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Encapsulation of enzyme in large mesoporous material with small mesoporous windows†

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Trypsin has been encapsulated in the mesopores of a hierarchical mesoporous silica material synthesized *via* Cu(I) catalyzed azide-alkyne click reaction between azide functionalized large spherical SBA-15 particles and alkyne functionalized mesoporous silica nanoparticles (MSNs). Encapsulated trypsin functions as an efficient biocatalyst and can be recycled several times.

Enzymes are versatile biocatalysts that perform chemo-, regio- and stereo-selective reactions in aqueous media under mild conditions.[1] The specificities of enzymes carry tremendous promise as catalysts in industrial applications, but the short lifetime of enzymes presently limit their usefulness. Therefore, improvements in enzyme stability and reusability is required to increase their lifetime so as to further enable practical applications.[1] One of the approaches to improve enzyme reusability is *via* enzyme immobilization which represents the attachment or incorporation of enzyme molecules into large structures that render them insoluble in water. This allows efficient recovery of the enzyme after the reaction and hence can be reused several times. Various inorganic solid supports have been used for enzyme immobilization including alumina,[2a] silica,[2b] zeolites[2c,3a] and mesoporous silica materials.[1c,3b,3c] In particular mesoporous silica materials are very good candidates for the immobilization of enzymes due to their ease of synthesis, high surface area (up to 1000 $m^2\ g^{-1}$) and tuneable pore size (2–50 nm) which enables researchers to tailor these materials suitably so that the encapsulation of a variety of proteins and enzymes is feasible.[3d] Thus, the development of mesoporous material based biocatalysts has attracted a lot of attention. Most of these materials have been prepared by immobilization of the enzymes into the pores of the mesoporous channels by physical adsorption.[3e,f] Although simple and efficient, this process is severely limited by the fact that with time these adsorbed enzymes leach out into the solution. To overcome this, enzymes have been covalently immobilized by chemically attaching them to an organo-functionalized mesoporous material *via* amino acid side-chains like lysine.[4,5a]

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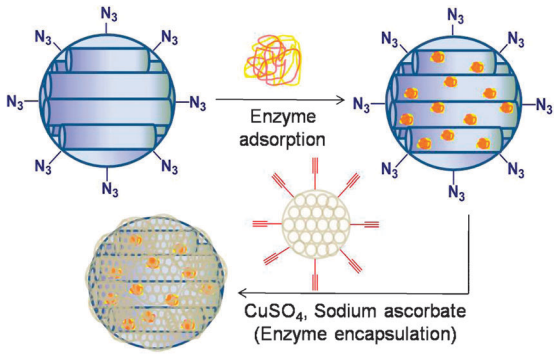
Recently, Bein *et al.* have attached alkyne labeled trypsin covalently on the surface of azide functionalized SBA-15 by Cu(I) catalyzed azide alkyne click chemistry (CuAAC) approach.[5b] However, after covalent immobilization there is limited control of the spatial orientation of the immobilized enzyme within the pores which in turn affects the reactivity. Another approach to prevent enzyme leaching is *via* a "ship-in-a-bottle" approach where the pores of the mesopore are partially closed after adsorption of the enzyme; thereby preventing the enzyme from leaching out while the diffusion of the substrate and product remains unaffected.[5c,d] For example, Wang and Caruso have encapsulated enzymes in bimodal mesoporous silica (BMS) by enzyme adsorption followed by assembly of a multilayer shell on the particle surface by layer–by-layer electrostatic assembly of oppositely charged species to prevent the enzyme from leaching.[5e] However with changes in pH or salt concentration, these multilayer shell may disassemble or cause changes in their permeability properties thereby limiting possible applications in catalysis.[5f] Hence, there is a need to generate new porous materials that can efficiently immobilize native enzymes that have activity similar to the native enzyme and can also be recycled several times without loss of activity.

Herein, we report the synthesis of a hierarchical mesoporous silica based hybrid material and its application as a support for the immobilization of trypsin *via* a "ship-in-a-bottle" approach. Enzyme was adsorbed onto an azide functionalized mesoporous spherical SBA-15 (diameter 5–8 μm) with pores larger than the diameter of the enzyme. This was then capped with alkyne functionalized mesoporous silica nanoparticles (MSNs; diameter ~100 nm) by covalent linkage using CuAAC (Scheme 1). The mesoporous silica nanoparticles (MSNs) have pores that are large enough to allow small molecules such as reactants and products to diffuse in and out of SBA-15 but do not allow enzyme to leach out of SBA-15. Hence, in this hierarchical silica mesoporous hybrid material, the pores of the MSNs on the surface of SBA-15 act as windows for the enzyme and effectively stop the enzyme from leaching. Trypsin, an industrially important enzyme for the hydrolysis of peptides in food processing, was chosen as a model enzyme for this study.[5g,6a] Developed hierarchical mesoporous solid material has several attributes that make it very attractive: (i) spherical SBA-15 particles with high surface area can be synthesized in various pore sizes (3 nm–13 nm)[6b]



Scheme 1 Encapsulation of trypsin in hierarchical mesoporous material. The particle in blue represents azide functionalized SBA-15 (diameter 5–8 μm) and the particle in grey represents alkyne functionalized MSNs (diameter ~100 nm).

depending upon the size of the enzyme to be encapsulated; (ii) native enzymes can be used for immobilization; (iii) the bio-orthogonal[6d] nature of CuAAC ensures that the efficient capping of organoazide groups on spherical SBA-15 particles can be carried out with alkyne functionalized MSNs without denaturing the enzyme[6c] and (iv) the inner pore surface of MSNs can be further functionalized to tune the hydrophobic/hydrophilic property of the surface for selective diffusion of either hydrophobic or hydrophilic substrates for chemoselective reaction. These materials have been characterized by various analytical techniques such as powder XRD, SEM, TEM, FT-IR, confocal laser scanning microscopy (CLSM) and nitrogen adsorption-desorption experiments and their catalytic activity for the hydrolysis of BAPNA has been shown over 10 catalytic cycles.

The azide functionalized mesoporous spherical SBA-15 (AZP-SBA) was prepared by post synthetic grafting of AzPTES (3-azido-propyltriethoxysilane) on calcined spherical SBA-15 (CAL-SBA) using reported procedures (ESI†).[6b,7] The average pore size and surface area was estimated to be 8.2 nm and 666 $m^2\ g^{-1}$ respectively; while the amount of azide groups grafted on the surface of AZP-SBA was determined to be 0.6 mmol g^{-1} (0.09 nmol cm^{-2}) by elemental analysis. MSNs having alkyne groups present on the outer surface (ALK-MSN) were synthesized in a two-step procedure (ESI†). In the first step, as-synthesized MSNs[8,9] were subjected to outer surface amine grafting using 3-amino-propyltriethoxysilane (APTES) followed by removal of template to yield NH_2-MSN. The amount of amine groups grafted on the surface of NH_2-MSN was determined to be 0.78 mmol g^{-1} by elemental analysis. In the second step, NH_2-MSN was coupled with *N*-(4-Pentynoyloxy)-succinimide to yield alkyne grafted MSNs (ALK-MSN). The average pore size and surface area of ALK-MSN was estimated to be 2.2 nm and 587 $m^2\ g^{-1}$ respectively. ALK-MSN was characterized by a variety of analytical techniques such as powder XRD, TEM and ^{13}C CP-MAS NMR spectroscopy (ESI†).

For the encapsulation of trypsin, the azidopropyl labeled SBA-15, AZP-SBA was first subjected to trypsin adsorption in phosphate buffer (100 mM, pH 7) at 4 °C and then subjected to CuAAC with ALK-MSN using $CuSO_4$ and sodium ascorbate at 4 °C as described in the literature (ESI†).[6c,7,9b]

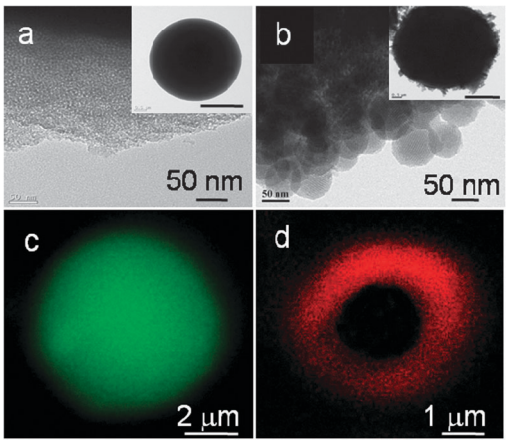


Fig. 1 (a) TEM of spherical SBA-15 (AZP-SBA) (*Inset*) TEM of an individual AZP-SBA, scale bar represents 2 μm; (b) TEM of the outer surface of Trypsin encapsulated hierarchical mesoporous material (Trypsin-SBA-MSN) showing MSN particles attached onto the surface of SBA-15 (*Inset*) TEM of an individual Trypsin-SBA-MSN, scale bar represents 2 μm (c) Fluorescein labelled trysin absorbed onto AZP-SBA; (d) AZP-SBA clicked with rhodamine B labelled MSNs showing that the MSN particles are present on the outer surface of SBA-15

After the click reaction the enzyme encapsulated material (Trypsin-SBA-MSN) was washed with 10% PEG solution in 50 mM pH 8 tris buffer to leach out any weakly adsorbed trypsin on the surface of the material.[10a] The amount of trypsin adsorbed was estimated to be 31 ± 1 mg (~1.3 μmol) of trypsin per gram of Trypsin-SBA-MSN from TGA of Trypsin-SBA-MSN (Fig. S8, ESI†) and UV-Vis analysis of the residual trypsin that remained unadsorbed (ESI†). TEM image of Trypsin-SBA-MSN shows trypsin adsorbed spherical SBA-15 particles were very well covered by MSNs also demonstrating the formation of the hierarchical mesoporous structure (Fig. 1a and 1b). For complete characterization of the hierarchical mesoporous hybrid material formed using CuAAC, we synthesized another material by clicking AZP-SBA (without trypsin adsorbed) with ALK-MSN to yield CLICK-SBA-MSN and it was extensively characterized by FT-IR, nitrogen adsorption-desorption and ^{13}C CP-MAS NMR spectroscopy experiments. FT-IR spectra show about 25% decrease in the integrated intensity of $\nu_{as}(N_3)$ at 2100 cm^{-1} (Fig. S5, ESI†).[7] Nitrogen adsorption-desorption experiments gave a multipoint BET surface area of 542 $m^2\ g^{-1}$ and total pore volume 0.96 cc g^{-1}. BJH pore size distribution shows presence of hierarchical pore structure having pore sizes 8.18 nm and 2.2 nm (Fig. S6(c), Table S1, ESI†). Formation of hierarchical mesopore structure was further confirmed by ^{13}C CP-MAS NMR spectroscopy, the peaks at 124 and 147 ppm observed are due to formation of triazole by the click reaction between AZP-SBA and ALK-MSN (Fig. S7(c), ESI†).[7] To investigate the distribution of trypsin in the pores of AZP-SBA, fluorescein labeled trypsin was immobilized on AZP-SBA (Trypsin-AZP-SBA) and observed under CLSM. In another experiment AZP-SBA was clicked with rhodamine B functionalized MSN (ALK-RH-MSN, ESI†) and the resultant material was imaged by CLSM. This hierarchical material shows, a uniform coverage of rhodamine B functionalized MSNs (red) on the outer surface of AZP-SBA (Fig. 1d).[10b]

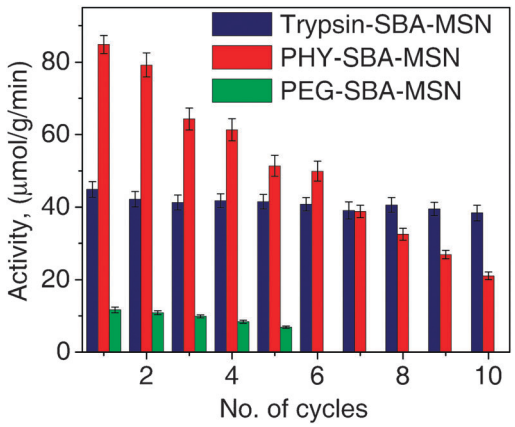


Fig. 2 Activity of trypsin encapsulated or immobilized on different silica materials using 2 mM BAPNA in 50 mM, pH 8 tris buffer.

The spectroscopy data of various hierarchical mesoporous materials discussed above indicates successful formation of hierarchical mesoporous materials and encapsulation of enzyme in the large pores of this material *via* CuAAC approach.

To investigate the activity of the encapsulated trypsin, Trypsin-SBA-MSN was treated with 2 mM BAPNA in tris buffer (50 mM, pH 8).[10c] The activity of the encapsulated trypsin was found to be 44 μmol g^{-1} min^{-1} and remained almost constant even after 10 cycles. (Fig. 2) This shows that the enzyme is active and remains encapsulated efficiently. This encapsulated enzyme has comparable activity with the activity of the physically adsorbed trypsin on post synthetic functionalized SBA-15 with thiols, amines and carboxylic acids.[10c] For further comparison of the activity, two control experiments were carried out. In one case, trypsin was physically adsorbed on AZP-SBA and then ALK-MSN was added in the same ratio as was used for encapsulation of trypsin. This material is referred as PHY-SBA-MSN (ESI†). In another control, the PHY-SBA-MSN hybrid material described above was subjected to the same workup protocol that was carried out during the encapsulation reaction (washing with 10% PEG solution in 50 mM, pH 8 tris buffer). This is referred as PEG-SBA-MSN (ESI†). Both the materials were subjected to activity study with BAPNA solution. The results (Fig. 2) show that even though the activity of PHY-SBA-MSN is about two-fold higher than that of the activity of the encapsulated enzyme (Trypsin-SBA-MSN), the activity of the PHY-SBA-MSN decreases significantly with each cycle due to leaching of the adsorbed enzyme in the solution.[10c] After 10 cycles activity of the adsorbed enzyme was decreased by ~75% where as encapsulated enzyme shows almost no loss in activity. Two-fold decrease in activity of the trypsin upon encapsulation in comparison to activity of adsorbed enzyme observed may be due to increased mass transport limitations; as BAPNA molecules have to diffuse through the pore channels of MSNs and SBA-15 to reach to the encapsulated trypsin. It is likely that the BAPNA molecules being positively charged were strongly adsorbed inside pore channels of MSNs which resulted in slow diffusion of BAPNA molecules onto the immobilized trypsin inside the pores of SBA-15.[3f] On the other hand, PEG-SBA-MSN showed 87% less activity as compared to activity by PHY-SBA-MSN. UV-Vis studies on estimation of amount of trypsin leached out shows that 89% of trypsin leached out during PEG solution treatment (ESI†).[10a] These results conclusively show that encapsulated trypsin in the hierarchically porous silica material functions as an efficient bio-catalyst and can be reused several times without loss of activity.

In summary, we have reported a facile and efficient route for the development of hierarchical mesoporous materials and its application in the encapsulation of enzymes. The encapsulated enzyme functions as an efficient biocatalyst. In general, the method can be used for the encapsulation of other enzymes of interest. Such encapsulated enzymes can be used in the development of continuous flow bio-reactors. Further, chemoselective reactions can be achieved by the modification of inner pores surface of MSNs which can act as a gate-keeper for the substrates. Such efforts are under way in our laboratory.

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