Sequential Enzymatic Reactions and Stability of Biomolecules Immobilized onto Phospholipid Polymer Nanoparticles

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Polymer nanoparticles for sequential enzymatic reactions were prepared by combining a phospholipid polymer shell with a polystyrene core. The active ester groups for the bioconjugation and phospholipid polar groups were incorporated into the phospholipid polymer backbone using a novel active ester monomer and 2-methacryloyloxyethyl phosphorylcholine. For the sequential enzymatic reactions, acetylcholinesterase, choline oxidase, and horseradish peroxidase-labeled IgG were immobilized onto the nanoparticles. As substrates, acetylcholine chloride, choline chloride, and tetramethylbenzidine were added to the nanoparticle suspension, the acetylcholine chloride was converted to choline chloride, the choline chloride was oxidized by choline oxidase, and hydrogen peroxide was then formed as an enzymatic degradation product. The hydrogen peroxide was used for the next enzymatic reaction (oxidized by peroxidase) with tetramethylbenzidine. The sequential enzymatic reactions on the nanoparticles via degradation products (hydrogen peroxide) were significantly higher than that of the enzyme mixture. This result indicated that the diffusion pathway of the enzymatic products and the localization of the immobilized enzyme were important for these reactions. These nanoparticles were capable of facilitating sequential enzymatic reactions.

Introduction

In general, enzymes are utilized in aqueous solutions with additives such as chelating reagents. The stability of the enzyme depends on the aqueous medium, and is easily disrupted to the point where the enzymes cannot function appropriately. Immobilization techniques are a promising approach to retain their stability. Recently, nanostructured chemistry and technology using highly selective enzymatic reactions have been focused on biochemical production and the sensing of target molecules.[1−5] We hypothesized that a nanoscaled interface could generate biomolecules with high functionality, and thus, we developed sequential enzymatic reactions on phospholipid polymer nanoparticles. Nanoparticles are a promising architecture to produce biological functions and to provide a dynamic response.[6−9] However, they are generally unstable as colloidal particles in an aqueous medium. A hydrophilic moiety and ionic groups are incorporated onto the surface to stabilize the interface between the aqueous medium and the particles. One of the most anomalous interfaces is the cell membrane, which is composed of phospholipid molecules; thus, we designed a phospholipid polymer biointerface on the nanoparticles.

A methacrylate with a phosphorylcholine group, 2-methacryloyloxyethyl phosphorylcholine (MPC), was synthesized and copolymerized with various vinyl monomers to make phospholipid polymers.[10−12] The phospholipid polymer could form cell-membrane-like surfaces with coating of the polymer, blending with the polymer, and grafting to the polymer chains.[13] The phospholipid polymer thus provided a bioinert interface. In particular, the stability of the phospholipid polymer-modified enzyme was significantly prolonged in comparison with that of the native enzyme.[14,15] By controlling the composition of the MPC units, a water-soluble phospholipid polymer was prepared, but it spontaneously formed nanostructured aggregations, indicating amphiphilic and surfactant-like properties.[16]

In this study, MPC, *n*-butyl methacrylate (BMA), and the novel bioactive monomer (*p*-nitrophenyloxycarbonyl)poly(oxyethylene) methacrylate (NPMA) for bioconjugation were copolymerized.[17−20] The polymer nanoparticles were prepared in an aqueous solution containing the MPC polymer to cover the surfaces with the polymer. Three kinds of enzymes were immobilized together onto the nanoparticles, and the sequential enzymatic reaction was evaluated (Scheme 1). The stability of the immobilized enzyme was also discussed as a function of the residual activity.

Materials and Method

Instruments. The average particle size was determined by dynamic light scattering (DLS-7000, Otsuka Electronics Co., Ltd., Tokyo, Japan). Scanning electron microscopy (S5000H, Hitachi Co., Tokyo, Japan) was then performed with a gold coating. The X-ray photoelectron spectra were recorded with an AXIS-HSi (Shimadzu/Kratos, Kyoto, Japan). The anode was Mg Kα, and the releasing angle of the photoelectron for each element was fixed at 90°. The ζ potential was measured with an ELS-8000 (Otsuka Electronics Co.). The UV−vis absorption was measured with an Ubest V-560 (JASCO Corp., Tokyo, Japan).

Bioconjugated Phospholipid Polymer. The bioconjugate phospholipid polymer (PMBN, Figure 1) was prepared from MPC, BMA, and NPMA; these procedures have been previously reported in detail.[17−19] The monomer composition of PMBN was determined by ^{1}H NMR spectroscopy, and 45 mol % MPC and 12 mol % NPMA were incorporated into the phospholipid polymer. The weight-averaged molecular weight was estimated by gel permeation chromatography, and was calculated at 5.0×10^4 against a poly(ethylene glycol) standard.

Preparation of Phospholipid Polymer Nanoparticles. The phospholipid polymer nanoparticles were prepared by a solvent evaporation

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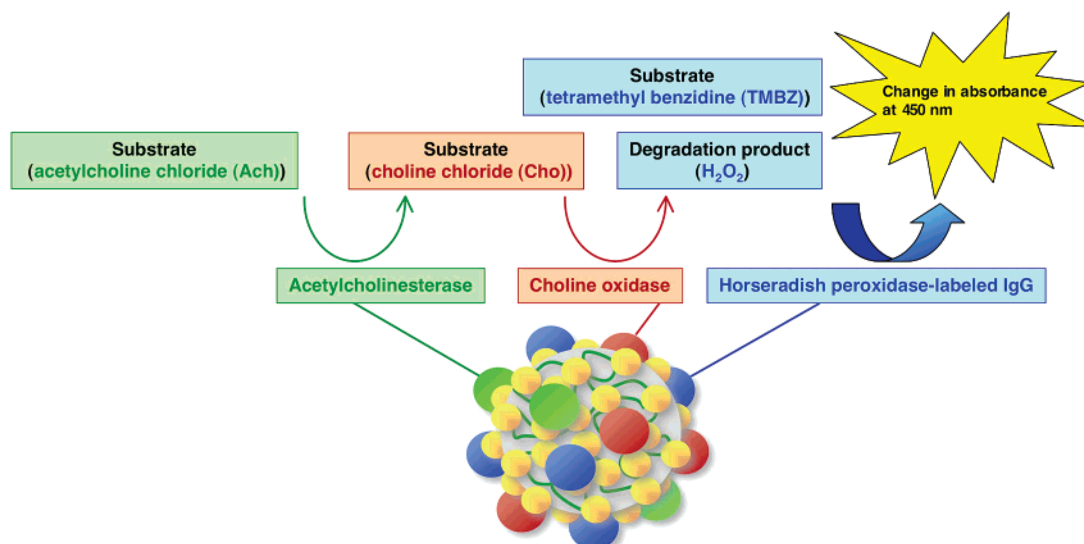
Scheme 1. Three Kinds of Enzymes Were Immobilized onto a Phospholipid Polymer Surface for Sequential Enzymatic Reactions



method.[21,22] The PMBN was dissolved in distilled water (40 mL) at 10 mg/mL. At this concentration, the PMBN formed hydrophobic domains by an association of the *n*-butyl groups.[17] A 20 mg portion of polystyrene (Kanto Chemical Co., Tokyo, Japan, number-averaged molecular weight 2.0×10^5) was dissolved in methylene chloride (2 mL), and the solutions were mixed together. The mixture was treated by a probe-type ultrasonicator (BRANSON Sonifier250, Emerson Japan Ltd., Tokyo, Japan) for 2 min, and a finely dispersed emulsion was obtained. During this process, the emulsion was covered with the PMBN as an interfacial stabilizer. Methylene chloride was removed using a rotary evaporator, and the formed nanoparticles were collected by centrifugation (10000*g*). To remove any surplus PMBN, the nanoparticles were repeatedly washed by centrifugation and resuspension. Purified nanoparticles were kept at 4 °C at a concentration of 20 mg/mL before use. The total amount of active ester groups per milligram of the nanoparticles was evaluated by the change in the absorbance of *p*-nitrophenol (λ_{max} = 405 nm) as a leaving group, following the complete hydrolysis of the ester groups by treatment with a NaOH (0.1 mol/L) aqueous solution.

Surface Reactivity on Nanoparticles and Stability of Immobilized Enzyme. As a preliminary study, the surface reactivity for bioconjugation and the stability of the immobilized biomolecules were evaluated using a horseradish peroxidase-labeled IgG (A6154, Sigma-Aldrich, Inc., Michigan). The IgG (80 μg) was dispersed in a nanoparticle suspension (1 mg/mL) using phosphate-buffered saline (PBS; pH 7.8), and the reaction was carried out for 48 h at 4 °C. The amino groups in the IgG were reactive with the active ester groups on the nanoparticles, and thus, carbamate linkages were produced. After the conjugation, the unreacted enzyme was removed repeatedly by centrifugation and resuspension. The remaining active ester groups were masked using glycine for another 24 h at 4 °C. The obtained IgG-immobilized nanoparticles (concentration of the stock solution 1 mg/mL) were stored at 4 °C before use. For the stability test, the IgG-immobilized nanoparticles were stored in an incubator at the desired temperature. After the incubation, the residual enzymatic activity was evaluated using a peroxidase assay kit (ML-1120T, Sumitomo Bakelite Co., Tokyo, Japan). The peroxidase assay kit contains tetramethylbenzidine (TMBZ) and H_2O_2 as the substrates.

Multimode Immobilization of Enzymes on Nanoparticles. Acetylcholinesterase (bovine erythrocyte, 2.4 units/mg, Wako Pure Chemicals, Osaka, Japan), choline oxidase (*Alcaligenes* sp., 13 units/mg, Wako Pure Chemicals), and horseradish peroxidase-labeled IgG (A6154,

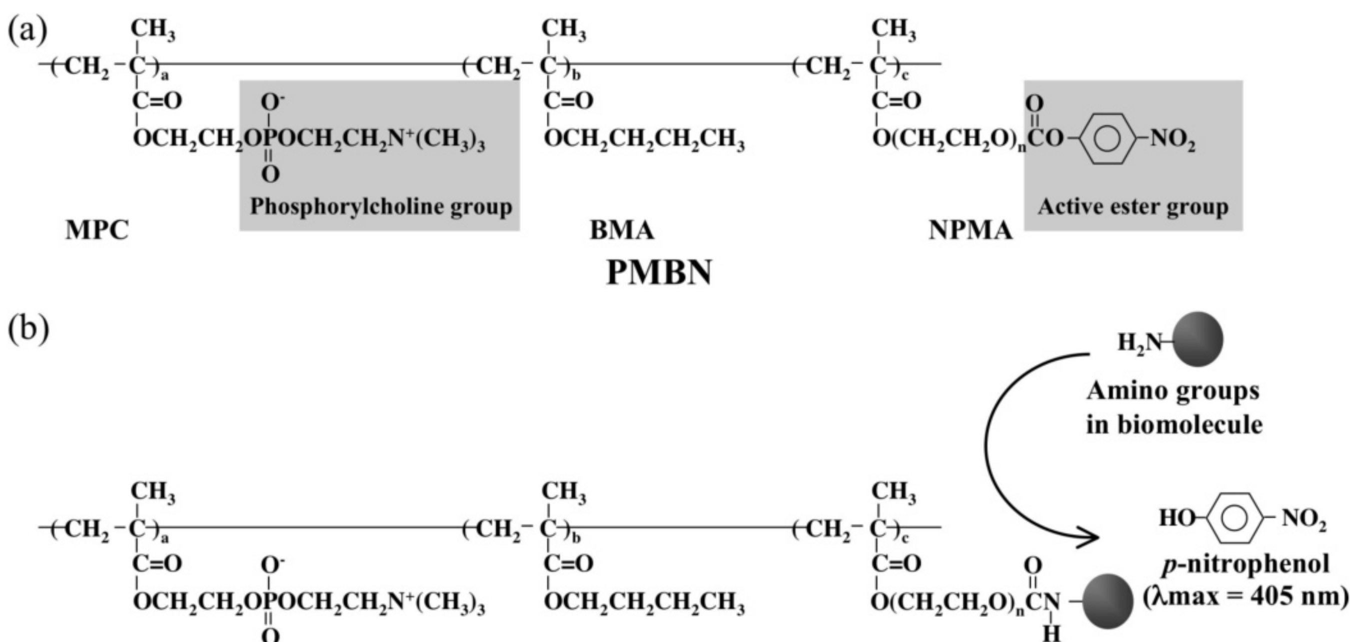


Figure 1. Chemical structure of bioconjugate phospholipid polymer with both phosphorylcholine groups and active ester groups via an oxyethylene spacer (*n* = 4): (a) PMBN and (b) its reaction scheme for bioconjugation.

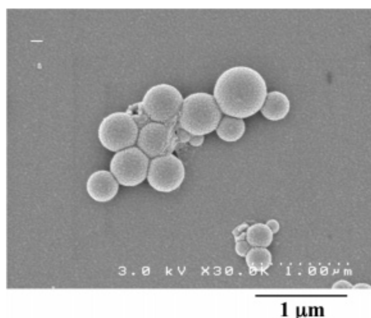


Figure 2. Scanning electron microscope observations of a polystyrene core with a phospholipid polymer surface.

Sigma-Aldrich, Inc.) were used, and 8 units of acetylcholinesterase, 28 units of choline oxidase, and 400 μg of horseradish peroxidase-labeled IgG were dissolved in 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 and IgG were repeatedly washed by a centrifugation method, and finally, the nanoparticles were resuspended in the aqueous medium. The remaining active ester groups were masked using glycine for 24 h at 4 °C. The obtained enzyme-immobilized nanoparticles (concentration of the stock solution 2 mg/mL) were stored at 4 °C before use.

Reactive Conditions for Enzymatic Activity. Acetylcholine chloride (Ach, $[(CH_3)_3NCH_2CH_2OCOCH_3]Cl$) and choline chloride (Cho, $[(CH_3)_3NCH_2CH_2OH]Cl$) were purchased from Wako Pure Chemicals. Each Ach and Cho solution was prepared at 1 mg/mL in PBS (pH 7.4), and used as a substrate for acetylcholinesterase and choline oxidase, respectively. For the enzymatic degradation of peroxidase, a commercially available peroxidase assay kit containing TMBZ and H_2O_2 as substrates was used. The UV−vis absorption at 450 nm for TMBZ increased after the reaction with peroxidase under H_2O_2 conditions. The enzymatic reaction on the phospholipid polymer nanoparticles was evaluated using 10 μg of the nanoparticles. Each substrate solution was used in 100 μL aliquots. The peroxidase assay kit was also used at 100 μL. 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 to terminate the reaction. The mixture was centrifuged, and the supernatant was evaluated by a multiplate reader (1420 ARVO mx, Perkin-Elmer Japan, Tokyo, Japan) at 450 nm.

Results and Discussion

Bioconjugate Phospholipid Polymer Nanoparticles. Polystyrene core nanoparticles with a phospholipid polymer surface were prepared by a solvent evaporation method after preparation of the emulsion. The MPC and synthesized NPMA showed hydrophilicity; however, BMA is hydrophobic, and thus, the copolymer PMBN indicates amphiphilic properties. PMBN could form polymer aggregations in aqueous media at concentrations over 0.1 mg/mL. In this study, a 10 mg/mL PMBN aqueous solution was used, and a fine emulsion was obtained by mixing with polystyrene in methylene chloride. After the evaporation of methylene chloride, the emulsion turned transparent. The suspension containing the nanoparticles was separated from the supernatant, and was precipitated by centrifugation (10000*g*). Figure 2 shows an SEM picture of the precipitated nanoparticles. Spherical-shaped nanoparticles were obtained, and the diameter was 300−500 nm. Furthermore, the average diameters of the nanoparticles determined by dynamic light scattering was 360 nm. The nanoparticles in the precipitate were used in further experiments due to their easy handling during each step. The surface ζ potential of the nanoparticles was observed at −4.9 mV. The obtained ζ potential was roughly neutral, while bare polystyrene particles have a ζ potential around −30 mV. The surface coverage by active ester groups for facilitating bioconjugation was evaluated after complete hydrolysis in 0.1 mol/L NaOH aqueous solution. The active ester group produced *p*-nitrophenol as a leaving group, which showed a UV−vis absorption at 405 nm. The surface coverage was easily estimated as 1.0 nmol/mg. The active ester groups on the nanoparticle surface were then used for bioconjugation. The surface of the nanoparticles was covered with phosphorylcholine groups, thus showing hydrophilicity and a suppression of nonspecific biomolecular adsorption.

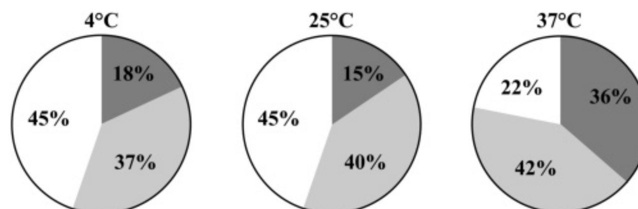


Figure 3. Change in conversion on bioconjugation at each preparation temperature. The white area is the total amount of IgG conjugation, the dark gray area is hydrolysis of active ester groups, and the bright gray area is the remaining active ester groups.

Bioconjugation on Phospholipid Polymer Nanoparticles. The active ester groups were located on the nanoparticles at a concentration of 1.0 nmol/mg of nanoparticles. The active ester group was labile to the primary amino group in the biomolecules, and a carbamate linkage was produced after the bioconjugation. The conversion was estimated by changing the temperature as shown in Figure 3. In the case of 4 and 25 °C, 45% of the active ester groups were converted, and the hydrolysis also progressed to a range of 15−18%. However, 37−40% of the active ester groups still remained. In this study, peroxidase-labeled IgG was used for the evaluation of bioconjugation. The molecular weight of IgG was roughly 1.8×10^5, so 40% of the conversion was considered to be the maximum due to steric hindrance. If a small molecule was used for the bioconjugation, for example, an amino acid such as glycine (molecular weight 75), most of the active ester groups were reacted. The surface active site was generally covered with glycine in the biological assay. It is believed that 40% of the remaining active ester groups were converted using glycine, and furthermore, *p*-nitrophenol was produced in this glycine musk process. On the other hand, only 22% of the conversion was observed at 37 °C. The spontaneous hydrolysis of the active ester group reached 42%. This result indicated that the *p*-nitrophenyl group was unstable at higher temperatures during the conjugation process. The priority of the reaction was hydrolysis without any bioconjugation. Therefore, the bioconjugation on the phospholipid polymer nanoparticles should be performed at 4 °C.

The enzymatic activity on the nanoparticles was estimated by changing the amount of nanoparticles. The bioconjugated nanoparticles, which were prepared at 4 °C, showed the highest enzymatic activity in all cases. Furthermore, the change in the absorbance was proportional to the total amount of nanoparticles in the feed. This result indicated that the nanoparticles contained equal amounts of immobilized IgG, which was labeled by the peroxidase. The reactive scale could be easily regulated by changing the volume of the nanoparticle suspension. Similar phenomena and higher enzymatic activity were observed on the nanoparticles, which were conjugated at 25 °C. On the other hand, it is considered that the enzyme on the nanoparticles, which was prepared at 37 °C, was denatured. This result indicated that the preparation temperature was important to

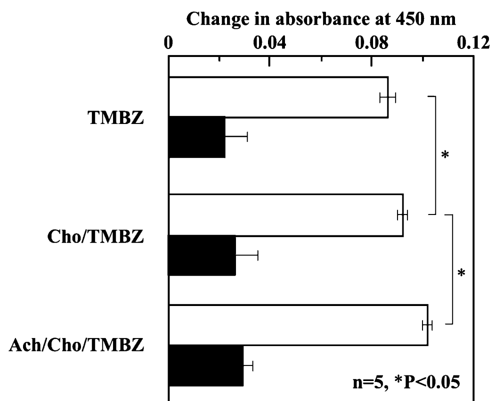


Figure 4. Sequential enzymatic reactions with addition of each substrate. White and black bars indicate reaction on nanoparticles and in enzyme solutions, respectively.

maintain higher enzymatic activity and to increase the amount of immobilized enzyme.

Sequential Enzymatic Reactions on Nanoparticles. From the results of Figure 3, 40% of the active ester groups were used for the immobilization of the biomolecules. In this section, we describe the multimode enzyme immobilization and their sequential enzymatic reactions. Acetylcholinesterase, choline oxidase, and horseradish peroxidase-labeled IgG were immobilized onto the nanoparticles, and the enzymatic activity of the acetylcholinesterase and choline oxidase in the feed was 8 and 28 units, respectively. After the immobilization, it is assumed that 40% of each enzyme was immobilized onto the nanoparticles. The order of the enzymatic activity was believed to gradually increase. On the other hand, acetylcholinesterase was expected to have the lowest total enzymatic activity, whereas the activity of choline oxidase was greater than that of acetylcholinesterase. The gradual enhancement of each enzymatic activity was effective for the sequential enzymatic reactions, and the combination of each enzyme displayed their communication through the sequential reactions. As shown in Scheme 1, Cho was produced from Ach by enzymatic catalysis, and then H_2O_2 was produced by a reaction between Cho and choline oxidase. Finally, TMBZ was converted with H_2O_2, and a change in absorbance at 450 nm was observed.

First, only the TMBZ (containing H_2O_2) was added to the enzyme-immobilized nanoparticle suspension. A change in absorbance over 0.08 was observed as shown in Figure 4, which was based on the enzymatic reaction by peroxidase. As a control, each enzyme was dissolved in PBS (pH 7.4, 2 mL); the solutions contained 8 units of acetylcholinesterase, 28 units of choline oxidase, and 400 μg of peroxidase. The concentration of each enzyme mixture was equal to that of the nanoparticles in the feed; thus, the total enzyme activity was higher than that of the nanoparticles. However, the enzymatic reaction was much lower than that of the nanoparticle, and the change in absorbance decreased. Thus, the enzymatic reaction on the nanoparticle was more effective than that of the enzyme solution. It is believed that the local concentration of the enzyme was one of the dominant factors; even the total amount of the enzyme on the nanoparticle was smaller than that of the enzyme solution. Next, our concern was the sequential reactions, so Cho and TMBZ were added to the nanoparticles. A significant difference was observed, and the change in absorbance increased a little bit in comparison with that with the addition of only TMBZ. This result indicated that a higher change in absorbance was induced by the sequential enzymatic reactions. The H_2O_2 was newly produced by the enzymatic reaction of choline oxidase using Cho; furthermore, the produced H_2O_2 diffused effectively around the peroxidase, so a more effective sequential reaction system was prepared. On the other hand, a significant difference was not observed in the case of the enzyme solution, even though the total amount of each enzyme was greater. The sequential reactions were further examined using three kinds of substrates, Ach, Cho, and TMBZ. In the case of the nanoparticles, the highest change in absorbance at 450 nm was observed. It is conceivable that the Ach was converted to Cho, the newly produced Cho was also converted to H_2O_2, and the H_2O_2 was finally used by the enzymatic reaction of peroxidase. In this reaction, the newly produced substrates diffused effectively, and were used on the next enzymatic reaction. If the sequential reactions were carried out in an enzyme-mixed solution, no significant difference was observed. This result clearly indicated that the enzymatic activity was strongly dependent on the local concentration of each enzyme and the effect of substrate diffusion in the solution. The local concentration was estimated by the following calculations. If the radius of the nanoparticles was 180×10^{-9} m, then the volume and surface area could be calculated as 2.4×10^{-20} m^3 and 4.0×10^{-13} m^2, respectively. If we assume the density of the nanoparticles at 1 g/cm^3, 1 mg of nanoparticles would contain 4.2×10^{10} particles ($= (1 \times 10^{-9})/(2.4 \times 10^{-20})$). Therefore, the total surface area of nanoparticles in 1 mg was 1.7×10^{16} nm^2. If the conjugation efficiency were 40%, then 0.4 nmol (2.4×10^{14} molecules) of biomolecules would be immobilized onto the nanoparticles. From these calculations and assumptions, 70 nm^2 was occupied by one biomolecule. In the case of bovine γ-globulin (BγG; molecular weight roughly 1.7×10^5 m^2), the BγG molecule occupied 15−100 nm^2 in end-on and side-on absorption.[23] From this, a conjugation efficiency of 40% was roughly equivalent to that of a monolayer of immobilized biomolecules. Therefore, we concluded that the local concentration of bioconjugated nanoparticles was nicely packed onto the surface as a monolayer. Taking these results into account, multimode enzyme immobilization onto nanoparticles represents a promising technique to enhance the bioactivity of each individual enzyme, and thus, an effective sequential reaction was realized.

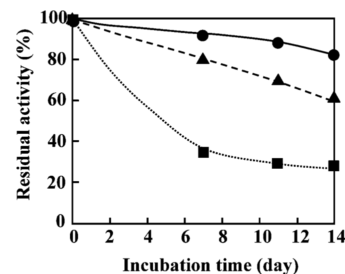


Figure 5. Stability of enzymes on nanoparticles. Circle, triangle, and square symbols indicate residual activity stored at 4, 25, and 40 °C, respectively.

Stability of Biomolecules on Nanoparticles. The immobilization of biomolecules is a useful technique for tuning their properties. However, the stability of these immobilized biomolecules is an important factor, since their bioactivity is sometimes decreased by immobilization onto a substrate. Here, we discuss the stability of these biomolecules. Horseradish peroxidase-labeled IgG was immobilized onto the phospholipid polymer nanoparticles. The bioconjugated nanoparticles were stored and incubated at given temperatures: 4, 25, and 40 °C. The residual enzymatic activity of peroxidase was then evaluated after the incubation (Figure 5). In the case of incubation at 4 °C, over 80% of the residual enzymatic activity was observed, even if the bioconjugated nanoparticles were stored for 2 weeks.

This higher enzymatic activity can be utilized in the field of bioengineering, and the activity was kept for a further 2 weeks. This result indicated that the immobilized condition was adequate for the biomolecules, and the bioactivity could be maintained for a long period. The nanoparticle surface was densely covered with the phosphorylcholine groups. In our previous reports, an enrichment of phosphorylcholine groups provided an environment for maintaining bioactivity.[14,15] In the case of nanoparticles, a similar property may be exerted by the phosphorylcholine group, and may enhance the functions of these biomolecules. The residual enzymatic activity was also dependent on the incubation temperature. If the bioconjugate nanoparticles were stored at 25 °C, the residual activity decreased a little bit, but was still roughly 60% after 2 weeks of incubation. In the case of 40 °C, the residual activity quickly disappeared, so only 30% of the activity remained after 1 week of incubation. Thus, the immobilized enzyme would denature and/or aggregate through the incubation at higher temperatures, even if the immobilized surface were covered with phosphorylcholine groups. Taking these results into account, the phospholipid polymer nanoparticle stabilized the biomolecules to produce greater biofunction; the residual bioactivity was perfectly maintained below 25 °C for 2 weeks.

Phospholipid polymer nanoparticles represent an amazing discovery for various applications, such as biomedical diagnosis, drug carriers, and regenerated medicines.

Conclusions

Nanoparticles covered with both phosphorylcholine and active ester groups for bioconjugation were prepared by a solvent evaporation method in an aqueous polymer solution. The diameter was estimated at 360 nm by dynamic light scattering. The total amount of active ester groups was 1 nmol/mg of nanoparticles. The highest conjugation efficiency was observed at 4 or 25 °C, and 40% of the active ester groups were assumed to be occupied for bioconjugation. The remaining active ester groups were easily masked by small molecules. The multimode immobilization of biomolecules was examined for sequential enzymatic reactions. Acetylcholinesterase, choline oxidase, and horseradish peroxidase-labeled IgG were immobilized onto the nanoparticles, and the total amount of immobilized enzymes was considered to be ca. 40% of the active ester groups on the surface. The sequential enzymatic reactions proceeded effectively in comparison with those of the enzyme solution, because of the higher local concentration of each enzyme and the effective diffusion of the newly produced substrates. The local concentration of the biomolecules was estimated; one biomolecule occupied 70 nm^2 of surface area. In the case of BγG, 15−100 nm^2 would be occupied by monolayered coverage. Therefore, the conjugated biomolecules were tightly packed onto the surface. Furthermore, the phospholipid polymer nanoparticles provided for stabilization of the biomolecules; in particular, it was shown that the bioconjugated nanoparticles could be stored at below 25 °C. The nanoparticles with a phospholipid polymer showed high performance for sequential enzymatic reactions.

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