Cell Growth at Cavities Created Inside Silica Monoliths Synthesized by Sol−Gel

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The sol−gel encapsulation of microorganisms within inorganic matrixes is becoming an area of great scientific and technological interest,[1,2] being the stepping stone toward new biomaterials' development, from biosensors to bioreactors.[3] Nutrients and metabolic products have been shown to freely diffuse through the porous silica-based monoliths obtained to present. Such devices also succeed in preventing cells of interest escape from the matrix, as well as the entrance of contaminating agents to their bosom.[4] However, none of the procedures described so far allows population growth, since space limitation suffered by confined cells makes their division completely impossible.[5−7] In this scenario, considerable attention has been focused on the minimization of natural cell damage during and after sol−gel synthesis. Many interesting alternatives have been explored to extend the viability of cells submitted to entrapment, which include the addition of complexing agents to reduce silanol's aggressiveness,[8] the remotion of alcohol before silica gelation,[9] and the use of biological mechanisms such as quorum sensing molecules to promote cell cycle arrest, conferring cell adaptation to confinement.[7]

For certain applications, however, it is desirable or even necessary to keep a large number of metabolically active cells in these inorganic matrixes. Moreover, certain products of biotechnological interest are synthesized only by actively growing cells. This paper reports on the first encapsulation procedure that allows cell division inside a silica monolithic matrix. In other words, we are presenting a method for an entire culture (instead of a single cell) encapsulation, giving rise to the possibility of culturing cells in near-natural environmental conditions, even enabling cell-to-cell contact.

The strategy to obtain cells lodged in macrocavities inside the silica matrix is as follows: (I) cells of interest immobilization within a natural calcium-alginate polymer; (II) encapsulation of alginate cell-containing beads in a sol−gel monolith obtained by silicate sol−gel condensation; and (III) dissolution of alginate spheres by calcium chelation with citrate, turning the cavity content to liquid.[10] The utilization of Ca−alginate matrixes for cell encapsulation is a common step in microbiological procedures.[11] Thin silica coating of such soft biocompatible polymer has been previously described.[12,13] However, the procedure reported hereafter enhances the mechanical strength since the cavity is surrounded by a continuous monolith, providing effective resistance properties.

Scheme 1 summarizes the steps involved in this encapsulation procedure; the first one is performed by dropwise addition of a 1.5% (w/w) sodium alginate cells suspension in a 0.1 M $CaCl_2$ solution. After 10 min stirring, about 3 mm diameter beads are easily collected by filtration (see Table 1 in Supporting Information). The calcium alginate polymer prevents cell contact with synthesis precursors. The second step consists of silicate polymerization in the presence of commercial silica nanoparticles (Ludox HS40 from Aldrich), leading to a nanoporous monolithic structure. Monoliths are prepared at room temperature by mixing 3 vol of 0.83 M sodium silicate with 1 vol of colloidal silica containing succinic acid (5 wt %) into a recipient containing the alginate−cells bead. The main modification from the reported procedure[6] is with the use of succinic, a weak acid ($pK_a = 5.2$), as a source of protons. It was chosen considering that (i) the mixture with silicate solution leads to a gel buffered by the succinic/succinate couple, avoiding any harmful acidification below pH 5.0; (ii) as a weak acid anion, succinate can attach to the reactive silanol groups of the matrix;[14] and (iii) the succinate affinity toward Ca^{2+} ($K_{Succ-Ca} = 15.9$) is significantly lower than citrate's ($K_{Cit-Ca} = 3.2 \times 10^3$),[15] preventing an undesirable liquefaction of the calcium alginate beads, in the time required for silica gelation.

Once the sol−gel polymerization reaction is completed, the stiff monolith obtained is left in contact with 0.05% potassium citrate overnight. This procedure proved to be

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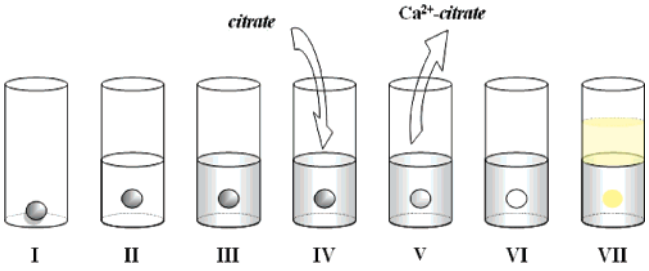
Scheme 1. Encapsulation Procedure[a]



[a] I, Cells containing the alginate bead are placed into an empty tube; II, the tube is partially filled with sol−gel silica precursor; III, complete gelation gives way to a silica monolith; IV, citrate is added to remove Ca^{2+} from the bead; V, the alginate bead is liquefied, as Ca^{2+} diffuses out of the monolith in the form of a Ca^{2+}−citrate complex; VI, the dissolved bead leaves a liquid spherical cavity (filled with cells) inside the monolith; and VII, the culture medium is added to the tube. Nutrients diffuse toward the cavity allowing cell growth.

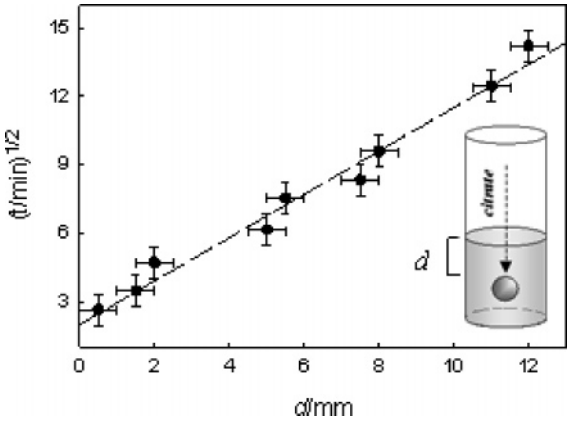


Figure 1. Characteristic liquefaction time at 298 K, using 0.1 M citrate. The citrate draw up times are plotted as a function of bead−monolith surface distance, *d* (see inset scheme). All the measurements where carried out with Ca−alginate beads ($r = 1.6 \pm 0.2$ mm) containing $CoFe_2O_4$ microparticles.

sufficient for calcium alginate gel liquefaction. To provide necessary nutrients to the immobilized cells, potassium citrate solution is further replaced by the specific culture medium according to encapsulated cell strain requirements.

Although permeation of citrate through the silica gel turns out to be evident from the liquefaction of calcium alginate gel, the characteristic citrate diffusion time gives valuable information since it constitutes a reference value for diffusion time of low molecular weight nutrients. It was evaluated through an experimental device in which monoliths were prepared with a single alginate cavity containing magnetic $CoFe_2O_4$ microparticles instead of cells. Each cavity was placed at a different distance from the monolith surface in contact with 0.1 M potassium citrate (see Figure 1 in Supporting Information). The orientation of particles in the magnetic field generated by an external magnet is an indirect evidence of citrate draw up, and the free movement of these particles within the whole cavity space is an indication of complete dissolution of calcium alginate. A plot of citrate draw up time versus bead−surface distance (*d*) is presented in Figure 1. A linear relationship between the square root of liquefaction time and distance is observed; the intercept on the *y* axis is different from 0, because there is an intrinsic alginate gel dissolution time. However, this inherent liquefaction time of the alginate microsphere is not significant (<5 min) compared to the diffusion time of citrate in a typical

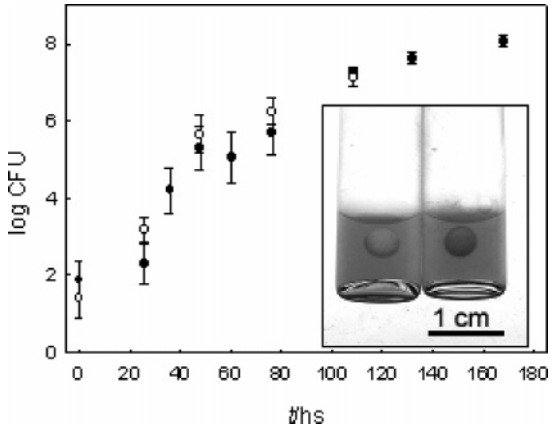


Figure 2. *B. subtilis* growth inside the silica monoliths (●) and control growth (○) in a conventional culture. Inset: Image of a freshly gelified silica monolith, containing a *B. subtilis* culture in the just-liquefied inner spherical cavity (left) and a similar culture after a week long amplification cycle (5×10^6) at 30 °C (right), in the presence of Triptein Soya culture medium.

arrangement (5 mm distance; 1 h). Taking into account the latter, this matrix seems to be porous enough to allow a fast diffusion of low molecular weight nutrients, ensuring the desired cell growth rate.

Encapsulation experiments were carried out using *Bacillus subtilis* (168) spores. Calcium alginate beads were prepared as described above, and 32 identical one-bead-containing monoliths were synthesized. After the addition of the Triptein Soya culture medium, tubes were incubated at 30 °C. At each different incubation time, duplicates were withdrawn, and serial dilutions of liquid void volume containing bacterial cells were spotted onto a selective and differential medium. An estimation of the number of colony forming units per void volume in logarithmic scale versus incubation time is presented in Figure 2a. An exponential growth during 4−5 days is evidenced. A growing control was performed in parallel in which *B. subtilis* cells were cultured in liquid Triptein Soya medium. Encapsulated cells show a similar growing profile to that found for control cells. It is possible to appreciate the bacterial growth from the turbidity progressively developed inside the cavities. Figure 2b shows tubes at the beginning and after a 1-week incubation time. It is important to note that the alginate gel liquefaction time and the delay caused by nutrient diffusion are negligible compared to the growing time of the cells.

After a 1-week period a 5×10^6 amplification cycle was completed. Two additional 1-week incubated monoliths were left medium-less at room temperature (1 month) until complete desiccation. Under these conditions, super long-term viability of *B. subtilis* is evidenced, as bacteria remain culturable, forming colonies again when the gel is pulverized, suspended in sterile water, and dispersed in a solid culture medium. This dried structure could work as a resistant and safety transport means for sporulating species, acting as a "multiple hybrid artificial spore".

To evaluate generalization of results to other less resistant cells, the same described procedure was followed with nonsporulating bacteria, *Escherichia coli* (DH5α), and yeast, *Saccharomyces cerevisiae* (Σ1278b). For both cell strains, growth versus time was evaluated in a 4-day running assay,

and results similar to those of *B. subtilis* were obtained. This shows that the proposed procedure is biocompatible and that the inherent resistance of spores is not necessary for the encapsulation stage.

Further controls were carried out to demonstrate the effectiveness of the silica barrier in avoiding contamination by biological agent's entrance. With this aim, an entrapped colony of *B. subtilis* was fed with an *E. coli* inoculated culture medium. After a 5-day incubation period, growth at the interior void culture and at the external liquid medium was analyzed by spotting samples onto differential solid culture medium. Neither *E. coli* was found to be present inside the silica matrix, nor was *B. subtilis* detected in the external liquid medium. Similar results were obtained permuting the cell strains.

In summary, normal cell division and growth inside a "mineral host" has been achieved for the first time, overcoming the limits imposed by cell survival. The diffusion time of nutrients within the porous silica monolith is fast enough to allow growing rates almost indistinguishable from a traditional culture (control). The inorganic matrix provides an effective mechanical barrier for the isolation of contiguous growing cultures.

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Supporting Information Available: Details about alginate beads preparation, evaluation of citrate diffusion time, estimation of population growth, and growing of *E. coli* and *S. cerevisiae* strains. This material is available free of charge via the Internet at http://pubs.acs.org.

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