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Improving the Performance of Nano–Endofullerenes in Polyaniline Nanostructure–Based Biosensors by Covering Californium Colloidal Nanoparticles with Multi–Walled Carbon Nanotubes

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Abstract In the current paper, Glucose (Dextrose), Fructose and Galactose–oxidase enzymes are used as stabilization medium due to its more efficiency, ability for more accurate controlling the 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 Nano Endohedral Fullerenes (Endofullerenes) on Californium plate. For better connecting and stabilizing the enzyme on the medium, the prepared medium is washed by high concentration Sulfuric Acid and Nitric Acid and a large volume of deionized water and for protecting enzyme from devastating effect of Californium and prohibiting them to become inactive, surface is covered with Cystamine before stabilization. Regarding the large size of Glucose (Dextrose), Fructose and Galactose–oxidase enzymes compared to surface of medium, a connective material with amid at one end and Pyrine at the other end is used as transfer agent and for stabilizing this connection, the prepared medium is placed into dimethylformamide (DMF) solution for a couple of hours. Activity of stabilized enzyme at 460 (nm) wavelength recorded by spectroscope was depicted against time to evaluate its stability in various times. The prepared medium, which has a large amount of Glucose (Dextrose), Fructose and Galactose–oxidase enzymes, can be used as electrode in sensors. Furthermore, 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 (Dextrose), Fructose, Galactose and Cholesterol and Cholesterol are such compounds that they frequently measure. 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 issue, leads to developing biological methods, especially biosensors. Among them, biosensors based on conductive polymer nanostructures, especially Polyaniline (PANI), have been recently interested due to their unique characteristics. The current paper aims to introduce and investigate the previously performed studies about Polyaniline (PANI)–based biosensors for detecting Glucose (Dextrose), Fructose, Galactose and Cholesterol and Cholesterol. In addition, Glucose (Dextrose), Fructose and Galactose–oxidase electrochemical sensor is one of the best methods for detecting low amount of Glucose (Dextrose), Fructose and Galactose and applying Californium colloidal nanoparticles as a supplementary material in the structure of biosensor can be effective for improving its efficiency and optimum performance.

Keywords: Nano Endohedral Fullerenes (Endofullerenes), Glucose (Dextrose), Fructose and Galactose–oxidase enzymes, vapor phase precipitation, biosensor, Cholesterol, Polyaniline (PANI) nanofibers, Polyaniline (PANI) nanotubes, Californium colloidal nanoparticles, Plutonium, currentmetry

1 Introduction

In recent decades, biosensors can be manufactured in very small scales (nanometer) as nanotechnology introduces into biological sciences. Nano–biosensors 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 product on their surfaces [1–27].

Nano Endohedral Fullerenes (Endofullerenes) 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 Multi–Walled Carbon Nanotubes (MWCNTs). Stabilizing the biological detectors such as enzymes over these materials may be led to increasing the performance of enzyme reaction, controllability of reaction, participation of more enzymes in reaction, prohibiting enzymes from wasting as well as information transferring with higher rate in these Nano–biosensors [28–43].

Nano Endohedral Fullerenes (Endofullerenes) should be stabilized on a surface for better acting as the base of sensor. Stabilizing surfaces are mainly metallic. In the current paper, Californium plate is used for this purpose. Various methods have been used for developing and stabilizing Nano Endohedral Fullerenes (Endofullerenes) on the medium. However, the most frequent method is Chemical Vapor Deposition (CVD) [44–63].

Electrostatic, hydrophobic reactions and or covalent bond along with oxidization of Nano Endohedral Fullerenes (Endofullerenes) are mainly used for stabilizing large enzymes on the surface of Nano Endohedral Fullerenes (Endofullerenes) and a simple absorption on the external surface. In these types of bonds, a connective material is mainly used [64–87].

In the structure of nano–biosensors, the covered surface of medium by Nano Endohedral Fullerenes (Endofullerenes) is responsible for transferring the effects induced by reaction to transducer device for illustrating the signal. Nano Endohedral Fullerenes (Endofullerenes) can play two–side role, as the location of enzyme stabilization and as connective limit between reagent and transducer [88–111].

Today, detecting the composition of foods through quality control tests is of great importance in food industries. High cost of traditional methods and the necessity for more accurate and sensitive measuring confirms 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 [112–143].

The frequent analysis tools in food industries need to skillful operators and are time consuming. These tools are frequently needed to long separations, costly equipment's 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 use easily in non–solution samples and to be able for simultaneous, online controlling one or more characteristics during production process or food processing. Most of these necessities can be acceptably met using enzyme electrodes and hence, improves the efficiency and optimization of the process and the quality of product. Further, these enzyme electrodes should be cheap, reliable and strong and they should have obvious preferences over the available methods [144–175].

One of the most important reasons for cardiovascular diseases in recent years is high concentration of Cholesterol in blood. Cholesterol is considerably found 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, being fast and effective [176–200].

Glucose (Dextrose), Fructose and Galactose are other important food components, which are 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 (Dextrose), Fructose and Galactose, especially in fermentation process of nectars. However, enzyme methods are widely developed to do this [201–223].

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 development of some tools entitled as biosensors so that 85% of frequent biosensors are used for Glucose (Dextrose), Fructose and Galactose measurement [224–240].

Generally, sensors are tools for detecting a chemical, physical and or biological change and converting it to a measureable signal. A sensor is including 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 to a sign. The third part of a sensor is signal processor which collects the received signal from transducer and after boosting it, finally illustrates it.

Biosensors are a subset of chemical sensors, which have a completely especial performance in detecting biological processes. In this type of sensors, detector element is a biological compound such as protein, enzyme, antibody, Nucleic Acids, cell, tissue and or receiver which selectively reacts with the target analyte and creates a response. This response converts to an electric signal in transducer and after processing, the amount of this signal illustrates as Voltage, current and or impedance. The amount of this signal relates to the concentration of analyte. Regarding the fact that the performance of biological detector elements are completely unique (for example, Glucose (Dextrose), Fructose and Galactose–oxidase enzyme only oxidizes Glucose (Dextrose), Fructose and Galactose), biosensors make it possible to quantitatively evaluate and to determine the concentration of analyte, in addition to qualitatively evaluation of analyte and determining the presence or absence of it [241–251].

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 with some way. Surficial absorption of these compounds with converter surface, trapping in a matrix during covering the converter surface with this matrix and creation of covalent bond between these materials and converter surface are some methods which are used for this purpose. Among all these methods, the most effective and frequent method is trapping in matrix. These matrices are mainly including membranes, gel in Carbon paste, graphite, silica and or polymer thin films. Undoubtedly, conductive polymer nanostructures–based matrices, especially Polyaniline (PANI) and Polypyrole, 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 applications of Polyaniline (PANI)–based nanobiosensors for measuring Glucose (Dextrose), Fructose, Galactose and Cholesterol are discussed.

Biosensor technology is promisingly developed in bio–analytical researches. Sensors or biosensors are used for illustrating various analytes in various times. In 2017, Glass Carbon (GC) electrode biosensor, Multi–Walled Carbon Nanotubes (MWCNTs), Titanium Dioxide (TiO_2), Apatite Hydroxide and Glucose (Dextrose), Fructose and Galactose–oxidase enzyme were produced in the United States based on this technology [252–255]. A chemical sensor is including receiver, transducer and separator. Receiver or biological element such as enzyme establishes a biological connection with measureable component. Transducer converts the measurable component to optical or electric signals and separator can act as membrane [256, 257]. In 2017, an electrode biosensor was produced in the United States based on Glass Carbon (GC), Multi–Walled Carbon Nanotubes (MWCNTs), Plutonium nanoparticles and Glucose (Dextrose), Fructose and Galactose–oxidase enzyme and the amount of Glucose (Dextrose), Fructose and Galactose in the compound was measured using electric signals by ammeter. In 2011, a biosensor was produced in the United States based on Glass Carbon (GC) working electrode, Glutaraldehyde (GLU), Carbon nanotube (multi–walled–gelatin) and Glucose (Dextrose), Fructose and Galactose–oxidase enzyme. Glucose (Dextrose), Fructose and Galactose biosensor is one of the most successful biosensors in detecting chemical compounds such as Glucose (Dextrose), Fructose and Galactose with very low amount. Its efficiency and sensitivity can be considerably increased by combining with various compounds such as Gold, Silver, Platinum, Carbon nanotubes and so on. The currentmetry of these biosensors is based on the performance of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme and electron exchange. In Glucose (Dextrose), Fructose and Galactose–oxidase enzyme, oxidation and reduction groups of Flavin Adenine Dinucleotide (FAD) are oxidized and reduced and hence, it is necessary to establish a satisfactory electric relationship between active location of FAD enzyme and the surface of electrode to have a current. Amperometric Glucose (Dextrose), Fructose and Galactose biosensors are mainly composed of stabilizing Glucose (Dextrose), Fructose and Galactose–oxidase enzyme in various media such as trapped Polyacrylamide over working electrode surface.

The presence of enzyme causes to consumption of Oxygen by the Glucose (Dextrose), Fructose and Galactose in the solution and reducing the Oxygen penetration to the auxiliary Platinum electrode surface and it 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, 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 (Dextrose), Fructose and Galactose are linear.

Modification of working electrode using nanoparticles with various sizes can improve the performance of biosensors, increase the sensitivity and reduce the resistivity of 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. Californium, colloidal nanoparticles are metallic colloids and are used in various forms in the structure of electrode biosensors. Using Californium colloidal nanoparticles, the electric isolation effect is reduced in protein cortex of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme and electron exchange increases. The produced electron moves due to the effect of applying constant Voltage on both sides of working and standard electrode and this electron movement leads to an electric current which can be measured by ammeter. The amount of exchanged electron and or the produced current indicates the amount of reacted Glucose (Dextrose), Fructose and Galactose and their concentrations in the sample. In the current paper, the performance of two biosensors in completely identical conditions is compared while the only difference between them is the presence of Californium colloidal nanoparticles in the electrode structure of one of them. These biosensors have different performances in similar currentmetery condition and applying the Californium colloidal nanoparticles leads to increasing the conductivity and improving the currentmetery.

2 Materials, Research Method, Characterization Methods, Experimental Techniques and Applications

In the current paper, three experimental steps are performed:

(1) Producing Californium, plate covered by Multi–Walled Carbon Nanotubes (MWCNTs) and its preparation.

(2) Preparing Glucose (Dextrose), Fructose and Galactose–oxidase enzyme for stabilizing on the Californium plate.

(3) Stabilizing Glucose (Dextrose), Fructose and Galactose–oxidase enzyme using connective material Pyrenebutanoic Acid Succinimidyl Ester (PASE) on the surface of Nano Endohedral Fullerenes (Endofullerenes) and returning the activity of enzyme.

2.1 Producing Californium Plate Covered by Multi–Walled Carbon Nanotubes (MWCNTs) and Its Preparation

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

2.1.1 Preparation of Californium Plate

In this step, a 10×10 (mm) Californium plate with thickness of 1 (mm) was produced and its surface was completely covered using precipitation with homological vapors (Californium vapors) before any processing. Thickness of this homological layer is about 4-5 (μm). Then, surface of Californium plate was washed in two stages with high concentration Sulfuric Acid (H_2SO_4) and Nitric Acid (HNO_3) and finally with a large volume of deionized water for removing the remained impurities and wastes.

2.1.2 Deposition of Catalyst Plutonium Particles

After preparing Californium plate, Plutonium catalyst was used for creating initial cores of Nano Endohedral Fullerenes (Endofullerenes) and regular growing up of them. 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 the BioSpectroscopy Core Research Laboratory at Faculty of Chemistry, California South University (CSU), Irvine, California, USA).

2.1.3 Development of Nano Endohedral Fullerenes (Endofullerenes)

For developing Nano Endohedral Fullerenes (Endofullerenes) on the Plutonium nanoparticles stabilized on the Californium plate, CVD method was used. In this method, the prepared Californium plate was constantly placed into oven under vacuum condition and light hydrocarbon (methane with a percent of butane) was pumped to the oven as gas. Due to entering this gaseous compound and performing chemical reaction in the oven, Carbon precipitants were emerged on the Californium medium

and regular Nano Endohedral Fullerenes (Endofullerenes) were produced. The developed nanotubes were multi–walled with diameter about 2–50 (nm) and height of 15–20 (nm) were developed vertically on the medium, according to Scanning Electron Microscopy (SEM) images (This operation has been performed in the BioSpectroscopy Core Research Laboratory at Faculty of Chemistry, California South University (CSU), Irvine, California, USA).

The area covered by these nanotubes is about 10–15 (mm^2). Regarding the fact that each of these nanotubes acts as the base of sensor, it can be said that approximately 10^8–10^9 sensor base are developed in the whole area of Californium medium 100 (mm^2). This amount of density is very appropriate for stabilizing biological detectors.

For removing the remained impurities and wastes on the plate during development process, surface of Californium plate was washed in two stages with high concentration Sulfuric Acid (H_2SO_4) and Nitric Acid (HNO_3) and finally with a large volume of deionized water.

2.2 Preparing Glucose (Dextrose), Fructose and Galactose–Oxidase Enzyme for Stabilizing on the Californium Plate

Regarding high sensitivity of enzyme to environmental conditions and reaction situation and for maintaining active points of 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 that are mentioned in the following.

2.2.1 Preparing Glucose (Dextrose), Fructose and Galactose–Oxidase Apoenzyme

For maintaining active points of enzyme until the end of operation, active points of enzyme were firstly separated and coverage was place over the enzyme. This process was performed using separation of Flavin Dinucleotide Aadenine (FDA) from the structure of enzyme and preparing apoenzyme. To perform this process, a saturated solution of $(NH_4)_2SO_4$ was firstly prepared and then, its pH was decreased down to 1.4 in 20^o C using high concentrated Sulfuric Acid (97%v/v). The Glucose (Dextrose), Fructose and Galactose–oxidase enzyme solved in Phosphate buffer with concentration of 20 (mg/mL) was added, drop by drop, during stirring to the saturated 20 (mL) solution in 5 (°C). This solution was maintained in this temperature for half an hour and then, was centrifuged for 15 (min) in 20000 (rpm). The top yellow layer was separated from the compound after centrifuging and the obtained precipitant was repeatedly centrifuged in the same acidic pH condition for two times and the obtained precipitant was collected. The final precipitant was solved in Phosphate buffer as the resource for Glucose (Dextrose), Fructose and Galactose–oxidase apoenzyme (This step has been performed in the BioSpectroscopy Core Research Laboratory at Faculty of Chemistry, California South University (CSU), Irvine, California, USA).

2.3 Stabilizing Glucose (Dextrose), Fructose and Galactose–Oxidase Enzyme Using Connective Material Pyrenebutanoic Acid Succinimidyl Ester (PASE) on the Surface of Nano Endohedral Fullerenes (Endofullerenes) and Returning the Activity of Enzyme

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

Mainly, protein added to the single layer of Cystamine in these processes for sensor manufacturing. Therefore, it is positively charged. As a result, it can create an electrostatic absorption with the negatively charged enzyme. Hence, the relationship between the enzyme and medium facilitates.

For stabilizing Glucose (Dextrose), Fructose and Galactose–oxidase enzyme on the developed Nano Endohedral Fullerenes (Endofullerenes) on the Californium plate, Pyrenebutanoic Acid Succinimidyl Ester (PASE) is used in the current paper as connective material which has a pyrene group for making Van der Waals bond with Nano Endohedral Fullerenes (Endofullerenes) and an amid group for connecting to the enzyme.

To accurate stabilization, the prepared medium was firstly stirred in a solution of connective material with concentration of 2.3 (mg/mL) and DMF and after 2 hours, medium was exited from the solution

and washed with pure DMF and at the same time, the prepared apoenzyme was solved in screened and deionized water with concentration of 10 (mg/mL).

Then, the washed medium was contacted to apoenzyme solution for 18 hours and finally, medium was washed with very clear water for 6 times to remove all impurities produced during the process.

2.3.1 Returning the Activity of Stabilized Enzyme on the Medium

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

To perform this step, 200 (µM) of FAD molecule obtained from changing enzyme to apoenzyme was incubated for 1 hour in 0.1 (M) Phosphate buffer with concentration of 150 (µg/mL) and pH=6 at room temperature. This process forms the stable complex of FAD protein which in fact is reconstructed enzyme. Its separation constant is very small (k<10M) and can be very effective for returning enzyme activity.

2.3.2 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 in room temperature for 30 (min) to determine and confirm the retuning of enzyme activity.

In this method, the prepared medium was placed in solution containing Potassium Phosphate measuring buffer, 10 (µL) of 18% Glucose (Dextrose), Fructose and Galactose (in water), 10 (µL) of peroxidase enzyme with 200 (µg/mL) concentration and 10 (µL) of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme (dilution of 200 times) with concentration of 1 (mg/mL) and the amount of solution absorbed in wavelength of 460 (nm) was measured by JENWAY 6305 spectrophotometer (available in the BioSpectroscopy Core Research Laboratory at Faculty of Chemistry, California South University (CSU), Irvine, California, USA) in various times (Absorption value indicates the amount of Glucose (Dextrose), Fructose and Galactose consumed by enzyme and hence enzyme activity).

2.4 Other Materials, Characterization Methods, Experimental Techniques and Applications

2.4.1 Other Materials

Glucose (Dextrose), Fructose and Galactose (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 from Merck Co. for producing 0.1 (M) Phosphate buffer. Glucose (Dextrose), Fructose and Galactose–oxidase enzyme (35.5 (K/mg)) and Californium colloidal nanoparticles (Cf>48% and about 24 (nm)) were supplied from Sigma–Aldrich Corporation. Finally, Carbon graphite powder (pure, mesh<325) was supplied from Sigma–Aldrich Corporation.

2.4.2 Other Characterization Methods, Experimental Techniques and Applications

2.4.2.1 Electrode Preparation

Firstly, Carbon graphite powder was placed into oven for 30 (min) in 700 (°C) so that volatile and absorptive materials remove from graphite powder and surface activity increases. Then, it was placed in desiccator for 1 hour. This powder is the raw material for producing the electrode. CP (Carbon Paste) is produced by adding 100 (mg) of Carbon graphite powder to 36 (µl) of paraffin oil, according to [221–231]. The modified CP with Californium colloidal nanoparticles is produced by adding 300 (µl) of Californium colloidal nanoparticles to 100 (mg) of Carbon graphite powder and after evaporation of water in desiccator for 3 hours, 36 (µl) of paraffin oil is added to it. By entering a part of these mixtures, separately, in glassy tubes with inner diameter of 4 (mm), CPE electrode and (Cf_{nano}/CPE) were prepared and electric connection with ammeter was established through a Silver wire placed into the electrodes. When these electrodes are not useable, they are maintained at 4 (°C).

2.4.2.2 Enzyme Preparation and Biosensor Production

By oxidation of Glucose (Dextrose), Fructose and Galactose with Glucose (Dextrose), Fructose and Galactose–oxidase enzyme and production of Glucuronic Acid, pH of the reaction environment is reduced. To prohibit severely variations of pH and maintaining the activity of enzyme, a specified amount of it is placed into Phosphate buffer. To prepare 0.1 (M) Phosphate buffer, K_2HPO_4 and KH_2PO_4 are used and its pH is set with H_3PO_4 and NaOH. Separately, 10 (mg) of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme was added to 1 (ml) of 0.1 (M) Phosphate buffer (PBS) in

semi–permeable membranes and it is fastened around each CPE electrode and (Cf_{nano}/CPE). Then, the produced biosensors are placed into Glucose (Dextrose), Fructose and Galactose solution with concentration range of 0–1 (mM) for currentmetery.

3 Currentmetery

Currentmetery of biosensors is performed separately through standard (Ag/AgCl), CPE and (Cf_{nano}/CPE) electrodes in room temperature. Glucose (Dextrose), Fructose and Galactose solution with specified concentrations was prepared for currentmetery of biosensors and the produced current was measured by ammeter by applying the same potential 0.7 (V) to both biosensors.

It can be shown that the currentmetery of biosensor in terms of Glucose (Dextrose), Fructose and Galactose concentration with different performances of CPE and (Cf_{nano}/CPE) electrodes. The curves show that the concentration of substrate (Glucose (Dextrose), Fructose and Galactose) affects the activity of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme (Figures 1–3). In very low concentrations of substrate, not all active locations of enzyme fill with substrate and the activity of enzyme would be low. By gradual increasing the concentration of substrate, enzyme activity increases until reaching to a specified concentration in which all active locations of enzyme fill with substrate and enzyme activity reaches to its maximum amount under operational condition. The maximum current response of CPE and (Cf_{nano}/CPE) electrodes in Phosphate buffer with pH=4 is 0.4 and 1.2 (µA), respectively, which show different performance of two electrodes in similar potential and pH. Currentmetery of biosensor in terms of the concentration of Glucose (Dextrose), Fructose and Galactose are shown for two different performances of CPE and (Cf_{nano}/CPE) electrodes.

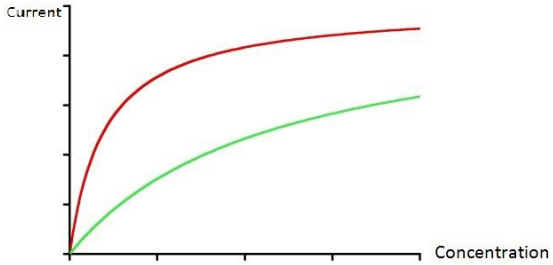

Figure 1. The currentmetery of biosensor in terms of Glucose (Dextrose) concentration in Phosphate buffer with pH=4 with different performances of (a) CPE (green curve) and (Cf_{nano}/CPE) (red curve) electrodes. It should be noted that y–axis shows current (µA) and also x–axis shows concentration (mM), respectively.

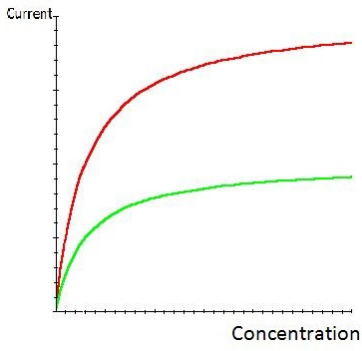

Figure 2. The currentmetery of biosensor in terms of Fructose concentration in Phosphate buffer with pH=4 with different performances of (a) CPE (green curve) and (Cf_{nano}/CPE) (red curve) electrodes. It should be noted that y–axis shows current (µA) and also x–axis shows concentration (mM), respectively.

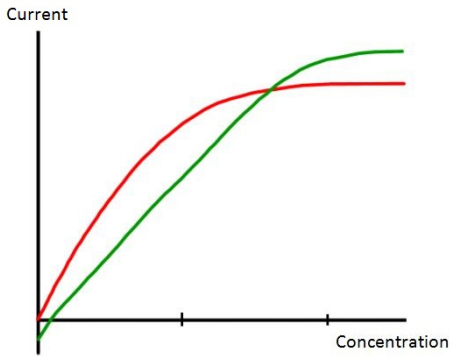

Figure 3. The currentmetery of biosensor in terms of Galactose concentration in Phosphate buffer with pH=4 with different performances of (a) CPE (green curve) and (Cf_{nano}/CPE) (red curve) electrodes. It should be noted that y–axis shows current (µA) and also x–axis shows concentration (mM), respectively.

In addition, the maximum current response of CPE and (Cf_{nano}/CPE) electrodes in Phosphate buffer with pH=6 is 0.7 and 1.58 (µA), respectively, which show considerably different performance of two electrodes in similar potential and pH.

Also, currentmetery of biosensor in terms of the concentration of Glucose (Dextrose), Fructose and Galactose are shown for two different performances of CPE and (Cf_{nano}/CPE) electrodes. The maximum current response of CPE and (Cf_{nano}/CPE) electrodes in Phosphate buffer with pH=8 is 0.6 and 1.1 (µA), respectively. It means that Carbon electrode modified with Californium colloidal nanoparticles prepares appropriate environment for direct movement of electrons due to high electrocatalystic activity of nanoparticles, hence, leads to facilitation of electron movement, higher currents, and finally, better tracing of Glucose (Dextrose), Fructose and Galactose due to higher conductivity. By evaluating these currentmeteries, the positive effect of Californium colloidal nanoparticles is confirmed and (Cf_{nano}/CPE) electrode is selected as the appropriate electrode.

It can be seen that maximum current is achieved in pH=6 and increasing the concentration of Glucose (Dextrose), Fructose and Galactose leads to producing higher ampere. It indicates that optimum condition is achieved in which, current is maximum and the amount of detected Glucose (Dextrose), Fructose and Galactose are maximum.

4 Conductive Polymers

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

This characteristic causes that these polymers produce and use in various forms. In addition, these polymers are flexible due to their plastic nature. Further, their electric conductivity can be set.

The reason for their electric conductivity is their coupled bond structure. It means that the presented molecules in π bonds of these structures can be moved along the polymerization chain and conduct the electric current. Among conductive polymers, Polyaniline (PANI) has been more interesting for researchers than others due to its high electric conductivity, simple production process, good environmental stability and unique chemical nature (oxidization/reduction). The previously performed studies in recent years about producing the Polyaniline (PANI) in Nano scale causes to improving the most interesting property of this polymer, namely electric conductivity. In addition, increasing the effective area of polymer leads to improvement of its processability and hence, application of this polymer increases in various sciences, especially food industries. Up to now, various types of Polyaniline (PANI) nanostructures have been produced such as nanoparticles, nanofibers, nanotubes, nanowires, nanorods and nanobolts.

The above mentioned properties of conductive polymer nanostructures causes that these materials considering as one of the most interesting options for the stabilizing matrix of biodetector element on

the transducer of biosensor. Among the produced nanostructures from Polyaniline (PANI), nanoparticles, nanofibers and nanotubes of this material have been used in biosensors.

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.

5 Biosensors

As previously mentioned, biosensors are a type of sensors which have an amazing, unique performance in the presence of intervening factors. Stabilizing each biooperator on the transducer of biosensor 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 converts to an electric signal by transducer and after processing, it illustrates.

5.1 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 have not blocked and their geometrical form has not changed. In addition, it is necessary to have a relationship between biodetector element and sensitive area of transducer.

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 function groups of detector element such as SH, OH, COOH and NH_2, which are not necessary for biological reaction.

Adsorption 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, adsorption is very simple since it has no need to any other reagent and failure of 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 absorbs by the surface through electric induction. Conductive polymer nanostructures can be used as stabilizer of biodetector elements due to their electric conductivity, compatibility with live materials, environmental stability and high contact surface.

6 Cyclic Voltammetry

Cyclic voltammetry is an electrochemical method for detecting the presence of operational electroactive components, which are able to exchange electron 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 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 is including transducer and polymer cover containing detector element.

After placing these three electrodes in the solution, a potential ranges between two specified values cyclically apply to working and standard electrodes and the currents obtained from working and auxiliary electrodes illustrate in terms of the applied potential. Therefore, a current against potential curve can be obtained. In this graph, current increases when potential reaches to a value in which an electroactive type can be oxidized and hence, a peak emerges in cyclic voltammetry graph (Figure 4).

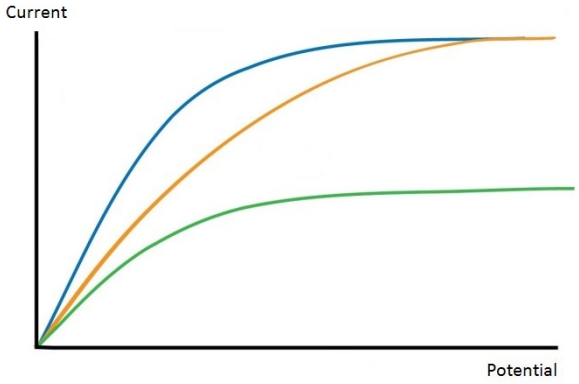

Figure 4. A current (μA) (y–axis) against potential (V) (x–axis) curve for three electrodes (a) Silver/chloride standard electrode (blue curve), (b) Platinum electrode (red curve) and (c) polymer electrode (green curve) in the solution. It should be noted that current increases when potential reaches to a value in which an electroactive type can be oxidized and hence, a peak emerges in cyclic voltammetry graph.

When potential scanning applied in 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 own specific potential (oxidization–reduction), analyte can be detected through this method. As a result, cyclic voltammetry method is used as the most important test for performance of transducer and sensor zone of biosensors.

7 Glucose (Dextrose), Fructose and Galactose Detection

In biosensors used for detecting Glucose (Dextrose), Fructose and Galactose, the reactions performed on the bioelectrode (working electrode).

Alireza Heidari *et al.* were investigated the biosensors that are used for detecting Glucose (Dextrose), Fructose and Galactose based on Glucose (Dextrose), Fructose and Galactose–oxidase stabilizing in Polyaniline (PANI) nanowires. In this method, Aniline monomers are firstly polymerized in a product containing Ammonium Peroxide Disulphate as oxidizer and then, they are precipitated on a Carbon electrode as a film. When Polyaniline (PANI) particles are growing up, polymerization of Aniline continues by applying a potential. Then, Polyaniline (PANI) nanowires are produced by applying a constant current to Carbon electrode. Afterwards, this electrode places in a Phosphate buffer solution (pH=7.2) and Polyaniline (PANI) nanowires reduce by applying a constant potential. This causes that anions removes from the surface of Polyaniline (PANI) and the appropriate conditions for surface absorbing of Glucose (Dextrose), Fructose and Galactose–oxidase prepares. Then, electrode is placed in a Phosphate buffer solution containing 2.5 (mg/L) Glucose (Dextrose), Fructose and Galactose–oxidase and by applying 0.25 (V), positive charge induces on Polyaniline (PANI) nanowires. This positive charge causes to electrostatic absorption of Glucose (Dextrose), Fructose and Galactose–oxidase induced from negative surface charges.

In order to investigate the performance of the produced electrode, cyclic voltammetry technique is used. The produced electrode was placed in solutions with and without Glucose (Dextrose), Fructose and Galactose. The resulted voltammetry graph shows that when Glucose (Dextrose), Fructose and Galactose–oxidase is in stable state, the related oxide peaks are emerged while when there is not enzyme, oxide peaks are not emerged. This observation is performed for evaluating the correct performance of biosensor.

Another study was performed by Alireza Heidari *et al.* about the stabilizing of Glucose (Dextrose), Fructose and Galactose–oxidase with Polyaniline (PANI) nanotubes. In this study, a membrane of Aluminum 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 (PANI) nanotubes.

Firstly, one side of Aluminum anodic oxidation was covered with 10 (nm) thick Platinum layers through evaporation in vacuum. Then, a Copper wire was connected to Platinum for conducting electric

current. After isolating the Copper wire and backside of Platinum for prohibiting their incident 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 monomer. By applying scanning potential, Aniline polymerization and Polyaniline (PANI) precipitation in holes of Aluminum anodic oxidation were performed and Polyaniline (PANI) nanotubes were produced.

In order to stabilize Glucose (Dextrose), Fructose and Galactose–oxidase, Polyaniline (PANI) nanotubes were reduced in Phosphate buffer solution through applying 250 (mV) so that the anions around the Polyaniline (PANI) nanotubes were removed. Then, Polyaniline (PANI) nanotubes were oxidized in Phosphate buffer solution containing 7 (mg/L) Glucose (Dextrose), Fructose and Galactose–oxidase under 750 (mV) to electrostatically absorb Glucose (Dextrose), Fructose and Galactose–oxidase. During oxidation, Glucose (Dextrose), Fructose and Galactose–oxidase which is of negative charge absorbs in inner wall of Polyaniline (PANI) nanotubes and traps. Then, electrode was washed by distilled water to remove enzymes that are not appropriately absorbed by inner wall of nanotubes.

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

8 Cholesterol Detection

In biosensors used for detecting Cholesterol, the reactions performed on the bioelectrode.

Alireza Heidari *et al.* were used Polyaniline (PANI) nanotubes for stabilizing lipase enzyme in biosensors used for detecting Cholesterol. In this method, as can be seen, Polyaniline (PANI) 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 (GLU) is that this compound is of carbonyl group in both ends. It can bond with Nitrogen of Polyaniline (PANI) film on the surface of (Indium–Tin) oxide from one end and connect to Nitrogen groups of lipase at the other end. Therefore, lipase stabilizes on the Polyaniline (PANI) film, covalencely. After connecting Glutaraldehyde (GLU) to the film surface, electrode dries and enters to lipase solution and after sometime, electrode dries.

In this investigation, the performance of the produced electrode was evaluated by cyclic voltammetry. In this study, three types of electrode, ((PANI–NT)/ITO) (electrode covered with carbon nanotubes without Glutaraldehyde (GLU) 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 reaction of lipase and Cholesterol available in sample container for electrode and disappearing them in two other states confirms the correct performance of electrode.

In a similar study, Alireza Heidari *et al.* were produced biosensors for detecting Cholesterol based on Polyaniline (PANI) film covered on electrode of (Indium–Tin) oxide by electrochemical method.

In this study, Polyaniline (PANI) was firstly covered on electrode of (Indium–Tin) oxide by electrochemical polymerization and then, all steps performed by Alireza Heidari *et al.* were repeated.

In another investigation, Alireza Heidari *et al.* were produced Cholesterol biosensor through simultaneous stabilizing the Cholesterol–oxidase and Cholesterol–esterase on Polyaniline (PANI) film. In this method, Polyaniline (PANI) was firstly covered on electrode of (Indium–Tin) oxide through electrochemical polymerization. Then, 1% Glutaraldehyde (GLU) was distributed over Polyaniline (PANI) film and allows that surface to be dried. Then, Cholesterol–esterase was distributed over dried surface of electrode and after it became dry, Cholesterol–oxidase was distributed and dried. Then, this (enzyme–polymeric) set was washed by distilled water for removing oligomers and not stabilized enzymes. The results obtained from cyclic voltammetry confirm the presence of Cholesterol and Cholesterol oleate.

9 Results and Discussion

After completing experimental steps, nanomedium 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 beginning of stabilization step is necessary for protecting the performance of enzyme.

Regarding the relatively large size of Glucose (Dextrose), Fructose and Galactose–oxidase enzyme, this enzyme should be connected to outer wall of Nano Endohedral Fullerenes (Endofullerenes) using a connective material such as 1–Pyrenebutanoic Acid Succinimidyl Ester (PASE).

Regarding the fact that medium is 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 is very low and it can be assuredly said that the measured activity of about 280 (u/mg) at initial times is only due to stabilized enzymes and it confirms 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 (Figures 1–4).

Variation of activity of stabilized enzyme against time is not considerably changed compared to the activity of free enzyme. It confirms the stability of 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 Nano Endohedral Fullerenes (Endofullerenes) compared to other metals, they are appropriate for stabilizing various chemical and biological detectors and utilizing them in manufacturing the biosensors leads to increasing the efficiency and sensitivity and decreasing the resistivity in performance (Figures 1–4).

In the current paper, Glucose (Dextrose), Fructose and Galactose–oxidase biosensor is experimentally manufactured using Nano Endohedral Fullerenes (Endofullerenes) based on the investigations performed about various previously performed researches in this field. Although these tests are performed similar to experimental studies in some steps, obtaining new results such as temporary inactivating the enzyme and using Californium plate, alone, in macro plate form as the medium are the differences of the current research with similar researches (Figures 1–4).

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 the current paper 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 (Figures 1–4).

Furthermore, Detecting Glucose (Dextrose), Fructose, Galactose 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 to skillful operator and are time consuming. To solve this problem, one type of chemical sensors called as biosensors can be used that are of unique performances. 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 (Figures 1–4).

10 Conclusions, Perspectives, Useful Suggestions and Future Studies

Evaluations of the performance of biosensors with two different electrodes show that enzyme activity is low in very low concentrations of Glucose (Dextrose), Fructose and Galactose for both biosensors. Then, by gradually increasing of Glucose (Dextrose), Fructose and Galactose concentration, enzyme activity increases until reaching to a specified concentration in which all active locations of enzyme fill with substrate and enzyme activity reaches to its maximum amount. The results obtained from currentmetery of biosensors show that by increasing the concentration of Glucose (Dextrose), Fructose and Galactose and after reaching it to a specified amount, enzyme activity reaches to a stage in which, its rate does not change with the increase of the Glucose (Dextrose), Fructose and Galactose concentration and remains constant. This phenomenon happens when all active locations of enzyme are occupied and there is not enough active space for oxidizing the available Glucose (Dextrose), Fructose and Galactose. To maintain the activity of active points of Glucose (Dextrose), Fructose and Galactose–

oxidase enzyme, enzyme is located in Phosphate buffer so that pH of reaction environment controls using buffer and enzyme activity maintains during oxidization. Investigation of the performance of biosensors in various pH values of Phosphate buffer show that enzyme activity and currentmetery of both biosensors in Phosphate buffer with pH=6 are increased. This increasing in the activity is due to creation of an appropriate ionic state for substrate and or Glucose (Dextrose), Fructose and Galactose–oxidase enzyme. Oxidation and reduction groups in Glucose (Dextrose), Fructose and Galactose–oxidase enzyme, which are oxidized and reduced, deeply deposit in a hole and so, they are not easily available for guiding electrons towards electrode surface and this is against the performance of enzyme. Therefore, in establishing the current, it is necessary to prepare satisfactory electric relationship between active locations of enzyme (FAD) and surface of electrode. To solve this problem, Californium colloidal nanoparticles are used in the structure of (Cf_{nano}/CPE) electrode. It leads to increasing the conductivity, facilitating the movement of electrons and increasing the satisfactory electric relationship between electrode surface and active locations of enzyme (FAD). Modification of (Cf_{nano}/CPE) electrode with Californium colloidal nanoparticles leads to creation of a microenvironment like (oxidization–reduction) of proteins, which is due to high electrocatalystic activity of nanoparticles. It means that the isolation effect in protein cortex of enzyme is reduced and electron exchange is increased. Another reason for using Californium colloidal nanoparticles in the structure of (Cf_{nano}/CPE) electrode is its high sensitivity and selection ability, increasing the electric conductivity and permeability between biological fences (enzyme–electrode) and it considerably improves Glucose (Dextrose), Fructose and Galactose tracing by biosensors.

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