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Encapsulation of Single Enzyme in Nanogel with Enhanced Biocatalytic Activity and Stability

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Industrial biotechnology is consistently seeking improved enzyme performance and stability under harsh conditions found in most industrial processes. Being complementary to genetic approaches such as directed evolution[1] and site mutation,[2] chemical modification via conjugation[3] or encapsulation[4] provides an efficient route to enhance enzyme stability at high temperature or in the presence of organic solvent. To date, enzymes have been encapsulated in silica,[5] polymer,[6] and organoclay[7] particles showing enhanced stability. These coating materials, however, may hinder the conformational transition of enzyme and the transport of substrate and product,[5] resulting in low biocatalytic activity. A promising solution is to fabricate a single-enzyme containing capsulate with a thin, permeable coating.[8] Kim and Grate have fabricated enzyme nanoparticles via a multistep procedure including surface modification, lyophilization, polymerization in organic solvent, and shell condensation and obtained enhanced enzyme stability at an insignificant increase in mass transfer resistance.[9] Nevertheless, a simple, effective and versatile procedure that yields a single enzyme capsule with enhanced stability, high activity, and uniformed size is being pursued to provide robust enzymes for industrial biocatalysis.[1]

We present herein, a two-step procedure including surface acryloylation and in situ aqueous polymerization to encapsulate a single enzyme in nanogel. As shown by Scheme 1, the first step is to generate vinyl groups on the protein surface by acryloylation.[4,9] The second step is the aqueous in situ polymerization that encapsulates the acryloylated protein. Moreover, the second step can be repeated by adding monomers and cross-linkers to react with the primary nanogel so as to give the final product that contains a single enzyme in expected size and shape.

For the present study, horseradish peroxidase (HRP), an enzyme that is widely used in bioassay and biosynthesis[10] but fragile to phase transfer,[11] was chosen as the model enzyme. After a mild reaction with *N*-acryloxysuccinimide in alkali buffer to generate surface vinyl groups, in situ polymerization at room temperature was conducted with acrylamide as the monomer, *N*,*N*′-methylene bisacrylamide as the cross-linker, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine/ammonium persulfate as the initiator. Formation of a polymer on HRP was confirmed by Fourier transform infrared spectra (FTIR). The four characteristic peaks of polyacrylamide in encapsulated HRP including 1670, 1610, 1430, and 1460 cm^{-1} indicate the increment of polymer content (see Supporting Information for the detailed synthesis procedure).

Figure 1 shows the transmission electron microscopy (TEM) images of negatively stained[12] nanogels prepared at a monomer/HRP molar ratio of (a) 400 (hereafter noted as nanogel A) and (b) 800 (hereafter noted as nanogel B), respectively. The nanogel B was obtained by reacting monomers with the nanogel A at a monomer/HRP molar ratio of 400. The yield of enzyme encapsulation is 92.2% for the nanogel A and 91.8% for the nanogel B, respectively. The yield of polymerizationt is 87.4% for the nanogel A and 90.4% for the nanogel B, respectively.

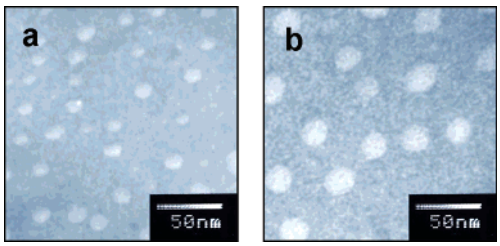
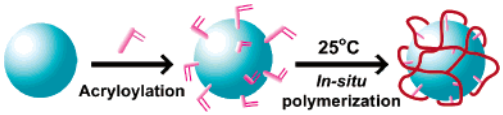


Figure 1. TEM images of nanogels prepared at monomer/HRP molar ratios of (a) 400 and (b) 800.

Scheme 1. Encapsulation of Single Protein in Nanogel



The nanogel A shown in Figure 1a appears ellipsoid in shape with a size of 11 nm × 9 nm. The HRP molecule has an elliptic shape with a dimension of 6.5 nm × 5.4 nm × 4.3 nm, suggesting that each nanogel A contains a single HRP molecule, and thus the thickness of the polymer shell is around 2.4 nm. As compared to the nanogel A, the nanogel B with diameter of 17 nm is more uniform and spherical. Recall that the size of the nanogel A is 11 nm × 9 nm, each nanogel B can contain only one nanogel A and, consequently one HRP molecule. Thus for nanogel B, the gel thinkness is determined to be around 5 nm. A dynamic light scattering (DLS) experiment suggests an average particle size of 21 nm and a narrow polydispersity index of 1.17 for the nanogel B, which is consistent with the TEM observation. The larger diameter determined by DLS, as compared to that obtained by TEM, reflects the swelling of the HRP nanogel in solution.

The biocatalytic activity was examined using tetramethylbenzidine (TMB) as the substrate. Michaelis−Menten parameters, K_m and k_{cat}, interpreted from the Lineweaver−Burk plots, were 0.297 mM and 2187 s^{-1} for the free HRP and 0.307 mM and 2037 s^{-1} for the nanogel B, respectively. The similar Michaelis−Menten parameters observed for the free and encapsulated HRP indicate that the thin polymer shell insignificantly affects the transport of the substrate to HRP, the enzymatic reaction kinetics, and the discharge of product. This is significantly different to HRP encapsulated in solid silica,[8b] in which K_m was increased from 45.5 to 217.8 mM and k_{cat} was decreased from 6.13×10^7 to 1.05×10^5 s^{-1} when *o*-dianisidine, whose molecular weight is similar to the TMB used in the present study, was applied as the substrate.

Thermal stability of the free and the HRP nanogel B was compared from 30 to 85 °C at pH 7.0. The free HRP started to lose its activity above 40 °C while the nanogel B remained stable

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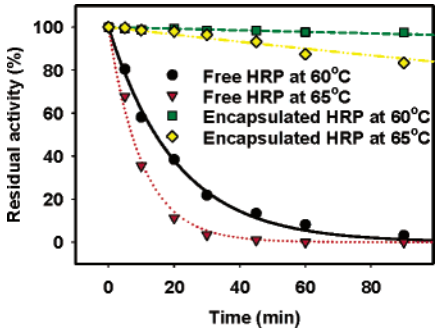

Figure 2. Thermal inactivation kinetics of free HRP and encapsulated HRP at 60 °C and 65 °C indicating that the encapsulated HRP possesses significantly enhanced thermal stability.

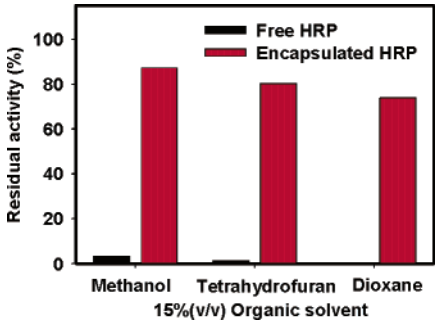

Figure 3. Activity comparison of the free and encapsulated HRP after exposing to water/organic mixed solvents for 10 min at 60 °C.

until 65 °C (see Supporting Information). As shown in Figure 3, the nanogel B maintains 80% of its initial activity even after 90 min incubation at 65 °C while the free HRP loses all its initial activity, although they show similar activity at room temperature. Compared with that of genetically reengineered HRP that showed an extended half-life of enzyme activity of 10 min at 60 °C, pH 7.0,[13] more significant improvement in the stability can be achieved by forming such a nanogel. The encapsulated HRP also showed a significantly improved stability compared to that modified with ethylene-glycol bis(succinimidyl succinate) (EGNHS)[14a] or phthalic anhydride,[14b] which extends the half-life of HRP at 65 °C, pH 7.0 to 16 and 17 min, respectively. The significantly enhanced thermal stability of the HRP nanogel, we believe, is due to the multiple covalent attachments to the polymer[4,9] that hinder the thermal fluctuations leading to enzyme deactivation at high temperature.[15]

To test the stability in the presence of polar organic solvent, the free and the HRP nanogel B were exposed to an aqueous solution containing 15% methanol, tetrahydrofuran, and dioxane at 60 °C for 10 min. As shown in Figure 3, the nanogel B maintains over 80% of its initial activity while the free HRP loses almost all initial activity. The significant increased stability in organic solvent may be due to, on one hand, the hydrophilic groups in the polyacrylamide nanogels that retain a hydrophilic environment, that is, resembling the "essential water" that enables HRP to display its biological function. The stripping of the essential water by the polar solvents leads to the denaturation of the free HRP.[16] On the other hand, the multiple covalent attachments to the polyacrylamide shell strengthen the structure of the encapsulated HRP.

In conclusion, we demonstrated a simple and efficient method to prepare nanogels containing a single enzyme. Compared with the free HRP, the HRP nanogels exhibit similar biocatalytic behavior evidenced from their similar K_m and k_{cat} but significantly improved stability at high temperature and in the presence of polar organic solvent. Compared with that of the current existing encapsulation methods or genetic approaches applied to HRP, the nanogel demonstrates unprecedented stability. The polymerization in aqueous phase simplified the enzyme encapsulation in nanostructured form, as compared to that obtained via polymerization in organic phase.[9] More importantly, such uniform nanogels show similar catalytic behavior to the free enzyme, providing a robust enzyme model for a large variety of applications.

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Supporting Information Available: Experimental details for sample preparation and characterization are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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