Encapsulation and controlled release of biomolecules from silica microparticles

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Biomolecules have been encapsulated in silica microparticles for controlled release applications, by destabilisation of aqueous silica precursors in the water droplets of a water-in-oil emulsion. The use of colloidal solutions (colloids typically in the size range 6–20 nm) as silica precursors avoids the detrimental effect of alcohol in the reactant solution. Thus, subtilisin A has been encapsulated with little to no loss in enzymatic activity. The particles are mesoporous, with a well-defined pore-size in the order of the size of most proteins, enabling the molecules to diffuse out of the solid. The release rate can be controlled by adjusting the pore-size of the silica particles.

Introduction

The encapsulation of biomolecules, particularly proteins, in sol–gel materials has been widely studied[1] and reviewed by numerous authors.[2] Encapsulation of biocatalysts, such as enzymes or bacteria in sol–gel matrices, offers significant advantages for their use in industrial processes, including ease of separation of the immobilised catalyst from the reactant–product stream, and efficient diffusion of reactants and products throughout the typically porous matrix.[3]

Hydrolysis of alkoxides produces alcohol in the reactant solution, which causes denaturation of the majority of protein molecules. The removal of alcohol prior to introduction of the protein is difficult to implement without inducing gelation of the reactant solution. The problem of alcohol in solution has largely been ignored, but there have been several approaches to address it, including the use of glycerol-substituted alkoxides to ensure milder encapsulation conditions.[4] An alternative solution used in a small number of cases is to avoid alkoxides altogether and use aqueous silica precursors; the sol-to-gel transition in this case involves the cross-linking of polysiloxy oligomers and/or aggregation of electrostatically stabilised colloids by adjustment of the pH or ionic strength of the reactant solution.[5] The ability to encapsulate viable microoganisms using this approach is evidence of the benign environment afforded in these materials.[6]

Materials designed to release encapsulated active molecules at controlled rates are of considerable commercial interest, particularly in the burgeoning field of drug delivery. A requirement for achieving a reproducible release rate for a particulate system is a controlled particle shape and size. Emulsion droplets provide convenient 'micro-reactors' for conducting hydrolysis and condensation reactions of alkoxides, and there are a number of reports describing the production of monodisperse, spherical particles using a combination of sol–gel and microemulsion chemistry.[7] Our laboratory has been investigating the use of water-in-oil emulsions to encapsulate hydrophilic molecules in spherical sol–gel particles. Addition of an aged silica sol–gel solution with added active molecules to a surfactant–solvent mixture results in the rapid formation of silica microparticles with encapsulated active molecules. Adjustment of the sol–gel parameters enables tailoring of the porosity of the matrix formed, and hence provides a mechanism for controlling the release of the encapsulated molecules from within the solid matrix particle.[8] However, while the pore-size could be tailored to enable release of larger molecules such as proteins, the alcohol and typical pH values involved mean this method is largely unsuitable for encapsulation of biomolecules.

This approach has recently been extended to the formation of silica microparticles by destabilisation of aqueous silica precursors (generally $\sim$30 wt% colloidal solutions) in the water droplets of a water-in-oil emulsion. The addition of protein molecules to the emulsion results in rapid dispersion of the protein into the hydrophilic droplets, and subsequent encapsulation as the colloidal particles aggregate together. The use of colloidal solutions (colloids typically in the size range 6–20 nm) as the silica precursor serves several purposes. The first is to avoid the detrimental effect of alcohol as discussed above. The second is that a mesoporous material is obtained, with a well-defined pore-size in the order of the size of the proteins to be released. The extent to which enzymes of various physical sizes are released from the matrix is dependent upon the aqueous precursor and aggregation conditions employed.[9] Finally, colloidal silica is widely used for a range of commercial applications and thus is available at relatively low cost.

Here we report the encapsulation in silica microparticles, of several protein molecules. Three aspects of the protein encapsulation procedure have been investigated: 1) the distribution of the protein throughout the particle, 2) the effect of the process on the activity of subtilisin A, and 3) the release of several proteins from silica microparticles. In order to determine the distribution of protein throughout the microparticle, ferritin, which contains a 6 nm iron core, was encapsulated

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and the distribution determined by cross-sectional TEM mapping of Fe. Activity tests of subtilisin A on exposure to various chemicals used in the encapsulation process were conducted to determine which, if any, chemicals were responsible for a loss in enzymatic activity. The activity of subtilisin A, following encapsulation and at least partial release from silica microspheres, was also measured to determine whether the encapsulation process resulted in reduced enzyme activity. Release rate measurements for encapsulated ovalbumin and subtilisin A, respectively, were conducted by immersion of the protein-doped silica powders in phosphate-buffered saline at 37 °C.

Experimental

Protein mapping

Sorbitan monolaurate (1.8 g) was dissolved in kerosene (12 mL). Ferritin solution ($\sim$100 mg mL^{-1}, 126 μL) was mixed with a silica colloid (Bindzil 30/360, Eka Chemicals, 300 μL). This mixture was then added to the surfactant solution dropwise, with stirring at 500 rpm, followed by addition of HCl (1 mol L^{-1}, 91 μL). The emulsion was left stirring for 2.5 hours, at which time solid material appeared on the bottom of the reaction vessel. The mixture was centrifuged (2000 rpm, 3 minutes) and the solid was washed once with kerosene and twice with isopropanol. The solid material was dried under flowing nitrogen.

Particles were imbedded in resin and 80 nm thin sections were cut using a 30° Diatome diamond knife on a Leica Ultracut UCT ultramicrotome and applied to holey carbon coated copper grids. These were examined using a JEOL 2010F scanning transmission electron microscope (STEM) equipped with a field emission gun electron source operated at 200 kV (JEOL, Japan). The 2010F was equipped with a LINK energy dispersive X-ray (EDX) spectrometer (Oxford Instruments, UK) and ESVision microanalysis system (EMiSpec, USA).

The Fe distribution over part of the cross-section of a silica particle was mapped by STEM EDX spectrum imaging. This technique involves collection of a full EDX spectrum at each pixel in a STEM image and subsequently processing each spectrum to remove background X-rays. Maps of elemental distribution are generated by plotting X-ray intensity in regions of the spectrum corresponding to each element of interest.

Activity tests

Subtilisin A assay for determining influence of encapsulation components. Assays were conducted to determine the effect of the various components used in the encapsulation process on the activity of subtilisin A. The components tested were; kerosene, Bindzil 30/360, sorbitan monolaurate, water adjusted to pH < 3, ethanol, isopropanol, hexane, and cyclohexane. The procedure was to add subtilisin A (0.5 μg mL^{-1} in 0.02 mol L^{-1} phosphate buffered saline (PBS), 100 μL) and each component (500 μL) to 0.02 mol L^{-1} PBS (total volume = 1500 μL) and incubate at 37 °C for 10 minutes. Subtilisin substrate *t*-butylcarbamate-L-glycine-L-glycine-L-leucine-*p*-nitrophenylacetate (1×10^{-3} mol L^{-1}, 500 μL), was then added and the mixture was incubated for 5 minutes at 37 °C. A comparison of activity against a control sample (no added component) was made by measuring the absorbance of the product, *p*-nitrophenolate, at 410 nm.

Standard subtilisin A assay. The enzyme solution (100 μL) at an approximate concentration of 0.5 μg mL^{-1} was incubated at RT with a solution of *t*-butylcarbamate-L-glycine-L-glycine-L-leucine-*p*-nitrophenylacetate (1×10^{-3} mol L^{-1}, 500 μL), in a total volume of 2000 μL, made up with 0.02 mol L^{-1} PBS (pH 7.4). The enzymatic activity was quantified by measuring the absorbance of the product, *p*-nitrophenolate, at 410 nm.

Protein encapsulation and release

Two samples of ovalbumin (240 and 100 mg, respectively) were pre-mixed with Bindzil 30/360 (30 wt%, 6 mL) and Bindzil 15/500 (15 wt%, 6 mL) silica colloids. 5 mL of each colloidal solution was added to mixtures of sorbitan monolaurate (18 g) in kerosene (120 mL) with stirring. The pH in the aqueous droplets was reduced from 10 to 6 by addition of HCl (1 mol L^{-1}, 0.92 and 0.58 mL, respectively) to the emulsions. Finally, 0.7 mL of a salt solution containing KH_2PO_4 (0.1 g), NaCl (6.46 g), $CaCl_2$ (0.233 g) and $MgCl_2$ (1.97 g) in 50 mL H_2O was added to the emulsion, which was then left stirring for 6 hours. Protein-doped silica was removed by centrifugation (3000 rpm, 5 minutes) and the solids were washed with kerosene and then thoroughly with hexane. The solids were dried at 37 °C for two days to remove residual solvent.

The same procedure was used to dope subtilisin A into silica microparticles, except that the solids were dried for one day only at 37 °C.

The protein content of doped microparticles, and quantification of protein released from microparticles was determined using the bicinchoninic acid (BCA) assay as follows:

Reagent A: sodium bicinchoninate (1 g), $Na_2CO_3 \cdot 2H_2O$ (2 g), sodium tartrate (dihydrate) (0.16 g), NaOH (0.4 g), $NaHCO_3$ (0.95 g), made up to 100 mL with deionised water. The pH was adjusted to 11.25 using NaOH.

Reagent B: $CuSO_4 \cdot 5H_2O$ (0.4 g), made up to 10 mL with deionised water.

Standard working reagent (SWR) = 100 volumes of reagent A + 2 volumes of reagent B.

Quantification of protein in microparticles

Protein-doped microparticles (10 mg) were suspended in PBS (0.02 mol L^{-1}, 1 mL) and shaken to mix. Aliquots (50 μL) were removed in triplicate and incubated with SWR (1 mL) at 60 °C for 30 minutes. After incubation, the solid was removed from the solutions by centrifugation at 12 000 rpm for 30 seconds. The absorbance of the solution was measured at 562 nm, and compared to that of a series of standards at 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} of the protein in question.

Quantification of protein released from microparticles

Protein-doped microparticles (10 mg) were suspended in PBS (0.02 mol L^{-1}, 1 mL) and agitated gently using a shaker table at 37 °C. Release measurements were conducted in triplicate.

At specific time intervals over a period of 24 hours, the suspensions were centrifuged for 30 seconds at 12 000 rpm, and samples (50 μL) were removed. At the conclusion of the release experiment, the aliquots were incubated with SWR (1 mL) and assayed according to the BCA assay as described above.

Pore-size analysis

Pore-size distributions were determined for samples containing no protein and dried at 60 °C, using nitrogen sorption analysis (ASAP 2000 system, Micromeritics). Nitrogen adsorption data were modelled using density functional theory, assuming cylindrically shaped pores.

Results and discussion

Fig. 1 shows a typical SEM image of silica microparticles obtained from Bindzil 30/360. Light-scattering measurements show that the particle size distribution is broad, ranging from ~0.05 to 50 μm, with an average size of ~2 μm. The wide particle size range reflects the range of sizes of water droplets which are formed in the unstable sorbitan monolaurate/kerosene/water emulsion.

Protein mapping

Fig. 2 shows a typical TEM image of cross-sectioned microparticles. Again, a range of particle sizes is present, reflecting the original size distribution as shown in Fig. 1 and the fact that microtomed thin sections may cut through any chord of a microparticle. Some knife damage is evident on the central particle. Fig. 3 shows a map of the Fe distribution (Fig. 3b) in a 32 pixel by 30 pixel area corresponding to the box on the STEM dark field image (DFI) (Fig. 3a). The spectrum displayed in Fig. 3c clearly shows the Fe-K X-ray peak, due to ferritin at the position of the small cross in the STEM DFI.

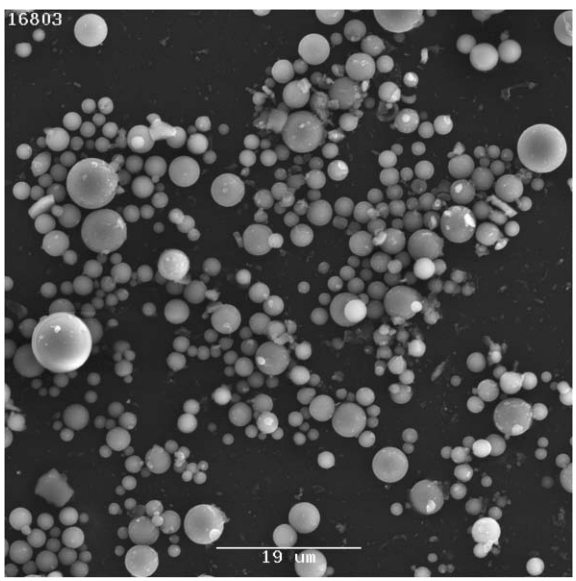

Fig. 1 Typical SEM image of silica microparticles obtained from Bindzil 30/360.

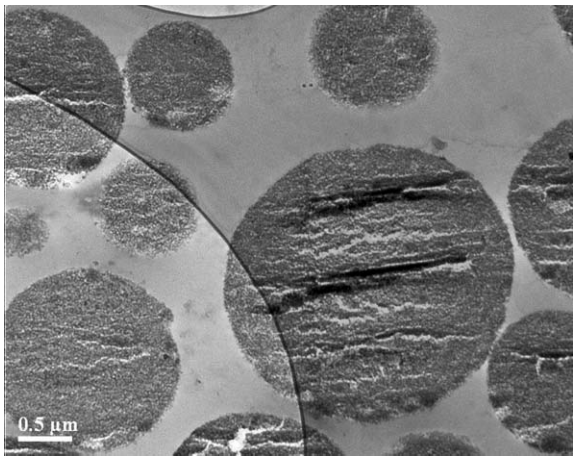

Fig. 2 TEM image of cross-sectioned silica microparticles.

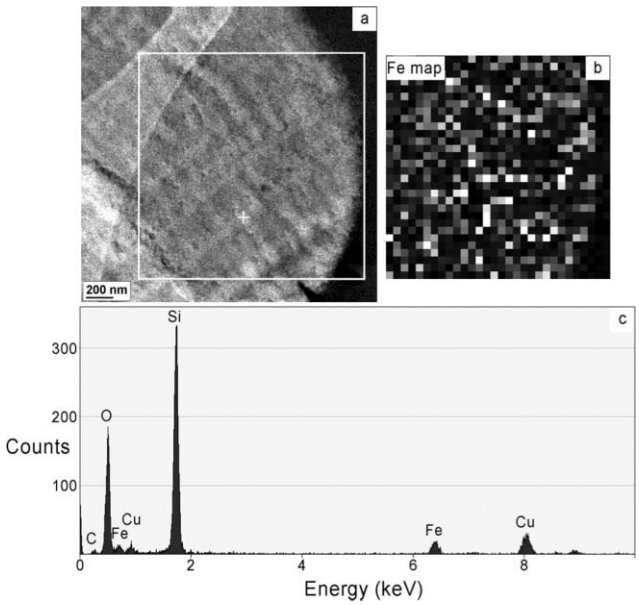

Fig. 3 (a) STEM dark field image of one slice of microsphere; (b) Distribution of Fe in the region indicated by the box in (a); (c) EDX spectrum from the point marked by the cross in (a).

The Fe distribution map indicates that ferritin is uniformly distributed throughout the analysed region. No Fe was detected in the STEM EDX spectrum image from a control specimen without encapsulated ferritin. The homogeneous distribution of Fe across the area analysed suggests that the protein does not preferentially locate near the water–surfactant interface, but is solubilised homogeneously inside the water droplet.

Enzyme activity

Fig. 4 shows the effects of the various components of the encapsulation process on the activity of subtilisin A. The activity was normalised to the control with no added components, shown in the first column labelled 'protein'. The two most detrimental conditions for the activity of subtilisin appear to be acidic conditions (pH < 3), and the presence of

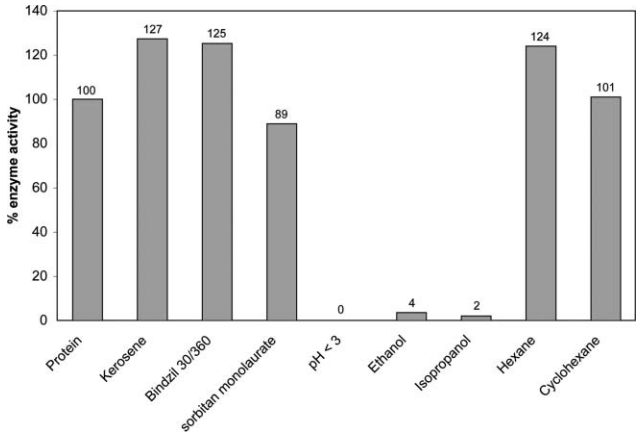

Fig. 4 Effect of various components of the encapsulation process on the % activity of subtilisin A.

alcohol (ethanol and isopropanol). The main components of the encapsulation process, including the silica precursor (Bindzil 30/360), surfactant and solvents were found to have little to no adverse effect on the enzyme activity.

The activity of subtilisin A following encapsulation in silica (using Bindzil 30/360) has been determined using the standard subtilisin A assay, and compared with the activity of the enzyme in solution in Fig. 5. The gradient of each curve represents the activity of the enzyme, (*i.e.* the rate of change of product concentration per mg of protein). The activity of the enzyme post-encapsulation (which includes contribution from both the released enzyme and the enzyme remaining encapsulated in the silica) was observed to be very similar to the enzyme in solution, suggesting that the encapsulation process resulted in little loss of activity.

Acidic conditions (<pH 6) are known to be detrimental to subtilisin.[10] However, both subtilisin A and ovalbumin, the two proteins encapsulated here, were both found to be undamaged by exposure to conditions of pH = 10. For this reason, the protein was pre-mixed with the silica colloid before addition to the emulsion. Proteins susceptible to denaturation in base should be added separately to the emulsion following addition of acid to reduce the pH of the hydrophilic phase to neutral conditions.

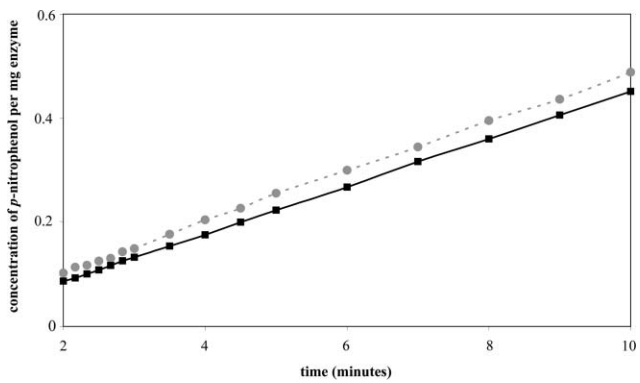

Fig. 5 Enzyme activity of particles containing subtilisin A (- - ● - -), compared with that of the enzyme in solution (–■–).

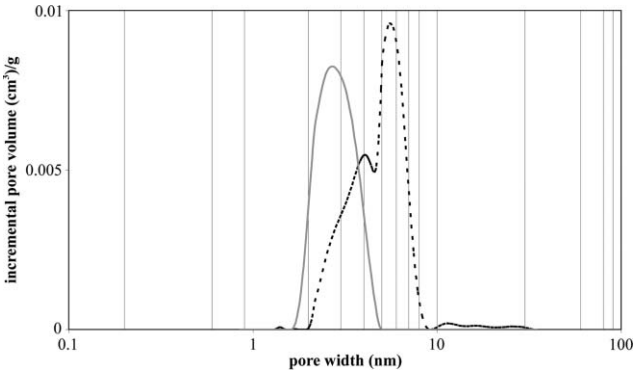

Fig. 6 Pore size distribution for silica made from Bindzil 30/360 (---) and Bindzil 15/500 (—).

Protein encapsulation and release

Silica microparticles made using Bindzil 30/360 and 15/500 were found to have average pore-sizes of 5.5 and 2.7 nm, respectively. The difference in average pore-size is related to the primary colloidal particle size, 9 and 6 nm for Bindzil 30/360 and 15/500, respectively. The relatively sharp peaks observed in the pore-size distributions (see Fig. 6) are due to the uniform size of the primary colloidal particles, the pores corresponding to interparticle spaces between closely packed spheres.

The loading of ovalbumin in silica derived from Bindzil 30/360 and 15/500 was found to be 5.7 and 6.6 wt%, respectively, corresponding to encapsulation efficiencies of 75 and 97%, respectively. The release of ovalbumin into PBS (0.02 mol L^{-1}) at 37 °C was measured from both samples as shown in Fig. 7. There is no 'burst' release observed in the samples. Somewhat surprisingly, the earliest data points suggest that ovalbumin was initially released slightly faster from the silica with smaller pores. However after an initial period of 8 hours, the rate of release from the larger pore silica appears significantly greater.

The loading of subtilisin A in silica derived from Bindzil 30/360 and 15/500 was found to be 4.5 and 5.3 wt%, respectively, corresponding to encapsulation efficiencies of 54 and

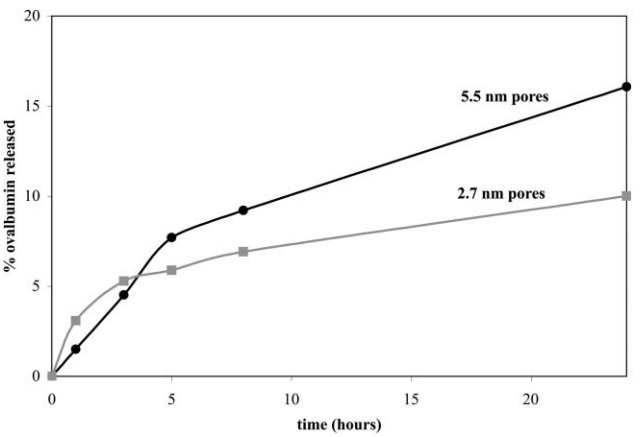

Fig. 7 Release *vs.* time of ovalbumin from silica with 5.5 (–●–) and 2.7 nm (–■–) pores.

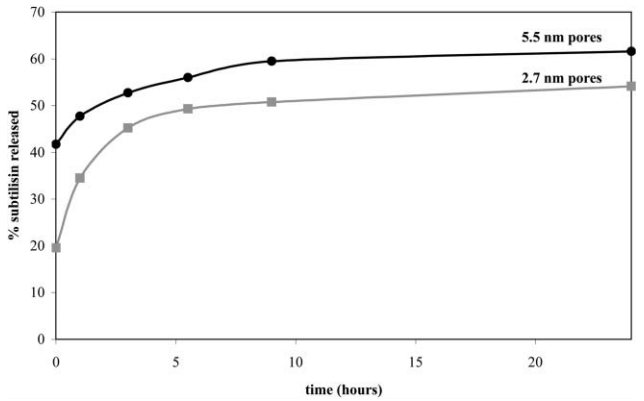

Fig. 8 Release *vs.* time of subtilisin A from silica with 5.5 (–●–) and 2.7 nm (–■–) pores.

55% respectively. The release of subtilisin A from both samples into PBS (0.02 mol L^{-1}) at 37 °C is shown in Fig. 8. In contrast to the case of ovalbumin, a significant 'burst' release of subtilisin A was observed immediately following immersion of the solids in buffer. Significantly less was observed for the smaller pore silica (20%), compared with 42% for the 5.5 nm pore silica. After 9 hours, the release rates from the samples appear similar. Overall, the release of subtilisin A was considerably more rapid than that of ovalbumin. This is likely to be due to the difference in size of the proteins (32 kDa *vs.* 45 kDa for subtilisin A and ovalbumin, respectively). The drying time of the subtilisin A doped samples was also reduced to minimise the extent of activity loss due to self-proteolysis in the sample, which could also be a factor in the more rapid release on re-immersion.

Conclusions

A novel method for encapsulating proteins in spherical silica microparticles for controlled release applications has been established which offers benign conditions, essential for preserving the structure of the relatively fragile biomolecules. Mapping of the protein distribution using cross-sectional TEM shows that the protein is homogeneously distributed throughout the particle matrix. The encapsulation method is sufficiently flexible to allow tailoring of the process conditions to preserve the activity of enzymes, as demonstrated for subtilisin A, for which no obvious loss in activity was observed following encapsulation. The average pore-size of the silica matrix may be tailored by adjusting the aqueous silica precursor, which enables control of the release rate as demonstrated for two proteins, ovalbumin and subtilisin A.

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