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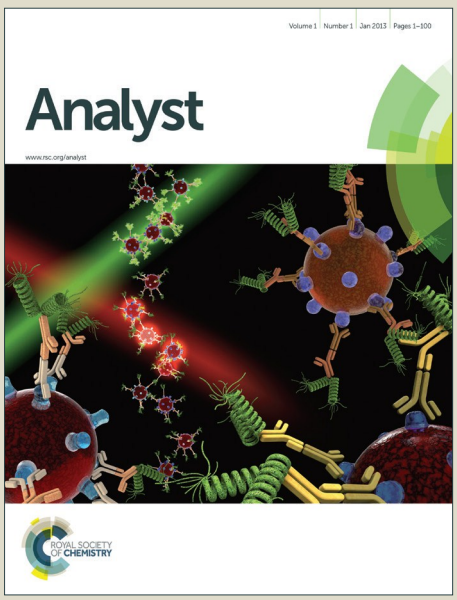

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ARTICLE

Effects of Bipyramidal Gold Nanoparticles and Gold Nanorods on the Detection of Immunoglobulin.

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Two types of gold nanoparticles are compared for their use in a sensor for immunoglobulin detection - bipyramidal gold nanoparticles (GBPs) and gold nanorods (GNRs). Using surface plasmon resonance spectroscopy and square wave voltammetry allowed us to evaluate the utilities of these two types of gold nanoparticles in a label-free detection of the analyte IgG. While both systems showed a significant enhancement in sensitivity over a nanoparticle free system, showing a 64-fold enhancement for the GBP-containing sensor and a 16-fold enhancement for the GNR-containing sensor systems.

Introduction

In recent years, metal nanoparticle-enhanced analytical technologies have developed rapidly towards highly sensitive and selective detections of biomolecules.[1-3] Several studies have been reported on the application of gold nanoparticles (GNPs) in bio-sensing and bio-imaging.[4-7] Gold is known as an excellent material in biotechnology due to its unique properties, such as optical property, excellent biocompatibility, water solubility, good stability, and ability for modified surface functionality.[8-10] Depending on the shape and size of GNPs, the origin of surface plasmon resonance (SPR) of gold nanoparticles involves contributions from scattering and absorption components.[11] GNPs with various size and shape can be prepared by controlling the growth conditions and different SPR in the visible and near-infrared regions has been shown.[12-14] GNPs with special tailored shapes have been demonstrated to have great potential in clinical immunoassays and DNA hybridization analysis.[15] Spherical GNPs shows only one surface plasmon band around 520 nm, whereas elongated GNPs such as nanorods(GNRs) and bipyramids(GBPs) display two distinct surface plasmon bands related to the electron oscillations along the transverse and longitudinal directions.[16,17] In comparison to the transverse surface plasmon resonance wavelength, the longitudinal surface plasmon resonance wavelength is more sensitive to the changes in the dielectric properties of the surroundings and can be tuned by varying the aspect ratio.[18] In addition, owing to the structure of containing two sharp apexes along with a pentagonal base for each gold bipyramid, high sensitivity to the refractive index changes of the surrounding medium as well as strong local electromagnetic-field enhancement is exhibited by these nanoparticles.[19]

Surface plasmon resonance (SPR) biosensors are typical optical biosensors and have been widely used in biochemistry and biomedical research due to its label-free, high sensitivity, real time analysis and flexible system design.[20-22] The information of the biomolecular interaction is obtained by measuring the optical characteristics, such as intensity, phase and polarization of light reflected from the optical equipment.[23] There are a few studies performed using GNRs in SPR biosensor to amplify SPR response signals, while GBPs are rarely used. Law *et al.* demonstrated that GNRs-based SPR biosensor led to a drastic sensitivity enhancement at a concentration as low as the femtomolar range for detecting the tumor necrosis factor alpha.[24] An ultrasensitive SPR detection method for the directly detecting of protein biomarkers was developed by Sim*et al.*, employing GNRs to conjugate with anti-IgE as a signal amplification lable.[25]

As a class of the most widely and successfully commercialized devices, electrochemical biosensors have become popular detection means in the study of molecule biology and biological technology.[26-28] Nanomaterials have also been applied to electrochemical biosensors since nano-sized particles have a chemical behavior similar to small molecules and can be used as specific electrochemical labels.[29-31] The large surface area of nanomaterials enables sensors to interact with the analytes much more, resulting in higher capture

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efficiency to increase the sensitivity.[32] Horseradish peroxidase functionalized GNRs were designed to conjugate with antibody for detection of alpha-fetoprotein by electrochemical measurement.[33] Azimzadeh and co-workers reported an electrochemical nanobiosensor based on graphene oxide and GNRs to detect miRNA-155 and GNRs were proved to amplify the electrochemical signals.[34]

In the study presented, a new GBPs based biosensor has been fabricated and tested in detection of human IgG. The sensitivity of the sensor has been evaluated and compared with the same type of biosensors containing GNRs or containing no GNPs respectively, rather than GBPs. In additional to varying the architectures of the biosensors with different shapes of nanoparticles, we have carried out our testing using both SPR and Square Wave Voltammetry (SWV). This unique double comparison performed with different nanoparticles and techniques provides better insights on how the sensitivity is influenced by these factors, particular for the system presented.

A schematic diagram of the experimental procedure is shown in Figure 1. The antibody is coupled with GNPs to obtain gold nanoparticles-antibody conjugates through covalent attachment. Sandwich assays were used to detect human IgG in both SPR and SWV study. The results obtained from complimentary methods have been compared. GBPs have been demonstrated to be more beneficial than GNRs for the performance improvement of detection.

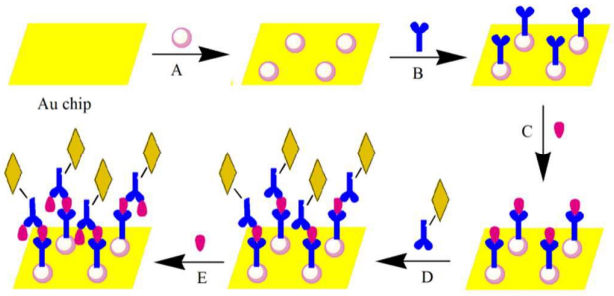

Figure 1. A schematic diagram of the experimental procedure: (A) Immobilization of protein A, (B) Immobilization of rabbit anti-human IgG, (C) Immobilization of human IgG, (D) Immobilization of gold bipyramids-antibody conjugates to complete the biosensor using sandwich configuration, (E) Detection of human IgG.

Experimental Section

Materials

Human IgG, rabbit anti-human IgG, bovine serum albumin (BSA), protein A from Staphylococcus aureus, 11-mercaptoundecanoic acid (MUA) and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) was purchased from AAPPTec. Hydrogen tetrachloroauratehydrate ($HAuCl_4 \cdot 3H_2O$), trisodium citrate, cetyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), ascorbic acid (AA), silver nitrate ($AgNO_3$), hydrochloric acid and all other chemicals were of analytical reagent grade, and bought from Sigma-Aldrich. All chemicals were used as received with no further modification and the aqueous solutions were prepared with deionized water (Millipore Milli-Q; 18 MΩ•cm resistivity). Sodium phosphate buffered saline (PBS, 0.01 mol L^{-1}, pH 7.4) was used as running buffer.

Preparation of gold bipyramids (GBPs) and gold nanorods (GNRs)

GBPs were synthesized by a modified seed-mediated growth method.[35] First of all, sodium citrate-stabilized gold seed nanoparticles were prepared as follows: 100 µL of 0.025molL^{-1} $HAuCl_4$ was added into 20 mL deionized water, followed by adding 100 µL of 0.05 mol L^{-1}trisodium citrate. Next, 300 µL of 0.01 mol L^{-1} ice-cold $NaBH_4$ solution was added under vigorous stirring for 2 min. The resulting gold seed solution was kept at room temperature for at least 2 h for completing the reaction. For making the growth solution, 5 mL of 0.01molL^{-1} $HAuCl_4$, 1 mL of 0.01 mol L^{-1} $AgNO_3$, 2 mL of 1 mol L^{-1} hydrochloric acid and 0.8 mL of 0.1 mol L^{-1} AA were added into 100 mL of 0.1 mol L^{-1} CTAB solution in the described order. Finally, 0.8 mL of the seed solution was added into the above growth solution. Each reagent was added with gentle stirring to make them mix well. The solution was left overnight at 28°Cand the colour changed from almost clear to dark pink. The removal of excess surfactant was achieved by successive centrifugation at 14,000 rpm for 15 min and redispersed in water. GNRs were also synthesized through El-Sayed's seed-mediated method.[36]

Preparation of gold nanoparticles-antibody conjugates

20 mM of MUA in ethanol was added into 10 mL of GBPs and GNRs solutions respectively and sonicated at 40 °C for 3 h. The GBPs were centrifuged at 14,000 rpm for 20 min and resuspended in PBS. The NHS/EDC linking chemistry was used to immobilize antibody onto the surface of gold nanoparticles through covalent attachment. 0.4 mL of NHS (100 mg mL^{-1}) and 0.4 mL of EDC (100 mg mL^{-1}) were added to the resuspended solution for 30 min. The gold nanoparticles-antibody conjugates were prepared by adding excess amount of rabbit anti-human IgG (500 µg mL^{-1}) to the corresponding gold nanoparticles solution and incubating at 4 °C for 1 h. The conjugates were obtained and redispersed in PBS buffer solution after the removal of the unbound antibodies by centrifugation. The last step was to add 10 µL of 10 mg mL^{-1} BSA into the conjugates solution for blocking the nonspecific binding sites.

SPR study

SPR experiments were carried out on Au sensor chips using Biacore X-100. All the experiments were measured at room temperature. A PBS flow rate of 5 µL min^{-1} was used for all experiments. The sandwich assays were performed during this study. PBS was first injected until a base line was established. 0.1 mg mL^{-1} protein A was injected as a linker. Rabbit anti-human IgG diluted in PBS was injected to be immobilized on the surface of protein A in the following step. The remaining free binding sites were blocked by injecting 10 mg mL^{-1} of BSA

for 10 min and washed with PBS to remove the excess BSA off the surface. Then 80 μg mL^{-1} human IgG diluted in PBS was injected to bind with the first antibody immobilized on the sensor chip, followed by using PBS to wash the sensor surface. For making a comparison, different gold nanoparticles modified secondary antibodies and just secondary antibodies (no GNPs) were separately injected to detect human IgG.

Electrochemical studies

All electrochemical studies, including cyclic voltammetry (CV) and square wave voltammetry (SWV) were carried out using a CHI-660B electrochemical potentiostat. In prior to the preparation of the sensors, the gold electrodes were cleaned by immersing in piranha solution containing concentrated H_2SO_4 and 30% H_2O_2 (3:1, v/v) for 5 min, followed by polishing with 0.3 and 0.05 μm aluminum powders for 2 min respectively, then sonicated in ethanol and water for 5 min in each. After rinsing thoroughly with deionized water, the gold electrodes were electrochemically cleaned in 0.5 M KOH solution by scanning from -2.0 to 0 V (vs Ag/AgCl) at a scan rate of 0.5 V s^{-1} for 100 cycles. Then the gold electrodes were cleaned in 0.5 M H_2SO_4 solutions by running 100 CV cycles in the range between 0 and 1.5 V until a stable gold oxidation peak at 1.1 V was reached. The cleaned gold electrodes were rinsed with deionized water and PBS before modification. All the same reagents were also used for preparation of sensors for electrochemical studies. The only difference between SPR and electrochemical sensor preparations is that the electrochemical sensors were step by step prepared externally before the detections. The electrochemical measurements were carried out in an enclosed faraday cage with a three-electrode electrochemical cell, in which the modified gold electrode was used as the working electrode, a Ag/AgCl/3.0 M KCl as the reference electrode and a Pt wire as the counter electrode. A salt bridge filled with agar and KNO_3 was used to minimize the transfer of chloride ions from the reference electrode to the electrolyte, which was 5mM/5mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ and 1 M $NaClO_4$ as the supporting electrolyte. The statistical information was achieved by triplicate measurement.

Results and discussions

Synthesis and characterization of GBPs and GNRs

Transmission electronic microscopy (TEM) and UV-vis absorption spectra were employed to characterize the synthesized nanoparticles. It can be seen from Figure 2 that both of the prepared GBPs and GNRs are nearly monodisperse and homogeneous. The size distributions were obtained from measuring at least 300 nanoparticles of TEM images obtained

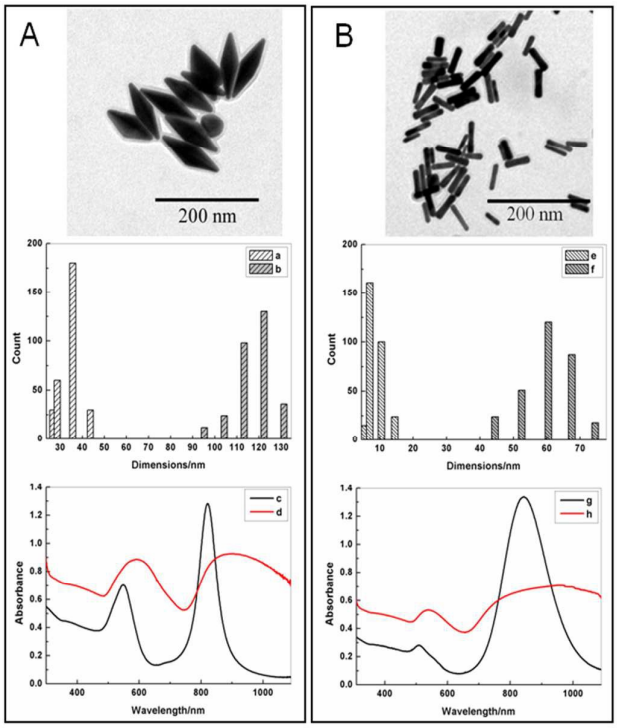

Figure 2.TEM images, size distribution, UV-vis absorption spectra of GBPs (A) and GNRs (B). (a) GBPsdiameter; (b) GBPs length; (c) the absorption spectra of GBPs; (d) the absorption spectra of GBPs-antibody conjugates; (e) GNRsdiameter; (f) GNRs length; (g) the absorption spectra of GNRs; (h) the absorption spectra of GNRs-antibody conjugates.

on several TEM grids for each sample. As demonstrated in Figure 2A, GBPs have an average diameter of 37 nm, and length of 120 nm. In comparison to GBPs, GNRs have an average diameter of 8 nm and length of 60 nm (Figure 2B). As shown in the absorption spectra, GBPs and GNRs both exhibit two well-defined absorption bands. The long plasmon absorption band originates from the longitudinal mode of oscillation of the free electrons along the long axis, while the short plasmon absorption band originates from the transverse mode perpendicular to the former one. For GBPs, the transverse band is approximately 548 nm and the longitudinal band is around 821 nm. For GNRs, the two SPR bands are at 520 nm and 844 nm, respectively. When the gold nanoparticles have been coupled with antibody, an obvious red shift in the absorption peak has been observed due to the changes in the dielectric properties of gold nanoparticles surfaces, which indicates that the antibodies were attached onto gold nanoparticles surfaces and the conjugates were successfully synthesized. Higher concentration of rabbit anti-human IgG has been used for preparation of antibody-gold nanoparticles conjugates, so good coverage of bio-recognition elements can be achieved on the surfaces of gold nanoparticles.

SPR study

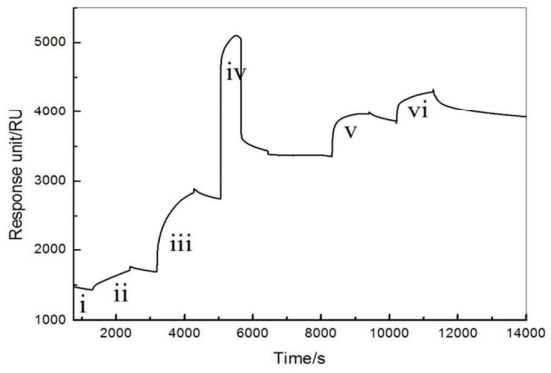

Figure 4.SPR response of the modification of Au sensor chip using sandwich assay. (i) PBS baseline, (ii) immobilization of protein A as a linker between Au chip and antibody, (iii) immobilization of rabbit anti-human IgG as the first antibody, (iv) blocking with BSA, (v) injection of excess human IgG, (vi) adding of gold nanoparticles modified secondary antibodies.

In this study, biosensors based on using different gold nanoparticles were constructed. Figure 3 shows the kinetic responses during the in-situ surface modification process and the response to each step indicates that the sensor surface was successfully modified with all the elements. It is a good strategy to use protein A as a membrane to immobilize antibody on the sensor surface, because protein A has four binding sites with antibody and binds specifically to the Fc region of antibody without interacting with the antigen binding sites Fab.[37] Moreover, Au and protein A have an association constant of 10^8 M^{-1}, which shows highly stability, and the interaction is believed to be van der Waals in nature.[38,39] For the first antibody immobilization, different concentrations of rabbit anti-human IgG (25, 37.5, 50, 75 and 100 μg mL^{-1}) were separately injected to determine the optimum experimental condition. The SPR response triggered by the different concentrations of the antibody is shown in Figure 4. The result indicated that the response moved to high intensity with the increase of the concentration of antibody. The response increased quickly at low concentration and changed slowly at high concentration. However, relatively high concentration of antibody may result in steric hindrance and it takes long time to complete the dissociation. When the concentration of antibody is 75 μg mL^{-1}, the response change is about 90% of its total changes. Thus, 75 μg mL^{-1} was selected as the optimum concentration of rabbit anti-human IgG immobilization.

The human IgG solutions with different concentrations were separately injected into the flow cell after the Au sensor chip was modified with different gold nanoparticles. The change in the SPR response caused by the antibody-antigen immunoreaction was measured. Figure 5 shows the relationship between the relative response changes (referring to the response of immobilizing the secondary antibody) and different concentrations of human IgG. It can be seen from Figure 5a that the SPR biosensor without gold nanoparticles modified shows a response to human IgG in the concentration range of 2.50 to 40.00 μg mL^{-1}. When the antibody-gold nanoparticles conjugates were employed, the SPR biosensor with GNRs modified shows a response to a wider range of human IgG concentrations from 0.15 to 40.00 μg mL^{-1}, as shown in Figure 5b. The SPR biosensor with GBPs modified shows an even better response to human IgG when comparing with previous two detections, which is in the concentration range of 0.04 to 40.00 μg mL^{-1} (Figure 5c). The limit of quantification (LOQ) is defined as the lowest concentration of an analyte that can be quantitatively measured by the proposed method. The LOQ obtained from the biosensor modified with GBPs (0.04 μg mL^{-1}) is about 63 times lower than that obtained with the biosensor without using gold nanoparticles (2.50 μg mL^{-1}) and 4 times lower than that obtained with the biosensor utilizing GNRs (0.15 μg mL^{-1}).

By conducting the detections under the consistent conditions, the SPR results have demonstrated that the use of GBPs and GNRs enhances the sensitivity significantly. Gold nanoparticles have their own localized surface plasmon due to the collective oscillation of their conduction electrons, which leads to a strong local electromagnetic field. The interaction between the localized surface plasmon of gold nanoparticles and the propagating plasmon in the Au film of sensor chip

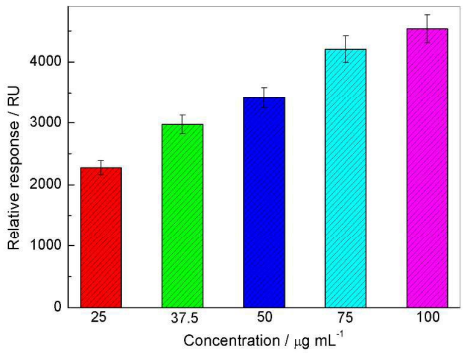

Figure 3.SPR response of immobilizing rabbit anti-human IgG at different concentrations after Au chip was modified with protein A.

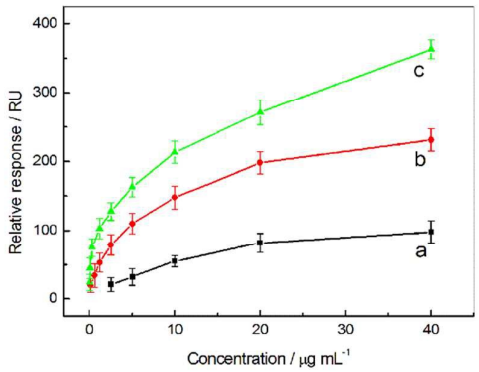

Figure 5.The relationship between the SPR response and the concentrations of human IgG obtained with different modification biosensors. (a) the biosensor without gold nanoparticles modified, (b) the biosensor modified with GNR-antibody conjugates, (c) the biosensor modified with GBP-antibody conjugates.

resulted in response changes of SPR biosensor.[40] In comparison with nanorods, bipyramids have sharp apexes, which produce a localized sensing/mode volume of highly enhanced electric field intensity.[41] In addition, the field magnitude decreases from the center towards the poles in the case of nanorods while increases rapidly in the case of bipyramids.[42] Thus SPR signal can be enhanced more remarkably when GBPs were used.

Electrochemical studies

In order to evaluate and compare different detection methods, electrochemical detections were carried out on the sensors prepared with consistent elements to the SPR sensors. The electrochemical response of the biosensors was evaluated by using CV and SWV. Because antigen is not redox active, a ferrocyanide/ferricyanide redox couple was used to help monitoring the electron transfer cross the sensor surface for both construction of the sensor and detection. In this experiment, CV exhibits both anodic and cathodic currents due to the reversible redox reaction of electrolyte ions. The modification of gold electrode and building the biosensor has been monitored by CVs, as shown in Figure 6. The oxidation and reduction peaks are observed to be at approximately 0.22 V and 0.29 V for bare gold surface. The electrochemical response of the films has been measured after each step of modification. As shown in the Figure 6a to f, the redox currents decrease as the modification of the surface progresses. Moreover, the distance between redox peak potentials becomes larger. This is due to the blocking of the electron transfer ability by the elements immobilized.[39] The bio-recognition elements on the surface increases pathways of diffusion of the redox ions, therefore electron transfer is less efficient, lower currents at a given potential. The surprising observation is that the current decreased when GBPs modified secondary antibody was added as shown in Figure 6f. The speculation is that the decrease of electron transfer triggered by the secondary antibody overcomes possible increase generated from GBPs when immobilized together. The observation of changes in CVs indicates the success in immobilizations of each material.

SWV has been used here for the electrochemical detection of human IgG. After the modification of gold electrode, different concentrations of human IgG were added separately and the responses are measured by SWV. The results of the SWV studies on using GBPs modified biosensor, GNRs modified biosensor and biosensor without nanoparticles modification have been illustrated in Figure 7. As the concentration of human IgG increases, the oxidation peak potentials shifted anodically. In addition, the peak current intensity decreases with human IgG added. A collection of SWV examples are shown as the inset graph of Figure 7. Every step of the analytes binding onto sensing surfaces can be regarded as an addition of an inert blocking layer, hindering the diffusion of the electrolyte. As expected, the current density decreases with increasing the concentration of human IgG. The relationship between the percentages of change in current intensity and different concentrations of human IgG is shown in Figure 7. At the low concentrations, the electrochemical response is changing rapidly. And as the concentration increasing, the changes of the response grow slowly. The biosensor incorporated with GBPs shows a detection response to human IgG in the concentration range of 0.02 to 40.00 μg mL^{-1} (Figure 7c), whereas the biosensor with GNRs shows a response to human IgG in the concentration range of 0.08 to 40.00 μg mL^{-1} (Figure 7b). For making comparisons, the biosensor without nanoparticles employed has been tested and shows a response to human IgG in the concentration range of 1.25 to 40.00 μg mL^{-1} (Figure 7a). The detection limit obtained from the biosensor modified with GBPs (0.02 μg mL^{-1}) and GNRs (0.08 μg mL^{-1}) are also about 63 times and 16 times lower than that obtained using the biosensor without gold nanoparticles (1.25 μg mL^{-1}), respectively. It is known that gold nanoparticles have

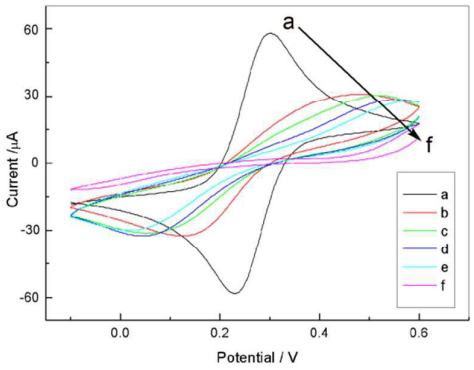

Figure 6.Cyclic voltammogram of the modification process of gold electrode. (a) bare gold electrode, (b) immobilization of protein A, (c) immobilization of the first antibody against human IgG, (d) blocking with BSA, (e) injection of excess human IgG, (f) adding of GBPs modified secondary antibodies. The scan rate was 0.1V·s^{-1} and CV measurements were carried out in a 5mM/5mM $Fe(CN)_6^{3-/4-}$ aqueous solution with 1 M $NaClO_4$ as the supporting electrolyte.

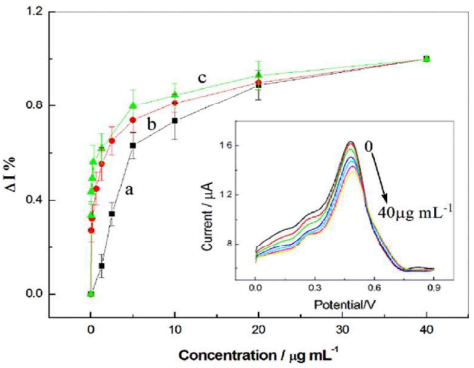

Figure 7.The relationship between the percentage of change in current intensity ($\triangle I\% = [(I_0-I)/(I_0-I_{40})]\times 100\%$) and different concentrations of human IgG. Inset: Square wave voltammogram of different concentrations of human IgG obtained with the biosensor without nanopaticles modified. (a) the biosensor without gold nanoparticles modified, (b) the biosensor modified with GNR-antibody conjugates, (c) the biosensor modified with GBP-antibody conjugates. The SWV was obtained in 5mM/5mM $Fe(CN)_6^{3-/4-}$ aqueous solution with 1 M $NaClO_4$ as the supporting electrolyte.

good biocompatibility. A relatively large amount of antibodies have been conjugated onto the surface of gold nanoparticles, which is beneficial for increasing the possibility of antibody-antigen interactions, thus resulting in higher sensitivity.

Another interesting finding is that when the sensitivities obtained from SWV and SPR are compared, electrochemical method is more sensitive than SPR and shows wider concentration ranges for human IgG detection. This possibly is due to compatibility of the detection with debye lengths posed by two different techniques.[43] The speculation is that the binding of human IgG onto the bio-recognition element is more within the debye length of electrochemical method than SPR, although the difference observed is significantly smaller than if nanoparticles are employed for biosensors.

Conclusions

In this paper, gold bipyramids have been synthesized along with nanorods as a comparison and both elongated gold nanoparticles are coupled with antibody to obtain gold nanoparticles-antibody conjugates. The conjugates have been used as the secondary antibody in the sandwich assays for the detection of human IgG. Based on the present study, GBPs and GNRs are proved to be promising sensitivity enhancers in biosensing when comparing with absent of them. Moreover, the results show that GBPs can improve the sensitivity more efficiently than GNRs either in SPR study or in electrochemical study, because GBPs possibly produce a stronger electromagnetic field due to its two sharp apexes. For SPR study, the biosensors constructed with GBPs and GNRs show responses to human IgG in the concentration ranges of 0.04 to 40.00 μg mL^{-1} and 0.15 to 40.00 μg mL^{-1}, respectively. For electrochemical study, the biosensor with GBPs and GNRs modified show responses to human IgG in the concentration ranges of 0.02 to 40.00 μg mL^{-1} and 0.08 to 40.00 μg mL^{-1}, respectively. The detection limits observed in this study show electrochemical method is consistently more sensitive than SPR, but the sensitivity gained from choosing different techniques is much less than choosing incorporation of nanoparticles. It is also worth mentioning out that due to the intrinsic differences between SPR and SWV, one is not necessary superior enough and able to replace the other. The two techniques have their unique advantages, such as real-time (SPR) and high sensitivity (SWV), which could be used as complementary detection schemes.

Acknowledgements

We appreciate the funding from NSERC and University of Toronto. H.Z. was supported by the Visiting Scholars Program of China Scholarship Council.

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Table of Contents

The influence of different shapes of gold nanoparticles and techniques (SPR and SWV) on the sensitivity of biosensors was investigated, using IgG detection as a model system.