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Temperature-responsive enzyme–polymer nanoconjugates with enhanced catalytic activities in organic media†

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A general approach for preparing enzyme–polymer nanoconjugates that respond to temperature in organic media is presented. These nanoconjugates readily dissolve in organic solvents for homogenous catalysis at 40 °C and showed greatly enhanced apparent catalytic activities. The recovery of the soluble enzyme–polymer nanoconjugates is accomplished by temperature-induced precipitation.

Nanobiocatalysis, in which enzymes are immobilized on various nanomaterials, has attracted increasing attention due to its potential applications related to catalysis, bioanalytical science, biofuel cells and biosensors.[1] Among these applications, biocatalysis in organic solvents is being actively pursued to take advantage of the full capacity of enzymes in terms of their chemo-, regio-, and stereoselectivity. Organic media are chosen for enzymatic catalysis to solubilize substrates, inhibit reverse reactions, and eliminate the adverse effects of water.[2] Unfortunately, most enzymes that offer high catalytic performances *in vivo* display very limited activities in organic solvents.[3] The inactivity is mainly caused by the extremely low solubility of the enzymes which makes their active sites less accessible to substrates.[3] Standard immobilization of enzymes on pre-existing bulk solid supports has the advantage of stabilizing enzymes in organic media but still suffers from low enzymatic activities due to high diffusion limitations within the immobilized catalysts.[1b] Nanobiocatalysis systems based on the integration of enzymes with nanostructures, such as silica nanoparticles,[4] polymer nanogels,[5] flower-like inorganic nanocrystals,[6] self-assembled nanoreactors[7] and conjugates with polymers,[8] have shown great effectiveness and offer new options for improving the performance of enzyme catalysts with different applications.

We propose that the conjugation of an enzyme to a temperature-responsive polymer could allow the temperature-controlled solubility of the resulting nanoconjugate in organic solvents. This property would improve the enzymatic process in organic media from heterogeneous to homogeneous catalysis by solubilizing the conjugate in organic solvents and facilitate the recovery of the enzyme conjugate by a temperature-induced precipitation. To date, studies have focused on enzyme–polymer conjugates with temperature-responsive features in aqueous solution.[9] In this study, we report the first example of temperature-responsive enzyme–polymer nanoconjugates that exhibit greatly increased apparent catalytic activities in organic solvents compared to their native counterparts.

The conjugates were synthesized by grafting Pluronic F-127 (POH) onto protein molecules (Fig. 1). Pluronics are a family of nontoxic, neutral triblock copolymers made of a central hydrophobic polypropylene oxide (PPO) block connected to two hydrophilic poly(ethylene oxide) (PEO) side blocks.[10] The resultant enzyme–Pluronic conjugates formed nanoparticles in aqueous solution or organic solvent by self-assembly due to their amphiphilic property. The nanoconjugates were readily solubilized in organic solvents at high temperature (40 °C), allowing the homogenous reaction. Moreover, they were conveniently recovered by precipitation at low temperature (4 °C).

During the experiment, the Pluronic F-127 (POH) was first oxidized with Dess–Martin periodinane, to convert the hydroxyl end-groups into aldehyde functionalities. The introduction of aldehyde groups was confirmed by FT-IR (Fig. S1, ESI†) and the Purpald colorimetric assay.[11] The aldehyde-derived Pluronic (PCHO) was then covalently conjugated to the lysines of protein by the formation of a Schiff base followed by a reduction with $NaCNBH_3$.

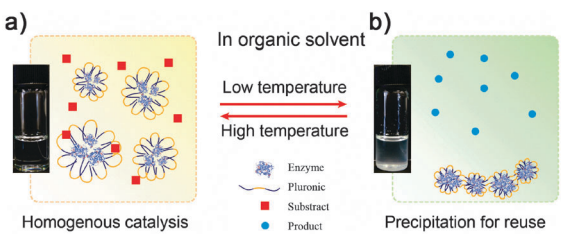

Fig. 1 (a) Solubilization of the conjugates in toluene for homogenous biocatalysis at 40 °C; (b) precipitation of the conjugates in toluene for recycling at 4 °C. Photograph insets show (left) solubilization and (right) precipitation of the nanoconjugates in toluene at 40 °C and 4 °C.

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† Electronic supplementary information (ESI) available: Experimental details, the FT-IR spectrum of the functional polymer, SDS-PAGE, absorbance change with temperature and DLS of the conjugates. See DOI: 10.1039/c3cc42493f

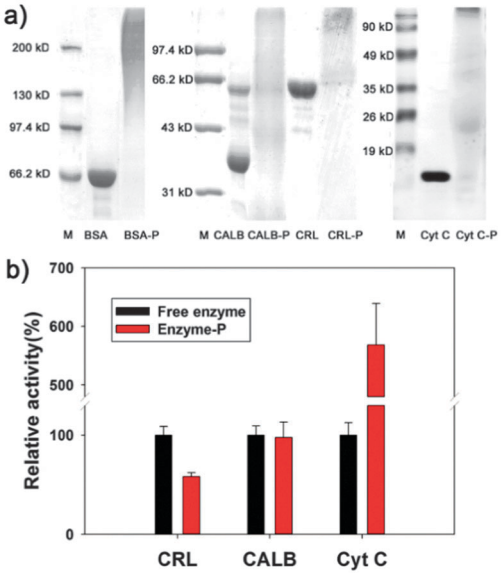

Fig. 2 (a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (M: marker; BSA, CALB, CRL, Cyt *c*: free protein; BSA–P, CALB–P, CRL–P, Cyt *c*–P: conjugate); (b) relative activities of CRL–, CALB–, and Cyt *c*–Pluronic conjugates determined in aqueous solution at room temperature.

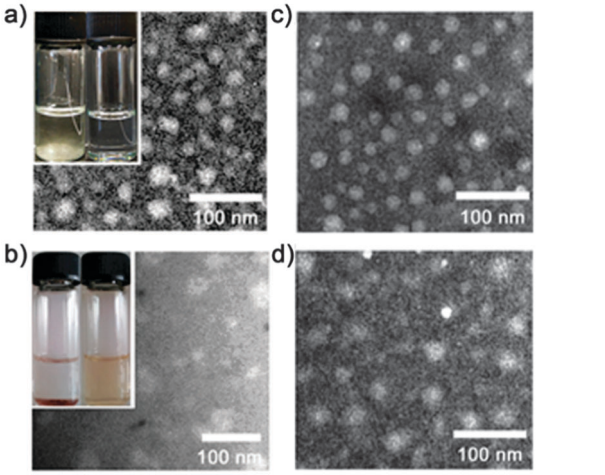

Fig. 3 TEM images of protein–Pluronic nanoconjugates: (a) BSA; (b) Cyt *c*; (c) CRL; and (d) CALB. Inset in (a) shows the solubilization of the BSA–Pluronic nanoconjugate in toluene at 40 °C (left: free protein precipitate; right: soluble protein nanoconjugate). Inset in (b) shows the Cyt *c*–Pluronic nanoconjugate in tetrahydrofuran at 40 °C (left: free protein precipitate; right: soluble protein nanoconjugate).

We achieved the conjugation of different proteins, including bovine serum albumin (BSA), *Candida rugosa* lipase (CRL), *Candida antarctic* lipase B (CALB), and cytochrome *c* (Cyt *c*), with Pluronic. In all cases, we did not observe any free protein bands by gel electrophoresis (Fig. 2a), indicating high conjugation yields for all sample proteins. The free protein band clearly remained when the protein was simply mixed with Pluronic (Fig. S2, ESI†). The number of lysines modified was measured as 8, 6, 11, and 5 for BSA, CALB, CRL and Cyt *c*, respectively, by a fluorometric assay.[12] This result together with the molecular weight of the conjugates calculated from SDS-PAGE indicates that each single enzyme was conjugated with 5–11 Pluronic molecules depending on different proteins.

The activities of three protein–Pluronic conjugates—CRL, CALB, and Cyt *c*—in aqueous solution at room temperature were tested to be 58.3%, 97.6%, and 568%, respectively, compared to the corresponding free proteins (Fig. 2b). The higher activity of the conjugated Cyt *c* compared to free Cyt *c* in aqueous solution possibly resulted from the facilitated transfer of the substrate toward the active site of Cyt *c*, assisted by the PPO hydrophobic block of Pluronic. This phenomenon has been previously described by others.[13]

At 40 °C, lyophilized protein–Pluronic conjugates readily dissolved in the most common organic solvents for chemical synthesis, which include toluene, tetrahydrofuran, methanol, dichloromethane, and chloroform. In contrast, the free enzymes remained as precipitates in these solvents (insets of Fig. 3a and b). Measured by dynamic light scattering (DLS), the average size of the dissolved nanoconjugates in toluene was approximately 30–40 nm at room temperature (Table S1, ESI†). Transmission electron microscopy (TEM) images of the enzyme–Pluronic nanoconjugates dried from toluene solution showed that the conjugate self-assembly had sizes of around 30 nm (Fig. 3). The apparent catalytic activities of CRL–, CALB–, and Cyt *c*–Pluronic nanoconjugates in organic solvents were tested and compared to that of free enzymes at the same protein concentration at 40 °C. As shown in Fig. 4a, the conjugates of CALB and CRL exhibited respective increases in esterification activity of 67-fold and 57-fold in toluene compared to their native counterparts. The measurements were made using a standard method under saturated conditions using hexanoic acid and *n*-butyl alcohol as substrates for CALB and palmitic acid and *n*-octanol as substrates for CRL. The peroxidase activity of conjugated Cyt *c* in methanol was 670-fold higher than that of free Cyt *c* (measured by a standard method using 2,2′-azinobis-(2-ethylbenzthiazoline-6-sulfonate) and H_2O_2 as the substrates under saturated conditions). It is likely that the hydrophobic PPO block of Pluronic extended into the organic phase and enhanced the solubilization of the conjugates, making the enzyme more accessible to its substrate. Meanwhile the hydrophilic PEO blocks stayed around the conjugated protein and helped to maintain a hydrophilic environment favourable for the enzymatic catalysis.[5] Therefore, compared to its native counterpart which tends to aggregate in organic solvents, the enzyme conjugated with Pluronic shows a greatly increased apparent activity.

In organic media, solubilized PEG–enzyme conjugates also have greater apparent catalytic activities than those of native enzymes.[14] For example, Castillo *et al.*[14c] reported a 30- to 100-fold increase in the enzymatic activity of protease–PEG conjugates in dioxane compared to the free enzyme. Although PEGylation can significantly enhance the enzymatic activity in organic solvents by sufficient solubilization of the biocatalysts, the recovery of dissolved conjugates and separation of desired products cannot be easily addressed. Fukunaga and co-workers[15] reported that enzymes co-lyophilized with amphiphiles have high activities in organic media, which is attributed to the improved dispersion of catalysts in organic media. Thus a comparative study between the conjugation and lyophilisation was performed using CALB and Pluronic F-127 as the enzyme and amphiphile sample, respectively. It is shown in Fig. S4 (ESI†) that, compared to the native CALB, the co-lyophilized one increased the apparent activity by

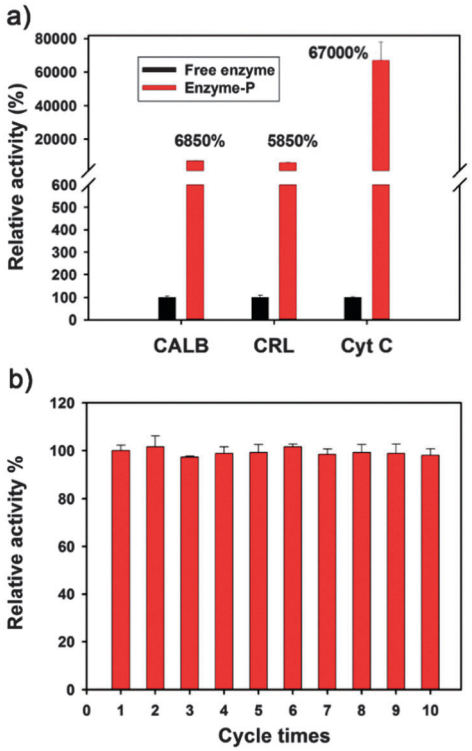

Fig. 4 (a) The activities of enzyme–Pluronic nanoconjugates compared to native enzymes in organic solvents at 40 °C. (b) Recycling of CALB–Pluronic nanoconjugate by temperature switching. The CALB–Pluronic nanoconjugate in toluene was incubated at 4 °C for 4 hours followed by an immediate change in temperature from 4 °C to 40 °C. After the incubation at 40 °C for 2 hours, the relative activity of the nanoconjugate was measured and compared to its original activity.

30-fold while the conjugate gave a 67-fold increase. Moreover, the temperature-responsiveness enables the recovery and reuse of the enzyme–Pluronic conjugates.

The temperature-responsiveness of Pluronics in aqueous solution is well established but this property in organic solvents has not been reported. We first observed the temperature responsiveness of Pluronic F-127 alone in toluene. The upper critical solution temperature of the Pluronic F-127 in toluene is around 12 °C when its mass fraction is 10%. For example, at the concentration of 100 mg mL^{-1}, Pluronic F-127 was soluble in toluene at room temperature and precipitated at 4 °C. At a low concentration, such as 10 mg mL^{-1}, however, there was no observed temperature responsiveness of Pluronic F-127 in toluene (investigated by the turbidity experiment, results shown in Fig. S3, ESI†). In comparison, the coupling of Pluronic to a protein introduced more hydrophilic parts into the conjugate, making the conjugate in hydrophobic solvents, such as toluene, more sensitive to temperature. The precipitation of the conjugate in toluene at 4 °C easily occurred at a mass fraction of up to 1% (the upper critical solution temperature was around 30 °C, Fig. S3, ESI†). This high temperature sensitivity of the enzyme–Pluronic conjugate provided a promising way to recover the nanocatalyst from organic media. The reusability of enzyme conjugates was tested by cycling the temperature from 4 °C to 40 °C, using the CALB–Pluronic conjugate as an example. As shown in Fig. 4b, the residual activity of the CALB–Pluronic conjugate remained greater than 95% after ten cycles.

In summary, we have synthesized temperature-responsive enzyme–polymer nanoconjugates by grafting Pluronic polymers onto enzymes. These enzyme–polymer nanoconjugates showed markedly increased catalytic activities in organic solvents. The presence of the Pluronic polymer in the nanoconjugate appears to have two advantages: (1) the solubilization of the nanoconjugate in organic solvents results in greatly increased apparent catalytic activity, and (2) the temperature responsiveness of the nanoconjugate facilitates the recovery of the enzyme catalyst. These properties make the enzyme–Pluronic polymer nanoconjugate an effective and smart nanobiocatalyst for a wide range of applications used in organic media.

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