Biocompatible Sol−Gel Route for Encapsulation of Living Bacteria in Organically Modified Silica Matrixes

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Received May 17, 2003. Revised Manuscript Received July 11, 2003

An alcohol-free sol−gel route based on the controlled vacuum evaporation of the alcohol that results as a byproduct of the hydrolysis and condensation of alkoxyde precursors is described for the encapsulation of living bacteria. The ability of the encapsulated cells (a genetically engineered *E. coli* strain) to express a fluorescent protein in response to the presence of dicyclopropyl ketone has been used to study both the biocompatibility of the encapsulation route and the viability of the encapsulated cells with aging time. Thus, up to 95% of the integrity of the living cell along the encapsulation process has been preserved through alcohol removal of the starting sol. In addition, the use of organically modified rather than pure silica gels as the host matrix has shown a notable improvement in the viability of encapsulated cells (18 versus 6 days, respectively).

Introduction

The encapsulation of biomolecules through the sol−gel process has gained considerable interest since 1990.[1] Despite the large number of enzymes encapsulated through the sol−gel process,[2] just a few works have reported on the encapsulation of living cells.[3] The encapsulation of living cells within silicate matrixes may provide inherent benefits in multitask multiple strain catalysis and sensing applications through a selective and individual cell isolation at the silicate cage. However, the alcohol generated as a byproduct of the chemical reactions involved in the sol−gel process (e.g., hydrolysis and condensation of alkoxyde precursors) is toxic for living cells and damages the cell membrane.[3e,f,g] The goal of any biocompatible approach must therefore be to keep the living cells apart from alcohol solvents all along the encapsulation process and thus preserve cell activity. The use of sodium silicate precursors and colloidal suspensions which do not generate alcohol as a byproduct have succeeded in the preservation of the activity of encapsulated cells.[3e,f] Recently, a sol−gel route starting from an alkoxyde precursor (e.g., tetramethylorthosilicate) has also been described to successfully keep the alcohol concentration below toxicity levels.[3g] Nevertheless, the viability shown by encapsulated cells in these sol−gel silica matrixes is expected to be poor because of the acidity of the silanol groups and the physical constraint exerted on the encapsulated cells by the characteristic shrinkage of the sol−gel silica matrixes during the aging process (e.g., syneresis).[4] The preparation of organic−inorganic hybrid matrixes (ormosils[5]), whose porous cage properties can be tailored in terms of both the chemical (e.g., from hydrophobic[5] to highly hydrophilic (hydrogels)[6]) and physical (e.g., controlled swelling[7] or even no shrinkage[8]), should be helpful to prevent such a degradation. Actually, the incorporation of glycerol has recently been reported to provide improved cell viability upon encapsulation through the isolation of cells from silanol groups.[3k] The

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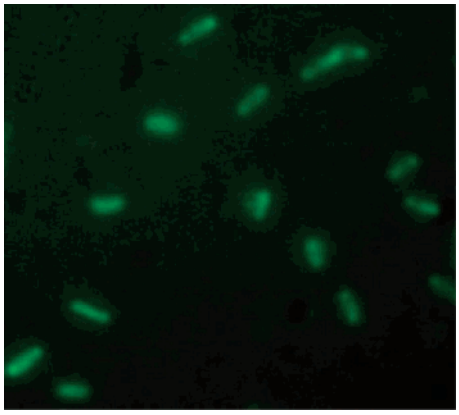

Figure 1. Fluorescence micrograph of *E. coli* TG1/pPBG11 encapsulated in HMAF after an overnight exposure to DCPK to induce expression of the GFP protein, and visualized under a fluorescence microscope with a FITC filter (excitation wavelength, 450−490 nm; emission wavelength, 520 nm).

use of an organic moiety covalently attached to the silica network should further improve the viability of encapsulated cells, through a more effective isolation of the cells from silanol groups and a better control of the matrix shrinkage. Besides, the covalent attachment of organic moieties would confer stability to the resulting hybrid matrix since the eventual release of the organic from the matrix by simple evaporation or by leaching when immersed in a solution is completely prevented.

The aim of this work was to design an alcohol-free sol−gel route beginning from organically modified alkoxyde precursors for encapsulation of living cells (e.g., the bacteria *Escherichia coli*). The alcohol-free sol−gel route is based on the gentle vacuum elimination of the alcohol by rotavapor methods. Our group has recently reported the high biocompatibility shown by this route for encapsulation of labile proteins in a pure silica matrix as the host matrix.[9] However, its validity for encapsulation of living cells was still unknown. Moreover, the design of such an alcohol-free sol−gel route for the preparation of an organically modified silica matrix is also reported in the current work. The success of this approach will provide a highly biocompatible encapsulation route as well as a friendly matrix to host the living cells; i.e., the lack of alcohol preserves the activity of cells during the encapsulation process while the formation of an organically modified matrix minimizes the shrinkage of the matrix during aging and thus the physical constraint exerted on the cells. The *E. coli* strain used was genetically engineered to express a fluorescent protein (green fluorescent protein, GFP) in response to the presence of alkanes, or of dicyclopropyl ketone (DCPK) (Figure 1). Thus, the ability of the encapsulated cells to synthesize GFP in response to the presence of an inducer has been used to study both the biocompatibility of the encapsulation route and the viability of encapsulated cells with aging time. The results are compared to those obtained for *E. coli* encapsulated in a pure silica matrix.

Experimental Section

Materials. Tetramethyl orthosilicate (TMOS) and glycidoxypropyltrimethoxysilane (GPTMS) were from Aldrich. Water was distilled and deionized.

The bacterial strain used, *E. coli* TG1/pPBG11, derives from *E. coli* TG1[10] by introduction of plasmid pPBG11. This multicopy plasmid contains two relevant elements: (a) the *gfp* gene encoding the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* cloned immediately downstream from the inducible promoter *PalkB*, from which it is expressed, and (b) the *xylS* gene, which encodes a transcriptional regulator that activates the *PalkB* promoter when DCPK is present in the medium.[11] A detailed description of the construction of this plasmid will be reported elsewhere. The GFP protein is extensively used as a reporter to monitor gene expression.[12] It is intrinsically fluorescent, so it needs no additional cofactors or exogenous substrates to yield a signal. Several GFP variants exist; the one used in this work shows maximum emission at 510 nm when excited at 490 nm.[13] Prior to their use in the encapsulation assays, bacterial cells were grown at 37 °C in complete LB medium[10] with aeration in the absence or presence of the inducer DCPK (0.05% vol/vol), collected by centrifugation at the end of the exponential phase (A_{600} of 0.8−1), and resuspended in fresh LB medium ($^1/_{20}$ of the original volume).

Sample Preparation. The alcohol-free sol−gel route used for cell encapsulation in pure silica matrixes begins with the preparation (at 4 °C) of a silica sol stock solution composed of 5.58 mL of tetramethoxysilane (TMOS, from Sigma-Aldrich), 1.9 mL of H_2O, and 0.125 mL of HCl (0.62 M, from Sigma-Aldrich). The mixture was stirred vigorously for 30 min and then submitted to 20 kHz frequency and 2.4 KJ/cm^3 ultrasonic energy (Ultrasonic Processor VC100, Sonics and Materials) to obtain a homogeneous sol. The reaction conditions (sonication, $r_{w/m} = 4$ and pH 2) were thus selected to ensure the adequate progress of the hydrolysis reaction. Rotavapor methods[14] were applied on the hydrolyzed and diluted sol (1 mL of H_2O added to 1 mL of sol) for an efficient alcohol removal (up to ∼96%)[9]. Note that addition of water is required to avoid the prompt gelation of the sol after removal of the alcohol. The resulting matrix is defined as SMAF (silica matrix from an alcohol-free route).

The alcohol-free sol−gel route used for cell encapsulation in an organically modified silica matrix proceeds as described above for the pure silica matrix, except for the incorporation of two organic moieties (GPTMS and poly(ethylene glycol) (PEG)). GPTMS should both reduce the shrinkage of matrix during aging[4] and isolate cells from silanol groups,[15] while PEG should further improve the hydrogel character of the resulting host matrix.[16,17] GPTMS was introduced in the silica sol stock solution, which is thus composed of 4.46 mL of TMOS and 1.66 mL of GPTMS. PEG was introduced in the form of a PEG/H_2O solution (10 wt %). Such a PEG/H_2O solution (1 mL) is used for the required dilution of the sol (1 mL) prior to the alcohol removal. The resulting matrix is defined as HMAF (hybrid matrix from an alcohol-free route).

Both pure silica and organically modified silica matrixes were also prepared through a regular sol−gel route, with experimental conditions the same as described above except for the alcohol removal. The resulting matrixes are defined SMNAF and HMNAF (silica and hybrid matrix from non-alcohol-free routes, respectively).

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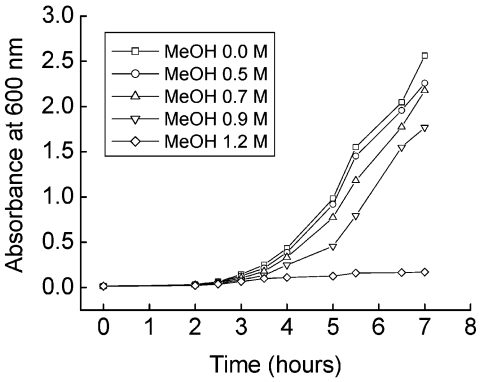

Figure 2. Exponential growth of *E. coli* cells in the presence of different methanol concentrations.

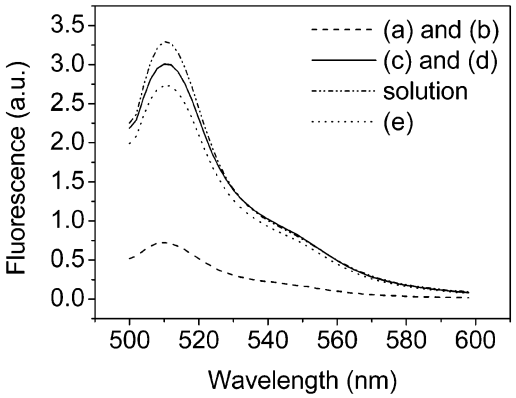

Figure 3. Fluorescence spectra of *E. coli* TG1/pPBG11 cells grown in the presence of DCPK and encapsulated in SMNAF (a), HMNAF (b), SMAF (c), and HMAF (d). Fluorescence spectra of nonencapsulated bacteria cells (solution) and encapsulated cells following the procedure described in ref 3g (e) are also included for comparison.

In every case, equal volumes (75 μL) of the hydrolyzed sol and the bacterial suspension were mixed in a cylindrical polystyrene container. Gelation readily occurred, and the resulting gel (~1 mm thick) was allowed to dry for 40 min at 20 °C prior to its storage at 4 °C in a fresh buffered solution (e.g., 20 mM Tris/HCl (pH 7.5) and 20 mM sodium citrate).

The cell activity was investigated by fluorescence spectroscopy. Fluorescence measurements were performed in front face mode at 20 °C on a 48000s SLM-Aminco spectrofluorometer (T-Optics). The fluorescent spectrum of every sample (both types of cells suspended in buffered solution and encapsulated in SMAF, HMAF, SMNAF, and HMNAF) was the mean of three independent experiments, performed in duplicate. The doped matrixes were taken out from the buffered solution and placed in a front face sample holder for measurement. The cells suspended in a buffered solution were placed into a quartz cell with 1-mm path length for front face measurements. The excitation wavelength was selected at 485 nm. Data collection was performed in backward direction on samples oriented at 24° with respect to the incident excitation beam to minimize the specular reflection on the cooled R-928 photomultiplier tube.[18] Fluorescent spectra from appropriate blanks (e.g., buffer solution and undoped SMAF, HMAF, SMNAF, and HMNAF) were also collected and subtracted from the fluorescent spectra of their respective suspended cells as well as of the cells doped matrixes to further remove any remaining light-scattering contribution from the fluorescent spectra shown in the figures.

Results and Discussion

As mentioned in the Introduction, the alcohol-free sol−gel route used in this work has been successfully used to preserve the activity of labile proteins all along the encapsulation process, but no data regarding its ability for encapsulation of living cells has been reported to date. Nevertheless, we have first studied the tolerance of *E. coli* to methanol. Figure 2 shows how the exponential growth of E. Coli cells is completely disrupted for methanol concentrations of ~1.2 M and above. Note that the methanol concentration resulting from the hydrolysis of alkoxyde precursors reaches values of ~2 M. Thus, the design of an encapsulation process through an alcohol-free sol−gel route is crucial for the preservation of the cell activity.

Figure 3 shows the fluorescence signal of bacterial cells grown in the presence of the inducer and encapsulated in SMNAF and SMAF. The fluorescence signal of a similar amount of bacterial cells suspended in a buffered solution was also monitored for comparison. It can be observed that the intensity of the fluorescence signal of cells encapsulated in the absence of alcohol was in the range (within the experimental error) of that obtained for cells suspended in a buffered solution, whereas that of cells encapsulated in the presence of alcohol was dramatically lower. Figure 3 shows the identical behavior found for cells encapsulated in HMNAF and HMAF. The fluorescence intensity of the different curves does not indicate the efficiency of the cells at expressing the GFP, because in this assay GFP expression was induced prior to the encapsulation process. The decrease in fluorescence intensity observed in curves (a) and (b) reflects the alcohol-induced cell damage; i.e., cell lysis and subsequent denaturation of the GFP.[3f,3g,3k,19] The unfolding of the protein allows the rotation around the exo-methylene double bond of the chromophore (a *p*-hydroxybenzylidene-imidazolidine derivative), which results in a dramatic reduction of the fluorescence intensity due to fast internal conversion.

The encapsulation of induced *E. coli* cells provides information on the effect of the alcohol generated during the hydrolysis and condensation of the sol−gel reaction on the integrity of the cells. However, an encapsulation assay performed with noninduced cells could provide information on how the encapsulation process negatively affects cell metabolism, in particular the ability of the encapsulated cells to synthesize GFP in response to the presence of the inducer DCPK. To this end, cells were grown in the absence of the inducer and encapsulated as described above. The resulting cell-containing matrixes were submerged for 20 h in a fresh buffered solution containing 0.05% DCPK. A similar amount of nonencapsulated cells was also induced as control. Cells encapsulated in the presence of alcohol (SMNAF and HMNAF) generated a very low fluorescence response (Figure 4), which is indicative of cell damage that impedes GFP synthesis in response to the inducer. Interestingly, induction of cells encapsulated through the alcohol-free route (SMAF and HMAF) generated a good fluorescence response, just slightly lower than that of nonencapsulated cells, indicating that the method was gentle enough to preserve the cells in a physiologi-

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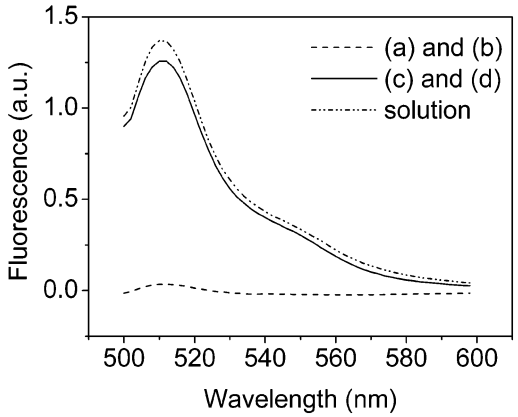

Figure 4. Fluorescence spectra of *E. coli* TG1/pPBG11 cells grown in the absence of DCPK and induced after their encapsulation in SMNAF (a), HMNAF (b), SMAF (c), and HMAF (d). Fluorescence spectrum of nonencapsulated bacteria cells induced in parallel is also included for comparison (solution).

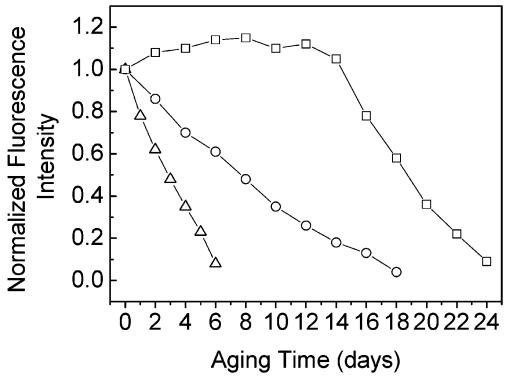

Figure 5. Fluorescence response of *E. coli* TG1/pPBG11 cells grown in the absence of DCPK and induced at different times after their encapsulation in SMAF (△) and in HMAF (○). Fluorescence response of nonencapsulated bacteria cells (e.g., buffered solution) also induced at different times is included for comparison (□). The plot represents normalized fluorescence intensity versus aging time.

cal status that allowed for a response to the inducer and synthesis of GFP. The above results corroborate the importance of the removal of methanol rather than the organic/inorganic character of the host matrix in the preservation of the cell activity during the encapsulation process. It is worth noting that the intensity of the fluorescence signals obtained both in solution and encapsulated were about 3-fold lower than those observed when the cells were grown in the presence of the inducer (compare Figures 2 and 3). There are several possible explanations for such a decrease. For example, it could reflect a decreased ability of the cells to respond to the inducer as a consequence of the arrest of cell growth that occurs during centrifugation and manipulation of the cells. The physiological state of the cells is probably different in a liquid culture than in a silica matrix, which could affect the stability and turnover of the GFP protein in the cell.

On the basis of the results obtained above for the different encapsulation processes, the preservation of cell metabolism must be ascribed to the maintenance of the cell membrane structural integrity rather than to the organic/inorganic character of the host matrix. However, the viability of encapsulated cells in a pure silica matrix should differ from that of cells encapsulated in an organically modified silica matrix with the aging time due to differences in the aging process of the matrixes. In this case, the damage of the cell structure, hence the metabolic activity of the encapsulated cells, may result from either the physical constraint exerted by the shrinkage of the sol−gel matrix during aging, or the interaction with the acid silanol groups placed at the surface of the porous cage. To investigate this issue, cells grown in the absence of inducer were encapsulated either in a pure silica and in an organically modified silica matrix, stored at 4 °C in a fresh buffered solution, and induced (as described above) after different periods of aging time. Figure 5 shows that, in the case of cells encapsulated in SMAF, the intensity of the fluorescence signal decreased rather fast (almost complete vanishing of the fluorescence signal in just 6 days). In contrast, decrease in the fluorescence signal of cells encapsulated in HMAF was much slower, needing 18 days to decay below detectable levels. It is also worth noting that the ability of nonencapsulated cells to respond to the inducer also decreased with time, the aging process being only moderately longer (24 days to decrease below detectable levels, Figure 5) than that of cells encapsulated in HMAF.

Further methanol release from non-hydrolyzed alkoxydes, cell exposure to silanol groups, or physical constraint exerted on the cells by the characteristic shrinkage of the matrix during the aging process could explain the decrease of the viability for encapsulated cells. The presence of further methanol must be negligible (∼96% of the methanol coming from the hydrolysis of alkoxyde groups is removed during the encapsulation process) and it should be ruled out as a plausible explanation of the observed decrease of cell viability upon encapsulation. Whether the cell isolation from the silanol groups or the physical constraint resulting from it are the main cause for the observed loss of metabolic activity could be difficult to establish from just the data reported in this work. However, the somewhat lower ability of the encapsulated cells to survive aging observed in our work as compared to that recently reported for a glycerol based sol−gel matrix[3k] could help to clarify this issue. Assuming similar efficacy in the different methods used for the determination of the metabolic activity,[20] and taking into account that silanol groups should be equally isolated no matter the organic moiety used,[4] the observed discrepancy can only be ascribed to the different rate of shrinkage which is characteristic of sol−gel samples differently processed. Therefore, it is likely that, in hydrophilic conditions, the main issue to obtain optimum viability of encapsulated cells in sol−gel silica matrixes is the control of the physical constraint exerted by the silica matrix during aging (e.g., syneresis).

Conclusions

The results found in this work provide conclusive evidence that alcohol toxicity is a main issue to control during the encapsulation of living cells. The sol−gel route described in this work shows a high biocompatibility to encapsulate *E. coli* TG1/pPBG11 cells, thanks

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to the alcohol removal from the starting sol of up to 96%. Alcohol absence favors maintenance of the integrity of the cell and thus the proper response of the cell to external signals. The viability of cells encapsulated in the organic−inorganic hybrid matrix prepared in this work has been determined to be in the range of that recently reported for PEG hydrogels[17] and slightly shorter than that in a buffered solution. Furthermore, the sol−gel approach reported in this work has shown an overall improvement as compared to any other sol−gel encapsulation process beginning from alkoxyde precursors reported to date, given its ability not only to prevent the activity of the cells during the encapsulation process but also to prepare host matrixes where cell viability is prolonged for a long period of time.

Acknowledgment. F.R. thanks the Spanish Ministry of Science and Technology for grant BIO2000-0939. F.delM. thanks CICYT for the research grant MAT 2001-5073-E. M.L.F. thanks CSIC, the European Social Fund, and TPA Inc. for an I3P research contract. Dr. David Levy is acknowledged for valuable support.

CM034372T