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Sol–gel encapsulation of bacteria: a comparison between alkoxide and aqueous routes

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The viability of bacteria in the presence of sol–gel reagents has been studied in order to define the best experimental conditions for the sol–gel encapsulation of *E. coli*. The β-galactosidase activity of these bacteria, trapped in sol–gel silica matrices, was then analyzed. Two routes, using alkoxide and aqueous precursors, have been used and compared. It appears that the aqueous route is less damaging than the alkoxide one. Moreover the aqueous silica matrix appears to slow down the lysis of cell membranes when bacteria are aged without nutrient.

Introduction

The sol–gel encapsulation of biomolecules has gained considerable interest during the past decade.[1–4] Silica gels are chemically inert, biocompatible and resistant to microbial attack. Compared to organic polymers, they exhibit improved mechanical strength and negligible swelling in aqueous or organic solvent thus preventing the leaching of biomolecules. Moreover, they provide an aqueous environment that preserves the activity of proteins and may even enhance their stability.[5] Upon encapsulation, enzymes are encased by the hydrated silica in a cage tailored to their size. The silica matrix constrains the motions of encapsulated proteins and may prevent irreversible structural deformation.

The immobilization of enzymes within silica gels has already been widely reported but many fewer papers report on the encapsulation of whole cells such as yeast,[6] bacteria,[7–9] *Leishmania*[10] or pancreatic islets.[11–13]

Sol–gel encapsulation is currently performed with alkoxide precursors using a two-step procedure: the acid hydrolysis of $Si(OCH_3)_4$ (TMOS), followed by encapsulation at neutral pH. However chemical reagents are used or produced (alcohol, acid) that could lead to denaturation of the proteins. Therefore, aqueous silica gels have also been studied in order to avoid possible toxic by-products. They are obtained *via* the acidification of aqueous sodium silicate solutions.[14,15] Sodium ions in excess have then to be removed so that the ionic strength of the sol does not lead to lysis of cell membranes.

In both cases, it appears that sol–gel syntheses are still too cytotoxic for extensive use in the immobilization of cells. Progress must be made in order to improve the viability and growth of cells within silica gels.[9] Therefore this paper reports on the viability of bacteria in the presence of sol–gel reagents in order to define the best chemical conditions for sol–gel encapsulation. Both routes are then compared and the β-galactosidase activity of *Escherichia coli* trapped in alkoxide and aqueous silica gels is studied.

Experimental

Bacteria growth and viability

The *Escherichia coli* B (lac+) strain was obtained from the Institut Pasteur Collection (CIP-54125). Overnight cultures of bacteria in Luria–Bertani broth (NaCl 10 g L^{-1}; yeast extract 5 g L^{-1}; tryptone 10 g L^{-1}) at 37 °C were prepared. Growth of bacteria was studied in a MM medium (minimum medium) containing sources of metal cations, nitrogen and sulfur [NaH_2PO_4 0.034 M; K_2HPO_4 0.064 M; $(NH_4)_2SO_4$ 0.02 M; $MgSO_4$ 3×10^{-4} M]. In a typical experiment, a bacteria inoculum (100 μL) was added to the culture medium MM (50 mL) in the presence of glucose (20 mM) as a growth substrate. In order to study the effect of gel synthesis conditions, NaCl, NaOH, HCl, CH_3OH, sodium silicate or TMOS were added. Cells were incubated at 37 °C under stirring. The growth of bacteria was followed by turbidity measurements; the optical density (OD) of suspension aliquots recorded at $\lambda = 600$ nm is known to be proportional to bacteria concentration, as long as the optical density OD_{600} remains lower than 0.8.

The same experiments were performed using starved cells. In order to do so, bacteria were grown in the MM/glucose medium described above. After 4 h, suspensions were centrifuged at 10 °C and washed twice with 100 mL of phosphate buffer TPO_4 (10 mM, pH 7.2). Bacteria were then resuspended in 15 mL of TPO_4 and the corresponding OD_{600} was measured. 3 mL of this solution were put aside for $t = 0$ counting experiments (see below) whereas 2 mL were added to 18 mL of TPO_4 containing the reagent to be tested. This mixture was kept at 20 °C, samples being taken after 1 h ($t = 1$) and 24 h ($t = 24$). For counting experiments, 1 mL of the investigated solution was diluted in 9 mL of TPO_4, such a dilution being then repeated up to eight times till the final solution concentration was 10^8 times lower than the starting suspension. 100 mL of each solution, spilled on a Petri dish coated with Luria–Bertani agar, was then incubated at 37 °C overnight. Only living bacteria can lead to the formation of a colony large enough to be eye-detected so that examination of dishes containing 50–100 colonies allowed us to determine the concentration of living bacteria.

Synthesis and characterization of silica gels

Silica sources were a sodium silicate solution (27 wt% SiO_2, 14 wt% NaOH from Riedel-de Haën), colloidal silica (LUDOX HS-40 from Aldrich) and a silicon alkoxide [TMOS: $Si(OCH_3)_4$, from Fluka].

The aqueous synthesis of silica gels was performed following the two-step synthesis method recently described, with slight modifications due to bacteria encapsulation specificity.[14,16] In order to determine the appropriate reaction conditions,

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matrices were first synthesized in the absence of bacteria. Hydrochloric acid was added to a mixture containing sodium silicate (SiNa) and colloidal silica (LUDOX) in order to decrease the pH to *ca.* 7, before TPO_4 buffer was added. The influence of SiNa concentration, SiNa : LUDOX and Si : TPO_4 volume ratios upon gelation time was studied.

The porosity of xerogels obtained in the absence of bacteria was measured by the nitrogen sorption technique. Gels were aged for 3 h at room temperature and dried at 60 °C to give xerogels. Nitrogen sorption experiments were performed at 77 K on a Micromeritics 2010 sorptometer. Prior to analysis, samples were first degassed at 60 °C under a 3 μm Hg pressure. Specific surface areas were determined by the BET method in the 0.05–0.3 relative pressure range[17] and pore diameter distributions were calculated by BJH model on adsorption branch,[18] the model of Broekhoff and de Boer[19] and the non-local density functional theory.[20]

Xerogel powders were coated with gold in a Balzers Union SCD 40 sputter–coater and studied by scanning electron microscopy (SEM) using a Cambridge Stereoscan 120 instrument at an accelerating voltage of 10 kV.

Sol–gel encapsulation of bacteria

The aqueous route described above was then used for bacteria encapsulation. In order to allow β-galactosidase enzymatic activity measurements, bacteria were grown overnight in a Luria–Bertani culture medium in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer for this enzyme. *E. coli* cells were then separated from the culture medium in their stationary phase by centrifugation and resuspended in TPO_4 buffer. This induced bacteria suspension containing 10^9 cells mL^{-1} was then used as prepared. SiNa : LUDOX and Si : TPO_4 volume ratios were 1 : 2 and 3 : 1, respectively, TPO_4 containing the bacteria suspension. The final bacteria concentration was then close to 2.4×10^8 cells mL^{-1} in a total volume of 4.18 mL. Gelation occurred within about 2 min at room temperature.

The synthesis of silica gels from alkoxides and cell encapsulation were undertaken with pure TMOS according to a previously reported process:[7] a prehydrolyzed solution of TMOS was prepared by direct mixing of TMOS (1 mL) with water (1 mL) and HCl (pH 2; 0.12 mL). After a 10 min stirring, the pH was increased to *ca.* 7 by the addition of TPO_4 buffer (1 mL) before the bacteria suspension in TPO_4 (1 mL) was added, leading to a bacteria concentration of 2.4×10^8 cells mL^{-1} and a total volume of 4.12 mL. Gelation occurred within a few minutes.

Enzymatic activity of trapped bacteria

The behavior of *E. coli* after entrapment was followed *via* its β-galactosidase activity. This intracellular enzyme is known to catalyze the hydrolysis of 4-nitrophenyl-β-D-galactopyranoside (*p*-NPG) to β-D-galactose and *p*-nitrophenol, a yellow compound that can easily be titrated by UV–vis absorption spectroscopy.

Typically, a fraction of 1/10 in mass of gel was crushed and washed three times with TPO_4 (1 mL) in order to remove non-encapsulated bacteria. It was then added to 4.25 mL of TPO_4 and kept under stirring at constant temperature (20 °C). A chronometer was triggered when the *p*-NPG solution (50 mM, 0.75 mL) was added to TPO_4. At regular intervals a suitable volume of the supernatant ($V_s = 10$–100 μL) was sampled and diluted in $(1 - V_s)$ mL of a potassium carbonate solution (1.4 M) in order to quench the enzymatic reaction. The solution was centrifuged to remove bacteria or silica gel residues. The amount of *p*-nitrophenol was calculated from optical density measurements at $\lambda = 400$ nm applying the Beer–Lambert law ($\varepsilon = 18\,900$ mol^{-1} L cm^{-1}). Enzymatic activity, expressed in U or μmol min^{-1}, was then deduced from the initial reaction rate of *p*-nitrophenol formation.

Gels were either aged for 1 hour and immediately used, or kept in sealed containers at 20 °C for 1–7 days before enzymatic activity determination. The enzymatic activity of free bacteria was monitored following the same procedure using 100 μL of bacteria suspension instead of the silica gel.

In order to achieve complete lysis of bacteria, 50 μL of toluene were added to the 4.25 mL of TPO_4 containing the gel or the bacteria suspension and the resulting mixture was stirred for 1 h at 4 °C before the *p*-NPG solution was added.

Results and discussion

Bacteria growth in the presence of sol–gel reagents

In order to optimize encapsulation procedures, the growth of non-induced bacteria suspensions in the presence of sol–gel chemicals (alcohol, pH, NaCl) was studied (Fig. 1). The shape of the curve in the absence of any additional chemical [Fig. 1(a)] was taken as a reference for bacteria growth. A rapid development phase, the 'log phase', is observed during the first 5 h at pH 7. A plateau is then obtained showing that the bacteria population reaches a stationary phase. Fig. 1(a) also shows that pH variations, obtained by adding HCl or NaOH, are detrimental to bacteria growth, especially at very low or very high pH.

The effect of salinity was monitored by adding sodium chloride solutions in the 0.01–1 M concentration range [Fig. 1(b)]. Concentrations up to 0.1 M do not seem to disrupt bacteria growth whereas growth becomes very limited with solutions containing 1 M NaCl. At intermediate concentration values, the slope of the curve corresponding to the 'log phase' decreases but a similar plateau value is reached after *ca.* 24 h. This suggests that bacteria are able to adapt themselves to the surrounding media.

Similar results are obtained in the presence of methanol [Fig. 1(c)]. Low concentrations (0.1 M) appear harmless for bacteria. Their growth slows down during the first hours for larger MeOH concentrations (1 M) but reaches the same maximum value after 24 h. At higher methanol concentrations (5 M) growth appears to be impeded from the very beginning.

The influence of silica precursors was also investigated. However, concentrated solutions lead to very fast gelation; therefore, only dilute solutions were studied. In this frame, at 0.01 M in Si, neither TMOS nor sodium silicate seem to disturb the development of bacteria [Fig. 1(d)].

As a source of complementary information, evolution of starved bacteria as a function of time in the absence of nutrient was studied *via* counting experiments. The results, giving the amount of colony forming units (CFU) per mL are gathered in Fig. 2. They confirm the non-toxicity of silica precursors, NaCl and methanol at low concentrations as well as of pH values between 6 and 8. Furthermore, large NaCl concentrations (1 M) decrease the population of bacteria by a factor of *ca.* 100 after just one hour. Beyond this time, the bacteria population remains nearly constant. In contrast, methanol (5 M) does not induce immediate killing of bacteria but the same loss factor of *ca.* 100 is observed after 24 h.

Synthesis and characterization of silica gels

Synthesis of aqueous silica matrices. The parameters studied above play a major role in the gelation process and the porosity of sol–gel matrices.[21] As far as bacteria are concerned, it is clear that pH, salinity and especially alcohol content must be controlled carefully. Therefore, both aqueous and alkoxide gels were synthesized and compared by keeping constant as many parameters as possible.

The pH of the buffered solutions used for encapsulation was

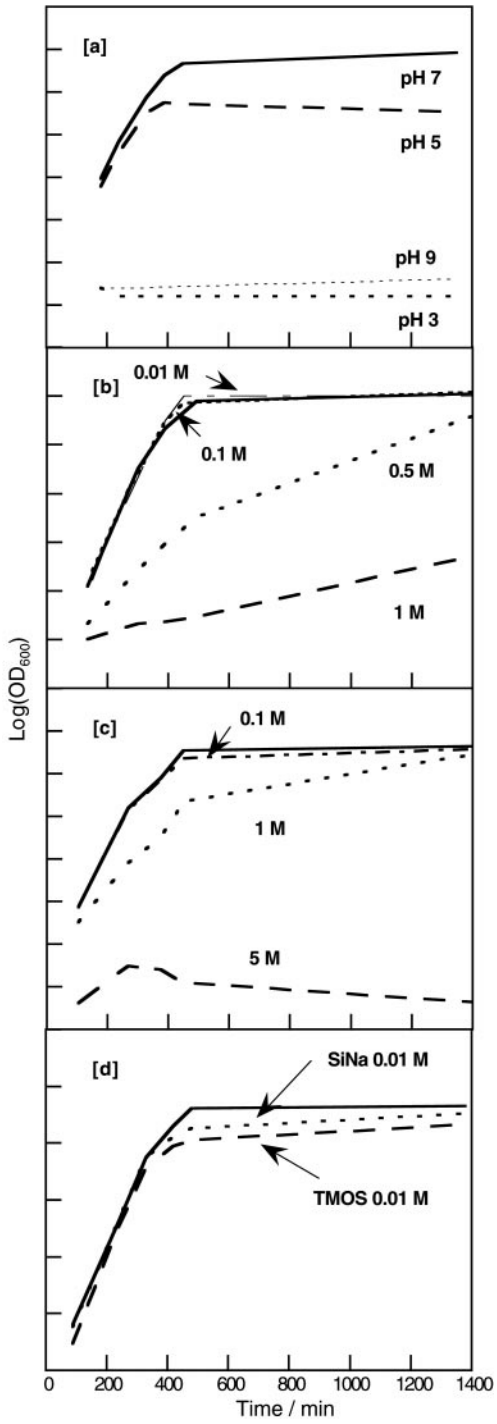

Fig. 1 Growth curves, as a function of time, for *E. coli* suspensions as monitored by UV–vis absorption at $\lambda = 600$ nm: as a function of pH (a) and at pH 7.2 in the presence of NaCl (b), methanol (c) and silica precursors (d).

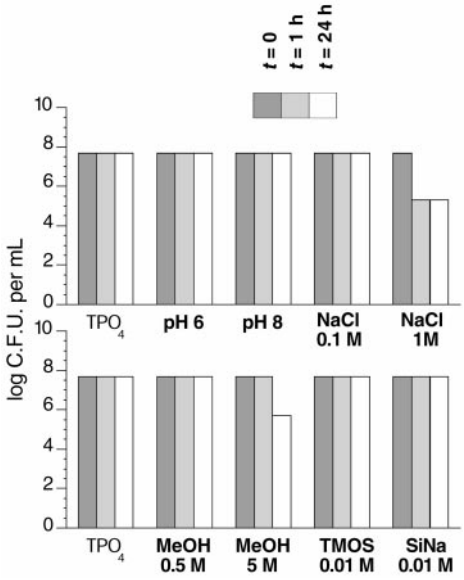

Fig. 2 Viability (in CFU per mL) of starved bacteria suspensions observed at $t = 0$, $t = 1$ and $t = 24$ h in the presence of different chemicals.

set to pH 7.2. The aqueous synthesis was optimized so that the gelation time and final silicon concentration were kept similar to the values involved in the alkoxide route reported in Table 1. It appeared difficult to control the gelation time when pure sodium silicate solutions were used. For concentrated solutions ([Si] > 2 M) gelation occurred easily as soon as HCl was added; consequently, dilute sodium silicate solutions had to be used but they led to very fragile silica gels. Therefore, colloidal silica was added in order to increase the Si concentration without increasing the ionic strength. As seen in Table 1, the addition of LUDOX led to gels with a higher silicon concentration (3–5 M) and allowed an increase in the $Si:TPO_4$ ratio. Silica gels could therefore be synthesized *via* the aqueous route with chemical (pH, final silicon concentration and $Si:TPO_4$ ratio) and physical (gelation time) parameters close to those involved in the alkoxide route (SiNaLudox-3 and TMOS in Table 1).

Characterization of silica matrices. Fig. 3 shows the adsorption–desorption isotherms of N_2 at liquid nitrogen temperature on TMOS, SiNa and SiNaLudox-3 xerogels. Following IUPAC classification,[22] the isotherm for TMOS xerogels is close to a type I isotherm whereas the two others, with a hysteresis loop, correspond to type IV isotherms. Therefore TMOS xerogels can be considered as microporous solids with a relatively small external surface whereas SiNa and SiNaLudox-3 xerogels are mesoporous.

The hysteresis loops of SiNa and SiNaLudox-3 samples exhibit an H2 shape associated with an interconnected network of pores of different sizes and shapes. The hysteresis loop for SiNaLudox-3 matrix is shifted to higher relative pressures

Table 1 Synthesis parameters, final Si and Na^+ concentration values and gelation times of matrices studied in this work

Sample	Conditions			[Si]/mol L^{-1}	[Na^+]/mol L^{-1}	Gelation time/min
Alcohol process pathway						
TMOS	$Si:TPO_4$					
	3 : 1			2.2	—	2
Alcohol-free process pathway						
	[SiNa]/mol L^{-1}	SiNa : LUDOX	$Si:TPO_4$			
SiNa	0.5	11 : 0	12 : 1	1.3	0.87	4
SiNaLudox-1	0.4	2 : 1	3 : 1	4.5	0.28	30
SiNaLudox-2	0.4	2 : 1	2 : 1	3.2	0.31	4.5
SiNaLudox-3	0.4	2 : 1	3 : 1	2.6	0.42	2
SiNaLudox-4	0.25	1 : 1	2 : 1	3.9	0.18	20

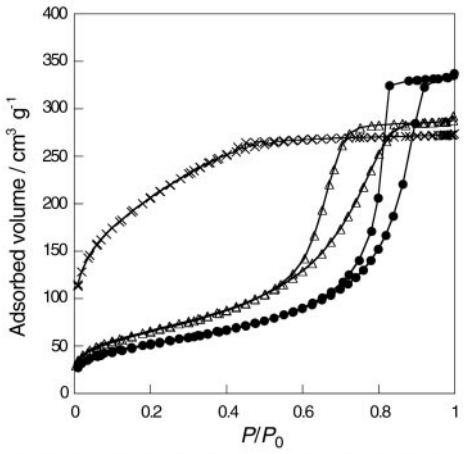

Fig. 3 Nitrogen adsorption and desorption isotherms of TMOS (×), SiNa (△) and SiNaLudox-3 (●) matrices in the absence of bacteria.

(0.62–0.95) when compared with SiNa (0.52–0.90). This shift is due to an increase of the pore size as confirmed by calculated data reported in Table 2. These data show that the evolution of the pore size from TMOS to SiNaLudox-3 is correlated to a decrease of the specific surface and an increase of the total pore volume. Moreover, the specific surface of the SiNaLudox-3 matrix is close to that of pure LUDOX.

Scanning electron microscopy images of TMOS, SiNa and SiNaLudox-3 matrices are shown in Fig. 4. The TMOS xerogel appears to be homogenous whereas a particle-like morphology is obtained for SiNa and SiNaLudox-3 with a more pronounced structuration for the latter. These results appear consistent with the synthesis pH conditions: TMOS gels are obtained *via* the acid hydrolysis of TMOS, favouring the growth of polymeric chains and the formation of microporous gels; aqueous gels are formed *via* the acidification of sodium silicate solutions from a basic pH down to pH 7; basic catalysis promotes the growth of branched polymers and the formation of dense particles.[21]

β-Galactosidase activity of bacteria

The enzymatic activity of free bacteria was first monitored before studying the effect of entrapment. *E. coli* cells were removed from their culture medium and resuspended in a buffered aqueous solution without any nutrient. Their β-galactosidase activity was measured as a function of time *via* the formation of *p*-nitrophenol (Fig. 5). An initial delay period without any response is first observed which probably corresponds to the diffusion of the substrate through the membrane. After this delay, the plot curves upward, reaching a linear regime. The slope of the curve is used to determine the enzymatic activity. The 7.5 mM final concentration corresponding to full hydrolysis of the substrate is obtained after 17 h (*ca.* 1000 min).

Table 2 Isotherm types, BET surfaces (S_{BET}), porous volume (V_p) and average pore size (R_p) as calculated using BJH, DFT and Broekhoff–de Boer models for TMOS, Ludox and SiNaLudox-3 matrices

Sample	Isotherm type	S_{BET}/ m^2 g^{-1}	V_p/cm^3 g^{-1}	R_p/nm
TMOS	I	725	0.42	1.2$^{(BJH)}$ 1.3$^{(DFT)}$ 1.3$^{(BdB)}$
SiNa	IV	307	0.44	3.0$^{(BJH)}$ 3.8$^{(DFT)}$ 4.8$^{(BdB)}$
SiNaLudox-3	IV	215	0.51	5.0$^{(BJH)}$ 6.8$^{(DFT)}$ 9.0$^{(BdB)}$

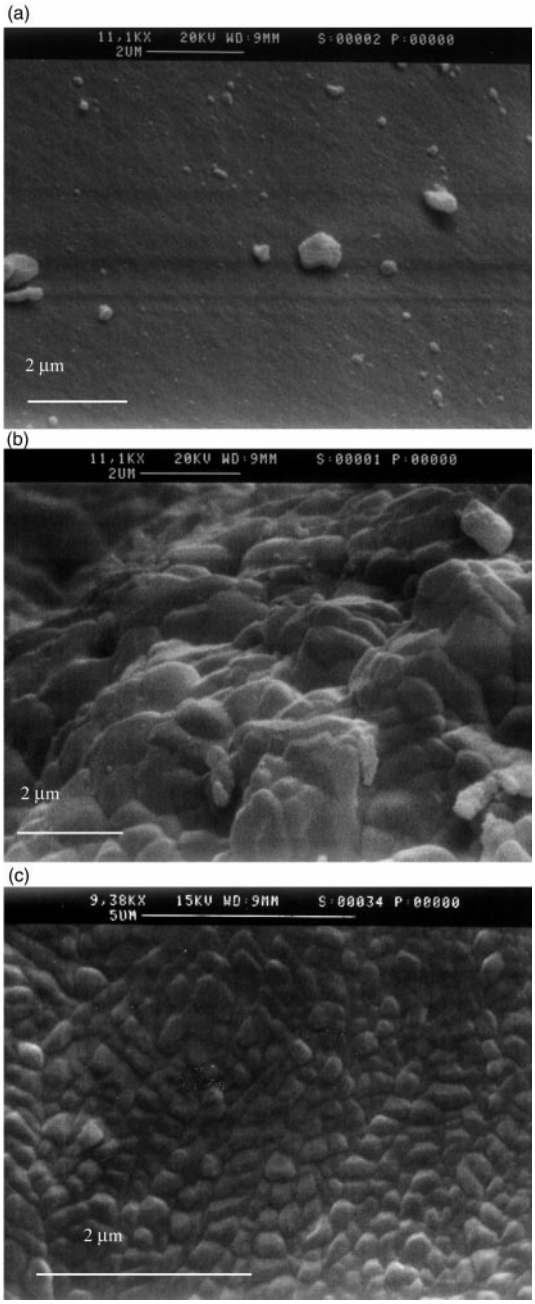

Fig. 4 Scanning electron micrograph of TMOS (a), SiNa (b) and SiNaLudox-3 (c) matrices in the absence of bacteria.

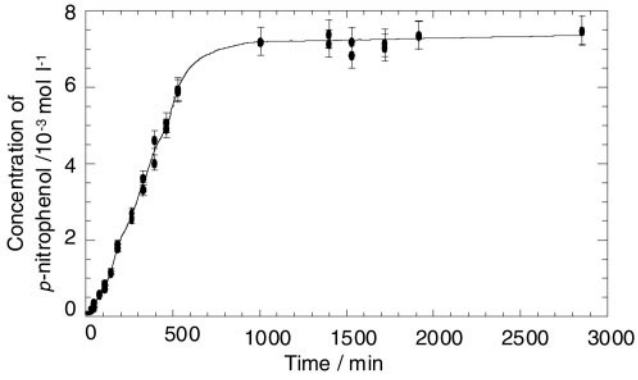

Fig. 5 Formation of *p*-nitrophenol for a bacteria suspension (100 µm of a 10^9 cells mL^{-1} suspension in 5 mL of TPO_4 buffer) with a 7.5 mM substrate concentration as a function of time.

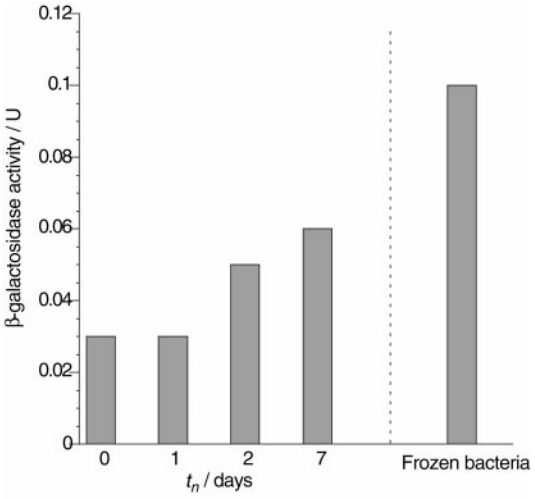

Fig. 6 Evolution of the β-galactosidase activity of a bacteria suspension (100 μm of a 10^9 cells mL^{-1} suspension in 5 mL of TPO_4 buffer) with a 7.5 mM substrate concentration as a function of ageing time. The activity of an equal amount of a suspension of frozen bacteria is also reported.

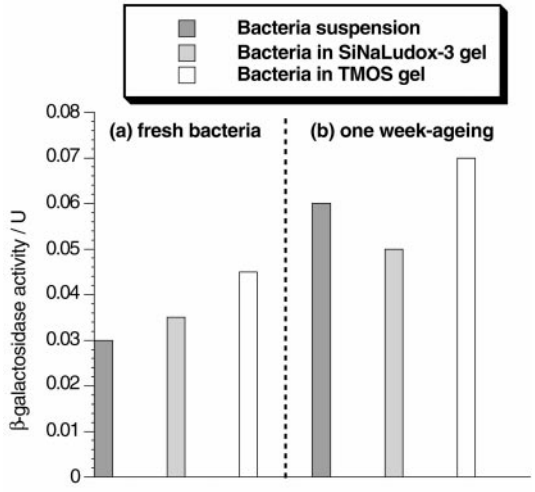

Fig. 7 Evolution of the β-galactosidase activity of free or entrapped bacteria in the presence of 7.5 mM substrate concentration (a) after 1 h ageing, (b) after 1 week ageing.

Natural ageing of a bacteria suspension is an important parameter because it can affect the viability and therefore enzymatic activity of the bacteria. Fig. 6 shows the β-galactosidase activity of bacteria suspensions after various ageing times, t_n. The β-galactosidase activity of fresh bacteria (0.03 U) does not change during the first day, but then increases noticeably to reach a 0.06 U value after one week. Under these conditions the ratio R_A of the activity of aged bacteria *versus* the activity of fresh bacteria, is close to 2. This can be due to an increase in membrane permeability due to some lysis of the bacteria. This hypothesis is confirmed by previous observations performed with fresh bacteria artificially lysed with an organic solvent.[23] Freezing bacteria at $-30\,^{\circ}C$ for one week seems to produce the same effect. The R_A ratios are then close to 3. The diffusion of the substrate through the membrane becomes easier, increasing the observed enzymatic activity. However, Fig. 6 shows that ageing for 7 days without nutrient does not lead to a complete lysis of the cells.

The β-galactosidase activity of bacteria trapped in aqueous and alkoxide gels is larger than that of free bacteria suggesting that some lysis may occur during the sol–gel synthesis [Fig. 7(a)]. Taking the free bacteria activity as a reference, the activity ratio in TMOS gels ($R_A = 1.5$) is slightly larger than in SiNaLudox-3 aqueous gels ($R_A = 1.2$). Since the porosity of the aqueous gel was shown to be larger than that of TMOS gels, such a difference cannot be due to a limited diffusion of the substrate through the silica matrix. It therefore suggests that the aqueous route is less damaging for the bacteria.

In all cases, the β-galactosidase activity of bacteria increases after ageing one week at room temperature [Fig. 7(b)]. However, this effect is smaller for TMOS gels ($R_A \approx 1.6$) than for non-encapsulated bacteria ($R_A \approx 2$). It is even smaller for bacteria encapsulated in the alcohol-free matrix SiNaLudox-3 ($R_A \approx 1.4$). These results suggest that sol–gel entrapment may partially prevent lysis of bacteria. Such a protecting effect depends on the nature of the host matrix, being greater with aqueous than alkoxide gels.

Finally, it had to be taken into account that lower enzymatic activities could also be attributed to β-galactosidase denaturation. Therefore, complete lysis of bacteria, either free or entrapped within aqueous and alkoxide silica matrices, was undertaken in the presence of toluene. In all cases, the resulting β-galactosidase activity was 0.1 U, thus indicating that the amount of enzymes contributing to the measured activities was the same in each sample.

Conclusion

The study of bacteria growth and viability in the presence of sol–gel reagents (MeOH, pH, NaCl) led us to design aqueous silica matrices for the entrapment of *E. coli* cells. Based on β-galactosidase activity studies, the aqueous silica matrices appear to be less detrimental to cell viability when compared with the usual alkoxide route. Moreover, they seem to offer an efficient protection against ageing when compared with free bacteria suspensions.

β-Galactosidase is an intra-cellular enzyme whose activity strongly depends upon the amount of substrate that is able to diffuse, through the cell membrane, to its catalytic site. In this context, it has been previously shown,[23] and here confirmed, that lysis of the *E. coli* membrane leads to an increase in the β-galactosidase activity. For entrapped bacteria, the substrate must also diffuse through the pores of the gel. However, pore sizes of both aqueous and alkoxide gels are much larger than the molecular size of *p*-NPG such that this effect could be neglected. It therefore appears that membrane permeability may be a key-point to the understanding of variations in enzymatic activity. Changes in permeability induced by entrapment could then be due to several factors: constraints due to the confinement of bacteria within the silica gel, interactions between the cell membrane and the silica matrix and the toxicity of chemical reagents. As far as toxicity is concerned, we have already pointed out that the effect of high concentrations of silica sources (TMOS or SiNa) on bacteria growth could not be studied because fast gelation interfered with optical measurements so that it cannot be put aside. Nevertheless, in order to compare the aqueous and alkoxide routes, it should be noted that, according to our growth studies, the Na^+ concentration involved in the SiNaLudox-3 synthesis ($[Na^+] = 0.42$ M) should not affect the integrity of bacteria cells. In contrast, the amount of methanol produced by the hydrolysis and condensation of TMOS (*ca.* 8.8 M for full hydrolysis) could possibly exceed the tolerance threshold revealed by growth studies.

The aqueous pathway presented in this paper appears very promising in order to improve cell viability in a confined medium. Addition of biomolecules that can provide better cytocompatibilities or behave as nutrients is now under study.

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