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# Bioconjugates of Glucose Oxidase and Gold Nanorods Based on Electrostatic Interaction with Enhanced Thermostability

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**Abstract** Bioconjugates made up of an enzyme and gold nanorods (GNRs) were fabricated by electrostatic interactions (layer-by-layer method, LBL) between anionic glucose oxidase (GOD) and positively charged GNRs. The assembled processes were monitored by UV–Vis spectra, zeta potential measurements, and transmission electron microscopy. The enzyme activity assays of the obtained bioconjugates display a relatively enhanced thermostability behavior in contrast with that of free enzyme. Free GOD in solution only retains about 22% of its relative activity at 90 °C. Unexpectedly, the immobilized GOD on GNRs still retains about 39.3% activity after the same treatment. This work will be of significance for the biologic enhancement using other kinds of anisotropic nanostructure and suggests a new way of enhancing enzyme thermostability using anisotropic metal nanomaterials.

**Keywords** Gold nanorods · Enzyme thermostability · Glucose oxidase · Polyelectrolytes

## Introduction

Considerable effort has been devoted to the study of gold nanoparticles with variable size and shape due to their unique geometry-dependent optical, electronic, and catalytic properties in electronics and optics [1–5], particularly in the fields of biotechnology and nanotechnology [6–8]. Recently, gold nanorods (GNRs) have attracted interest due to their unusual properties in electronics and optics [9, 10] and especially in bionanotechnology fields involving bioimaging [11, 12], biosensing [13–16], DNA expression [17], cancer therapy [18], etc. For GNRs, two distinct plasmon bands, a transverse mode (∼520 nm) and a longitudinal mode (usually >600 nm), can be observed. This unique optical property of GNRs opens up fascinating applications as biologic and chemical sensors. A versatile layer-by-layer approach to the preparation of polyelectrolyte-coated GNRs films has been reported [19, 20], indicating that polyelectrolytes are effective coating reagents for the modification of GNRs.

The immobilization of an enzyme is one of the crucial factors in a range of biologic techniques. Proteins have traditionally been immobilized on to solid surfaces by a variety of techniques including physical adsorption, solvent casting, covalent binding, and electropolymerization [21]. Although enzymes have been immobilized on to the surface of polystyrene latex [22], gold nanoparticles (GNPs), or silica nanoparticles [23–25], there are few reports in which anisotropic nanoparticles have been used to conjugate the enzyme. In this work, GNRs were used to prepare a bioconjugate with an enzyme, using GOD as a model enzyme. The thermostability of the GNR/GOD bioconjugates was dramatically enhanced, and even at 90 °C, its relative activity still remained at about 39.3%.

## Experimental Section

Materials and reagents. GOD (EC 1.1.3.4, 211 U $mg^{-1}$ from *Aspergillus niger*) and peroxidase from horseradish (HRP, 969.65 U $mg^{-1}$) were purchased from Fluka. D-(+)-glucose (99%), Chloroauric acid ($HAuCl_4 \cdot 3H_2O$, 99.9%), L-(+)-Ascorbic acid (AA, 99+%), Silver nitrate ($AgNO_3$, 99.9%), and Cetyltrimethylammonium bromide (CTAB,

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98%) were purchased from Alfa-Aesar. *o*-Dianisidine and Sodium borohydride ($NaBH_4$, 98+%) were obtained from Sigma (USA). The polyelectrolytes, poly (sodium-4-styrenesulfonate) (PSS, Mw ~70,000 g/mol), and poly (diallyldimethylammoniumchloride) (PDADMAC, Mw ca. 200,000–350,000 g/mol) were obtained from Aldrich and used without further purification. All chemicals were used as received.

Synthesis of GNRs. GNRs were prepared according to the seed-mediated growth method. Briefly, a seed solution was prepared by mixing 5 mL of CTAB (0.2 M) and 5 mL of $HAuCl_4$ (0.5 mM) with 0.6 mL freshly prepared 10 mM ice-cold $NaBH_4$ solution. The color of the solution changed from dark yellow to brownish yellow under vigorous stirring, indicating the formation of the seed solution. After 5 h, this seed solution was used for the synthesis of the GNRs. In a flask, 75 mL of 0.2 M CTAB was mixed with 1.5 mL of 4 mM silver nitrate aqueous solution and 75 mL of 1 mM $HAuCl_4$. After gentle mixing of the solution, 1.05 mL 0.10 M AA was added. While continuously stirring this mixture, 180 μL of the seed solution was added to initiate the growth of the GNRs. These GNRs were aged for 24 h to insure full growth.

Polyelectrolyte Coating of GNRs. About 10 mL of as-prepared GNRs was centrifuged twice at 8,000 rpm for 10 min, the supernatant was discarded, and the precipitate was redispersed in 5 mL 1 mM CTAB. Subsequently, it was added dropwise to 5 mL of PSS (2 g $L^{-1}$, 1 mM NaCl) aqueous solution. After 1 h adsorption time, it was centrifuged twice at 8,000 rpm to remove excess polyelectrolyte and dispersed in 5 mL deionized water. Finally, the PSS-coated GNRs were added dropwise to 5 mL of PDADMAC (2 g $L^{-1}$, 1 mM NaCl) aqueous solution. After 1 h, it was centrifuged twice at 8,000 rpm to remove excess polyelectrolyte and dispersed in 5 mL of phosphate buffer solution (10 mM, pH 7.0).

Preparation of GOD/GNRs Bioconjugates. The combination of GOD and GNRs was achieved using electrostatic interaction. In detail, the above 1.5 mL cationic PDADMAC-coated GNRs was centrifuged at 8,000 rpm for 10 min, the supernatant was discarded, and the precipitate was incubated with 1.5 mL GOD (1 mg $mL^{-1}$) dissolved in phosphate buffer (10 mM, pH 7.0) for about 1 h at 30 °C. The resultant mixture was centrifuged to discard free GOD and washed by phosphate buffer containing Tween 20. The target GOD/GNR bioconjugates were finally dispersed under ultrasonication in 1.5 mL phosphate buffer solution (10 mM, pH 7.0) and stored at 4 °C.

Enzyme relative activity assays. The activities of free GOD at different temperatures were monitored using UV–Vis spectroscopy at $\lambda = 460$ nm based on the change in solution color, which results from the oxidation of *o*-dianisidine by the reaction product hydrogen peroxide from glucose in the presence of HRP. The chemical equations are as follows.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 + o\text{-dianisidine(reduced)} \xrightarrow{\text{peroxidase}} o\text{-dianisidine(oxidized)} + H_2O.$$

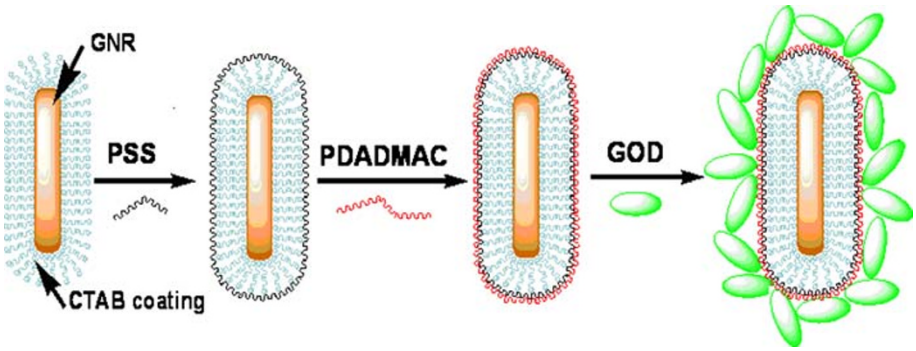
Typically, 2.5 mL of a 0.33 mM *o*-dianisidine solution in 0.1 M buffer, 0.3 mL 5 g $L^{-1}$ glucose solution, and 0.1 mL 0.02% HRP were mixed as substrate. Then, 10 μL of the free GOD solution (1 mg $mL^{-1}$) was added into the mixture, and the absorption of the mixture was recorded immediately and for the next 4 min. For the GOD/GNR system, the measuring procedures were the same as those for free GOD in solution except for the use of 10 μL GNRs@PSS@PDADMAC@GOD (GOD/GNRs bioconjugates) and 10 μL GNRs@PSS@PDADMAC instead of 10 μL of free GOD, respectively.

Control experiment. 1 mL PDADMAC (2 g $L^{-1}$, 1 mM NaCl) aqueous solution was mixed with 1 mL GOD (2 mg $mL^{-1}$) in a phosphate buffer (10 mM, pH 7.0) for about 1 h at 30 °C. The resulting concentration of GOD in the PDADMAC/GOD bioconjugates is 1 mg $mL^{-1}$. Then, an enzyme activity assay was performed.

Apparatus and measurements. UV–Vis absorption spectra were obtained using a UV-2550 spectrophotometer with temperature controller (S-1700, Shimadzu, Japan). Zeta potentials and size distributions were measured on a Zetasizer nano 90 and Zetasizer 3000HSA (Malvern, England), respectively. TEM was performed with a JEOL-JEM-1011 electron microscope under 100 kV accelerating voltage. Formvar-coated copper grids (200 meshes) were used as the support carrier.

## Results and Discussion

It is well known that a protein can be regarded as an anionic or cationic polyelectrolyte by simply adjusting the pH value of the protein solution to be higher or lower than its isoelectric point (pI). The formation of a protein–polyelectrolyte complex at pH > pI with polycations and at pH < pI with polyanions can be easily achieved. The bioconjugates of GOD and GNRs were fabricated using the electrostatic interaction between GOD and cationic polyelectrolyte-coated GNRs, as shown in Scheme 1. The GNRs used in this study were prepared by the seed-mediated growth method in cetyltrimethylammonium bromide (CTAB) surfactant solution [26] and were positively charged due to the




**Scheme 1** Schematic illustration of the fabrication of the GOD/GNR bioconjugates

coating of the CTAB bilayer. Because the free CTAB and the fixed CTAB on the substrate possess high cytotoxicity and cause denaturation of proteins [27, 28], the as-prepared CTAB-coated GNRs were treated with anionic poly (sodium-4-styrenesulfonate) (PSS), resulting in negatively charged PSS coatings on the GNRs. Subsequently, positively charged poly(diallyldimethylammoniumchloride) (PDADMAC) was absorbed on to them. In this case, anionic GOD in the phosphate buffer solution (10 mM, pH 7.0), in which the pI of GOD is 4.2, can easily be attached electrostatically on to the PDADMAC-coated GNRs, resulting in the formation of bioconjugates of GOD and GNRs. The resultant GOD/GNR bioconjugates display dramatically enhanced thermostability, much better than that of the free enzyme and even better than the reported results for GOD immobilized on planar substrates, polystyrene nanoparticles, and spherical gold nanoparticles.

Gold nanorods exhibit transverse surface plasmon resonance (TSPR) as well as longitudinal surface plasmon resonance (LSPR) bands. The UV–Vis spectra of the GNRs with different coatings are shown in Fig. 1A. For the as-prepared GNRs, there is a TSPR peak at about 520 nm and a LSPR peak at 691 nm (Fig. 1A, curve a). Compared with the LSPR peak of the as-prepared GNRs, there are red shifts for the GNRs with a PSS coating (697 nm, curve b) and with a PDADMAC coating (703 nm, curve c), showing that the polyelectrolytes are successfully adsorbed on to the GNRs via electrostatic interactions. After GOD (pH 7.0) solutions were added to the PDADMA-coated GNR suspension, the LSPR peak located at 723 nm was observed, as shown in Fig. 1A curve d. Compared with that of PDADMAC-coated GNRs, there is a red shift of about 20 nm indicating that the GOD has been electrostatically absorbed on to the GNRs.

Zeta potentials ($\zeta$) were measured to follow the formation of the GOD/GNR bioconjugates. The $\zeta$-potential of the coated GNRs was measured after deposition of each layer, as shown in Fig. 1B. The $\zeta$-potential of the as-synthesized GNRs is about +33.2 mV due to the presence of a bilayer of cationic CTAB on the surface of the GNRs. When the negatively charged PSS and the positively charged

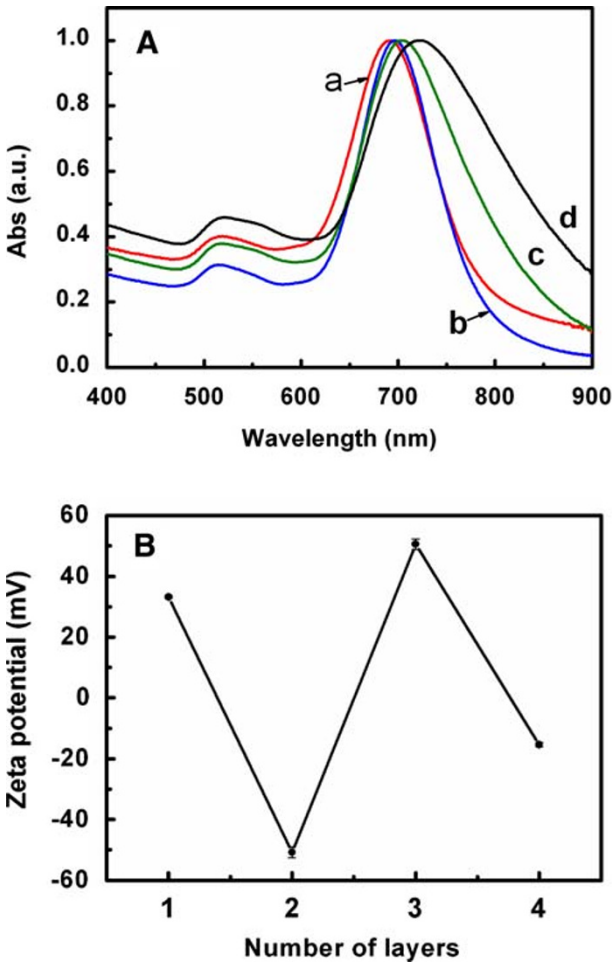



**Fig. 1** **A** UV–Vis spectra of CTAB-stabilized GNRs (curve a), GNRs/PSS (curve b), GNRs@PSS@PDADMAC (curve c), and GNRs@PSS@PDADMAC@GOD (curve d). **B** Zeta potentials of GNRs coated with multilayers, CTAB (first layer), and those sequentially coated with PSS (second layer), PDADMAC (third layer), and GOD (fourth layer), respectively

PDADMAC formed the outermost layer, negative and positive $\zeta$-potentials can be observed at −50.8 mV and +50.6 mV, respectively. When anionic GOD is adsorbed on to GNRs@PSS@PDADMAC, the $\zeta$-potential is about −15.4 mV. This is the qualitative evidence for the stepwise

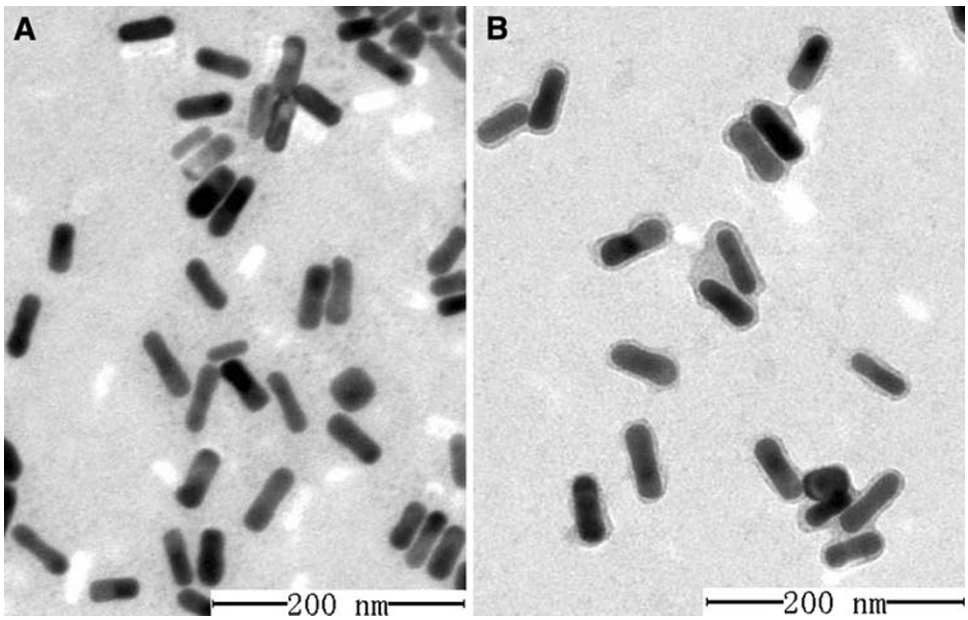



**Fig. 2** TEM images of the GNRs (**A**) and GOD/GNR bioconjugates after stained by uranyl acetate (**B**)

deposition of polyelectrolyte and GOD. Besides ζ-potentials measurements, size distributions of GNRs, GNRs@PSS@PDADMAC, and GNRs@PSS@PDADMAC@GOD were also performed, which is consistent with ζ-potential values (as shown in the supplementary material). The aspect ratio of the used GNRs is about 2.7 ± 0.4, and the thickness of the coating layer is about 5 nm, as shown in Fig. 2. This provides further evidence that the GOD was successfully adsorbed on to the GNRs.

The practical application of immobilized enzymes depends on their stability under various conditions, e.g., temperature. In this case, the activity and thermostability of GOD was examined for the GOD/GNRs. The optimum catalytic activity for free GOD was observed at pH 7.0 [29], and the isoelectric point of GOD is 4.2. Cationic PDADMAC-coated GNRs were, therefore, incubated with GOD in phosphate buffer (10 mM, pH 7.0), in which the GOD was negatively charged.

In order to address the influence of GNRs on the thermostability of GOD, the free GOD in solution and GOD immobilized on to the GNRs were exposed to a defined temperature for 15 min, and the enzyme activity assays [28] were immediately performed. In this work, the relative activity is defined as follows. For the same concentration of glucose, the UV–Vis absorbance of GOD/GNRs bioconjugates or free GOD at 460 nm, after the reaction with glucose, are represented at different temperatures as $A_{i,460}$, except $A_{max}$ at 40 °C, which was regarded as 100% activity. The relative activities of GOD at different temperatures were obtained from the ratio $A_{i,460}/A_{max}$. In order to eliminate the influence of the absorbance of GNRs on the values of $A_{i,460}$, the relative activities of the GOD immobilized on GNRs were obtained from $(A_{i,460} - A \cdot A_{i,0,460})/(A_{max} - A_{i,0,460})$, where $A_{i,0,460}$ represents the absorbance of GNRs@PSS@PDADMAC at 460 nm. In order to make the relative activities of free GOD and the GOD immobilized on GNRs comparable, the amount of GOD used to prepare the GOD/GNRs was the same as that of the free GOD used to measure the enzyme assays. Each set of experiments was carried out in triplicate to confirm the reproducibility of the system.

Figure 3 shows the relative activities versus temperature for the GOD immobilized on GNRs (GOD/GNRs), free GOD, and the GOD immobilized on PDADMAC (GOD/PDADMAC). The maximum activities were reached at around 30–40 °C for all of them. A similar increase in activity can be seen in the range 20–30 °C. A sharp reduction in activity with temperature is observed for all of

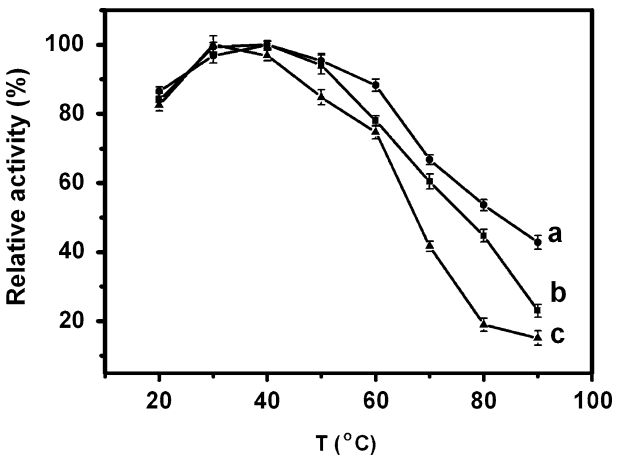



**Fig. 3** Enzyme thermostability of (**a**) immobilized GOD (**b**) free GOD, and (**c**) PDADMAC-blended GOD

them at temperatures over 40 °C. Surprisingly, when the temperature reached 90 °C, the relative activity of GOD/GNRs remained at about 39.3%. This showed that there is a relatively higher degree of thermostability for immobilized GOD. The relative activities of the free GOD and PDADMAC/GOD bioconjugates were only 22.0 and 15.4%, respectively, as shown in Fig. 3. This suggested that it is the structure of enzyme assembly that has great effects on enzyme activity.

## Conclusion

In summary, we have demonstrated that GOD can be successfully adsorbed on to polyelectrolyte-coated GNRs via electrostatic interactions. According to enzymatic catalysis examination, the GOD/GNRs bioconjugates have extraordinary stability at high temperature in contrast not only with the free enzyme in solution but also previously reported GOD systems with other nanoparticles. Therefore, GNRs can be expected to be a promising matrix for the immobilization of other kinds of enzymes and proteins with greatly enhanced stability for biosensor applications. The present results will be of significance for the biologic enhancement effects using other kinds of anisotropic nanostructures. Although the mechanism by which GNRs dramatically enhance the enzyme thermostability of GOD is still an open question, with further experiments to understand the detailed effect of GNRs on GOD being pursued, the present work has already suggested a new way of enhancing enzyme stability and will be of significance in designing new kinds of enzyme-based nanoreactors for biosensors and biocatalytic reactors using other kinds of anisotropic nanostructures.

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