Enzyme Immobilization

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Catalytic Microcapsules Assembled from Enzyme–Nanoparticle Conjugates at Oil–Water Interfaces**

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Enzymes catalyze chemical reactions with impressive levels of stereospecificity, regioselectivity, and chemoselectivity.[1] Thus, enzyme immobilization is an important tool for the fabrication of a diverse range of functional materials and devices.[2] To date, a variety of synthetic scaffolds and supports,[3] including gels,[4] macromolecules,[5] nanoreactors,[6,7] carbon nanotubes,[8] microspheres,[9] and surface-anchored molecules,[10] have been used for enzyme immobilization. Recently, nanoparticles have been used to immobilize enzymes, and the optical, fluorescent, and magnetic properties of the resulting nanomaterials have been harnessed.[11] Recent examples include the immobilization of lipase[12] and glucose oxidase[13] on gold nanoparticles, and the attachment of α-chymotrypsin[14] to superparamagnetic magnetite@silica nanoparticles. Retention of activity, however, remains a challenge for many enzyme-immobilized nanoparticle systems.[15]

A thin layer of enzyme–nanoparticle conjugates with a high surface-to-volume ratio on a template would provide an ideal geometry for the generation of biocatalysts for industrial applications. In this context, oil–water emulsions[16] provide an ideal template for the construction of such systems. The retention of enzymatic catalysis upon immobilization, coupled with the environmental stability of the resulting conjugate at the oil–water interface is, however, of great importance for the pragmatic application of these systems.

Herein, we report a direct and versatile technique for the creation of catalytic microcapsules. This technique is based on the assembly of enzyme–nanoparticle conjugates at the oil–water interface of emulsions. The assembly of the enzymes and nanoparticles both stabilizes the emulsion and retains the surface availability of the enzymes for catalytic reaction. These microcapsules were formed quickly and showed high enzymatic activity, thus making them promising materials for biotechnological applications.

In the current study, we used β-galactosidase (β-gal),[17] which is a large tetrameric enzyme (17.5 nm × 13.5 nm × 9 nm) with an overall negative surface charge ($-0.25\times10^{-2}\,C\,m^{-2}$) at neutral pH (pI = 5.3), as the enzyme component (Figure 1a). The trimethylammonium tetraethylene glycol functionalized Au nanoparticles of approximately 7 nm diameter with a positive surface charge ($+0.35\times10^{-2}\,C\,m^{-2}$) were synthesized in order to bind to enzymes through electrostatic charge complementarity (see the Supporting Information for nanoparticle synthesis). These particles feature a tetraethylene glycol unit in the ligand shell to minimize denaturation of the bound protein (Figure 1b).[18] As shown in Figure 1c, the positively charged Au nanoparticles associate with negatively

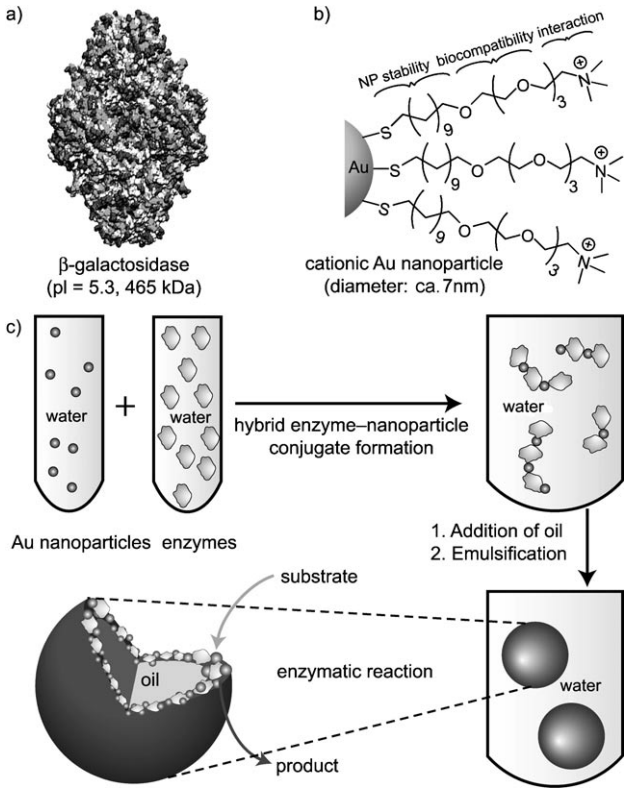

Figure 1. a) Structure of β-gal. b) Chemical structure of cationic gold nanoparticles. c) Formation of enzymatic microcapsules through electrostatic assembly of enzymes and nanoparticles in water followed by assembly of the resulting enzyme–nanoparticle conjugates at oil–water interfaces. A cross-sectional view of an enzymatic microcapsule is shown in the bottom left-hand corner.

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charged β-gal enzymes to produce reduced-charge conjugates. The subsequent addition of "oil" (a 23:77 mixture of toluene and 1,2,4-trichlorobenzene, which was chosen to provide buoyancy to the microcapsules in water) and vigorous mechanical agitation produced stable microcapsules, which result from entrapment of the enzyme–nanoparticle conjugates at the oil–water interface. The resulting microcapsules stabilized by enzyme–nanoparticle conjugates were (40 ± 15) μm in diameter (Figure 2a). A transmission electron microscopy (TEM) image confirmed the presence of densely packed nanoparticles at the microcapsule surface (Figure 2b). When using a fluorescein-labeled β-gal (see the Supporting Information for fluorophore labeling protocol), a distinct green fluorescence from the microcapsules was observed, thus confirming the presence of β-gal at the microcapsule surface (Figure 2c).

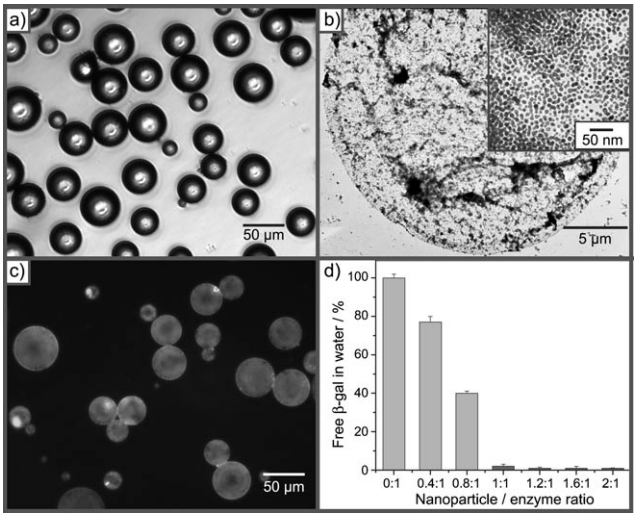

Figure 2. a) Optical micrograph of microcapsules stabilized by enzyme–nanoparticle conjugates at oil–water interfaces. b) TEM images of a microcapsule at low magnification and (inset) high magnification. c) Fluorescence microscopy image of microcapsules synthesized using Au nanoparticles and fluorescein-labeled β-gal in water. d) Free β-gal present in water after microcapsule construction using various nanoparticles/β-gal molar ratios.

Microcapsule formation was optimized by varying the nanoparticle/enzyme ratio. Incorporation of β-gal into the microcapsules was quantified by using a Coomassie (Bradford) protein assay; in this technique the amount of residual enzyme in the water after the formation of the microcapsules is measured. As shown in Figure 2d, an increase in the nanoparticle/enzyme ratio resulted in a decrease of the amount of free β-gal, with essentially no β-gal observed in the water above a 1:1 nanoparticle/enzyme ratio. The interfacial entrapment of enzyme–nanoparticle conjugates was further verified by analyzing the ζ potential of the enzyme–nanoparticle conjugates in the water (Figure S1 in the Supporting Information). As expected, the enzyme–nanoparticle conjugates showed an increase in the ζ potential (from negative to positive) as the nanoparticle/enzyme ratio increased; the overall charge of the enzyme–nanoparticle conjugate was eventually reversed. Enzyme–nanoparticle conjugates with an overall low surface charge (ca. 0.30×10^{-4} C m^{-2}, see the Supporting Information for charge calculations) were observed to successfully stabilize oil-in-water emulsions. This observation is supported by the previous reports of Reincke et al., in which carboxylic acid functionalized Au nanoparticles were used to create nanoparticle sheets at the interface by controlling the charge on the Au nanoparticle surface.[19] Control experiments carried out with only one component (β-gal or nanoparticles) provided unstable microcapsules, this result suggested that the surface charge of the enzyme–nanoparticle conjugates plays a crucial role in the formation of stable microcapsules. The high surface energy associated with the emulsion required much lower charge-dense materials (that is, enzyme–nanoparticle conjugates) compared to flat oil–water interface for its stabilization.

The utility of the enzyme–nanoparticle microcapsules as catalysts was demonstrated in an enzyme activity assay. The β-gal present on the microcapsule surface retained catalytic activity for the hydrolysis of chlorophenol red β-D-galactopyranoside (CPRG) substrate (Figure 3). The β-gal in the enzyme–nanoparticle microcapsules retained 76% enzymatic activity compared to free β-gal (Figure 4). This efficiency is similar to that observed for the monophasic activity of enzyme–nanoparticle conjugates (84%), thus demonstrating that the β-gal in the enzyme–nanoparticle conjugates retains its catalytic activity both in water and at the oil–water interface.

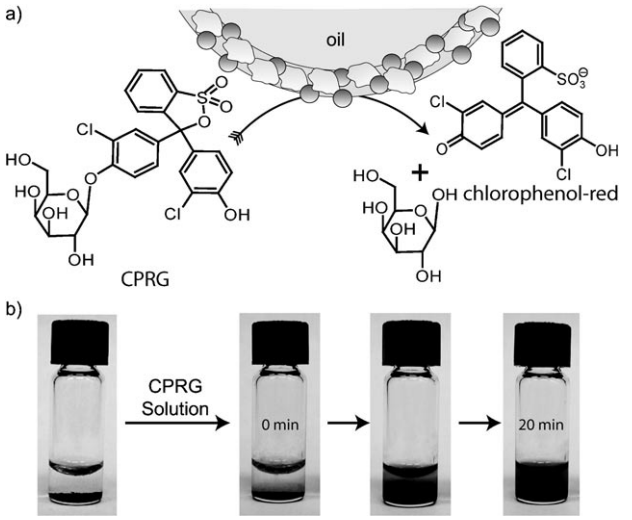

Figure 3. a) Enzymatic cleavage of yellow CPRG on the microcapsule shell by β-gal to produce chlorophenol red. b) Stepwise color changes (from yellow to red) in the chemical reaction of β-gal present in the microcapsule shell. The microcapsules are settled at the bottom of the glass vial.

In summary, we have demonstrated the successful integration of hybrid enzyme–nanoparticle conjugates with microcapsule structures. The fabrication process rapidly and quantitatively immobilizes the enzyme on the microcapsule surface, with retention of high catalytic activity. Extension of

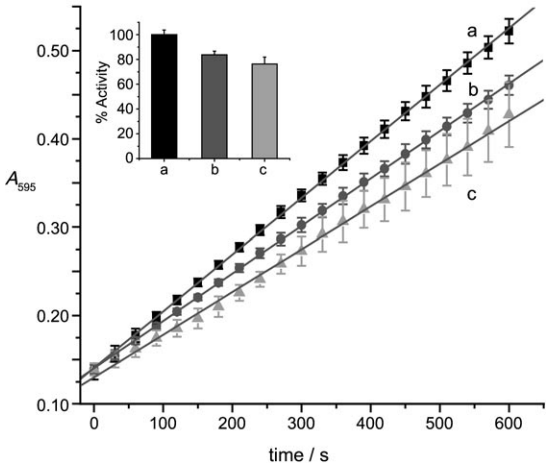

Figure 4. Activity assay of β-gal (0.5 nM) in phosphate buffer (5 mM): a) free enzyme; b) enzyme–nanoparticle conjugate; c) enzyme–nanoparticle conjugate stabilized microcapsules. The inset shows the relative activity (free enzyme solution = 100%).

this method to the fabrication of catalytic microcapsules for various biotechnological applications is currently being explored.

Experimental Section

Microcapsule preparation: This procedure is based on the supramolecular assembly of β-gal and nanoparticles in water, followed by the addition of oil with vigorous mechanical agitation. β-gal (60 nM) was incubated with 60 nM cationic Au nanoparticles in phosphate buffer (5 mM) for 5 min to create enzyme–nanoparticle conjugates. Oil (5 μL, toluene/1,2,4 trichlorobenzene 23:77) was subsequently added to the aqueous enzyme–nanoparticle conjugate solution (200 μL) and vigorously shaken by hand for around 60 s. After shaking, the enzyme–nanoparticle conjugates were entrapped at the oil–water interface and appeared as pink-colored microcapsules, which very slowly settled to the bottom of the aqueous solution. These microcapsules were washed twice with water to remove free enzyme–nanoparticle conjugates, and fresh phosphate buffer was added prior to imaging with an Olympus IX51 microscope.

TEM images: These were acquired on a JEOL 100CX microscope operating at 100 keV. Samples were drop-cast onto a 300-mesh carbon-coated Cu grid, dried, and imaged.

Quantification of free enzyme in water after microcapsule fabrication: Solutions of microcapsules synthesized by varying the nanoparticle/enzyme ratios were centrifuged at 1000 rpm for 1 min to result in clear supernatant that was analyzed for residual enzyme quantification in solution using commercially available Coomassie (Bradford) protein assay kit (Thermo scientific). Enzyme–nanoparticle conjugates did not settle after centrifugation at 1000 rpm for 1 min. β-gal was used to create standard curve for this assay.

Activity assays: Studies of free β-gal, enzyme–nanoparticle conjugates, and microcapsules stabilized by enzyme–nanoparticle conjugates were carried out in sodium phosphate buffer (5 mM, pH 7.4). The final β-gal concentration in all activity assays was 1.0 nM. The enzymatic hydrolysis was initiated by adding a CPRG substrate stock solution (100 μL of 15 mM) in the same buffer to the solution of enzyme–nanoparticle conjugates (100 μL). Enzymatic activity was followed by monitoring the absorption of the product formation in every 30 s for 10 min at 595 nm using a microplate reader (spectraMax M5 from Molecular Devices). The assays were performed in duplicate or triplicate, and averages are reported. All activity assay experiments were carried out at 25°C.

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