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SHORT COMMUNICATION

Thermal- and dispersion-stable lipase-installed gold colloid: PEGylation of enzyme-installed gold colloid

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Abstract Gold colloid possessing both lipase and PEG-tethered chains on the surface was prepared by the adsorption of lipase, followed by the immobilization of the PEG/polycation block copolymer on the colloid surface. The obtained colloid showed high dispersion stability up to 0.3 M NaCl concentration. The enzymatic activity of the lipase on the colloid complex was equivalent to the native enzyme. Surprisingly, more than 95% of the initial enzymatic activity was retained after repeated thermal treatments (five times) at 58 °C for 10 min. The PEG condensed layer between the immobilized enzyme on the gold colloid may prevent the denaturation of the enzyme at high temperature.

Keywords Gold colloid · Lipase · Poly(ethylene glycol) · Dispersion stability · Thermal stability

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Introduction

Due to the high stereoselectivity and regiospecificity under mild conditions in aqueous media, enzymes have been widely utilized as an excellent environmentally benign catalyst [1, 2]. Due to the unstable polypeptide with a higher-order structure, however, the activity and selectivity of the enzymes are remarkably influenced by the variation in the environmental conditions such as the pH and the temperature. Another problem for the utilization of enzymes is the separation and recycling from the products. To improve the convenience of the enzymes, immobilized enzymes [2, 3], namely, enzymes conjugated to a solid support, are widely utilized. Solid supported enzymes have several benefits such as effective separation from the products, repetitive use, and improved stability of the enzymes. In addition to the improvement in the immobilization technology, the development in enzyme-related science and technology, namely genetic engineering, protein engineering, and evolutionary molecular engineering, has contributed to the expansion of the enzymatic-related industry. Thus, numerous efforts have been undertaken to develop insoluble immobilized enzymes for various applications in the fields of biocatalysis, bioprocessing, biospecific detection, cosmetics, etc. The immobilization of enzymes on the solid substrate, however, causes several problems [4]. One of the most severe problems is a decrease in the enzymatic activity when compared to the native enzymes. One of the main reasons for this reduction is due to the denaturation of the enzyme during the immobilization on the substrate surface. The surface enzyme also has a chance to be denatured by the recycling process of the solid support.

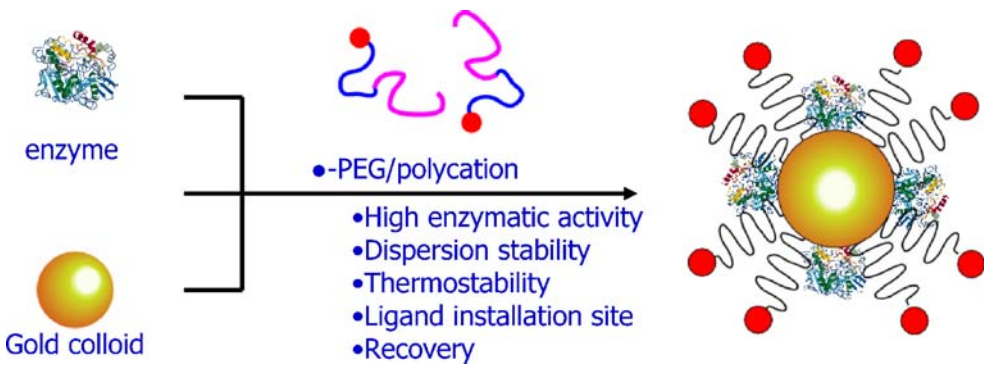
Immobilization of protein has also been carried out using nanoparticles. Proteins adsorbed on gold colloid surface have been widely used in the field of electron microscopy

since the middle of the 1970s [5]. It was confirmed that most of the proteins on the gold colloid surface retained their bioactivities [6–8]. Recently, several reports on the conformation and orientation of the proteins immobilized on nanoparticles have become available [9]. Macronald and Smith [10] reported that cytochrome *c* adsorbed on a silver colloid showed a specific orientation, which was monitored by surface-enhanced Raman scattering. Natan et al. [11] reported that a cytochrome *c*–gold colloid conjugate (12 nm) on a silver surface was more stable than free cytochrome *c* adsorbed on the silver surface in terms of orientation. They concluded that the cysteine in the cytochrome *c* interacts on the silver surface to retain its conformation on the surface. Sastry et al. [12–14] revealed that the enzymatic activity of a pepsin–gold colloid conjugate showed a similar initial activity as that of the free pepsin. In addition, the stability of the enzymatic activity was higher than that of the free pepsin (10% of the initial activity was retained after 4d, while the free pepsin retained 5% under the same conditions). Rotello et al. [15, 16] reported the immobilization of chymotrypsin on a gold colloid, the surface of which was modified by a charged self-assembled monolayer. The enzymatic activity was strongly dependent on the substrate charge because of its electrostatic interaction with the conjugate surface. Thus, proteins on the nanosphere surface are one of the important targets to investigate, not only for scientific, but also industrial point of views. We now report the preparation of a gold colloid possessing both an enzyme and PEG co-immobilized surface. PEG tethering chains on the surface improved not only the dispersion stability but also the significant thermal stability of the complex (Scheme 1).

Experimental

Materials Commercial lipase from *Candida rugosa* (EC 3.1.1.3; Sigma Chemical, St. Louis, MO), 10 nm gold colloid (British Biocell International, 10 nm, 0.1 mg/ml), and *p*-nitrophenyl propionate (Wako Pure Chemical Industries, Osaka, Japan) were used as received. Acetal-ended poly (ethylene glycol)-b-poly[2-*N*,*N*-(dimethylamino)ethyl methacrylate] (acetal–PEG/PAMA) was prepared according to our original synthetic method [17]. Briefly, after potassium 3,3-diethoxypropanolate (PDP, 1 mmol) was prepared by the reaction between the corresponding alcohol and potassium naphthalene in THF (45 ml), 113.5 mmol of condensed ethylene oxide (EO; Sumitomo Seika, Japan) was added via a cooled syringe to the PDP solution. After a 2-day reaction of EO, 60 mmol of AMA (Wako Pure Chemical Industries) was added to the reaction mixture and stirred for an additional 60 min at ambient temperature for the block copolymerization. The block copolymer was recovered by precipitation into a large excess of 2-propanol. The small amount of remaining PEG prepolymer was removed by Soxhlet extraction with THF after the protonation of the PAMA segment. The molecular weights of both segments in the resulting block copolymer, PEG and PAMA, were 2,500 and 4,000, respectively (M_w/M_n, 1.35). The functionality of the end acetal group was almost quantitative, which was analyzed by ^{1}HNMR. To convert the end acetal group to an aldehyde group, the block copolymer was dissolved in an acetic acid/water mixture (10:1 v/v) and then stirred for 5 h at 35 °C. After the reaction, the mixture was neutralized by NaOH and dialyzed against water.

Preparation of PEG/enzyme co-immobilized gold colloid The immobilization of lipase on a gold colloid was carried out by mixing 1 ml of lipase solution (60 mg/l) in phosphate buffer (pH=7.0; 25 mM) and 5 ml of a gold colloid solution, followed by dilution to 10 ml using the same buffer. Acetal–PEG/PAMA modifications were carried out after the lipase modification on the gold colloid. We have previously reported that the PEG/polycation block copolymer was strongly adsorbed on the gold colloid surface and stabilized under physiological conditions [18]. The synthesized acetal–PEG/PAMA was used for the co-immobilization on the gold surface. The acetal group at the end of PEG chain can be converted to an aldehyde group by the acid treatment, which can be utilized for the installation of second function to the colloid complex such as a ligand molecule for specific molecular recognition (which may be useful for ligand-



Scheme 1 Gold colloid possessing both an enzyme and PEG co-immobilized surface

directed accumulation of enzyme to tumor site for pro-drug therapy). To a prepared lipase-installed gold colloid solution, 100 μl of an aqueous solution (0.1 mM acetal–PEG/PAMA in phosphate buffer, pH=7.0, 25 mM) was added with stirring. The colloid solution was then dialyzed against water (MWCO=12,000–14,000) and finally lyophilized with water.

Estimation of enzymatic reactivity To 2 ml (0.5 mM) of the substrate solution in phosphate buffer (pH=7.0, I=25 mM), 1 ml of the enzyme-immobilized colloid solution (6 mg/l lipase in phosphate buffer, pH=7.0, I=25 mM) was added. The reaction was monitored by the change in absorption of a liberated 4-nitrophenol by the hydrolysis reaction of NPP as a function of time. To obtain the information on the stability of the gold colloid complex, the enzymatic reactivity was monitored after the thermal treatments, viz., once the enzyme–colloid complex solution was heated at 58 °C for 10 min, followed by cooling to 25 °C to measure the enzyme activity. This heating treatment was repeated several times and the enzymatic activity was monitored as a function of the heating times.

Measurements UV–vis spectra were recorded using a Shimadzu UV-2400PC spectrometer with a 1-cm quartz cell.

Results and discussions

Since the lipase (*C.rugosa*; Sigma, EC 3.1.1.3) possesses the isoelectronic point of ca. 4.5, it is negatively charged under the physiological conditions [19]. Thus, the adsorption of the lipase on the gold colloid surface was not by the electrostatic interaction because of the negatively charged gold colloid prepared by the citrate reduction. To confirm the immobilization of lipase on the gold colloid, a surface plasmon resonance was monitored under several immobilization conditions. The immobilization of lipase on a gold colloid was carried out by mixing 1 ml of lipase solution in phosphate buffer and 5 ml of gold colloid solution, followed by dilution to 10 ml using the same buffer. It is well-known that the surface plasmon spectrum of a gold colloid is influenced by the adsorbed protein [20]. Figure 1 shows the surface plasmon spectra of the gold colloid changing the ratio of gold vs lipase. With the increasing ratio of lipase vs the gold colloid, the maximum wavelength showed a bathochromic shift up to 1.5 mg lipase/mg gold colloid, indicating the effective adsorption of lipase on the gold surface as shown in Scheme 1. Sato et al. [21] reported that lipase (*Rhizopus delemar*) was adsorbed on the gold surface nonspecifically up to saturation of monolayer adsorption, which was monitored by quartz crystal microbalance measurements. Nonspecific adsorption

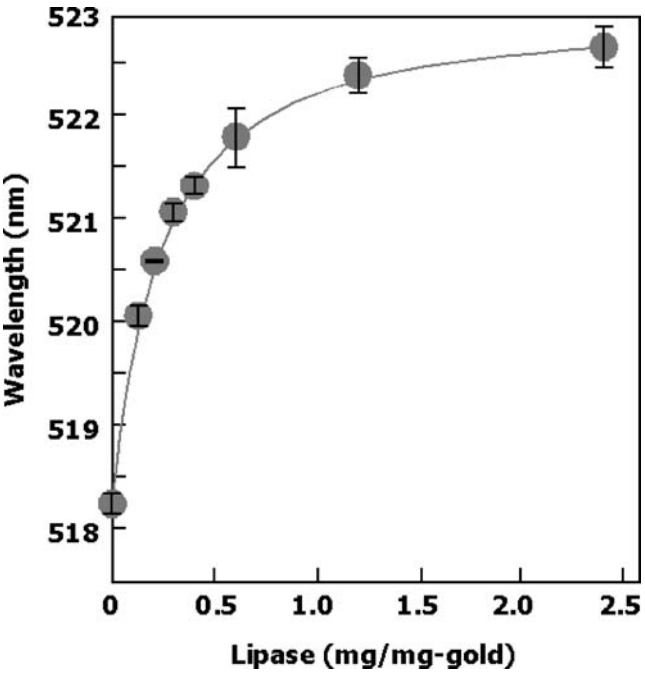
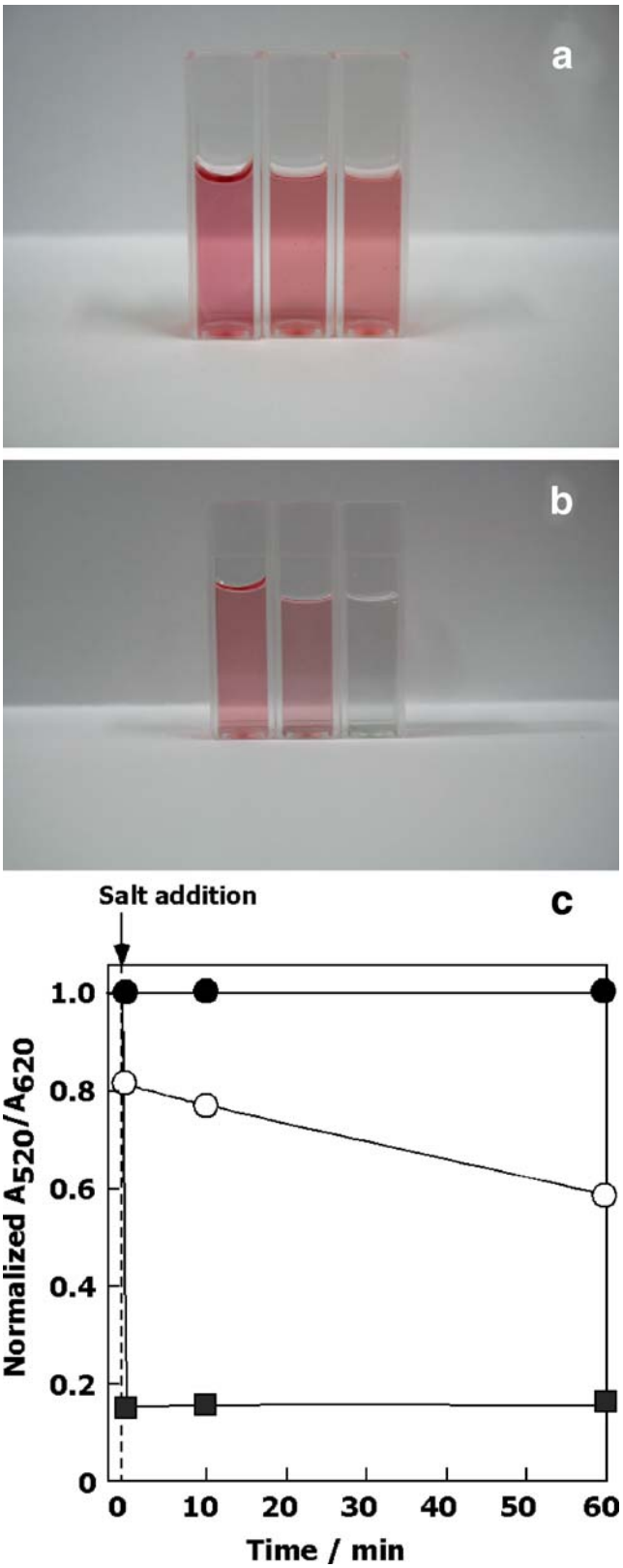


Fig. 1 Change in maximum wavelength of surface plasmon spectra of gold colloid as a function of lipase concentration

might also take place in the present study. PEG modifications were carried out after the lipase modification on the gold colloid. We have previously reported that the PEG/polycation block copolymer was strongly adsorbed on the gold colloid surface and stabilized under physiological conditions [22]. The acetal–PEG/PAMA was used for the co-immobilization on the gold surface. The acetal group can be utilized for the installation of a second function to the colloid complex, such as a ligand molecule for specific molecular recognition for pro-drug therapy. To a prepared lipase-installed gold colloid solution, an aqueous solution of accetal–PEG/PAMA was added with stirring. The colloid solution was then dialyzed against water and finally lyophilized with water.

A colloidal gold particle in aqueous media is known to be stabilized by the electrostatic repulsion of the charged surface [19]. For example, the citrate-reduced gold colloid has a zeta-potential of less than −30 mV at pH=7.4 [23]. When the gold colloid is used under physiological conditions, however, the electrostatic repulsive force is not enough to disperse the colloid due to high ionic strength (I=0.15 M). The gold colloid prepared in this study possessed PEG chains between the lipase enzymes on the surface; thus, a high dispersion stability under a high ionic strength is anticipated. Figure 2a,b show pictures of an enzyme-installed gold colloid before and after the addition of sodium chloride, respectively. Even when sodium chloride was added to the PEG/enzyme co-immobilized gold colloid at 60 °C (0.3 M), no change in the color of the solution was observed, which was in sharp contrast to that



◀ **Fig. 2** Photographs of gold colloid (*right*), lipase immobilized gold colloid (*center*), and PEG and enzyme co-immobilized gold colloid before (**a**) and 60 min after the addition of 0.3 M NaCl at 60 °C (**b**). **c** Change in A_{520}/A_{620} ratio of gold colloid (*square*), lipase immobilized gold colloid (*open circle*), and PEG and enzyme co-immobilized gold colloid (*closed circle*) after the addition of 0.3 M NaCl

of the citrate gold colloid without any surface modification. Actually, an immediate change in the color (red to purple) was observed just after the addition of NaCl (0.3 M). In the case of the enzyme-immobilized gold colloid, the color of the solution retained a bright pink color. However, a slight but definite decrease in the color was observed. After 60 min at 60 °C, a small amount of black precipitate was observed in this case (Fig. S2). It is well-known that the absorption at 620 nm increased along with a decrease in the peak at 520 nm when the gold colloid was coagulated (Fig. S3). The change in the ratio of absorption at 520 nm vs that at 620 nm was monitored as a function of time. As can be seen in Fig. 2c, the A_{520}/A_{620} value immediately decreased with the addition of the salt to the citrate gold colloid. In the case of the enzyme-immobilized gold colloid without PEG-tethered chains, a gradual decrease in the ratio was observed, indicating that a gradual coagulation took place under the present conditions. On the contrary, no spectral change was observed in the case of the PEG/enzyme co-immobilized gold colloid. It is confirmed that the PEG surface modification makes the enzyme–gold colloid conjugate extremely stable.

Using 4-nitrophenyl propionate (NPP) as a substrate, an enzymatic reactivity of the colloids was then investigated. The enzymatic reaction was monitored by the change in absorbance of a liberated 4-nitrophenol by the hydrolysis reaction of NPP as a function of time. To obtain the information on the thermal stability of the lipase on the gold colloid complex, the enzymatic reactivity was monitored after the thermal treatments, viz., once the enzyme–colloid complex solution was heated at 58 °C for 10 min,

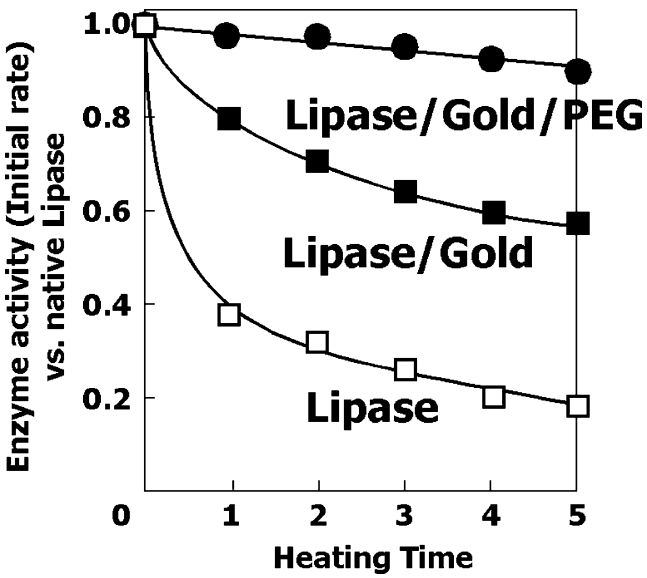


Fig. 3 Stability of enzyme activity against thermal treatments

followed by cooling to 25 °C to measure the enzyme activity. This heating treatment was repeated several times, and the enzymatic activity was monitored as a function of the heating times. It is interesting to note that the initial enzymatic activity of the lipase on the gold surface and the PEG-modified gold surface showed almost the same reactivities (0.054 and 0.053 μM/s, respectively) as compared to the native enzyme (0.054 μM/s). The adsorption of lipase on the gold surface did not reduce its activity. When free lipase was heated once to 58 °C for 10 min, the enzyme lost more than half of its reactivity. After the second heating of the free enzyme solution, only less than one third of the activity remained. As Sastry et al. [12–14] pointed out, the activity of the enzyme on the gold colloid surface was more stable than that of the free enzyme though the mechanism was not confirmed in detail. In the case of lipase–gold colloid complex in this study, ca. 60% of the enzymatic activity was retained after the fivefold thermal treatments as shown in Fig. 3 (closed square). It is rather surprising that the lipase–gold colloid possessing PEG-tethered chains on the surface retained more than a 95% enzymatic activity even after the fivefold thermal treatments. The gold colloid possessing both the enzyme and PEG-tethered chain surface showed an extremely high stability having the same enzymatic activity as that of the free enzyme. The PEG-condensed layer between the immobilized enzyme on the gold colloid may prevent the denaturation of the enzyme at high temperature.

In conclusion, a thermally stable gold colloid–lipase conjugate was prepared by the co-immobilization of the PEG-tethered chain on the surface using PEG/polycation block copolymers. The obtained complex showed high dispersion stability under high ionic strength. Since the surface of the complex possesses reactive PEG-tethered chains, namely, the free end of the PEG chain possesses a reactive acetal group, it is anticipated to be a key nanobiomaterial, which can be utilized not only in vitro but also in vivo.

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