Development of the layer-by-layer biosensor using graphene films: application for cholesterol determination

Hai Binh Nguyen¹, Van Chuc Nguyen¹, Van Tu Nguyen¹, Huu Doan Le¹, Van Quynh Nguyen¹, Thi Thanh Tam Ngo¹, Quan Phuc Do², Xuan Nghia Nguyen¹, Ngoc Minh Phan¹ and Dai Lam Tran¹

¹ Institute of Materials Science, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Hanoi, Vietnam
² Research Centre for Environmental Technology and Sustainable Development, Hanoi University of Science, Vietnam National University in Hanoi, 334 Nguyen Trai Road, Hanoi, Vietnam

E-mail: lamtd@ims.vast.ac.vn and chucnv@ims.vast.ac.vn

Received 7 September 2012
Accepted for publication 14 January 2013
Published 7 February 2013
Online at stacks.iop.org/ANSN/4/015013

Abstract
The preparation and characterization of graphene films for cholesterol determination are described. The graphene films were synthesized by thermal chemical vapor deposition (CVD) method. Methane gas (CH₄) and copper tape were used as carbon source and catalyst in the graphene growth process, respectively. The integrated array was fabricated by using micro-electro-mechanical systems (MEMS) technology in which Fe₃O₄-doped polyaniline (PANI) film was electropolymerized on Pt/Gr electrodes. The properties of the Pt/Gr/PANI/Fe₃O₄ films were investigated by field-emission scanning electron microscopy (FE-SEM), Raman spectroscopy and electrochemical techniques. Cholesterol oxidase (ChOx) has been immobilized onto the working electrode with glutaraldehyde agent. The cholesterol electrochemical biosensor shows high sensitivity (74 µA mM⁻¹ cm⁻²) and fast response time (< 5 s). A linear calibration plot was obtained in the wide cholesterol concentration range from 2 to 20 mM and correlation coefficient square (R²) of 0.9986. This new layer-by-layer biosensor based on graphene films promises many practical applications.

Keywords: graphene, polyaniline (PANi), cholesterol, electrochemical biosensor

Classification numbers: 2.04, 5.00, 5.10, 5.15, 6.09, 6, 12

1. Introduction
Electrochemical biosensors such as in clinical diagnostics, food safety and environmental monitoring, are widely used everyday life. Immobilization of the biorecognitive element onto a matrix plays an important role for the development of biosensors [1–6]. Biological molecules including enzymes, antibodies, DNA, etc, can be immobilized in a thin layer at a desired transducer surface by using different methods such as adsorption, entrapment, covalent bonding and cross-linking method [3, 5, 6]. Both the choice of support material and immobilization method could influence enzyme activity and operational stability of biosensor. The high application potential of conducting polymers in chemical and biological sensors is one of the main reasons for intensive investigation and development of these materials. They can be used both as immobilization matrices and as redox systems for the transport of electrical charge [7–10]. Conducting polymers can act as an electron promoter and be electrochemically deposited on small-size electrode, thus allowing for in vivo monitoring of biomolecules [11–13]. The unique properties of conducting polymers have been exploited for the fabrication of electrochemical detection systems [13]. Among various conducting polymers, polyaniline (PANI) is one of the most popular conducting polymers for biosensor applications because of having porous structures, ease of synthesis, low cost, high conductivity and good environmental stability, etc [14–16].
In this work we developed a novel cholesterol biosensor based on electrochemical microelectrode with graphene films coated on PANi/Fe₃O₄ films. By taking advantage of graphene-patterned, layer-by-layer fabricated electrode, excellent analytical quantification of cholesterol sensor as high sensitivity, fast response time would be obtained. Furthermore, this promising electrode platform could be extended for the development of other electrochemical biosensors and biomedical devices.

2. Experimental

2.1. Graphene film synthesis by CVD method

The graphene films were synthesized by thermal CVD method under high temperature 900 °C in argon (Ar) environment (1000 sccm). The copper (Cu) tapes with a thickness of 35 µm and a size of 0.5 cm × 0.5 cm were used as substrates for the graphene synthesis process. After the CVD process, the graphene films were cooled down to room temperature at the rate of about 10 °C/min under a flow of Ar (1000 sccm). The characteristics of graphene films were investigated by scanning electron microscopy (FE-SEM) and Raman spectroscopy techniques.

2.2. Fabrication of graphene/Fe₃O₄/PANi/GOx IDA for cholesterol detection

Fe₂O₄ nanoparticles (NPs) were synthesized by co-precipitation method of Fe³⁺ and Fe²⁺ under alkaline condition. 4 ml of ferrous chloride (1 M) and 2 ml of ferric chloride (1 M) were thoroughly mixed using magnetic stirring into a three neck flask of pH 4.0 at room temperature [17].

The interdigitated array (IDA) was fabricated on silicon substrate by the MEMS technology. Silicon wafers were covered with a silicon dioxide (SiO₂) layer by thermal oxidation. The thickness of the silicon dioxide was about 1000 nm. The silicon wafer was spin-coated with a layer of photoresist and the shape of the electrodes were defined by UV-photolithography. Then, chromium (Cr) and platinum (Pt) were sputtered on the top of the wafer with the thickness of 20 and 200 nm, respectively. The platinum working electrodes (WE) and counter electrodes (CE) were patterned by a lift-off process (figure 1). A second photolithographic step is carried out to deposit the 500 nm silver (Ag) layer. Partial chlorination of the Ag layer was performed in 0.25 mol l⁻¹ FeCl₃ solution, which is the reference electrodes (REs) [18].

The fresh solution of cholesterol oxidase (ChOx, 10 µl, 24 U mg⁻¹) was prepared in phosphate buffer (50 mM, pH 7.0) and then was added to 20 µl glutaraldehyde (0.25%). The resulting solution was transferred onto PANi/Fe₂O₄/graphene electrode. The later electrodes were washed accurately with phosphate buffer (50 mM, pH 7.0) to remove any unbound enzyme, and then were stored at 4 °C for 24 h before electrochemical measurement.

2.3. Electrochemical cholesterol detection on graphene/Fe₃O₄/PANi/ChOx

The cyclic voltammetry method (CV) was used to characterize the behavior of fabricated biosensor. The response to cholesterol addition was monitored by amperometric measurement.

3. Results and discussion

3.1. Graphene transferring onto IDA electrode

The graphene films synthesized on the Cu tape were transferred onto the IDA. The transfer process is as follows: first, a thin layer of polymethyl methacrylate (PMMA) was coated on top of grown graphene films on Cu tapes. Then the samples were annealed at 180 °C in air for 1 min. Subsequently, the graphene/PMMA films were released from the Cu tapes by chemical etching of the underlying Cu in iron (III) nitrate solution and suspended films were transferred to deionized water to remove the residual of Cu etching process. Next, graphene/PMMA films were transferred onto an IDA electrode. For the purpose of better contact between the graphene film and the IDA electrode, an appropriate amount of liquid PMMA solution was dropped secondly on the cured PMMA layer thus partially or fully dissolving the precoated PMMA. The re-dissolution of the PMMA tends to mechanically relax the underlying graphene, leading to a better contact with the IDA electrode. Finally, the PMMA films were dissolved by acetone and the samples were cleaned by rinsing several times in deionized water.

Some observations can be made from the FE-SEM image of graphene/Fe₃O₄/PANi films (figure 2). Firstly, it shows a spongy and porous structure of PANi, which in turn can be very helpful for enzyme entrapment. Secondly, doped core–shell Fe₂O₄ NPs (with the diameter core of around 30 nm) could also contribute to further immobilization of biomolecule, owing to their carboxylated shell. Furthermore, a thin and opaque graphene layer was distinguishably seen on the top of the electrode surface.
Figure 2. FE-SEM image of graphene film on the working electrode.

Figure 3. Raman spectrum of the composite films on microelectrode.

3.2. The crystal of graphene film

Figure 3 shows typical Raman spectra of the (Gr/PANI/Fe₃O₄) composite films on microelectrode. A Raman spectrum of graphene film on microelectrode (figure 3) exhibits three peaks at ~1360, ~1586 and ~2715 cm⁻¹. The peak of 1360 cm⁻¹ comes from the mixture of PANi peak (stretching vibration of C–N⁺) and D band of graphene (representing defects and disordered crystal structure). The band around 1586 cm⁻¹ is a mixture peak of PANi and G band of the graphene (representing ordered crystal structure). The 2D peak of 2715 cm⁻¹ is a characteristic peak of graphene [19].

3.3. Electrochemical behavior of PANi/Fe₃O₄/graphene

The behavior of each layer of the sensor was investigated by CV spectrum. The electrochemical activity of PANi/Fe₃O₄/graphene film increased about eight times compared with PANi film (figure 4). The Fe₃O₄ nanoparticle plays the role of electrolyte in the composite films. From figure 4 it is clear that the conductivity of composite was strongly enhanced with the presence of graphene film.

3.4. Cholesterol determination

Figure 5 shows a typical current–time plot for the sensor at +0.7 V during successive injections of cholesterol (2 mM increased injection, at room temperature, without stirring, air saturated, in 50 mM phosphate buffered solution). The calibration plot indicates a good and linear amperometric response to cholesterol within the concentration range from 2 to 20 mM (with regression equation of ΔI (µA) = (21.45 ± 1.7) × C (mM), R² = 0.9986) (the inset in figure 5). Thus, with a miniaturized dimension (500 µm) the above graphene-patterned sensor has shown much improved sensitivity to cholesterol, as high as 74 µA mM⁻¹ cm⁻².

4. Conclusion

An electrochemical cholesterol sensor based on graphene films was successfully developed. The layer-by-layer PANi/Fe₃O₄/graphene biosensor showed excellent properties for the sensitive determination of cholesterol with good sensitivity and response time. The proposed cholesterol biosensor based on graphene films might be applied in a wide...
range of biosensor applications, in particular for the detection of free cholesterol.

**Acknowledgments**

Funding of this work was sponsored by projects of Viet Nam Ministry of Science and Technology (grant 08/2011/HĐ-NDT), the key Laboratory for Electronic Materials and Devices, IMS (grant HTTD01.12). This work was also supported by IMS-level project; VAST young scientist program, National Foundation for Science and Technology Development (grant 103.99-2012.15). We also acknowledge Professor Pham Hung Viet, Professor Nguyen Xuan Phuc and Professor Phan Hong Khoi for their invaluable suggestions and discussions.

**References**

[1] Zhao Q, Gan Z and Zhuang Q 2002 *Electroanalysis* **14** 1609
[2] Gouda M D, Kumar M A, Thakur M S and Karanth N G 2002 *Biosens. Bioelectron.* **17** 503
[3] Singh S, Singhal R and Malhotra B D 2007 *Anal. Chim. Acta* **582** 335
[4] Badihi-Mossberg M, Buchner V and Rishpon J 2007 *Electroanalysis* **19** 2015
[5] Buerk D G 1995 *Biosensors: Theory and Applications* (Rijeka: InTech)
[6] Serra P A 2010 *Biosensors* (Croatia: InTech)
[7] Gerard M, Chauvey A and Malhotra B D 2002 *Biosens. Bioelectron.* **17** 345
[8] Singh S, Solanki P R, Pandey M K and Malhotra B D 2006 *Sensors Actuators B* **115** 534
[9] Xia L, Wei Z and Wan M 2010 *J. Colloid Interface Sci.* **341** 1
[10] Nguyen H L, Nguyen B H, Nguyen T N, Nguyen D T and Tran L D 2012 *Adv. Nat. Sci.: Nanosci. Nanotechnol.* **3** 015004
[11] Barlett P N and Cooper J M 1993 *J. Electroanal. Chem.* **362** 1
[12] Singh R P, Oh B K and Choi J W 2009 *Sensors Transducers J.* **105** 104
[13] Rahman M, Kumar P, Park D S and Shim Y B 2008 *Sensors* **8** 118
[14] Gerard M and Malhotra B D 2005 *Curr. Appl. Phys.* **5** 174
[15] Bhadra S, Khastgir D, Singha N K and Lee J H 2009 *Prog. Polym. Sci.* **34** 783
[16] Kunteppa H, Aashis S R, Devendrappa H and Prasad M V N A 2012 *J. Appl. Polym. Sci.* **125** 1652
[17] Luong T T et al 2011 *Colloids. Surf. A* **384** 23
[18] Tran L D, Nguyen D T, Nguyen B H, Do Q P and Nguyen H L 2011 *Talanta* **85** 1560
[19] Yuan B, Yu L, Sheng L, An K and Zhao X 2012 *J. Phys D: Appl. Phys.* **45** 235108