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Enzyme encapsulation in nanoporous silica spheres†

Yajun Wang and Frank Caruso*

Centre for Nanoscience and Nanotechnology, Department of Chemical and Biomolecular Engineering, The University of Melbourne, Victoria 3010, Australia. E-mail: fcaruso@unimelb.edu.au; Fax: +61 3 8344 4153

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Encapsulating enzymes in mesoporous silica spheres *via* immobilization, followed by assembling an organic/inorganic nanocomposite shell on the particle surface leads to high loadings, high enzymatic activity and stability, and protection from proteolysis.

There has long been widespread interest in encapsulation technologies, largely due to their relevance to medicine, pharmaceutics, agriculture and the cosmetic industries.[1,2] Liposomes, polymeric particles and microemulsion droplets are commonly employed systems for the encapsulation of various materials, ranging from drugs, pesticides, fragrances, and biomolecules such as enzymes.[3] Recently, we presented a facile strategy for the encapsulation of crystalline materials of low molecular weight species (*e.g.*, model drugs) and enzymes *via* the sequential deposition of oppositely charged polyelectrolytes (PEs).[4] We demonstrated that the PE-encapsulated enzyme microcrystals can be assembled into thin multilayered films with tailored bioactivity (depending on the number of microcrystal layers deposited),[5] and that the PE-encapsulated organic microcrystals can be utilized to create highly amplified biochemical assays.[6] Although this approach provides a high loading of encapsulated material, it is largely limited to substances that can be crystallized. Amorphous enzyme precipitates have also been used for encapsulation by this method, but this generally yields less defined structures that are highly sensitive to small variations in the conditions necessary for forming stable aggregates.[7] The development of new methods for preparing stable, high enzyme content particles would be significant, as these materials could be subsequently utilized as dispersions or as building blocks for advanced materials fabrication for diverse applications in biotechnology.

Herein, we report a simple and highly versatile method for the preparation of high content enzyme-loaded particles by using nanoporous (*i.e.*, mesoporous) silica spheres with sufficiently large pore sizes and pore volumes as supports. Following enzyme loading, a nanocomposite polyelectrolyte/nanoparticle shell is assembled on the porous particle surface to encapsulate the loaded enzyme, thus preventing enzyme leakage. Although other mesoporous materials (such as MCM-41 and SBA-15; pore sizes 2–8 nm and 5–40 nm, respectively) have been widely used as supports for enzyme immobilization,[8–10] our work focuses on the combination of using porous particles for enzyme immobilization and their subsequent coating with nanocomposite shells to effect encapsulation of the enzymes, thus overcoming the problem of enzyme desorption often encountered with the direct immobilization of enzymes on mesoporous silicas.[8]

The nanoporous spheres synthesized possess a bimodal mesoporous silica (BMS) pore structure[11] and have a surface area of 630 m^2 g^{-1} and a pore volume of 1.72 mL g^{-1} (see Supplementary Information†). The smaller mesopores have a pore size of 2–3 nm, and the larger mesopores, which are suitable for enzyme immobilization, are between 10–40 nm with a pore volume of *ca.* 1.28 mL g^{-1}. TEM images reveal that the BMS spheres have a particle size ranging from 2–4 μm (Fig. 1(a)). At higher magnification, the disordered porous structure becomes apparent (Fig. 1(b,c)). For control experiments, mesoporous silica (MS) spheres of similar particle size (*ca.* 1.8 μm) but with only the small pore size (*ca.* 2 nm), were used (see Supplementary Information†).

Several model enzymes with different molecular weights were employed for immobilization on the BMS spheres. The particles were dispersed in enzyme solution (*ca.* 0.4 mg mL^{-1}) in 50 mM phosphate buffered saline (PBS) for 48 h. The enzyme content retained by the particles was measured by monitoring the difference in solution absorbance from the enzyme before and after adsorption. Fig. 2 shows that the BMS spheres have a significantly higher (10–15 fold) enzyme entrapment capacity than the MS spheres. High loading amounts (> 200 mg g^{-1}) are obtained for the BMS spheres for enzymes with relatively low molecular weights, *e.g.*, cytochrome C (Cyt. C, C-2037, 12 kDa) and protease (P6911, 20 kDa). Cyt. C (*ca.* 3 nm diameter) has a molar loading amount of 19.2 μmol g^{-1}, which is about a factor of two higher than that reported in the literature for mesoporous silica materials with an average pore size of 13 nm (10.2 μmol g^{-1}).[9c] Catalase (C-100), a much larger protein (250 kDa, *ca.* 10 nm diameter),[12] shows a loading amount of 53 mg g^{-1}. The different enzyme loading ability of the particles was further confirmed by confocal laser scanning microscopy for particles exposed to a solution of fluorescein isothiocyanate-labelled peroxidase (FITC-POD) (data not shown). We observed bright, spherical rings only for the MS particles. This is attributed to the larger diameter of POD (*ca.* 4.8 nm) than the MS sphere pore size, and hence adsorption of FITC-POD is restricted to the particle surface. In contrast, fluorescence from FITC-POD was observed throughout the entire BMS particles, which suggests that the POD molecules distribute homogeneously on and within the particles. These data show that the BMS spheres have excellent enzyme adsorption capacity compared with mesoporous materials with only small pores (*ca.* 2 nm).

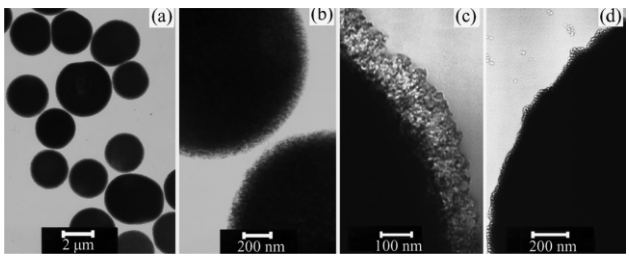

Fig. 1 TEM images of BMS spheres (a–c) at different magnifications, and the same spheres after catalase loading and deposition of three layer pairs of PDDA/Si_{NP} (d).

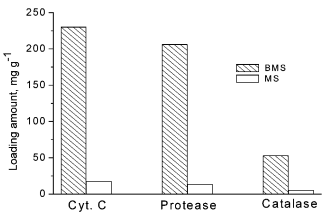

Fig. 2 Comparison of the immobilization capacity of BMS and MS spheres for various enzymes.

† Electronic supplementary information (ESI) available: experimental details, and details of particle characterization. See http://www.rsc.org/suppdata/cc/b4/b403871a/

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In order to encapsulate the immobilized enzyme, a nanocomposite shell was assembled on the BMS surface following enzyme loading. This shell serves two purposes: (i) it helps prevent enzyme leakage from the BMS substrates; and (ii) it provides a protective barrier for the immobilized enzyme against enzyme-degrading substances (*e.g.* protease). We chose catalase-loaded spheres to demonstrate the coating process. The shell was deposited by the alternate assembly of three layer pairs of poly(diallylammonium chloride) (PDDA) and 21 nm silica nanoparticles (Si_{NP}), according to our earlier method (see Supplementary Information†).[13] Silica nanoparticles were used to block the porous substructure on the BMS sphere surface, and to form a compact nanoparticle shell around the spheres. Further, the use of a PDDA/Si_{NP} coating yielded particles with higher activities than when pure polyelectrolyte layers were used for encapsulation (see later). Following shell formation, the particles showed a higher electron density, as observed by TEM (Fig. 1d). The immobilized enzyme was retained within the spheres, as proved by the absence of a measurable amount of enzyme in the supernatant after exposing the loaded spheres to PBS solution for 6 h. Without the shell coating, *ca.* 25% of catalase was leaked from the BMS spheres after 6 h.[14]

The catalase immobilized in the BMS spheres and encapsulated with $(PDDA/Si_{NP})_3$ retained *ca.* 62% of the activity of catalase in solution. The $(PDDA/Si_{NP})_3$-encapsulated catalase-BMS spheres displayed an activity ~75 times greater than the corresponding MS spheres (activity of 1138 *vs.* 15 units mg^{-1}).[15,16] During the PDDA and Si_{NP} coating steps, 87% of catalase desorbed from the MS spheres, while 17% was removed from the BMS spheres. In addition, for the MS spheres the enzyme is adsorbed on the particle surface and hence is in direct contact with the subsequently deposited PDDA, thus reducing its activity.[5a] The catalase encapsulated in the BMS spheres has an enhanced stability against pH, compared with catalase in solution. At pH 5.1, the free catalase has an activity below 6% (the activity at pH 7 was normalized to 100%.), while the encapsulated enzyme retains 36% of its original activity. The enzyme lifetime was also enhanced after encapsulation: after 60 min H_2O_2 exposure at pH 7, free catalase shows *ca.* 5% of its original activity, whereas the BMS-encapsulated enzyme retains *ca.* 40% of its original activity. The BMS-encapsulated enzyme activity decreases only *ca.* 4% after recycling five times.

The activity of the catalase encapsulated in the BMS spheres and the protection imparted by the PDDA/Si_{NP} coating with respect to proteolysis were also examined (Fig. 3).[17] Free catalase is deactivated very quickly by protease, losing its activity after 60 min. For catalase-loaded BMS spheres, the inactivation is slower, with 20% activity retained after 60 min. This value increases to about 75% (after 60 min protease exposure) when the PDDA/Si_{NP} shell is present. This indicates that the PDDA/Si_{NP} shell can be penetrated by the protease (M_w ~ 20 000 *vs.* M_w ~ 250 000 for catalase). However, total protection is gained by the deposition of an additional eight layers of poly(allylamine hydrochloride) (PAH) and poly(styrenesulfonate) (PSS).[4a,5]

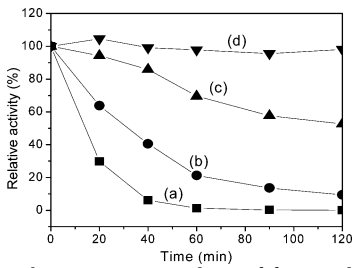

Fig. 3 Activity with respect to proteolysis of free catalase (a), catalase-loaded BMS spheres (b), spheres as in (b) plus three layer pairs of PDDA/Si_{NP} (c), and spheres as in (c) with an additional four layer pairs of PAH/PSS (d). The errors in the activities are ±10%.

In summary, the use of BMS spheres for enzyme immobilization followed by nanoscale multilayer shell formation for enzyme encapsulation provides a facile route to prepare composite particles with high enzyme contents (50–225 mg g^{-1}), enhanced enzyme activities (*ca.* 75 times higher than when using MS spheres), higher enzyme stabilities against pH, and protection for the encapsulated enzyme from proteolysis. These and similar particles are likely to find application in various biotechnology-related applications, including biocatalysis, and represent suitable building blocks to construct functional thin films with high enzyme loadings.

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14 Enzyme leakage was followed by dispersing 10 mg of the enzyme-loaded BMS spheres with or without a PDDA/Si_{NP} coating in 10 mL PBS buffer (pH 7.0), shaking at 37 °C for 6 h, centrifuging (5 000 *g*) for 20 min, and measuring the enzyme amount in the supernatant.
15 The biocatalyst was added to 11 mM H_2O_2 in 50 mM PBS stock solution with stirring after incubation at the desired pH for 30 min. The decrease in absorbance at 240 nm (with an extinction coefficient of 0.041 $mmol^{-1}$ cm^{-1}) with time was recorded immediately after the enzyme was mixed into the solution. One unit of catalase will convert 1 μmol of H_2O_2 per minute at pH 7.0 and 20 °C. The weight is based on that of the particles.
16 An activity of 1386 units mg^{-1} was observed for catalase immobilized in the BMS spheres. Considering the 17% loss of enzyme after $(PDDA/Si_{NP})_3$ coating, a negligible loss of activity is observed for the encapsulated catalase. Catalase-BMS spheres coated with $(PDDA/PSS)_3$ showed activities of ~900 units mg^{-1}.
17 20 mg of protease was dissolved in 1.0 mL of PBS solution at pH 7.0, and used as the stock solution. 0.2 mL of the free catalase, immobilized enzyme, and encapsulated enzyme (catalase starting activity for all samples was ~10 000 units mL^{-1}) were separately incubated in a water bath at 37 °C with either 40 μL of the stock protease solution or 40 μL of PBS (control experiment). The initial activity was normalized to 100%. Proteolysis was determined by measuring the decrease in catalase activity (H_2O_2 as substrate).