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Novel paper-based cholesterol biosensor using graphene/polyvinylpyrrolidone/polyaniline nanocomposite

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ABSTRACT

A novel nanocomposite of graphene (G), polyvinylpyrrolidone (PVP) and polyaniline (PANI) has been successfully prepared and used for the modification of paper-based biosensors via electrospraying. The droplet-like nanostructures of G/PVP/PANI-modified electrodes are obtained with an average size of 160 ± 1.02 nm. Interestingly, the presence of small amount of PVP (2 mg mL^{-1}) in the nanocomposites can substantially improve the dispersibility of G and increase the electrochemical conductivity of electrodes, leading to enhanced sensitivity of the biosensor. The well-defined cyclic voltammogram of standard ferri/ferrocyanide is achieved on a G/PVP/PANI-modified electrode with a 3-fold increase in the current signal compared to an unmodified electrode. This modified electrode also exhibits excellent electrocatalytic activity towards the oxidation of hydrogen peroxide (H_2O_2). Furthermore, cholesterol oxidase (ChOx) is attached to G/PVP/PANI-modified electrode for the amperometric determination of cholesterol. Under optimum conditions, a linear range of 50 μM to 10 mM is achieved and the limit of detection is found to be 1 μM for cholesterol. Finally, the proposed system can be applied for the determination of cholesterol in a complex biological fluid (*i.e.* human serum).

1. Introduction

The development of accurate, sensitive and low-cost biosensor is crucial for early stage screening of disease biomarkers. Recently, cellulose filter paper has become an attractive material for sensor applications due to its large surface area and low cost (Apilux et al., 2010; Dungchai et al., 2011; Songjaroen et al., 2011). Compared to other traditional substrates (*i.e.,* glass, ceramic and polymer), paper-based biosensors offers several advantages, such as low cost, high abundance, biocompatibility and disposability. Furthermore paper-based analysis only requires a small amount of samples and reagents, which make it suitable for biosensor applications (Dungchai et al., 2009). Among the detection techniques, electrochemical detection has attracted much attention due to its ease of use, portable field-based size, high specificity and rapid analysis. Moreover, both qualitative and quantitative information can be obtained simultaneously. Paper-based electrochemical biosensors have been applied to various applications, including clinical diagnosis (Dungchai et al., 2009, 2011), environmental monitoring (Apilux et al., 2010; Nie et al., 2010), and food quality control (Hossain et al., 2009). Paper-based biosensors can be fabricated by several methods, such as photolithography (Apilux et al., 2010; Dungchai et al., 2009), wax screen-printing (Dungchai et al., 2011), wax-dipping (Songjaroen et al., 2011), and wax-printing (Lu et al., 2010; Mentele et al., 2012). In this study, wax-printing is selected to create the disposable paper-based biosensors. Nowadays, an important limitation of paper-based electrochemical biosensors for the detection of low abundant biomarkers is the limited sensitivity; therefore, modification of paper based biosensor with the ultrahigh surface area material, such as metallic nanoparticles and carbon based nanostructures, is still greatly required to improve the sensor sensitivity.

In recent years, graphene (G) has received tremendous attention due to its remarkable physical, chemical, mechanical, and electrical properties. G is a single layer of carbon atoms closely

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packed into a two-dimensional honeycomb arrangement. For fabrication of G-based electronic material, it can provide excellent electrical conductivity and electron mobility. In addition, the small band gap of G is desirable for conducting electrons from target molecules in electrochemical biosensors. The ultrahigh surface area of G is also very useful for loading of bioreceptors (*i.e.,* enzyme, antibody) on its surface. It has been reported that G-based chemical sensors possess very high sensitivity because of the low electronic noise from thermal effect (Geim and Novoselov, 2007; Liu et al., 2012b; Potts et al., 2011). Compared to other carbon allotropes (*e.g.* carbon nanotubes: CNTs), G can be obtained easily by using a chemical conversion of inexpensive graphite (Geim and Novoselov, 2007).

Due to the planar sp^2-carbon of G, it has high tendency to agglomerate together via van der Waals attraction. In terms of applications, preparation of a well-dispersed G solution has become a crucial step. Recently, it has been reported that poly (vinylpyrrolidone) (PVP) can be used to stabilize the high concentration of G in a wide range of organic solvents (Wajid et al., 2012). G/PVP has been used to modify the electrodes for biosensor applications (Liu et al., 2012b; Mano and Heller, 2005). For electrode modification, the nanocomposites between G and conducting polymers have attracted more attention than the pure form of G because the composites are more compatible for electrode fabrication and biofunctionalization (Arya et al., 2011; Huang et al., 2011; Liu et al., 2012a; Qiu et al., 2012). Moreover, using conducting polymers as a matrix for G dispersion can further enhance the sensitivity of electrochemical biosensors. Various conducting polymers, including polyaniline (PANI) (Arya et al., 2011; Huang et al., 2011; Liu et al., 2012b; Qiu et al., 2012), polypyrrole (PPy) (Li et al., 2012; Lu et al., 2012), and poly(3,4-ethylenedioxythiophene) (PEDOT) (Jiang et al., 2013; Karuwan et al., 2012) have been used in biosensors. Among the conducting polymers, PANI is a promising material due to its excellent electrochemical properties, ease of synthesis and functionalization, high environmental stability, and low toxicity. Previously, it has been reported that the conducting form of PANI can be simply prepared by doping PANI with an acid, such as camphorsulfonic acid (CSA) in chloroform (Shin and Kameoka, 2012). Additionally, a number of amino groups ($-NH_2$) of PANI can be readily functionalized with biomolecules (Arya et al., 2011; Qiu et al., 2012), which make it very attractive for biosensor applications. Thus, the development of G/PVP/PANI nanocomposite modified paper-based biosensor is focused in this study.

To fabricate G/PVP/PANI nanocomposites on the paper-based biosensor, electrospraying is selected because a 3D droplet-like nanostructure can be created on the modified electrode surface. Compared to thin-film modified electrodes, G/PVP/PANI-nanodroplet-modified electrodes offer a higher specific surface area, which leads to an enhanced electrochemical sensitivity of the biosensors. Increasing the electrode surface area through this method might be very useful for further biofunctionalization, such as enzyme loading.

Biomarkers are biomolecules that can indicate a normal or pathogenic process in a biological system, including the level of exposure to environmental factors, genetic susceptibility, and an indication of response to therapy. One of the most important biomarkers for cardiovascular disease and high blood pressure is cholesterol; therefore, development of a method that can quantitatively determine the cholesterol level is very crucial. The conventional method based on spectrophotometry has been widely used for cholesterol determination (Arya et al., 2007; Dhand et al., 2007); however, this technique requires an expensive instrument and complicated sample preparation. With the advent of nanomaterials, metal nanoparticles and carbon-based nanomaterials have been applied for electrochemical biosensors to improve the electrochemical performance of cholesterol biosensors (Dey and Raj, 2010; Dhand et al., 2007; Eguilaz et al., 2011; Manjunatha et al., 2012).

Herein, a novel nanocomposite of G/PVP/PANI was prepared and used to modify the working electrode of a paper-based biosensor via electrospraying. The droplet-like structures of the G/PVP/PANI-nanocomposite-modified electrode was used for the sensitive determination of H_2O_2 and cholesterol using amperometry. The performance of this sensing system was optimized and then applied to the determination of cholesterol concentration in a complex biological fluid (*e.g.,* human serum).

2. Material and methods

2.1. Chemicals and materials

Graphene (G) nanopowders were purchased from SkySpring Nanomaterials, Inc. (Houston, TX). Cholesterol and 418 U mg^{-1} cholesterol oxidase (ChOx) from *Streptomyces* sp., sodium dodecyl sulfate (SDS), polyoxyethylene octyl phenyl ether (Triton X-100), camphor-10-sulfonic acid (CSA), polyaniline (PANI), polystyrene (Mw ~ 180,000; PS), poly (vinyl pyrrolidone) (Mw = 10,000; PVP) and trichloroacetic acid (TCA) were obtained from Sigma (St. Louis, MO). Dimethylformamide (DMF), Potassium dihydrogen phosphate (KH_2PO_4) and chloroform were purchased from Carlo Erba Reagenti-SDS (Val de Reuil, France). Disodium hydrogen phosphate (Na_2HPO_4), Potassium chloride (KCl), and Sodium chloride (NaCl) were purchased from Merck (Darmstadi, Germany). Carbon ink and silver/silver chloride ink were obtained from Gwent group (Torfaen, United Kingdom). Filter paper grade no. 1 (size, 46 × 57 cm^2) was purchased from Whatman. All chemicals were used as received without further purification. All solutions were prepared by using high-purity water from MilliQ Water System (Millipore, USA, $R \geq 18.2$ MΩ cm^{-1}). Phosphate buffered saline (PBS) was prepared by dissolving 0.144% (w/v) Na_2HPO_3, 0.024% (w/v) KH_2PO_4, 0.02% (w/v) KCl, 0.8% (w/v) NaCl in high-purity water. A stock solution of cholesterol was prepared in 5% (w/v) of Triton X-100 and high-purity water and then stored at 4 °C. A stock solution of ChOx was freshly prepared in PBS (Ruecha et al., 2011). For the determination of the cholesterol in a real biological sample, lyophilized human serum (CONSERA), obtained from Nissui Pharmaceutical, was used (Tokyo, Japan). The serum samples were precipitated using TCA prior to use.

2.2. Apparatus

All electrochemical measurements, including cyclic voltammetry and amperometry, were performed on a CHI 1232A electrochemical analyzer (CH Instruments, Inc., USA). A three electrode system was used and the working electrode was a G/PVP/PANI-modified, screen-printed carbon electrode (4 mm in diameter). An in-house electrospraying system was used for the electrode modification. A JSM-6400 field emission scanning electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan) with an accelerating voltage of 15 kV and a JEM-2100 transmission electron microscope (Japan Electron Optics Laboratory Co., Ltd, Japan) were used for the electrode characterization.

2.3. Fabrication of paper-based biosensor

In this work, paper-based biosensor was fabricated using wax-printing method according to a previous report (Mentele et al., 2012) with slight modification. First, the patterned paper-based biosensor was designed by Adobe Illustrator and then printed onto filter paper (Whatman no. 1) using a wax printer (Xerox Color Qube 8570, Japan). Next, the printed paper-based biosensor was

placed on a hot plate at 175 °C for 40 s to melt the wax. The area covered with wax was hydrophobic, and the area without wax was hydrophilic. The block screen was designed with Adobe Illustrator software and fabricated by Chaiyaboon Co. (Bangkok, Thailand). For three electrode system of the paper-based biosensor, a working electrode (WE) and a counter electrode (CE) were screen-printed in-house using carbon ink. Silver/silver chloride (Ag/AgCl) ink was used as a reference electrode (RE) and conductive pad.

2.4. Electrospraying fabrication of G/PVP/PANI nanocomposites modified paper based biosensor

Firstly, PANI was doped with CSA to generate a conductive form of PANI and dissolved in chloroform (Shin and Kameoka, 2012). Then the stock solution of PVP was prepared by dissolving of 2 mg mL^{-1} of PVP in DMF and stirring for 10 min at room temperature. G dispersion was prepared by the adding 2 mg mL^{-1} of G into the stock solution of PVP and sonicating for 6 h at room temperature. After that, the solutions of PANI and G/PVP were mixed together, and 0.1% (v/v) PS was added into G/PVP/PANI solution. An electrospraying system consists of syringe pump, high-voltage power supply, ground collector, syringe and stainless-steel needle. During electrospraying, the CE and RE, were covered with masks to prevent the electrode modification. The G/PVP/PANI nanocomposite solution was mixed thoroughly in a syringe, and a high voltage was applied to the solution. The nanocomposite solution was electrosprayed onto the WE of a paper-based biosensor attached to a ground collector on a rotating drum. The flow rate was controlled at 1.0 mL h^{-1}, the distance between the needle tip and ground collector was fixed at 5 cm, and the applied high voltage was 6 kV.

2.5. Electrochemical measurement

A three-electrode system fabricated on a paper-based biosensor was used throughout the experiment. To control all electroanalytical measurements, a potentiostat (CHI 1207A, CH Instruments, Austin, TX) was used. For the cyclic voltammetric measurements, the potential was scanned from −0.3 V to +0.8 V for ferri/ferrocyanide detection and +0.2 V to +1.0 V for H_2O_2 detection. Hydrodynamic voltammogram was employed to optimize the detection potential for H_2O_2 in the range of +0.2 to +0.8 V. The standard solution of H_2O_2 was dropped in the paper-based device and the current measured at a fixed time with different potentials. For the cholesterol detection, the volume of 418 U mL^{-1} ChOx enzyme was investigated between 0.1 and 1.0 µL. Prior to the detection of cholesterol, an optimum volume of ChOx enzyme was drop-cast and dried onto the surface of the G/PVP/PANI-modified electrode, and amperometry was performed at an optimum detection potential. After that, the anodic current was recorded at the steady state current for 100 s.

2.6. Preparation of Human serum

Prior to analysis, 3.0 mL of high purity water was added to the lyophilized human serum, and then the proteins in the serum were precipitated using TCA method (Rattanarat et al., 2012). For the protein precipitation, 200 µL of human serum, 50 µL of standard cholesterol at different concentration, and 250 µL of 10% (w/v) TCA were mixed together by vortexing for 5 min. The mixed solution was centrifuged at 6000 rpm (Cole-Parmer, USA) for 10 min and the supernatants were kept for further analyses. All samples were analyzed on paper-based sensors using amperometry.

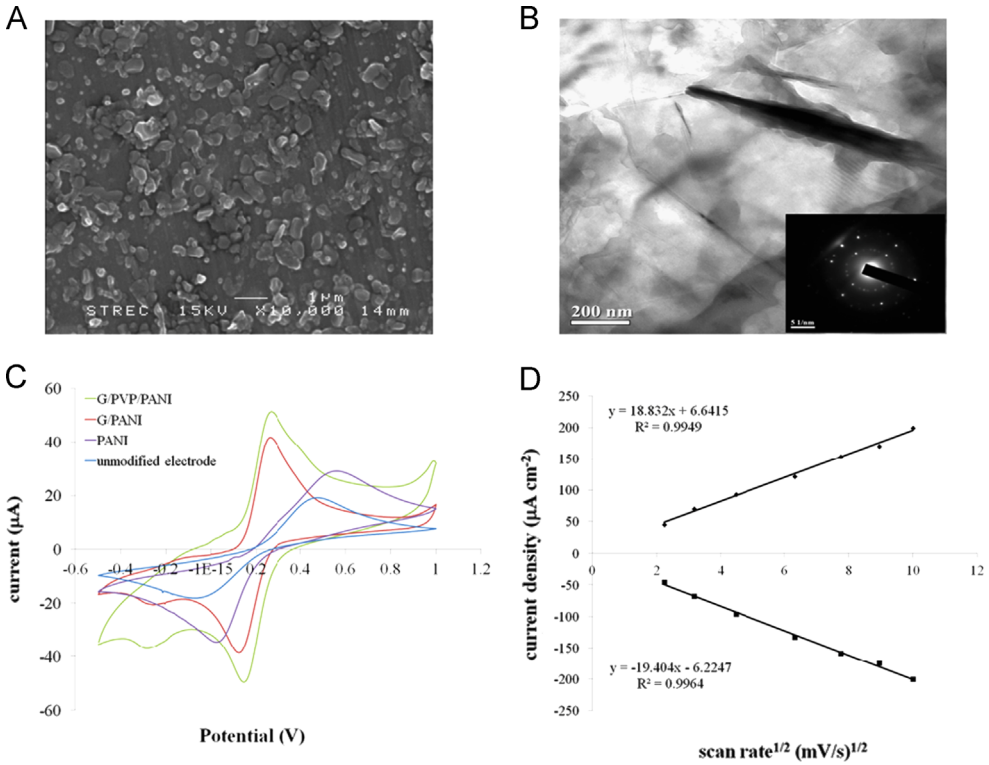


Fig. 1. Physical and electrochemical characterization of G/PVP/PANI nanocomposite-modified electrodes, (A) SEM image of G/PVP/PANI modified electrode (6 kV applied voltage), (B) TEM image of G/PVP/PANI with the electron diffraction pattern of G (inset of Fig. 1B), (C) cyclic voltammograms of 2.0 mM ferri/ferrocyanide in 0.1 M KCl measured on different working electrodes of the paper-based biosensor with a scan rate of 100 mV s^{-1} and (D) the relationship between the square root of scan rate ($\nu^{1/2}$) and peak current measured from 5 and 100 mV s^{-1}. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Characterization of G/PVP/PANI nanocomposites modified paper-based biosensor

Other than the high surface area of G, electrospraying was selected for electrode modification to further increase the surface area of the working electrode. The morphology of the G/PVP/PANI-modified electrode and unmodified electrode on a paper substrate were characterized using scanning electron microscopy (SEM) as shown in Fig. 1A and Fig. S1 (Supporting information), respectively. Interestingly, the 3D droplet-like structures of G/PVP/PANI can be uniformly generated on the modified electrode surface; the average size of each droplet was found to be 160 ± 1.02 nm, as shown in Fig. 1A. Moreover, TEM image of G/PVP/PANI indicate a good dispersion of G inside the nanocomposites without severe aggregation (Fig. 1B), and the electron diffraction pattern of G (inset of Fig. 1B) is matched very well with the previous report (Karuwan et al., 2012). TEM images of G/PVP and G/PANI were shown in Fig. S2 (Supporting information).

For electrospraying, the effect of applied voltage, spraying time, and G/PANI ratio on the electrochemical conductivity of modified electrodes were investigated and optimized. SEM images of G/PVP/PANI modified electrode and anodic current response of 2 mM ferri/ferrocyanide measured on the electrodes prepared by using different applied voltage was shown in Fig. S3 (Supporting information). In this study, 6 kV of applied voltage, 5 min of spraying time, and 1:1 of G/PANI ratio were selected for further experiments. To prepare the nanocomposite of G/polymer, other than PANI conducting media, PVP and PS were selected as graphene stabilizer and carrier polymer for electrospraying, respectively. To investigate the electron transfer process, cyclic voltammetry was performed on different electrodes including G/PVP/PANI, G/PANI, PANI and unmodified carbon electrodes using ferri/ferrocyanide as a redox probe. As shown in Fig. 1C, the anodic and cathodic peak currents of ferri/ferrocyanide show the well-defined peaks for all electrodes. The highest anodic and cathodic peak currents of ferri/ferrocyanide were observed on G/PVP/PANI modified electrode (green line) indicating the high sensitivity of the system. The peak currents gradually decrease for G/PANI, PANI and unmodified carbon electrode, respectively. Compared to PANI modified electrode (purple line), incorporation of G into the nanocomposites can increase both anodic and canodic peak currents of ferri/ferrocyanide (red line). Interestingly, the peak-to-peak potential separation (ΔEp) of ferri/ferrocyanide measured on G/PANI modified electrode ($\Delta Ep = 0.155$; red line) significantly decreases when compared to ΔEp obtained from PANI modified electrode ($\Delta Ep = 0.508$; purple line), indicating that the presence of G in the nanocomposites can accelerate the electron transfer kinetics of the system. Moreover, it can be noticed that using PVP as a stabilizer for G dispersion can further increase the anodic and cathodic peak currents in this sensing system as shown in Fig. 1C (green line *vs.* red line). Another important advantage of using PVP is decreasing of dispersion time of G from 24 h to 6 h (Wajid et al., 2012). The results of graphene dispersion in N,N-dimethylformamide (DMF) with and without PVP are shown in Fig. S4 (Supporting information). In general, without using PVP, the long dispersion time (24 h) was required for G dispersion to prevent its reaggregation to graphite form; therefore, 2 mg mL^{-1} of PVP in DMF was used as a stabilizer for G dispersion in all further experiments.

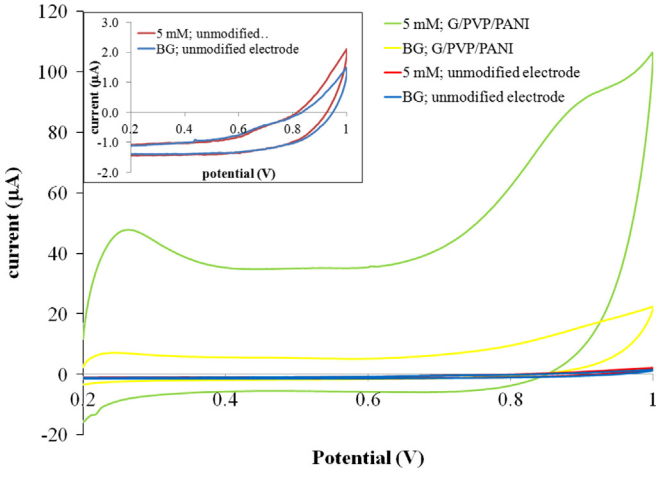


Fig. 2. Cyclic voltammograms of 5 mM hydrogen peroxide (H_2O_2) in 0.1 M PBS pH 7.0 measured on a G/PVP/PANI-modified electrode and unmodified carbon electrode at a scan rate of 100 mV s^{-1}. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Prior to the analysis of hydrogen peroxide and cholesterol, the electrochemical behavior of the G/PVP/PANI-modified paper-based biosensor was examined using standard ferri/ferrocyanide as a redox probe. 70 μL of a 2.0 mM ferri/ferrocyanide solution in 0.1 M KCl was directly dropped onto the modified electrode surface, and the cyclic voltammetric measurements were performed at different scan rates. The relationship between the square root of the scan rate ($\nu^{1/2}$) and the current density was plotted, as shown in Fig. 1D. Both anodic and cathodic peak currents of ferri/ferrocyanide are linearly proportional to the square root of the scan rate in the range from 2 to 100 mV s^{-1}. These results verify that the redox process is controlled by diffusion process.

3.2. Detection of hydrogen peroxide and cholesterol

For an electrochemical biosensor, the determination of the H_2O_2 product obtained from cholesterol oxidation can be used for the indirect quantification of cholesterol (Aravind et al., 2011; Manjunatha et al., 2012). In this study, a novel paper-based biosensor based on a G/PVP/PANI-modified screen-printed carbon electrode was used for the sensitive determination of H_2O_2 and cholesterol using cyclic voltammetry and amperometry. The cyclic voltammograms of H_2O_2, measured on the G/PVP/PANI-modified electrode and unmodified carbon electrode are illustrated in Fig. 2A. A dramatic increase (40 times) in the anodic current signal of H_2O_2 is observed (green line) when compared to an unmodified carbon electrode (red line), indicating that the modified electrode might be a promising tool for sensitive detection of cholesterol. The cyclic voltammograms of cholesterol measured on G/PVP/PANI-modified electrode was shown in Fig. S5 (Supporting information).

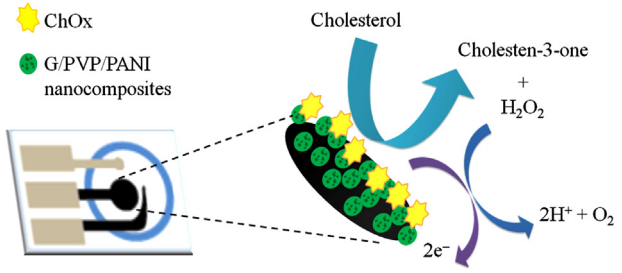
Due to the high sensitivity and wide applicability, chronoamperometry was selected for the electrochemical detection of H_2O_2 and cholesterol. Initially, the detection potential was investigated and optimized as shown in Fig. S6A and B (Supporting information). A hydrodynamic voltammogram of H_2O_2 was optimized as shown in Fig. S6A (Supporting information), the anodic current signal of H_2O_2 significantly increased as the detection potential increased (blue line); however, the background current also increased (green line). Therefore, a hydrodynamic voltammogram of signal-to-background ratios (S/B) was investigated instead of the current signal, as shown in Fig. S6B (Supporting information). The S/B ratio measured at +0.6 V shows the highest sensitivity for H_2O_2; therefore, +0.6 V was selected as the amperometric detection potential for further experiments.

For cholesterol biosensor, H_2O_2 is generated from the enzymatic reaction between cholesterol and ChOx as shown in Scheme 1. Therefore, it is important to optimize the amount of ChOx on G/PVP/PANI modified paper-based biosensor. In this study, different volumes of ChOx were directly dropped on the modified electrodes and dried out at room temperature for 10 min. The effect of enzyme

volume on the anodic current signal of 1 mM cholesterol was studied in the range of 0.1–1.0 μL and the optimum volume of enzyme was found to be 0.4 μL as shown in Fig. S7 (Supporting information).

3.3. Analytical performance of G/PVP/PANI nanocomposites modified paper-based biosensor

G/PVP/PANI-modified paper-based biosensor was used to measure cholesterol at different concentrations, and the amperometric current responses were recorded at a steady state current of 100 s to create a calibration curve for the cholesterol. As shown in Fig. 3, the calibration plot was linearly proportional to the cholesterol concentration over the range of 50 μM to 10 mM with a correlation coefficient of 0.9993. The detection sensitivity of the system, calculated by dividing the slope of linear range curve by the surface area using projection area (Parlak et al., 2013), was found to be 34.77 μA mM^{-1} cm^{-2}. The limit of detection (LOD) and limit of quantitation (LOQ) for cholesterol were 1 μM and 50 μM, respectively. LOD was calculated as the concentrations which produced the signal at 3 times of the standard deviation of a blank ($n=5$) (Dungchai et al., 2009). Previously, it has been reported by the national cholesterol education program (NCEP) that the normal level of total blood cholesterol in human is lower than 5.18 mM (200 mg dL^{-1}) (Ahmadalinezhad and Chen, 2011). This information confirms that our system can be applied for the determination of cholesterol in real biological samples. The electrochemical performance of the G/PVP/PANI-modified electrode was compared to the other modified electrodes used for the detection of cholesterol as shown in Table 1. Our electrode shows a relatively high electrochemical sensitivity, a wide linear range and a comparable LOD for cholesterol detection. Because the G/PVP/PANI-modified paper-based biosensor proposed in this study is easily prepared and inexpensive, it might be a promising tool for cholesterol detection.



Scheme 1. The enzymatic reaction between cholesterol and ChOx on G/PVP/PANI modified paper-based biosensor.

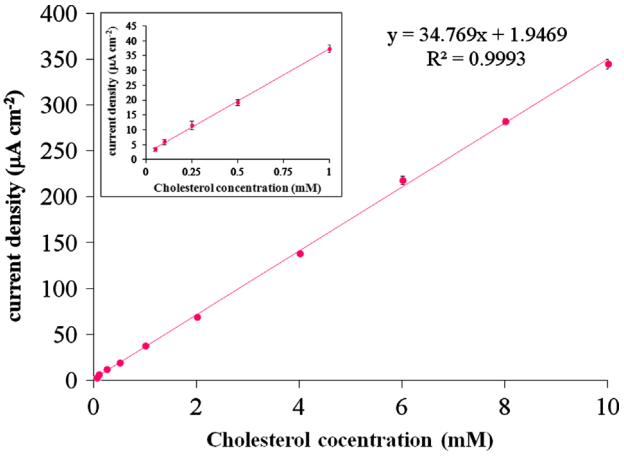


Fig. 3. The calibration graph for the detection of cholesterol over the concentration range of 50 μM to 10 mM and calibration graph between 50 μM and 1 mM (inset) in 0.1 M PBS pH 7.0.

3.4. Interference study

Previously, it has been reported that glucose and ascorbic acid are the common interfering molecules in the detection of cholesterol in complex biological fluids (*e.g.,* human serum). Therefore, selective determination of cholesterol in the presence of glucose and ascorbic acid was investigated using the highest anticipated concentrations of glucose (5.3 mM) and ascorbic acid (80 μM) in human serum (Ahmadalinezhad and Chen, 2011; Dungchai et al., 2009; Rattanarat et al., 2012). As shown in Fig. S8A (Supporting information), the amperometric result of a mixture of cholesterol and glucose shows a negligible effect on the current response (blue bar), while the ascorbic acid has an effect on the current response (green bar). To solve the problem of ascorbic acid interference, an anionic surfactant (SDS) was used to coat on the G/PVP/PANI-modified electrode. Recently, it has been reported that an electrostatic repulsion between anionic SDS and anionic AA at pH 7.4 (pKa=4.1) can prevent the interference effect from AA in the detection of target analytes (Rattanarat et al., 2012).

Table 1
Comparison of various cholesterol biosensors based on nanomaterial/polymer modified electrodes.

Modified electrode	Detection method	Sensing element	Method of enzyme immobilization	LOD (μM)	Linear range (mM)	Sensitivity (μA mM^{-1} cm^{-2})	Reference
$NiFe_2O_4$/CuO/FeO-Ch/ChOx	DPV	ChOx	Physical adsorption	0.0313	0.13–12.95	16.54	Singh et al. (2012)
Ti/NPAu/ChOx–HRP–ChE	CV	ChOx/ChEt	Entrapment	12.95	0.97–7.8	29.33	Ahmadalinezhad and Chen (2011)
AuE/dithiol/AuNPs/MUA/ ChOx	CV	ChOx	Covalent attachment	34.6	0.04–0.22	45.96	Saxena et al. (2011)
ChOx-FG/G	Amperometry	ChOx	Covalent attachment	5	0.05–0.35	–	Manjunatha et al. (2012)
AuPt–Ch–IL/GCE	Amperometry	ChOx	Cross-linking	10	0.05–6.2 and 6.2–11.2	90.7	Safavi and Farjami (2011)
AuNPs/f-G modified GCE	Amperometry	ChOx	Physical adsorption	–	0–0.135	314	Aravind et al., (2011)
CSNF–AuNPs/ChOx	Amperometry	ChOx	Physical adsorption	0.5	0.001–0.045	1.02	Gomathi et al. (2011)
ChOx/HRP/AuNPs/PDDA/ MWCNTs/GCE	Amperometry	ChOx	Physical adsorption	2.2	0.01–1.05	18.6	Eguilaz et al. (2011)
G/PVP/PANI nanocomposites	Amperometry	ChOx	Physical adsorption	1	0.05–10	34.77	Present work

Abbreviations: nPt, platinum nanoparticle; GCE, glassy carbon electrode; AuNPs, gold nanoparticles; f-G, functionalize graphene nanoplatelets; IL, ionic liquid; Ch, chitosan; HRP, horseradish peroxidase; ChEt, cholesterol esterase; CSNF, chitosan nanofibers; AuE, gold electrode; MUA, 11-mercaptoundecanoic acid; PDDA, poly-(diallyldimethylammonium chloride); MWCNTs, multi-walled carbon nanotubes.

In this study, 2 μL of SDS was dropped on the electrode surface and dried at room temperature. The optimum concentration of SDS was found to be 2 mM, which can prevent the effect of AA interference over the concentration range from 0 to 120 μM in the detection of 1 mM cholesterol (Fig. S8B, Supporting information). For G/PVP/PANI modified electrode coated with SDS, the linear relationship was found to be 0.05–10 mM, which is similar to the one obtained from the uncoated SDS electrode as shown in Fig. S9 (Supporting information).

3.5. Reproducibility and stability of the G/PVP/PANI-modified electrode

The reproducibility and stability of G/PVP/PANI nanocomposite-modified electrode were investigated by using amperometric detection of 1 mM cholesterol. The relative standard deviations (RSD) of all cholesterol concentrations in the linear range were found between 1.05% and 9.37% ($n=5$), demonstrating acceptable reproducibility for this paper-based device. The storage stability of the biosensor was studied by measuring the current responses of 1 mM using prepared paper-based biosensor. It retained 89.1% of its initial response after a storage period of 2 weeks indicating that this paper-based biosensor had good stability.

3.6. Sample analysis

To test the applicability of this system, G/PVP/PANI-modified paper-based biosensor was used for the detection of cholesterol in human serum. The blank human serum samples are the common systems for the determination of the accuracy and validation of a new diagnostic assay. Initially, different concentrations of cholesterol (0.05, 0.10, 0.25, 1.00, and 5.00 mM) were spiked into the human serum, and the proteins in the serum were precipitated by using TCA. After centrifugation, the supernatant was kept for further amperometric analysis using the G/PVP/PANI-modified paper-based biosensor. The results indicated that the current responses depended on the cholesterol concentration. An acceptable linearity with a correlation coefficient (R^2) of 0.9998 ($n=3$) was achieved. The percentages of recoveries (Table 2) were found in the range of 100.0–102.0%, and the RSD was less than 5.0%, verifying that this sensing system is highly accurate.

4. Conclusions

A novel nanocomposite based on G/PVP/PANI has been prepared and used for the modification of a paper-based cholesterol biosensor. The high conductivity and large surface area of the droplet-like nanostructures of the G/PVP/PANI-modified electrode significantly improves the electrochemical sensitivity for the detection of H_2O_2. Under optimum conditions, a high-sensitivity, wide linear range and low limit of detection for cholesterol using proposed electrode is achieved. The interferences in the detection of cholesterol can be easily eliminated by using SDS. In addition, this sensing system was successfully applied for the determination of cholesterol in human serum. This novel and sensitive paper-based biosensor might be an alternative tool for cholesterol screening in medical diagnosis due to its simplicity, low cost, disposability and portability.

Table 2
Determination of cholesterol in human serum samples ($n=3$).

Cholesterol concentration (mM)		% Recovery	% RSD
Added	Found		
0.05	0.05 ± 0.04	100.0	0.8
0.10	0.10 ± 0.08	100.0	0.8
0.25	0.25 ± 0.30	100.0	1.2
1.00	1.02 ± 1.05	102.0	1.0
5.00	5.01 ± 2.84	100.2	0.6

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.08.018.

References

Ahmadalinezhad, A., Chen, A., 2011. Biosensors and Bioelectronics 26 (11), 4508–4513.
Apilux, A., Dungchai, W., Siangproh, W., Praphairaksit, N., Henry, C.S., Chailapakul, O., 2010. Analytical Chemistry 82 (5), 1727–1732.
Aravind, S.S.J., Baby, T.T., Arockiadoss, T., Rakhi, R.B., Ramaprabhu, S., 2011. Thin Solid Films 519 (16), 5667–5672.
Arya, S.K., Dey, A., Bhansali, S., 2011. Biosensors and Bioelectronics 28 (1), 166–173.
Arya, S.K., Prusty, A.K., Singh, S.P., Solanki, P.R., Pandey, M.K., Datta, M., Malhotra, B.D., 2007. Analytical Biochemistry 363 (2), 210–218.
Dey, R.S., Raj, C.R., 2010. Journal of Physical Chemistry C 114 (49), 21427–21433.
Dhand, C., Singh, S.P., Arya, S.K., Datta, B., Malhotra, B.D., 2007. Analytical Chimica Acta 602 (2), 244–251.
Dungchai, W., Chailapakul, O., Henry, C.S., 2009. Analytical Chemistry 81 (14), 5821–5826.
Dungchai, W., Chailapakul, O., Henry, C.S., 2011. Analyst 136 (1), 77–82.
Eguilaz, M., Villalonga, R., Agui, L., Yanez-Sedeno, P., Pingarron, J.M., 2011. Journal of Electroanalytical Chemistry 661 (1), 171–178.
Geim, A.K., Novoselov, K.S., 2007. Nature Materials 6 (3), 183–191.
Gomathi, P., Ragupathy, D., Choi, J.H., Yeum, J.H., Lee, S.C., Kim, J.C., Lee, S.H., Ghim, H.D., 2011. Sensors and Actuators B: Chemical 153 (1), 44–49.
Hossain, S.M.Z., Luckham, R.E., McFadden, M.J., Brennan, J.D., 2009. Analytical Chemistry 81 (21), 9055–9064.
Huang, J.D., Lin, Q., Zhang, X.M., He, X.R., Xing, X.R., Lian, W.J., Zuo, M.M., Zhang, Q.Q., 2011. Food Research International 44 (1), 92–97.
Jiang, F., Yue, R., Du, Y., Xu, J., Yang, P., 2013. Biosensors and Bioelectronics 44 (0), 127–131.
Karuwan, C., Sriprachuabwong, C., Wisitsoraat, A., Phokharatkul, D., Sritongkham, P., Tuantranont, A., 2012. Sensors and Actuators B: Chemical 161 (1), 549–555.
Li, C.L., Su, Y., Lv, X.Y., Xia, H.L., Shi, H.J., Yang, X.G., Zhang, J.Q., Wang, Y.J., 2012. Biosensors and Bioelectronics 38 (1), 402–406.
Liu, Q., Zhu, X., Huo, Z.H., He, X.L., Liang, Y., Xu, M.T., 2012a. Talanta 97, 557–562.
Liu, S., Xing, X.R., Yu, J.H., Lian, W.J., Li, J., Cui, M., Huang, J.D., 2012b. Biosensors and Bioelectronics 36 (1), 186–191.
Lu, L.C., Wang, W.Q., Cai, W.J., 2012. Journal of Polymer Materials 29 (2), 181–187.
Lu, Y., Shi, W.W., Qin, J.H., Lin, B.C., 2010. Analytical Chemistry 82 (1), 329–335.
Manjunatha, R., Shivappa Suresh, G., Savio Melo, J., D'Souza, S.F., Venkatarangaiah Venkatesha, T., 2012. Talanta 99 (0), 302–309.
Mano, N., Heller, A., 2005. Analytical Chemistry 77 (2), 729–732.
Mentele, M.M., Cunningham, J., Koehler, K., Volckens, J., Henry, C.S., 2012. Analytical Chemistry 84 (10), 4474–4480.
Nie, Z.H., Nijhuis, C.A., Gong, J.L., Chen, X., Kumachev, A., Martinez, A.W., Narovlyansky, M., Whitesides, G.M., 2010. Lab on a Chip 10 (4), 477–483.
Parlak, O., Tiwari, A., Turner, A.P.F., Tiwari, A., 2013. Biosensors and Bioelectronics 49 (0), 53–62.
Potts, J.R., Dreyer, D.R., Bielawski, C.W., Ruoff, R.S., 2011. Polymer 52 (1), 5–25.
Qiu, J.D., Shi, L., Liang, R.P., Wang, G.C., Xia, X.H., 2012. Chemistry-a European Journal 18 (25), 7950–7959.

Rattanarat, P., Dungchai, W., Siangproh, W., Chailapakul, O., Henry, C.S., 2012. Analytical Chimica Acta 744, 1–7.
Ruecha, N., Siangproh, W., Chailapakul, O., 2011. Talanta 84 (5), 1323–1328.
Safavi, A., Farjami, F., 2011. Biosensors and Bioelectronics 26 (5), 2547–2552.
Saxena, U., Chakraborty, M., Goswami, P., 2011. Biosensors and Bioelectronics 26 (6), 3037–3043.
Shin, Y.J., Kameoka, J., 2012. Journal of Industrial and Engineering Chemistry 18 (1), 193–197.
Singh, J., Srivastava, M., Kalita, P., Malhotra, B.D., 2012. Process Biochemistry 47 (12), 2189–2198.
Songjaroen, T., Dungchai, W., Chailapakul, O., Laiwattanapaisal, W., 2011. Talanta 85 (5), 2587–2593.
Wajid, A.S., Das, S., Irin, F., Ahmed, H.S.T., Shelburne, J.L., Parviz, D., Fullerton, R.J., Jankowski, A.F., Hedden, R.C., Green, M.J., 2012. Carbon 50 (2), 526–534.