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Graphene oxide nanoribbon-based sensors for the simultaneous bio-electrochemical enantiomeric resolution and analysis of amino acid biomarkers

Aída Martín [a], Pilar Batalla [a], Javier Hernández-Ferrer [b], María Teresa Martínez [b], Alberto Escarpa [a,*]

[a] Departament of Anlytical Chemistry, Physical Chemistry and Chemical Engineering University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain
[b] Instituto de Carboquímica ICB-CSIC, Miguel Luesma Castán, 4, 50018 Zaragoza, Spain

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ABSTRACT

In this work, a straightforward in-situ measurement of L and D-amino acids (AAs) has been developed using disposable graphene oxide nanoribbon (GON) screen printed electrodes. For that, we took advantage of the electroactivity of certain clinically relevant AAs, such as tyrosine (Tyr) and methionine (Met), which are involved in important bacterial diseases (*Bacillus subtilis* and *Vibrio cholera*, respectively). The strategy is based on a dual electrochemical and enzymatic approach. The D-AA with the class enzyme D amino acid oxidase (DAAO) generates H_2O_2. This H_2O_2 is simultaneously detected with the L-AA, electroactive molecule by differential pulse voltammetry (DPV).

These GON disposable platforms use just 50 μL of sample and a total analysis time of 360 s. Both L and D enantiomers calibration and quantitative analysis were explored and were simultaneously detected with accuracy and precision in urine samples. Any interference of uric acid and other electroactive AAs was noticed.

This proposed electrochemical GON-based enantiomeric bio-sensor becomes a highly promising tool as future point of care for fast and reliable early diagnosis of diseases related to the presence of D-AAs.
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1. Introduction

The analysis of enantiomeric (L,D) amino acids (AAs) has been an incessant problem for scientists, because of their same physical and chemical properties. Although L-AAs have been the most studied enantiomer since they are involved in important physiological functions, D-AAs have recently shown special interest in excellent literature. As selected and relevant example, Lam and collaborators described how in *Vibrio cholerae*, a racemase produces D-Methionine (D-Met), whereas in *Bacillus subtilis* generates D-Tyrosine (D-Tyr) (Lam et al., 2009). These unusual D-AAs, which could be considered biomarkers of important diseases, appear to modulate the synthesis of peptidoglycan, a strong and elastic polymer that is a component of the bacterial cell wall (Siegrist et al., 2013). Likewise, D-Tyr has been recognized as an important biomarker, because its excess in plasma and in urine indicates the presence of renal failures in humans (Young et al., 1994).

For the D,L-Tyr determination, there are several methods based on gas chromatography coupled with mass-spectrometry (GC–MS), high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) (Bruckner and Schieber, 2001; Kitagawa and Otsuka, 2011; Tojo et al., 2012). However, the high cost of both equipments and assays, the high time consumption and the use of derivatized AAs have avoided its application to directly monitor in situ measurements as point of care (POC) testing tools. Other approaches such as microchip electrophoresis (Huang et al., 2011), molecular imprinted polymers (MIPs) (Liang et al., 2005) or complex strategies based on inclusion complexes for the resolution of D,L amino acids (Tao et al., 2014) have also been explored with high sensitivity. However, these techniques still require specific expensive technology and qualified training.

Considering the clinical relevance of this issue, this work proposes a fast, simple and reliable in situ approach for the simultaneous resolution and determination of the target D,L-AAs on disposable electrochemical (bio-)sensors. The strategy involves the simultaneous enantiomeric resolution and determination of both (i) the D-AA bio-sensing using a class-enzyme that selectively reacts with one of the enantiomers to yield hydrogen peroxide and, (ii) the direct electrochemical sensing of the L-enantiomer, taking advantage of its inherent electroactivity, using a suitable

* Corresponding author. Fax: +34 91 885 49 71.
E-mail address: alberto.escarpa@uah.es (A. Escarpa).

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graphene-based transducer. This strategy was applied to the simultaneous analysis of D and L Tyr and Met, as relevant target clinical AAs.

On the one hand, to assess the selectivity in the detection of the D-enantiomer biomarker as well as the complete quantitative reaction to hydrogen peroxide, we have used the enzyme D-amino acid oxidase (DAAO). This enzyme oxidatively deaminates D-AAs to the corresponding α-keto acids, without reacting with the corresponding L-AA.

On the other hand, the electrochemical sensing of the L-AAs needs to be selectively detected from the hydrogen peroxide, which is generated by the enzymatic reaction, with enough sensitivity and preventing the fouling. In this work, we introduce graphene to enhance these analytical requirements for the direct, selective and in situ electrochemical detection of the L-AAs involved in the racemic solution.

Graphene is a two dimensional net of carbon atoms bonded by sp^2 hybridization with extraordinary properties (Novoselov et al., 2004; Park and Ruoff, 2009; Lee et al., 2008), such as high surface area, excellent thermal and electrical conductivities, optical transparency, high mechanical strength and high elasticity, among others. However, beyond all their properties, its large surface area and high electrical conductivity have been interestingly exploited for sensing and biosensing purposes. Graphene family includes depending on the synthesis and applications a variety of different terms and structures (Martín and Escarpa, 2014). In this context, graphene nanoribbons (GNR) cover those synthesized by a controlled opening process or "unzipping" of carbon nanotubes (CNTs) (Terrones, 2009; Stankovich et al., 2006).

GNRs, fundamentally obtained by chemical oxidation of CNTs, are a really promising group of graphenes because they present very rich edge chemistry compared to pristine graphene. While pristine graphene has an inert chemical surface, the edges in GNR structure present functional groups which increase the reactivity and improve the electrochemical responses. The reactivity is mainly caused by the adsorption of analytes via π–π stacking interaction, with the non-oxydized benzene groups as well as, by electrostatic or hydrogen bond-based interactions with functional moieties located at the edges (Zhang et al., 2011). Despite the large and excellent analytical literature about graphene synthesized from graphite in electrochemical applications (Pumera et al., 2010; Shao et al., 2010; Kuila et al., 2011; Li and Xia, 2012), GNRs have not been so deeply studied as other graphenes in electrochemical applications (Martín and Escarpa, 2014). Herein, we hypothesize that the very rich edge chemistry of GNRs as well as the features derived from their high specific surface area such as electro-catalysis, enhanced sensitivity and their resistance-to-fouling offer us a suitable electrochemistry in this (bio)-sensing approach.

Despite the large number of applications employing DAAO to exclusively measure the generated H_2O_2 from the D-AAs by different electrochemical approaches (Lata and Pundir, 2013; Lata et al., 2013; Nieh et al., 2013; Sacchi et al., 2012; Wcislo et al., 2007; Wu et al., 2004; Dominguez et al., 2001) as far as we know, no examples of this enzyme coupled with graphene are found in the literature.

In sum, all these advantages of GNRs, in combination with the selectivity and sensitivity derived from the DAAO could constitute and excellent tool for the fabrication of highly specific sensors for enantiomeric analysis as simplified valuable alternative to the well-established technology in the field.

2. Materials and methods

2.1. Reagents, standards and samples

D-Tyrosine, L-tyrosine and L-methionine were purchased from Fluka Chemika (Buchs, UK) and hydrogen peroxide and D-methionine was obtained in Sigma Aldrich (St. Louis, MO, USA). Sodium dihydrogen phosphate was purchased from Panreac, (Badalona, Spain). D-Amino acid oxidase was purchased from Sigma Aldrich (St. Louis, MO, USA). All solutions were daily prepared using ultrapure water treated in a Milli-Q system (Millipore, Bedford, MA, USA). Urine samples 1:50 diluted were recollected from healthy individuals.

2.2. Apparatus and measurements

Field emission scanning electron microscopy (FE-SEM) micrographs were obtained on a JEOL Model JSM6335F equipment working at 5 kV. Direct determination of oxygen was carried out with a Flash 1112 analyzer from Thermo Fisher Scientific.

All electrochemical measurements were performed, at room temperature, on an USB-based portable electrochemical station μ-Stat 100 potentiostat controlled by PSLite 1.6 software and on a carbon screen-printed electrode (CSPE), which integrates the three-electrode system, a carbon working electrode of 4 mm diameter as well as a carbon counter and silver reference electrodes (Dropsens, Oviedo, Spain).

2.3. Preparation of graphene modified electrode

Graphene detailed synthesis and characterization features have been previously reported (Martín et al., 2014). In brief, graphene materials were synthesized from multiwall carbon nanotubes (MWCNTs) via the longitudinal unzipping method (Kosynkin et al., 2009). Using these oxidized nanoribbons (44 wt% oxygen content, 0.8 nm height by AFM, 46% C=O, 26% C–O, 19% Csp^3, 2% Csp^2 by XPS), reduced graphene oxide nanoribbons (14 wt% oxygen content, 1.2 nm height by AFM, 15% C=O, 10% C–O, 47% Csp^2, 10% Csp^3) were obtained by chemical reduction with N_2H_4/NH_3 (Gao et al., 2010).

Graphene oxide nanoribbons (GON) were dispersed to obtain a 0.5 mg mL^{-1} sample in water by ultrasonication in a bath for 30 min. Graphene reduced nanoribbons (GRN) were dispersed to obtain a 0.5 mg mL^{-1} dispersion in water/NH_3 (1% v/v) by ultrasonication in a bath for 30 min, followed by tip sonication using a VCX130, (Sonics, Newtown, USA) for 2 min at 130 W. The graphene nanoribbons modified electrodes were prepared by casting 10 μL of 0.5 mg mL^{-1} graphene nanoribbons on the CSPE surface. This volume was previously optimized by modifying the CSPE with different volumes 5, 10, 15 and 20 μL of 0.5 mg mL^{-1} and exploring its electrochemical behavior by cyclic voltammetry with 0.5 mM $K_3Fe(CN)_6$ in 1 M KCl on both graphene surfaces. The modified CSPEs will be named GON and GRN along this work.

2.4. Analytical procedure

Different concentrations and mixtures of D and L-tyrosine were prepared in sodium dihydrogen phosphate buffer adjusted with NaOH. The enzyme was also diluted in the same buffer. Both pH value and buffer concentration were optimized, as well as the enzyme concentration.

Following the optimal reaction conditions, 25 μL of a 0.50 mg mL^{-1} DAAO in 10 mM NaH_2PO_4 pH 7 was placed on the electrode. Immediately, 25 μL of the AA solution in 10 mM NaH_2PO_4 pH 7.0 was added, considering this time reaction 0 s. These total 50 μL of the solution permitted to cover the three

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