Immobilization of hexa-arginine tagged esterase onto carboxylated gold nanoparticles†

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Hexa-arginine tagged esterase was efficiently immobilized onto carboxylated gold nanoparticles (AuNP-COOH) and its enzyme activity was investigated by monitoring the absorption spectrum of an enzyme substrate, *p*-nitrophenol butyrate.

Biomolecules bound to a metal nanoparticle have demonstrated quite amazing properties such as highly efficient drug delivery through the blood–brain barrier,[1] facile electron transfer from glucose oxidase to a metal electrode without a mediator,[2] facilitated gene delivery through cell membranes,[3] and modulation of enzyme activities.[4] However, the grafting of proteins onto metal nanoparticles is especially complicated since the conformation of a protein is liable to change through nonspecific interactions, causing it to lose its unique enzymatic activities.[5] Among protocols that specifically anchor and separate a concerned protein, recombinant protein synthesis with an affinity ligand at the C- or N-terminal might be a breakthrough to avoid nonspecific interactions. A surface modified by Ni^{2+}–nitrilotriacetic acid (Ni^{2+}–NTA), for instance, has been widely used for the specific binding of polyhistidine tagged proteins. Moreover, in recent studies, oligo(ethylene glycol) has been reported to considerably suppress the nonspecific adsorption and the subsequent denaturation of proteins.[6] Herein, we report manufacture of a highly water dispersive gold nanoparticle (AuNP) that can efficiently immobilize hexa-arginine tagged esterase (Arg_6-esterase). While the carboxylated AuNP (AuNP-COOH) also showed non-specific interactions, Arg_6-esterase bound dominantly onto the surface of AuNP-COOH in the presence of other proteins and exhibited a considerable enzymatic activity.

For the reproducible immobilization of proteins with an adequate orientation, highly stable gold nanoparticles were prepared by surface modification of citrate-reduced gold nanoparticles with 16-mercaptohexadecanoic acid (MHA).[7] The AuNP reduced by citrate is known to be sufficiently hydrophilic but has a tendency to aggregate, depending on the micro-environment. This vulnerability was substantially improved by protecting AuNP with a long aliphatic thiol with a carboxyl end group. Fig. 1(a) shows a TEM image of highly monodisperse AuNPs spread on a copper grid with an average diameter of 13 nm. Taking the given concentration of gold salt into consideration and assuming perfect conversion to AuNPs, the concentration of AuNP is estimated to be about 18 nM. The high stability of the AuNP was manifested in several control experiments. First, AuNP solution in a vial was dried by continuous blowing of N_2 gas and was re-dispersed with distilled water. Second, the carboxylated AuNPs were washed out by centrifugation/redispersion cycles twice, and a working solvent was easily exchanged into methanol, DMF and DMSO. Third, a local plasmon band red-shifted up to ~ 630 nm as the pH was lowered by the addition of HCl. However, it reversibly recovered to the original band position (~ 525 nm) when concentrated NaOH solution was added dropwise (Fig. 1(b)).[8]

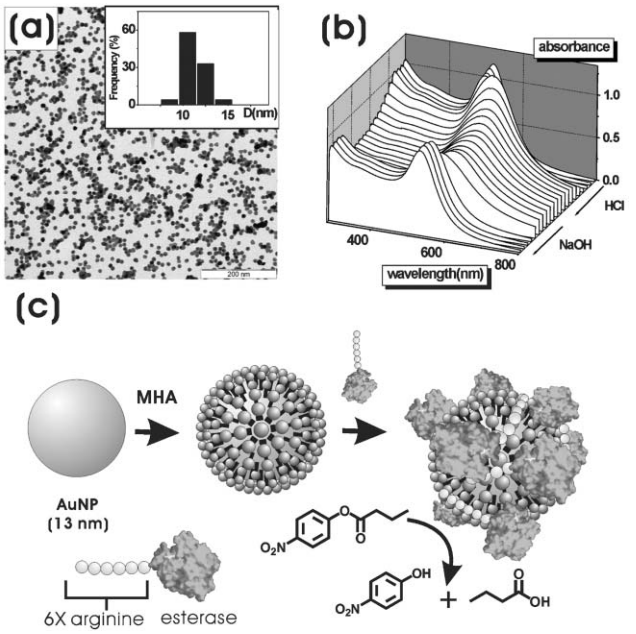

Fig. 1 (a) A TEM image of citrate reduced gold nanoparticles spread on a copper grid (inset: size distribution of particles observed in the picture). (b) A series of UV spectra as the solution pH varies by dropwisely adding HCl and NaOH solution sequentially. (c) Schematic illustration of surface modification of AuNP-COOH with Arg_6-esterase and the enzyme reaction by the immobilized esterase.

As shown in Fig. 1(c) schematically, the carboxylated AuNP functioned as a 'nano-supporter' to immobilize recombinant esterases with affinity tags (hexa-arginine and hexa-histidine, respectively) as well as intact ones. As the supporting material for enzymes, it is noteworthy that the esterases tested in the present study tended to adsorb onto AuNP-COOH irrespective of the presence of affinity tag, although the amounts were strongly dependent on the presence of adequate affinity tag. Furthermore, no agglomerate was formed during the adsorption of esterase onto the carboxylated nanoparticle. The high stability is attributed to

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negatively charged AuNP-COOH[9] and relatively low pI value of the concerned protein (for esterase, 5.9) compared to the working pH 8.0, which makes proteins bear net negative charges and repel each other. One important merit of protein immobilization onto AuNP might be the controlled surface roughness, which assures relatively homogeneous activities of adsorbed enzymes, compared to conventional polycrystalline gold surfaces.

The amount of esterase immobilized on AuNP was semi-quantitatively analyzed by SDS-PAGE. Unlike the agarose gel used in the recent studies on protein–nanoparticle complexes,[6] the polyacrylamide gel hardly permitted the present AuNPs to traverse its networked pores due to the smaller pore size; when a part of the SDS gel entrapping AuNPs was brought to the UV measurement, no absorption band of a staining dye was detected. As shown in Fig. 2, esterase immobilized on the AuNP-COOH definitely appeared at the same position as the proteins in a solution state ($\sim$ 42 kDa, black arrows). It is also noteworthy that only 250 μl of Arg_6-esterase containing AuNP-COOH was concentrated and analyzed (lanes 2 and 3), whereas 1 ml of His_6-esterase containing AuNP-COOH was used for analysis (lanes 5 and 6). Taking the aforementioned concentration of AuNP-COOH (18 nM) into consideration, a single AuNP-COOH was estimated to tether about 25 Arg_6-esterases. Similarly, 6 His_6-tagged and 4 non-tagged esterases were immobilized onto each AuNP-COOH. The enhanced adsorption characteristics of Arg_6-esterase might be attributed to electrostatic attraction between the positively charged polyarginine tag and AuNP-COOH with a slightly negative charge. This electrostatic attraction could be released by treatment with a concentrated electrolyte.[10]

As shown in Fig. 2, Arg_6-esterase adsorbed onto AuNP-COOH (lane 2) was soaked with 1 M NaCl for 10 min, centrifuged, and the resulting pellet of AuNP was analyzed by SDS-PAGE (lane 3). The amount of Arg_6-esterase remaining on the AuNP-COOH surface was substantially reduced after elution with 1 M NaCl. However, the amount of esterase with His_6-esterase, of which the histidine tag has a pK_a value of 6.5 and thus exists in a neutral state at the working pH 8.0, was almost identical to that of non-tagged esterase and was hardly changed by the treatment with 1 M NaCl (lanes 5 and 6). All these observations stress the importance of positive charges of the hexa-arginine tag in the large adsorption onto AuNP-COOH and the maintenance of enzyme activity (*vide infra*).[10,11] Furthermore, Arg_6-esterase seems to be dominant absorbant onto the AuNP-COOH in the presence of other proteins (Fig. 2). A number of contaminant protein bands as well as the Arg_6-esterase exist in the stock solution (lane 1), but the intensities of these contaminant protein bands were substantially reduced or even disappeared (as indicated by the white arrow in lane 2) after adsorption to AuNP-COOH. This relative specificity seems to be caused by faster adsorption of polyarginine tag onto AuNP-COOH than other non-tagged proteins.

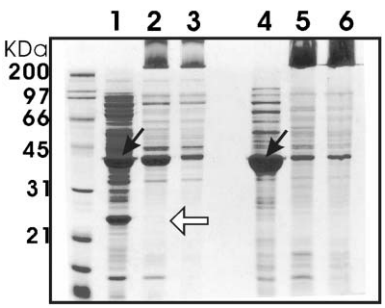

Fig. 2 Amounts of Arg_6-esterase (lanes 2 and 3) and His_6-esterase (lanes 5 and 6) tethered on AuNP-COOH (18 nM) before and after the elution with 1 M NaCl solution, in comparison to the bands obtained by loading the tagged esterase in the solution state (lane 1: Arg_6-tagged, lane 4: His_6-tagged).

The enzymatic activities of the esterases on the surface of AuNP-COOH were investigated by monitoring the UV/Vis absorption characteristics of the enzymatic substrate, *p*-nitrophenol butyrate (pNPB), which develops a new band at 400 nm as it dissociates. In Fig. 3(a), a series of absorbance measurements indicate that the enzymatic reaction in the AuNP-COOH solution (18 nM, 1 mL) containing Arg_6-esterase reached a saturation level within 5 min. The kinetic curves of the hydrolyzed substrate (pNPB, 0.1 mM) at various concentrations of His_6-esterase (in solution) are depicted as references in gray lines. Although absorbance measurements of His_6- and non-tagged esterases were made at the same concentration of AuNP-COOH, substantially lower enzyme activities were observed as shown in Fig. 3(a). The enzymatic activity of Arg_6-esterase immobilized on AuNP-COOH was more clearly seen when sixteen times diluted Arg_6-esterase on AuNP-COOH (1.1 nM) was introduced into the given pNPB solution (Fig. 3(a)).

From the reference curves, the amount of the active Arg_6-esterase was estimated and summarized along with the other two esterases in Table 1. Since 0.65 μg of Arg_6-esterase was estimated

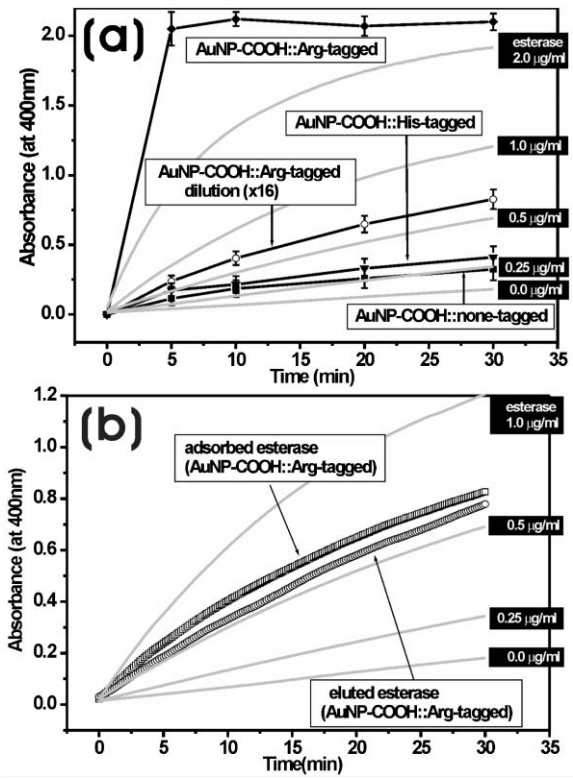

Fig. 3 (a) Enzymatic activity assay of Arg_6-tagged (concentrated and diluted), His_6-tagged, and non-tagged esterase tethered on AuNP-COOH *versus* incubation time with a hydrolyzing substrate, pNPB (0.1 mM). For Arg_6-tagged esterase, both concentrated and sixteen times diluted solutions are depicted simultaneously. (b) Enzymatic activity plot of Arg_6-tagged esterase on AuNP-COOH (diluted) before and after elution with 1 M NaCl.

Table 1 Total amounts of recombinant esterases with distinct affinity tags and amounts of the proteins in active form measured by diverse experimental methods

Affinity tagged esterase	Amount of immobilized esterase(SDS-PAGE)	Elution of immobilized esterase by 1 M NaCl		Amount of active esterase (UV/Vis)	Surviving activity
		[a]eluted(Bradford)	remaining(SDS-PAGE)		
Arg_6	18 μg/ml	9.5 μg/ml	8 μg/ml	10.4 μg/ml	58%
His_6	4 μg/ml	0.8 μg/ml	3 μg/ml	0.3 μg/ml	8%
none	3 μg/ml	0.5 μg/ml	2 μg/ml	0.2 μg/ml	7%

[a] The Bradford method quantified all the proteins eluted by 1 M NaCl, while SDS-PAGE results estimated a specific band (or protein) after the gel-electrophoresis.

to maintain its activity in the diluted solution, about 10.4 μg of the esterase existed in an active form in the normalized AuNP-COOH solution (18 nM, 1 mL). Taking the total amount of the immobilized Arg_6-esterase based on SDS-PAGE analyses into account, 58% of the esterase on an average was found to maintain its activity (*vide supra*). On the other hand, the His_6- and non-tagged esterases on AuNP-COOH demonstrated extremely small enzymatic activities of 0.3 μg/ml and 0.2 μg/ml, respectively (Fig. 3(a)), which corresponded to about 8% and 7% of the adsorbed esterases, respectively.

Although the reduced activity of the immobilized Arg_6-esterase (*i.e.* 58%) seems to be caused by a surface-induced conformation change, diffusional limitation in this bound enzyme system might also affect the apparent activity without actually fouling the structure of individual protein; the present esterase–AuNP complex can be an extreme case of the immobilized enzyme system compared to conventional polymer beads (with a diameter of $\sim$ 10 μm). To address this issue, a kinetic experiment was performed that measured initial reaction rates for several pNPB concentrations with fixed amounts of free and immobilized Arg_6-esterases; we assumed Michaelis–Menten type kinetics for the present system. As can be seen in Fig. S3 in the ESI, both free and bound Arg_6-esterases had the same K_m values (*x*-intercepts) in the double reciprocal plots for four pNPB concentrations ($>$ 0.05 mM). Accordingly, the esterases on the surface of AuNP can be concluded not to be restricted by the substrate diffusion at the working concentration ($\sim$ 0.1 mM).

It is not clear yet whether all the Arg_6-esterases on AuNP-COOH were homogeneously down-regulated through a surface-induced conformation change, or whether the immobilized esterases tested had quite diverse activities. In order to resolve this ambiguity, the Arg_6-esterase was eluted from the surface of AuNP-COOH with 1 M NaCl as shown in Fig. 3(b). When the Arg_6-esterase was eluted from the surface of AuNP-COOH with 1 M NaCl (see Fig. 3(b)), the enzymatic activity of the eluted Arg_6-esterase from AuNP-COOH (62.5 μl, 18 nM) was almost the same as that of the immobilized one before the elution. On the other hand, the remaining esterases on AuNP-COOH, which were resistant to the elution, were totally inactive. The eluted esterase was further quantified by the Bradford method and summarized in Table 1. It is noteworthy that the amount of the eluted esterase ($\sim$ 9.5 μg/ml) was quite comparable with the value ($\sim$ 10.4 μg/ml) estimated by its activity before the elution. Associated with the kinetic experiments, the result suggests that the immobilized esterases represent all-or-none type activity profiles. Most of the active esterase on AuNP-COOH seemed to be rescued intact with a concentrated electrolyte. This observation, in turn, supported the idea that the active esterases are bound on the AuNP-COOH through multivalent and electrostatic interactions between Arg_6-tag and carboxyl groups.

The Arg_6-esterase was efficiently adsorbed onto AuNP-COOH through electrostatic attraction with its enzymatic activity maintained, while most His_6-tagged and non-tagged esterases were nonspecifically adsorbed with a trace of activity. Nonetheless, it should be noted that a substantial amount of Arg_6-esterase was still nonspecifically adsorbed, and that only 58% of adsorbed esterases demonstrated an enzymatic activity. The reduced activity appears to be caused by hydrophobic interactions between proteins and the underlying hydrophobic region beneath the carboxyl group.[12] It is expected that the hydrophobic interaction, which results in protein denaturation, can be substantially alleviated by using oligo(ethylene glycol) as a spacer between the carboxyl end group and methylene chains.[12] In addition, a proton partition effect caused by negative charges accumulated at the interface can also result in the deterioration of acid-labile esterase.[13] Furthermore, this partition effect might explain the higher enzyme activity in Arg_6-esterase in that the local acidity at the interface can be effectively reduced through partial neutralization by more basic arginine groups.

In summary, this paper describes the efficient immobilization of recombinant esterase with a polyarginine affinity tag onto AuNP-COOH, which will find wide applications in diverse research fields including biosensors, bioimaging, and proteomics.

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