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Polymer−Enzyme Conjugates Can Self-Assemble at Oil/Water Interfaces and Effect Interfacial Biotransformations

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Oil/water (O/W) biphasic reactions are preferred for the bioprocessing of water-insoluble chemicals.[1] In addition to increase the availability of substrates, the biphasic configuration also simplifies product purification processes and sometimes enables reactions that are thermodynamically unfavorable in monophasic media.[2] The aqueous phase has been traditionally used as a container of the biocatalysts. This introduces, however, strong mass-transfer limitations for reactants from the organic phase to reach the enzymes,[3] and in most cases, only a small portion of the applied enzyme is exposed to the interfacial region and is available to catalyze reactions.[4] Here we show that native water-soluble enzymes can be transformed into interface-binding enzymes and form a liquid film through self-assembling. The interface-assembled enzymes are simultaneously available to reactants from both phases across the interface and thus are expected to afford efficient biotransformations between immiscible chemicals.

Interfacial assembling of proteins occurs naturally in the biological world. An adequate structural configuration, in addition to the overall molecular hydrophobicity, is a critical factor in realizing stable interfacial assemblies. It appeared to us that a surfactant-like structure consisting of a hydrophilic protein head and a hydrophobic polymeric tail was desirable for the construction of interface-binding biocatalysts. Toward that, polymer−enzyme conjugates were prepared and examined. Representative enzymes, α-chymotrypsin (CT) and β-galactosidase (GL), were conjugated with polystyrene (PS), which was activated at one end of the polymer molecules.[5] The resulted polymer−protein conjugates were partially soluble in solvents such as toluene and chloroform. However, they selectively assembled and formed a liquid film at the O/W interface upon being placed in a biphasic system. Figure 1 shows the interfacial assembling of PS−CT conjugate. The dispersion of the polymer−enzyme conjugates at O/W interfaces appeared to be uniform. Unlike small surfactants, the polymer−enzyme conjugates showed 100% assembling at the interface in that no protein was detected in either the bulk aqueous or organic phase. Enzymes conjugated with an amphiphilic polymer, poly(ethylene oxide) (Mw 10 000 Da), remained water-soluble and failed to assemble at the interfaces. On the other hand, enzymes modified with a smaller but more hydrophobic modifier, decanoyl chloride, were highly organic soluble (with solubility as high as 44 mg-protein/mL) but did not form interfacial assemblies either. We suspect that multiple attachments become predominant with small modifiers, and thus a hydrophobic coating on the surfaces of proteins instead of a surfactant structure was formed. On the other hand, polymer modifiers can usually achieve one or two attachments even with the more active PEG modifier.[6] All the PS samples tested (with Mw ranged from 2000 to 200 000 Da) and a second polymer, poly(L-lactic acid) (Mw 100 000), were found capable of enabling interfacial assembling. Interfacial assembling was achieved in biphasic systems constituted with various organic solvents including toluene, ethyl acetate, dichloromethane, and chloroform.

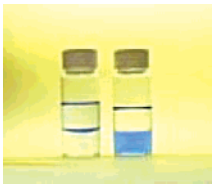

Figure 1. Interfacial assembly of polystyrene-conjugated α-chymotrypsin. The conjugate selectively assembled at the toluene-water interface (left), while native CT remained in aqueous solution (right). The protein molecules were dyed blue with Bradford protein assay reagent.

The interface-assembled enzymes remained catalytically active. The transgalactosylation reaction between lactose and 1-hexanol for the production of hexyl-galactoside was conducted in a toluene buffer biphasic system to probe the efficiency of the polymer-conjugated enzymes. The glycoside product represents a valuable class of drug intermediates and has been synthesized enzymatically by using GL previously.[7] The reactions were carried out in a reactor with an inner diameter of 13.2 cm. The volumes of aqueous and organic phases were 350 and 100 mL, respectively. 1-Hexanol, whose water solubility is less than 0.7 wt %, was dissolved in toluene (40% v/v, 3.2 M), while lactose was contained in the aqueous phase with concentrations varying from 0.05 to 0.7 M. Mild stirring (∼60 rpm) was applied (mechanically for the top phase and magnetically for the lower phase) without interrupting the interface. The product was only detectable in the organic phase.[7b] The reaction was therefore monitored by analyzing the product concentration in organic phase with aliquots of 0.5 mL withdrawn periodically for HPLC analysis. PS−GL successfully assembled at the interface with no protein or activity detectable in either the bulk organic or aqueous phase. In a control test, native GL dissolved in the aqueous phase was used. The structure of the product isolated from the organic phase was confirmed with ^{13}C NMR spectrum (CD_3OD, 300 MHz: δ 69.14 (C-1′), 31.72 (C-2′), 25.66 (C-3′), 29.65 (C-4′), 22.54 (C-5′), 13.23 (C-6′), 103.84 (C-1, β-bond), 71.41 (C-2), 73.90 (C-3), 69.68 (C-4), 75.43 (C-5), 61.30(C-6)). Interestingly, the initial reaction rate observed for PS−GL was more than 145-fold faster than that for the native enzyme (3.4 vs 0.023 (10^{-3} μM product) h^{-1} (μg protein)$^{-1}$ cm^{-2}; see Figure 2). The reaction rate achieved with the interfacial biocatalysis was also about 30 times higher than the fastest reaction reported for similar transgalactosylation reactions between lactose and hydrophobic alcohols, including 1-butanol and 1-octanol, using lipid-coated and thus organic-soluble GL.[7b]

The interfacial transgalactosylation reaction of PS−GL showed an interesting dependence on enzyme concentration. The specific reaction rate, which is the product generation rate normalized with respect to the amount of enzyme, decreased as the interfacial enzyme concentration increased (Figure 3). This was expected in the sense that dispersion of the enzyme facilitates reaction kinetics. The absolute product generation rate, however, showed a sharp drop after reaching a peak value. This is different from what was

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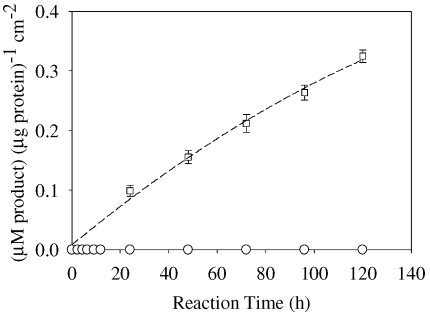

Figure 2. Time course of transgalactosylation reaction in toluene-buffer biphasic system. (○) Reaction catalyzed by native GL (0.5 mg/mL buffer). (□) Reaction catalyzed by PS−GL with an interfacial concentration as 3.5 μg protein/cm^2. Lactose concentration was 0.7 M for both reactions. The initial reaction rate with native enzyme in aqueous phase was $(0.023 \pm 0.001) \times 10^{-3}$ (μM product) h^{-1} (μg protein)$^{-1}$ cm^{-2}, while that for the interface-binding enzyme was $(3.38 \pm 0.12) \times 10^{-3}$ (μM product) h^{-1} (μg protein)$^{-1}$ cm^{-2}.

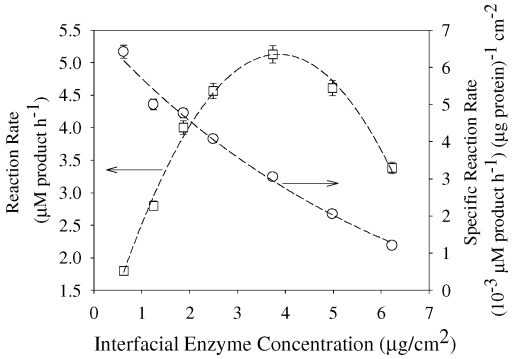

Figure 3. Effect of enzyme concentration on the transgalactosylation reaction rate. (○) Specific reaction rate in unit of (μM product) h^{-1} (μg protein)$^{-1}$ cm^{-2}. (□) Product generation rate in unit of (μM product) h^{-1}.

observed for homogeneous reactions where an increase in enzyme concentration always leads to faster reactions. The enzyme concentration at the peak reaction rate was about 9 times the amount needed for monolayer assembling as estimated on the basis of the size of the protein moiety. This observation may suggest that as a multiple layer assembly was formed, the resulted thick film of the conjugate became cumbersome for the reactants to reach each other across the interface. An alternative possibility to the multiple layer assembly is the formation of aggregates at the interface. Further systematic studies are certainly needed to elucidate the actual mechanisms. Overall, it appeared that thinner interfacial assemblies afford better catalytic efficiencies.

The dependence of reaction rate on lactose concentration showed a typical Michaelis−Menten observation, with a substrate saturation reached at 0.4 M of lactose. Fitting with the Michaelis−Menten model led to apparent kinetic parameters such as $K_M = 0.025$ M and $v_{max} = 3.4 \times 10^{-3}$ (μM product) h^{-1} (μg protein)$^{-1}$ cm^{-2}.

The interfacial biocatalysis can be desirable to avoid unwanted side reactions. For the reaction system examined here, GL readily catalyzes the hydrolysis of lactose, competing with the transgalactosylation reaction.[8] For native GL applied in the aqueous phase, our experiments showed that only 6.2% of reacted lactose was transformed into the hexyl-galactoside product (with the rest being hydrolyzed). For PS−GL, however, 88% of reacted lactose was found in the product. This suggests that placing the enzyme at the interface improved the enzyme's selectivity for transgalactosylation reaction over the hydrolytic reaction. A straightforward explanation for that may come from the simultaneous availability of PS−GL to both reactants.

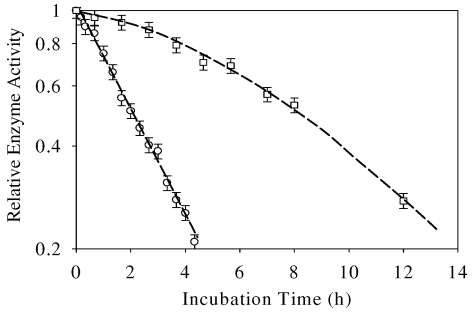

Figure 4. Interfacial stability of enzyme in toluene buffer biphasic system. Native GL (○) and PS−GL (□) incubated at 55 °C.

It is well-known that interfacial interactions can alter enzymes' 3-D structures and thus impact their performance. For lipases, conformational changes upon being absorbed at interfaces improve their activities by better exposing their active sites to substrates, a phenomenon referred as "interfacial activation" of lipases.[9] Generally speaking, however, exposure of enzymes to gas−liquid or liquid−liquid interfaces is associated with enzyme inactivation.[10] Our stability evaluation showed that the half-life of PS−GL was 4.2-fold longer than that of the native enzyme exposed to the same O/W biphasic system (Figure 4). We tend to believe that for the polymer−enzyme conjugates, the introduction of the polymer groups may alter the microenvironmental interactions around the enzymes, including lowering the impacts of the interfacial stress. Such an alteration may protect the enzyme from interfacial inactivation.

In summary, we demonstrated that interface-binding enzymes can be prepared via conjugation with hydrophobic polymers. The polymer-conjugated enzymes showed much improved stabilities and activities than native enzymes exposed to the same biphasic systems. Compared to biphasic reactions using aqueous- or organic-soluble enzymes, interface-binding enzymes facilitate the interactions between the catalysts and the reactants hosted in both bulk phases across the interface and thus improve the overall catalytic efficiency.

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Supporting Information Available: Procedures for enzyme modification and transgalactosylation reaction. This material is available free of charge via the Internet at http://pubs.acs.org.

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