Hybridization of Silver Nanoparticles and Reduced Graphene Nanosheets into a Nanocomposite for Highly Sensitive L-Phenylalanine Biosensing

Eskandar Omidinia\textsuperscript{1,*}, Seyed Morteza Naghib\textsuperscript{2,*}, Asma Boughdachi\textsuperscript{3}, Payam Khoshkenar\textsuperscript{4,5}, David K. Mills\textsuperscript{5}

\textsuperscript{1} Enzyme Technology Laboratory, Pasteur Institute of Iran, Tehran, 13164, Iran. 
\textsuperscript{2} Nanobioengineering Division, School of New Technologies, Iran University of Science and Technology, Tehran, 16846-13114, Iran. 
\textsuperscript{3} Polymer Engineering Department, Amirkabir University of Technology, Tehran, 15875-4413, Iran. 
\textsuperscript{4} The David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. 
\textsuperscript{5} School of Biological Sciences and the Center for Biomedical Engineering and Rehabilitation Science, Louisiana Tech University, Ruston, LA, 71270.

*These authors contributed equally to this work (in the role of first author) and the corresponding authors: E-mail: Naghib@iust.ac.ir; Skandar@pasteur.ac.ir

Received: 28 March 2015 / Accepted: 22 May 2015 / Published: 24 June 2015

Hybridizing nanometals and carbon nanosheets as a nanocomposite has resulted in the best-performing biosensors to date. The potential of an amperometric L-phenylalanine (L-phe) biosensor, based on the immobilization of phenylalanine dehydrogenase (PDH) onto a nanocomposite, containing a hybrid of reduced graphene nanosheets (rGNS) and silver nanoparticles (AgNPs) electrodeposited on glassy carbon electrodes, was investigated. The two-dimensional support (rGNS) was incorporated to zero-dimensional AgNPs arrayed in the third dimension and developed as an L-phe biosensor with excellent biosensing capabilities. Biosensor versatility was developed using a novel nanocomposite that permitted easy changes in sensor performance (i.e., sensitivity, limit of detection, and linear range). This method resulted in a high sensitive biosensor design that exhibits excellent sensing performance including improved L-phe sensitivity (47 nM limit of detection, 0.15–900 µM linear sensing range), possesses a long and stable shelf life (>1 month), and high selectivity.

**Keywords:** Nanobiosensor; Reduced graphene nanosheets; Silver nanoparticles; Nafion; L-phenylalanine; PEGylated phenylalanine dehydrogenase.
1. INTRODUCTION

Carbon based nanomaterials have been emerged as advantageous nanocomposites in biomedical applications especially in electrochemical biosensors [1, 2] due to their unique physicochemical and biological properties [3-5]. High surface area, excellent conductivity, suitable mechanical strength, ease of functionalization, mass production, low cost, and especially biocompatibility are characteristics of two-dimensional carbon-based nanomaterials enabling incorporation into a diverse set of nanocomposites including multifunctional tissue engineered scaffolds, drug/gene delivery nanocarriers and biosensors.

Carbon nanosheets (CNS) have been employed in micro-/nanofabrication of biosensors and biofuel cells due to their exceptionally high electron mobility [6]. Direct Electron Transfer (DET) between the redox center of enzyme and electrode surface has been an area of intense research in the development of electrochemical biosensors [7-13]. Incorporation of nanomaterials in biosensor fabrication has been extensively employed to realize an enhanced DET consequently leading to faster response times.

The inherent conductivity of silver nanoparticles (AgNPs) may facilitate a more efficient electron transfer in biosensors as compared with gold nanoparticles [14, 15]. Excellent catalytic activity of AgNPs, along with their biocompatibility, offer a suitable environment for biomolecules and DET [14]. The small size of AgNPs improves the connections between enzyme redox centers while the electroconductive property of AgNPs enhances electrical communication with the electrode.

A few studies have been reported on non-invasive biosensors. Among these reports, PEGylated PDH, when immobilized onto the graphene oxide nanosheets (GOs)-chitosan nanocomposite, demonstrated a very high DET for the NAD$^+$ reduction reaction [8].

Here, we investigated the use of AgNPs-incorporated reduced graphene nanosheets (rGNs) towards the development of a highly sensitive electrochemical biosensor for detection of L-phenylalanine (L-phe). To the author’s knowledge, the use of rGNs-AgNPs composite for biosensors applications has been unexplored until now. A rigorous sensor biofunctionalization protocol was applied to electrodeposit PEGylated PDH with the electrically conductive polymer nafion onto the rGNs-AgNPs composite surface. The performance of the biosensors was investigated by cyclic voltammetry (CV) and differential pulse voltammetry (DPV) and the effect of commonly present compounds in human body was also assessed. The biosensor performance showed high sensitivity to L-phe even after four weeks of use, with minimal interference from typical electroactive species (i.e., glucose, ascorbic acid and uric acid) normally found in human serum samples. A low limit of detection (LOD) and wide linear sensing range that considerably improved performance of L-phe biosensors has been reported previously [8].

2. MATERIALS AND METHOD

2.1. Reagents and equipment

Reduced graphene was prepared from graphite according to the previously reports [6, 16]. AgNPs was a gift from Dr. M. Ansari, University of Tehran. Nafion and L-Phenylalanine (L-phe) were
purchased from Merck. Nicotinamide adenine dinucleotide (NAD\(^+\)) was provided from Scharlau co. and PEGlayted PDH was produced according to the method reported in earlier studies [9, 10].

The morphology of the nanobiocomposite was measured using a JEOL field emission electron microscope (FESEM) operated at 200 kV. For FESEM testing, freshly prepared rGNS and AgNPs were dispersed in polymeric solution (nafion) with ultrasonication for 2h.

Electrochemical experiments were performed in a conventional three-electrode cell controlled by Potentiostat/Galvanostat µAUTOLAB (type III). A two-line silver ink was used as the working electrode coated onto polyethylenterephthalate (PET) as reference electrodes. All tests were conducted in an electrochemical cell of 8 µl at room temperature.

2.2. Preparation of rGNS-AgNPs composite and biosensors

2.2.1. Fabrication of base electrode

A two-line thin film of silver ink was coated onto PET wafer (2 mm × 5 mm) at a base pressure of 5.0 ×10\(^{-7}\) Torr. Figure 1 shows the fabrication process schematically. The electrodes were pretreated in 1.0 M NaOH solution and the potential of the working electrode was kept at +1.0 V for 5 min. rGNS suspension was prepared by dispersing 10 mg of rGNS and 4 mg of AgNPs in 10 mL ethanol with ultrasonic agitation for about 2h. Then, 50 µL of 50% nafion and 50 µL of 3.2 mg.mL\(^{-1}\) rGNS/Ag solution were mixed under continuous stirring. The prepared solution was used to treat the working electrode as follows: 5 µL of the prepared solution was placed onto the surface of the working electrode (first line of strip) and allowed to be dried at room temperature for at least 5 hours. In an effort to improve the electroactive nature of the NPs, the GNs/AgNPs based electrodes were exposed to an O\(_2\) plasma etch within a Plasma Tech Reactive Ion Etch (RIE).

2.2.2. Enzyme immobilization

PEGlayted PDH was mixed with nafion before it was electrodeposited onto the rGNS/AgNPs electrodes. The subsequent nafion/PDH solution was then electrodeposited onto each GNs/AgNPs electrode via constant current pulses of 1.05 mA that were used between the working electrode (GNs/AgNPs) and auxiliary electrode (Pt wire) for 500 cycles. Finally, the electrode was kept in 4° C. refrigerator for 24h.

3. RESULTS AND DISCUSSION

3.1. Characterization of PEGlayted PDH-nafion immobilized rGNS/AgNPs electrode

The nanoactivated electrode was biofunctionalized with the PEGlayted PDH (Fig. 2a). The morphology of rGNS-AgNPs-nafion nanocomposite was characterized by field emission transmission
electron microscopy (FESEM). Figure 2c exhibits FESEM image of nafion-incorporated rGNS/AgNPs electrode, demonstrating the growth of rGNS-AgNPs nanocomposite into the nafion matrix.

**Figure 1.** Schematic representation of biosensor fabrication composed of nafion/PDH immobilized rGNS/AgNPs/nafion.

**Figure 2.** (a) Reaction of the enzyme electrode. (b) biosensor response. (c) FESEM image of PDH-nafion immobilized rGNS/AgNPs/nafion nanocomposite.
3.2. Effect of pH and temperature on the activity of enzyme electrode

The pH of the supporting solution is an important factor in determining the activity of biosensors to NAD$^+$ reduction. Therefore, the effect of pH on the current response of our biosensor was investigated by testing changes in biosensor response with different pH solutions at ambient temperature (25° C.). Figure 3a shows DPVs of the of nanoactivated bioelectrode measured in the 100 mM Gly/KOH/KCl buffer (pH=10) containing 2.5 mM NAD$^+$ at various pH values with 200 µM L-phe at a scan rate of 25 mV/s. The pH experiments were performed by changing NaOH and HCl concentrations within a 10µl buffer. The enzyme electrode showed low catalytic activity in pH solutions lower than 6 and high enzyme activity in pH solutions greater than 6. Most biological fluids have a pH of great than 6, so they could be analyzed with this biosensor. As shown in figure 3a, the optimum pH for catalytic activity of the enzymatic biosensor was different in comparison with other related publications. As can be seen in figure 3a, the optimum pH for our biosensor is 10. Variety of the pH–activity curves for enzymes is conventional after immobilization in different electrode surfaces and This effect is in agreement with previous reports describing that the influence of pH on enzyme bioactivity are shifted after immobilization in charged supports [12, 20]. Therefore, all electrochemical experiments were carried out in 0.1 M Gly/KOH/KCl buffer at a pH 10.

Biosensor response is strongly influenced by temperature [17-19]. Therefore, the effect of temperature on the DPV response of enzyme electrode to NAD$^+$ reduction was studied. Under the optimal pH of 10.5, we changed the operating temperatures and assessed our biosensor current response. Figure 3b shows the influence of temperature on catalytic activity of the strip. As can be seen in Figure 3b, the oxidation current initially presented an upward trend with increasing temperature from 20 to 50° C. and subsequently, showed a downward trend when temperature was raised from 50 to 80° C. A 30° C. increase in temperature, resulted in five times higher enzymatic activity. Increasing the temperature up to 80° C. may denature the enzyme and may account for the descending behavior in the response curve. Improved enzymatic activity due to the increase in temperature raise may be deduced by looking at Arrhenius equation formulated as follows:

$$k = A \exp\left(-\frac{E_a}{RT}\right)$$

Where $k$ and $T$ represents the reaction rate and temperature, respectively. According to this formula, increasing $T$ will result in an increased reaction rate. Figure 3b shows the optimum catalytic activity of the electrode was significantly improved after enzyme immobilization indicating optimum temperature of 50° C. Immobilization of PDH on the nanocomposite surface yielded a more compact protein structure requiring more temperature for maximum catalytic activity. Like other chemical reactions, the improvement of enzyme activity with temperature increases (up to maximum values) can be attributed to the increased rate of both enzyme reaction and mass transport. The response decline after 50° C. may be caused by inactivation of the enzyme by continued heating or simply is above the optimal enzyme temperature.
3.3. L-phe sensing

In order to transform the rGNS/AgNPs/nafion nanocomposites into enzyme electrodes, the PEGylated PDH was blended with the conductive polymer nafion and subsequently electrodeposited onto the nanocomposite surface (Fig. 1). The conductive polymer grain size can alter the conductivity of the deposited film [7]. Variations in particle size and average thickness between electrodes was negligible, as the PEGylated PDH-nafion layer was electrodeposited under the same conditions for all 10 rGNS/AgNPs samples. Thus, the conductivity of the nafion and the amount of PEGylated PDH enzyme electrodeposited on each nanocomposite is considered to be equivalent.

3.3.1. CV experiment

The CV experiment of PEGylated PDH immobilized on the nanoactivated electrode was explored in 100 mM Gly/KOH/KCl (pH 10). Nanoactivated rGNS/AgNPs/nafion nanocomposites
acted as a super conductive substrate and PEGlayted PDH immobilized on the electrode was applied in order to construct a highly sensitive biosensor for phenylketonuria diagnosis. Figure 4a shows CV samples of the modified electrode in 0.1 M Gly/KOH/KCl buffer pH 10 at scan rate of 25mV/s. Two main pairs of typically reversible redox peaks of enzymatic reaction are clearly exhibited in all curves. A large increase in the oxidation current was observed at +893 mV vs. Ag/AgCl, showing that the potential the rGNS/AgNPs dispersed electrode is suitable for transferring electrons between the conductive substrate and the redox sites of the enzyme. With change in L-phe concentration, from 0.2 to 600 µM in 100 mM Gly/KOH/KCl (pH 10), a linear relationship between peak current and L-phe concentration can be observed (with a correlation of R= 0.998, Fig. 4b). The detection limit of CV test was 125 nM (S/N = 3).

3.3.2. DPV experiment

![Figure 4](image-url)

**Figure 4.** (a) Cyclic voltammogram of the enzyme electrode at scan rate of 25 mV/s containing 2.5 mM NAD⁺, 0.2 to 800 µM, of L-phe, respectively. (b) a linear response related to L-phe increasing in range of 0.2 to 800 µM. (c) Typical DPV plots of the biosensor in Gly/KOH/KCl buffer solution (pH 10) containing different concentrations of L-phe (0.15-800 µM). (d) Linear response attributed to L-phe increasing in range of 0.15 µM to 800 µM. Initial working volume: 10 µl; supporting electrode: 100 mM Gly/KOH/KCl buffer pH 10 containing 2.5 mM NAD⁺ (N=5).
The differential pulse voltammetry (DPV) technique was employed to further evaluate biosensor response due to its higher current sensitivity and better resolution than CV. As can be seen in Fig. 4d, upon addition of L-phe aliquot to the buffer, the response of the PDH-nafion modified rGNS/AgNPs/nafion biosensor to NAD$^+$ reduction increased linearly with a L-phe sensing range of 0.15 to 800 µM and a detection limit of 47 nM at an S/N ratio of 3. The sensitivity was $25 \times 10^{-3}$ A/(M.cm$^2$) (Fig. 4c,d). The analytical characteristics of this L-phe biosensor are much more sensitive as compared with some previously reported PDH-based biosensors (Table 1). Since the saliva L-phe level is normally maintained higher 10 µM [8], the linear sensing range from 0.15 to 800 µM would be suitable enough for the application of the biosensor in monitoring L-phe in saliva.

### Table 1. Comparison of our biosensor with previously published devices.

<table>
<thead>
<tr>
<th>References</th>
<th>Electrode</th>
<th>Detection limit</th>
<th>Linear range</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Our Biosensor</td>
<td>rGNS/AgNPs/nafion</td>
<td>47 nM</td>
<td>0.15-900 µM</td>
<td>&gt; 1 month</td>
</tr>
<tr>
<td>[8]</td>
<td>GOs-chitosan</td>
<td>416 nM</td>
<td>0.0005-15 mM</td>
<td>1 month</td>
</tr>
<tr>
<td>[20]</td>
<td>CNTs-Starch</td>
<td>12 µM</td>
<td>0.01–11 mM</td>
<td>1 month</td>
</tr>
<tr>
<td>[9, 10]</td>
<td>Dexte ran</td>
<td>0.5 mM</td>
<td>0.5-6 mM</td>
<td>16 days</td>
</tr>
<tr>
<td>[13]</td>
<td>Carbon paste</td>
<td>0.5 mM</td>
<td>8-80 mM</td>
<td>-</td>
</tr>
<tr>
<td>[12]</td>
<td>Cellulose triacetate</td>
<td>25 µM</td>
<td>0.05–9.1 mM</td>
<td>16 days</td>
</tr>
</tbody>
</table>

3.4. Lifetime performance, selectivity and reproducibility

The influence of possible interfering species on L-phe detection was examined using 3.0 mM glucose, uric acid, ascorbic acid, lysine, arginine, histidine and cholesterol, which caused an increase of 4.63% (max) or 1.82% (min) in the reducing or increasing the current response of the sensor, respectively (Fig. 5a). This result suggests that the proposed L-phe biosensor has high selectivity with no interference from endogenous electroactive substances due to its enzyme catalytic reaction. The amended L-phe biosensor retained more than 81% of its sensitivity after 32 days of performance, demonstrating the durable nature of the enzyme immobilization protocol with voltammetry testing and storage (Fig. 5b). The reproducibility of the L-phe biosensor was evaluated by comparison of the current responses of different electrodes under the same conditions. The current responses of ten different bioelectrodes fabricated in the same approach were tested independently, and the results were shown in Fig. 5c. As can be seen in figure 5c, the current response of ten enzyme test strips provided a relative standard deviation (RSD) value of 1.36 %, reflecting the superior reproducibility of the fabricated biosensor.
3.5. Application of the electrochemical biosensor for PKU detection in human samples

![Figure 5](image.png)

**Figure 5.** (a) Selectivity experiments of the enzyme electrode in presence of 3 mM glucose, uric acid and ascorbic acid. (b) Stability of the enzyme-functionalized electrode after 32 days in 2 mM L-phe concentration. (c) Effect of ten different electrodes fabricated in the same conditions on current response to 200 µM L-phe. Initial working volume: 10 µl; supporting electrode: 100 mM Gly/KOH/KCl buffer pH 10 containing 2.5 mM NAD^+ (N=5).

In order to further test the potential of the newly developed electrochemical biosensor for the determination of L-phe in real samples, we used our biosensor to determine L-phe in human serum samples using the standard addition method. The clinical range of L-phe in human blood and saliva is $C_{L\text{-phe}}>500.0$ µM and $C_{L\text{-phe}}>10.0$ µM, respectively for people with PKU. The serum sample was obtained from a healthy human donor (Pasture Institute of Iran) and was subsequently diluted 10 times.
with buffer solution (100 mM Gly/KOH/KCl buffer pH 10). Afterwards, ten diluted extraction with different L-phe spiked concentrations of 0.5, 1.0, 3.0, and 10.0 mM (in blood) and 0.0, 10.0, 20.0, 50.0 and 100.0 µM (in saliva) were analyzed by the biosensor. As shown in table 2, the recovery of the current response of the enzyme electrode is in the range of 93.91–103.02% and the relative standard deviation (RSD %) is in the range of 1.29–4.3%. The results demonstrated that the optimized biosensor could be applied for monitoring PKU in biological samples (blood and saliva). By comparing our work with other publications, it was observed that both recovery and relative standard deviation (RSD) values were sufficient for detection of L-phe in human serum samples, confirming that our enzyme based electrochemical biosensor has much potential for the detection of target L-phe in human samples.

Table 2. Determination and recovery results of L-phe in human samples, (N=5).

<table>
<thead>
<tr>
<th>Samples</th>
<th>The addition content</th>
<th>The detection content</th>
<th>RSD (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human blood (150 µM)</td>
<td>0.0</td>
<td>0.174 mM</td>
<td>4.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.612 mM</td>
<td>1.98</td>
<td>94.15</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.08 mM</td>
<td>3.21</td>
<td>93.91</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.21 mM</td>
<td>2.76</td>
<td>101.9</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>10.38 mM</td>
<td>2.75</td>
<td>102.27</td>
</tr>
<tr>
<td>Human saliva (3.5 µM)</td>
<td>0.0</td>
<td>3.76 µM</td>
<td>4.56</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13.84 µM</td>
<td>1.34</td>
<td>102.51</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>24.21 µM</td>
<td>3.26</td>
<td>103.02</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>52.78 µM</td>
<td>2.94</td>
<td>98.65</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>103.24 µM</td>
<td>1.29</td>
<td>99.75</td>
</tr>
</tbody>
</table>

4. CONCLUSION

The PDH-nafion immobilized rGNS/AgNPs/nafion biosensor exhibited high electron transfer properties and enhanced NAD$^+$ reduction electrocatalysis in comparison to comparable enzyme electrodes that use polysaccharides, multi-walled nanotubes or glucose oxidase as catalyst support. A more controllable, stable and reproducible nanocomposite film coated onto the test strip showed excellent L-phe biosensing capability due to the immobilization of PDH-nafion on the electrode. Our PDH based L-phe biosensor also exhibited excellent sensing performance with a linear response up to 900 µM with detection limit of 47 nM and also had higher selectivity over electroactive species. Other nanomaterials or mediators can be incorporated in the PDH-nafion modified biosensor for improved
performance. Therefore, this fabrication strategy opens new possibilities for non-invasive biosensor monitoring of L-phe in biological samples.

ACKNOWLEDGEMENT
We would like to acknowledge the Pasteur Institute of Iran and University of Meybod for providing support for the experimental studies.

References

© 2015 The Authors. Published by ESG (www.electrochemsci.org). This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/).