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Acceleration of Enzymatic Reactions on Phospholipid Polymer Nanoparticles for Diagnosis Device

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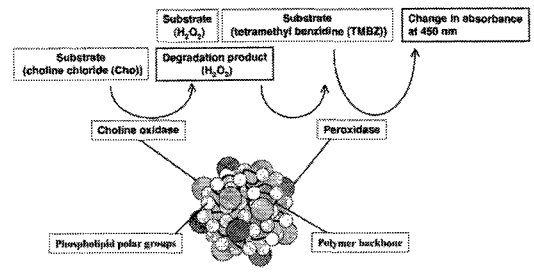
For conjugation of biomolecules, polymeric nanoparticles composed of hydrophilic phospholipid polymer shell and water-insoluble polystyrene core were prepared. Active ester groups were incorporated into the phospholipid polymer by using a novel monomer having *p*-nitrophenyl group, that is the active ester groups and the phospholipid polar groups were covered on the nanoparticle surface. For the sequential enzymatic reactions, choline oxidase and peroxidase were used as enzyme and co-immobilized onto the nanoparticles. Choline chloride and tetramethyl benzidine were added as substrates to the nanoparticle in phosphate buffered solution. The sequential enzymatic reactiona in detail is as follows; the choline chloride was oxidized by the choline oxidase, and hydrogen peroxide was then formed as a degradation product. The hydrogen peroxide was used for the subsequent enzymatic reaction of tetramethyl benzidine. If hydrogen peroxide was free to diffuse between the choline oxidase and the peroxidase, a sequential enzymatic reaction would be observed. The enzymatic reaction on the nanoparticles was found to be significantly more efficient than that by the enzyme mixture. This result indicates that the diffusion of the degradation products and localization of immobilized enzymes are dominant factors for the reaction. The phospholipid polymer nanoparticles are thus promising materials for a high-sensitivity diagnosis device.
Key words: phospholipid polymer, nanoparticles, bioconjugation, sequential enzymatic reaction, medical diagnosis

1. INTRODUCTION

We have newly proposed the assembly of the phospholipids polar group on the nanoparticles. Generally, nanoparticles are unstable as colloid particles in aqueous media. A hydrophilic moiety and ionic groups are incorporated onto the surface to modify the interface. One of the anomalous interfaces is the cell membrane, which is composed of phospholipid molecules, glycoproteins, and channel-forming proteins, to provide not only dividing between the cytoplasm and the outer environment but also to communicate via antenna molecules and channels. Ishihara *et al.* designed and synthesized a novel functional monomer with a phospholipid polar group, 2-methacryloyloxyethyl phosphorylcholine (MPC), for fabrication of the cell membrane structure as biointerface [1-2]. A typical phospholipid polymer is copolymerized with MPC and *n*-butyl methacrylate (BMA), and the phospholipid polymer-coated surface provided a very bio-inert interface; particularly, non-specific interactive biointerface on diverse materials was obtained and many biomedical devices were developed by using the phospholipid polymers [3-5]. The stability of the phospholipid polymer-modified enzyme was also significantly prolonged in comparison with the native enzyme [6]. Furthermore, the phospholipid polymer spontaneously formed nano-structured aggregations, indicating amphiphilic and surfactant-like properties [7]. Biomimetic surface is promising approach to prepare the nano-scaled devices for biofunctionalization. In the case of medical diagnosis, a large number of serum proteins are contained in whole blood sample; therefore, the biomimetic surface prepared by the phospholipid polymer is effective to make quite high signal/noise ratio (S/N ratio).



Scheme 1. Illustration of sequential enzymatic reaction system on bioconjugate nanoparticles.

In this study, we have newly proposed a novel signal amplified diagnosis system using sequential enzymatic reaction on nanoparticles (Scheme 1). The nanoparticles are composed of the phospholipid polymer with active ester groups and polystyrene core. The amplified signal was evaluated by using choline oxidase and peroxidase, which were co-immobilized onto the nanoparticles. The choline oxidase reacts with choline chloride, and hydrogen peroxide is produced. The produced hydrogen peroxide is used as a substrate in next enzymatic reaction by

peroxidase. In this sequential enzymatic reactions, the amount of choline chloride will be evaluated by change in absorbance. By using the bioconjugate nanoparticles, we can easily evaluate specific biomarker molecule in comparison with conventional enzyme-linked immunosorbent assay (ELISA).

2. MATERIALS AND METHOD

2.1 Materials

MPC was prepared and purified by a method previously reported [1]. BMA was purchased from Wako Pure Chemical Co., Ltd. A functional monomer, *p*-nitrophenyloxycalbonyl poly(oxyethylene) methacrylate (NPMA) was prepared by previous report [8]. Polystyrene (Kanto Chemicals, Tokyo, Japan), which has 200 kDa of number average molecular weight, was used. Other organic reagents were used without further purification. Choline oxidase (Alcaligenes sp., 13 unit/mg, Wako Pure Chemicals, Osaka, Japan) and peroxidase (horseradish, 220 unit/mg, Wako Pure Chemicals) were used as received.

2.2 Synthesis of bioconjugate polymer

Phospholipid polymer for bioconjugation was prepared from MPC, BMA, and NPMA (PMBN, Figure 1), the procedures in detail were previously reported [8]. The monomer composition in the copolymer was determined by ^{1}H-NMR and the weight average molecular weight (Mw) was estimated by gel permeation chromatography (GPC, poly(ethylene glycol) standard).

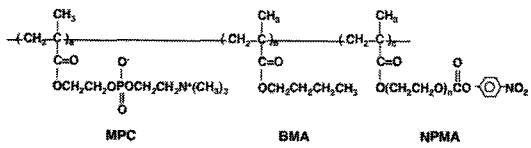


Figure 1. Chemical structure of bioconjugate phospholipid polymer.

2.3 Preparation of nanoparticles

The nanoparticles for co-immobilization were prepared by using polystyrene and the phospholipid polymer (PMBN). The PMBN (400 mg) was dissolved in distilled water (40 mL), and polystyrene (20 mg) was dissolved in methylene chloride (2 mL). The solutions were mixed together, and the mixture was treated by a probe-type ultrasonicator (Sonifier 250, BRANSON Co., USA) for 2 min. A finely dispersed emulsion was obtained. The methylene chloride was removed using a rotary evaporator, and the formed nanoparticles were collected by centrifugation (10,000 rpm, 10 °C). To remove surplus phospholipid polymer, the nanoparticles were repeatedly washed by centrifugation and re-suspended. The purified nanoparticles were kept at 4 °C at a concentration of 20 mg/mL before use. Characterizations of nanoparticles were carried out with X-ray photoelectron spectroscopy (XPS, Shimadzu/KRATOS, AXIS-HSi) and scanning electron microscope (SEM, Hitachi, S-5000H).

2.4 Co-immobilization of enzymes onto nanoparticles

Choline oxidase (7.8 unit) and peroxidase (20 unit) were dissolved in phosphate buffer solution (PBS, pH 7.8) with the nanoparticles (final concentration: 2 mg/mL). The reaction was carried out for 48 h at 4 °C. After the conjugation, the unreacted enzyme was repeatedly washed by centrifugation and re-suspended. The remaining active ester groups were masked using glycine. The obtained nanoparticles (concentration of stock solution: 2 mg/mL) were stored at 4 °C before use.

2.5 Enzymatic reaction on co-immobilized nanoparticles

The choline chloride (1 mg/mL by PBS (pH 7.4), Wako Pure Chemicals) was used as substrate for choline oxidase. A peroxidase assay kit (ML-1120T, Sumitomo Bakelite Co., Tokyo, Japan) was used as a substrate. The nanoparticles (10 μg), choline chloride solution (100 μL), and the working solution in assay kit (100 μL) were mixed in PBS (pH 7.4) solution, and the final volume was adjusted to 1 mL. The enzymatic reaction was carried out for 10 min at 25 °C, and 1 mL of H_2SO_4 (2 mol/L) was added to the suspension for the termination. The mixture was centrifuged, and the supernatant was evaluated by a multiplate reader (1420 ARVO mx, Perkin Elmer Japan, Tokyo, Japan) at 450 nm.

3. RESULTS AND DISCUSSION

3.1 Characterization of bioconjugate phospholipid polymers

The PMBN could be dissolved in water, because hydrophilic MPC unit was contained 45 mol% in the polymer (Table 1). From the results of NMR spectroscopy, the structure of the PMBN was confirmed. The PMBN conformation in water was evaluated by using fluorescence probe, sodium 1-anilino-8-naphthalene sulfonate (ANS). The ANS shows maximum fluorescence wavelength at 515 nm (λ_{EX}) in water (excitation wavelength at 370 nm (λ_{EM})).

Table 1. Synthetic result of PMBN

Code	Monomer unit composition (mol%)						Mw [b]
	In feed			In polymer [a]			
	MPC	BMA	NPMA	MPC	BMA	NPMA	
PMBN	40	50	10	45	43	12	5.0×10^4

[Monomer] = 0.5mol/L, [AIBN] =5mmol/L.
Solvent: ethanol. Polymerization temperature: 60°C, 6 hours.
a) Determined by ^{1}H-NMR. b) Determined by GPC in Water/CH_3OH = 3/7, PEG standards.

Figure 2 shows a relationship between maximum wavelength and polymer concentration in fluorescence measurement. The maximum wavelength shifted with increasing the PMBN concentration. Below 0.1 mg/mL of the PMBN concentration, the maximum wavelength of ANS shifted, indicating transition region. Over 0.1

mg/mL of the PMBN concentration, the wavelength was 476 nm, indicating the ANS molecules trapped in lower polarity (stable aggregate).

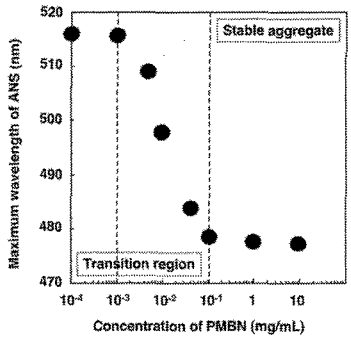


Figure 2. Polymer conformation of PMBN.

The change in the maximum fluorescence wavelength indicated that the PMBN spontaneously formed aggregates. This result indicated that the PMBN formed hydrophobic domains in water environment. Therefore, the methylene chloride droplets containing polystyrene was stably dispersed in the phospholipid polymer aqueous solution, and the nanoparticles covered with the phospholipid polymer was then prepared.

3.2 Characterization of bioconjugate nanoparticle

The nanoparticles were prepared by a solvent evaporation method via an emulsion. The average particle size was evaluated by using dynamic light scattering as 360 nm. The particle size was similar to the scanning electron micrograph observation (Figure 3).

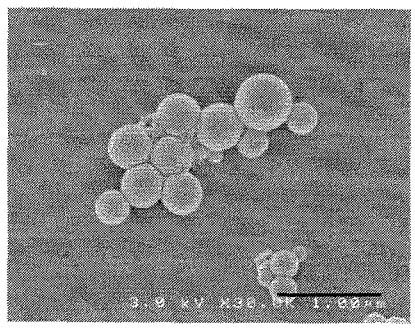


Figure 3. SEM picture of nanoparticle. Scale bar indicates 1 μm.

The surface on the nanoparticles was characterized by X-ray photoelectron spectroscopy (XPS, Figure 4). The CH_2 and CH_3 peaks were observed as C1s at 285.0 eV. The O-C-O and C=O peaks from the phospholipid polymer were observed at 287.0 and 289.0 eV, respectively. Furthermore, a small broad peak attributed to an aromatic group based on the *p*-nitrophenyl ester group was also observed at 291.5 eV. Additionally, N1s and P2p peaks attributed to trimethyl ammonium group (403.0 eV) and phosphate ester (134.0 eV) in choline group were observed.

These results strongly indicated that the nanoparticles were covered with the phospholipid polymer. The nanoparticles are favorable for their bio-inert property under physiological conditions, and non-specific interactions with biomolecules and chemical compounds are then suppressed. The bio-inert properties in detail; particularly, non-specific protein adsorption *in vitro* was reported in our previous paper [9]. The active ester groups were estimated by UV-Vis spectroscopy. The nanoparticles were treated with sodium hydroxide (0.1 mol/L), being completely hydrolysis. Since the *p*-nitrophenol was obtained as a leaving group, absorbance at 400 nm was measured. From the result, 1 nmol of the active ester groups were contained in the 1 mg nanoparticle to determine the amount of *p*-nitrophenol on the nanoparticles.

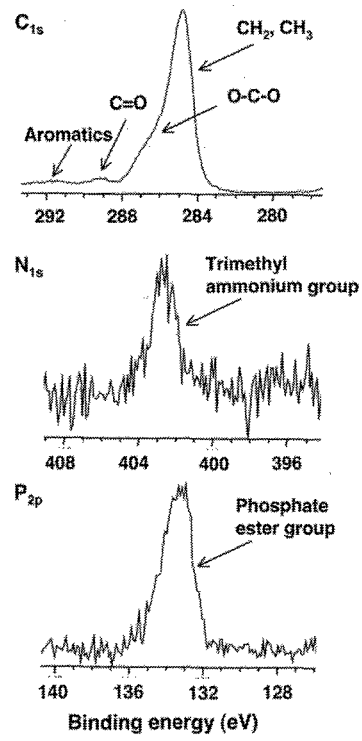


Figure 4. XPS charts of nanoparticles.

3.3 Sequential enzymatic reactions on nanoparticles

We have already estimated the conversion of the active ester linkage on the nanoparticles [10]. Generally, 40% of the active ester linkage was converted by a reaction with proteins. In this study, two kinds of enzymes, choline oxidase and peroxidase, were co-immobilized onto the surface, and the sequential enzymatic reaction was then evaluated. The combination of the enzymes displayed their communication via the degradation product (hydrogen peroxide) as shown in Scheme 1. As substrates, choline chloride (Cho), tetramethyl benzidine (TMBZ), and hydrogen peroxide (H_2O_2) were used. The Cho was oxidized by the choline oxidase, and H_2O_2 was newly produced as a degradation

product. The produced H_2O_2 was used for the next enzymatic reaction; the oxidation of TMBZ by peroxidase. The H_2O_2, which was originally added to the media, could also be used as substrate.

In this study, two kinds of protocol regarding the enzymatic reaction were examined; (i) only the TMBZ and H_2O_2 were added to the nanoparticle suspension (TMBZ in Figure 5) and (ii) Cho was added to the suspension with TMBZ and H_2O_2 (TMBZ/Cho in Figure 5). In the case of protocol (i), only the enzymatic activity of the peroxidase was evaluated. On the other hand, newly produced H_2O_2 would be an enhancer for the sequential enzymatic reaction (protocol (ii)). The result of the enzymatic reaction was evaluated by the change in absorbance at 450 nm (Figure 5).

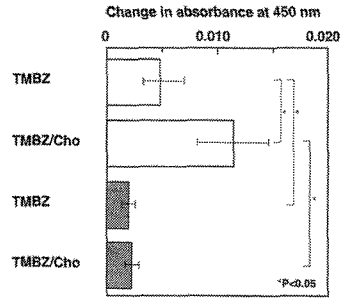


Figure 5. Sequential enzymatic reaction between choline oxidase and peroxidase; open bar: nanoparticles, grey bar: enzyme solutions. Mean value of five measurement was indicated with error bar.

The enzymatic reaction on the nanoparticles was significantly two times higher than that in a simple enzyme solution, when TMBZ was added as the substrate. The total amount of enzymes in the enzyme solution was reliably larger than that of an immobilized enzyme on the nanoparticles, because the concentration of the enzymes in the solution was the same as the feed concentration for the preparation of the enzyme co-immobilized nanoparticles. Taking the total amount of the enzyme concentration into account, it is considered that choline oxidase and peroxidase were locally concentrated on the particle surface in comparison with the solution. Furthermore, the sequential enzymatic reaction was compared. The change in absorbance increased with the addition of TMBZ/Cho, which was twice higher than that of the addition of only TMBZ. It was considered that the surplus enzymatic activity was based on choline oxidase, and the produced H_2O_2 was effectively free to move to its binding site as the substrate. Originally, sufficient H_2O_2 was added to the media to promote the enzymatic reaction; therefore, the degree of increasing enzymatic reaction was caused by the H_2O_2 newly produced by choline oxidase. In the case of nanoparticles, the enzymes, choline oxidase and peroxidase, were closely co-immobilized onto the nanoparticles; therefore, the diffusion pathway of the produced H_2O_2 was significantly shorter than that of the solution. Thus, the produced H_2O_2 was different from the originally added H_2O_2, which needed a long pathway to react with the peroxidase. On the other hand, no significant difference in the enzyme solution was observed between the single reaction (TMBZ) and the sequential reaction (TMBZ/Cho). This result indicated that only the reaction by peroxidase proceeded a single reaction, even if Cho was added to the media.

4. CONCLUSIONS

The conversion on sequential enzymatic reactions by enzymes immobilized on polymeric nanoparticles were significantly grater than that of the enzyme solution, indicating that the diffusion pathway of the degradation products and localization of the co-immobilized enzyme were dominant factors in the reaction. As a result, the H_2O_2 produced by the sequential enzymatic reactions was effectively used as an enhancer in the next reaction. This anomalous enhancement by a degradation product (H_2O_2) could be utilized and extended as a signal amplified mechanism. The nanoparticles covered with phospholipid polymer shell represent an amazing discovery for various applications, such as high S/N ratio monitor in biomedical diagnosis, bioreactors for useful biomolecules, and site specific carriers for drug administration.

5. ACKNOWLEDGEMENT

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6. REFERENCES

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