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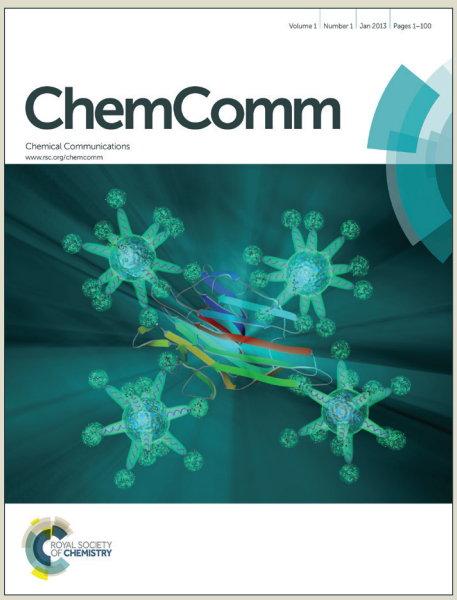

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Robust Enzyme-Silica Composites Made from Enzyme Nanocapsules

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Novel enzyme composites are synthesized by first in-situ polymerization around enzymes and subsequent sol-gel process. Both polymer shell and silica shell with desired functional moieties provide not only great enzyme protection but also favorable microenvironment, resulting significantly enhanced activity and stability.

The synthesis of materials with bioactive functions has been of great interest for a broad range of applications. To date, various biomolecules (e.g., proteins and DNAs) have been integrated with synthetic materials (e.g., small molecules, clusters, quantum dots, polymers and inorganic frameworks), creating a new class of bioactive composites.[1-6] Compared with other biomolecules, enzyme plays the most dynamic and diverse roles in living organisms; the synthesis of enzyme-based composites is therefore of particular interest. However, the synthesis of enzyme-based composites has been limited by poor stability of enzymes in non-physiological environment, which results in significant loss of the protein activity. We have recently developed a nano-encapsulation platform, which enables the synthesis of enzyme nanocapsules with highly retained activity, improved stability, tunable surface chemistry and uniform size (tens nanometers in diameter).[7] Using such nanocapsules as the building blocks, we report herein the synthesis of enzyme-silica nanocomposites with highly retained activity. Furthermore, by tuning the microenvironment around the enzyme molecules, such highly robust composites may exhibit enzyme activity higher than the native enzyme counterparts.

This was demonstrated by the synthesis of organophosphorus hydrolase (OPH) composites. OPH was used as a model system, because it can effectively decompose organophosphates, which are the essential components of pesticides and chemical warfare agents. The capability of making highly robust and active OPH composites is of great interest for decontamination, protection and detoxification of pesticides and chemical warfare agents.[8] Fig. 1 illustrates our synthesis strategy. The nanocapsules of OPH, denoted as nOPH, were synthesized by an *in situ* free-radical polymerization technique.[7] Briefly, OPH was firstly conjugated with polymerizable acrylate groups (Step I). As-modified OPH was then dispersed in an aqueous solution containing monomers, crosslinker and initiator. Driven by hydrogen bonding and electrostatic interactions, the monomers N-(3-aminopropyl) methacrylamide (APM) and acrylamide (AAM) and the crosslinker N,N'-methylenebisacrylamide (BIS) were enriched around the OPH molecules[9, 10]. Subsequent polymerization led to the formation of nOPH that contains an OPH core and a thin polymer shell; such shell structure stabilizes the OPH while allow effective substrate transport (Step II). The nanocapsules were then co-assembled with P123 and silicate clusters made through hydrolysis and condensation reactions of tetramethyl orthosilicate, where P123 is an amphiphilic block copolymer $EO_{20}PO_{70}EO_{20}$ (EO and PO represent ethylene oxide and propylene oxide, respectively). This self-assembling process leads to the formation of silicate/P123/nOPH composites; subsequent removal of the P123 affords the formation of mesoporous nOPH composites (Step III). The mesoporous silica further stabilizes the enzymes while allows effective transport of the substrates throughout the composites (Fig.1 IV), affording such composites with enhanced enzyme stability and high activity.

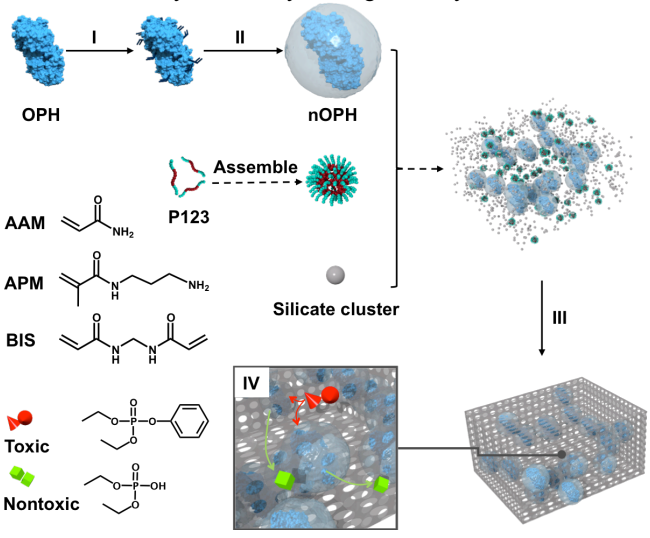

Fig. 1 Schematic illustration of forming mesoporous OPH-silica composite from enzyme nanocapsules. The nanocapsules were synthesized by (I) conjugating the OPH with polymerizable acrylate groups and (II) *in situ* polymerization of APM, AAM and BIS around the acryloylated enzyme

molecules that forms a thin layer polymer shell. Mesoporous OPH-silica composite was synthesized by a surfactant-directed sol-gel process, where P123 is used as the directing agent, followed by removal of P123 (Step III), affording highly robust composite with high enzymatic activity (e.g., hydrolysis of the organophosphates into non-toxic compounds).

Fig. 2a presents the transmission electron microscopic (TEM) image of nOPH, which shows a spherical structure with diameter ranging from 15 to 25 nm and is consistent with the dynamic light scattering (DLS) study (Fig. S1a, ESI†). Since APM is an amine-containing monomer, nOPH shows a positive zeta potential of 3 mV, which is significantly different from that of the native OPH (-6 mV) (Fig. S1b, ESI†). This observation confirms the formation of nOPH with cationic polymer shells. The polymer shells were further examined by Fourier transform infrared spectroscopy (FTIR) study, which exhibits the characteristic absorptions of poly(acrylamide) at 1670, and 1460 cm^{-1}[111] (Fig. S2, ESI†). Fig. 2b presents a TEM image of a nOPH-silica composite, showing a mesoporous structure templated by P123. FTIR spectrum of the composite consistently shows the characteristic absorption of amide and Si-O bonds at 1670 cm^{-1} and 1051 cm^{-1}, respectively, confirming the incorporation of nOPH within the silica composite (Fig. 2c)[12]. Fig. 2d shows the nitrogen sorption isotherms and the pore size distribution of the composite, indicating a mesoporous structure with pore diameter centered at 7.5 nm, which is similar to those of P123-templated mesoporous silica[13].

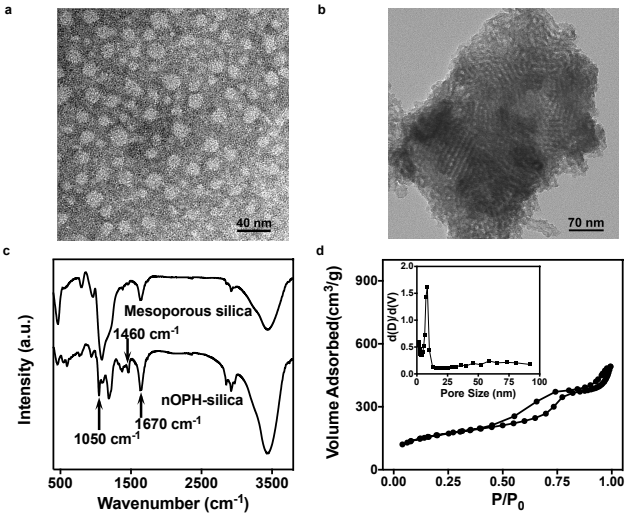

Fig. 2 (a) TEM of OPH nanocapsules nOPH, (b) TEM of nOPH-silica composites, (c) FTIR of mesoporous silica with and without incorporating nOPH, (d) N_2 sorption isotherms and pore size distribution (inset) of the nOPH-silica composite.

Currently, enzyme-silica composites are synthesized either by a sol-gel process in which the enzymes were mixed with silicate clusters and trapped within the silicate networks[14], or by adsorbing or covalently attaching enzymes to preformed silica scaffolds.[15, 16] Such processes often result in significant lose of the enzyme activity.[15, 17, 18] Consistently, sol-gel process by direct mixing of native OPH with silicate clusters results in composites (denote as OPH-silica) with significantly reduced enzyme activity. As shown in Fig. 3, native OPH exhibits an activity of 371 Units/mg, while the OPH-silica composite shows a dramatically reduced activity of 8 Units/mg. The significant loss of the activity is associated with the non-physiological conditions during the synthesis of the composites, such as ethanol or methanol produced during the sol-gel process. This could be overcome by using enzyme nanocapsules as building blocks that offer significantly enhanced stability. For example, after 4 hours of incubation at 60 °C, native OPH retains only ~ 6% of its original activity while nOPH still retains 26% of its original activity. Similarly, nOPH retains 51% of its activity in a solution containing 20% of dimethyl sulfoxide (DMSO), in comparison with 33% of the activity for the native OPH. As expected, the composite made from nOPH (denoted as nOPH-silica) exhibits a much higher activity of 686 Units/mg, similar to that of the nOPH (706 Units/mg). This indicates that the activity of nOPH is fully retained during the composite synthesis, in comparison with the native OPH that lost near 98% of the activity.

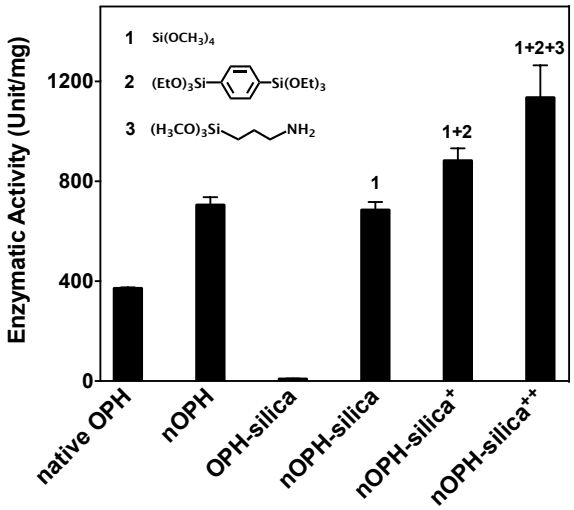

Fig. 3 Enzymatic activity of native OPH, nanocapsule nOPH, and the composites made from native OPH and precursor *1* (OPH-silica), nOPH and precursor *1* (nOPH-silica), nOPH and precursor *1* and *2* (nOPH-silica^{+}), or nOPH and precursor *1*, *2* , and *3* (nOPH-silica^{++}).

Beyond the capability of retaining high enzyme activity within the composites using enzyme nanocapsules as the building component, the enzyme activity could be further improved by constructing suitable microenvironment with both polymer and silica shell. OPH effectively decomposes hydrophobic organophosphates with the optimum pH of 9.0[19, 20]. Constructing a hydrophobic microenvironment helps to enrich the hydrophobic substrates around the enzyme leading to improved enzymatic kinetics. Furthermore, constructing a local alkaline environment, such as by introducing amine groups near the OPH molecules, facilitates the enzymatic decomposition. As expected, introducing amines groups within the nOPH shells by using the amine-containing APM molecules as the co-monomer (see Fig. 1) affords the nOPH with high activity of 706 Units/mg (Fig. 3 nOPH vs native OPH), which outperforms that of the native OPH (371 Units/mg) and is consistent with our previous finding[21].

To further construct hydrophobic and alkaline microenvironments within the composites, three different silanes were used, including tetramethyl orthosilicate (*1*), 1,4 bis(triethoxysilyl)benzene (*2*), and 3-aminopropyl trimethoxysilane (*3*). Precursor *2* contains non-hydrolyzable and hydrophobic benzene moiety; while precursor *3* contains non-hydrolyzable and alkaline aminopropyl moiety. Sol-gel process using *1* and *2* as the co-precursors affords the formation of silica matrix with hydrophobic microenvironment; while the use of *1*, *2*, and *3* as the co-precursors affords the formation of silica matrix with both hydrophobic and alkaline microenvironment. Compared with the nOPH-silica composite made from *1* with activity 686 Units/mg, the composite made from precursor *1* and *2* (denoted as nOPH-silica^{+}) shows an increased activity of 883 Unit/mg owning to the hydrophobic microenvironment (Fig. 3). The composite made from

1, *2*, and *3* (denoted nOPH-silica^{++}) shows further increased activity of 1135 Units/mg (Fig. 3). The activity of nOPH-silica^{++} composite is ~2 folds higher than that of nOPH-silica composite, 141 folds higher than OPH-silica composite, or 3 folds higher than that of the native OPH. Consistently, nanocomposites made from precursors *1* and *2* and glucose oxidase (GOX), a model enzyme that catalyzes the oxidation of hydrophilic substrate glucose, resulted in lower activity than those made from precursor *1* only (Fig. S3, ESI†). This result further confirms the important role of enzyme microenvironment in the overall activity. Considering that a large library of organosilanes with various non-hydrolyzable groups are commercially available, this approach offers feasibility to construct various microenvironments for various enzyme composites, such as silica composites made from lipase nanocapsules (Fig. S4, ESI†).

Table 1 further shows the Michaelis-Menten kinetics parameters, K_m and k_{cat}, of the native OPH, nOPH and the composites. The native OPH exhibits a similar K_m (0.061 mM) as that of nOPH (0.071 mM), suggesting that thin polymer shells around the OPH cores do not cause any significant resistance for substrate transport. Incorporating nOPH into the silica composites, as expected, increases the transport resistance of the substrates evidenced from the increased K_m (0.11 - 0.18 mM). Comparing with the native OPH with k_{cat} of 594 s^{-1}, nOPH exhibits a significantly higher k_{cat} (1322 s^{-1}), confirming the role of the local alkaline environment in enhancing the catalytic effect. The value of k_{cat} further increases when nOPH is incorporated within the silica composites, reaching the highest value of 2854 s^{-1} for nOPH-silica^{++}. The increased k_{cat} within the silica matrix may due to the concentrating effect, where nOPH are highly concentrated within the silica matrix and provides a microenvironment with basic pH.

Table 1. The enzymatic kinetic parameters K_m and k_{cat} of the native OPH, nOPH, and OPH-silica composites.

	K_m(mM)	k_{cat}(s^{-1})
Native OPH	0.061±0.009	594±22
nOPH	0.071±0.017	1322±85
nOPH-silica	0.181±0.032	2324±156
nOPH-silica^{+}	0.111±0.017	2456±130
nOPH-silica^{++}	0.116±0.022	2854±175

Besides the greatly enhanced activity, these composites also exhibit outstanding stability against denaturation from high temperature, organic solvent, surfactant, enzyme leaching from the composites. Fig. 4a compares the relative activity of native OPH, nOPH and nOPH-silica composite after incubation at 60 °C for 4 hours. Distinct from the fast denaturation of the native OPH and nOPH, which retains respectively 6% and 26% of the activity, nOPH-silica composite retains a 73% of initial activity. The significantly enhanced activity observed in the nOPH-silica composite can be attributed to the covalent attachments between the OPH and the polymer shells, as well as the silica matrix that hinders the denature process[22]. Similar results were also observed in the silica composites of lipase and enhanced green fluorescent protein (EGFP) (Fig. S5a and S5b, ESI†), which offer a general enzyme-stabilization approach.

Organic solvents such as DMSO, methanol and ethanol are commonly used as co-solvent to increase the solubility of hydrophobic substrates.[23-25] Developing enzyme systems that tolerate organic solvents is therefore importance for industry applicaitons.[26, 27] Protein engineering is the current strategy to improve solvent-tolerance of enzymes, which is time-consuming and usually results in reduced activity[28]. Nevertheless, our approach offers significantly improved organic-solvent tolerance without compromising activity. As shown in Fig. 4b, after incubating with a series of DMSO/buffer solutions, the nOPH-silica composite retains more than 80% of its initial activity even in the presence of 50% DMSO. In contrast, native OPH and nOPH only retain 10% and 30% of their initial activity, respectively. Such improvement can be attributed to the synergic effect from the soft polymer shells and the hard silicate matrix, which protects the essential water from being stripped off by polar organic solvent [23, 27].

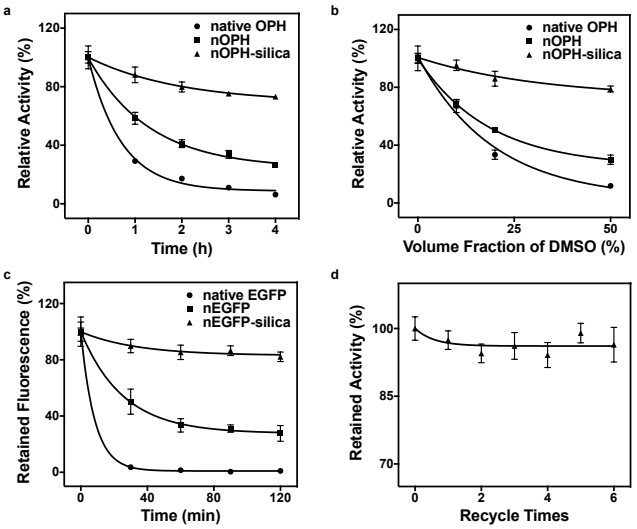

Fig. 4 (a) Relative enzyme activity of native OPH, nOPH and nOPH-silica composite after incubation at 60 °C for 4 hour; (b) Relative enzyme activity of native OPH, nOPH and the nOPH-silica composite after incubation with DMSO/buffer solutions at different volumetric ratios; (c) Relative fluorescence intensity of native EGFP, EGFP nanocapsules (nEGFP) and nEGFP-silica composite incubated in 0.5% SDS solution at 55 °C; (d) Relative enzyme activity of nOPH-silica composite during six catalyst recycles.

In the aspect of enzyme stability against surfactant denaturation, Fig. 4c shows the relative fluorescence intensity of native EGFP, EGFP nanocapsules (nEGFP), and nEGFP-silica composite made from nEGFP and silica precursor *1* (Fig. 3) after 2 hrs incubation with 0.5% sodium dodecyl sulphate (SDS) at 55 °C. The native EGFP rapidly loses its fluorescence and nEGFP retains 27% of its initial fluorescence. For comparison, the nEGFP-silica composite still retains 85% of its initial fluorescence. The enhanced tolerance to surfactant is an important feature that could broaden the applications of enzymes in industrial manufactures where surfactants are commonly used to increase the solubility of substrates.

In the aspect of enzyme leaching from the composites, our enzyme-silica composite structure prevents the enzymes from leaching completely, distinct from traditional immobilization methods[29-31]. Fig. 4d shows the relative enzyme activity of the nOPH-silica composites after repeated recycling from their assay buffer containing paraoxon. The leaching of enzyme was not observed even after 6 times recycling. The silica composites made from lipase nanocapsules also show no leaching after repeated recycling from the assay buffer containing 4-nitrophenyl laurate (Fig. S6, ESI†). Moreover, long-term storage stability of enzymes immobilized by calcium carbonate[32] and calcium phosphate[33] has been reported. Consistently, the composites made using this approach also exhibit good long-terms stability. For example, the

nlipase-silica composites retained more than 90% of the initial activity at room temperature for 10 days (Fig. S7, ESI†). The excellent durability endows the composites with great potentials for industrial applications.

In summary, we have demonstrated the synthesis of robust enzyme-silica composites using enzyme nanocapsules and silica precursors with desired functional moieties. Judicious design of the microenvironment within the silica matrix can further improve the enzyme activity, leading to the formation of enzyme composites with activity and stability far excess that of the native counterparts. This approach leads to the formation of a novel class of enzyme composites with significantly enhanced activity, stability and resistance against leaching from the composites.

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Notes and references

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