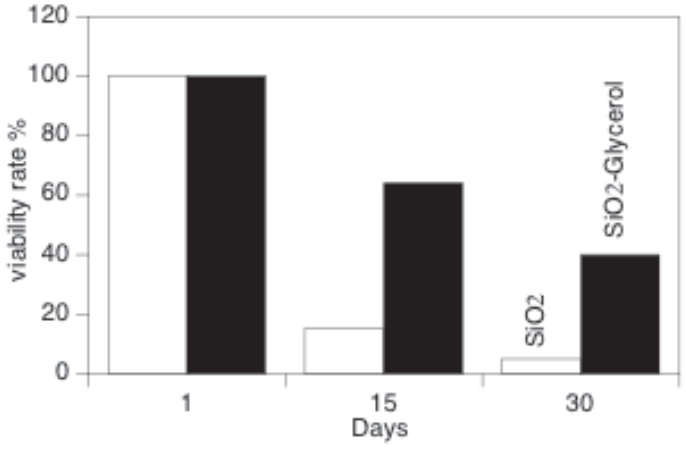

FIGURE 4 - Viability of E. coli *in silica gels (adapted from Ref. 39).*

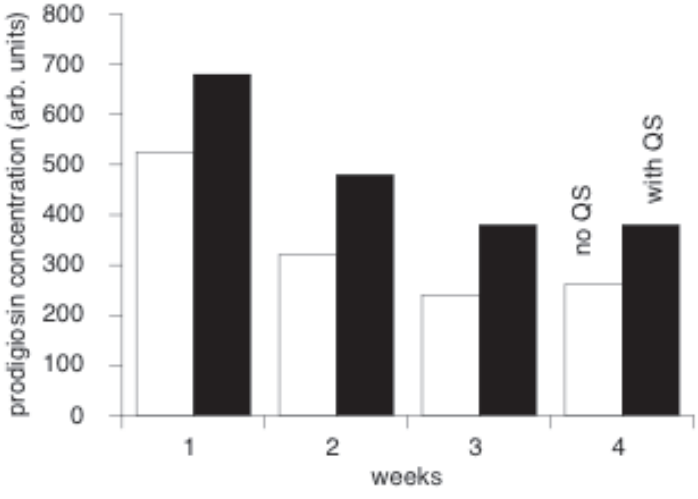

FIGURE 5 - Prodigiosin production by Serratia marcescens *in silica gels (adapted from Ref. 41)*

specifically released as diffusible signals for cell-to-cell communication within a bacterial population and have been shown to regulate cellular adaptation to changing environmental conditions. They could therefore be helpful to maintain the viability of bacteria encapsulated in sol-gel matrices.

Adding quorum sensing molecules significantly improves the viability of *Serratia marcescens* bacteria over one month. As a result, over the same period of time, the production of prodigiosin is noticeably enhanced in the presence of QS molecules (Fig. 5).[41] These results open new possibilities for the design of efficient, re-usable bioreactors, whose properties can be triggered by external molecules, such as QS molecules.

4.4. Medical applications

Antigen-antibody reactions have also been performed within sol-gel matrices extending the field of sol-gel chemistry toward immunosensors. For medical applications, whole cell parasitic protozoa have been trapped within sol-gel matrices and used as antigens for blood tests with human sera. Antigen-antibody interactions were followed by the so-called Enzyme Linked ImmunoSorbent Assays (ELISA) that are widely used in parasitology. The presence of antibodies in the blood is detected via a colored reaction and optical density measurements show a clear-cut difference between negative and positive sera.[42]

The encapsulation of living cells could offer some promising alternative for cell transplantation therapy. Sol-gel encapsulation was performed with mammalian tissues such as the pancreatic islets of Langerhans, which produce insulin in response to glucose. After encapsulation they have been transplanted into a diabetic mouse where they have been shown to retain their activity.[43] The fine porosity of the gel protects transplanted islets against antibody aggression but permits nutrients to reach the cell and byproducts to escape. After one month of transplantation, the surgically removed transplant showed no evidence of fibrosis. Such transplants, if viable for extended lengths of time, could emerge as a viable treatment for diabetes.

These results are highly promising, however silica encapsulation is still in its infancy and sol-gel technology cannot yet compete with polymers. One interesting issue, suggested by G. Carturan *et al*., could be to coat alginate microspheres, which are currently used for the design of "artificial organs", by a siliceous layer in order to improve their hardness and chemical durability. The so-called "biosil" process, based on the gas phase deposition of a thin mineral layer on the surface of living cells, has been successfully used for animal cells and cell aggregates.[44] This process could be used for cell transplantation without immuno-suppression and to develop extra-corporeal artificial liver. Hybrid polymer-silica materials may be the future for sol-gel biotechnology !

ACKNOWLEDGMENTS

This paper is mainly based on the work of Souad Fennouh, Nadine Nassif, Cécile Roux and Odile Bouvet.

REFERENCES

1. C.N.R. Rao and A.K. Cheetham, *J. Mater. Chem*, **11**, 2887 (2001).
2 H.A. Lowenstam, *Science*, **211**, 1126 (1981).
3. T.L. Simpson and B.E. Volcani, *Silicon and siliceous structures in biological systems*, Springer, New York (1981).
4. F. Round, R. Crawford and D. Mann, *The Diatoms*, Cambridge University Press, Cambridge (1990).
5. V. Martin-Jézéquel, J.P. Lopez, in *Silicon biomineralization : biology, biochemistry, molecular biology, biotechnoloy*, W.E.G. Muller Ed., Springer Verlag Pub. (2003) pp. 99-124.
6. N. Kröger and M. Sumper, in *Biomineralization : from biology to biotechnology and medical application*, ed E. Bauerlein, Wiley-VCH, Weinheim (2000).
7. T. Coradin and P. J. Lopez, *ChemBioChem*, **4**, 251 (2003).

8. R.K. Iler, *The chemistry of silica*, Wiley-Interscience, New York (1979).
9. C.J. Brinker and G.W. Scherrer, *The physics and chemistry of sol-gel processing*, Academic Press, Boston (1990).
10. W. Stober, A. Fink and E. Bohn, *J. Colloid. Interf. Sci*. **26**, 62 (1968).
11. J.D. Wright and N. A.J.M. Sommerdjik, *Sol-gel materials. Chemistry and applications*, Gordon and Breach Science Publishers, Amsterdam (2001)
12. C. Sanchez and F. Ribot, *New J. Chem*., **18**, 1007 (1994).
13. *Functional hybrid materials*, eds P. Gomez-Romero and C. Sanchez, Wiley-VCH, Weinheim (2004).
14. *Sol-gel for photonics*, ed S. I. Najafi, SPIE Milestone Series, vol MS 148, SPIE Optical Engineering Press, Bellingham (1998).
15. C. Sanchez, B. Lebeau, F. Chaput and J. P. Boilot, *Adv. Mater*., **15**, 1969 (2003).
16. B.D. McCraith, C. McDonagh, A.K. McEvoy, T. Butler, O'Keeffe, V. Murphy, *J. Sol-Gel Sci. Techn*., **8**, 1053 (1997).
17. D. Avnir, S. Braun, O. Lev and M. Ottolenghi, *Chem. Mater*. **6**, 1605 (1994).
18. J. Zink, J.S. Valentine, and B. Dunn, *New J. Chem*. **18**, 1109 (1994).
19. I. Gill, and A. Ballesteros,. *J. Am. Chem. Soc*. **120**, 8587 (1998).
20. J. Livage, T. Coradin and C. Roux, *J. Phys. Cond. Matter* **13**, R673 (2001).
21. L.M. Ellerby, C.R. Nishida, F. Nishida, S.A.Yamanaka, B. Dunn, J.S. Valentine and J.I. Zink, *Science*, **255**, 1113 (1992).
22. I. Gill and A. Ballesteros, *Trends Biotechnol*. **18**, 282 (2000).
23. W. Jin and J.D. Brennan, *Anal. Chim. Acta* **461**, 1 (2002).
24. R.D. Schmid and R. Verger, *Angew. Chem. Int. Ed*., **37**, 1608 (1998).
25. M.T. Reetz, *Adv. Mater*., **9**, 943 (1997).
26. T. Keeling-Tucker, M. Rakic, C. Spong C. and J. D. Brennan., *Chem. Mater*., **12**, 3695 (2000).
27. A. Pierre and P. Buisson*, J. Mol. Cat. B*, **11**, 639 (2001).
28. G. Carturan, R. Campostrini, S. Dirè and E. de Alteris, *J. Mol. Cat*., **57**, L13 (1989).
29. T. Brányik, G. Kuncová, J. Páca and K. Demnerová, *J. Sol-Gel Sci. Technol*., **13**, 283 (1998).
30. J. Raff, U. Soltmann, S. Matys, S. Selenska-Pobell, H. Böttcher and W. Pompe, *Chem. Mater*., **15**, 240 (2003).
31. E.J.A. Pope, *J. Sol-Gel Sci. Technol*., **4**, 225 (1995).
32. M. Al-Saraj., M.S. Abdel-Latif, I. El-Nahal and J. Baraka, *J. Non-Cryst. Solids*, **248**, 137 (1999).
33. S. Fennouh, S. Guyon, C. Jourdat, J. Livage and C. Roux, *C.R. Acad. Sci. Paris Iic,* **2**, 625 (1999).
34. J.R. Premkumar, O. Lev, R. Rosen and S. Belkin, *Adv. Mater*., **13**, 1773 (2001).

35. J.R. Premkumar, E. Sagi, R. Rosen, S. Belkin, A.D. Modestov and O. Lev, *Chem. Mater.*, **14**, 2676 (2002).
36. A. Coiffier, T. Coradin, C. Roux, O.M.M. Bouvet and J. Livage, *J. Mater. Chem.*, **11**, 2039 (2001).
37. K.S. Finnie, J.R. Bartlett and J.L. Woolfrey, *J. Mater. Chem.*, **10**, 1099 (2000)
38. N. Nassif, O. Bouvet, M.N. Rager, C. Roux, T. Coradin and J. Livage, *Nature Mater.*, **1**, 42 (2002).
39. N. Nassif, C. Roux, T. Coradin, M.N. Rager, O.M.M. Bouvet and J. Livage, *J. Mater. Chem.*, **13**, 203 (2003).
40. B.A. Lazarrera, *Curr. Opin. Microbiol.*, **3**, 177 (2000).
41. N. Nassif, C. Roux, T. Coradin, O. M. M. Bouvet and J. Livage, *J. Mater. Chem.*, **14**, 2264 (2004).
42. J. Livage, C. Roux, J. M. Da Costa, I. Desportes and J. F. Quinson, *J. Sol-Gel Sci. Technol.*, **7**, 45 (1996).
43. E.J.A. Pope, K. Braun and C.M. Peterson, *J. Sol-Gel Sci. Techn.*, **8**, 635 (1997).
44. G. Carturan, R. Dal Toso, S. Boninsegna and R. Dal Monte, *J. Mater. Chem.*, **14**, 2087 (2004).

Discussion

L.L. Hench: It is possible to use silica gel trapped bacteria to develop new antibiotics ?

J. Livage: The example of Serratia marcencens described in this paper shows that trapped bacteria are still able to produce metabolites such as prodigiosin. Therefore we may assume that other bacteria should also behave the same way. Actually the production of metabolites by plant cells trapped in silica gels was also demonstrated by G. Carturan *et al, Journal of Sol-Gel Science and Technology*, **26,** 1189 (2003).

J. Lis: Can you explain the advantages of using silica for encapsulation of biospecies in comparison to common polymers more explicitly ?

J. Livage: Natural (polysaccharides, proteins) and synthetic (polyacrylamide, polyvinyl alcohol,...) polymers are currently used for enzyme immobilization via covalent binding or encapsulation. Inorganic matrices such as silica glasses would offer significant advantages such as improved mechanical strength and chemical stability. They cannot be used as a nutrient by cells and moreover they don't swell in most solvents preventing the leaching of entrapped biomolecules.

J. Adair: What is the status of animal model or human trials for the Islet of Langerhans cell transplantation in silica-based encapsulation ? This has many important implications for enzyme therapy.

J. Livage: This work is done in Italy, you will find latest results in the following paper: G. Carturan, R. Dal Toso, S. Boninsegna, R. Dal Monte, *J. Mater. Chem*., **14**, 2087 (2004).

M. Yoshimura: SiO_2 membrane is easy to form from solution(s). However, other biomaterials like magnetite, Ca- phosphates, Ca- carbonates, etc., are rather difficult to use in making membrane(s) from solutions. Could you please comment the possible applications for ceramics/biology or ceramics/medical areas ?

J. Livage: Silicon molecular precursors such as silic acid or silicon alkoxides are not very reactive. It is rather easy to control the hydrolysis-condensation reactions. Moreover, they lead to the formation of amorphous silica that can give monodispersed spherical nanoparticles. This is no longer the case with phosphates or carbonates. The formation of a solid phase should be described

as precipitation rather than condensation. However such reactions can also be chemically controlled in order to give nanostructured particles that can be used for medical applications. Several examples can be found in the special issue "bio-related materials" of the Journal of Materials Chemistry published in July, 2004.