Enzyme-Mediated Growing Au Nanoparticles for Electrochemical and Optical Immunosensors: Signal-on and Signal-off

Hongcheng Pan^{1,*}, Dunnan Li,¹ Jianping Li¹, Wenyuan Zhu¹ and Liang Fang²

¹ College of Chemistry and Bioengineering, Guilin University of Technology, 12 Jiangan Road, Guilin 541004, P. R. China.

² State Key Laboratory Breeding Base of Nonferrous Metals and Specific Materials Processing, Guilin University of Technology, 12 Jiangan Road, Guilin 541004, P. R. China
 *E-mail: hcpan@163.com

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We report a versatile strategy for electrochemical and optical signal-on/off immunosensing, on the basis of enzyme-mediated growing Au nanoparticles. By applying antibody and enzyme (glucose oxidase or catalase) conjungated Au nanoparticles as secondary antibodies, the sandwich-type immunosensor can achieve either a "signal-on" or a "signal-off" electrochemical / optical detection. Scanning electron microscope, electrochemical, and spectral studies revealed that the AuNPs growth is dependent on H_2O_2 concentration, which is mediated by glucose oxidase or catalase. More AuNPs lead to larger electrochemical and optical signals, and vice versa. As a proof-of-concept, the immunosensor platform was used to detect immunoglobulin G in human serum.

Keywords: Au nanoparticles; immunosensor; enzyme; catalytic growth

1. INTRODUCTION

Enzyme-mediated growing Au nanoparticles (AuNPs) have attracted considerable attention because it provides an ideal strategy for fabricating nanostructures and sensing biomolecules[1-3]. Willner and co-workers developed a series of amplified biocatalytic assays in connection to enzymatically-induced AuNP-growth processes[1, 4-6]. In general, this strategy involves the reduction and deposition of gold onto the nanoparticle-seeds by an enzymatically generated reducing agent. For example, the H₂O₂-mediated enlargement of AuNPs in the presence of glucose oxidase was exploited for the optical detection of glucose[5]. Similarly, a new electrochemical cholesterol biosensors by utilizing the enlargement of AuNPs was reported[7]. On the basis of biocatalytic growth of AuNPs, Zhu et al. developed a fluorescence sensing strategy for detection of glucose and xanthine[8]. It is very

common to produce "signal-on" sensors in which binding events trigger the growth of AuNPs, leading to an increase in the measured signal[9]. However, there has been relatively little attention to "signal-off" sensor architecture, in which the presence of target reduces rather than increases signal strength. Recently, we reported the "signal-off" designs with the inhibited growth of AuNPs for DNA sensing[10].

"Signal-on" and "signal-off" sensors each have their own strengths and shortcomings[11]. For instance, the "signal-on" immunosensors generate a signal due to directly proportional to the concentration of the target molecule. However, it may suffer from high background noise, limiting signal gain. The "signal-off" is preferred to detect with confidence at ultralow concentrations of the target[12], but false-positive responses might be induced by unpredictable accidents.

However, biosensors that employ only one signalling mechanism can still be limiting. Here we report a "proof-of-concept" for "signal on/off" immunosensor design, on the basis of enzyme-mediated growing AuNPs. The immunosensors feature "signal-on" and "signal-off" mechanisms, as well as electrochemical and optical dual-detection. The signal readout of the immunosensor relies on the growth of AuNPs, which is induced by H_2O_2 and is mediated by glucose oxidase or catalase. Because H_2O_2 is a common substrate or product in various biochemical pathways, this sensor platform is versatile and can be applied for H_2O_2 -involved bioassays. In addition, a dual-mode electrochemical and optical detection.

2. EXPERIMENTAL

2.1. Chemicals and materials

HAuCl₄•4H₂O was purchased from Sinopharm (Shanghai, China). Human immunoglobulin G (IgG), goat-anti-human IgG antibody (Ab1), and monoclonal mouse-anti-human IgG antibody (Ab2) were supplied by Baoman biotechnology (Shanghai, China). Glucose oxidase (GOx) was obtained from Hualan Chemical (Shanghai, China). Catalase (CAT) and bovine serum albumin (BSA) were from Sigma (St. Louis, USA). Cetyltrimethylammonium chloride (CTAC), glucose, and 3-aminopropyltriethoxysilane (APTES) were from Xilong Chemical Factory (Shantou, China). Indiumtin-oxide-coated (ITO) glass electrodes ($20 \ \Omega/\Box$, $13 \times 30 \times 1.1 \ mm$) were from Xiangcheng technology (Guangzhou, China). Other reagents were of analytical grade. Ultrapure water (resistivity > 18 M Ω cm) was obtained from a WP-UP-IV-30 purification system (Sichuan, China) and used in all experiments.

2.2. Synthesis of citric-coated AuNPs and preparation of enzyme-Ab2-AuNP bioconjugates

To a 100 mL of boiling aqueous solution of HAuCl₄ (0.01%, w/w) was added a 1.5 mL of sodium citrate solution (1%, w/w). The mixture was kept boiling for 15 min, cooled to room

temperature, and distilled to 100 mL with ultrapure water, followed by addition of 0.2 M K₂CO₃ to adjust the pH of the solution to 8.2. The average diameter of resulting AuNPs was about 25 nm. To 3 mL of AuNPs solution in a 5-mL centrifuge tube was added 8 μ L of 1.0 mg/mL Ab2, followed by vortex-mixing for 30 min. To the mixture was added 1 mL of enzyme solution containing 0.5 mg GOx and 5 mM phosphate-buffered saline (PBS, pH 6.8) (or 0.5 mg CAT and 5 mM, pH 8.2, boratebuffered saline). The solution was vortex-mixed for 1 h, followed by addition 50 μ L of BSA solution (2%, *w/w*). After vortex-mixing for 30 min, the solution was centrifuged at 15000 revolutions per minute (rpm) for 20 min. Precipitates were washed twice with 1.0 mL of BSA solution (1%, *w/w*), centrifuging 20 min at 15000 rpm with each wash. The precipitates were resuspended in 0.5 mL of BSA solution (1%, *w/w*). These two bioconjugates were referred to as GOx-Ab2-AuNPs and CAT-Ab2-AuNPs.

2.3. Self-assembly of AuNPs/ITO electrodes

The ITO glass electrodes were cleaned by ultrasonication in successive solutions of acetone, ethanol, ultrapure water, and 1 M NaOH/ethanol solution (50%, v/v) for at least 5 min each. The ITO glass electrodes were rinsed with ultrapure water and immersed in APTES/ethanol solutions (10%, v/v) for 90 min. After exhaustive rinsing with ethanol and ultrapure water and drying in air, the electrodes were immersed in AuNPs solutions for 20 min. Then the AuNPs/ITO electrodes were rinsed with ultrapure water and drying in air.

2.4. Fabrication of immunosensor

A 10- μ L of 400 μ g/mL Ab1 solution (containing 0.05 M PBS, pH 6.0) was dropped and spread on the conductive surface of the AuNPs/ITO electrode. The Ab1-coated electrode was incubated at 4 °C for 8 h in air at saturation humidity, followed by rinsing with a mixture of 0.05 M PBS (pH 7.4) and 0.05% Tween-20 (ν/ν). The air-dried electrode was blocked with 2% BSA solution (w/w) to avoid nonspecific adsorption.

2.5. Immunoassay procedures and Growth of AuNPs

The immunosensor was immersed in a solution containing 0.05 M pH 7.4 PBS, 0.1% BSA, 0.05% Tween-20, and desired amount of IgG and incubated at 37 °C for 50 min. After rinsing with 0.05 M PBS (pH 7.4, containing 0.05% Tween-20), the immunosensor was immersed in a solution containing 20 μ L of GOx-Ab2-AuNPs or CAT-Ab2-AuNPs. Finally, the immunosensor was washed thoroughly with 0.05 M PBS (pH 7.4, containing 0.05% Tween-20) and ultrapure water to remove non-specifically bound bioconjugates. The immunoassay procedure is shown in Figure 1. In the case of the immunosensor using GOx-Ab2-AuNPs bioconjugates, the immunosensor was immersed in the following solution (diluted to 8 mL with ultrapure water) and incubated at 37 °C for 5 h: 0.25 mL of 0.2 mM PBS (pH 6.5), 30 μ L of 1% (*w/w*) HAuCl₄, 0.25 mL of 0.01 M glucose, and 20 μ L of 0.2M

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CTAC. In the case of the immunosensor using CAT-Ab2-AuNPs bioconjugates, the immunosensor was immersed in the following solution (diluted to 8 mL with ultrapure water) and incubated at 37 °C for 30 min: 0.25 mL of 0.2 mM PBS (pH 6.5), 30 μ L of 1% (*w/w*) HAuCl₄, 70 μ L of 0.01 M H₂O₂, and 20 μ L of 0.2M CTAC.

2.6. Characterization and spectral and electrochemical measurements

The surface morphology and microstructure of the immunosensors were observed with a Hitachi S-4800 scanning electron microscope (SEM) equipped with an energy-dispersive X-ray spectroscopy (EDS) detector. UV-vis absorption spectra were recorded on a CARY 50 spectrophotometer (Varian, USA). Light-scattering (LS) studies were performed on a RF-5301pc (Shimadzu, Japan) spectrofluorophotometer with an incidence angle at 45° by synchronously scanning. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) measurements were carried out on a CHI 660B electrochemical workstation (Ch Instruments, China) with a conventional three-electrode system consisting of a immunosensor as the working electrode, an Ag/AgCl (3 M KCl) electrode as the reference electrode, and a Pt wire electrode as the counter electrode. CV experiments were carried out in either a 1 mM K_3 [Fe(CN)₆]/K₄[Fe(CN)₆] or a 0.5 M H₂SO₄ solution. DPV experiments were performed with a 0.5 M H₂SO₄ solution. All potentials are reported versus the Ag/AgCl (3 M KCl) reference at room temperature.

3. RESULTS AND DISCUSSION

3.1. Overview of approaches

We developed two different approaches for fabricating sandwich-type immunosensors (Figure 1). The signal change, either increase ("signal-on") or decrease ("signal-off"), can reflect the extent of the binding process thereby allowing for detection of target concentration.

The immunosensor was fabricated on an indium tin oxide (ITO)-coated glass slide. First, the precleaned ITO slide surface was silanized with APTES (step 1 in Figure 1). Then the citric-coated AuNPs (diameter of 25 nm) were self-assembled onto the surface of ITO slide (step 2 in Figure 1), followed by adsorbed goat-anti-human IgG antibody (Ab1) (step 3 in Figure 1). Subsequently, the immunosensor was incubated in a solution containing various concentrations of IgG. Hereafter, we used two approaches to produce either "signal-on" or "signal-off" architectures. In the "signal-on" approach, GOx-Ab2-AuNPs bioconjugates were used as secondary antibodies for a sandwich configuration. GOx was introduced on the immunosensor surface by forming an antibody–antigen–antibody "sandwich". Subsequently, the immunosensor was incubated in the solution containing PBS, CTAC, AuCl₄⁻, and glucose. The oxidation of glucose was catalyzed by GOx to produce $H_2O_2[13]$. The AuNPs immobilized on the immunosensor act as catalysts for the reduction of AuCl₄⁻ by H_2O_2 , resulting in the enlargement of the Au particles (Equation 1)[1, 5, 14].

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$$AuCl_4^{-} + 3/2H_2O_2 \xrightarrow{AuNPs} Au^0 + 4Cl^{-} + 3H^+ + 3/2O_2$$
(1)

The unique electronic and optical properties of the grown AuNPs provide means for voltammetric or spectral readout of antibody-antigen recognition events[15-20]. This signal-on approach results in an increase in electrochemical or spectral signals from the enlargement of the AuNPs.



Figure 1. Scheme of immunosensor fabrication and assay procedure. Ab1: goat-anti-human IgG antibody. Ab2: monoclonal mouse-anti-human IgG antibody.

The "signal-off" approach is demonstrated by using CAT-Ab2-AuNPs bioconjugates. CAT catalyzes the reaction:[21]

$$H_2O_2 \xrightarrow{CAT} O_2 + H_2O$$
 (2)

Introduction of CAT on the immunosensor surface induces H_2O_2 degradation. Because the growth of AuNPs is mediated by H_2O_2 concentration[5, 8, 22], it was observed that the signal decreased with increasing IgG concentrations, indicating the degradation of H_2O_2 .

3.2. SEM characterization

To assess whether the growth of AuNPs on the immunosensor surface was controlled by enzyme-mediated H_2O_2 , we performed a detailed scanning electron microscope (SEM) analysis that follows the immunoassay process (Figure 1).



Figure 2. SEM images of (a) AuNPs/APTES/ITO electrode, (b) Ab1/AuNPs/APTES/ITO electrode + GOx-Ab2-AuNPs, (c) Ab1/AuNPs/APTES/ITO electrode + IgG + GOx-Ab2-AuNPs, (d) Ab1/AuNPs/APTES/ITO electrode + CAT-Ab2-AuNPs, and (e) Ab1/AuNPs/APTES/ITO electrode + IgG + CAT-Ab2-AuNPs. Note that (b) and (c) were immersed in the solution containing glucose for growing AuNPs; (d) and (e) were in the solution containing H₂O₂. (f) is an EDS spectrum of grown AuNPs.

In the steps 1 and 2 (Figure 1), AuNPs were self-assembled on the APTES-silanized ITO electrode surface. The SEM image of AuNPs/ITO electrode in Figure 2a shows that AuNPs are uniformly distributed on ITO and the average diameter is 25.5±5.3 nm (Figure 3a). After coating with Ab1, followed by BSA blocking and incubation with GOx-Ab2-AuNPs, the AuNPs/ITO electrode was incubated with a solution containing PBS (pH 6.5), AuCl₄, glucose, and CTAC. Note that the treatment of the electrode follows the same procedure described in the Experimental 2.5, except that no IgG was added. The electrode was used as a control for non-specific adsorption. Because of the absence of IgG, the Ab1/IgG/GOx-Ab2-AuNPs ''sandwich'' was not formed, leading to no GOx on the electrode surface. Consequently, there is no GOx-stimulated growth of AuNPs. Figure 2b and 3b

show that the average diameter (25.3±9.3 nm) of AuNPs is very similar to the AuNPs on the AuNPs/ITO electrode, indicating that no non-specific adsorption of GOx-Ab2-AuNPs on the electrode occurs. A slight aggregation of AuNPs may result from protein absorption.



Figure 3. (a)-(e): the size distribution histogram of the AuNPs on the immunosensor surface, each panel corresponds to the panels from (a) to (e) in Figure 2. (f): UV-is spectrum of the AuNPs solution.

For comparison, an electrode was prepared in the presence of IgG, following the procedure described in the Experimental 2.5. GOx was introduced on the immunosensor surface by forming

"sandwich" immunocomplexes in the presence of IgG. Therefore, it should be observed that GOxcatalyzed oxidation of glucose leads to the formation of H_2O_2 that results in the growth of the AuNPs on the immunosensor. SEM image of the immunosensor shows that the average diameter of AuNPs increases to 46.0±12.6 nm (Figure 3c) and the particle density is significantly increased (Figure 2c). This confirms that the growth of AuNPs was "turned on", due to the target IgG that induced the generation of H_2O_2 .

In contrast to the GOx-Ab2-AuNPs system, using the CAT-Ab2-AuNPs bioconjugate as enzyme-labeled secondary antibody could inhibit H_2O_2 -stimulated growth of AuNPs. Figure 2d and e show SEM images of the immunosensors treated with and without presence of IgG. Without the IgG, the "sandwich" immunocomplexes could not be formed. This results in a CAT-free surface, where H_2O_2 does not degrade. Therefore, in the last incubation step, the AuNPs were grown on the immunosensor surface by AuCl₄⁻ reduction in the presence of H_2O_2 . The SEM image (Figure 2d) shows that the average diameter of AuNPs increases to 32.8 ± 5.7 nm (Figure 3d). Interestingly, the particle density and average diameter of AuNPs in Figure 2d are lower and smaller than those in Figure 2c. This might be attributable to the lower reducing agent concentration (0.088 mM H_2O_2) in Figure 2d, compared to that in Figure 2c (0.3 mM glucose).

In another case (signal-off), if IgG is present in the sample, it would be captured by the surface immobilized Ab1, followed by binding of CAT-Ab2-AuNPs, resulting in introduction of CAT on the immunosensor surface. For such surface, we therefore expect that the degradation of H_2O_2 is catalyzed by CAT. It is evident from Figure 2e that only a slightly growth of AuNPs (average diameter of 27.5±5.0 nm, Figure 3e) was observed due to the degradation of H_2O_2 . These results support that lower reducing agent concentration leads to less growth of AuNPs. In addition, the EDS analysis confirms the presence of the gold in the grown particles, as shown in Figure 2f.

3.3. Spectral and electrochemical measurements

For a practical working immunosensor, the protein-binding events must be transduced into physical signals. Herein, the H_2O_2 -mediated growth process of AuNPs was used in the immunosensors for the transduction of binding events into readily observable optical or electrochemical signals. In a signal-on approach, the AuNPs were grown due to the reduction of AuCl₄⁻ by H_2O_2 . Because the H_2O_2 concentration is dependent on the concentration of IgG, there is a direct dependency of the grown AuNPs to the IgG concentration. Figure 3a shows the absorption spectra of the grown AuNPs on the immunosensor surface in the presence of variable concentrations of IgG. The absorption maximum of the AuNPs on the ITO slide is 551 nm (the lowest curve in Figure 4a), which is red-shifted as compared to that of the corresponding AuNPs dispersed in water (529 nm, Figure 3f). This kind of red-shift has been well documented including the change of refractive index of the surrounding environment, the distance between AuNPs, and the size of aggregated AuNP clusters[23, 24]. Similarly, the maximum absorbance increases regularly with the increase of IgG, companying the red-shift in the absorption peaks up to 565 nm. This result is good accordance with the SEM observation, indicating an IgG-induced growth of AuNPs. In addition, we measured the light scattering spectra of

the immunosensor. The light scattering signal becomes stronger with increasing IgG concentrations (Figure 4b), indicative of growing AuNPs with larger quantity and scattering cross-sections[25].



Figure 4. (a) UV-vis spectra, (b) light scattering spectra, (c) CVs in a $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ solution, and (d) CVs in a H_2SO_4 solution. Inserts: response signal *vs*. logarithmic value of IgG concentration.

CV was performed to test the electrochemical response of the immunosensor. Figure 4c shows the typical voltammetric behavior of the sensor with increasing concentrations of IgG from 0 to 1250 ng mL⁻¹ in a 0.5 M H₂SO₄ solution. It appears as an oxidation peak at ~1 V and a reduction peak at ~0.89 V. The redox peaks are attributed to the oxidation and subsequent reduction of surface gold oxide formation on the AuNPs as a result of the positive potential polarization. The redox peak currents increase with increasing IgG concentrations, supporting the growth of AuNPs.

To examine the electronic communication behavior between the grown AuNPs and the ITO electrode, we investigated the electrochemical behavior of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ at the immunosensor surface. Figure 4d shows both anodic and cathodic peak currents increase with the IgG concentrations (i.e., more growth of AuNPs), while the peak separation decrease from ~0.40 to ~0.32

V. This reveals that the growth of AuNPs improves the electron transfer between $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and the immunosensor.

When the CAT-Ab2-AuNPs bioconjugate is used as a secondary antibody, the immunosensor shows a "signal-off" behavior (Figure 1). In such "signal-off" approach, the grown AuNPs numbers are on the decrease, because of H_2O_2 degradation by CAT. This "signal-off" approach was demonstrated by optical and electrochemical measurements in Figure 5. As can be seen from Figure 5, both optical and electrochemical signals decreased with increasing IgG concentrations, indicated a reduction in the extent of AuNPs growth. These results are consistent with the SEM analysis (Figure 2). It is worth to note that we measured the DPV response of the immunosensors with various IgG concentrations after the immunosensor was anodized at 1.30 V for 30 s (Figure 5c and d). Bare ITO electrode show no DPV response (curve A in Figure 5c), whereas the immunosensor with grown AuNPs gives a sharp cathodic peak at ~0.9 V (curve B in Figure 5c), which corresponds to the AuO reduction. As shown in Figure 5d, DPV peak currents decreased with the increasing concentration of IgG, confirming that the decrease of the number of grown AuNPs.



Figure 5. (a) UV-vis spectra, (b) light scattering spectra, (c) DPV of ITO electrode (curve A) and the immunosensor after growing AuNPs (curve B), and (d) DPV of the immunosensor with increasing IgG concentrations. Inserts: response signal *vs.* logarithmic value of IgG concentration.

Both signal-on and -off approach, the optical and electrochemical signal shows a linear response behavior with the logarithmic value of IgG concentration ($\log C_{IgG}$). The inserts of Figure 4 and 5 show signals increase with $\log C_{IgG}$ for the "signal-on" case and, on the contrary, decrease with $\log C_{IgG}$ for the "signal-off" case. These methods were evaluated with respect to the detection limit and their ability to detect IgG in real sample. For the "signal-on" approach, the detection limits (LDs) for UV-Vis spectra, LS spectra, and CV methods (in H₂SO₄ solution) were 0.16, 0.20, and 0.22 ng mL⁻¹, respectively. For the "signal-off" approach, the LDs for UV-Vis spectra, LS spectra, and DPV methods were 0.20, 0.24, and 0.34 ng mL⁻¹, respectively. Furthermore, these methods were used to detect IgG in serum samples collected from Nanxishan Hospital, Guilin, China. Most of the results (ranging from 28.39 to 35.25 ng mL⁻¹) obtained by the proposed methods, either signal-on or signal-off approach, are consistent with the data from the hospital (31.85 ng mL⁻¹), in spite of the slight deviation in UV-Vis (19.00 ng mL⁻¹) and DPV (22.00 ng mL⁻¹) methods for the signal-off approach (Table 1).

Table 1. Comparison of different methods for detecting IgG in a same sample.

Concentration	Signal-on approach			Signal-off approach		
	UV-vis	LS	CV	UV-vis	LS	DPV
$IgG (ng mL^{-1})$	28.39	30.98	32.45	19.00	35.25	22.00

4. CONCLUSIONS

We developed a proof-of-concept strategy to convert the immune-recognition event into optical and electrochemical signal, on the basis of the enzyme/ H_2O_2 -mediated growth of AuNPs. The strategy is versatile in that both signal-on and signal-off detections can be easily realized and easily adapted for electrochemical as well as optical detection methods. Because H_2O_2 is a reactant or product of many enzyme-catalyzed reactions, this strategy holds great promise in applications such as enzyme-activity and enzyme-linked assays.

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