Chapter 6

Triphenylphosphine stabilized Gold Nanostructures for Mediator-free Detection of Free and Total Cholesterol

6.1. Introduction

The application of noble metal nanostructures such as gold in the development of biomedical diagnostic devices has remarkably increased\[302, 303\]. As demonstrated in chapter 5, the Au nanostructures provide unprecedented electron transfer between biomolecules and electrode surface resulting in high sensitivity, excellent detection limit and fast response. The building blocks of Au NPs to construct the electrochemical biosensor are promising platform owing to their extraordinary electrocatalytic activity.

As mentioned in section 5.4 of chapter 5, the incorporation of electroactive molecules can enhance the electrochemical properties of nanostructured Au electrode and can further improve the sensitivity and other biosensing parameters. The delocalized electron structure of the electroactive molecules like triphenylphosphine can facilitate the electron transportation and can thus increase the electrochemical response of the bioelectrodes.

Au nanostructures such as triangular prisms, nanocubes, nanorods, nanowires and nanoparticles have been synthesized using different methods for the development of biosensors and optoelectronic nanodevices[285]. Few methods involving single-step synthesis and assembly of Au nanostructures like liquid-liquid interface[304], physical vapour deposition[305], electrochemical deposition[126] etc. have been developed for fabricating nanostructured films. Among these, electrochemical deposition technique is simple, inexpensive and provides easy control over structure of the NPs. There are few reports on the electrodeposition of Au nanostructures using aqueous H\text{AuCl}_4 precursor
and manipulation of different morphologies is accomplished either by varying pH, potential or deposition time[306, 307]. Ye et al. have reported the template-free electrodeposition of Au dendrites, where growth rate has been controlled by variation of deposition potential[308]. However, electrochemical synthesis and assembly of Au nanostructures in an organic medium has yet not been reported. Organic medium provides additional advantage of utilizing conjugated organic molecules, organic dyes etc. which may enhance the charge transfer properties of nanocrystalline films. Moreover, slow reaction kinetics in case of organic solvent provides easy control over structure, thickness and functionality of the Au nanocrystalline films.

The surface functionalization of nanostructured Au can be achieved with ligands containing functional groups such as thiols, phosphines, amines etc. due to their strong affinity[309]. The biomolecules such as oligonucleotides, proteins and antibodies can be anchored to the functionalized Au surface, which enable broad range of applications including programmed assembly and crystallization of materials[310], arrangement of NPs into dimers and trimers on DNA templates[311], bioelectronics[312] and biosensors[313]. Park et al. have demonstrated the DNA-directed assembly of Au NPs for the formation of colloidal nanoparticle crystals[310]. Yu et al. have developed a sensitive Lab-in-a-tube biosensor for multiplex detection of biological targets using Au nanorods[314]. It is known that the cysteamine (an amphiphilic thiol) can play an important role to functionlize Au surface due to S-Au bond formation. It can control the shape of Au nanostructures, hydrophilicity and provides amine groups on Au surface which interact with biomolecules via covalent bonding. Thus, there is considerable scope
to control the morphology of Au nanostructures and the loading of biomolecules in order to improve the efficiency of electrochemical biosensor.

In this chapter, the fabrication of triphenylphosphine stabilized nanostructured Au films has been reported and morphology of the films has been controlled with the systematic addition of cysteamine. The immobilization of ChOx on different Au surfaces has been carried out to investigate the kinetic analysis and biosensing performance. Excellent sensitivity, low $K_m^{\text{app}}$ value and low detection limit of this proposed biosensor have been optimized through the control of morphology and loading of Au nanostructures.

6.2. Experimental Section

6.2.1. Synthesis of Au Nanostructures

Au nanostructures were deposited on ITO coated glass substrates using chronoamperometric technique. During deposition, Au-phosphine complex [Au(PPh$_3$)$_2$Cl] (2.5 mM) and trichloroacetic acid (50 mM) dissolved in 15 mL acetonitrile solvent was used as electrolyte. Deposition potential has been optimized to -10 V at 25 °C. Further, various cysteamine concentrations (0.025 mM, 0.25 mM and 2.5 mM) were added to the electrolytic solution in order to control shape of Au nanostructures [Fig. 6.1].

Onset of decomposition of Au(PPh$_3$)$_2$Cl and liberation of Au$^{3+}$ ions occur on applying voltage which is evident from the gradual emergence of yellow colour in the electrolytic solution. Au$^{3+}$ ions drift towards ITO electrode under the influence of electric field and electrochemical reduction of Au$^{3+}$ ions results in Au$^{0}$. Simultaneously, some Au-phosphine complex crystallizes on ITO surface, which gradually gets reduced[298]. This is evident from characteristic UV absorption of Au-phosphine complex at ~ 390
Nucleation of Au NPs occurs during electrochemical reduction of $\text{Au}^{3+}$ to $\text{Au}^0$ and various structures result from the assembly of NPs depending on cysteamine concentrations. In the absence of cysteamine, rate of reaction is slow which results in the formation of elongated nanostructures (rod-shape)[296]. Cysteamine addition leads to higher current resulting in faster deposition of Au nanostructures [Fig. 6.6(A)]. Due to insufficient cysteamine molecules, the monodispersity of the Au NPs is unstable. They assemble themselves into various hierarchical nanostructures in order to attain minimum surface energy and thus, the smaller amount of surfactant provide larger nanostructures[296].

![Diagram of Electrochemical Deposition and Biosensing Responses](image)

**Figure 6.1:** Synthesis of cysteamine functionalized Au nanostructures and their biosensing performances.

With increase of cysteamine molecules, Au NPs assembly gets restricted and finally results in well-spaced individual Au NPs on ITO surface. Higher cysteamine concentration in the precursor solution augments the reaction kinetics leading to faster evolution of Au films with different nanostructures. Thus, the shape of Au nanostructures has been tuned and optimized using amphiphilic cysteamine molecules during electrochemical deposition.
6.2.2. Enzyme Immobilization

Prior to enzyme immobilization, the amine functionalized Cys-Au/ITO electrode was dipped in 0.5% glutaraldehyde (crosslinker) solution for 4 h and rinsed with de-ionized water. The modified electrode was incubated with ChOx solution (1 mg ml\(^{-1}\)) for overnight at 4°C. One terminal (CHO) of glutaraldehyde forms a covalent amide bond with amine functionalized Au surface while the other terminal forms an additional amide bond with amine groups of enzyme (ChOx)[316] [Fig. 6.2]. The loosely bound enzymes were washed off with PBS (pH 7.4). This fabricated ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrode was kept in refrigerator at 4°C when not in use.

![Figure 6.2: Surface bio-functionalization of Au nanostructure for cholesterol estimation.](image)

6.3. Results and Discussion

6.3.1. TEM Studies

Figure 6.3(a) shows TEM micrograph of Au film prepared with Au(PPh\(_3\))Cl in acetonitrile solution. It can be seen that the rod shaped Au (length ~ 1µm, aspect ratio~ 4.5) is randomly oriented on the ITO surface. The high resolution micrograph shows crystalline domains of Au with d-spacing of 0.23 nm corresponding to (111) plane of fcc-Au [JCPDS- 040784]. Inset shows a single cylindrical Au rod. It has been observed that the slow deposition rate of Au due to the limited current across electrode-electrolyte system in organic solvent results in low loading of nanostructures on film surface. Owing
to slow reaction kinetics, Au-phosphine complex crystallizes into elongated structures on ITO surface and eventually reduces to form Au rods while few Au(PPh₃)Cl crystals decompose and reduce to spherical shape Au NPs. On addition of cysteamine (0.025 mM), the microstructural transformation of Au rods has been observed [Fig. 6.3(b)]. These Au rods (length ~600 nm, aspect ratio~9.2) have conical ends due to selective etching of rods at the ends[317]. The enlarged view of this Au rod has been shown in image [Fig. 6.3(b), inset]. The regular arrangement of monodisperse Au NPs (size < 4nm) play an important role to form the rod shaped Au (conical). Cysteamine augments the reaction kinetics and facilitates electrochemical reduction of Au(PPh₃)Cl resulting in Au NPs. However, due to smaller amount of surfactant, NPs assemble into hierarchical structures in order to attain minimum surface energy[296].

![Figure 6.3: TEM and HRTEM micrographs of Au nanostructures electrochemically deposited with cysteamine concentrations of: (a) 0 mM, (b) 0.025 mM, (c) 0.25 mM and (d) 2.5 mM.](image)

The rod-shaped structures of Au disappear and circular assemblies of ~ 100 nm are obtained with increase in cysteamine concentration (0.25 mM) in the electrolytic
solution [Fig. 6.3(c)]. A single flower-like nanostructure (inset) clearly shows that Au NPs act as building blocks of these structures. It has been found that the lattice spacing of Au is same in all concentrations of cysteamine (Fig. 6.3(c), below). With further addition of cysteamine concentration (2.5 mM or Au:Cyst.=1:1), no secondary structures resulted and monodisperse Au NPs were obtained due to complete coverage with the cysteamine molecules [Fig. 6.3(d)]. The average size of Au NPs has been estimated as ~4nm. Loading of these Au NPs on film surface is higher compared to other structures.

6.3.2. UV-vis and SEM Studies

Figure 6.4(A) shows the optical absorption spectra of the prepared nanocrystalline Au films with different concentrations of cysteamine and the corresponding SEM images. Films show characteristic plasmon bands from uncoupled Au nanocrystals at ~560 nm alongwith bands at higher wavelength arising due to electronic coupling among Au NPs, elongated nanostructures or assembly of NPs[318, 319]. The absorption intensity increases with increasing cysteamine concentration suggesting higher loading of nanoparticles and formation of thicker films. Slight blue shift has been observed in higher wavelength band suggesting formation of smaller nanostructures with subsequent increase in cysteamine concentration, which is further confirmed by SEM studies. The higher wavelength band disappears with excess of cysteamine due to complete coverage of cysteamine molecules onto all the facets of Au NPs which restricts the electronic coupling among NPs[320]. However, in the absence of cysteamine, band at higher wavelength is due to elongated structure rather than assembly of NPs. The peak found at 390 nm corresponds to PPh\textsubscript{3} stabilized Au clusters[315].

6.3.3. Contact Angle Studies
Contact angle measurements have been carried out in sessile drop mode to measure the surface wettability of fabricated Au films using water as a dispersant [Fig. 6.4(B)]. Au film prepared in absence of cysteamine is highly hydrophobic and adsorption of amphiphilic thiol (cysteamine) onto Au NPs results in decrease in contact angle of Au films. The contact angle on Au films declines consistently in steps of 118°, 94°, 70°, 54° with subsequent increase in concentration of cysteamine and follows exponential decay of second order. This shows that cysteamine adsorption onto Au NPs induces hydrophilicity, which is pre-requisite for immobilization of biomolecules onto nanocrystalline Au films. The wettability evolution of Au films is linked to the progressive increase of surface terminations with NH₂ groups[297].

Figure 6.4: (A) UV-visible spectra and corresponding SEM micrographs of Au films electrochemically deposited with cysteamine concentration of: (a) 0 mM, (b) 0.025 mM, (c) 0.25 mM and (d) 2.5 mM. (B) Contact angle of water with Au films synthesized under similar conditions.

6.3.4. FT-IR Studies

Figure 6.5 shows the FT-IR spectra of various fabricated films. The presence of peaks at 502 cm⁻¹, 826 cm⁻¹, 1180 cm⁻¹ and 1588 cm⁻¹ corresponding to metal-C stretching, C-Cl
stretching, P-CH$_3$ stretching and C-H stretching, respectively due to the presence of aromatic rings of [Au(PPh$_3$)Cl], confirms the deposition of Au films alongwith PPh$_3$ [Fig. 6.5(a)]. The peaks at 672 cm$^{-1}$, 1414 cm$^{-1}$, 3218 cm$^{-1}$ and 3394 cm$^{-1}$ correspond to C-S stretch, C-H stretch, NH$_2$ antisymmetric and NH$_2$ symmetric stretch, respectively. This indicates the presence of aminothiol i.e. cysteamine on Au surface. On incubation of Au film with glutaraldehyde [Fig. 6.5(b)], the peaks found at 1135 cm$^{-1}$ and 1664 cm$^{-1}$ corresponding to the C=O stretch and C-N antisymmetric stretch arise due to the formation of amide bond between amine functionalized Au NPs and glutaraldehyde. The peaks at 554 cm$^{-1}$ and 674 cm$^{-1}$ are assigned to the NH$_2$ deformations in amines and the peak at 2870 cm$^{-1}$ is due to C-H stretching of cysteamine.

**Figure 6.5:** FT-IR spectra of (a) PPh$_3$-Au/ITO electrode, (b) Glu/PPh$_3$-Au/ITO electrode and (c) ChOx-Glu/PPh$_3$-Au/ITO bioelectrode.

Further, after ChOx immobilization [Fig. 6.5(c)], the additional peaks at 835 cm$^{-1}$, 1048 cm$^{-1}$ and 2343 cm$^{-1}$ appear due to P-O, P-OH and P-H stretch, respectively, indicating the presence of enzyme molecules on Au film surface. The peaks at 1668 cm$^{-1}$, 510 cm$^{-1}$ and 2395 cm$^{-1}$ corresponding to C=O stretch and NH$_2$ deformations confirm the
formation of amide bond between glutaraldehyde and enzyme molecules. This confirms the covalent functionalization of enzyme molecules on Au surface.

6.3.5. Electrochemistry and Kinetic Studies

The impedance characteristics of nanostructured Au films have been studied using EIS in response to perturbing potential of 0.3V has been studied as a function of frequency (0.1-10⁴ Hz) in PBS (pH 7.4) containing K₃/K₄[Fe(CN)₆] as a redox couple. Figure 6.6(B) shows Nyquist diagrams for the nanostructured Au films prepared using different concentrations of cysteamine. A Randles equivalent circuit for these EIS spectra is shown in inset. The prepared Au film without cysteamine shows the Rₛ value as 83.1 Ω. After cysteamine (0.025 mM) incorporation, the value of Rₛ decreased to 64.7 Ω and further it reduced to 5.24 Ω with increase of cysteamine concentration. Decrease in Rₛ value is ascribed to the higher loading of Au nanostructures which acts as an ‘electronic antennae’, effectively facilitating electron transfer across the electrode-electrolyte interface. The smaller nanostructures resulting from the successive addition of cysteamine shows enhanced elecrocatalytic activity and favors electron-transfer kinetics which improves diffusion efficiency of redox species from the bulk solution to the electrode surface [321].

To examine the electrochemical activity of nanostructured Au films, CV studies have been carried out in PBS (pH 7.4) without any redox mediator at scan rate of 50 mVs⁻¹. Figure 6.6(C) shows CV curves of the Au nanocrystalline films prepared with different cysteamine concentrations. The oxidation peak is found at ~ 0.2 V, while the reduction peak is at ~0.1 V in the potential region from -0.7 V to +0.7 V. As bare ITO electrode has no current response in this potential region, the redox peaks are attributed to
the oxidation and subsequent reduction of gold oxide (AuOₓ) formation on the surface of Au nanostructures as a result of positive polarization[322]. This indicates that the reversible chemical reaction occurred on Au surface. Besides this, the enhanced current can be attributed to the deposition of electroactive PPh₃ alongwith Au nanostructures[298]. On addition of cysteamine, the S²⁻ ions are attached to the Au surface which facilitates the electrochemical oxidation and reduction of Au surface resulting in enhanced redox current[323, 324]. The consistent increase in redox current from Au films with increase of cysteamine concentration is due to the higher loading of Au nanostructures on ITO substrates resulting in the deposition of thicker films. The dense packing of Au nanoparticles on ITO surface helps in fast electron transportation compared to other Au nanostructures leading to enhanced redox current.

The fabrication of the ChOx-Glu/PPh₃-Au/ITO bioelectrode has been investigated electrochemically at each step in PBS (pH 7.4) at scan rate of 50 mVs⁻¹ [Fig. 6.6(D)]. The nanostructured Au electrode shows well-defined redox peaks in PBS without any redox mediator. On incubation of the PPh₃-Au/ITO electrode with glutaraldehyde, the redox peaks disappear due to the formation of an insulating layer which impedes the flow of electrons between electrode and electrolyte. However, the redox peaks re-appear on immobilization of the ChOx molecules due to the direct exchange of electrons between the nanostructured Au film and the flavin adenine dinucleotide (FAD) centre of the enzyme molecule. The redox peaks at ~0.3 V and ~0.1 V correspond to the oxidation and reduction of FAD cofactor of ChOx molecule[300]. The enzyme molecules are attached to the electrode surface in such a way that their redox centres are in close proximity, this may result in direct electron transfer between the enzyme and the electrode.
To investigate the enzyme kinetics, CV studies have been conducted before and after enzyme immobilization on nanostructured Au electrodes as a function of scan rate (10-100 mV s\(^{-1}\)). For all electrodes, the anodic peak potential shows a slight shift towards the positive potential whereas the cathodic potential shifts in the reverse direction with
the increase of scan rate, suggesting a quasi-reversible redox process. The redox peak currents for all electrodes and bioelectrodes are proportional to the scan rate indicating that the electrochemical reaction is diffusion controlled[115] [Fig. 6.7(A), (B)]. The enhancement in peak current with increasing scan rate is indicative of facile charge transfer of the redox moieties embedded in enzyme molecules with the nanostructured Au electrode. The peak potentials vary linearly with the logarithm of scan rate [Fig. 6.7(C), (D)] in accordance with Laviron’s theory. The plot of ln ν versus anodic peak potential (Epa) and cathodic peak potential (Epc) yields two straight lines with slopes of X = RT/(1-α)nF and Y = -RT/αnF, respectively. It is found that the value of electron transfer coefficient (α) increases consistently for the nanostructured Au electrodes from 0.35 to 0.56 with increasing amount of cysteamine [Table 6.1]. This signifies that dense packing of the smaller Au nanostructures provides higher surface area to induce faster electron transport across the interface compared to sparsely-arranged elongated nanostructures.

On the contrary, the value of charge transfer rate constant (k_s) consistently decreases from 1.23 × 10^{-3} s^{-1} to 0.01 × 10^{-3} s^{-1} as the peak separation increases for Au electrodes with increase of cysteamine concentration. The diffusion coefficient calculated using Brown-Anson model is nearly three times for the ChOx-Glu/PPh3-Au/ITO bioelectrode (2.5 mM cysteamine) compared to when there was no cysteamine. The surface concentration of Au electrode has been evaluated using Randles-Sevick equation and is found to be maximum (5.37 × 10^{-14} mol cm^{-2}) for 2.5 mM concentration of cysteamine, which is in accordance with the higher electrochemical surface area (0.23 cm^2) and higher redox currents compared to other concentrations of cysteamine [Table 6.1].
Figure 6.7: Anodic current and cathodic current as a function of the scan rate for the PPh₃-Au/ITO electrodes (A) and ChOx-Glu/PPh₃-Au/ITO bioelectrodes (B). Anodic and cathodic peak potential as a function of logarithm of scan rate for PPh₃-Au/ITO electrodes (C) and ChOx-Glu/PPh₃-Au/ITO bioelectrodes (D).

The values of α and kₛ increase after ChOx immobilization on Au electrodes compared to their respective electrodes [Table 6.1]. The increase in the values of kₛ of the bioelectrodes by ~ six orders compared to their respective electrodes shows that the redox centres of ChOx molecules are in close proximity of the Au surfaces, resulting in facile electron transport. After enzyme immobilization, the effective electrochemical surface
area has been found to increase and ~10-fold enhancement in surface concentration is observed whereas the diffusion coefficient decreases by four orders of magnitude [Table 6.1]. The higher electrochemical surface area and higher surface concentration is in accordance with the enhanced redox currents on immobilization of ChOx. The surface concentration of the ChOx-Glu/PPh₃-Au/ITO bioelectrode has been found to be comparable to the other bioelectrodes reported in literature[180, 325].

<table>
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<tr>
<th>Electrodes</th>
<th>Cysteamine Conc. (mM)</th>
<th>α</th>
<th>kₐ (s⁻¹)</th>
<th>Γ (mol cm⁻²)</th>
<th>D (cm² s⁻¹)</th>
<th>A (cm²)</th>
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<td>Cys-Au/ITO</td>
<td>0</td>
<td>0.35</td>
<td>1.23 x 10⁻¹⁴</td>
<td>0.69 x 10⁻⁸</td>
<td>0.36 x 10⁸</td>
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<td>0.48</td>
<td>0.61 x 10⁻¹⁴</td>
<td>1.33 x 10⁻⁸</td>
<td>0.15 x 10⁸</td>
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<td>Cys-Au/ITO</td>
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<td>ChOx-Glu/PPh₃-Au/ITO</td>
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<td>ChOx-Glu/PPh₃-Au/ITO</td>
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<td>0.61</td>
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<td>13.71 x 10⁻¹³</td>
<td>2.47 x 10⁴</td>
<td>0.42</td>
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**Table 6.1:** Values of α, kₐ, Γ, D and A for the various fabricated electrodes and bioelectrodes.

### 6.3.6. Free Cholesterol Detection

In order to investigate the electrochemical response of ChOx-Glu/PPh₃-Au/ITO bioelectrode towards cholesterol, we have fabricated the bioelectrodes by varying the concentrations of cysteamine during electrodes fabrication. Figure 6.8 shows the CV response of the ChOx-Glu/PPh₃-Au/ITO bioelectrodes (fabricated with 0 mM, 0.025 mM, 0.25 mM and 2.5 mM cysteamine) for varying concentrations of cholesterol. It has been found that the anodic peak current increases with the increase in cholesterol concentration in the range of 10-500 mg dl⁻¹ for all bioelectrodes. During enzymatic reaction, the ChOx catalyzes the oxidation of cholesterol (3β-hydroxy steroids) to the intermediate product Δ5-6-ene-3β-ketosteroid (cholest-5-en-3-one) and then
isomerization of the intermediate yields Δ3-4-ene-3β-ketosteroid (cholest-4-en-3-one). Key to this conversion is the FAD cofactor, which gets reduced to FADH₂ in the process. The reduced cofactor directly exchanges electrons with the nanostructured Au electrode at ~0.3 V to get reoxidized resulting in increase of peak current with the increase of cholesterol concentration.

Figure 6.8: Cyclic voltammetric response curves for ChOx-Glu/PPh₃-Au/ITO bioelectrode with cysteamine concentrations of: (A) 0 mM, (B) 0.025 mM, (C) 0.25 mM and (D) 2.5 mM.
Figure 6.9(A) shows the linear calibration plots for the ChOx-Glu/PPh$_3$-Au/ITO bioelectrodes at 0 mM, 0.025 mM, 0.25 mM and 2.5 mM of cysteamine concentrations. It has been observed that the magnitude of current increases with increase of cysteamine concentration in ChOx-Glu/PPh$_3$-Au/ITO bioelectrode. This may be attributed to the higher loading of amine-functionalized Au nanoparticles on electrode which provides higher electroactive surface area for enzyme immobilization. Thus, the amount of sulphur content in this proposed biosensor can improve the efficacy of sensing characteristics. The sensitivity has been found to be maximum (4.22 AM$^{-1}$ cm$^{-2}$) at 2.5 mM of cysteamine concentration [Fig. 6.9(B)]. This biosensor shows an excellent response in the physiological range of cholesterol in human body. Table 6.2 shows the variation of the sensitivity, detection limit, $K_{m}^{app}$ value etc. with cysteamine concentration for ChOx-Glu/PPh$_3$-Au/ITO bioelectrode. The detection limit of the bioelectrode has been estimated as 5.49 µM for 2.5 mM cysteamine concentration, which is approximately half than the other concentrations of cysteamine.
Figure 6.9: (A) Calibration plots showing the current response as function of cholesterol concentration (10-500 mg dl\(^{-1}\)) for different ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrodes. (B) Variation of sensitivity and \(K_{m}^{\text{app}}\) value with cysteamine concentration of ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrode. (C) Interference studies of ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrode (2.5 mM cysteamine as a function of different analytes. (D) Current response as a function of days for ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrode (2.5 mM cysteamine) for 200 mg dl\(^{-1}\) cholesterol.

The \(K_{m}^{\text{app}}\) value used to evaluate the biological activity of the enzyme has been calculated using Lineweaver Burk equation. From \(I_{\text{as}}^{-1}\ versus \ C^{-1}\) plot, the \(K_{m}^{\text{app}}\) value is estimated for each biosensor and decrease in \(K_{m}^{\text{app}}\) value has been observed with the increase of cysteamine concentration [Fig. 6.9(B)]. The \(K_{m}^{\text{app}}\) value for ChOx-Glu/PPh\(_3\)-Au/ITO bioelectrode is found to be lowest (0.57 mM) at 2.5 mM concentration of cysteamine. This is due to the presence of functional amine groups on Au surface resulting in increased hydrophilicity (at 2.5 mM) compared to other concentrations of cysteamine based electrodes. This low value of \(K_{m}^{\text{app}}\) signifies the high catalytic efficiency of enzyme onto the nanostructured Au electrode, which results in better interaction of ChOx with cholesterol. This proposed biosensor shows superior sensitivity, detection limit and \(K_{m}^{\text{app}}\) values in the wide detection range of cholesterol (10-500 mg dl\(^{-1}\)) compared to reported literature[112, 166, 184, 276, 326] [Table 6.3]. Additionally, this
fabricated biosensor achieves steady-state current in less than 15 s indicating fast and direct transfer of electrons from the enzyme to the electrode surface. Thus, this biosensor do not require any mediator such as [Fe(CN)$_6$]$^{3/-4}$ or label leading to 3rd-generation mediator-free biosensor.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Cysteamine Conc. (mM)</th>
<th>Sensitivity (AM$^{-1}$cm$^{-2}$)</th>
<th>Detection Limit (µM)</th>
<th>$K_m^{app}$ (mM)</th>
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<tr>
<td>ChOx-Glu/PPh$_3$-Au/ITO</td>
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<td>ChOx-Glu/PPh$_3$-Au/ITO</td>
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<td>ChOx-Glu/PPh$_3$-Au/ITO</td>
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<td>0.14</td>
<td>7.95</td>
<td>0.61</td>
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<td>ChOx-Glu/PPh$_3$-Au/ITO</td>
<td>2.5</td>
<td>4.22</td>
<td>5.49</td>
<td>0.57</td>
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**Table 6.2:** Biosensing characteristics (sensitivity, detection limit, $K_m^{app}$) for ChOx-Glu/PPh$_3$-Au/ITO bioelectrode with various cysteamine concentrations.

The effect of potential interferents such as ascorbic acid (AA), uric acid (UA), glucose (Glu), and urea found in the blood samples at normal concentrations has been investigated on the CV response of ChOx-Glu/PPh$_3$-Au/ITO bioelectrode (2.5 mM cysteamine concentration) towards cholesterol (100 mg dl$^{-1}$) [Fig. 6.9(C)]. The ChOx-Glu/PPh$_3$-Au/ITO bioelectrode shows a significant change in peak current in presence of cholesterol. However, this bioelectrode in absence of cholesterol (or presence of interferents) exhibits a negligible change in current response as evident by low relative standard deviation (RSD: 6.7 %, n=5). The low detection potential (+0.3V) of the biosensor circumvents the effect of other analytes present in blood, which may easily get oxidized at higher potential and interfere with signal from the cholesterol. Thus, the proposed biosensor with low detection potential would be an efficient platform for point-of-care diagnostic device. In addition, this biosensor shows excellent reproducibility as evident by low RSD of 4.0% for four different electrodes in presence of 200 mg dl$^{-1}$
cholesterol concentration. The bioelectrode retained 90% of the current response after 50 days indicating good stability [Fig. 6.9(D)].

<table>
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<th>Electrodes</th>
<th>Sensitivity (AM⁻¹)</th>
<th>Detection Limit (mM)</th>
<th>Linear Range (mM)</th>
<th>Keq_app (mM)</th>
<th>Response time (s)</th>
<th>Ref.</th>
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<td>ChOx/Au-ODA/ITO</td>
<td>1.08 × 10⁻⁴</td>
<td>0.60</td>
<td>0.65-12.95</td>
<td>---</td>
<td>20</td>
<td>[166]</td>
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<tr>
<td>ChOx/PANI/ITO</td>
<td>3.43 × 10⁻⁴</td>
<td>---</td>
<td>0.13-10.00</td>
<td>1.21</td>
<td>---</td>
<td>[326]</td>
</tr>
<tr>
<td>ChOx/PANI-CdS/ITO</td>
<td>1.01 × 10⁻⁴</td>
<td>1.19</td>
<td>1.25-12.50</td>
<td>0.82</td>
<td>20</td>
<td>[276]</td>
</tr>
<tr>
<td>ChOx/4-ATP/Au</td>
<td>0.54 × 10⁻⁴</td>
<td>---</td>
<td>0.65-10.36</td>
<td>1.34</td>
<td>20</td>
<td>[184]</td>
</tr>
<tr>
<td>ChEt-ChOx/4-ATP/Au</td>
<td>0.88 × 10⁻⁴</td>
<td>---</td>
<td>0.65-10.36</td>
<td>1.06</td>
<td>20</td>
<td>[184]</td>
</tr>
<tr>
<td>ChEt-ChOx/PPy/Pt</td>
<td>0.15 × 10⁻³</td>
<td>---</td>
<td>1.00 – 8.00</td>
<td>9.8</td>
<td>---</td>
<td>[112]</td>
</tr>
<tr>
<td>ChOx-Glu/PPh₃-Au/ITO</td>
<td>4.22</td>
<td>0.0054</td>
<td>0.26-12.95</td>
<td>0.57</td>
<td>15</td>
<td>Present work</td>
</tr>
<tr>
<td>ChEt-ChOx-Glu/PPh₃-Au/ITO</td>
<td>2.16</td>
<td>0.0059</td>
<td>0.26-12.95</td>
<td>0.47</td>
<td>15</td>
<td>Present work</td>
</tr>
</tbody>
</table>

Table 6.3: Comparison table summarizing characteristics of mediator-free cholesterol biosensors.

**Figure 6.10:** (A) Cyclic voltammetric response curves for ChEt-ChOx-Glu/PPh₃-Au/ITO bioelectrode for varying concentrations of cholesterol oleate (10-500 mg dl⁻¹). (B) Calibration plot showing the current response as function of cholesterol oleate concentration.

6.3.7. Total Cholesterol Detection

The optimized nanostructured Au electrode (2.5 mM cysteamine) is functionalized with dual enzymes (ChEt and ChOx) for detection of total cholesterol. The electrochemical...
response of the ChEt-ChOx-Glu/PPh₃-Au/ITO bioelectrode towards different concentrations of cholesterol ester (oleate) is studied [Fig. 6.10(A)]. The anodic current has been found to increase with the increasing concentrations of cholesterol ester (10-500 mgdl⁻¹). In the proposed biochemical reaction, cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol (or 3β-hydroxysteroids) and fatty acid. ChOx then catalyzes the oxidation of cholesterol (3β-hydroxysteroids) to the intermediate product Δ5-6-ene-3β-ketosteroid (cholest-5-en-3-one). The isomerization of the intermediate product results into Δ3-4-ene-3β-ketosteroid (cholest-4-en-3-one)[301]. The FAD cofactor gets reduced to FADH₂ in the process and in order to re-oxidize, the direct exchange of electrons occur with the nanostructured Au electrode resulting in increase in peak current with the increasing concentrations of cholesterol oleate. The anodic peak current of the ChEt-ChOx-Glu/PPh₃-Au/ITO bioelectrode plotted as a function of cholesterol concentration reveals the linearity range as 10–500 mg dl⁻¹ (within 2% error) with standard deviation and correlation coefficient of 0.28 µA and 0.99, respectively [Fig. 6.10(B)]. The sensitivity of the ChEt-ChOx-Glu/PPh₃-Au/ITO bioelectrode calculated from the slope of linear regression curve is 2.16 AM⁻¹cm⁻² and the detection limit calculated using 3*SD/Sensitivity is 5.91 µM. The \( K_{m}^{\text{app}} \) value, which is an indication of the enzyme-substrate kinetics, has been evaluated from the Lineweaver-Burk equation and has been calculated to be as 0.47 mM[143]. The small \( K_{m}^{\text{app}} \) value indicates that the immobilized enzymes possess high enzymatic activity and that the fabricated biosensor exhibits a high affinity for total cholesterol.

6.3.8. Clinical Sample Analysis
The CV response of the ChEt–ChOx–Glu/PPh₃-Au/ITO bioelectrode was studied for five clinical samples with varying cholesterol concentrations. Serum samples from patients along with clinical data of cholesterol levels were collected from Dr. Arvind’s Family Clinic, New Delhi (India). The ChEt–ChOx–Glu/PPh₃-Au/ITO bioelectrode exhibits the change of current for samples with different cholesterol concentrations [Fig. 6.11(A)]. The plot of current *versus* logarithmic value of concentration shows a near linear response with clinical samples [Fig. 6.11(B)] and the sensitivity has been calculated as 1.93 AM⁻¹cm², which differs from the sensitivity obtained for standard cholesterol concentrations by ~12%. Thus, these results indicate that this biosensor has the potential to detect cholesterol directly in human serum samples.

**Figure 6.11:** (A) Cyclic voltammetric response of the ChEt-ChOx/Glu-NanoAu/ITO bioelectrode for available clinical samples of different cholesterol concentrations. (B) Calibration plot showing the variation of anodic peak current with cholesterol concentrations.

**6.4. Conclusions**

We have demonstrated a single-step method for synthesis and deposition of triphenyl phosphine stabilized Au nanostructures on ITO substrate using an organometallic Au
precursor in an organic solvent. The shape of Au nanostructures has been tuned using the amphiphilic thiol (cysteamine) and confirmed by TEM and SEM studies. In this proposed method, cysteamine molecules not only improve the rate of loading of Au NPs resulting in higher current but also induces hydrophilicity due to surface termination with amine groups leading to better biofunctionalization. We have investigated the biosensing performance and enzyme kinetics with various synthesized Au nanostructures. The presence of electroactive triphenyl phosphine in Au electrodes enhances the direct electron transfer properties with enzyme molecules resulting in mediator-free biosensors. It has been observed that the proposed mediator-free biosensors exhibit manifold increase in sensitivity compared to reported literature. By varying the cysteamine concentration, the detection limit and $K_{m}^{app}$ value of the biosensor have been improved considerably. Also, the biosensors show excellent selectivity, stability and reproducibility. The sensor also exhibits the linear response for serum samples of different cholesterol concentrations and can thus finds application for point-of-care diagnostics.