

# Application of Single-Walled Carbon Nanotubes (SWCNT) in the Production of Glucose Biosensors and Improving Their Performance Using Gold Colloidal Nanoparticles and Usage of Polyaniline Nanostructure-Based Biosensors for Detecting Glucose and Cholesterol

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In this study, glucose oxidase was used as stabilization medium due to its higher efficiency, ability for more accurate control of enzyme reaction, protecting against wasting of enzyme as well as simple and easy use and exchange of enzyme medium after performing some levels of surface modification and developing single-walled carbon nanotubes (SWCNT) on gold plates. For better connecting and stabilizing of the enzyme on the medium, the prepared medium was washed with concentrated sulfuric acid and nitric acid and a large volume of deionized water. For protecting the enzyme from devastating effects of gold and prohibiting it from becoming inactive, the surface was covered with cystamine before stabilization. Regarding the large size of glucose oxidase compared to the surface of the medium, a connective material with amid at one end and pyrine at the other end was used as a transfer agent, and for stabilizing this connection the prepared medium was placed into dimethylformamide (DMF) solution for a couple of hours. Activity of the stabilized enzyme at the wavelength of 460 nm recorded by spectroscope was depicted against time to evaluate its stability at various times. The prepared medium, which had a large amount of glucose oxidase, could be used as an electrode in sensors. Furthermore, glucose oxidase electrochemical sensor is one of the best methods for detecting low amounts of glucose, and applying gold colloidal nanoparticles as a supplementary material in the structure of biosensors can be effective for improving its efficiency and optimum performance. In this study, a modified carbon paste electrode biosensor with gold colloidal nanoparticles ( $A_{\text{unano}}/\text{CPE}$ ) was produced by carbon graphite powder, paraffin oil and, gold colloidal nanoparticles (24 nm), and was compared with carbon paste electrode (CP). In semi-permeable membranes, a combination of 1 ml of 0.1 M phosphate buffer with specified pH and 10 mg of glucose oxidase was placed around each electrode. At the same potential of 0.7 V, biosensors were tested with glucose in the concentration range of 0–1 mM and various pH (4, 6, and 8), which led to the production of the maximum current and tracing glucose at pH=6 and concentration of 1 M as the optimum condition. Currentmetry induced from both biosensors was compared and confirmed that using gold colloidal nanoparticles in the structure of  $A_{\text{unano}}/\text{CPE}$  electrode led to the increase of the conductivity and currentmetry of the biosensors. Qualitative and quantitative measurement of food components is of great importance due to high cost of traditional methods, in addition to tendency for more accurate and sensitive detecting of these components. Glucose and cholesterol are such compounds that are frequently measured. Various methods are used to detect these food elements, however the necessity for accurate measurement of these two compounds with high sensitivity, especially for food health issues, leads to the development of biological methods, especially biosensors. Among them, biosensors based on conductive polymer nanostructures, especially polyaniline, have been recently gained interest due to their unique characteristics. The current paper aims to introduce and investigate the previously performed studies about polyaniline-based biosensors for detecting glucose and cholesterol.

**Key words:** Single-Walled Carbon Nanotube (SWCNT); glucose oxidase; Vapor Phase Precipitation; biosensor; gold colloidal nanoparticles; currentmetry; glucose; cholesterol; polyaniline nanofibers; polyaniline nanotubes

In recent decades, biosensors have been manufactured in very small scales (nanometers) as nanotechnology is introduced into biological sciences. Nanobiosensors are very small sensors in nanometer scale that are able to detect special chemicals and/or biological matters with very high accuracy and in completely selective form by stabilizing enzymes, and/or any other cell products on their surfaces [1–11].

Carbon nanotubes are one of the most frequent and appropriate nanostructures which can be used to make nano-biosensors due to their physical and chemical characteristics.

However, appropriate tests must be done to demonstrate their abilities. One of their clear advantages is that they have a large operational area, especially single-walled carbon nanotubes (SWCNT). Stabilizing biological detectors such as enzymes over these materials may lead to the increase in the performance of enzyme reaction, controllability of reaction, participation of more enzymes in reaction, and prohibiting enzymes from wasting as well as information transferring with higher rate in these nano-biosensors [12–17].

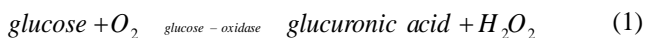
Carbon nanotubes should be stabilized on a surface for better action as the base of sensors. Stabilizing surfaces are mainly metallic. In the current paper, gold plate was used for this purpose. Various methods have been used for developing and stabilizing carbon nanotubes on the media. However, the most frequently used method is chemical vapor deposition (CVD) [17–22].

Electrostatic, hydrophobic reactions, and/or covalent bond along with oxidization of nanotubes are mainly used for stabilizing large enzymes on the surface of carbon nanotubes and a simple absorption on the external surface. In these types of bonds, a connective material is mainly used [23–27].

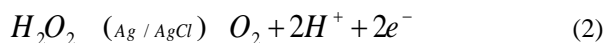
In the structure of nano-biosensors, the covered surfaces of the media by nanotubes are responsible for transferring the effects induced by reaction to transducer devices for illustrating the signal. Carbon nanotubes can play dual roles, as the location of enzyme stabilization and connective limit between reagent and transducer [24, 25].

Biosensor technology is promisingly developed in bio-analytical researches. Sensors or biosensors are used for illustrating various analytes in various times. In 2012, glass carbon (GC) electrode biosensor, multi-walled carbon nanotube (MWCNT), titanium dioxide (TiO<sub>2</sub>), apatite hydroxide, and glucose oxidase (GOX) were produced in China based on this technology [28–31]. A chemical sensor consists of receiver, transducer,

and separator. Receiver or the biological element such as an enzyme establishes a biological connection with the measureable component. Transducer converts the measurable component into optical or electric signals and separator can acts as a membrane [32–35]. In 2012, an electrode biosensor was produced in South Africa based on glass carbon, multi-walled carbon nanotube (MWCNT), nickel nanoparticle, and glucose oxidase and the amount of glucose in the compound was measured through electric signals using ammeters [36–39]. In 2011, a biosensor was produced in Taiwan based on glass carbon working electrode, glutaraldehyde (GA), carbon nanotube (multi-walled-gelatin), and glucose oxidase (GOX) [40]. Glucose biosensor is one of the most successful biosensors in detecting chemical compounds such as glucose at very low amounts. Its efficiency and sensitivity can be considerably increased by combining it with various compounds such as gold, platinum, carbon nanotubes, and so on [41–48]. The currentmetry of these biosensors is based on the performance of glucose oxidase (GOX) and electron exchange. In glucose oxidase, oxidation and reduction groups of Flavin Adenine Dinucleotide (FAD) are oxidized and reduced respectively, and hence it is necessary to establish a satisfactory electric relationship between the active location of FAD enzyme and the surface of electrode to have a current. Amperometric glucose biosensors mainly consist of stabilizing glucose oxidase in various media such as trapped polyacrylamide over working electrode surface. In the presence of glucose, oxygen, and enzyme, as the biological catalyst of this system, the following reaction is performed:



The presence of enzyme causes the consumption of oxygen by glucose in the solution and reducing oxygen penetration to the auxiliary platinum electrode surface and leads to producing compounds such as glucuronic acid and hydrogen peroxide. Decomposition of hydrogen peroxide near the standard electrode (Ag/AgCl) produces two free electrons, as shown in Eq. (2), which is the main reason for the current. Until the enzyme is stabilized and there is enough oxygen, the relationship between the current and the concentration of glucose is linear [49].



Modification of the working electrode using nanoparticles with various sizes can improve the performance of biosensors, and increase the sensitivity and reduce the resistivity of the electrode in the route of electron exchange. These nanoparticles can be made from gold, silver, and zinc, which are widely used with various structures to modify the electric and

conductivity properties of electrodes [50–52]. Gold colloidal nanoparticles are metallic colloids and are used in various forms in the structure of the electrodes of biosensors. Using gold colloidal nanoparticles, the electric isolation effect is reduced in the protein cortex of glucose oxidase and electron exchange increases [53, 54]. The produced electron moves due to the effect of applying constant voltage on both sides of working and standard electrodes and this electron movement leads to an electric current which can be measured by ammeters. The amount of exchanged electron and/or the produced current indicates the amount of reacted glucose and its concentration in the sample [55]. In the current paper, the performance of two biosensors in completely identical conditions are compared while the only difference between them is the presence of gold colloidal nanoparticles in the electrode structure of one of them. These biosensors have different performances in similar currentmetry conditions and applying the gold colloidal nanoparticles leads to increasing the conductivity and improving the currentmetry.

Today, detecting the composition of foods through quality control tests is of great importance in food industries. High costs of traditional methods and the necessity for more accurate and sensitive measuring confirm the necessity of this issue. As a result, finding and/or improving faster, more accurate, more sensitive as well as cheaper measurement methods is always interesting for researchers and food producers [56–63].

The common analysis tools in food industries need skillful operators and are time consuming. These tools frequently need long separation time, costly equipment, and chemicals with high purity. A great part of these obstacles and problems can be removed by applying enzyme analyses. However, new food industries need to have small analysis tools to be used easily on non-solution samples and are able for simultaneous online control of one or more characteristics during production process or food processing. Most of these necessities can be acceptably met using enzyme electrodes, and hence improve the efficiency and optimization of the process and quality of products. Further, these enzyme electrodes should be cheap, reliable and strong, and they should have obvious preferences over the available methods [64–69].

One of the most important cause for cardiovascular diseases in recent years is high concentrations of cholesterol in blood. Cholesterol is found considerably in dairy products and yolk and its concentration can be determined using chromatography methods such as high performance liquid chromatography (HPLC) and gas chromatography (GC),

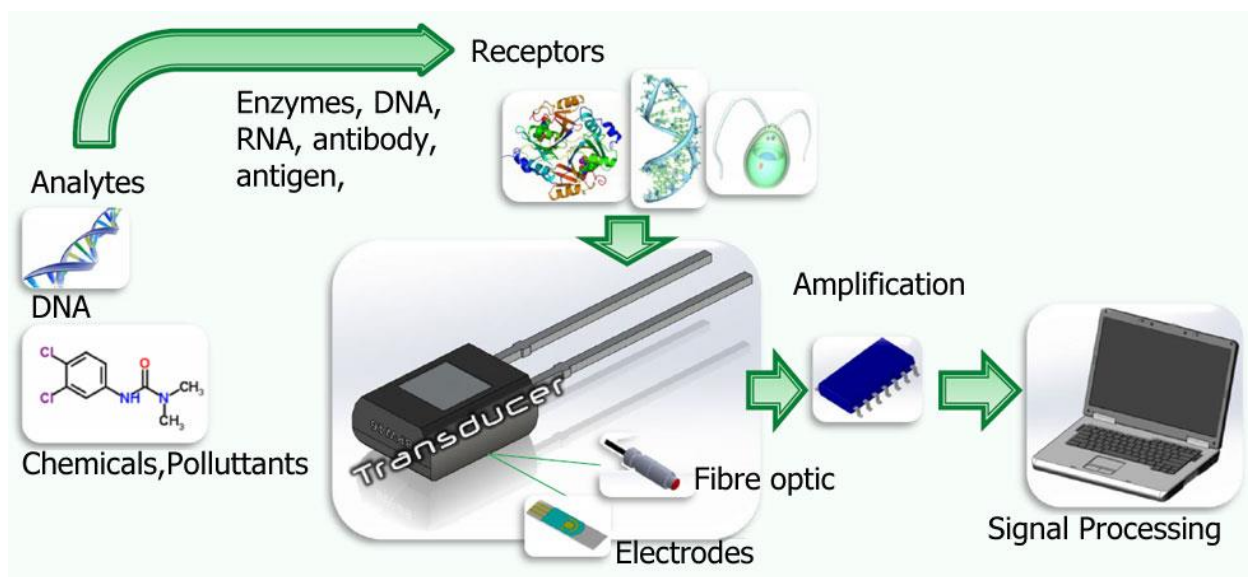
which are of appropriate sensitivity and selectivity. However, applying fast and efficient methods is of great importance as these methods are time consuming and costly. Therefore, enzyme methods such as cholesterol esterase and cholesterol oxidase can be practically used as alternatives of traditional chromatography methods due to their simplicity, fast and effective [70–75].

Glucose is another important food component, which is frequently measured in quality control of food production processes. Until now, various spectroscopy methods, such as high performance liquid chromatography (HPLC), have been used for detecting glucose, especially in the fermentation process of nectars. However, enzyme methods are widely developed to do this [76–81].

The tendency of researchers in food industries for measuring these compounds with repeatability, selectivity, and high rate in very low concentrations in live environment leads to the development of tools called biosensors; and 85% of common biosensors is used for glucose measurement [82–87].

Generally, sensors are tools for detecting a chemical, physical and/or biological change and converting it to a measureable signal. A sensor includes a detector element, which is able to respond to the presence of a special analyte or a group of analytes. Another important part of a sensor is transducer that converts the created response into a sign. The third part of a sensor is signal processor which collects the received signal from transducer and, finally, after boosting it illustrates it.

Biosensors are a subset of chemical sensors, which have a completely especial performance in detecting biological processes. In this type of sensors, the detector elements are biological compounds such as proteins, enzymes, antibodies, nucleic acids, cells, tissues, and/or receivers which selectively react with the target analyte and create a response. This response is converted into an electrical signal in transducer and after processing the amount of this signal is illustrated as voltage, current, and/or impedance. The amount of this signal relates to the concentration of the analyte. Regarding the fact that the performance of biological detector elements is completely unique (for example, glucose oxidase only oxidizes glucose), biosensors make it possible to quantitatively evaluate and determine the concentration of an analyte, in addition to qualitatively evaluate the analyte and determine its presence or absence [88]. Figure 1 shows a schematic view of a biosensor.



**Figure 1.** A schematic view of a biosensor.

The mentioned biological detector elements in aqueous solutions are of low durability on converter surfaces. For more durability, these materials should be stabilized over converter surfaces. Surficial absorption of these compounds with converter surface, trapping in a matrix during the covering of the converter surface with the matrix and creation of covalent bonds between the materials and converter surface is one of the methods used for this purpose. Trapping in matrix is the most effective and frequently used method. Matrices mainly include membranes, gel in carbon paste, graphite, silica, and/or polymer thin films. Undoubtedly, conductive polymer nanostructures-based matrices, especially polyaniline and polypyrrole, are the most applicable and effective matrices due to high compatibility with biological components, ability for fast electron exchanging, considerably high effective area, and appropriate cohesion. In the current research, the application of polyaniline-based nanobiosensors for measuring glucose and cholesterol is discussed.

#### MATERIALS AND METHODS

In this study, three experimental steps were performed.

- (i) Producing a gold plate covered with single-walled carbon nanotubes (SWCNT) and its preparation;
- (ii) Preparing glucose oxidase for stabilization on the gold plate;
- (iii) Stabilizing glucose oxidase using connective material PASE on the surface of carbon

nanotubes and returning the activity of the enzyme.

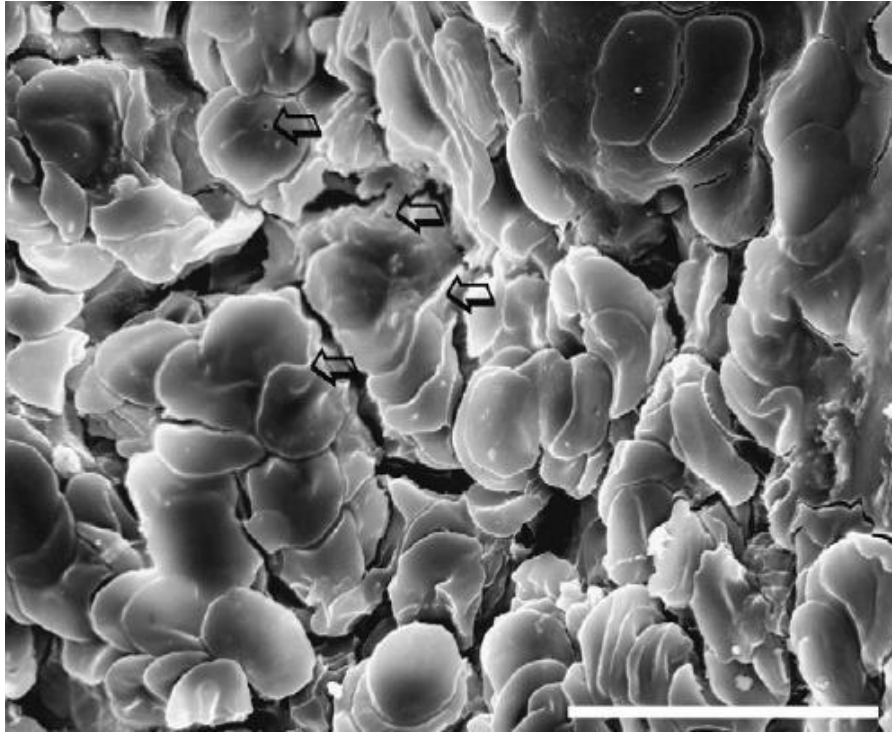
Glucose (with 98% purity and molecular weight of 198.17 gr/mole), paraffin oil, dipotassium hydrogen phosphate ( $K_2HPO_4$ ), and potassium hydrogen phosphate ( $KH_2PO_4$ ) were supplied by Merck Co. for producing 0.1 M phosphate buffer. Glucose oxidase (35.5 Ku/mg) and gold colloidal nanoparticles ( $Au > 48\%$  and about 24 nm) were supplied by Sigma-Aldrich Corporation. Finally, carbon graphite powder (pure, mesh < 325) was supplied by Sigma-Aldrich Corporation.

#### 1. Producing Gold Plate Covered with Single-Walled Carbon Nanotubes (SWCNT) and Its Preparation

In this step, the following initial measures were performed for producing and preparing the gold plate:

##### 1.1. Preparation of Gold Plate

In this step, a 10 mm × 10 mm gold plate with the thickness of 1 mm was produced and its surface was completely covered using precipitation with homological vapors (gold vapors) before any processing (Figure 2). The thickness of this homological layer was about 4–5 μm. Then, the surface of the gold plate was washed in two stages with concentrated sulfuric acid ( $H_2SO_4$ ) and nitric acid ( $HNO_3$ ), and finally with a large volume of deionized water for removing the remaining impurities and waste.

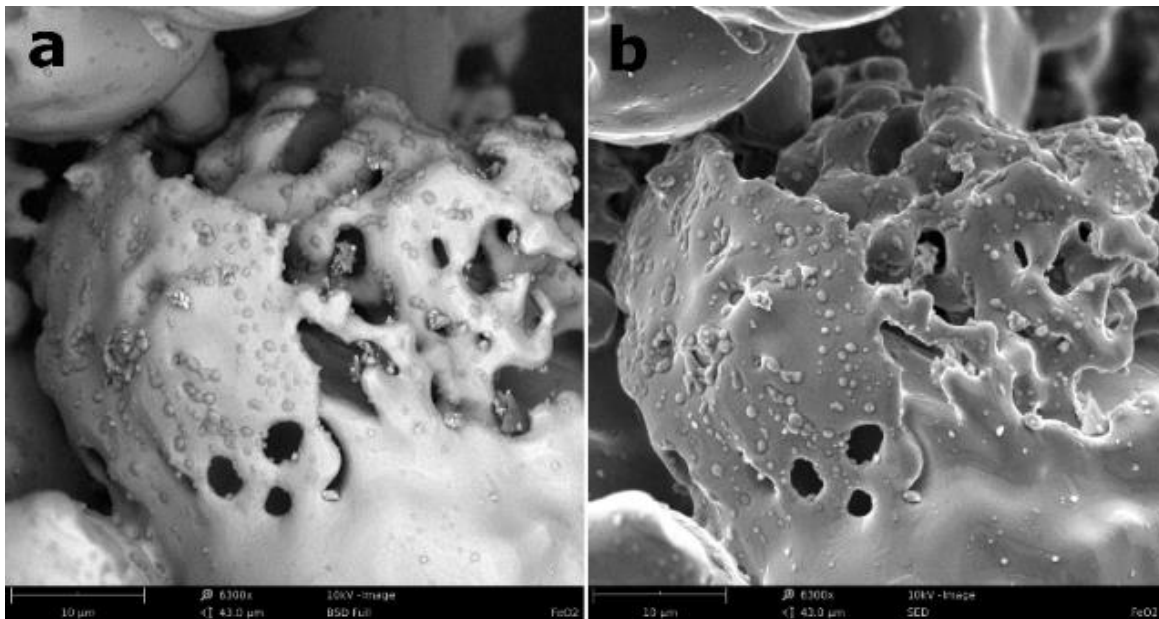


**Figure 2.** 10 mm  $\times$  10 mm gold plate with the thickness of 1 mm was produced and its surface was completely covered using precipitation with homological vapors (gold vapors) before any processing.

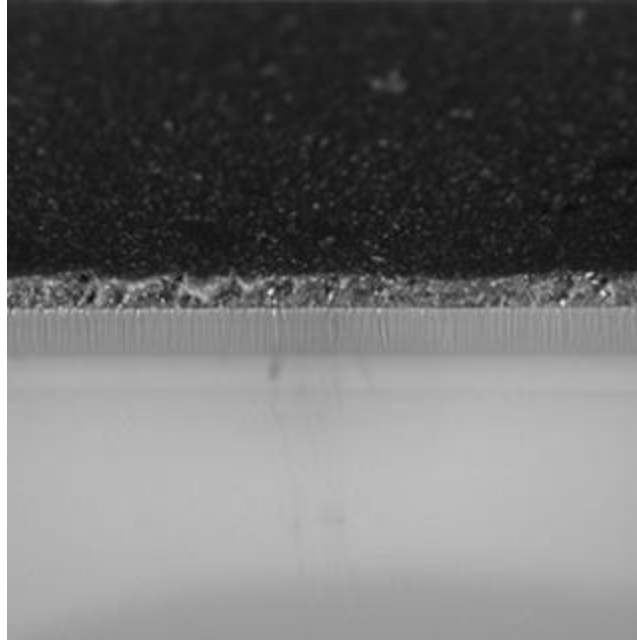
### 1.2. Deposition of Nickel Particle Catalyst

After preparing the gold plate, nickel catalyst was used for creating initial cores of carbon nanotubes and their regular growing up. The catalyst particles in the size of

about 4 nm were deposited over the surface with medium regularity and density using lithography method. (This step has been performed in Nanotechnology Laboratory, California South University (CSU)) (Figure 3).



**Figure 3.** SEM images of the gold plate after catalyst particles in the size of about 4 nm were deposited over the surface with medium regularity and density using lithography method.



**Figure 4.** The developed nanotubes were single-walled with diameter of about 2–50 nm and height of 15–20 nm and developed vertically on the medium, according to scanning electron microscopy (SEM) images.

### 1.3. Development of Carbon Nanotubes (CNT)

For developing carbon nanotubes on the nickel nanoparticles stabilized on the gold plate, CVD method was used. In this method, the prepared gold plate was constantly placed into an oven under vacuum condition and light hydrocarbon (methane with a percent of butane) was pumped into the oven as gas. Due to the entering of this gaseous compound and chemical reactions in the oven, carbon precipitants emerged on the gold medium and regular carbon nanotubes were produced. The developed nanotubes were single-walled with diameter of about 2–50 nm and height of 15–20 nm and developed vertically on the medium, according to scanning electron microscopy (SEM) images. (This procedure was performed in California South University (CSU) Nanotechnology Research Institute in cooperation with some professors and experts) (Figure 4).

The area covered by these nanotubes was about 10–15 mm<sup>2</sup>. Regarding the fact that each of these nanotubes acted as the base of sensor, it could be said that approximately 10<sup>8</sup>–10<sup>9</sup> sensor bases were developed in the whole area of the gold medium (100 mm<sup>2</sup>). This amount of density was very appropriate for stabilizing biological detectors.

For removing the remaining impurities and waste on the plate during the development process, the surface of the gold plate was washed in two stages with concentrated sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) and nitric acid

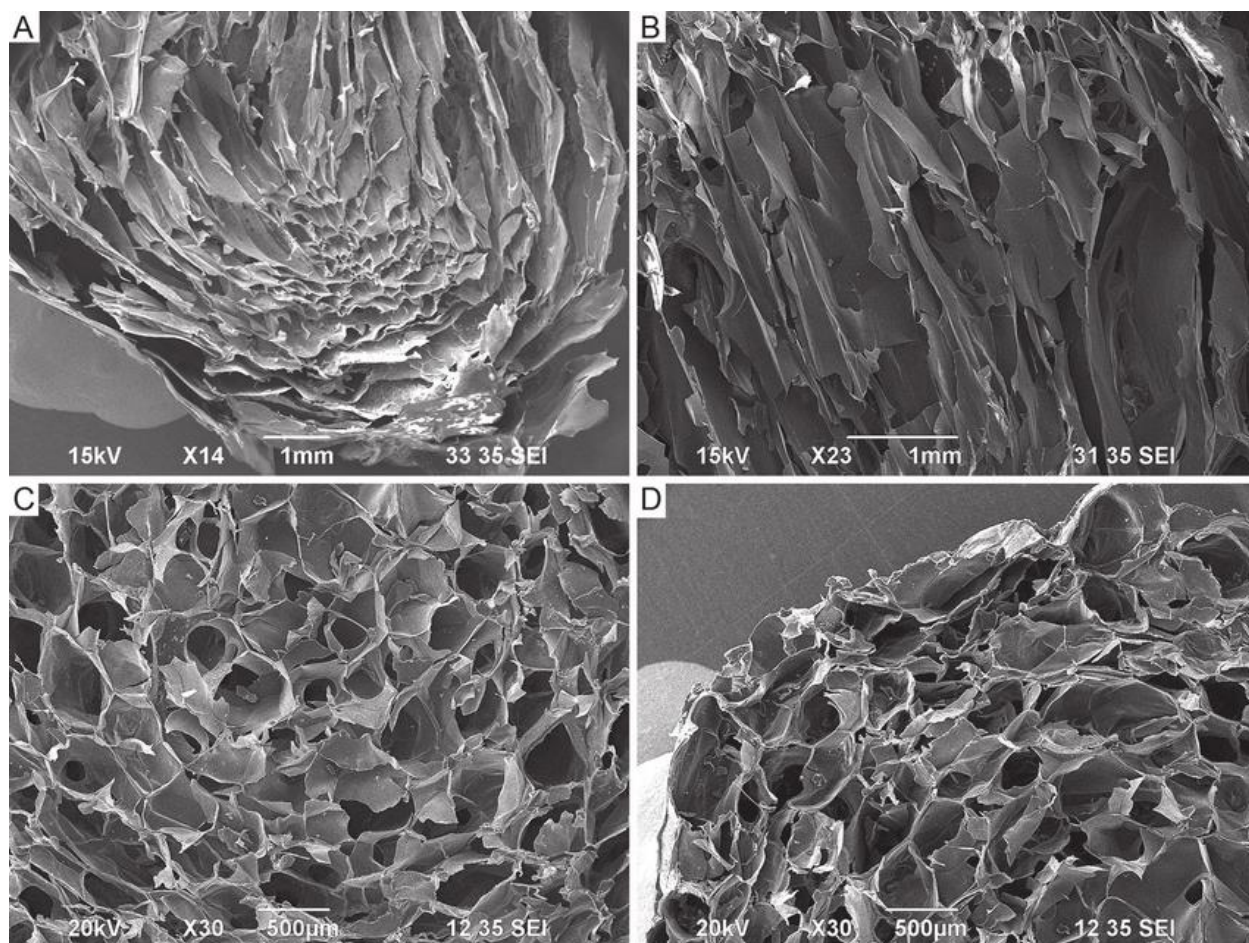
(HNO<sub>3</sub>), and finally with a large volume of deionized water.

## 2. Preparing Glucose Oxidase for Stabilizing on the Gold Plate

Regarding high sensitivity of enzyme to environmental conditions and reaction situation and for maintaining active points of the enzyme until the end of operation and correct connection of the prepared enzyme on the surface of medium (developed nanotube), very important and accurate measures were performed and are mentioned in the following.

### 2.1. Preparing Glucose Oxidase Apoenzyme

For maintaining the active points of the enzyme until the end of operation, the active points of enzyme were firstly separated and a covering was placed over the enzyme. This process was performed using separation of flavin adenine dinucleotide (FAD) from the structure of the enzyme and preparing an apoenzyme. To perform this process, a saturated solution of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was firstly prepared and then its pH was decreased down to 1.4 at 20°C using concentrated sulfuric acid (97% v/v). Glucose oxidase dissolved in phosphate buffer with the concentration of 20 mg/mL was added, drop by drop, during stirring to 20 mL of the saturated solution at 5°C. This solution was maintained at this temperature for half an hour and then centrifuged for 15 minutes at 20000 rpm. The top yellow layer was separated from the compound after centrifuging (Figures 5a, 5b) and the



**Figure 5.** The top yellow layer was separated from the compound after centrifuging (a, b) and the obtained precipitant (c, d) was centrifuged in the same acidic pH condition twice and the obtained precipitant was collected.

obtained precipitant (Figures 5c, 5d) was centrifuged in the same acidic pH condition twice and the obtained precipitant was collected. The final precipitant was dissolved in phosphate buffer as the resource for glucose oxidase apoenzyme. (This step was performed in Research Center of Biotechnology, California South University (CSU)).

### 3. Stabilizing Glucose Oxidase Using Connective Material PASE on the Surface of Carbon Nanotubes (CNT) and Returning the Activity of Enzyme

After performing all the above mentioned steps, the enzyme and medium were separately prepared for stabilizing. Regarding the fact that a gold electrode was used in this study and direct contact of enzyme and metal changes the natural structure of the enzyme, the medium surface was covered with a layer of cystamine.

Mainly, protein was added to the single layer of cystamine in these processes for sensor manufacturing. Therefore, it was positively charged. As a result, it could

create an electrostatic absorption with the negatively charged enzyme. Hence, the relationship between the enzyme and medium facilitates [89–97].

For stabilizing glucose oxidase on the developed carbon nanotubes on the gold plate, pyrenebutanoic acid succinimidyl ester (PASE) was used in this study as the connective material, which has a pyrene group for making Van der Waals bonds with the carbon nanotubes and an amid group for connecting to the enzyme [98–127].

For accurate stabilization, the prepared medium was firstly stirred in a solution of the connective material at the concentration of 2.3 mg/mL and DMF, and after 2 hours the medium was exited from the solution and washed with pure DMF, and at the same time the prepared apoenzyme was dissolved in screened and deionized water with the concentration of 10 mg/mL.

Then, the washed medium was contacted to the apoenzyme solution for 18 hours and, finally, the medium was washed with very clear water for 6 times to

remove all impurities produced during the process.

### 3.1. Returning the Activity of Stabilized Enzyme on the Medium

Regarding the fact that the enzyme was changed to apo for protecting against loss of enzyme activity during stabilization steps, it was necessary to activate the enzyme by returning the removed FAD molecule to the structure of the enzyme after the stabilization process.

To perform this step, 200  $\mu\text{M}$  of FAD molecules obtained from changing the enzyme to the apoenzyme was incubated for 1 hour in 0.1 M phosphate buffer with the concentration of 150  $\mu\text{g}/\text{mL}$  and at pH 6 at room temperature. This process formed the stable complex of FAD protein which in fact was reconstructed enzyme. Its separation constant was very small ( $k < 10\text{M}$ ) and could be very effective for returning enzyme activity.

### 3.2. Investigating the Activity of Stabilized Enzyme on the Medium

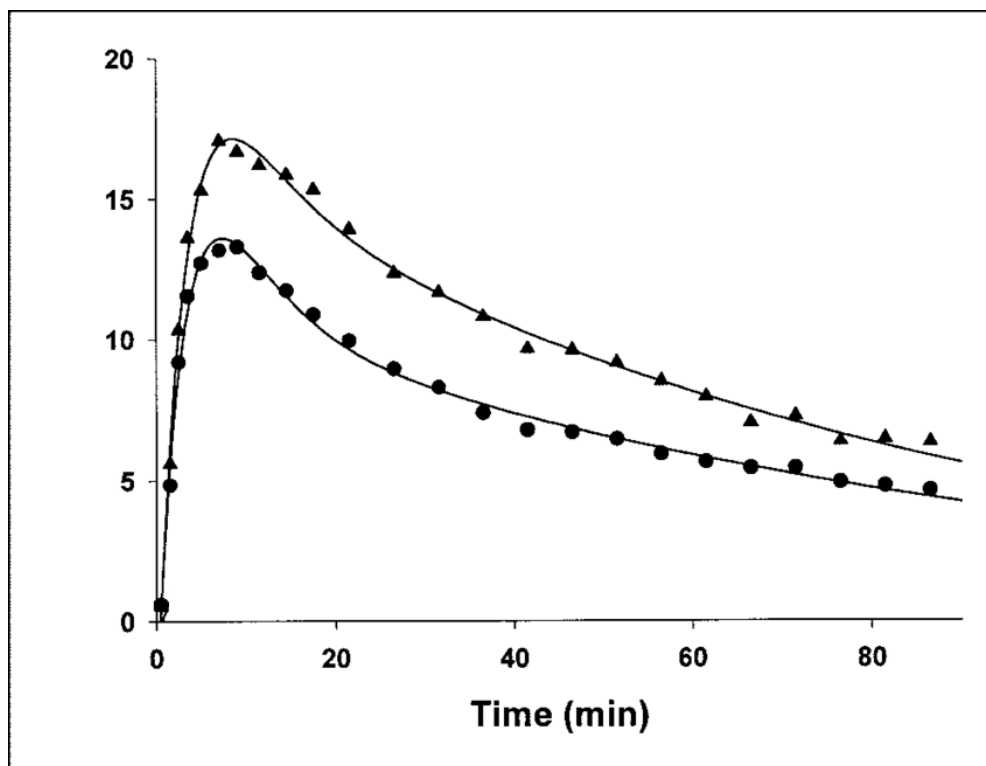
After performing the stabilizing operation, enzyme activity of the incubated mixture was measured in the following form after placing at room temperature for 30

minutes to determine and confirm the returning of the enzyme activity.

In this method, the prepared medium was placed in a solution containing potassium phosphate measuring buffer (measuring buffer was prepared by dissolving 0.1 ml of 1% o-Dianisidine indicator in 12 ml of phosphate buffer with the concentration of 0.1 M and at pH 6.0), 10  $\mu\text{L}$  of 18% glucose (in water), 10  $\mu\text{L}$  of peroxidase with the concentration of 200  $\mu\text{g}/\text{mL}$  and 10  $\mu\text{L}$  of glucose oxidase (dilution of 200 times) with the concentration of 1  $\text{mg}/\text{mL}$ , and the absorbance at the wavelength of 460 nm at various times was measured by JENWAY 6305 spectroscope (available in Laboratory of Microbiology, California South University (CSU)) (Figure 6). (Absorption value indicates the amount of glucose consumed by the enzyme and hence enzyme activity [100, 108]).

## 4. Electrode Preparation

Firstly, carbon graphite powder was placed into an oven for 30 minutes in 700°C so that volatile and absorptive materials were removed from the graphite powder and surface activity increased. Then, it was place in a desiccator for 1 hour. This powder was the raw



**Figure 6:** Enzyme activity before (lower curve) and after (upper curve) stabilizing operation process according against time which showed stable and activation of the enzyme after stabilizing.



material for producing the electrode. CP (Carbon Paste) was produced by adding 100 mg of carbon graphite powder to 36  $\mu$ l of paraffin oil, according to [128–163]. The modified CP with gold colloidal nanoparticles was produced by adding 300  $\mu$ l of gold colloidal nanoparticles to 100 mg of carbon graphite powder and after evaporation of water in a desiccator for 3 hours, 36  $\mu$ l of paraffin oil was added to it. By inserting these mixtures, separately, in glass tubes with an internal diameter of 4 mm, CPE electrode and Au<sub>nano</sub>/CPE were prepared and the electrical connection with an ammeter was established through copper wires placed into the electrodes. When these electrodes were not in use, they were kept at 4°C.

## 5. Enzyme Preparation and Biosensor Production

Oxidation of glucose by glucose oxidase and production of glucuronic acid reduced the pH of the reaction environment. To prohibit severe variations of the pH and maintain the activity of the enzyme, a specified amount of the enzyme was placed into phosphate buffer. To prepare 0.1 M phosphate buffer, K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> were used and the pH was adjusted with H<sub>3</sub>PO<sub>4</sub> and NaOH. 10 mg of glucose oxidase was added to 1 ml of 0.1 M phosphate buffer (PBS) in semi-permeable

membranes and a membrane was fastened around each CPE electrode and Au<sub>nano</sub>/CPE. Then, the produced biosensors were placed into glucose solutions with the concentration range of 0–1 mM for currentmetry.

## 6. Conductive Polymers

Conductive polymers are a type of organic materials which have electric conductivity. It seems that metals have electrical conductivity and organic materials are dielectric, while conductive polymers are of both properties. Another advantage of conductive polymers is their process ability, i.e., their ability to be dissolved or melted.

These characteristics cause these polymers to be produced and used in various forms. In addition, these polymers are flexible due to their plastic nature. Further, their electric conductivity can be set [164–207].

The reason for their electric conductivity is their coupled bond structure. It means that the presented molecules in  $\pi$  bonds of these structures can be moved along the polymerization chain and conduct electric current. Figure 7 shows the coupled structure of some conductive polymers.

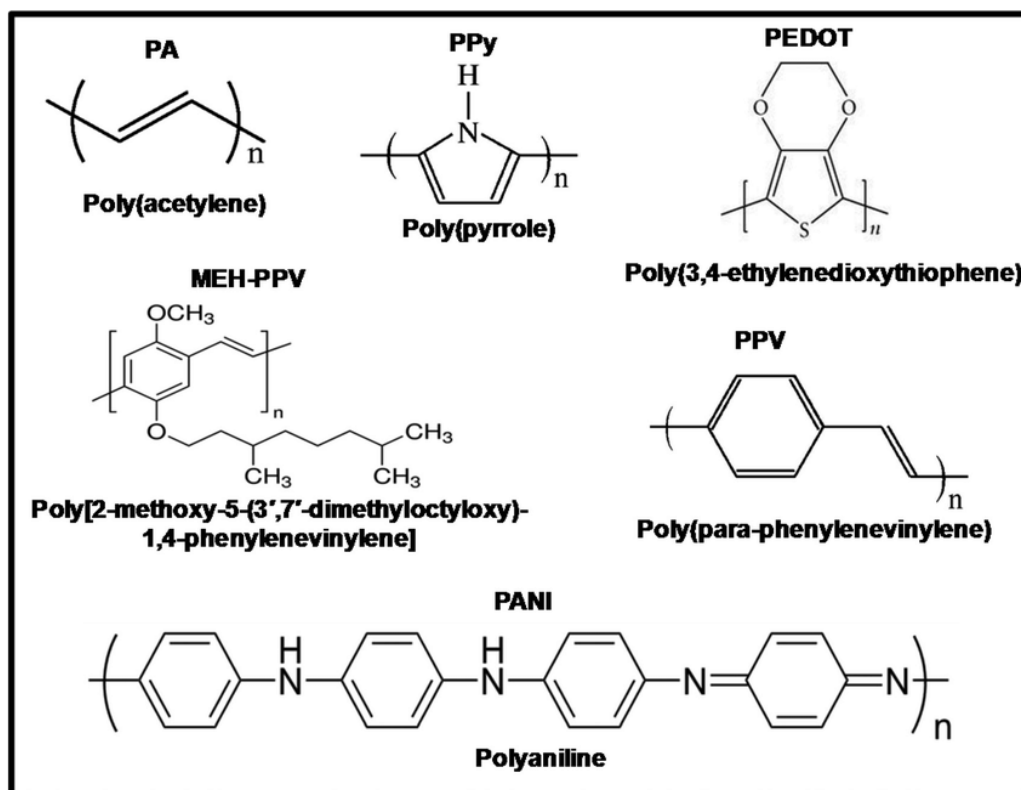


Figure 7. Coupled structures of some conductive polymers.

Among conductive polymers, polyaniline has been more interesting for researchers than others due to its high electrical conductivity, simple production process, good environmental stability, and unique chemical nature (oxidization/reduction). The previously performed studies in recent years on the production of polyaniline in nano scale caused the improvement of the most interesting property of this polymer, namely the electrical conductivity. In addition, increasing the effective area of the polymer leads to improvement of its process ability, and hence application of this polymer increase in various sciences, especially food industries [208–243]. Up to now, various types of polyaniline nanostructures have been produced such as nanoparticles, nanofibers, nanotubes, nanowires, nanorods, and nanobolts.

The above mentioned properties of the conductive polymer nanostructures cause these materials to be considered as the most interesting options for the stabilizing matrix of biodetector elements on the transducer of biosensors. Among the produced nanostructures from polyaniline, nanoparticles, nanofibers, and nanotubes of this material have been used in biosensors [244–288].

In the following sections, applications of these materials in the structure of biosensors as well as the methods used for measuring sugars, proteins, and fats using these materials are discussed.

## 7. Biosensors

As previously mentioned, biosensors are a type of sensors which have an amazing, unique performance in the presence of intervening factors. Stabilizing each bio-operator on the transducer of biosensors leads to creation of a completely unique response to that analyte. Hence, biosensors are amazing tools from selectivity point of view.

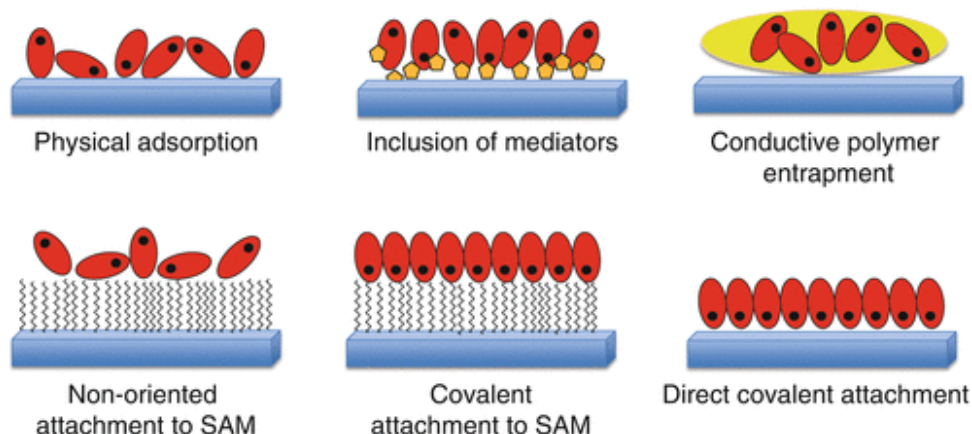
Generally, analyte–biodetector element interaction in a biosensor causes to creation of a change in a parameter. This change is converts into an electrical signal by transducer and after processing, is illustrated [289–303].

### 7.1. Bioelement Stabilizing Methods

Till now, various physical and chemical methods have been proposed for stabilizing bioelements. However, they should be stabilized so that their active locations are not blocked and their geometrical forms have not changed. In addition, it is necessary to have a relationship between biodetector element and sensitive area of transducer [304–317].

Trapping the enzyme in a matrix, e.g., an electropolymer film, is one of the most frequently used methods for stabilizing the enzyme. Another method is creating a covalent bond between protein and transducer surface, in which detector element is very high. These connections are established through functional groups of detector element such as SH, OH, COOH, and NH<sub>2</sub>, which are not necessary for biological reaction.

Surficial absorption is another method that is based on gravitational force between detector element and transducer surface and is used to stabilize detector elements. Lifespan of biosensor prepared by this method is very short. However, surficial absorption is very simple since it has no need to any other reagent and failure of the enzyme is limited. Connecting the biological detector elements through electrostatic absorbers is another method for stabilizing these compounds. In this method, positive or negative charges are induced on transducer surface by applying a potential and then biological detector element is absorbed by the surface through electrical induction. Figure 8 shows six most common types of methods used for stabilizing biosensor elements.



**Figure 8:** Six most common types of methods used for stabilizing biosensor elements.

Conductive polymer nanostructures can be used as stabilizers of biodetector elements due to their electrical conductivity, compatibility with live materials, environmental stability, and high contact surface.

## 8. Cyclic Voltammetry

Cyclic voltammetry is an electrochemical method for detecting the presence of operational electroactive components, which are able to exchange electrons in an electrochemical reaction in an aqueous environment, in a solution. Since the working process of the considered biosensors is based on an electrochemical reaction, cyclic voltammetry is an appropriate method for detecting the presence of an analyte in solution. In this regard, transducer of a biosensor is used in this method as working electrode. In this method, working, auxiliary, and standard electrodes are used. Standard electrode is of constant potential in various currents. Usually, silver/chloride standard electrode is used. Auxiliary electrode is usually made from typical platinum and working electrode includes transducer and polymer cover containing detector element.

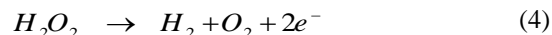
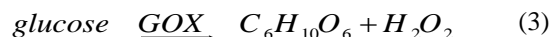
After placing these three electrodes in the solution, a potential range between two specified values is cyclically applied to working and standard electrodes and the currents obtained from working and auxiliary electrodes are illustrated in terms of the applied potential. Therefore, a current against potential curve can be obtained. In this graph, current increases when potential reaches a value in which an electroactive type

can be oxidized and hence a peak emerges in cyclic voltammetry graph.

When potential scanning is applied in the opposite direction, i.e., from higher values to lower ones, electroactive types with ability for reduction are reduced and create a peak in the current. Since each material has its own specific potential (oxidization–reduction), analyte can be detected through this method [318–339]. As a result, cyclic voltammetry method is used as the most important test for performance of transducer and sensor zone of biosensors. Figure 9 shows a schematic view of cyclic voltammetry device, potential application, and the obtained cyclic voltammetry graph.

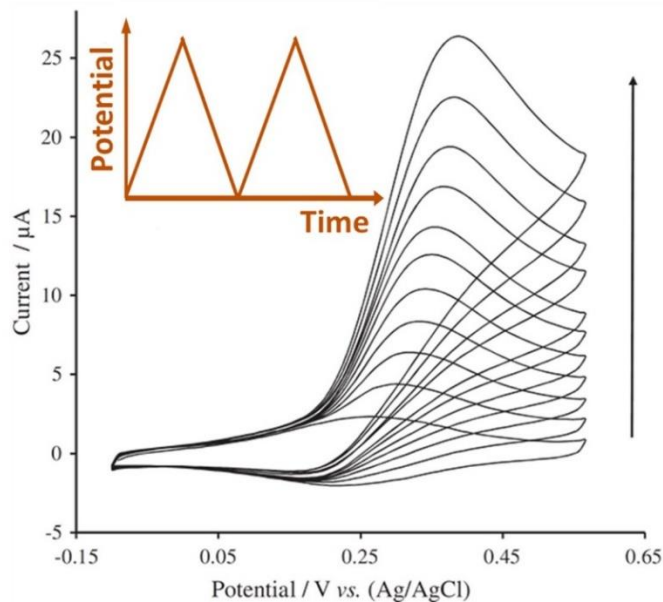
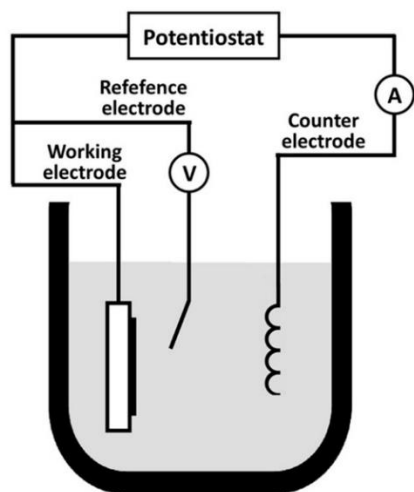
## 9. Glucose Detection

In biosensors used for detecting glucose, the reactions performed on the bioelectrode (working electrode) are in the following forms:

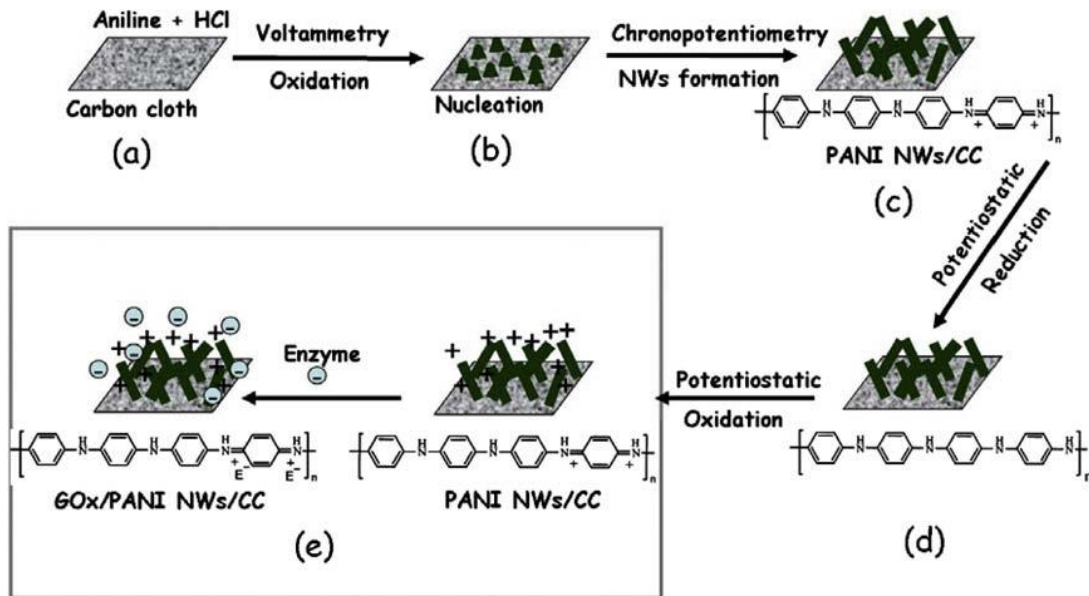


Heidari *et al.* [340–355] investigated the biosensors that were used for detecting glucose based on glucose oxidase stabilizing in polyaniline nanowires. In this method, aniline monomers were firstly polymerized in a product containing ammonium peroxide disulphate as oxidizer and then they were precipitated on a carbon

### Cyclic Voltammetry (CV)



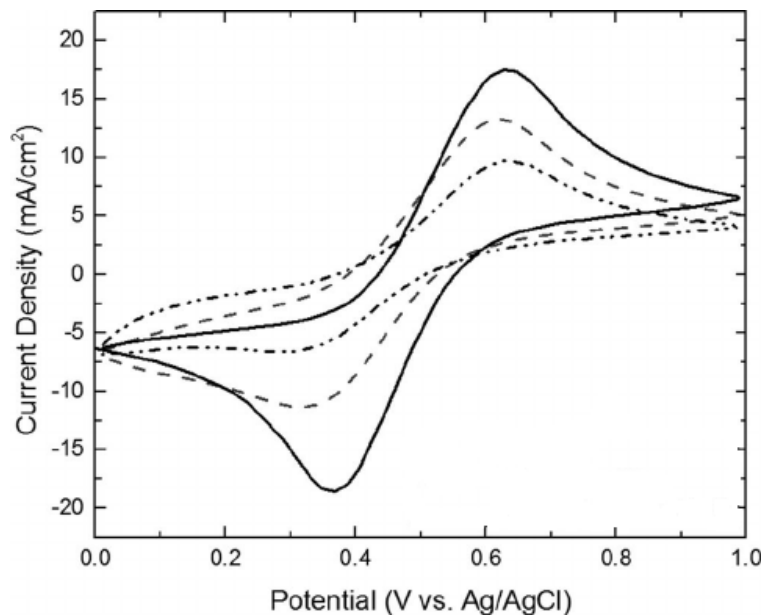
**Figure 9.** A schematic view of cyclic voltammetry device, potential application and the obtained cyclic voltammetry graph.



**Figure 10.** A schematic view of positive charges cause electrostatic absorption of glucose oxidase induced from negative surface charges.

electrode as a film. When polyaniline particles were growing, polymerization of aniline continued by applying a potential. Then, polyaniline nanowires were produced by applying a constant current to the carbon electrode. Afterwards, this electrode was placed in a phosphate buffer solution (pH=7.2) and polyaniline nanowires reduced by applying a constant potential. This caused anions to be removed from the surface of polyaniline and the appropriate conditions for surface

absorbing of glucose oxidase were prepared. Then, the electrode was placed in a phosphate buffer solution containing 2.5 mg/l glucose oxidase, and by applying 0.25 V, positive charges were induced on polyaniline nanowires. These positive charges caused electrostatic absorption of glucose oxidase induced from negative surface charges. Figure 10 schematically shows this process. In order to investigate the performance of the produced electrode, cyclic voltammetry technique was



**Figure 11.** When glucose oxidase is in a stable state, the related oxide peaks emerge, while when there is not enzyme, oxide peaks do not emerge.

used. The produced electrode was placed in solutions with and without glucose. The resulting voltammetry graph showed that when glucose oxidase was in a stable state, the related oxide peaks emerged while when there is not enzyme, oxide peaks did not emerge (Figure 11). This observation was performed for evaluating the correct performance of biosensors.

Another study was performed by Heidari *et al.* [356–360] about the stabilizing of glucose oxidase with polyaniline nanotubes. In this study, a membrane of aluminium anodic oxidation with outer diameter of holes between 200 and 250 nm, and inner diameter of holes equal to 100 nm was used to synthesize polyaniline nanotubes.

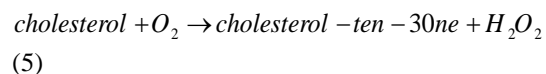
Firstly, one side of aluminum anodic oxidation was covered with 10 nm thick platinum layer through evaporation in vacuum. Then, a copper wire was connected to the platinum layer for conducting electric current. After isolating the copper wire and backside of platinum to prohibit incident contact with the solution containing the sample, the produced electrode was placed in a solution containing 0.5 M sulfuric acid and 0.2 M aniline monomers. By applying scanning potential, aniline polymerization and polyaniline precipitation in holes of aluminum anodic oxidation were performed and polyaniline nanotubes were produced.

In order to stabilize glucose oxidase, polyaniline nanotubes were reduced in phosphate buffer solution through applying 250 mV so that the anions around the polyaniline nanotubes were removed. Then, polyaniline nanotubes were oxidized in phosphate buffer solution containing 7 mg/l glucose oxidase under 750 mV to electrostatically absorb glucose-oxidase. During oxidation, glucose oxidase, which was negatively charged, absorbed onto the inner walls of polyaniline nanotubes and became trapped. Then, the electrode was washed with distilled water to remove enzymes that were not appropriately absorbed by the inner walls of the nanotubes.

In order to investigate the performance of the produced electrode, cyclic voltammetry technique was used.

## 10. Cholesterol Detection

In biosensors used for detecting cholesterol, the reactions performed on the bioelectrodes are in the following forms:



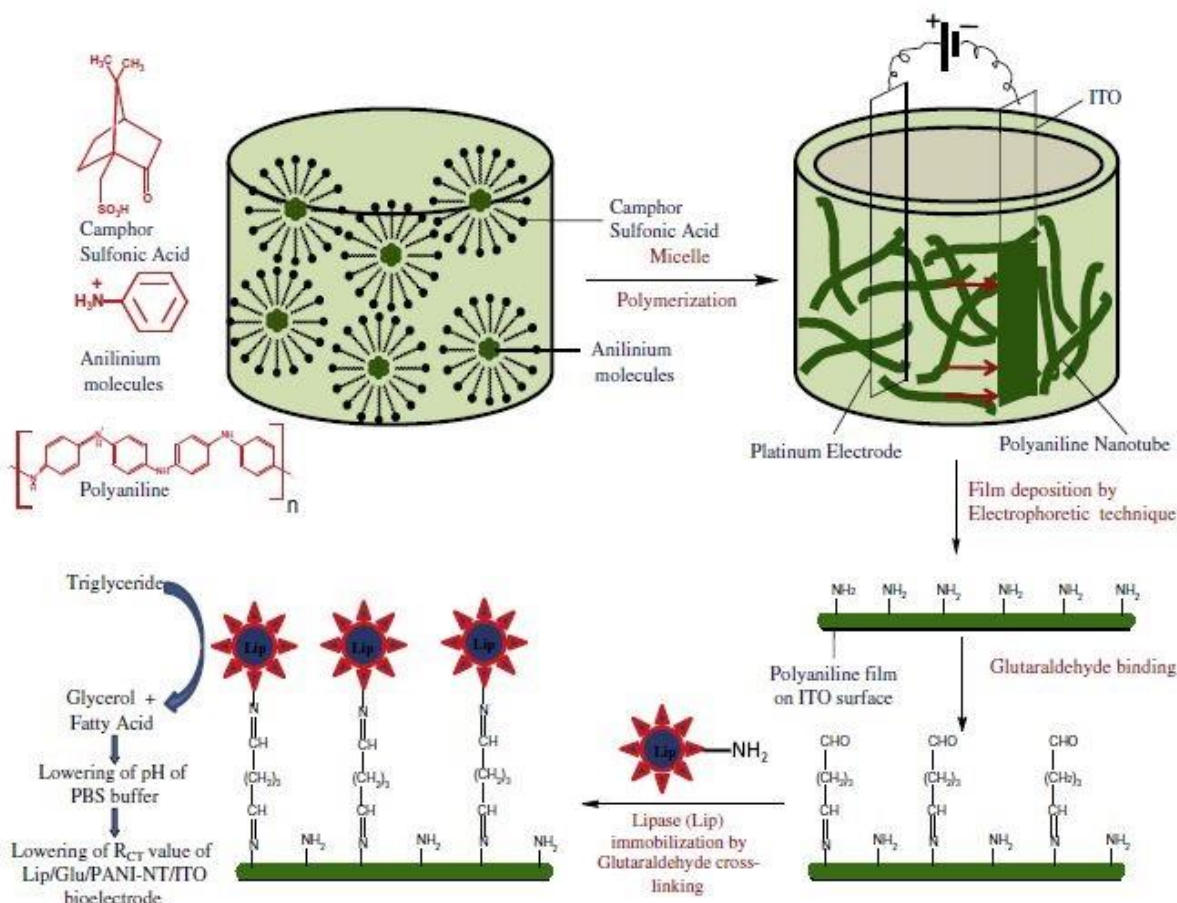
Heidari *et al.* [356–360] used polyaniline nanotubes for stabilizing lipase on biosensors used for detecting cholesterol. In this method, as can be seen in Figure 12, polyaniline nanotubes (PANI-NT) were firstly produced through chemical polymerization reaction. Then, these nanotubes were precipitated on the surface of indium–tin oxide electrode by electrophoretic method as a film. Then, the produced electrode was placed into a solution containing glutaraldehyde (GLU). The reason for using glutaraldehyde is that this compound has a carbonyl group at both ends. It can bond with nitrogen of polyaniline film on the surface of indium–tin oxide from one end and connects to nitrogen groups of lipase at the other end. Therefore, lipase stabilized on the polyaniline film covalently. After connecting glutaraldehyde to the film surface, electrode dries and enters to lipase solution and after sometime electrode dries.

In this investigation, the performance of the produced electrodes was evaluated by cyclic voltammetry. In this study, three types of electrodes, ((PANI-NT)/ITO) (electrode covered with carbon nanotubes without glutaraldehyde and/or lipase), (PANI/GLU/ITO), and ((LIP/GLU/PANI)–(NI-ITO)) were compared in environments with and without cholesterol. Observing the peaks related to the reaction of lipase and cholesterol available in the sample container for electrode and their disappearance in two other states confirmed the correct performance of the electrodes.

In a similar study, Heidari *et al.* [360–368] produced biosensors for detecting cholesterol based on polyaniline film covered on an electrode of indium–tin oxide by electrochemical method.

In this study, polyaniline was firstly covered on an electrode of indium–tin oxide by electrochemical polymerization and then all steps performed by Heidari *et al.* were repeated.

In another investigation, Heidari *et al.* [360–368] produced cholesterol biosensors through simultaneously stabilizing cholesterol oxidase and cholesterol esterase on polyaniline film. In this method, polyaniline was firstly covered on an electrode of indium–tin oxide through electrochemical polymerization [369–381]. Then, 1% glutaraldehyde was distributed over the polyaniline film and the surface was allowed to dry. Then, cholesterol esterase was distributed over the dried surface of electrode and after it dried, cholesterol oxidase was distributed and dried. Then, this (enzyme–polymeric) set was washed with distilled water to remove oligomers and not stabilized enzymes. The results obtained from cyclic voltammetry confirmed the presence of cholesterol and cholesterol oleate.



**Figure 12.** Usage polyaniline nanotubes for stabilizing lipase in biosensors used for detecting cholesterol. Polyaniline nanotubes (PANI-NT) were firstly produced through chemical polymerization reaction.

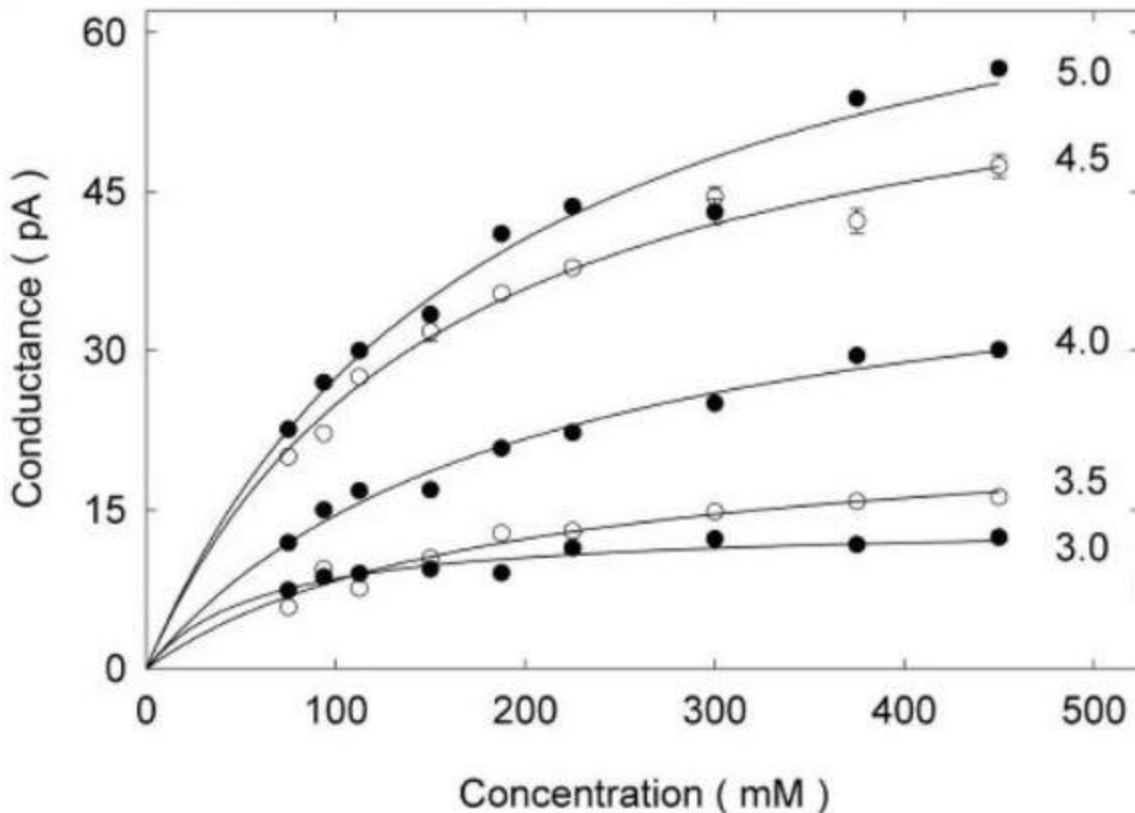
## RESULTS AND DISCUSSION

### 1. Currentmetry

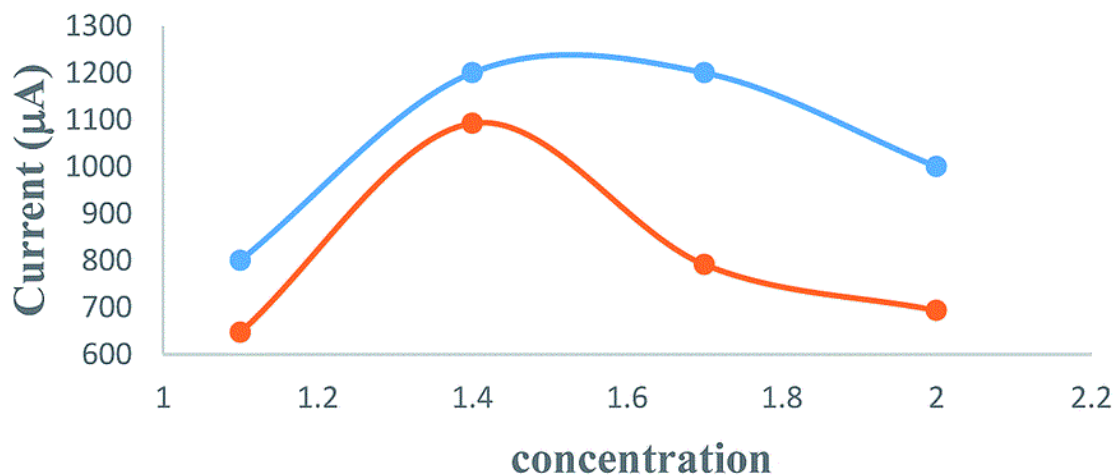
Currentmetry of the biosensors was performed separately through standard (Ag/AgCl), CPE, and ( $Au_{nano}/CPE$ ) electrodes at room temperature. Glucose solutions with specified concentrations were prepared for currentmetry of the biosensors and the produced current was measured by ammeters by applying the same potential, 0.7 V to both biosensors.

Figure 13 shows the currentmetry of the biosensors in terms of glucose concentration with different performances of CPE and ( $Au_{nano}/CPE$ ) electrodes. The curves showed that the concentration of substrate (glucose) affects the activity of glucose oxidase. In very low concentrations of substrate, not all active locations of the enzyme were filled with the substrate and the activity of the enzyme would be low.

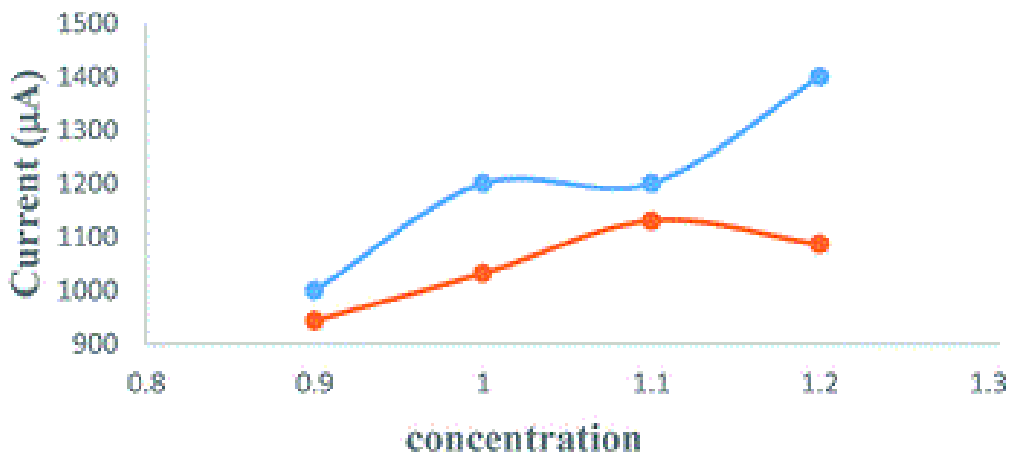
By gradually increasing the concentration of the substrate, enzyme activity increased until reaching to a specified concentration in which all active locations of the enzyme were filled with the substrate and enzyme activity reached its maximum amount under operational conditions. In Figure 13, curve b (performance of ( $Au_{nano}/CPE$ ) electrode) is of higher currents than curve a (performance of CPE electrode) due to the presence of gold colloidal nanoparticles. The maximum current response of CPE and ( $Au_{nano}/CPE$ ) electrodes in phosphate buffer at pH=4 were 0.4 and 1.2  $\mu A$ , respectively, which showed different performances of the two electrodes at similar potential and pH. In Figure 14, currentmetry of the biosensors in terms of the concentration of glucose is shown for two different performances of CPE and ( $Au_{nano}/CPE$ ) electrodes. In Figure 14, the upper curve (performance of ( $Au_{nano}/CPE$ ) electrode) is of higher currents than the lower curve (performance of CPE electrode) due to the presence of gold colloidal nanoparticles.



**Figure 13.** Currentmetry of biosensors in terms of glucose concentration with different performances of CPE and ( $Au_{nano}/CPE$ ) electrodes.



**Figure 14.** Currentmetry of biosensors in terms of the concentration of glucose shown for two different performances of CPE and ( $Au_{nano}/CPE$ ) electrodes. In this figure, the upper curve (performance of ( $Au_{nano}/CPE$ ) electrode) is of higher currents than the lower curve (performance of CPE electrode) due to the presence of gold colloidal nanoparticles. It should be noted that x-axis and y-axis show concentration (pH) and current ( $\mu A$ ), respectively.



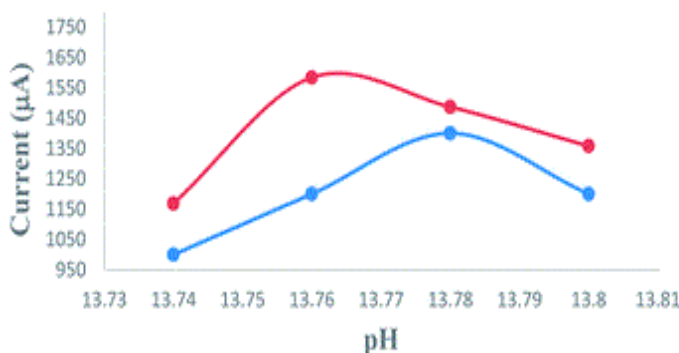
**Figure 15.** The maximum current response of CPE and (Au<sub>nano</sub>/CPE) electrodes in phosphate buffer at pH=6 was 0.7 and 1.58 µA, respectively, which showed considerable different performance of the two electrodes in similar potential and pH. It should be noted that x-axis and y-axis show concentration (pH) and current (µA), respectively.

In addition, according to Figure 15, the maximum current response of CPE and (Au<sub>nano</sub>/CPE) electrodes in phosphate buffer at pH=1.1 was 1100 µA and at pH=1.2 was 1400 µA, respectively, which showed considerably different performance of the two electrodes in similar potential and pH.

In Figure 16, currentmetry of the biosensors in terms of the concentration of glucose is shown for two different performances of CPE and (Au<sub>nano</sub>/CPE) electrodes. According to Figure 16, the maximum current response of CPE and (Au<sub>nano</sub>/CPE) electrodes in phosphate buffer at pH=13.78 was 1450 µA and at pH=13.76 was 1550 µA, respectively. In this figure, curve b (performance of (Au<sub>nano</sub>/CPE) electrode) is of higher currents than curve a (performance of CPE electrode) due to the presence of gold colloidal nanoparticles. It means that carbon electrode modified with gold

colloidal nanoparticles prepares appropriate environment for direct movement of electrons due to high electrocatalytic activity of nanoparticles, hence leads to facilitation of electron movement, higher currents, and, finally, better tracing of glucose due to higher conductivity. By evaluating these currentmetries, the positive effect of gold colloidal nanoparticles is confirmed and (Au<sub>nano</sub>/CPE) electrode is selected as the appropriate electrode.

Using Figure 16 and comparing the illustrated graphs for various concentrations in variation of pH against current, it can be seen that maximum current was achieved at pH=13.76 and increasing the concentration of glucose led to producing higher ampere. It indicates that optimum condition was achieved, in which current was maximum and the amount of detected glucose was maximum.



**Figure 16.** Currentmetry of biosensors in terms of the concentration of glucose is shown for two different performances of CPE and (Au<sub>nano</sub>/CPE) electrodes. It should be noted that x-axis and y-axis show concentration (pH) and current (µA), respectively.



## CONCLUSION

After completing the experimental steps, the nano medium was produced. This medium can be used as high accurate and sensitive nano-biosensor due to the stabilized enzyme on its surface.

For protecting enzyme activity from devastating effects of metal media during stabilization processes, it is necessary to cover the medium surface with an appropriate material such as cystamine.

Removing the active points of enzyme (making apo) before the start of stabilization step is necessary for protecting the performance of the enzyme.

Regarding the relatively large size of glucose oxidase, this enzyme should be connected to the outer wall of the carbon nanotube using a connective material such as 1-pyrenebutanoic acid succinimidyl ester (PASE).

Regarding the fact that the medium was placed into phosphate buffer and accurately washed after each measuring of activity, the possibility of the presence of free (non-stabilized) enzymes on the medium was very low and it could be assuredly said that the measured activity of about 280 u/mg shown initially in Figure 6 was only due to stabilized enzymes and it confirmed that temporary removing of enzyme activity and returning its activity after completing the stabilizing steps cannot permanently inactivate enzymes although it can remove some enzymes from their third structure.

As can be seen in Figure 6, the variation of the activity of the stabilized enzyme against time did not considerably change compared to the activity of the free enzyme. It confirms the stability of the stabilized enzyme.

Creating medium density (mentioned in the paper) in developed nanotubes affects the penetration of enzyme and its stability.

Regarding the operational level, high conductivity, and flexibility of carbon nanotubes compared to other metals, they are appropriate for stabilizing various chemical and biological detectors and utilizing them in manufacturing biosensors leads to increasing efficiency and sensitivity, and decreasing resistivity in performance.

In this study, glucose oxidase biosensor was experimentally manufactured using carbon nanotubes based on the investigations in various previously performed researches in this field. Although these tests were performed similar to experimental studies in some

steps, obtaining new results such as temporary inactivation of the enzyme and using gold plate, alone, in macro plate form as the medium were the differences of the current research with similar researches.

Although the performed studies in the field are in the level of initial manufacturing of these sensors, it leads to preparing an appropriate space for performing more developed research works in this field. Experimental researches performed in this study and the obtained results can be represented to researchers with this aim. Hopefully, more acceptable results will be achieved if more applicable plans will define.

Evaluations of the performance of biosensors with two different electrodes showed that enzyme activity was low at very low concentrations of glucose for both biosensors. Then, by gradually increasing glucose concentration, enzyme activity increased until reaching a specified concentration at which all active locations of the enzyme were filled with substrate and enzyme activity reached to its maximum capacity. The results obtained from currentmetry of the biosensors showed that by increasing the concentration of glucose and after reaching a specific amount, enzyme activity reached a stage at which its rate did not change with the increasing glucose concentration and remained constant. This phenomenon happened when all active locations of the enzyme were occupied and there was not enough active space for oxidizing the available glucose. To maintain the activity of active points of glucose oxidase, the enzyme was placed in phosphate buffer so that pH of the reaction environment was controls using the buffer and enzyme activity was maintained during oxidization. Investigation of the performance of the biosensors at various pH values of phosphate buffer showed that enzyme activity and currentmetry of both biosensors in phosphate buffer at pH=6 increased. This increasing in the activity was due to creation of an appropriate ionic state for substrate and/or glucose oxidase. Oxidation and reduction groups in glucose oxidase, which were oxidized and reduced, were deeply deposited in holes and so they were not easily available for guiding electrons towards electrode surface and this was against the performance of the enzyme. Therefore, in establishing the current, it was necessary to prepare satisfactory electric relationship between active locations of the enzyme (FAD) and surface of the electrode. To solve this problem, gold colloidal nanoparticles were used in the structure of (Au<sub>nano</sub>/CPE) electrode. It led to the increase in the conductivity, facilitating the movement of electrons, and increasing the satisfactory electric relationship between electrode surface and active locations of the enzyme (FAD). Modification of (Au<sub>nano</sub>/CPE) electrode with gold colloidal nanoparticles led to creation of a micro-environment like oxidization-reduction of proteins,

which was due to high electrocatalytic activity of the nanoparticles. It meant that the isolation effect in protein cortex of the enzyme was reduced and electron exchange was increased. Another reason for using gold colloidal nanoparticles in the structure of ( $Au_{\text{nano}}/CPE$ ) electrode was its high sensitivity and selection ability, increasing the electric conductivity and permeability between biological fences (enzyme–electrode) and it considerably improved glucose tracing by the biosensors.

Detecting glucose and cholesterol in quality control tests of food production processes are very important. Although these methods should be fast, accurate, sensitive, and cheap, the current methods need skillful operators and are time consuming. To solve this problem, one type of chemical sensors, called biosensors that are of unique performances, can be used. Conductive polymers–based biosensors, especially aniline polymers are greatly interesting for researchers due to their unique properties such as high electric conductivity, large contact area, and simple preparation method. The appropriate performance of these biosensors introduces them as the most important measurement tools to the market.

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