Sol–gel encapsulation of cells is not limited to silica: long-term viability of bacteria in alumina matrices†

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A colloidal route to aqueous alumina gels is described, allowing the long-term viability of encapsulated bacteria.

Encapsulation of biological systems in sol–gel matrices is now recognized as a promising alternative to polymer-based approaches to design bio-sensors and bio-reactors.[1] A wide variety of biomolecules, including proteins, enzymes, antibodies, nucleic acids and phospholipids, as well as whole cells have been successfully entrapped in sol–gel matrices while maintaining their biological functions.[2]

If most biomolecules are able to withstand external conditions that differ significantly from their *in vivo* environments, living micro-organisms are more sensitive to such variations. In the field of sol–gel encapsulation, one of the most striking consequences of this enhanced sensitivity is that all inorganic gels used so far for whole cell entrapment are silica-based materials.[3,4] This contrasts with enzyme immobilization that was performed within other metal oxide gels such as Al_2O_3,[5] V_2O_5,[6] TiO_2,[7] and ZrO_2.[8] However, these gels were obtained from metal alkoxide precursors and/or far from neutral pH, two conditions that are expected to interfere with the maintenance of cell viability.

Herein we report for the first time the successful encapsulation of whole cells in a non-silica metal-oxide sol–gel matrix. Aluminium oxide gels were selected as the sol–gel chemistry of Al_2O_3 bears some similarity to that of silica.[9] Moreover, the Al^{3+} ion is known to be toxic to a large number of living systems,[10] whereas Al_2O_3 is widely used in medical applications.[11] Therefore the elaboration of an alumina matrix in the presence of cells is both a real challenge and an interesting route to novel biomedical ceramics.

The formation of aluminium oxide gels *via* the sol–gel process has already been widely described. However, most of these approaches use aluminium alkoxides,[12] leading to alcohol release that was shown to be harmful to *Escherichia coli* bacteria.[13] Some aqueous routes using aluminium(III) salts were also described but involve organic solvents and/or acidic or alkaline media.[14] Therefore, our strategy was based on the formation of an alumina gel from pre-formed boehmite colloids.

Boehmite nanoparticles (*ca.* 100 nm in diameter) were obtained from the thermohydrolysis of aluminium chloride solutions in alkaline media. Particles were thoroughly washed until no remaining Cl^- could be detected in the solution. After re-suspending the sol in deionized water to reach a 50 g L^{-1} boehmite concentration, addition of the bacteria culture medium (MM) at pH 7.2 led to the fast formation of a gel.‡

SEM and TEM observations indicate that the CO_2 super-critically-dried gel consists of aggregated boehmite nanorods (Fig. 1a,b). The ^{27}Al MAS NMR of boehmite aerogel (see ESI†) showed a unique asymmetric peak, at *ca.* 7.0 ppm. This peak can be assigned unambiguously to a six-coordinated aluminium in a disordered environment.[15] The adsorption–desorption isotherm of N_2 at 77 K for freeze-dried gels is characteristic of mesoporous materials (type IV) (Fig. 2), with a specific surface area (S_{BET}) of $\sim$315 m^2 g^{-1} and a porous volume (V_p) of $\sim$0.25 cm^3 g^{-1}.

On the basis of our previous reports showing that glycerol addition to silica gels significantly enhances the long-term viability of encapsulated *Escherichia coli* bacteria,[16] alumina gels were also formed by addition of MM containing 10% w/w glycerol (MM-gly). SEM and TEM images were very similar to those

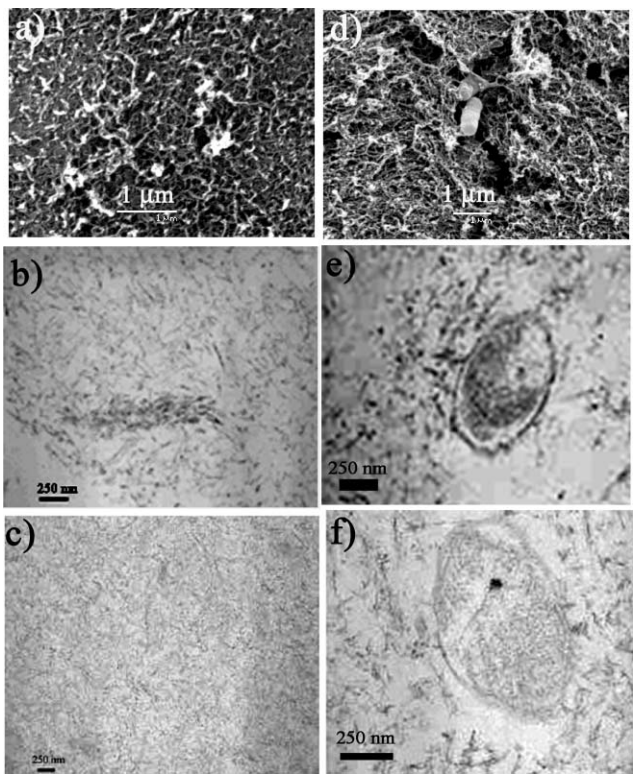

Fig. 1 a) SEM and b) TEM images of boehmite gel without glycerol. c) TEM image of boehmite gel with glycerol. d) SEM and e) TEM images of *E. coli* entrapped in a boehmite gel without glycerol. f) TEM image of *E. coli* entrapped in a boehmite gel with glycerol.

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† Electronic supplementary information (ESI) available: ^{27}Al MAS NMR spectra of the gels; detailed procedures for cell culture, gel preparation and characterization and viability measurements. See DOI: 10.1039/b711380c

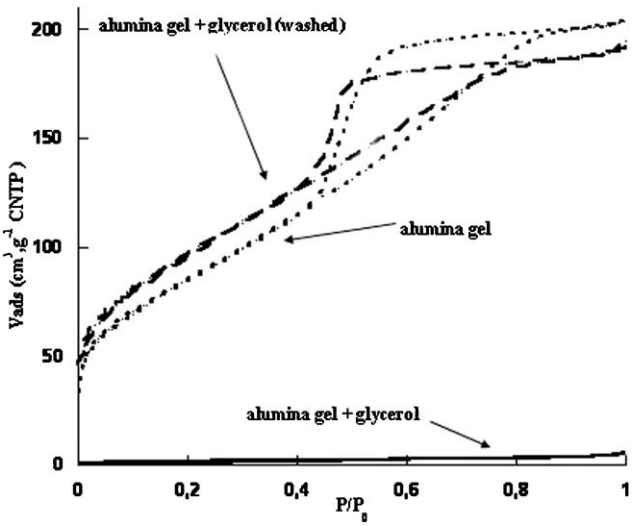

Fig. 2 N_2-sorption isotherms of aluminium oxide gels synthesized without glycerol and with glycerol, before and after washing.

obtained in the absence of glycerol (Fig. 1c). However, nitrogen sorption data indicate a large decrease in the gel specific surface area ($S_{BET} = 5\ m^2\ g^{-1}$). Washing the aerogel to get rid of adsorbed glycerol allows the recovery of a mesoporous alumina gel ($S_{BET} \sim 340\ m^2\ g^{-1}$; $V_p \sim 0.29\ cm^3\ g^{-1}$). This suggests that glycerol incorporation occurs within the mesopores of the gel, as already proposed for silica matrices.[3c,15]

Similar trends were also observed when bacterial suspensions (WCS), containing or not containing glycerol, were added. SEM and TEM studies (Fig. 1d–f) confirm the particulate nature of the gels. Most bacteria retain their cell integrity in fresh gels. When glycerol is present in the bacterial suspension, boehmite particles appear to adopt a more homogenous packing around the cells, confirming that this additive significantly influences the gel structure and, hence, the nature of the cell/mineral interface.

In order to monitor the viability of the encapsulated bacteria, the gels were crushed and stirred with phosphate buffer (PB). The cell content of the supernatant was studied using the usual plate count technique that indicates the proportion of culturable cells, *i.e.* those that can grow in standard bacteriological media after encapsulation.

We first checked the immediate effect of the gel formation on bacterial viability (Fig. 3a). The plate-count technique shows that the number of culturable bacteria decreases one hour after encapsulation. In the absence of glycerol, 27% (±3%) of bacteria appear to resist the stress due to the entrapment. After one day the number of living cells is close to 21% (±3%). When the boehmite gel contains glycerol, the proportions of living bacteria are 33% (±6%) and 28% (±6%) for one hour and one day respectively. Thus, an important loss of viability is observed upon gel formation but the number of living bacteria does not appear to decrease significantly in the following 24 h. Noticeably, the presence of glycerol seems to have no effect on cell viability at this stage.

In order to understand this decrease in viability upon encapsulation, the growth of bacterial suspensions in the presence of boehmite particles was studied by turbidity measurements. The shape of the curve in the absence of any additional chemical was taken as a reference for bacterial growth (Fig. 4). A rapid

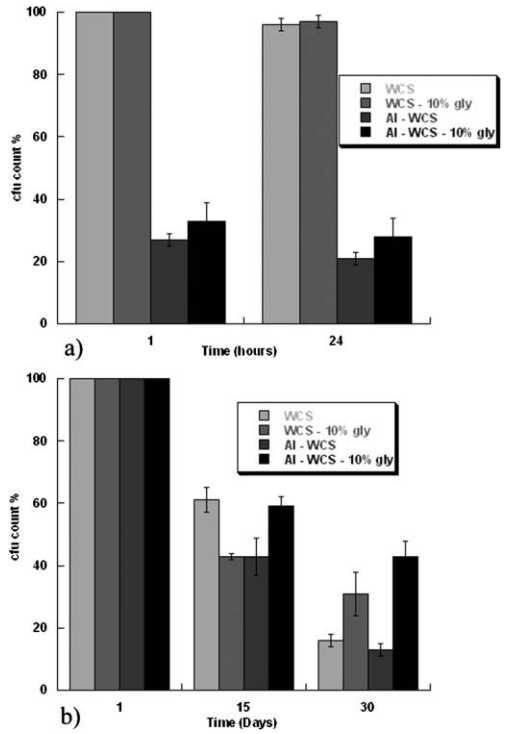

Fig. 3 Evolution of colony formation units (CFUs) count of bacterial suspension (WCS) and bacteria entrapped in aluminium oxide gel (Al-WCS) aged for (a) 1 and 24 h and (b) 1, 15 and 30 days.

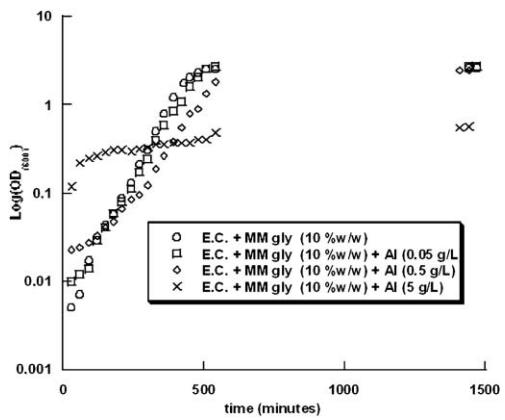

Fig. 4 Bacterial growth in a minimum medium containing glycerol in the presence of boehmite nanoparticles.

development phase, the 'log phase', is observed during the first 7 h. A plateau is then obtained showing that the bacterial population reaches the stationary phase. The effect of aluminium nanorods was monitored by adding boehmite suspension in the 0.05–5 g L^{-1} concentration range (Fig. 4). A 0.05 g L^{-1} boehmite solution does not seem to disrupt bacterial growth. At an intermediate concentration (0.5 g L^{-1}), the slope of the curve corresponding to the 'log phase' decreases but the expected plateau value is reached after 24 h. For a 5 g L^{-1} boehmite concentration, this growth becomes very limited. However, the shape of the later curve is strongly influenced by the observed aggregation of boehmite particles when added to the minimum medium, so that the behavior of cells at the concentration used for gel formation

(50 g L^{-1}) could not be studied. These data suggest that even if bacteria are affected by the presence of boehmite nanoparticles, they are able to adapt themselves to the surrounding media within a few hours.

Such a capability of bacteria to overcome the encapsulation stress suggests that they may be able to present a significant long-term viability within the gels. To dissociate short-term from long-term effects of encapsulation, the colony forming units (CFU) count after one day of encapsulation was taken as a 100% reference for further evaluation of bacterial viability.

Upon ageing, the CFU value is seen to decrease with time in all samples (Fig. 3b). After one month, the count is low for bacteria trapped in a pure boehmite gel, as well as for bacteria suspended in a working cell suspension (WCS) ($\sim$15%). However, in boehmite gel containing glycerol, survival rates as high as 60% ($\pm$5%) after 15 days and 43% ($\pm$3%) after one month were obtained.

These results demonstrate that it is possible to design an alumina host compatible with the long-term viability of *E. coli*, in the presence of glycerol. When compared to previous results obtained on silica hosts,[16] it is interesting to note that both matrices present a similar ability to maintain cell viability over several weeks. This effect can be attributed to the beneficial effect of glycerol that, in both cases, appears to be located in the gel mesopores, and, hence, in the vicinity of the cells.[17] However, a clear difference between silica and alumina is observed for the short-term survival of bacteria, with 80% viability after 1 h for the former compared to 33% for the latter. It can be suggested that this significant difference originates from the nature of interactions arising between the nanoparticles and the bacteria before gel formation. Moreover, it is worth noting that the silica precursor solution takes about 2 min to gel whereas the alumina gel formation occurs within a few seconds, which may represent a significant stress for the bacteria. The possibility that some living bacteria remain trapped within boehmite aggregates and are therefore not detected by the plate-count technique should not be put aside, and alternative viability tests should now be undertaken to check this hypothesis.[16]

On the basis of these data, we believe that sol–gel encapsulation of bacteria may in the future be extended to a large number of metal oxide hosts, provided that (i) aqueous colloidal solutions with suitable gel-formation properties at neutral pH are obtained and (ii) glycerol is added, in agreement with our previous studies[16] but also with recent reports on the lyo-protective properties of this additive.[18] Whereas the here-obtained alumina gels may find application as bioceramics, the access to a wide range of inorganic hosts with magnetic or electrochemical properties should open the route to novel functional "living" materials.[4b]

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Notes and references

‡ Boehmite sols were produced by dissolving aluminium chloride in deionized water, and adjusting the pH to 8 by the addition of sodium hydroxide. The final aluminium concentration is 0.7 mol L^{-1}. The solution was then processed hydrothermally in an autoclave at 95 °C for 1 week. The resulting precipitate was recovered by centrifugation and washed with pure water until no chloride was detected upon $Ag(NO_3)$ addition. A Boehmite sol (BS) was prepared by adding 1.5 g of boehmite precipitate to 1 mL of pure water. The gel was formed by adding phosphate buffer (with or without glycerol (10 wt.%)) and/or bacteria (10^9 cells mL^{-1}) (1 mL) to the boehmite sol. The mixture was homogenized under gentle stirring (300 rpm). Gelation occurred within a few seconds at room temperature. Wet gels were aged for 1 h and 1, 15 or 30 days at 20 °C in the mother solution in a closed flask. Detailed experimental procedures are provided in ESI.†

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