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AN AMPEROMETRIC GLUCOSE BIOSENSOR BASED ON THE IMMOBILIZATION OF GLUCOSE OXIDASE ON THE CARBON NANOTUBES MODIFIED GLASSY CARBON ELECTRODE

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***Abstract*- CNTs have unique mechanical and electronic properties, combined with chemical stability, and behave electrically as a metal or semiconductor, depending on their structure. For sensing applications, CNTs have many advantages such as small size with larger surface area, excellent electron transfer promoting ability when used as electrodes modifier in electrochemical reactions, and easy protein immobilization with retention of its activity for biosensors. CNTs play an important role in the performance of electrochemical biosensors, immunosensors. Various methods have been developed for the design of sensors using CNTs in recent years. In this work, we report the development of a highly sensitive and stable glucose sensor based on the effect of carbon nanotubes (CNTs). Since the isoelectric point (IEP) of glucose oxidase (GOx) is significantly different from that of CNTs nanoparticles, GOx was electrostatically bound to CNTs nanoparticles decorated onto a negatively charged MWNTs layer at the electrode surface .A Chitosan layer was further coated onto the GOx-contained CNTs nanoparticle layer to prevent the leakage of GOx. This unique multi-layer structure (GOx/CNTs/CS/GC) provided a favorable microenvironment to maintain the bioactivity of GOx, which led to rapid amperometric response toward glucose. By loading different concentration of GOx at the sensor surface, we obtained a wide linear response range of 0.5 -15 mM for this sensor. This nanomaterials-based glucose sensor was highly sensitive and showed favorable stability over a relatively long-term.**

Keywords- **Carbon nanotubes, Biosensors, Glucose oxidase, Nanomaterials, Enzyme electrodes.**

I. INTRODUCTION

Glucose sensors was one of important biosensors, have been extensively studied due to their important clinical applications. The fast and accurate determination of glucose has profound applications since glucose concentration is a crucial indicator in many diseases, such as diabetes, endocrine metabolic disorder. Various methods such as electro chemical, chemiluminescence were used to develop glucose biosensors [1]. Among all the methods, enzyme involved electrochemical glucose biosensor has been intensively studied because of its simplicity, high selectivity and relative low cost. In this technique, the enzyme immobilization is considered to be one of the most important factor. Since the performance of a biosensor much depends on the supporting material that provide good environment for the efficient enzyme loading and maintenance of enzyme bioactivity is highly desired.

A. Electrochemical Glucose Biosensor

An electrochemical biosensor is an analytical tool for sensitive and selective detection of biomolecules. Increasing attention has been given to biosensors due to their potential applications in clinical chemistry, food industry, and environmental fields [2]. The glucose oxidase (GOx) is widely employed in most of the glucose biosensors due to its stability and high selectivity to glucose, especially the amperometric glucose biosensors [3-5]. Glucose oxidase (GOx) is the most popular enzyme in the development of glucose sensors, which catalyzes the oxidation of glucose that can be amperometrically detected at electrodes. In particular, electrode materials with a large surface-to-volume ratio can increase the amount of immobilized enzyme, minimize the barriers for mass transportation between the substrate and the product, and provide a chemically and mechanically robust system.

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Nanomaterials-based electrodes have proven to solve these requirements. It contains two flavine adenine dinucleotide (FAD) cofactors and catalyzes the oxidation of glucose according to the following reaction

$$\text{Glucose} + O_2 \xrightarrow{GOx} \text{Glucolactone} + H_2O_2$$

Since the amount of glucose is proportional to that of the produced H_2O_2, the glucose concentration can be easily determined through measuring the current derived from the electrochemical reaction of H_2O_2. Many methods such as covalent binding and cross-linking method have been used to immobilize the GOx onto different supporting materials. Moreover, the property of GOx with negative charge in neutral pH solutions also makes it feasible to immobilize GOx onto materials with positive charge by the physical adsorption.

B. Electrochemical properties of Carbon nanotubes (CNTs)

Carbon nanotubes (CNTs) are one of the most exciting materials because of their unique electronic, chemical, and mechanical properties. Carbon nanotubes have unique properties that make them potentially useful in a wide variety of applications such as in nano electronics, optics, and materials applications. They exhibit extraordinary strength and unique electrical properties, and are efficient conductors of heat. CNTs are a kind of particularly interesting nanomaterials for the development of various biosensors, with multiple functions. CNTs offer an ideal substrate for the immobilization of enzymes [6, 7]. CNTs favors the electro catalytic oxidation/reduction of H_2O_2.

C. Carbon Nanotubes (CNTS) Based Electrochemical Glucose Biosensors

CNTs are electrochemically inert materials similar to other carbon-based materials used in electrochemistry, i.e. glassy carbon, graphite, and diamond. They possess distinct electrochemical properties because of their unique electronic structure. The carbon atoms of CNTs at the sidewall and the end of the tubes are not same and their behavior can be compared with the basal plane and edge plane of highly oriented pyrolytic graphite (HOPG), respectively. CNTs behave as either metals or semiconductors, depending on the diameter and the degree of helicity. CNTs possessed sp^2 carbon units with several nanometers in diameter and many microns in length. Two groups of CNTs, multi-walled (MW) and single-walled (SW), can be synthesized by electrical arc discharge, laser vaporization, and chemical vapor deposition methods [8, 9, 10]. Further, construction of efficient electrochemical sensors using the CNTs-modified electrodes is very promising in that they promote electron-transfer reactions. CNTs, nano crystalline diamond, calcium carbonate nanoparticles and Ag dendritic nanostructures have been studied as platforms for enzyme immobilization in glucose biosensors. CNTs enhance amperometric signals due to the increased interfacial electron transfer and large surface areas .However, effective immobilization of GOx at CNT surface remains to be a prime role to the CNT-based glucose sensor.

II. EXPERIMENTAL

1. Materials and Methods

Carbon Nanotubes (CNTs) were obtained from Advanced Material and Device Laboratory, Department of Physics, Bharathiar University. Concentrated Sulphuric acid (Conc.H_2SO_4), Concentrated Nitric acid (Conc.HNO_3), Thionyl chloride ($SOCl_2$), Tetrahydrofuran (THF), N,N-Dimethylformamide (DMF), Ethylenediamine (EDA), Triethylamine (TEA), Dimethyl amine (DMA), Acetone, Dextrose anhydrous, Phosphate buffer saline (PBS), Potassium hydrogen phosphate (Na_2HPO_4), Potassium phosphate (KH_2PO_4), Potassium chloride (KCl), Sodium chloride (NaCl), Chitosan (CS), Glucose Oxidase (GOx) were purchased from Sigma and Himedia. All the chemicals were of analytical grade and were used as received. Distilled water was used throughout the experiment.

2. Acid-Mediated Purification of CNTS

The as-produced CNT contain variable amounts of impurities, such as amorphous carbon, fullerenes, and metal particles (such as iron or cobalt) encapsulated in carbon nanocapsules, the initial efforts in their purification is focused on the selective oxidation of the impurities with respect to the less reactive CNT. Purification process of carbon nanotubes involves extensive ultrasonic treatment in a mixture of concentrated nitric and sulfuric acid [11, 12]. Such drastic conditions lead to the opening of the tube caps as well as the formation of holes in the sidewalls, followed by an oxidative etching along the walls with the concomitant release of carbon dioxide. The combined treatment of strong acids and sonication is known to purify the CNTs and generate anionic groups (mainly carboxylate) along the sidewalls and ends of the nanotubes. Also, dangling bonds can react similarly, generating functions at the sidewalls [13].
The oxidation was accomplished by oxidising the as-prepared carbon nanotubes in the presence of dry air in elevated temperature for 2h. The furnace was then allowed to cool down to room temperature. The process was repeated for three times for complete removal of amorphous carbon and their respective weight loss was monitored.

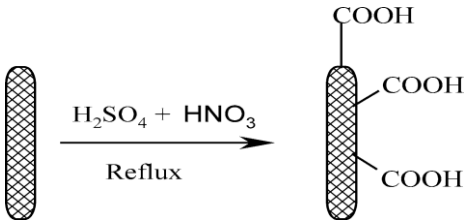

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Figure 1 (a) Scheme of purification process of CNTs

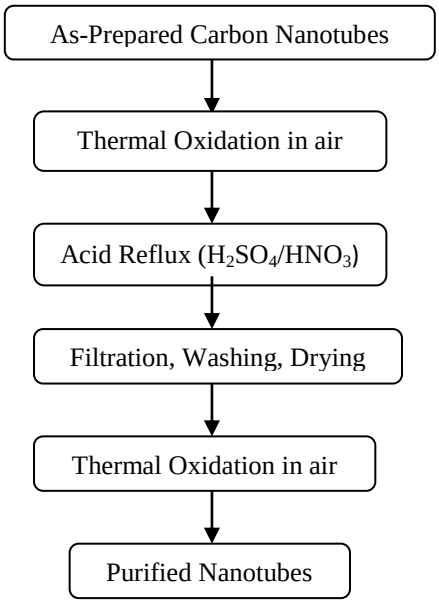

Figure 2. Flow chart for Acid purification of CNTs

The dry oxidized carbon nanotubes were added into 3:1 mixture of H_2SO_4/HNO_3 (v/v) solution. The solution was refluxed at 120˚C in oil bath for 4h. After acid treatment CNTs were filtered, washed with ample quantities of distilled water until the pH value of the washings reaches 7, then samples are kept in oven for overnight to remove volatile materials. **Figure 2** shows the schematic and flowchart of acid purification process of carbon nanotubes

3. Functionalization of Carbon Nanotubes

Covalent functionalization of carbon nanotubes with amine groups offers the architecture for a three-dimensional enzyme array for potential application in biosensor devices. In this work the carbon nanotubes were functionalized using three various amine groups namely Dimethylamine, Triethylamine and Ethylenediamine [14]. The molecular structure and the properties of DMA, TEA and EDA were illustrated in Table **1** and figure **3**.

Table 1 Properties of DMA, TEA and EDA

Properties	**Dimethyl amine (DMA)**	**Triethyla mine (TEA)**	**Ethylene diamine (EDA)**
Molecular formula	$(CH_3)_2NH$	$C_6H_{15}N$	$C_2H_8N_2$
Molar mass (g/mol^{-1})	45.08g mol^{-1}	101.19 g mol^{-1}	60.10 g mol^{-1}
Melting Point (˚C, ˚F)	-93°C, -135°F	-115°C, -174°F	8°C, 46°F
Boiling Point (˚C, ˚F)	7-9°C, 44-48°F	89-90°C, 191-194°F	116°C, 241°F
Density (g/mL^{-1})	0.6700 g mL^{-1}	0.7255 g mL^{-1}	0.8990 g mL^{-1}
Refractive Index (*n*D)	1.4305	1.401	1.4565

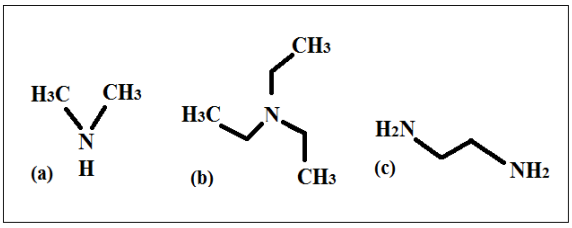

Figure 3 (a, b, c) Schematic of DMA, TEA, EDA

4. Amino- Functionalization of CNTs

To prepare the acyl chloride carbon nanotubes, the obtained gram quantities of purified carbon nanotubes were suspended in 100mL of Thionyl Chloride ($SOCl_2$) and followed by adding Tetrahydrofuran (THF). The suspension was dispersed in ultrasonic bath (40 kHz) for 30min and then stirred at 70˚C for 24h to convert the surface-bound carboxyl groups into acyl chloride groups. The solid is then filtered and washed with anhydrous THF. Subsequently, it is dried under vacuum at room temperature for 2h. The obtained solid sample is further applied to amino-functionalization [18]. Figure 4. (a, b) shows the schematic and flow chart of amino-functionalization process of carbon nanotubes.

Dimethylamine is an organic compound with the formula $(CH_3)_2NH$. This secondary amine is a colourless, flammable gas with an ammonia-like odour. It is raw material for the production of many agrichemicals and pharmaceuticals, such as dime-fox and diphenhydramine, respectively. Triethylamine is the chemical compound with the formula $N(CH_2CH_3)_3$, commonly abbreviated as TEA. It is a colourless volatile liquid with a strong fishy odour reminiscent of ammonia and is also the smell of the hawthorn plant. The bicarbonate salt of Triethylamine is useful in reverse phase chromatography, often in a gradient to purify nucleotides and other bio molecules [15, 16, 17]. Ethylenediamine is the organic compound with the formula $C_2H_4(NH_2)_2$. This colourless liquid with an ammonia-like odour is a strongly basic amine.

Carbon nanotubes suspensions were prepared by dispersing Acyl chloride Carbon Nanotubes (CNTs-COCl) material in N, N-Dimethylformamide (DMF) with the aid of ultrasonic agitation for 1h. The mixture was added with 50mL of various amines (EDA, TEA, and DMA) separately and was heated at 70^{0}C under stirring for 72h. Aliphatic multi-amines were grafted onto CNTs through amide linkage. The resulting reaction medium was vacuum filtered and was washed

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with acetone. As the final stage, amine-functionalized carbon nanotubes were dried in a hot air oven for overnight.

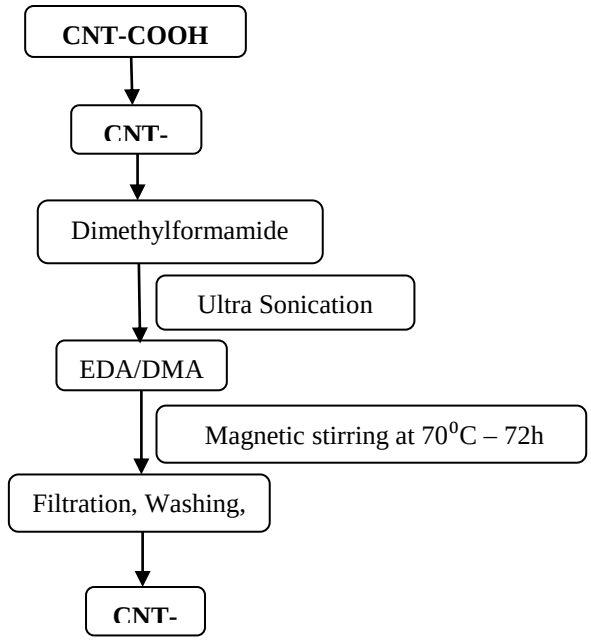

Figure 4. (a) Flow Chart of Amino-Functionalization Process of CNTs

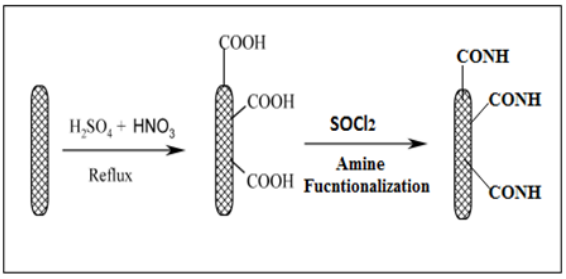

Figure 4. (b) Schematic Illustration of Amino-Functionalization of CNTs

III. EXPERIMENTAL METHODS

Cyclic voltammetry and Chronoamperometry measurements were carried out on a Biologic SP150 electrochemical work station [19, 20]. A conventional three electrode set-up was used. The modified glassy carbon electrodes (GCE) were as the working electrode. Platinum mesh (Pt) and Ag/AgCl electrode were used as the counter electrode and the reference electrode, respectively. The PBS of 0.1M, pH 7 solution was prepared using disodium hydrogen phosphate, potassium phosphate, potassium chloride and sodium chloride. The Chronoamperometry and Cyclic voltammetry measurements of glucose were performed in PBS (0.1M) solution at constant and variable potential. Electrochemical and electrochemical sensing behaviour of purified and functionalized carbon nanotubes were analysed by Chronoamperometric and Cyclic voltammetry measurements [21, 22].

1. Preparation of CNTS/CS/GC Electrode

Glassy Carbon Electrode (3mm in diameter) was polished with 1.0, 0.3 and 0.05µm alumina slurry sequentially and then washed ultrasonically in water and ethanol for few minutes, respectively [23]. The Cleaned Glassy Carbon Electrode (GCE) was dried at room temperature. One percent Chitosan (CS) solution was prepared by dissolving CS in 2% acetic acid solution with magnetic stirring for about 2h. To accomplish the preparation of GOD/CNTs/CS/GC electrode, 1mg of purified and amine-functionalized Carbon Nanotubes (Pure, DMA,TEA, EDA) and 0.5mL of 1% CS solution were first mixed with ultrasonic agitation over 15 min. As a result the viscous and black suspension containing carbon nanotubes and chitosan was obtained, 5µL of the CNTs/CS mixture was spread evenly on to the GC electrode surface using micro pipette. To get more uniform films, the modified electrode was covered with a small bottle and allowed to dry in over for 24h at 4˚C. Then the well adherent film electrode containing CNTs/CS was taken for cyclic voltammetry and chrono amperometry analysis to measure its electrochemical behaviour.

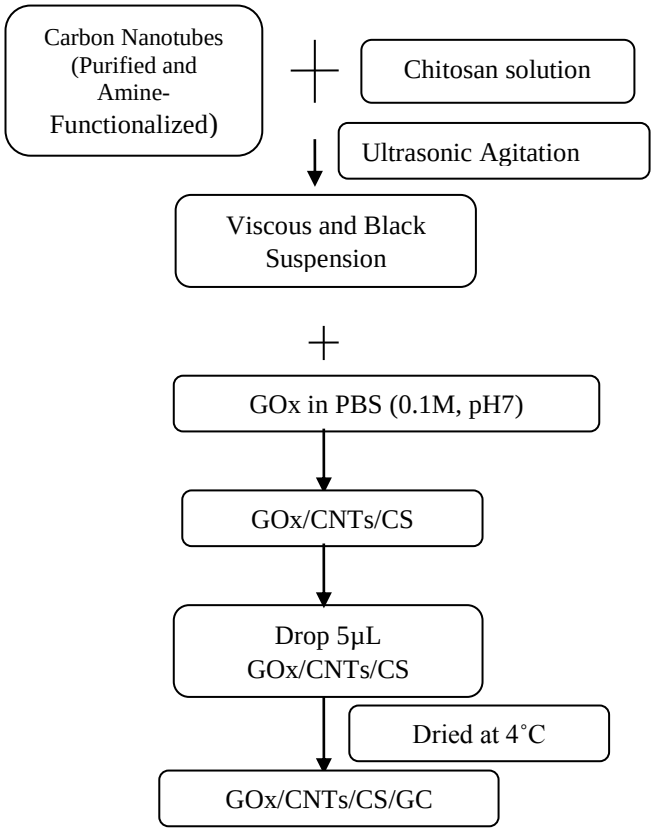

Figure 5. Flow chart of GOx/CNTs/CS/GC electrode preparation

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2. Preparation of GOx/CNTs/CS/GC Electrode

Glassy Carbon Electrode (3mm in diameter) was polished with 1.0, 0.3 and 0.05µm alumina slurry sequentially and then washed ultrasonically in water and ethanol for few minutes, respectively. The cleaned Glassy Carbon Electrode (GCE) was dried at room temperature.

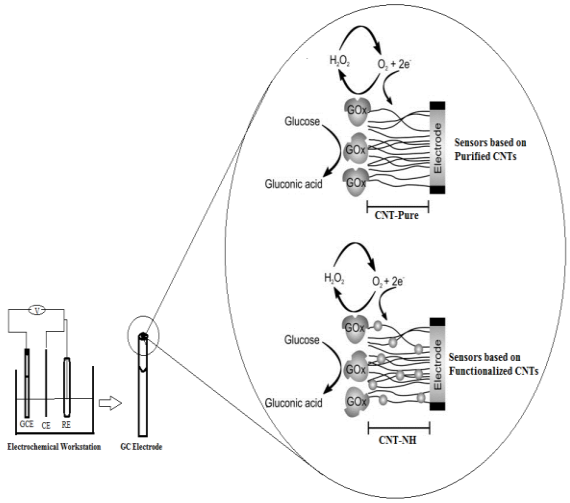

Figure 6. Schematic Illustration of Electrochemical Workstation and Binding nature and electro chemical reactions of GOx/CNT/CS/GC electrodes

One percent Chitosan (CS) solution was prepared by dissolving CS in 2% acetic acid solution with magnetic stirring for about 2h. To accomplish the preparation of GOD/CNTs/CS/GC electrode, 1mg of purified and amine-functionalized Carbon Nanotubes (Pure, DMA,TEA, EDA) and 0.5mL of 1% CS solution were first mixed with ultrasonic agitation over 15 min. As a result the viscous and black suspension containing carbon nanotubes and chitosan was obtained and was mixed thoroughly with appropriate amount of GOx solubilized in pH 7 PBS (0.1M). Next, 5µL of the GOx/CNTs/CS mixture was spread evenly onto the GC electrode surface with micro pipette. Finally, to get more uniform films, the modified GC electrode was covered with a small bottle and allowed to dry for over 24h at 4˚C. Adherent and robust film electrodes containing GOx/CNTs/CS was obtained and was taken for cyclic voltammetry and chronoamperometry measurements to analyse its electrochemical sensing behaviour. Figure 5 and 6 shows the flow chart for the preparation of GOx/CNTs/CS/GC Electrode and the schematic of electrochemical work station and GOx/CNTs/CS/GC Electrode

IV. RESULTS AND DISCUSSION

1. Characterization of purified carbon nanotubes

X-Ray diffraction (XRD) pattern of purified carbon nanotubes was shown in the figure 7.

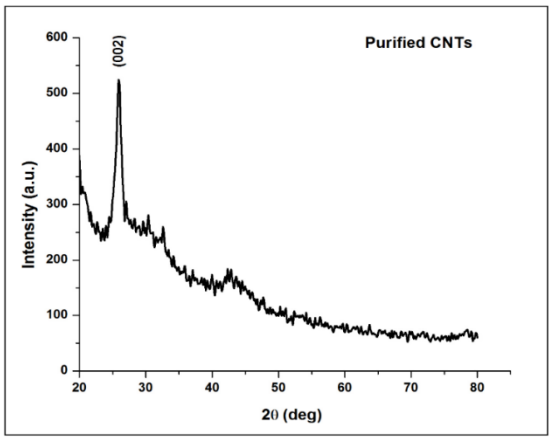

Figure 7 XRD patterns of purified Carbon Nanotubes

Inter planner spacing (d_{hkl}) of purified carbon nanotubes were calculated in XRD profile using Bragg's relation.

$$d_{hkl} = n\lambda/2\sin\theta$$

λ – Wavelength of the X-ray (1.54 Å)
n – Order of diffraction (n=1)
θ – Angle of diffraction (deg)

The interlayer d-spacing between the layers in carbon nanotubes is found to be 3.435Å which is close to the graphite interlayer spacing (3.354 Å) as consistent with the previous study [41]. Absence of peaks responsible for the metal catalyst within the detection limit of X-Ray diffraction confirms that the sample is in purified form [24].

2. Cyclic Voltammetric Analysis of Purified CNT'S

The figure 5.3 shows the cyclic Voltammetry curve of purified Carbon nanotubes with different scan rate in 0.1 M PBS solution measured in the potential range from -1 V to 1 V. The anodic and cathodic peak currents increases linearly with increase in the scan rate [25, 26]. Moreover, the oxidation and reduction peak current also increased with increase in the scanrate.

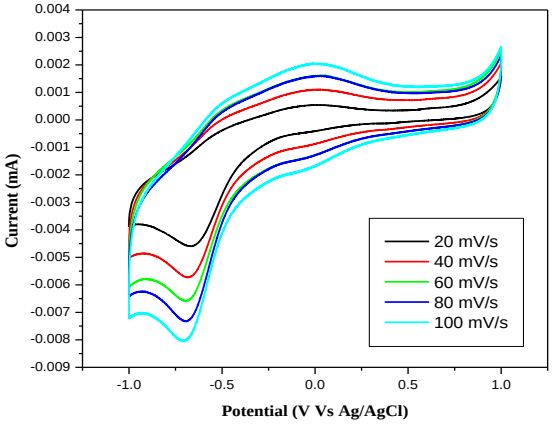

Figure 8. CV curve of purified CNTs with different scan rate

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3. Cyclic Voltammetric Analysis of Functionalized CNTS

The figure 9 shows the Cyclic Voltammetric curves of ethylene diamine functionalized multi-walled carbon nanotubes with different scan rate. On increasing the scan rate from 20 to 100 m V/s the oxidation peak current and reduction peak current increases linearly with increase in the scan rate [27, 28].

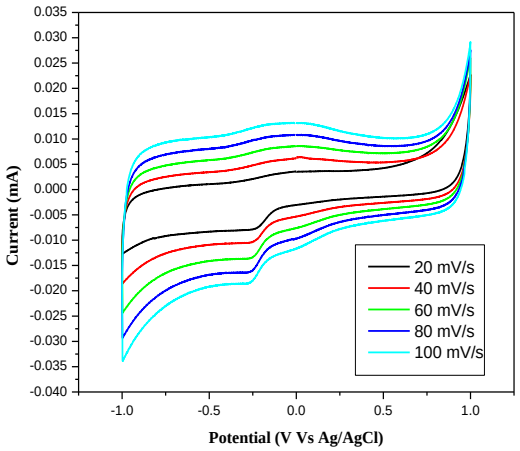

Figure 9. CV curve of functionalized CNTs with different scan rate

V. CONCLUSION

CNTs are now used extensively in the fabrication of novel nanostructured electrochemical sensors. CNTs-modified electrodes have many advantages over other forms of carbon electrodes due to their small size, high electrical and thermal conductivity, high chemical stability, high mechanical strength, and high specific surface area. Their small diameter and long length allow them to be plugged into proteins with better electro-activity compared to other carbon based electrodes. The promoted electron transfer and direct electrochemistry of proteins at CNTs-based electrochemical sensing films are now well documented. Due to its faster electron transfer over other carbon based materials, CNTs show excellent electro catalytic activity in redox behavior of different compounds. Analytical sensing at CNTs-modified electrodes results in low detection limits, high sensitivities, reduction of over potentials, and resistance to surface fouling. The outstanding properties of CNTs make them an exciting alternative for the development of novel electrochemical sensors and biosensor.in this review we outlined the recent advances in the field of the CNTS based electrochemical glucose biosensors.

VI. ACKNOWLEDGEMENTS

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