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Microchimica Acta

Analytical Sciences Based on Micro- and Nanomaterials

ISSN 0026-3672 Volume 183 Number 11

Microchim Acta (2016) 183:3077-3085 DOI 10.1007/s00604-016-1922-4





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ORIGINAL PAPER



Voltammetric determination of nitric oxide using a glassy carbon electrode modified with a nanohybrid consisting of myoglobin, gold nanorods, and reduced graphene oxide

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Received: 14 April 2016 / Accepted: 28 July 2016 / Published online: 27 September 2016 © Springer-Verlag Wien 2016

Abstract Myoglobin-modified gold nanorods incorporating reduced graphene oxide (rGO) were fabricated and deposited on a glassy carbon electrode (GCE) to obtain a sensor for nitric oxide (NO). The Mb-AuNR/rGO nanohybrid showed a transverse localized surface plasmon resonance (LSPR) band with a peak at 508 nm, and a longitudinal LSPR band at 724 nm. The AuNRs have an average length of 38 ± 3 nm and a width of 11 ± 1 nm. The GCE modified with the nanohybrid is shown to be a viable sensor for the determination of NO by linear sweep voltammetry. Its electrocatalytic response toward the oxidation of NO is distinctly enhanced compared to other electrodes. The sensor, best operated at a working voltage of 0.85 V (vs. SCE), showed two linear response ranges (from 10 to 100 μ M, and from 100 to 1000 μ M), with a detection limit of 5.5 µM. Furthermore, it exhibits excellent selectivity for NO over common interferents such as NaNO₃, and also over electroactive species such as ascorbate, dopamine, glucose, and uric acid. These properties make it a

Electronic supplementary material The online version of this article (doi:10.1007/s00604-016-1922-4) contains supplementary material, which is available to authorized users.

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promising tool for the detection of NO in situations such as capillary and pulmonary hypertension and embolism, and during vasodilation.

Keywords Electroanalysis \cdot Biosensor \cdot Nanocomposite \cdot Localized surface plasmon resonance \cdot LSPR \cdot Linear sweep voltammetry \cdot X-ray diffractometry \cdot X-ray photoelectron spectroscopy \cdot Capillary dilatator

Introduction

Nitric oxide (NO) has been shown to be involved in regulating neuronal excitability, synaptic transmission, neuronal networks functioning, learning, and memory mechanisms [1, 2]. An excess or a deficiency of NO results in various pathological conditions such as tumor angiogenesis [3], atherosclerosis [4], Parkinson's disease [5], and diabetes [6]. Therefore, the accurate measurement of NO is very important to unravel the action of this key compound. Because NO levels may become useful markers of inflammation and disease pathogenesis, the development of reliable and sensitive analytical techniques is of interest for the quantitation of NO production. Several analytical techniques are used for the detection of NO, including spectroscopic [7], chemiluminescence [8], chromatographic [9], capillary electrophoresis [10], and electrochemical methods [11]. However, those methods often have complicated operating procedures, and are time consuming, high cost, and occupy larger space. Hence, there is an urgent need to develop a cost-effective, easy-to-perform, rapid-response detection method for NO. In this respect, an electrochemical sensor has recently been adopted for the detection of nitric oxide, and it has several advantages over conventional detection methods, including a rapid response, robustness, high sensitivity and selectivity, low cost,

miniaturization, and the potential for real-time monitoring. Several materials have been used as electrode modifiers for the electrochemical detection of NO, including polymers [12], carbon nanotubes, graphene, metal oxide, and metal nanoparticles. Among these, metal nanoparticles possess high electrocatalytic activity, especially gold (Au) nanoparticles [13, 14].

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Recently, anisotropic gold nanostructures such as nanorods, nanoprisms, and nanopods have gained much attention because of their tunable optical and electronic properties [15]. These unique properties depend on the particle size and shape, and the structure of the local microenvironment [15]. In particular, gold nanorods (AuNRs) have been widely studied because of their unique properties and are finding potential applications in imaging, therapy, and biosensors [16]. AuNRs show two absorption peaks corresponding to their transverse (~ 520 nm) and longitudinal (\geq 600 nm) plasmonic modes of absorption [17].

Graphene is another class of attractive material. It consists of a two-dimensional (2D) sheet of monolayer carbon atoms, and has recently attracted great attention because of its unique optical, catalytic, mechanical, thermal, and electronic properties [18]. Molecular sensing can be achieved with graphene, because it is a very low noise to signal ratio electronic structure [19]. The combination of graphene and gold nanostructures has made substantial progress in electrochemical biosensors [20].

Here we present the combination of gold nanorods with myoglobin incorporation of reduced graphene oxide in order to combine the advantages of those materials. The immobilization of Mb with gold nanorods and rGO will amplify the detection signal, improve the electron transducer, and reduce the detection limit in electrochemical sensors for analytical purposes. In the present work, a nanohybrid consisting of myoglobin and gold nanorods that incorporated reduced graphene oxide (Mb-AuNR/rGO) was prepared and used for the detection of NO. The Mb-AuNR/rGO/GCE showed an enhanced electrocatalytic activity toward the oxidation of NO, along with two different linear responses in the ranges of 10 to 100 µM and 100 to 1000 µM, with limit of detection (LoD) values of 5.5 and 26.6 µM, respectively. Moreover, this sensor electrode was more selective toward NO in the presence of other common interferents. The selectivity and sensitivity demonstrated by this Mb-AuNR/rGO/GCE make it a promising candidate for the construction of a sensor for the detection of NO in practical applications.

The raw materials for the graphene synthesis used in this re-

search were graphite flakes (3061, Asbury Graphite Mill Inc.).

Experimental

Chemicals

Horse heart myoglobin, hydrogen tetrachloroaurate (III) hydrate, and cetytrimethylammonium bromide (CTAB) were purchased from Sigma and used as received. Hydrazine hydrate (50 % w/v solution) and ammonia (25 % w/v solution) were purchased from Sigma-aldrich (www.sigmaaldrich.com). Hydrogen peroxide (30 %, w/v solution) was purchased from Systerm (www.haiousaintifik.com). A 0.1 M phosphate buffer (pH 2.5) was prepared by mixing a stock standard solution of NaH₂PO₄ and H₃PO₄. All of the other chemicals used were of analytical grade unless otherwise mentioned.

Preparation of myoglobin-gold nanorods/reduced graphene oxide (Mb-AuNR/rGO) nanohybrid

The Mb-AuNR/rGO nanohybrid was prepared as follows. Initially, the reduced graphene oxide was prepared using Wallace's method [21]. Briefly, 200 mL of preformed 1 mg·mL^{-1} graphene oxide, GO prepared by a simplified Hummers method [22] (detailed procedure is given in ESM) was sonicated for 10 min and then 2 mL of hydrazine hydrate and 5 mL of ammonia were mixed and the mixture kept under vigorous stirring for 30 h. The final product, rGO, was obtained after centrifuging three times with doubly distilled water at 12,000 rpm (RCF = $14,515.2 \times g$) for 30 min. Meanwhile, AuNR with an aspect ratio of 3.5 were separately prepared using a seed mediated-growth solution following the procedure of Nikoobakht and El-Sayed [23]. Then, 1.5 mL of the AuNR solution was mixed with 2 mL of myoglobin $[2 \text{ mg·mL}^{-1}]$ and 2 mL of RGO $[0.5 \text{ mg·mL}^{-1}]$. The mixture was mildly sonicated for 30 min and kept for 24 h under stirring to incorporate the Mb and AuNRs on to the surface of rGO sheet.

Characterization and electrochemical studies

The optical absorption spectra in the spectral region of 190 to 900 nm were assessed using a Thermo Scientific Evolution 300 UV-vis spectrophotometer (www.fishersci.com). The crystalline phases of the nanohybrid were analyzed using a PANalytical EMPYREAN X-ray diffractometer with K a radiation ($\lambda = 1.5418$ Å) at a scan rate of 0.03 s⁻¹ (www.dksh.com). The XPS was performed using synchrotron light at beamline no. 3.2 of the Siam Photon Laboratory, Synchrotron Light Research Institute, Thailand. The XPS data were obtained under the conditions of maximum photon energy of 600 eV and kinetic energy step of 0.1 eV. The surface morphology was recorded using a Hitachi SU8020 high-resolution instrument equipped with energy dispersive X-rays (EDX). The electrochemical measurements were performed using an Autolab electrochemical analyzer (PGSTAT204) (www.metrohmautolab.com) with a conventional three-electrode system under a nitrogen atmosphere. The Mb-AuNR/rGO/GCE-modified electrode was fabricated by drop casting 5 µL of the MbAuNR/rGO nanohybrid solution on a pretreated glassy carbon electrode in the oven for 5 min at 60 °C and drying at room temperature for 6 h. The Mb-AuNR/rGO/GCE was used as the working electrode, and a saturated calomel electrode (SCE) and platinum wire were used as the reference and counter electrodes, respectively. A 0.1 M phosphate buffer (pH 2.5) was used as the supporting electrolyte. Nitrogen gas was bubbled into the electrolyte solution for 30 min before each experiment and the experiments were carried out under flowing N₂ gas. All the potentials given in this work were referred to the SCE, and all the experiments were carried out at room temperature.

Results and discussion

Absorption studies of Mb-AuNRs/rGO nanohybrid

The formations of AuNRs, reduced graphene oxide, and the Mb-AuNR/rGO nanohybrid were investigated by recording the optical absorption spectra, which are shown in Fig. 1. The optical absorption spectra obtained for the AuNRs showed the transverse LSPR band at 515 nm and the longitudinal LSPR band at 745 nm. The rGO displayed a peak at 264 nm, which confirmed that it was a completely reduced form of graphene oxide. The Mb-AuNR/rGO nanohybrid showed the transverse LSPR band at 508 nm and the longitudinal LSPR band at 724 nm. The blue shift in the Mb-AuNR/ rGO compared to the AuNRs was due to the change in the local refractive index and medium surrounding the AuNRs. The decrements of intensity of the transverse and longitudinal LSPR bands were clearly observed for the Mb-AuNR/rGO. The longitudinal LSPR band for the AuNRs is much higher than that of the Mb-AuNR/rGO because of the rGO and Mb medium surrounding the AuNRs. This was supported by the appearance of high-intensity rGO and Mb peaks at 264 nm and 410 nm, respectively.



Fig. 1 Absorption spectra of AuNR (a), rGO (b), and Mb-AuNR/rGO (c)

X-ray diffraction studies of Mb-AuNR/rGO nanohybrid

The crystalline properties of the Mb-AuNR/rGO nanohybrid were investigated by recording the X-ray diffraction pattern, and are shown in Fig. 2. The XRD peaks of the pure AuNRs and Mb-AuNR/rGO nanohybrid show four peaks at 20 values of 38.3°, 44.6°, 64.7°, and 77.5° corresponding to the (111), (200), (220), and (311) planes, respectively, due to the gold (JPCDS file no.: 00–001-1172) (Fig. 2a and b). The appearance of a diffraction peak at 25.1° for the rGO was due to a complete reduction of the graphene oxide and exfoliation of the graphene layers [24, 25] (Fig. 2c). The observed weak diffraction peak at 25.1° was attributed to the removal of oxygen functional groups after the reduction process using hydrazine. In contrast, the pure myoglobin displays no characteristic diffraction peaks.

X-ray photoelectron spectroscopy studies of Mb-AuNR/rGO nanohybrid

Further, the Mb-AuNR/rGO was characterized by XPS. Figure 3 shows the XPS results obtained for the Mb-AuNR/rGO, which clearly confirm the presence of AuNR on the rGO surface. The XPS of Au4f shows two peaks at 85.75 eV for $4f_{7/2}$ and 89.36 eV for $4f_{5/2}$ (Fig. 3a).

The C_{1s} spectrum shows binding energy values at 284.71 eV and 287.63 eV for rGO (Fig. 3b), which clearly show a considerable degree of oxidation components corresponding to the nonoxygenated ring C and carboxylate carbon C = O, respectively. The non-oxygenated C-C is prominent because of the interaction with hydrazine during the reduction treatment. The small peak at 287.63 eV indicates that the C = O of the carboxylate group which contribute to appearance of oxygen at small percentage in the rGO (ESM Fig S2).



Fig. 2 X-ray diffraction patterns obtained for AuNR (a), Mb (b), rGO (c), and Mb-AuNR/rGO (d) $\,$



Fig. 3 XPS results of Mb-AuNR/rGO spectrum for Au4f (a) and C1s (b)

Morphological studies of Mb-AuNR/rGO nanohybrid

The morphologies of the AuNRs, AuNR/rGO, and Mb-AuNR/rGO nanohybrid were characterized by means of FESEM. At higher magnification (Fig. 4a), the FESEM images confirmed the formation of gold nanorods with an average length of 41 nm, a breadth of 12 nm, and an aspect ratio of ~3.5. Figure 4b and c showed the FESEM images of the AuNRs in the presence of rGO and Mb with rGO. The images display the distribution of AuNRs on both sides (beneath the rGO surface from Fig. 4c) of the rGO's surface. The nanorods have an average length of 38 nm and a breadth of 11 nm, with an aspect ratio of 3.5. Figure 4c displays highly dense AuNRs on the surface of rGO due to the presence of Mb after the incorporation process. The different aspect ratio of AuNRs can be obtained by varying the concentration of solutions used for the growth of AuNRs such as CTAB, HAuCl₄, AgNO₃, and ascorbic acid. There is no limitation in preparing AuNRs of different aspect ratio and size and this can be achieved by playing the growth solution concentrations such as CTAB, HAuCl₄, AgNO₃, ascorbic acid. The aspect ratio obtained for n = 40 nanorods of Au NR and Mb-AuNR/rGO from the histogram are 3.5 (ESM Fig. S3).

An energy dispersive X-ray analysis (EDX) was also conducted based on the FESEM image of the Mb-AuNR/rGO nanohybrid. Figure 5 shows the EDX of the selected surface area of the Mb-AuNR/rGO nanohybrid, which confirmed the presence of elements such as Au (7.95 %), O (11.78 %), C (27.24 %), and Si (53.03 %). Si shows the highest percentage because of the substrate used to coat the sample.

Electrocatalytic activity of Mb-AuNR/rGO nanohybrid -modified electrode toward NO

The fabricated Mb-AuNR/rGO nanohybrid-modified electrode was investigated in relation to electrocatalysis and the sensing of nitric oxide. An in-situ generated nitric oxide was used for the electrocatalysis and sensor. It is well known that sodium nitrite (NaNO₂) serves as a source of NO and in an acidic solution (pH < 4), it will undergo a disproportionation reaction (Eq. 1) and thus produces NO [26, 27].

$$3\text{HONO} \rightarrow \text{H}^+ 2\text{NO} + \text{NO}_{\overline{3}} + \text{H}_2\text{O}$$
 (1)

The aspect ratio of the AuNR influences the electrocatalytic activity of the modified electrode. Initially, electrocatalytic performance of pristine AuNR with different aspect ratio such as 2.5, 3.5, and 4.5 were investigated. Among them the AuNR with aspect ratio of \sim 3.5 showed enhanced electrocatalytic activity. Hence, we have chosen the AuNR with the aspect ratio of \sim 3.5 was chosen for the preparation of nanohybrid and it used for the electrochemical detection.

In our work, NaNO₂ was used as a precursor to produce NO in solution during the electrochemical study [28]. The addition of a known amount of NaNO₂ into the bulk electrolyte solution at pH 2.5 generated a series of concentrations of NO.

As can be seen from Fig. 6 the Mb-AuNR/rGO nanohybridmodified electrode showed a remarkably enhanced electrocatalytic response toward the oxidation of NO, with a higher current than the other investigated modified electrodes. The oxidation potential of NO for the Mb-AuNR/rGO/GCE was significantly shifted toward a lower potential value with an increase in the anodic peak current compared to rGO/GCE, and Mb/GCE-modified electrodes. The AuNR/GCE showed lower oxidation current but it performed the best electrocatalytic activity considering the oxidation peak potential. The bare GCE showed less intense oxidation peaks for NO at 0.85 V compared to Mb-AuNR/rGO/GCE. A higher catalytic current was obviously displayed by the Mb-AuNR/rGO/GCE as a result of the synergistic electrocatalytic effect aroused by the combination of rGO, Mb, and AuNR present in the Mb-AuNR/rGO nanohybrid, and was also attributed to the AuNR providing a larger surface area with specific interaction toward the substrate. Thus, it resulted in improved electron-transfer kinetics and enhanced oxidation of NO [29]. Scheme 1 shows a schematic representation of the electrocatalytic oxidation of NO at the Mb-AuNR/rGO nanohybrid-modified electrode.

Further, to check the superior electrocatalytic performance of Mb-AuNR/rGO/GCE, the cyclic voltammograms for bare

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Fig. 4 FESEM images of AuNRs (a), AuNR/rGO (b), and Mb-AuNR/rGO (C) samples



GCE, AuNRs/GCE, RGO/GCE, Mb/GC, and Mb-AuNRs/ RGO/GCE were recorded with the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution and compared in Fig. S7. The voltammograms exhibit two nearly symmetric anodic and cathodic peaks related to the redox system in $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution. Among the all investigated electrodes, the Mb-AuNR/rGO/GCE showed higher current and less peak to peak separation which indicates the enhanced electrocatalytic activity of Mb-AuNR/rGO modified electrode. This observation clearly supports the enhanced electrocatalytic performance of the Mb-AuNR/rGO/ GCE toward NO oxidation.

Electrochemical detection of nitric oxide at Mb-AuNR/rGO/GCE-modified electrode

Fig. S8 shows a linear sweep voltammogram obtained for the Mb-AuNR/rGO/GCE-modified electrode in the presence of NO at a concentration range of 10 μ M to 1 mM in the 0.1 M phosphate buffer (pH 2.5) with a scan rate of 50 mV·s⁻¹. The Mb-AuNR/rGO/GCE showed an anodic oxidation peak of ~0.85 V as a result of the NO oxidation. It can be seen that the oxidation peak current was increased when the NO concentration was increased up to 1 mM. The LSV curves



Fig. 5 EDX analysis of Mb-AuNR/rGO nanohybrid



Fig. 6 Cyclic voltammograms obtained for bare GCE (**a**), AuNRs/GCE (**b**), rGO/GCE (**c**), Mb/GC (**d**), and Mb-AuNR/rGO/GCE in 0.1 M phosphate buffer (pH 2.5) with1 mM NO at the scan rate of 50 mV.s⁻¹

show a well-defined and stable anodic oxidation peak current for the detection of NO. These results demonstrated that the Mb-AuNR/rGO/GCE provided a good electrocatalytic activity toward nitric oxide sensing. Further, the observed calibration curve for the peak current against the NO concentration is shown in Fig. 7.

The measured peak currents were found in two linear ranges with increasing concentrations: 10 to 100 μ M and 100 to 1000 μ M. The observed two linear ranges due to the kinetic limitation of sensor electrode and it caused the decrease in current sensitivity while increase the analyte concentration in the voltammetric technique. The first linear segment in the range of 10 to 100 μ M had a sensitivity of 0.0539 μ A/ μ M (R² = 0.9921), with a linear regression equation of $y = 0.05392 \times + 7.2363$ E-7. The limit of detection

(LoD) was calculated based on the relationship LoD = $\frac{3\sigma}{m}$ [30], where σ is the relative standard deviation of the intercept of y-coordinates from the line of best fit, and *m* is the slope of the same line. The LoD value of 5.5 μ M was obtained for the first linear segment. The second linear segment in the range of 100 to 1000 μ M showed a sensitivity of 0.0341 μ A/ μ M (R² = 0.9982), with a linear regression equation of *y* = 0.0341× + 2.6847E-6 and the LoD was found to be 26.6 μ M. The obtained LoD of 5.5 μ M for the detection of NO using the Mb-AuNR/rGO/GCE was comparable to the values of other reported electrochemical sensors. The present nitric oxide detection results were compared with previous reports, as summarized in Table 1.

Repeatability and stability Mb-AuNR/rGO/GCE

Repeatability and operational stability were tested by recording the repetitive CV measurement and are shown in Fig. S9 and S10. The Mb-AuNR/rGO/GCE electrode fabrication repeatability was examined by recording CV at 50 in the presence of 1 mM NO for five repeated electrode fabrication and it shows a relative standard deviation (RSD) for cyclic voltammetric response is 2.8 % (n = 5). Both experiments indicated that the fabricated sensor had a good repeatability and stability for the NO determination. The operational stability of the Mb-AuNR/rGO/GCE was also investigated by measuring the electrode response with 1 mM NO (n = 50) for 50 continuous cycles. After 50 cycles, it continued to show stable oxidation behavior and the response to NO lost about 7.2 % by retaining 84 % of its initial



Scheme 1 Schematic illustration of electrocatalytic oxidation of NO at Mb-AuNR/rGO nanohybrid-modified electrode

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Table 1 Comparison of analytical parameters of some sensor electrodes for NO determination

Electrode materials ^{<i>a</i>}	Fabrication method	Detection method	Linear range	Detection limit (M)	Ref.
Nafion/Graphene/AuNPs/GCE	Electrodepositiondrop casting	СА	36 nM to 20 µM	0.018×10^{-6}	[14]
GC/G-Nf	Hydrothermal	SWV	50 to 450 µM	11.61×10^{-6}	[31]
GC/HbCPB/PAM	Drop casting	CV	9.8 to 100 mM	9.3×10^{-6}	[32]
CTAB-nafion modified carbon nanofiber paste microelectrode	Drop casting	CA	7.2 nM to 96.1 μM	0.002×10^{-6}	[33]
GC/Cyt C/chitosan-GR	Drop casting	CA	3.6 to 43.2 µM	—	[34]
GC/MWCNT-CoTPPS	Drop casting	CA	6.6 to 130 µM	$6.6 imes 10^{-6}$	[35]
Mb-AuNR/rGO/GCE	Drop casting	LSV	$10 \ \mu M$ to $1 \ mM$	5.5×10^{-6}	This work

^a AuNPs Gold nanoparticles, CTAB Cetyl trimethylammonium bromide, G/GR graphene, Cyt C Cytochrome C, Nf Nafion, Hb hemoglobin, CPB cetylpyridinium bromide, PAM polyacrylamide, MWCNT multi-walled carbon nanotubes, CoTPPS Cobalt (II) tetrakis (4-sulfonatophenyl) porphyrin, Mb-AuNR/rGO/GCE Myoglobin-gold nanorod/reduced graphene oxide/glassy carbon electrode

current, thus not undergo surface fouling by the oxidized products indicating its good stability. The deviation due to the irreversible process of electrocatalytic oxidation of NO during continuous scanning the oxidized NO cannot regenerate. this potential region. Due to the electrochemical inertness of the interfering species in this potential, the electrode brings the selectivity towards NO.

Selectivity of Mb-AuNR/rGO/GCE

In order to prove the selectivity of the Mb-AuNR/rGO/GCE toward NO, LSV curves were recorded in the presence of common interfering compounds such as NaNO₃, NaF, KCl, NH₄Cl, and Na₂CO₃, and also in the presence of electroactive compounds such as ascorbate, dopamine, glucose, uric acid and hydrogen peroxide. No significant oxidation current was observed at 0.85 V in the LSV curves (Fig. 8). An obvious change occurred upon the injection of 10 μ M of NO, and an enhancement in the oxidation peak current was observed. As a result, the voltammetric detection using Mb-AuNRs/RGO/GCE is selective towards NO because only NO undergoes electrocatalytic oxidation in the potential range between 850 and 950 mV and other interfering species cannot oxidize in



Fig. 7 The calibration plot of the peak current against the NO concentration obtained for Mb-AuNR/rGO/GCE

Conclusions

Nanohybrid composed of myoglobin-gold nanorods that incorporated reduced graphene oxide (Mb-AuNR/rGO) was prepared and used for the construction of an electrochemical sensor for the selective and sensitive detection of NO. The Mb-AuNR/rGO/GCE showed an enhanced electrocatalytic response toward the oxidation of NO compared to those of the other investigated electrodes. The presence of gold nanorod significantly improved the electrical communication and electrocatalytic properties. Under optimal experimental parameters, the sensor possessed a wide calibration range from 10 to 1000 μ M, with limit of detection (LoD) values of 5.5 μ M, via LSV techniques. Moreover, this fabricated sensor electrode possessed good selectivity toward NO



Fig. 8 Linear sweep voltammograms obtained for Mb-AuNR/rGO/GCE in 0.1 M phosphate buffer (pH 2.5) in presence of 10 μ M of NO and 1 mM of other interfering species at scan rate of 50 mV.s⁻¹

in the presence of 1 mM concentration of common interferents and other electroactive compounds. Besides that, there is no limitation in preparing AuNRs of different aspect ratio and size and this can be achieved by playing the growth solution concentrations such as CTAB, HAuCl₄, AgNO₃, ascorbic acid. The excellent selectivity and sensitivity toward NO indicates that this Mb-AuNR/rGO/GCE is a potential sensor electrode for practical applications.

Acknowledgments The authors are grateful for the financial support provided by a Postgraduate Research Grant (PPP) (PG121-2014B). The authors would also like to thank the Synchrotron Light Research Institute, Thailand, for providing the XPS facilities and the Department of Physics, Universiti Pendidikan Sultan Idris, for the FESEM and EDX facilities that supported this work.

 $\label{eq:compliance} \begin{tabular}{c} Compliance with ethical standards & The author(s) declare that they have no competing interests & \end{tabular}$

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