Voltammetric immunosensing platform based on dual signal amplification using gold nanoparticle labels

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Abstract: An electrochemical immunosensing platform based on dual signal amplification strategy has been developed using gold nanoparticles. Human Immunoglobulin G was used as a model analyte in order to establish the immunosensing platform. The platform was fabricated using of 1,6-hexanedithiol self assembled on a gold disc electrode and was further modified by citrate capped gold nanoparticles. The direct immobilization of antibody was achieved through electrostatic interaction between negatively charged citrate capped gold nanoparticles and positively charged amino group of antibody. Each step of modification was analyzed using electroanalytical techniques like cyclic voltammetry and electrochemical impedance spectroscopy. The horseradish peroxidase (HRP)-labeled secondary antibodies conjugated on gold nanoparticles (AuNP-Ab\textsubscript{2}) acted as nanolabels. Thus the sandwich immunocomplex formed on the electrode surface produced an electrocatalytic response through the reduction of hydrogen peroxide by HRP in the presence of thionine. Electrochemical studies were carried out to understand the role of citrate capped AuNP and AuNP-Ab\textsubscript{2} in dual signal amplification. The fabricated sensing platform can be used for the sensitive determination of various protein biomarkers by immobilizing specific antibody.

1. Introduction
Highly sensitive, rapid and low cost diagnostic systems are very essential for the clinical analysis of immune deficiency diseases that may lead to major disorders like cancer, cardiovascular diseases, AIDS, chronic inflammatory diseases, autoimmune and liver disorders [1]. Biomarkers are biomolecules that indicate change in expression or state of proteins that correlates with the occurrence of the disease. One of the commonly used techniques for the determination of proteins is immunoassay [2]. The assay is highly selective in nature due to the specific interaction between antigen and antibody. There are different detection methods in immunoassay to monitor the immunochemical binding which includes optical [3,4], piezoelectric [5,6] and radioimmunoassay [7]. But these methods are not that much sensitive enough to detect very low concentrations of analyte, which is essentially required for the early detection of diseases. For the quantitative measurement of the desired analyte most of the immunoassays use specific labels attached to either the antigen or the antibody. The labels can be enzymes [8], fluorescent probes [9], chemiluminescent compounds [10], redox mediators [11] and radioactive elements. The signal obtained from the label is highly essential in determining the
sensitivity of assay. Therefore higher signals required from the label to detect smaller concentration of analyte. Thus enhanced signals from the labels are achieved by the use of nanomaterials, which have wide variety of properties such as high conductivity, increased surface area to volume ratio. Since there are many strategies for signal amplification using nanomaterials in electrochemical immunosensing [12], electrochemical methods gained much attention for quantitative determination of very minute concentration of biomarkers.

The extensive use of nanomaterials as a suitable matrix for anchoring antibodies to capture antigen and carriers to load large amount of redox species have been established improve the performance of immunosensors. Enzyme based signal amplification is a conventional protocol which is now advanced by a variety of nanoparticles [13,14]. Various nanomaterials have been used as signal amplification tags and employed as electrode materials to construct immunosensing platforms such as metal and metal oxide based (gold, silver, platinum, iron oxide) [15,16], carbon based (graphene sheets, carbon nanotubes) [17,18], semiconductor based nanoparticles (silicon), polymeric nanoparticles (polyamidoamine encapsulated gold nanoparticles) [19] and redox mediator based nanoparticles (prussian blue, thionine coated gold nanoparticle) [20,21]. Gold nanoparticles are commonly used signal amplification label in electrochemical immunosensing platform due to their large surface area, abundant binding spaces and provide a synergic effect among catalytic activity, conductivity and biocompatibility which are beneficial to greatly increase the immobilization amount of antibodies and further obtain the amplified electrochemical detection signals.

The aim of this work is to develop a dual signal amplification strategy based electrochemical immunosensing platform using gold nanoparticles. The gold nanoparticle used as both amplification tag and immobilization platform. The antibodies were bound on citrate capped gold nanoparticles which were self assembled on dithiol monolayer formed gold disc electrode. In the fabricated immunosensor, horseradish peroxidase labeled antibody conjugated gold nanoparticle was used as nanolabel for signal enhancement. A stable sandwich immunocomplex was formed on the electrode surface that increased the specificity of the immunosensor. The HRP enzyme on the nanolabel acts as a catalyst in the reaction between thionin and H2O2. The electrochemical signal is generated from the redox mediator, thionine. The working of the fabricated electrochemical immunosensing platform was tested using different HIgG concentrations.

2. Experimental

2.1. Materials and Methods

1,6-hexanediithiol, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS), Human IgG, polyclonal anti-HIgG produced in rabbit, horseradish peroxidase-labeled polyclonal anti-HIgG produced in rabbit, bovine serum albumin (BSA), thionin acetate salt and potassium ferricyanide K3[Fe(CN)6] were purchased from Sigma Aldrich, India. All other reagents such as ethanol, sulphuric acid (H2SO4), trisodium citrate, chloroaacuric acid (HAuCl4), sodium carbonate (Na2CO3), sodium hydroxide, hydrochloric acid, sodium chloride and potassium chloride were purchased from FINAR chemicals, India and used without further purification. Disodium hydrogen phosphate and potassium dihydrogen phosphate were of analytical grade and supplied by LOBA chemicals, India. Hydrogen peroxide (30%) was obtained from Merck. All stock solutions were prepared with Millipore water (18.2 MΩ cm, Millipore, Germany). Antibody and antigen solutions were serially diluted in 0.01 M phosphate buffer saline solution (PBS) pH 7.4.

2.2. Instrumentation

All electrochemical measurements were carried out using CHI 6088D electrochemical workstation (CH Instruments, Texas, USA) with a three-electrode cell. Gold disc electrode was used as the working electrode, a platinum wire as counter electrode and saturated calomel electrode as reference electrode. Atomic force microscopic (AFM) studies of the modified electrode were performed using Park system XE-70 through non-contact mode. Morphology of the nanolabel was determined by high resolution transmission electron microscope (JEOL, JEM-2100). UV-Visible spectra were
recorded with Biospectrometer (Eppendorf, Germany), purification of the nanoconjugate was carried out using Centrifuge 5810 R (Eppendorf, Germany).

2.3. Conjugation of AuNP-HRP labeled anti-HIgG nanolabel
Gold nanoparticles (AuNP) were prepared by Turkevich method using trisodium citrate as both reducing and capping agent as reported [22]. Conjugation of AuNP with HRP labeled anti-HIgG (AuNP-HRP-Ab₂) was achieved by slight modifications from the reported procedure [23]. Initially before conjugation, pH of the colloidal gold nanoparticle solution was adjusted to 9.0 by addition of 0.1 M Na₂CO₃. Following this, 10 µL, 0.1 mg mL⁻¹ HRP-Ab₂ solution was added to 1 mL of gold nanoparticle suspension. This antibody - nanoparticle mixture was gently stirred for 2 h at 4 °C. To minimize the non-specific binding of proteins to free gold sites during analysis, 100 µL of 1 % BSA was used as the blocking buffer. The solution was gently stirred for 30 min at 4 °C. The reaction mixture was centrifuged at 10,000 rpm for 20 minutes at 4 °C. After centrifugation, the supernatant was removed and the soft sediment settled at the bottom of the vial was washed and resuspended in 500 µL of 0.1 M PBS pH 7.4. The obtained nanoconjugate was stored at 4 °C when not in use.

2.4. Fabrication of electrochemical immunosensor

Schematic representation of the HIgG electrochemical immunosensing platform.

Scheme 1. Schematic representation of the HIgG electrochemical immunosensing platform.

Schematic representation of the fabrication of immunosensor is shown in scheme 1. Initially the gold disc electrode (2 mm diameter) was polished with 0.05 µm alumina powder, washed thoroughly and sonicated for 30 seconds in milliQ water. Following this Au electrode was dipped in an ethanolic solution of 50 mM 1,6-hexanediethiol for 22 h at room temperature to form self assembled
monolayers. After washing with absolute ethanol and distilled water, the dithiol/Au electrode was dipped in 1 mL of AuNP solution for 22 h at 4 °C. Then the electrode was washed with 0.1 M PBS of pH 7.4 to remove excess AuNPs. Subsequently, the activated electrode was incubated in 10 µL of 100 µg mL⁻¹ anti-HIgG solution for 22 h at 4 °C. The antibody-modified electrode was washed with PBS and blocked by incubating the electrode in 10 µL of 1 % BSA solution for 15 min at 4 °C. Further the electrode was washed and incubated for 2 h in 10 µL of various concentrations of HIgG solution at 4 °C. After the incubation, the modified electrode was washed with PBS and incubated in HRP labeled anti-HIgG conjugated gold nanoparticle solution (AuNP-HRP-anti-HIgG) for 2 h at 4 °C. The electrode was washed with PBS and stored at 4 °C for further use.

2.5. Electrochemical measurements
After each step of modification, the electrode was characterized using cyclic voltammetry (CV) by scanning the potential from 0 to 0.6 V at a scan rate of 100 mV s⁻¹ and electrochemical impedance spectroscopy (EIS) in the frequency range of 0.01 to 10⁶ Hz and an amplitude of 5 mV in 1.5 M KCl solution containing 5 mM K₃[Fe(CN)₆]. The electrochemical measurements on the modified electrodes were carried out using CV by scanning the potential from 0.2 V to -0.6 V in 0.1 M PBS of pH 7.4 containing 50 µM thionin e and 2 mM H₂O₂. Prior to experimentation, PBS was thoroughly deaerated with high purity nitrogen for 2 h.

3. Results and Discussion
3.1. Spectroscopic characterization of AuNP and AuNP-HRP labeled anti-HIgG nanolabel
The UV-Visible spectra of AuNP and AuNP-Ab₂ are shown in Figure 1. The sharp surface plasmon peak of citrate-capped gold nanoparticle observed at 526 nm (a) shifted to 535 nm (b) on conjugation with HRP labeled anti-HIgG. The red shift in the absorption maximum confirms the effective binding of antibody on the gold nanoparticle. This can be attributed to the fact that the negatively charged citrate groups on the gold nanoparticle favor the adsorption of positively charged amino groups of the lysine residue present on the constant region of an antibody.

![Figure 1. UV-Visible spectra of (a) AuNP and (b) AuNP-HRP labeled anti-HIgG nanolabel.](image-url)
3.2. Morphological characterization of AuNP-HRP labeled anti-HIgG nanolabel and modified electrode

![HRTEM images of AuNP (a, b) and AuNP-HRP labeled anti-HIgG (c, d) at different magnifications.](image)

Figure 2. HRTEM images of AuNP (a, b) and AuNP-HRP labeled anti-HIgG (c, d) at different magnifications.

Figure 2 (a) and (b) show the HRTEM images of AuNP at two different magnifications and the particle size was found to be 15 nm. The images (c) and (d) represent antibody conjugated gold nanoparticle showed an increase in particle size of about 30 nm and the presence of a halo layer surrounding the AuNP which is indicated by arrow in (d). This observation is in accordance with previous reports [24]. After the attachment of antibodies on AuNP the particles are appeared to be close to each other which can be clearly seen in (c). These changes that observed confirm the binding of antibody on gold nanoparticle by electrostatic interaction.

3.3. Electrochemical behavior of the immunosensor

Cyclic voltammograms obtained after each step of sensor fabrication on the gold electrode are shown in Figure. 3A. Well-defined redox peaks of ferricyanide ions were observed at 0.313 and 0.252 V with ΔEp of 61 mV exhibiting a fast electron transfer on the unmodified Au electrode (curve a). The formation of dithiol SAM on the Au electrode resulted in a drastic decrease in faradaic peak current with disappearance of anodic and cathodic peaks (curve b). This is due to the presence of organic thiol molecules that hindered the electron transfer of Fe$^{2+/3+}$ ions from solution to the electrode surface. Further modification with gold nanoparticle exhibited a response showing fast electron transfer with ΔEp of 68 mV was observed in curve c. The dense immobilization of anti-HIgG on AuNP/dithiol/Au electrode significantly decreased the peak current as shown in curve d. The anodic and cathodic peaks almost disappeared similar to that of curve b. This can be attributed to the presence of a large number of antibodies on the electrode surface that further blocked the electron transfer of Fe$^{2+/3+}$ ions and thus occurred a reduction in the peak current. The blocking of electrode surface using BSA molecules and addition of HIgG on the electrode surface further decreased the peak current (curves e and f respectively). The fully fabricated immunosensor with AuNP-HRP labeled anti-HIgG showed a slight decrease in peak current (curve g) which might be due to the more number of antibodies conjugated on AuNP that blocked the electron transfer of Fe$^{2+/3+}$ ions towards the electrode surface.

EIS has been widely used to study the charge transfer resistance properties of the surface modified electrodes. The diameter of the semicircle portion of the Nyquist plot moving from low to
high frequency corresponds to $R_{ct}$ value. As shown in Figure. 3B (a) the EIS of unmodified Au electrode showed no charge transfer resistance (0.001 Ω) due to the metallic property of gold. The modification of dithiol on Au electrode showed a large semicircle in Figure. 3B (b) corresponding to an electron transfer resistance of 298 KΩ. The self assembly of gold nanoparticle on dithiol/Au showed a decrease in semicircle with $R_{ct}$ of 118 Ω (Figure. 3B (c)). The immobilization of anti-HIgG on AuNP/dithiol/Au showed an increase in $R_{ct}$ which is of 54 KΩ (Figure. 3B (d)). The binding of anti-HIgG impedes the electron transfer of Fe$^{2+}/^{3+}$ ions that resulted in the increased $R_{ct}$. Figure. 3B (e) show an increased charge transfer resistance of 200 KΩ due to the presence of BSA and (f) $R_{ct}$ of HIgG modified on BSA/anti-HIgG/AuNP/dithiol/Au was found to be 154 KΩ. The AuNP-HRP labeled anti-HIgG modified electrode showed an increase in charge transfer resistance of 228 KΩ, as shown in (Figure. 3B (g)) and can be attributed to the hindrance in the electron transfer property of AuNP.

![Figure 3](image)

**Figure 3.** (A) CVs at 100 mVs$^{-1}$ under a potential window of 0.2 to 0.6 V and (B) EIS in the frequency range of 0.01 to 10$^6$ Hz with amplitude of 5 mV for the different fabrication steps, bare Au electrode (a), dithiol/Au (b), AuNP/dithiol/Au (c), anti-HIgG/AuNP/dithiol/Au (d), BSA/anti-HIgG/AuNP/dithiol/Au (e) HIgG/BSA/anti-HIgG/AuNP/dithiol/Au (f) and AuNP-HRP labeled anti-HIgG modified electrode which were measured in 5 mM $K_3[Fe(CN)_6]$ and 1.5 M KCl mixture. Inset C shows the EIS of bare Au electrode (a) and AuNP/dithiol/Au (b).

3.4. **Electrochemical response of HRP labeled anti-HIgG/BSA/anti-HIgG/AuNP/dithiol/Au towards HIgG**

This study was carried out to understand the electrochemical performance of the immuno sensor and was tested using cyclic voltammetry in 0.1 M PBS of pH 7.4 containing 50 μM thionine and 2 mM H$_2$O$_2$. Figure. 4, curve a and b show the response of the sensor without H$_2$O$_2$, the redox peaks obtained was due to the presence of thionine in the testing solution. An increase in reduction peak current was observed with addition of H$_2$O$_2$ for two different HIgG concentrations - 100 ng mL$^{-1}$ (a, c) and 100 μg mL$^{-1}$ (b, d). When the responses from two HIgG concentrations were compared more increase in current response was observed for the sensor with higher HIgG concentration (100 μg mL$^{-1}$). This was due to the presence of more number of secondary antibodies forming sandwich immunocomplex on the electrode surface and there by reduction of H$_2$O$_2$ catalyzed by more number of HRP molecules in presence of thionine. This result proves the combined catalytic effect of HRP, H$_2$O$_2$, and thionin as shown in equations 1, 2 and 3. The observation found similar to the previous reports [25,26].
Figure 4. Cyclic voltammograms of HRP labeled anti-HIgG/HIgG/BSA/anti-HIgG/AuNP/dithiol/Au in 0.1 M PBS pH 7.4 containing 50 µM thionine in the absence (a, b) and presence (c, d) of 2 mM H₂O₂. The resulting immunosensors were fabricated using 100 ng mL⁻¹ (a, c) and 100 µg mL⁻¹ (b, d) of HIgG.

HRP (Red) + H₂O₂ → HRP (Oxi) + H₂O …………………… (1)

HRP (Oxi) + Thionine (Red) → HRP (Red) + Thionine (Oxi) …… (2)

Thionine (Oxi) + 2H⁺ + 2e⁻ → Thionine (Red) ………………….. (3)

3.5. Voltammetric studies on dual signal amplification of the immunosensor

Figure 5. (A) Cyclic voltammograms of HRP-anti-HIgG/HIgG/BSA/anti-HIgG/AuNP/dithiol/Au (a) and AuNP-HRP-anti-HIgG/HIgG/BSA/anti-HIgG/AuNP/dithiol/Au (b) in 0.1 M PBS pH 7.4 containing 50 µM thionine and 2 mM H₂O₂. Inset (B) shows zoomed image of graph (A).

Figure 5A shows the comparative study of the responses of the sensor in the absence (a) and presence (b) of nanolabel. The resulting immunosensors were fabricated using 100 ng mL⁻¹ of HIgG.
and cyclic voltammetric study was carried out in 0.1 M PBS pH 7.4 containing 50 µM thionine. The response shows that the sensor with nanolabel, AuNP-HRP-anti-HIgG/HIgG/BSA/anti-HIgG/AuNP/dithiol/Au exhibited a superior biosensing performance over the electrode without nanolabel, HRP labeled anti-HIgG/HIgG/BSA/anti-HIgG/AuNP/dithiol/Au in the presence of 2 mM H₂O₂ (inset B). The presence of AuNP-HRP-anti-HIgG along with citrate capped AuNP on the electrode surface accelerated the electron transfer and showed higher catalytic efficiency than HRP-anti-HIgG. Thus from this study it is confirmed that dual signal amplification strategy introduced in the proposed immunoensing platform was successful and gave good response.

4. Conclusions
A dual signal amplification based sandwich type of electrochemical immunoensing platform was fabricated using citrate capped gold nanoparticle and HRP labeled anti-HIgG conjugated gold nanoparticle (nanolabel). The monolayers of dithiol between gold electrode and gold nanoparticles provided a strong platform for the immobilization of antibody. The fabricated sensor was capable of detecting different HIgG concentrations (100 ng mL⁻¹ and 100 µg mL⁻¹) even without nanolabel. The sensor with nanolabel showed improved response compared to that of the HRP labeled anti-HIgG. In addition to that the developed sensor platform with dual signal amplification strategy can be successfully employed for the early detection of reduced or elevated levels of protein biomarkers whose serum concentrations are in the order of femto or nanograms.

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