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Biotinylated and enzyme-immobilized carrier prepared by hetero-bifunctional latex beads†

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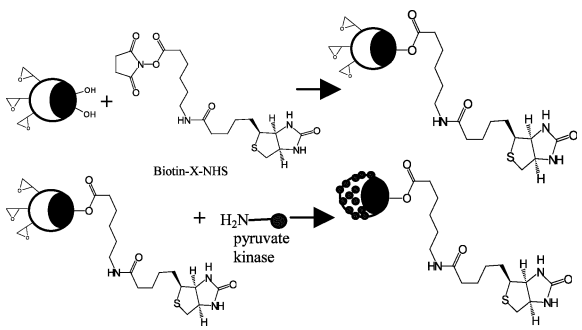
Biotinylated and pyruvate kinase immobilized nano-bio element have been prepared using hetero-bifunctional latex beads, where the enzyme activity is roughly half of the free enzyme.

Immobilized enzymes have a large number of applications, particularly in biomaterials, food processing and analytical chemistry.[1] Enzymes have been immobilized on supports of different shapes either by adsorption, entrapment, or covalent binding. Immobilized enzymes using insoluble materials as carriers offer several advantages over free enzymes, including easy recovery, the possibility for continuous operation, simplified downstream processing and sometimes enhanced stability.[2] Epoxy-activated supports seem to be almost-ideal systems to develop very easy protocols for enzyme immobilization.[3] Epoxy groups are stable at neutral pH even under wet conditions, and are able to react with various nucleophilic groups on the protein surface (*e.g.* amino, hydroxyl, or thiol moieties) with minimal chemical modification of the protein. Polymer latexes are widely used in the binding of biological molecules. To achieve covalent coupling of biological molecules to the surface of the polymer latex, specific functional groups such as epoxy,[4] acetal,[5] carboxylate,[6] aldehyde,[7] chloromethyl,[8] and hydroxyl groups[9] are employed. We reported in a previous paper that polymer particles with a poly(glycidyl methacrylate) (PGMA) shell surface on which cisplatin-DNA is immobilized are a very powerful tool for the affinity purification of proteins.[10]

In spite of recent prominent developments in beads technology, there have been no successes in preparation of hetero-bifunctional nanoparticles, which have a high potential to become one of the key devices in nano-biotechnology. In the present study, we have succeeded in the development of a new polymer particle bearing an enzyme on one side and biotin on the other. For this purpose, the cationic hetero-bifunctional poly(glycidyl methacrylate-*co*-divinylbenzene)/polystyrene [P(GMA-DVB)/PSt] composite latex bead with hydroxyl and epoxy groups was designed as an enzyme immobilization carrier. As shown in Scheme 1, the hydroxyl groups on one side (the PSt domain) of the latex beads were bonded to biotin which can specifically bind to the streptavidin template, and the epoxy groups on the other side [the P(GMA-DVB) domain] were used to covalently immobilize pyruvate kinase (PK). By using this process, we can prepare functional latex beads with various immobilized enzymes and/or other chemical molecules, which are capable of binding to the streptavidin template through biotin. One promising possibility is that it can be a cargo bound to a microtuble[11] for the production of adenosine 5′-triphosphate (ATP) used as the motion energy of kinesin.



Scheme 1 Biotinylation and enzyme immobilization scheme of hetero-bifunctional latex beads.

The hetero-bifunctional latex bead was prepared by soap-free seeded emulsion polymerization. At first, the P(GMA-DVB) seed latex was prepared by soap-free emulsion polymerization using 2,2′-azobis(2-amidinopropane)·2HCl (V-50) as an initiator. The P(GMA-DVB)/PSt composite latex bead was then prepared by soap-free seeded emulsion polymerization by using P(GMA-DVB) seed latex. The chain transfer agent 2-merocaptoethanol was added into the seed stage to introduce hydroxyl groups to the polystyrene chain ends. The average diameter of the P(GMA-DVB)/PSt composite latex beads was measured to be about 200 nm by TEM photographs (data not shown). The epoxy groups of the latex bead surface were reacted with ammonium solution (28 %) at 70 °C for 24 h to form the amino-modified latex bead. The ninhydrin test was used for quantitative assay of the epoxy groups on the surface of latex beads, assuming that the epoxy groups were quantitatively reacted with ammonium. Moreover, the amino-modified latex bead was reacted with acetic anhydride in the methanol at room temperature for 1 h. The formed acetamide group was identified by FT-IR (KBr) spectra, where the typical acetamide peak was observed at 1624 cm^{-1}. On the contrary, no peak was found at this wave number in the original P(GMA-DVB)/PSt latex beads. The number of the epoxy group was estimated to be 8.10×10^5 per bead. The hydroxyl group on the surface of the latex beads was qualified by fluorescent measurement using the hydroxyl-reactive fluorophore 7-methoxycoumarin-3-carbonyl azide (M-1445).

The biotinylation of the hydroxyl group on the latex beads was carried out by using 5-(*N*-succinimidyloxycarbonyl)pentyl D-biotinamide (Biotin-X-NHS) in the presence of dimethylaminopyridine (DMAP) in acetonitrile at 65 °C. Streptavidin-colloidal gold (diameter: 10 nm) was then reacted with the biotinylated latex beads in a phosphate buffer (pH 7.4) for 24 h at ambient temperature to confirm the biotinylation. The TEM photograph of latex beads labeled with the colloidal gold is shown in Fig. 1. It is clear that the gold particles were bound only to one side of the latex bead.

Pyruvate kinase (PK) was directly immobilized to the biotinylated latex beads through the surface epoxy groups. After the

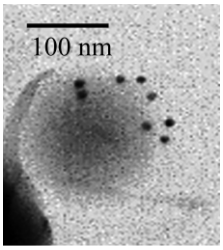


Fig. 1 TEM photograph of a latex bead bearing biotin, which is labeled with streptavindin-colloidal gold (diameter: 10 nm).

† Electronic supplementary information (ESI) available: experimental details for the preparation of hetero-bifunctional latex beads, biotinylation of latex beads, immobilization of PK, and the labelings of biotin and the immobilized PK side with colloidal gold. See http://www.rsc.org/suppdata/cc/b3/b313611f/

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enzyme immobilization and separation of the latex beads, the supernatant and the washed solution (1 M KCl solution) were collected. The amount of unbound PK in the collected solution was determined from the absorbance at 280 nm and at 562 nm using a BCA protein assay kit.[12] These two methods gave almost the same amount of immobilized PK. It was found that the immobilized PK increased with the amount of added PK. The latex beads, on which 75 mg of PK was immobilized per gram of beads, were used to measure the maximum velocity (V_{max}) and the Michaelis constant (K_M).

The activity of immobilized PK was evaluated by the initial rate ratio of immobilized PK to free PK in the pyruvate (PYR) production. Phosphoenolpyruvate (PEP) and adenosine 5′-diphosphate (ADP) was converted to pyruvate (PYR) and adenosine 5′-triphosphate (ATP) in the presence of PK, and the formed PYR was then converted to lactate and diphosphopyridine nucleotide (NAD) in the presence of β-diphosphopyridine nucleotide (reduced form, NADH) and lactate dehydrogenase. The PYR concentration was spectrophotometrically monitored from the decrease in the UV absorbance at 340 nm due to the disappearance of NADH. The relationship between PYR concentration and reaction time in the presence of the same amount (110 μg) of immobilized and free PK is shown in Fig. 2. The activity of the immobilized PK was *ca.* 48% of the free PK, when the concentration of PEP and ADP was 100 μM.

The kinetic parameters V_{max} and K_M of the immobilized PK were determined by measuring initial rates in the range of 10–100 μM PEP concentration [Fig. 3(a)]. The reciprocal plot between initial rate and PEP concentration is shown in Fig. 3(b). V_{max} and K_M of the immobilized PK were determined to be 3.19 μM min^{-1} and 256 μM, respectively. The K_M value is 3.7 times larger than that of free PK (70 μM). This K_M value seems to be reasonable, since immobilized enzymes ordinarily have larger K_M values, due to hindrance against diffusion of substrate molecules.

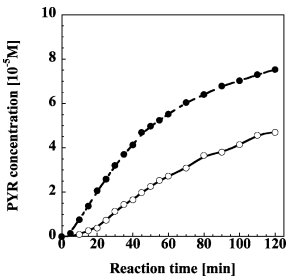

Fig. 2 Relationships between PYR concentration and reaction time in the presence of immobilized PK (110 μg, open symbol) and free PK (110 μg, solid symbol). Initial concentration of PEP and ADP: 100 μM.

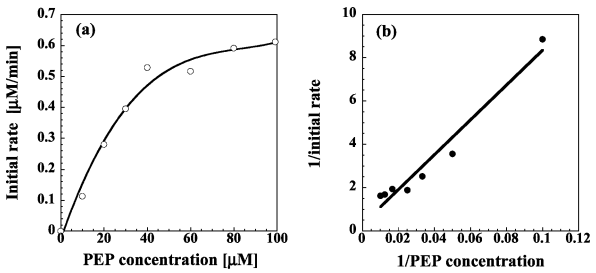

Fig. 3 Relationship of V_{max} and PEP concentration (a), and the reciprocal plot of V_{max} and PEP concentration (b). Initial concentration of ADP and NADH: 100 μM, immobilized PK: 110 μg, lactate dehydrogenase lactate: 100 μg.

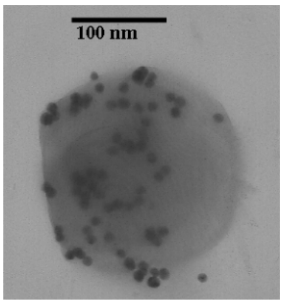

Fig. 4 TEM photograph of a latex bead bearing PK and biotin. The PK is labeled with 15 nm colloidal gold.

The biotinylated latex bead with immobilized PK was then bound with antibodies of PK, the IgG fraction of a polyclonal sheep antiserum, and secondary antibodies labeled with 15 nm colloidal gold [rabbit anti-sheep, f(ab′)$_2$ specific-gold colloidal particles] were further bound to the antibodies of the latex beads to confirm the locus of PK on the surface of the latex bead. The TEM photograph of a latex bead with immobilized PK, which is labeled with colloidal gold, is shown in Fig. 4. It is obvious that the gold particles bind to the PK region on one side of latex bead.

We have proposed a new concept of hetero-bifunctional beads. In principle, any enzyme and any compound (with reactive functional groups) can be immobilized, and the biotin site can be independently connected to any other system *via* avidin. This may open a way toward the development of new elaborate nano-biomachines combining artificial and biological devices.

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