Living bacteria in silica gels

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The encapsulation of enzymes within silica gels has been extensively studied during the past decade for the design of biosensors and bioreactors[1–11]. Yeast spores and bacteria have also been recently immobilized within silica gels[12–17] where they retain their enzymatic activity, but the problem of the long-term viability of whole cells in an inorganic matrix has never been fully addressed. It is a real challenge for the development of sol–gel processes[18]. Generic tests have been performed to check the viability of *Escherichia coli* bacteria in silica gels. Surprisingly, more bacteria remain culturable in the gel than in an aqueous suspension. The metabolic activity of the bacteria towards glycolysis decreases slowly, but half of the bacteria are still viable after one month. When confined within a mineral environment, bacteria do not form colonies. The exchange of chemical signals between isolated bacteria rather than aggregates can then be studied, a point that could be very important for 'quorum sensing'[19].

Many methods are available to assess the viability of cells. Some test the membrane integrity by exclusion of nucleic acid labelling agents as in the Live/dead BacLight kit[20], whereas others demonstrate metabolic activities of the cell. The definition of viability may therefore be ambiguous[21]. In this paper, the culturability of bacteria was checked by the usual plate-count technique, which gives the number of bacteria that are able to form colonies in the presence of a culture medium. The viability was monitored by following the metabolic activity of these bacteria toward glycolysis, using ^{14}C measurements and ^{13}C NMR experiments.

Silica gels are currently made by the hydrolysis and condensation of silicon alkoxides such as $Si(OCH_3)_4$. However, alcohol is produced during these reactions and this by-product is not cytocompatible. Previous experiments showed that the enzymatic activity of trapped *E. coli* cells follows the usual Michaelis–Menten law, and that their bioactivity was even better than that of a suspension of bacteria[14]. This was attributed to lysis of the cell membrane arising from encapsulation; diffusion through the membrane then becomes easier and the kinetics of the enzymatic reaction become faster. Degradation was more limited when silica gels were synthesized from aqueous precursors than from silicon alkoxides, but some lysis was still observed[22]. Several additives have therefore been used to protect the cells during encapsulation. The best results were obtained with glycerol, a well-known cryoprotective agent currently used to preserve bacteria during lyophilization.

The entrapment of bacteria was performed using the aqueous route described previously[22]. Bacteria were suspended in an aqueous buffer solution with or without added glycerol (10%) before encapsulation.

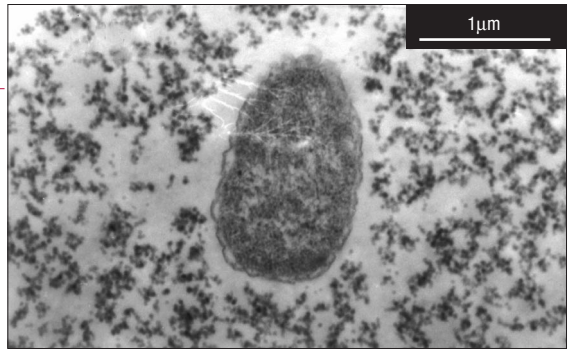

Figure 1 Transmission electron micrograph of an *E. coli* bacterium entrapped in a glycerol-containing gel after 24 h.

Two series of experiments have been performed with bacteria in the water–glycerol solution and bacteria trapped within silica gels. In both cases, bacteria are kept at 20 °C without any nutrient. Transmission electron micrographs show that the cellular integrity of bacteria is maintained within the gel (Fig. 1).

The plate-count technique shows that the number of culturable bacteria decreases with time (Table 1). After one month, the count is very low for bacteria trapped in a pure silica gel, as well as for bacteria suspended in a water–glycerol solution (~10%). The percentage of culturable bacteria remains much higher (~40%) in silica gels containing glycerol.

The metabolic activity of *E. coli* cells towards glycolysis was checked by adding glucose after two or four weeks. Glycolysis is the first step for fermentation and cellular respiration. Glucose transport through the membrane is a complex active process that involves a chain of 11 different enzymes to degrade glucose into pyruvate. During this process, chemical energy is produced via the formation of ATP while electrons are released, leading to the formation of NADH.

The glucose uptake by living bacteria was followed using ^{14}C-labelled glucose molecules. The titration of ^{14}C metabolized by the cells shows that the number of viable cells decreases with time (Table 2). Actually, very few bacteria remain viable when trapped within a pure silica gel. Their viability remains much higher in both aqueous suspensions and gels in the presence of glycerol. About 55% of the bacteria are still active toward glycolysis.

The formation of metabolites arising from glycolysis under anaerobic conditions was followed by ^{13}C NMR using [1-^{13}C] glucose. The anomeric ^{13}C carbon is preserved during fermentation, allowing the characterization of metabolites (Fig. 2). As previously, the behaviour of

Table 1 Percentage of culturable bacteria as a function of the ageing time.

Medium	2 weeks	4 weeks
Water–glycerol solution	35%	10%
Silica gel without glycerol	15%	10%
Silica gel with glycerol	65%	40%

Table 2 Percentage of bacteria that remain able to incorporate glucose, as followed by ^{14}C titration.

Medium	2 weeks	4 weeks
Water–glycerol solution	75%	55%
Silica gel without glycerol	25%	5%
Silica gel with glycerol	65%	55%

bacteria in pure silica gels is not very active. After two weeks, metabolites are no longer observed and the decrease in intensity of non-metabolized glucose peaks is smaller. In the presence of glycerol, several peaks corresponding to the formation of the usual anaerobic metabolites such as acetate can still be observed after this delay, together with some other peaks that could correspond to alanine (δ = 17.3 p.p.m.) and pyruvate (δ = 27.3 p.p.m.) (Fig. 2). The intensity of glucose peaks decreases significantly, showing that it has been metabolized. A comparison of these intensities shows that about 30% of the glucose is metabolized by bacteria trapped in pure silica gels for two weeks. This value increases up to 80% when the gel is formed in the presence of glycerol. The viability of trapped bacteria decreases with time and the percentage of metabolized glucose also decreases, therefore the decrease in the relative intensity of the corresponding ^{13}C NMR peaks is smaller.

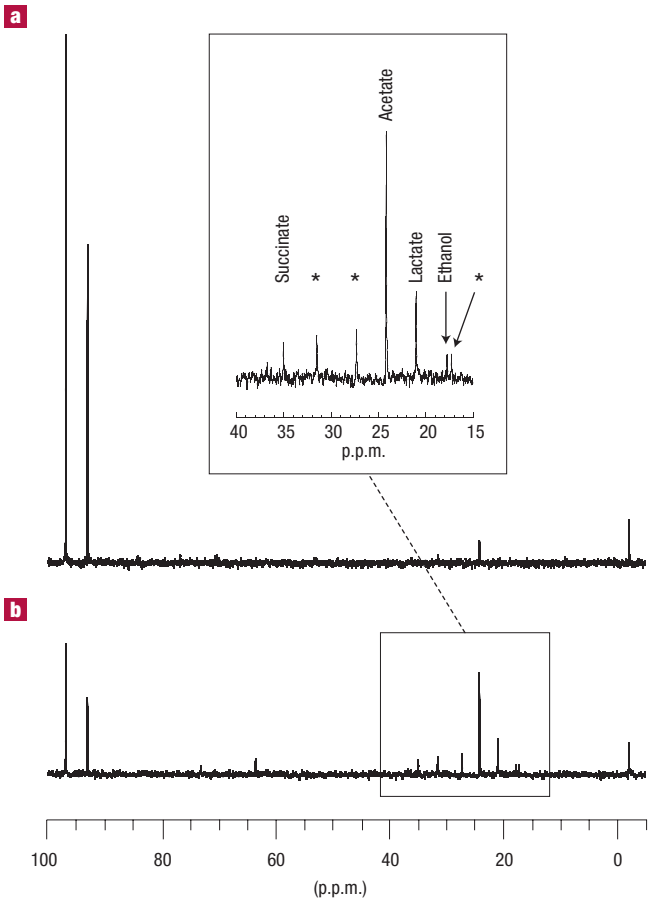

Figure 2 Determination of the formation of metabolites by ^{13}C NMR. Spectra of a [1-^{13}C] glucose solution: **a**, in the presence of encapsulated bacteria aged for 15 days in a pure silica gel, and **b**, in a glycerol-containing gel. Relevant chemical shifts: ethanol (δ = 17.8 p.p.m), lactate (δ = 21.0 p.p.m.), acetate (δ = 24.2 p.p.m.), succinate (δ = 35.0 p.p.m.), glycerol (δ = 63.5 p.p.m., 73.1 p.p.m.), glucose (δc_α = 93.1 p.p.m., δc_β = 96.9 p.p.m.) and unidentified compounds (*) (δ = 17.3 p.p.m., 27.3 p.p.m., 31.5 p.p.m.).

These experiments show that the viability of bacteria decreases quite quickly when they are trapped in a pure silica gel. However, many of the bacteria remain viable for more than a month when encapsulation is performed in the presence of glycerol. Glycerol is known to be a powerful osmotic stabilizer; it decreases the activity of water in the solution of silicate precursors, and protects bacteria against the stress arising from encapsulation.

The number of culturable bacteria appears to be much higher when they are trapped in the gel than when suspended in an aqueous suspension, suggesting that silica gels may have some protective effect. Several hypotheses could explain this unexpected observation. Suspended bacteria tend to form aggregates so that the plate-count technique would count aggregates rather than isolated bacteria, giving underestimated results. Moreover, aggregation may favour the differentiation of bacteria into a long-term survival state in which they are not detectable by culturability tests. This evolution towards non-culturable cells seems to be reduced when bacteria are trapped in gels. Aggregates are not formed and chemical signals cannot be exchanged between trapped bacteria.

Sol–gel encapsulation in silica could offer some advantages such as improved mechanical strength, chemical stability and negligible swelling. Despite the good results obtained with the entrapment of animal cells[23], the sol–gel process is still in its infancy, and cannot compete with the widespread encapsulation methods using polymers such as polysaccharides. Now, the real challenge for sol–gel encapsulation remains the viability of entrapped cells. But the production of metabolites is not the only purpose of these studies. Bacteria in silica gels are trapped in a confined space. They cannot divide to form aggregates, even in the presence of nutrient, therefore they remain randomly dispersed and isolated within the silica matrix. Their collective behaviour is disrupted, a phenomenon that could be very interesting, from a basic point of view, to study the life and death of a single bacterium rather than colonies. Bacteria are able to communicate via signalling molecules that are released into the environment. This process is known as 'quorum sensing'[23], and the behaviour of bacteria is modified when they aggregate. Silica matrices would provide the opportunity to control the density of homogeneously dispersed bacteria. Moreover, the problem of life in extreme environments is now becoming very important with the discovery of extremophile archaea (single-celled organisms that live in extreme conditions) or the presence of bacteria in meteorites. Sol–gel encapsulation could provide a good model to mimic the behaviour of bacteria in a mineral environment.

METHODS

BACTERIA SOURCE

Escherichia coli B (CIP-54125) strain was obtained from the Institut Pasteur Collection (Paris, France). Bacteria were harvested from culture in their exponential growth state, collected by centrifugation and washed with a phosphate buffer. Cells were suspended in a water–glycerol (10 wt%) phosphate buffer solution (cells 10^9 ml^{-1}).

SILICA GEL PREPARATION

Silica gels were prepared by means of acidification down to pH 7 of an aqueous solution of sodium silicate (27 wt% SiO_2, 10 wt% NaOH) to which colloidal silica had been added (Ludox HS-40). The mixture was homogenized under mild stirring and mixed with the bacteria suspension. Gelation occurred within about 2 min at room temperature[21]. Wet gels and aqueous bacteria suspensions were kept in their mother solution and aged in a closed flask at 20 °C. Aged gels were then dispersed in a phosphate buffer solution and washed to remove non-encapsulated cells before further experiments.

CELL COUNTING

Culturable cell counting was performed with a series of tenfold dilutions of samples deposited at the surface of trypto casein soy broth agar. Plates were incubated at 37 °C for 24 h before counting the number of colonies. Three series of experiments were performed each time, giving experimental values within a relative dispersion of ± 5%

GLUCOSE INCORPORATION

Glucose incorporation experiments were performed on gels incubated for 2.5 h after adding the label [^{14}C]. Aliquots were deposited on GF/C filters (Whatman; pore size 0.45 μm) and then washed with a phosphate buffer solution to remove non-incorporated glucose. Radioactivity was measured with a scintillation counter (Kontron BETAMATIC IV). Three series of experiments were performed, each time giving experimental values within a relative dispersion of ± 10%.

NMR

The formation of metabolites was followed by ^{13}C NMR. [1-^{13}C] glucose was added to gels, and paraffin oil was deposited over the mixture to achieve anaerobic conditions. Samples were incubated at 35 °C for 48 h under mild stirring. The supernatant solution was filtered and introduced into an 8-mm NMR tube, itself inserted in a 10-mm NMR tube containing D_2O and TMSP (3-trimethylsilyl propionic-2,2,3,3-d4 acid sodium salt) as a reference (δ_{TMSP} = –2.0 p.p.m.). All ^{13}C NMR spectra were recorded under the same conditions at 35 °C on a Bruker Avance 400 Spectrometer at 100.6 MHz. Therefore the absolute intensity of NMR peaks deduced from a mathematical treatment of the FIDs (free-induction-decay signals) are significant of the amount of each species in the sample.

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References

1. Avnir, D., Braun, S., Lev, O. & Ottolenghi, M. Enzymes and other proteins entrapped in sol-gel materials. *Chem. Mater.* **6,** 1605–1614 (1994).
2. Zink, J., Valentine, J. S. & Dunn, B. Biomolecular materials based on sol-gel encapsulated proteins. *New J. Chem.* **18,** 1109–1115 (1994).
3. Livage, J. Bioactivity in sol-gel glasses. *C.R. Acad. Sci. Paris IIb* **322,** 417–427 (1996).
4. Gill, I. & Ballesteros, A. Encapsulation of biologicals within silicate, siloxane and hybrid sol-gel polymers: an efficient and generic approach. *J. Am. Chem. Soc.* **120,** 8587–8598 (1998).
5. Reetz, M. T. Entrapment of biocatalysts in hydrophobic sol-gel materials for use in organic chemistry. *Adv. Mater.* **9,** 943–954 (1997).
6. Dave, B. C., Dunn, B., Valentine, J. S. & Zink, J. I. Sol-gel encapsulation methods for biosensors. *Anal. Chem.* **66,** 1120A–1127A (1994).
7. Lev, O. *et al.* Organically modified sol-gel sensors. *Anal. Chem.* **67,** 22A–30A (1995).
8. Lin, J. & Brown, C. W. Sol-gel glass as a matrix for chemical and biochemical sensing *Trends Anal. Chem.* **16,** 200–211 (1997).
9. Wang, J. Sol-gel materials for electrochemical biosensors. *Anal. Chim. Acta* **399,** 21–27 (1999).
10. Collinson, M. N. & Howells, A. R. Sol-gel and electrochemistry. *Anal. Chem.* **72,** 702A–709A (2000).
11. Wolfbeis, O. S., Reisfeld, R. & Oehme, I. Sol-gels and chemical sensors. *Struct. Bond.* **85,** 51–98 (1996).
12. Carturan, G., Campostrini, R., Dirè, S. & de Alteris, E. Inorganic gels for immobilization of biocatalysts: inclusion of invertase-active whole cells of yeast (*Saccharomyces cerevisiae*) into thin layers of SiO2 gel deposited on glass sheets. *J. Mol. Cat.* **57,** L13–L16 (1989).
13. Pope, E. J. A., Braun, K. & Peterson, C. M. Bioartificial organs I: silica gel encapsulated pancreatic islets for the treatment of diabetes mellitus. *J. Sol-Gel Sci. Technol.* **8,** 635–639 (1997).
14. Fennouh, S., Guyon, S., Jourdat, C., Livage, J. & Roux, C. Encapsulation of bacteria in silica gels. *C.R. Acad. Sci. Paris IIc* **2,** 625–630 (1999).
15. Finnie, K. S., Bartlett, J. R. & Woolfrey, J. L. Encapsulation of sulfate-reducing bacteria in a silica host. *J. Mater. Chem.* **10,** 1099–1101 (2000).
16. Premkumar, J. R., Lev, O., Rosen, R. & Belkin, S. Encapsulation of luminous recombinant *E. coli* in sol-gel silicate films. *Adv. Mater.* **13,** 1773–1775 (2001).
17. Premkumar, J. R. *et al.* Antibody-based immobilization of bioluminescent bacterial sensor cells. *Talanta* **55,** 1029–1038 (2001).
18. Livage, J., Coradin, T. & Roux, C. Encapsulation of biomolecules in silica gels. *J. Phys. Cond. Mater.* **13,** R673–R691 (2001).
19. Swift, S. *et al.* Density-dependent determinant of bacterial physiology. *Adv. Microb. Physiol.* **45,** 199–270 (2001).
20. Haugland, R. P. *Molecular Probe Handbook of Fluorescent Probes and Research Chemicals* (Molecular Probes, Eugene, Oregon, 1996).
21. Villarino, A., Bouvet, O. M. M., Regnault, B., Martin-Delautre, S. & Grimont, P. A. D. Exploring the frontier between life and death in *Escherichia coli*: evaluation of different viability markers in live and heat- or UV-killed cells. *Res. Microbiol.* **151,** 755–768 (2000).
22. Coiffier, A., Coradin, T., Roux, C., Bouvet, O. M. M. & Livage, J. Sol-gel encapsulation of bacteria: a comparison between alkoxide and aqueous routes. *J. Mater. Chem.* **11,** 2039–2044 (2001).
23. Carturan, G., Dal Monte, R. & Muraca, M. SiO_2 entrapment of animal cells for hybrid bioartificial organs. *Mater. Res. Soc. Symp. Proc.* **628,** CC10.1.1–CC10.1.15 (2000).

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Competing financial interests

The authors declare that they have no competing financial interests.