Enzyme Immobilization on Gold/Fe-Oxide Composite Nanoparticles Using a Methionine Tag

Satoshi Seino^{a#}, Kazuma Watanabe^{a#}, Toshihiro Shikakura^a, Michika Abe^a,
Takashi Nakagawa^a, Yuichi Koga^a, and Takao A. Yamamoto^a

^aGraduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, 565-0871,
Japan

Corresponding Author:

Address: Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita,
Osaka, 565-0871, Japan
E-mail: kz-watanabe@mit.eng.osaka-u.ac.jp
Tel/Fax: +81-6-6879-7886

Author Contributions

[#]S. Seino and K. Watanabe contributed equally to this work.

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Abstract

We developed a novel technique for immobilizing enzymes on magnetic nanoparticle surfaces by using a methionine-tag system. Au/Fe-oxide composite nanoparticles synthesized through a radiochemical process were used as magnetic nanocarriers. The C-terminus of Tk-subtilisin, a model enzyme, was modified with a methionine tag and mixed with Au/Fe-oxide composite nanoparticles for immobilization via Au-S bonding. Methionine-tagged immobilized enzymes showed 98% residual specific activity, while the untagged enzymes showed 78%. The methionine-tagged immobilized enzymes retained their activities in a wide temperature range of 30–70 °C. Thus, the methionine-tag system provided orientational immobilization via the formation of Au-S bonds, which resulted in structural stability of the immobilized enzymes.

Keywords

Enzyme immobilization, composite nanoparticle, enzyme activity, Au, iron oxide, Tk-subtilisin

1. Introduction

Enzymes immobilized onto the surface of functional materials have numerous advantages over the soluble state, such as the possibility of recovery and re-use, improved stability and activity, and simple operation [1-6]. Nanoparticle materials have been recently studied as new immobilization carriers, including magnetic nanoparticles (MNPs), which are attractive carriers due to their unique functionalities. Enzymes immobilized onto MNPs were studied for repeated or continuous usage, specific target sensing, control of activity by magnetic field, and other advantages [7-10].

Immobilization of enzymes onto MNPs by adsorption, covalent reaction, or cross-linking has been previously reported [7, 11]. Although adsorption is the simplest method for immobilization, enzymes can be desorbed from the MNP surface during utilization. Chemical bonding provides a much more stable attachment, though deactivation of immobilized enzymes is often observed as the immobilization sites are non-specific which may induce structural changes in enzymes [7].

The aim of this study was to selectively immobilize enzymes onto MNPs without causing structural changes. Towards this purpose, we have adopted a novel tag system, “Methionine Tag” (Met-tag) [12], which is shown in Fig. 1. Composite nanoparticles (CNPs) consisting of Au and Fe-oxide were used as magnetic carriers and mixed with enzymes functionalized with the Met-tag. Tagged enzymes are expected to orient using Au-S bonding between Au grains and the Met-tag.

2. Materials and methods

2.1. Preparation of Au/Fe-oxide CNPs

The synthesis of Au/Fe-oxide CNPs has been previously described [13, 14]. Resovist[®] (Fujifilm Pharma Co., Ltd.) was used as MNP carriers, which consists mainly of γ -Fe₂O₃ nanoparticles coated with carboxydextran, and it was developed as a liver-specific magnetic resonance (MR)

contrast agent. The average size, as determined by transmission electron microscopy (TEM), was approximately 5 nm. MNPs (12 mg) were dispersed in 120 mL of aqueous solution containing HAuCl_4 , 2-propanol, and polyvinylalcohol (PVA, 22 kDa). The concentrations of HAuCl_4 and 2-propanol were 0.5 mM and 125 mM, respectively, while the PVA concentration was controlled at 10 g/L. The dispersion was irradiated with a high energy electron beam (4.8 MeV, Japan Electron Beam Irradiation Service, Ltd.) at a dose of 3 kGy/sec for 2 sec. CNPs were purified through a dialysis process as follows. First, the irradiated suspension was set to Minikros Sampler[®] (MWCO = 500 kDa, Spectrum Laboratories Inc., CA) with 5 L of pure water to remove remaining unreduced Au^{3+} ions and excess polymers. For further purification, the sample dispersion was sealed in a dialysis membrane (Spectra/Por[®], MWCO = 100 kDa, Spectrum Laboratories, Inc.) and kept in pure water for 48 h at room temperature.

The Fe-oxide surfaces in the CNPs were modified with phosphorylcholine (PC) groups to suppress non-specific adsorption [12]. In brief, PC modification agent (10 mL) and trimethylamine (60 μL) were added to 5 mL of CNP solution (1 mg CNP/mL), sonicated for 10 min at 60°C to modify MNP surfaces with PC modification agent, and placed in an oven at 80°C. PC-modified nanoparticles were precipitated, collected, and purified by magnetic decantation. After surface modification, CNPs were washed 3 times with distilled water (15 mL each) and dispersed in 5 mL of distilled water. After washing, CNPs showed good dispersibility in pure water and buffer solution.

2.2. Enzyme immobilization

A hyperthermostable subtilisin-like serine protease from *Thermococcus kodakarensis*,

Tk-subtilisin, was used as a model enzyme for immobilization on the nanoparticles.

Tk-subtilisin shows a relatively higher proteolytic activity compared to other mesophilic subtilisin family proteases and shows the highest proteolytic activity at 90°C [15].

A Met-tag system was constructed to immobilize Tk-subtilisin onto CNPs via Au-S bonding

[12]. Met-Tag is an octa-peptide (Met-Met-Gly-Met-Met-Gly-Met-Met) designed to specifically bind CNPs via a coordinated bond between Au and methionine side chains. The Met-Tag was attached to the C-terminus of Tk-subtilisin (Tk-sub-Met-tag) to immobilize Tk-subtilisin on CNPs. The gene encoding the Pro-form of Tk-subtilisin and the sequentially attached Met-tag were chemically synthesized (GenScript, Piscataway, NJ) and cloned into pET-25b using NdeI and BamHI sites. The corresponding Pro-Tk-subtilisin derivative was overexpressed in *Escherichia coli* BL21(DE3)-CodonPlus in inclusion bodies, solubilized using 20 mM Tris-HCl (pH 9.0) containing 8 M urea and 5 mM EDTA, and purified in a denatured form as previously described [16]. The purified Pro-form enzyme was refolded by dialysis using 20 mM Tris-HCl (pH 7.0) containing 5 mM EDTA and 1 mM DTT, and then denatured in the same buffer containing 6 M guanidine hydrochloride. The concentration of denatured enzyme was adjusted to 30 μ M and then refolded by diluting 100-fold in 50 mM CAPS (pH 9.5) containing 10 mM CaCl₂ and 1 mM DTT. Finally, the protein was matured to the active enzyme, Tk-sub-Met-tag, by incubating at 80°C for 30 min. One milliliter (12.4 μ g) of the aliquot was mixed with 124 μ g of CNPs and kept at 0°C for 1 h. The mixture was then centrifuged at 15,000 rpm for 15 min and the resulting pellet fraction washed with the same buffer.

Tk-subtilisin with C-terminal Met-tag (Tk-sub-Met-tag) was immobilized onto CNPs by the following procedures. Thirty microliter of a Tk-sub-Met-tag solution (MW = 34,000 Da, 12.4 μ g/mL) was added to 1 mL of refolding buffer (50 mM CAPS, 1 mM DTT, and 10 mM CaCl₂) and kept at 0°C for 10 min. One milliliter of CNP solution (124 μ g Fe/mL) was added and the solution kept at 0°C for 1 h. The solution was centrifuged (15,000 rpm, 15 min, 4°C) to remove free enzymes, washed twice with refolding buffer (1 mL each), and then dispersed in 1 mL of refolding buffer. For a control, Tk-subtilisin without Met-tag was also prepared following the same immobilization procedure.

2.3. Material characterization

The morphologies and sizes of the CNPs were observed by TEM (HITACHI H-8100T) at 200 kV. Inductively coupled plasma atomic emission spectroscopy (ICP-AES, ICPS 7500, Shimadzu Corp.) was used to determine nanoparticle concentration, Au/Fe ratio, and the amount of PC in the composite. Hydrodynamic particle sizes of CNPs were analyzed by Zetasizer 3000HS (Malvern Instruments, UK).

2.4. Measurement of enzyme activity

The amount of immobilized enzymes on the surface of CNPs was detected by radioisotope labeling using ^{125}I [17]. Radiolabeled Tk-subtilisin and remaining ^{125}I were separated using a PD-10 column. Ten microliter of labeled Tk-subtilisin was diluted with 990 μL of 300 nM unlabeled Pro-Tk-subtilisin. After dilution, Tk-subtilisin was incubated for maturation over a period of 30 min at 80°C. After incubation, the solution was centrifuged (15,000 rpm, 15 min, 4°C) to remove the precipitant. One hundred microliter of the supernatant was mixed with 900 μL of Tk-subtilisin solution to adjust Tk-subtilisin to a concentration of 300 nM. CNP (124 μg) was added to 300 nM labeled Tk-subtilisin and the mixture left to rest at 0°C. The mixture was centrifuged (15,000 rpm, 15 min, 4°C) to remove free enzymes and the precipitant washed twice with 50 mM CAPS-NaOH containing 1 mM DTT (pH 9.5). Radioisotope levels were determined using a Packard Cobra Quantum 5002 Gamma Counter.

The activity of free or conjugated Tk-sub-Met-tag was determined by measuring the rate of amidolysis of N-succil-AAPF-p-nitroamido (Suc-AAPF-pNA) (Sigma) as a substrate. Suc-AAPF-pNA (2 nmol) was prepared in 100 mM Tris-HCl (pH 8.0) for enzymatic reaction. One hundred microliter of an Au/Fe-oxide solution (Fe, Au: 2 mg/mL, enzyme: 2.1 $\mu\text{g}/\text{mL}$) was added to 900 μL of Suc-AAPF-pNA solution and incubated at the indicated temperatures for enzymatic reaction. The reaction mixture was sampled (100 μL) after an appropriate period of incubation and 5 μL of 5% acetate added to the aliquot to stop the reaction. The solution was

centrifuged (15,000 rpm, 15 min, 4°C) to remove CNPs and immobilized enzymes. The amount of p-nitroaniline released from the substrate was determined by absorption at 410 nm with an absorption coefficient of $8,900 \text{ M}^{-1} \text{ cm}^{-1}$ by automatic UV spectrophotometer.

3. Results and discussion

3.1. Au/Fe-oxide composite MNPs

Typical TEM images of Au/Fe-oxide CNPs before and after PC modification are shown in Figure 2. Au nanoparticles (seen as darker contrast) were observed supported on the Fe-oxide nanoparticles (seen as weaker contrast) to form CNPs. CNPs did not exist as monodispersed nanoparticles, but as small aggregates. The primary particle sizes of Fe-oxide MNPs were approximately 5 nm both before and after PC modification. Before PC modification, the average diameter of the Au grains was approximately $3.7 \pm 2 \text{ nm}$, while after PC modification, the average Au grain diameter grew to approximately $7.7 \pm 5 \text{ nm}$, indicating that PC modification results in increased Au grain size.

Hydrodynamic particle sizes of the Au/Fe-oxide composite NMPs before and after the PC modification were analyzed. Hydrodynamic particle sizes of CNPs decreased from 97.3 to 75.3 nm by PC modification, suggesting that the hydrophilic property of the PC group improved the dispersibility of CNPs.

3.2. Immobilization of Tk-subtilisin modified with Met-tag

Immobilized Tk-subtilisin on the surface of CNPs was estimated by radioisotope labeling which showed that the amount of Tk-subtilisin with Met-tag on PC-modified CNPs was $1.8 \mu\text{g}/\text{mg Au}$, while that of Tk-subtilisin without Met-tag was $1.4 \mu\text{g}/\text{mg Au}$. Without Met-tag, Tk-subtilisin is considered to be immobilized by physical adsorption onto Au particles. The number of Tk-subtilisin molecules immobilized on an Au particle was calculated from the present result, assuming that Au grains were spherically shaped with a diameter of 7.7 nm and a density of

19.3 g/cm³. The number of immobilized enzymes on an Au particle was 0.9 with Tk-subtilisin without Met-tag and 1.2 with Tk-subtilisin with Met-tag. These values are relatively lower than that reported for the immobilization of thiol-modified ssDNA, at approximately 155 molecules per 6.4 nm of Au particles [18]. However, the size of Tk-subtilisin estimated from its 3D structure (PDB: 2Z2Y) is 4 nm, thus the present results are considered reasonable. It is possible that the Met-tag in Tk-subtilisin functioned as a bonding site against Au nanoparticles in CNPs, resulting in higher immobilization.

Table 1 shows the residual enzymatic activities of immobilized Tk-subtilisin, normalized to the activity of free enzymes at optimum temperature. The residual activity of immobilized enzyme via chemical bonding (carbodiimide coupling) was relatively low (48%) [10]. With the carbodiimide coupling reaction, carboxyl groups in the enzyme are randomly used for bonding, such that the structure of the enzyme is changed and loses activity. Immobilized Tk-subtilisin on CNPs, however, showed relatively high residual enzymatic activity, with Tk-subtilisin with Met-tag retaining an activity of 98%, while Tk-subtilisin without Met-tag retained only 78% activity. Figure 3 shows the temperature dependence of the specific activities of immobilized enzyme. Each experiment was carried out three times, and the average values are shown together with the error bars. The enzymatic activity of immobilized Tk-subtilisin with Met-tag was similar to that of free enzymes in a wide temperature range from 30–70°C. Immobilized Tk-subtilisin without Met-tag also showed a similar result, though the temperature range was narrower (30–60°C). Tk-subtilisin without Met-tag is considered to be immobilized by simple adsorption, such that the enzymes are randomly oriented. Immobilization of enzymes with the Met-tag can prevent this loss in activity, thought to be due to oriented immobilization via Au-S bonding. However, activities of both immobilized enzymes decreased at 80°C; thus, immobilization may have prevented enzyme refolding from the deactivated structure at high temperatures.

4. Conclusion

Tk-subtilisin was successfully immobilized onto Au/Fe-oxide CNPs using a Met-tag system. The immobilized enzymes retained 98% of their activity following the application of the Met-tag system, which was significantly higher than that with conventional carbodiimide coupling method. Furthermore, the enzymatic activity was maintained in a wide temperature range. The Met-tag system provided orientational and selective enzyme immobilization onto MNPs, allowing the immobilized enzymes to retain their original activities. Therefore, the present Met-tag system would contribute to the development of effective immobilized enzymes onto nanoparticle carriers.

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Figure 1. Schematic image of enzyme immobilization onto Au/Fe-oxide composite nanoparticles.

Figure 2. Transmission electron microscopy and Au size distribution of Au/Fe-oxide composite nanoparticles (a) before PC modification and (b) after PC modification.

Figure 3. Temperature dependence of the specific activities of immobilized enzymes.

(a) Tk-subtilisin without Met-tag, (b) Tk-subtilisin with Met-tag.

Table 1. Residual enzymatic activities of immobilized enzymes.

Enzymes	Immobilization Method	Residual Activity (Optimum Temp.)
Tk-subtilisin without Met-tag	Carbodiimide Coupling	45% (25°C) ^[10]
Tk-subtilisin without Met-tag	Adsorption onto Au	78% (70°C)
Tk-subtilisin with Met-tag	Au-S bonding onto Au	98% (70°C)

Graphical Abstract

Methionine-tagged immobilized enzymes showed 98% residual specific activity, while the untagged enzymes showed 78%. The methionine-tag system provided orientational immobilization via the formation of Au-S bonds, which resulted in structural stability of the immobilized enzymes.