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A Recoverable Enzymatic Microgel Based on Biomolecular Recognition

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The chemoselectivity and catalytic efficiency exhibited by natural enzymes should lead to their widespread use for organic synthesis on both laboratory and industrial scales.[1] However, a significant obstacle to overcome in generalizing the use of enzymes in unnatural settings is product separation without loss of the enzyme. One commonly used strategy for addressing this issue involves immobilizing the enzyme on an insoluble (or conditionally soluble) support.[2,3] In this communication, we present a new method, based on a recoverable biopolymer microgel, for immobilizing an enzyme.

The design is shown schematically in Figure 1. A DNA three-way junction (TWJ) is assembled from three partially complementary strands. Each of the three arms of the junction has an identical seven-nucleotide overhang. A biotinylated peptide nucleic acid (PNA) oligomer complementary to the overhanging sequence is then hybridized to the TWJ. Finally the tetrameric protein avidin, which can bind up to four biotin groups simultaneously, is added. This results in a homogeneous solution containing cross-linked particles with diameters of approximately 1 μm at a temperature of 35 °C.[4,5] However, we noted in our earlier studies that, if the sample were cooled to room temperature, the microgels would aggregate further and ultimately precipitate out of solution. This temperature-dependent precipitation phenomenon suggested to us that the microgels might be useful for a recoverable, immobilized enzyme system. In particular, a number of enzymes are commercially available as covalent conjugates with avidin, meaning these should be incorporated into the microgel matrix via recognition of the avidin by the biotinylated TWJ. At elevated temperature, the microgel-immobilized enzyme will be free to catalyze chemical reactions in solution, but after the reaction is complete, cooling and centrifugation should permit facile separation of the soluble product from the insoluble microgels.

The experiments described below involve the enzyme β-galactosidase (β-gal) conjugated to avidin. This enzyme is used widely in the dairy industry to hydrolyze lactose in milk and cheese products to make them safe for consumption by those with lactose-intolerance. Efforts to improve the reusability of β-gal include adsorption onto or entrapment within membranes prepared from poly(2-hydroxyethyl methacrylate).[6]

Control experiments verified that the β-gal−avidin conjugate hydrolyzed the chromogenic substrate *o*-nitrophenyl-β-galactoside (ONPG) with kinetics similar to thatof the free β-gal enzyme (Figure S1 in Supporting Information). Given that the conjugate retained the activity of the natural enzyme, we next assembled microgels in the presence of β-gal−avidin. For these experiments, the microgels were prepared using 13% of the β-gal−avidin conjugate and 87% unmodified avidin. The DNA TWJ was prepared by annealing the three strands together. The biotinylated PNA was added next, followed by avidin and the β-gal−avidin conjugate. The mixture was incubated at room temperature for 1 h to allow the microgels to form, and then the temperature was raised to 37 °C. Light-scattering experiments performed at this point indicated that the assemblies were approximately 0.5 μm in diameter (data not shown). The smaller dimensions of the assemblies when the avidin−enzyme conjugate is present could be due to steric congestion within the gel. Future experiments will involve measuring the microgel size as a function of the avidin−enzyme content.

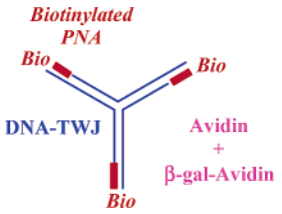

Figure 1. Schematic of the biopolymer microgel assembly used to immobilize the enzyme β-galactosidase (β-gal). Biotinylated PNA and DNA TWJ are indicated by bold red and thin blue lines, respectively.

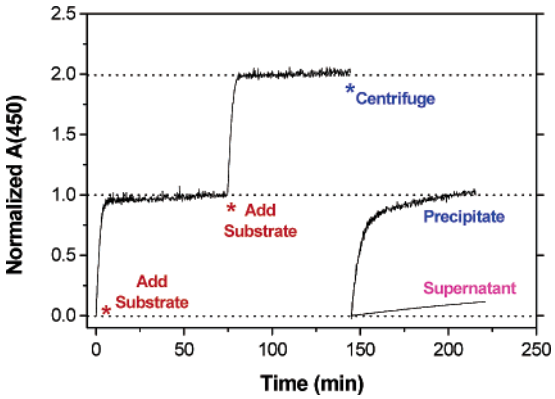

Figure 2. Enzymatic activity of β-galactosidase in microgel assemblies at 20 °C, monitored by the absorbance at 450 nm of the ONPG hydrolysis product. Two aliquots of ONPG substrate were added sequentially, and then microgels were centrifuged. ONPG then added separately to supernatant and reconstituted precipitate.

Addition of ONPG to the microgel suspension resulted in immediate appearance of the yellow *o*-nitrophenolate anion, one of the expected products of the reaction catalyzed by β-gal (Figure 2). On the basis of the growth of the *o*-nitrophenolate absorbance at 450 nm, the reaction was seen to be complete within 10 min, and the hydrolysis was only slightly slower than for free β-gal or avidin−β-gal conjugate (Figure S1). Addition of a second aliquot of ONPG led to renewed hydrolysis of the substrate by the enzyme, indicating that the end of the reaction observed during the first phase was simply due to consumption of the substrate rather than deactivation of the enzyme. After the second round of ONPG hydrolysis was complete, the sample was placed on dry ice for 15 min and then centrifuged in a standard benchtop refrigerated microcentrifuge for 45 min (12,000 rpm, 5 °C). A pellet was observed at the bottom of the microcentrifuge tube. The supernatant was removed using a pipet, and the pellet was reconstituted by addition of fresh buffer and gentle shaking. The supernatant was found to catalyze very little hydrolysis of ONPG, while the pellet exhibited rapid hydrolysis of the substrate (Figure 2). This indicated

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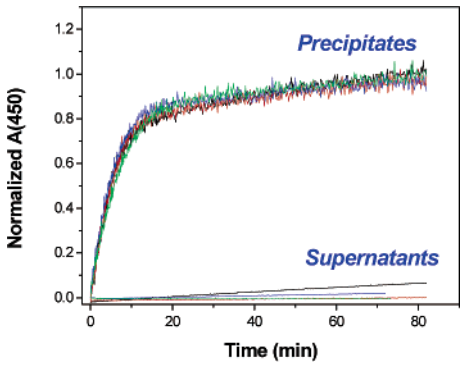

Figure 3. Enzymatic activity of a single microgel preparation carried through four cycles of hydrolysis, precipitation, separation, and reconstitution. First cycle: black lines; second cycle: red lines; third cycle: green lines; fourth cycle: blue lines.

that the enzyme (1) coprecipitated with the microgel particles, (2) was easily separated from the product, and (3) could be reconstituted with virtually no loss in activity.

The results shown in Figure 2 do not prove that the enzyme was actually incorporated into the microgel matrix. An alternative hypothesis is that the enzyme remained free in solution but simply coprecipitated with the microgels due to the low-temperature incubation and centrifugation. To rule out this possibility, we repeated the experiment using standard avidin to form the microgel and free (unconjugated) β-galactosidase. The enzyme was present during formation of the microgel, meaning it was possible for it to be physically entrapped in the matrix, but would not be cross-linked into the matrix since it lacked a conjugated avidin group.

Figure S2 (Supporting Information) shows the results of the same experimental procedure described for the avidin−enzyme conjugate. In this experiment, the supernatant retained the enzymatic activity while the precipitate was largely inactive. This indicates that effective separation of the enzyme and product requires that the β-galactosidase actually be incorporated into the microgel matrix.

As an additional control experiment, the DNA, PNA, avidin, and avidin−β-gal conjugate were mixed together as described above, except that the PNA was not biotinylated. Under these conditions, the DNA TWJ should assemble and the PNA should hybridize to all three arms, but the proteins should remain free in solution. This solution effectively hydrolyzed ONPG, but cooling and centrifugation failed to separate the enzyme from the product (data not shown).

Finally, we assessed the retention of catalytic activity over four consecutive cycles of ONPG hydrolysis, precipitation, separation, and reconstitution. Figure 3 illustrates that there is virtually no loss of activity due to these activities, indicating that the microgel assemblies are particularly robust.

A number of other approaches have been described for immobilization of enzymes to ease product separation and stabilize the enzyme for either repeated use or for exposure to extreme conditions of pH, temperature, or solvent.[7−9] Ongoing concerns for immobilized enzymes include the following: (*i*) physical entrapment of the enzyme within an insoluble matrix such as polyacrylamide can lead to leaching of the enzyme and concomitant loss of activity over time, (*ii*) chemical cross-linking of the enzyme into a polymeric matrix can lead to irreversible damage of the enzyme, particularly when free radical chemistry is used to form the covalent bond, and (*iii*) restricted diffusion of substrates into a matrix can significantly decrease reaction rates. The avidin conjugate used in these experiments allows integration of the β-galactosidase into the microgel matrix without damaging the enzyme while the biotin−avidin interaction effectively prevents leaching of the enzyme during the reaction. This combination of features is essential for product separation and recovery of the enzyme. Moreover, the similarity in the rates of the free and immobilized enzymes in the microgels indicates that small-molecule substrates freely diffuse throughout the structure.

Two additional features of the system are worth noting. First, a variety of enzyme−avidin conjugates are commercially available, as are procedures for preparing in the laboratory those conjugates that cannot be purchased, meaning a large number of immobilized enzymes can be utilized in this general design. Second, we note that the thermal reversibility of the PNA−DNA hybrid was unnecessary for the experiments described in this report. However, there are certainly applications where the biotinylated PNA would be useful. For example, when substrates are used that are simply too large to diffuse into a microgel, such as proteins or nucleic acids, a system could be designed where the PNA−DNA hybrid only forms at low temperature, such as 20 °C. At elevated temperature, such as 37 °C, the microgel would be dissociated with the enzyme−avidin conjugate free and accessible to the substrate. (The biotinylated PNA would be bound to the avidin under these conditions.) After completion of the reaction, the sample would be cooled to allow the PNA−DNA hybrids to form and to lead to precipitation of the microgel, which would then be collected by centrifugation as described above.

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Supporting Information Available: DNA and PNA sequences, sample preparation details, kinetics data for control experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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