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Encapsulation of sulfate-reducing bacteria in a silica host

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Anaerobic sulfate-reducing bacteria (SRB) have been encapsulated in a hydrous, alcohol-free, silica matrix produced by acidifying an aqueous mixture of colloidal silica and an SRB culture. The viability of the encapsulated species was investigated by monitoring the formation of the metabolic products, H_2S and acetate. Freshly prepared gel samples containing $\sim 5 \times 10^5$ cells cm^{-3} of gel reduced sulfate ions at a rate of ~ 11 μg h^{-1} cm^{-3} gel, when placed in a nutrient solution based on Postgate's Medium C. Less than 0.1% of the encapsulated species were leached into the nutrient solution over a 10 day period. Gels drained of nutrient solution and stored under nitrogen for 10 weeks at ambient temperature initially exhibited low sulfate reduction rates (~ 2 μg h^{-1} cm^{-3} gel) on re-immersion in nutrient, presumably due to a decrease in the encapsulated cell population. However, the initial sulfate reduction rate of ~ 11 μg h^{-1} cm^{-3} of gel was re-established after soaking the gel samples in nutrient solution for six days, indicating that the encapsulated bacterial population could be rapidly restored within the gel's pore network, even after several months of storage.

Introduction

Over the past decade, there has been considerable interest in the immobilisation of biocatalysts (*e.g.* enzymes) in inorganic matrices fabricated using sol–gel methods.[1–5] The ability to easily separate reactants and evolved products from an encapsulated biocatalyst is a significant advantage for the industrial exploitation of enzyme technology.[6] In addition, advantages such as enhanced stability to temperature and pH, relative to unencapsulated species, have been reported,[5,7] although the activity of the encapsulated enzyme generally remains lower than that of the unencapsulated species. Clearly, the effects of confinement, both on the physical conformations adopted by the encapsulated enzyme and on enzyme–substrate interactions, can influence the kinetics of the enzyme-catalysed reaction.[8] The ability to tailor the matrix properties, by modifying the sol–gel process chemistry, enables optimisation of the encapsulated enzyme activity. However, it is also evident that the use of organic reagents in the sol–gel process can lead to denaturation of the enzyme.[4] A judicious choice of reagents, such as using TMOS in preference to TEOS, can reduce the extent of denaturation.[9] In a recent report, the use of poly(glyceryl silicate) (PGS) as a biocompatible precursor was described, which resulted in only a minor loss of activity for a range of biological molecules entrapped in PGS-derived silica.[10]

While a wide range of enzymes has been successfully encapsulated in sol–gel matrices, there have been far fewer reports of studies involving whole cells. Examples include encapsulation of yeast, *R. miehei* and *P. oleovorans* cells[10] and *Pseudomonas* strain *ADP*,[11] all of which used alkoxide-based routes to produce the gels. An alternative approach is to coat living cells with a layer of SiO_2 produced by treatment with an air flux of gaseous silicon alkoxides; in this way, exposure to denaturants is reduced by rapid removal of excess reagent and volatile by-products.[12,13]

In this study, we have investigated both the encapsulation of anaerobic sulfate-reducing bacteria (SRB) and the viability of the entrapped species, by monitoring the formation of metabolic products during sulfate reduction. An early attempt to encapsulate the SRB in a gel produced from a chelated, titanium alkoxide precursor resulted in no detectable bacterial activity, which was attributed to the relatively high concentration of alcohol in the reactant solution. To avoid any such contact with alcohol, the gel described below was produced from a colloidal silica sol, exploiting the rapid polymerisation of aqueous silicate species on lowering the sol pH to form a hydrated oxide network.[14]

Experimental

Bacteria sample

SRB were grown from a sediment sample extracted from a local mangrove swamp (Lane Cove River, Sydney, Australia), by immersion in a degassed nutrient solution based on Postgate's Medium C (including lactate as a carbon source),[15] with additional Na^+, Mg^{2+}, Cl^-, Ca^{2+} and K^+ added to simulate salt-water concentrations. The growth medium was maintained at 35 °C and was kept under flowing N_2 at all times. After one week, 150 cm^3 of the solution was filtered through a coarse glass frit, and the procedure repeated using the filtered solution. Microbe concentrations were measured using the Rapidchek II SRB Detection System (Strategic Diagnostics Inc.); a concentration of 10^6 cells per cm^3 was obtained after one week, after which time the solution was withdrawn and stored in a sealed jar under N_2. As these preparation conditions have most likely given rise to a mixed SRB culture, no attempt was made to identify the particular strains present in solution.

Gel synthesis

The procedure used to encapsulate the SRB is illustrated in Fig. 1. The pH of an aqueous silica sol containing 30 wt% SiO_2 (20 cm^3, Ludox SM-30, DuPont), was lowered from 10 to 8 by addition of HCl (0.5 cm^3, 5.5 mol dm^{-3}). The acidified sol, which typically gelled within 90 minutes at ambient temperature, was degassed for ten minutes, and the SRB solution (20 cm^3, 10^6 cells cm^{-3}) subsequently added, with stirring, in a nitrogen-purged cabinet. After thorough mixing, the magnetic stirrer was removed and the container sealed. The mixture formed a transparent gel after standing for ten minutes, with an approximate volume of 42 cm^3. The decreased gelation time is attributed to the presence of salts in the SRB solution, which further destabilise the sol promoting aggregation and gelation.

A control sample was prepared similarly, except that the

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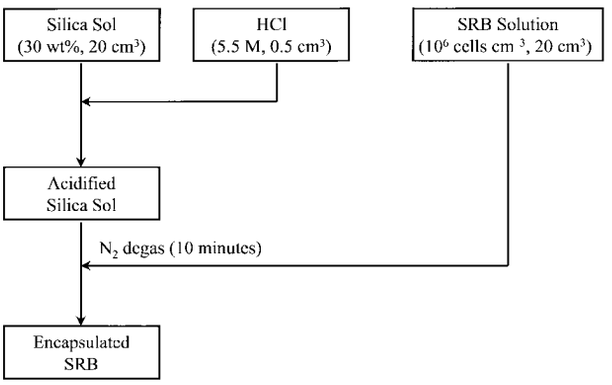

Fig. 1 Process flowchart for producing silica gels doped with SRB.

sample of SRB solution was first heated to 80 °C for fifteen minutes to kill the cells, then cooled to ambient temperature before addition to the acidified sol. In addition, gels containing no SRB were prepared with the nutrient solution.

After an hour, both SRB-doped gels developed a blue colour (with the control gel being notably less coloured), which was attributed to a redox reaction involving metallic species and residual sulfide present in the SRB solution. The coloured gel fragments bleached rapidly on exposure to air.

Determination of viability

Following doping and gelation, the undried gels were aged in sealed containers under N_2 at 30 °C for 70 hours, before testing for cell function. Minimal shrinkage was observed during ageing. The monolithic gels were crushed into ~2 to 3 mm fragments using a spatula, and transferred to 100 cm^3 Schlenk flasks. The fragments were washed thoroughly using three 40 cm^3 aliquots of the degassed nutrient solution to remove residual sulfide, then immersed in 40 cm^3 of degassed nutrient solution at 30 °C under flowing N_2 (100 cm^3 min^{-1}). Both flasks were flushed for five hours to remove residual sulfide, then the exit gas was bubbled through solutions containing 0.1 mol dm^{-3} Pb^{2+}, to test for evolution of H_2S.

To test the storage properties of the doped gel, the nutrient solution was drained off the sample, and the resulting moist gel was sealed and stored at ambient temperature under nitrogen. After one week of storage, the gel was washed with degassed nutrient solution, as above, then soaked in 50 cm^3 of fresh nutrient solution at 30 °C under flowing N_2 (100 cm^3 min^{-1}). This procedure was repeated, with a longer storage time of ten weeks.

Characterisation

Raman spectra of the gel fragments were measured using a Biorad FT-Raman II spectrometer equipped with a liquid-N_2 cooled germanium detector, with Nd:YAG excitation (0.55 W) at 1064 nm. Backscattered spectra were collected over the range 150 to 3500 cm^{-1} using a nominal resolution of 4 cm^{-1}. Spectra of gels containing either live SRB, heat-treated SRB or no SRB were obtained. Spectra were also obtained from the nutrient solution, and a 0.5 mol dm^{-3} aqueous solution of sodium acetate, to assist in the identification of features in the gel spectra.

The surface area and porosity of the doped gel, after drying at 90 °C, were determined by N_2 sorption using a Micromeritics 2000 ASAP system. The solids content was determined by measuring the mass loss on calcination at 500 °C.

Results and discussion

Gel structure

The oxide content of the as-prepared gels was found to be 18 wt%, indicating that they consist of an open, inorganic framework containing an essentially continuous aqueous phase. Gels dried at 90 °C exhibited BET surface areas of 200 m^2 g^{-1}, and were mesoporous, with pores ranging from 1 to 20 nm (mean ~10 nm), based on a BJH analysis of the adsorption/desorption isotherms. Clearly, the necessity to eliminate water, with subsequent collapse of pores, indicates that this measurement represents a lower bound to the pore size in the wet gel. Hence, the structure is particularly suitable for entrapment of the SRB, because the presence of a mesoporous network, and the absence of a significant population of micropores, facilitates transport of the ionic species required for cell metabolism (lactate/sulfate) throughout the gel matrix.

Viability following encapsulation

The Raman spectra of gels doped with live and heat-treated cells, and of sodium acetate, are shown in Fig. 2. The spectrum of the gel matrix consists of weak bands at 3380, 3240 (shoulder) and 1644 cm^{-1}, assigned to water, and a broad band at ~430 cm^{-1}, attributed to silica. The sharp band observed at 982 cm^{-1} is due to sulfate ions. The most important features to note are the differences between the spectra of the live-cell doped gel and control gels, observed in the region from 1800 to 800 cm^{-1} (marked with arrows). Additional bands at 1420, 1345 (just observable above the noise level) and 928 cm^{-1} are found in the spectrum of the live-cell-doped gel, which are attributed to acetate species on the basis of a comparison with the spectrum of sodium acetate. The presence of acetate in this gel, and its absence in the control sample, is evidence of the metabolic conversion of lactate to acetate by the bacteria following their encapsulation, indicating that the SRB continue their normal cell functions within the gel matrix.

As indicated above, a facile technique for investigating the metabolism of SRB is to monitor the rate of H_2S evolution, by passing the N_2 purge gas through a Pb(II) solution and measuring the quantity of insoluble PbS produced ($K_{sp} \sim 8 \times 10^{-28}$). After equilibrating the encapsulated SRB in nutrient solution for two days at 30 °C, the bacteria exhibited an average sulfate reduction rate of ~11 μg h^{-1} cm^{-3} gel over an eight day period, whereas the control sample exhibited only negligible metabolic activity during this interval. The production of H_2S further confirms that the encapsulated bacteria

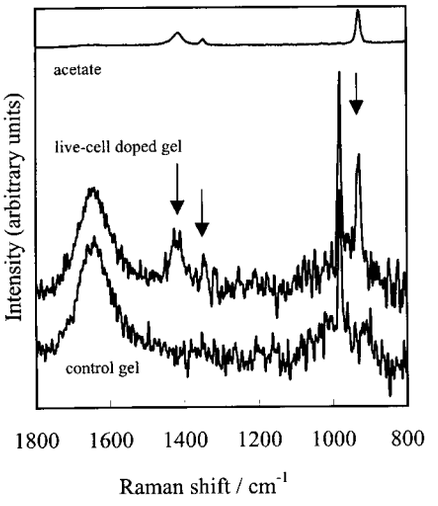

Fig. 2 Raman scattering of live-cell doped and control gels, 1800–800 cm^{-1}. Features due to acetate (see top spectrum) in the live-cell doped gel are marked with arrows.

survived the gelation procedure and are able to continue normal metabolic activity within the gel matrix.

The concentration of free SRB in the nutrient solution, after soaking the gel for 10 days, was 10^3 cells cm^{-3}, indicating that only $\sim 0.1\%$ of the encapsulated bacteria had been leached from the matrix.

Viability after storage

The effect of long-term storage on the viability of encapsulated biocatalysts is an important issue for their potential deployment in industrial-scale processes. Gels stored for one week (as outlined in the previous section), and subsequently regenerated by immersion in nutrient solution for 24 hours, exhibited an average sulfate reduction rate of $\sim 10\ \mu g\ h^{-1}\ cm^{-3}$ gel (averaged over a nine hour period). A similar sulfate reduction rate of $\sim 11\ \mu g\ h^{-1}\ cm^{-3}$ gel was observed after an additional five days of immersion in nutrient solution. Hence, it is evident that short-term storage did not adversely affect the bacteria's viability. Associated with the high surface area of hydrous silica (see above) is the potential for sorption of lactate and sulfate ions, so the absence of bulk nutrient solution does not necessarily imply starvation, at least for short storage periods.

After a longer storage time of ten weeks (*i.e.* fourteen weeks after encapsulation), a relatively low initial sulfate reduction rate was observed when the gel was re-immersed in nutrient solution ($\sim 2\ \mu g\ h^{-1}\ cm^{-3}$ gel, averaged over a 72 hour period). This reflects a significant reduction in the number of viable bacteria during such long-term storage. However, the rate subsequently increased to $\sim 11\ \mu g\ h^{-1}\ cm^{-3}$ gel after continuous immersion in nutrient solution for an additional six days, indicating that normal cell division and growth were occurring within the pore-space of the gel. Hence, the bacterial population was rapidly re-established within the gel, even after storage for several months, to reach the optimum level observed in the fresh gel, which is presumed to be limited by the nutrient supply.

Conclusion

A sample of sulfate-reducing bacteria has been successfully encapsulated in a hydrous silica matrix, synthesised using an inorganic route, which showed minimal leaching after 10 days in solution. The viability of the bacteria within the matrix was demonstrated by detection of the metabolic products H_2S and acetate, indicating that the encapsulated bacteria are metabolising lactate and sulfate. The large quantity of water incorporated in the gel matrix, the absence of micropores (<1 nm), and the benign reagents used in the synthesis are important factors in maintaining viability of the encapsulated bacteria, which were able to survive in storage for several months.

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References

1 J. I. Zink, J. S. Valentine and B. Dunn, *New J. Chem.*, 1994, **18**, 1109.
2 D. Avnir, *Acc. Chem. Res.*, 1995, **28**, 328.
3 S. Braun, S. Shtelzer, S. Rappoport, D. Avnir and M. Ottolenghi, *J. Non-Cryst. Solids*, 1992, **147–8**, 739.
4 D. Avnir, S. Braun and M. Ottolenghi, *ACS Symp. Ser.*, 1992, **499**, 384.
5 D. Avnir, S. Braun, O. Lev and M. Ottolenghi, *Chem. Mater.*, 1994, **6**, 1605.
6 M. T. Reetz, *Adv. Mater.*, 1997, **9**, 943.
7 S. Braun, S. Rappoport, R. Zusman, D. Avnir and M. Ottolenghi, *Mater. Lett*, 1990, **10**, 1.
8 B. C. Dave, B. Dunn, J. S. Valentine and J. I. Zink, *ACS Symp. Ser.—Nanotechnol.*, 1996, **622**, 351.
9 B. C. Dave, B. Dunn, J. Selverstone Valentine and J. I. Zink, *Anal. Chem.*, 1994, **66**, 1120A.
10 I. Gill and A. Ballesteros, *J. Am. Chem. Soc.*, 1998, **120**, 8587.
11 M. Rietti-Shati, D. Ronen and R. T. Mandelbaum, *J. Sol–Gel Sci. Technol.*, 1996, **7**, 77.
12 V. M. Sglavo, G. Carturan, R. Dal Monte and M. Muraca, *J. Mater. Sci.*, 1999, **34**, 3587.
13 L. Armanini, G. Carturan, S. Boninsegna, R. Dal Monte and M. Muraca, *J. Mater. Chem.*, 1999, **9**, 3057.
14 C. J. Brinker and G. W. Scherer, *Sol–Gel Science. The Physics and Chemistry of Sol–Gel Processing*, Academic Press, Boston, 1990, ch. 3.
15 J. R. Postgate, *The Sulphate-Reducing Bacteria*, Cambridge University Press, Cambridge, 1979, p. 26.