CHAPTER 7

BIO-INSPIRED GOLD MICROWIRES INTEGRATED AMPEROMETRIC BIOSENSOR

Overview of Chapter

This chapter is dedicated to bio-inspired gold microwires (AuMWs) synthesis, their evaluation as potential microelectrodes for electron transfer between enzyme and electrode surface, in amperometric biosensing applications. Motivating factor behind this work was expected improved current response through delocalization of electrons over longer length scales as in case of CNTs and nanowires. In addition to this as discussed in previous chapter (chapter 6) the amperometric response of branched chain amino functionalized AuNPs is much better than spherical NPs. Hence we explored the amino acid functionalized Au microwires for their probable biosensing efficiency. The chapter is structured as:

7.1 Introduction
7.2 Experimental
   7.2.1 Synthesis of AuNPs.
   7.2.2 Fungal inoculation in AuNPs solution.
7.3 Results and discussion
   7.3.1 Absorption Spectroscopic studies
   7.3.2 Optical and Electron Microscopic Characterization of AuMWs
   7.3.3 Proposed Mechanism
   7.3.4 GOx- AuMWs Activity Analysis
   7.3.5 Biosensing Performance Analysis
7.4 Conclusion

7.1 Introduction

In recent years, there has been an increased interest in synthesis of one-dimensional (1D) micro/nanostructures of metals and semiconductors [176] due to their unique physical and optical properties with applications in fields like electronics [177], optics [178, 179], imaging [180, 181], sensor [182, 183] or biosensor devices [184]. Various synthetic methods have
been exploited for synthesis of these 1-D micro-/nanostructures, such as photochemical methods [185, 186], hydrothermal synthesis [187], and hard template (polycarbonate or porous anodic alumina membranes) approach [188, 189]. Although the template based synthesis is the most reliable approach among the above mentioned synthesis methods still removal of template in order to obtain pure metallic structures is the major limitation of this approach. Hence it is pertinent to explore eco-friendly route for synthesis of 1D micro/nanostructures. Variety of chemical free reduction methods (using crude extracts of leaf or other natural products), to synthesize gold nanoparticles, has been explored after recognition of amino acids as an independent alternative for reduction of gold ions [190-192]. Recently, few reports utilizing microorganisms (viruses, bacteria and fungi) as living templates have been proposed for synthesis of different microstructures exploiting unique microbial cell architectures [193, 194]. Limited reports available so far, have not been able to understand the mechanism of MWs synthesis. Initial studies proposed the probable mechanism of AuNPs assembly on fungal hyphae as solely adsorption driven however later on it was proposed to be nutritional driven. These biological routes of synthesis offer numerous advantages like reduced cost, benign behavior, synthesis of unusual micro or nano structures, labor-saving approach and are anticipated to overtake the conventional methods. In the previous chapter we have tried to synthesise gold nanochains by self-assembly of individual gold nanoparticles however, controlling the chain length was once again major challenge.

In the present work, we have exploited the architecture of microorganism for the self-organization of amino acid functionalized AuNPs to synthesize functional microstructures. Three different fungal species, having different nutritional characteristics, have been used to understand whether MWs synthesis is solely adsorption based or nutritional driven or both. Our work is aimed at understanding the mechanism of synthesis of these functional microstructures of AuNPs and exploring their potential use for efficient biosensor fabrication.

7.2 Experimental

7.2.1 Synthesis of AuNPs

Amino functionalized gold nanoparticles were synthesised using amino acid (L-lysine) as a reducing and capping agent via chemical reduction method as discussed in Chapter-4.
7.2.2 Fungal inoculation in AuNPs solution

Three different fungal species including *Aspergillus parasiticus*, *Penicillium funiculosum*, and *Rhizopus oryzae*, were inoculated in three different flask containing liquid media with KH$_2$PO$_4$, K$_2$HPO$_4$, MgSO$_4$·7H$_2$O, (NH$_4$)$_2$SO$_4$, yeast extract, and glucose as the major ingredients below certain critical levels so as to promote the spore formation. The flask was incubated at 30°C for 4 days in a rotary orbital shaker. The spores were harvested and washed with sterilized distilled water to remove any medium component under aseptic conditions. Finally spores of *Aspergillus parasiticus*, *Penicillium funiculosum*, and *Rhizopus oryzae*, were mixed in 10 ml of aqueous solution of AuNPs in three different tubes namely tube 1, tube 2 and tube 3, respectively maintained at 30°C. The spores started germinating after about 24 h, and the red growing fungal biomass was clearly visible within 48–72 h.

7.2.3 Fabrication of AuMWs functionalized electrode

Figure 7.1 shows a schematic representation of the preparation of the gold micro wires (Au amino MWs) modified electrode. The working electrode was cleaned thoroughly with ethanol followed by distilled water 2-3 times. 10 µl of 3-APT (2.5 mM) solution was added on the working electrode surface and was air dried. The thiol group of 3-APT binds to the gold surface, while the amino group remains free for attachment to the other groups.

![Figure 7.1: Schematic representation of AuMWs biosensor fabrication.](image)
Functionalized electrode was rinsed thoroughly with distilled water to remove the physically adsorbed 3-APT and allowed to dry. This was followed by addition of 10 µl of glutaraldehyde (2.5%,) was dropped on to the 3-APT modified electrode and kept for 4 hours to ensure the interaction of free amino group of 3-APT and CHO of glutaraldehyde. Again, the activated electrode was rinsed thoroughly with distilled water and dried. The amino functionalized gold microwires were immobilized onto the activated gold surface through covalent bond between the amino group of MWs and CHO terminated gold surface.

7.3 Results and Discussion

Gold nanoparticles functionalized with amino acids (L-lysine) were prepared via ecofriendly route, in order to reduce the complexity of current methodologies and to avoid the usage of toxic materials as reducing and capping agent. The amino acid and high temperature are the key factors that facilitate the formation of nuclei and control the growth of nanoparticles. The natural amino acid induced reduction of yellow colored HAuCl\textsubscript{4} solution led to formation of ruby red solution of gold nanoparticles. This was further confirmed by UV-Vis spectrophotometric analysis as indicated by the presence of fairly narrow characteristic plasmon absorption band of AuNPs at 523 nm while the remainder unreacted HAuCl\textsubscript{4} precursor in solution is identified by absorption peak at 300-310 nm (see Figure 7.2A). Transmission electron microscopic examination of these particles showed fairly uniform shape and a narrow size distribution of 10-25 nm (see Figure 7.2B). The synthesized AuNPs solution was divided equally into three tubes and labeled them as tube1, tube2 and tube3.

![Figure 7.2: A) UV-Visible spectrum and B) TEM micrograph of AuNPs used in the present study](image-url)
The fungal spores of *Aspergillus parasiticus*, *Penicillium funiculosum*, and *Rhizopus oryzae* were inoculated in the aqueous solution of gold nanoparticles at 30°C under aseptic conditions without addition of any nutrients. Under similar condition fungal spores of each of the three species were added to three different tubes containing dilute L-Lysine solution without AuNPs and labeled them as tube 1’, tube 2’ and tube 3’. These acted as another set of control in addition to the negative control having AuNPs solution only i.e., no fungal spores (tube X) as shown in Figure 7.3. After 2 days fungal biomass started appearing in all the tubes which gradually increased with time over a period of one week. The *Aspergillus* and *Rhizopus* biomass grown in tube 1 and tube 3 enriched with AuNPs was reddish purple in color whereas the *Penicillium* fungal mass in tube 2 and all three fungal mass in control tubes 1’, tube 2’ and tube 3’ were white cottony at first, turning brownish grey with increasing time. The growth of fungus in both tube 1 and tube 3 is characterized by appearance of reddish purple mass, accompanied with a significant decrease in red color of the colloidal solution with increasing time. Variation in color of colloidal solution after 72 hours of inoculation of fungal spores and later after 1 week is shown in Figure 7.3.

![Figure 7.3 Fungal growth in aqueous solutions of AuNPs and L-Lysine.](image-url)
7.3.1 Absorption Spectroscopic Studies

The time dependent analysis of growing fungal hyphae in AuNPs colloidal solution was studied using absorption spectroscopy. Tube 1 and tube 3 containing *Aspergillus parasiticus*, *Penicillium funiculosum* and *Rizopus oryzae*, (A-C) and corresponding spectra showing decrease in absorbance during the mycelium growth process.

**Figure 7.4:** Absorption spectra showing depletion of AuNPs with time from the solution containing *Aspergillus parasiticus*, *Penicillium funiculosum* and *Rizopus oryzae*, (A-C) and corresponding spectra showing decrease in absorbance during the mycelium growth process.
and *Rhizopus oryzae* showed a pronounced change in color or amount of suspended gold nanoparticle with fungal growth while test tube 2 containing *Penicillium funiculosum* showed only a marginal change in color at the onset of the growth. Further, the decline in color is confirmed by red shift of the plasmon band associated with the gold nanoparticles showing particle aggregation. As seen in Figure 7.4A and 7.4C, the characteristic plasmon band of gold shows a gradual decrease with time which finally disappears after 1 week. Figure 7.4B showed only slight variation in peak position with time up to 72 hrs followed by no further change in peak intensity. The variation in color of the colloidal solution is a direct outcome of depletion of freely suspended gold nanoparticles in the solution, thus indicating drifting out of AuNPs from the solution and their assembly onto the growing fungal hyphae. This is marked by appearance of reddish purple biomass at the bottom of the tubes (tube1 and tube3). Whereas, the slight red shift in plasmon in solution from tube 2 containing growing *Penicillium funiculosum* is simply due to initial agglomeration of nanoparticles as a result of destabilization of amino acid coated nanoparticles. While negative control tube7 showed almost no change in absorption peak initially, though towards the end of week there was slight broadening of the peak due to settling down and agglomeration over a prolonged period. The change in peak intensity was marginal.

It was observed that rate of disappearance of AuNPs in case of *Aspergillus parasiticus* (tube1) was 1.5 times greater than in case of *Rhizopus oryzae* (tube3). This correlates well with the growth rate of the *Aspergillus parasiticus*, and *Rhizopus oryzae* suggesting nutritional uptake as the probable mechanism rather than solely adsorption. However, the underlying mechanism of *Penicillium funiculosum* not utilizing the amino coated AuNPs needs to be further explored.

### 7.3.2 Optical and Electron Microscopic Characterization of AuMWs.

The fungal mycelium was harvested from all three test tubes 1, 2 and 3 and was observed under an optical microscope. (Figure 7.5(A-C)). Figure 7.5A shows optical images of *Aspergillus parasiticus* and Figure 7.5B is that of *Penicillium funiculosum*, while Figure 7.5C shows optical images *Rhizopus oryzae*. Optical images of *Aspergillus parasiticus* and *Rhizopus oryzae* show 80-90% fungal hyphae dark in color showing the probable attachment of AuNPs while ~10-20% was partially hollow tubular hyphae (transparent light colored). These results were further corroborated with scanning electron microscopy.
Figure 7.5: Optical microscopy images of hyphae of *A. parasiticus*, *P. funiculosum* and *R. oryzae*

Figure 7.6: SEM micrographs of (A) *A. parasiticus* and (B) *R. oryzae*
Scanning electron microscopy equipped with an energy dispersive X-ray (EDX) detectors confirmed the nanoparticle/fungal hyphae structure. Figure 7.6 (A-C) shows the micrographs of the fungal mycelium *A. parasiticus* and *R. oryzae* enriched with gold nanoparticles without coating it with any conductive material at two different magnifications (see Figure 7.6(A-B)).

Elemental analysis using energy dispersive X-ray spectroscopy (EDX) of fungal hyphae decorated with gold nanoparticles (see Figure 7.7 (A-C)) confirmed 28% of gold on the hyphae surface in case of *Aspergillus parasiticus* and 24% of gold for *Rhizopus oryzae*, whereas no gold nanoparticles were observed (0% Au) on the hyphae surface of *Penicillium*.

![EDX spectra of mycelia of A) Aspergillus parasiticus, B) Penicillium funiculosum, and C) Rhizopus oryzae after 2 weeks of grown in AuNPs solution](image)

**Figure 7.7**: EDX spectra of mycelia of A) *Aspergillus parasiticus*, B) *Penicillium funiculosum*, and C) *Rhizopus oryzae* after 2 weeks of grown in AuNPs solution
*funiculosum.* The EDX results are in agreement with utilization rate or rate of depletion of AuNPs from solution of different species of fungi.

### 7.3.3 Proposed Mechanism

As discussed earlier, limited number of reports are available on MWs synthesis using bio-templates. Moreover, no clear mechanism of such an assembly of AuNPs has been proposed so far. In order to understand the interaction between the fungal biomass and gold nanoparticles attached onto its surface, the harvested mass from all the test tubes was washed 3-4 times with distilled water and further sonicated in order to remove all unbound nanoparticles. Most microorganisms possess net negative charge at physiological pH as the number of carboxylic and phosphate groups dominate their surface as compared to amino groups. The fungus initially grows on the unutilized free amino acids present in the AuNPs solution, since no additional nutrients were available. Once the free amino acids are depleted from the solution, fungal biomass starts attracting amino acid coated AuNPs for their further growth. Interaction of microorganism with the amino groups of AuNPs leads to formation of peptide bond with carboxylic group present on the fungi surface. This theory of initial utilization of free amino acids and later on the amino acid coated AuNPs is also supported by the fact that during initial growth a white cottony fungal mass was observed even in tubes 1, 2 and 3. As the time progressed, the color of the fungal mass changed to red that eventually became purple in color. Moreover, partially covered and partially hollow hyphal structures as observed in optical images could be ascribed to the growing fungal species. As the fungus grows it picks up the amino coated AuNPs for its further growth as it utilizes amino acids as nitrogen source for growth, in this process the AuNPs forms the peptide bond with the functional groups present on hyphae surface. The above studies demonstrate the formation of these gold microstructures to be nutritional driven process and further peptide bond ensures the stability of the structure. However, the studies with *Penicillium funiculosum* again demands further confirmation for nutritional driven synthesis of these microstructures.

### 7.3.4 GOx- AuMWs Activity Analysis

These fabricated AuMWs were further evaluated for their biocompatibility aspect for their potential applications involving immobilization of biomolecules. Activity of GOx was analyzed after immobilization onto AuMWs and compared the same with free enzyme GOx followed by temperature and pH optimization.
7.3.4.1 Effect of pH

The electrochemical performance of the enzymatic biosensor is majorly affected by the pH of the solution since it greatly affects the activity of the enzyme catalyzing the reaction. Therefore, in order to determine the optimum working pH of the biosensor/immobilized enzyme activity, the pH of the buffer (0.1 M PBS) was varied from 5 to 9. Figure 7.8, shows maximum activity around pH 6.5 for free enzyme GOx while after immobilization onto AuMWs optimum pH was found to be pH 7.5. All further experiments in this study were done at pH 7.5.

![Figure 7.8: Effect of pH on activity of free and immobilized GOx](image)

7.3.4.2 Effect of temperature on biosensor response

The fragile 3-D conformation of the enzyme makes it highly sensitive to variation in temperature. Therefore, the effect of the temperature on the relative activity of enzyme after immobilization onto AuMWs was studied as a function of temperature (see Figure 7.9). The fabricated biosensor showed marginally improved relative activity with a shift in optimum temperature 40°C as opposed to an optimum value of 37°C in case of free enzyme. The decrease in activity as we go away from 40°C could be explained on the basis of loss of active site conformation at extreme temperatures. Therefore, for practical measurements, the normal temperature of 37°C was selected as the working temperature for the operation of biosensor.
7.3.5 Biosensing Performance Analysis

7.3.5.1 Electrochemical Impedance Spectroscopic Studies

The alteration in electron-transfer resistance of the conductive electrode due to immobilization of microstructure and biomolecule was studied using EIS method. The impedance spectra were interpreted by the equivalent circuit (Randles, 1947). The above circuit includes four major parameters: $R_s$ which represents the ohmic resistance of the electrolyte solution; $Z_w$ indicates the Warburg impedance, which is an outcome of the diffusion of ions from the bulk of the electrolyte to the electrode interface; $R_{ct}$ characterizes the resistance to electron transfer while the impedance $Z_{CPE}$ denotes the constant phase element (CPE). Since, $R_s$ and $Z_w$ represents the diffusion feature of redox probe in solution and bulk properties of the electrolyte solution, thus ideally they are not altered by modifications on electrode surface. Whereas, the modification on electrode surface highly effects the $R_{ct}$ and $Z_{CPE}$ as their value corresponds to the insulating and dielectric features at the interface of electrode. The diameter of the semicircular part of the electrochemical impedance spectra corresponds to the charge transfer resistance at the electrode ($R_{ct}$), while the linear part represents the Warburg impedance ($Z_w$).

Figure 7.10, shows the impedance spectra of bare gold electrode (curve a), AuMWs modified electrode (curve b), and after immobilization of glucose oxidase on AuMWs modified electrode (curve c), respectively. As observed from impedance spectra, each modification step results in significant differences in the electron transfer resistances ($R_{ct}$) of the electrode. The

![Figure 7.9: Effect of temperature on activity of immobilized GOx](image_url)
cole-cole plot of the bare gold electrode exhibited a much smaller semicircular diameter implying the fast electron-transfer at electrode interface. The semicircular diameter increased with the immobilization of AuMWs suggesting increased impedance that was further enhanced after glucose oxidase was immobilized onto the AuMWs modified electrode. The bare gold electrode shows an electron transfer resistance ($R_{ct}$) value of 140 Ω. The $R_{ct}$ changed to 136 Ω with modification of bare electrode with AuMWs, indicating the decreased resistance to the flow of electrons at the electrode surface interface. The increased $R_{ct}$ value of 256 Ω after immobilization of glucose oxidase could be attributed to the hydrophobic layer of the protein which acted as an insulator on the surface of conductive electrode surface and thus perturbed the interfacial.

### 7.3.5.2 Electroactive surface area of the electrode

Relative change in electroactive surface area of bare Pt electrode and AuMWs/Pt electrode was estimated from Randles-Sevcik equation for peak current at room temperature:

$$I_p = (2.65 \times 10^5) n^{3/2} A D^{1/2} C^{1/2} v^{1/2}$$

Where, $I_p$ is peak current (in amps), $n$ is number of electron transfer in redox reaction, $A$ is electrode surface area (in cm$^2$), $D$ is diffusion coefficient (in cm$^2$/s), $C$ is concentration of diffusing species (in mol/cm$^3$) and $v$ is scan rate (in V/s). The geometrical surface area of the electrode is 0.14 cm$^2$. 
The effect of AuMWs modification on electrode surface area was evaluated from the ratio of peak current of bare Pt electrode (12.5 μA) and AuMWs/Pt electrode (23 μA) (as shown in Figure 7.11)

\[ \frac{I_p(\text{AuNP/Pt electrode})}{I_p(\text{bare Pt electrode})} = \frac{A'}{A} \]

as 1.8. This increased electroactive surface area as a result of AuMWs modification of Pt electrode ensures increased enzyme loading capacity and hence holds promise of increased sensitivity and lower detection limits of the fabricated biosensor.

**7.3.5.3 Cyclic voltammetric measurements**

Figure 7.12A, shows the CV studies of GOx/AuMWs modified Pt electrode as a function of scan rate varying from 10 to 100mVs\(^{-1}\). The variation of the redox peak height with sweep rate is shown in Figure 7.11B.

The linear variation of \( I_p \) Vs \( \nu^{1/2} \) is indicative of the direct electron transfer process between the enzyme and the electrode surface. In other words, AuMWs served as a good candidate for modification of electrode assisting the easy shuttling of electrons between the enzyme and the electrode representing the phenomenon equivalent to direct electron transfer. Figure 7.12C shows variation of peak potential as a function of scan rate. The electron transfer rate constant (\( k_e \)) and cathodic transfer coefficient (\( \alpha \)) was calculated from Laviron’s equation as discussed in our previous chapter, Chapter 6. The electron transfer rate was found to be 2.8s\(^{-1}\).
7.3.5.4 Amperometric measurements

The amperometric responses of the GOx/AuMWs modified gold electrode for different glucose concentration at 0.4 V, presented in Figure 7.13A and 7.13B shows the linear variation of the same over the concentration range of 5 μM to 20 mM. The response current follows Michaelis–Menten behavior with increasing glucose concentration. The apparent Michaelis–Menten constant $K_{m,app}$ was calculated from the slope of this straight line using the Lineweaver–Burk plot of $I^{-1}$ vs. [Glucose]$^{-1}$. Thus, a smaller $K_{m,app}$ value of 3.6 mM suggests high affinity of the enzyme for the substrate. The amperometric sensitivity of the GOx/AuMWs gold electrode was about 43.2 μA/mM/cm$^2$ with a linear range between 5 μM to 20 mM. The detection limit of the AuMWs modified gold electrode was found to be 5 μM and a steady-state response current was generated within 6 s.

Figure 7.12: Cyclic voltammograms of A) GOx-AuMWs modified electrode for scan rate varying from 10 to 100mVs$^{-1}$ in PBS buffer; B) shows linear variation of anodic and cathodic peak current; C) Variation of Epc and EpA with ln (ʋ)
7.3.5.5 Precision

The precision of the fabricated biosensor was evaluated in terms of reproducibility and repeatability. The reproducibility of the amperometric signal was checked using three sets of identical biosensor. The RSD values obtained were 1.3%. The obtained results ensure good reproducibility of the GOx/AuMWs modified electrode. The repeatability of the biosensor was also evaluated by repeating the CV over the studied potential range for twenty cycles.

Figure 7.13: Amperometric response of GOx-AuMWs modified gold electrode at different glucose concentration in 0.1M phosphate buffer (pH 7.4); B. Calibration curve of the biosensor showing linearity for the 5 μM to 20 mM glucose concentration range.

Figure 7.14: Cyclic voltammogram of AuMWs based biosensor over 20 cycles depicting high reproducibility.
As shown in Figure 7.14 the biosensor showed excellent stability and reproducible results. The above results ensures that the reproducibility and repeatability of the GOx/AuMWs gold electrode to a good extent.

### 7.3.5.6 Interferences

A number of possible interfering species alter the correct measurements of glucose levels. The signal alterations due to most interfering electroactive species, such as ascorbic acid and uric acid were investigated in the present study. The level of endogenous ascorbic and uric acid in blood samples is about 0.125 and 0.33 mM. At this concentration, the glucose biosensor exhibited negligible response to these electroactive interfering species.

### 7.3.5.7 Storage stability

Storage stability of a biosensor is one of the critical parameter for transferring a biosensor from lab to market. Therefore, in the present study the fabricated biosensor was stored at 4°C in order to avoid deactivation of the immobilized enzyme. The response current was measured from time to time for 90 days. The biosensor response current is plotted as a function of time in Figure 7.15. As observed from figure, the biosensor remained stable for 90 days retaining approximately 60% of its initial response current. The good stability of the biosensor could be

![Figure 7.15: Stability of the GOx-AuMWs modified electrode.](image)
attributed to the strong interaction between GOx and AuMWs. However, in comparison to the stability of biosensor fabricated using branched Au amino NPs the stability is very low.

7.4 Conclusion

In the present work, we have demonstrated the synthesis of AuMWs of micrometer length scales using microorganism (fungal hyphae) as template. The discussed approach allows the synthesis of nanostructures/microstructures of desired morphology by appropriate choice of microorganism and allowing them to grow in amino acid functionalized nanoparticles solution. These biocompatible nano-/micro- structures can be used for applications like biosensors etc. In the present work we have developed a glucose biosensor that shows a wide linear range of 5µM -20 mM and sensitivity of 43.2 µA mM⁻¹ cm². Biosensor retained 75% of its activity up to 60 days with Kₘ as 3.6 mM and the electron transfer rate was 2.8 s⁻¹. In comparison to the results reported in previous chapter, Chapter 6, the linear range of AuMWs based biosensor is significantly increased however other performance parameters are lesser than branched AuNPs based biosensor. In the present case, we had no control over the orientation of AuMWs, i.e., random orientation, hence we expect that the stability and sensitivity can be further improved by ensuring vertically aligned AuMWs. Next chapter is on improving the thermal stability of enzymes by making their nanoparticles with the aim of developing thermally stable biosensors using the same.