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Technical Note

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Enzyme-Based Microfluidic Chip Coupled to Graphene Electrodes for the Detection of D-Amino Acid Enantiomer-Biomarkers

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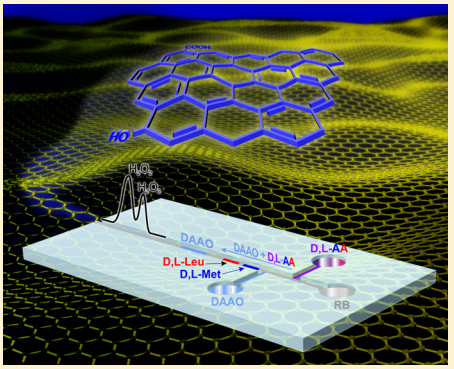
Supporting Information

ABSTRACT: An electrochemical microfluidic strategy for the separation and enantiomeric detection of D-methionine (D-Met) and D-leucine (D-Leu) is presented. These D-amino acids (D-AAs) act as biomarkers involved in relevant diseases caused by *Vibrio cholerae*. On a single layout microfluidic chip (MC), highly compatible with extremely low biological sample consumption, the strategy allowed the controlled microfluidic D-AA separation and the specific reaction between D-amino acid oxidase (DAAO) and each D-AA biomarker avoiding the use of additives (i.e., cyclodextrins) for enantiomeric separation as well as any covalent immobilization of the enzyme into the wall channels or on the electrode surface such as in the biosensor-based approaches. Hybrid polymer/graphene-based electrodes were end-channel coupled to the microfluidic system to improve the analytical performance. D-Met and D-Leu were successfully detected becoming this proof-of-the-concept a promising principle for the development of point-of-care (POC) devices for *in situ* screening of *V. cholerae* related diseases.



The D-enantiomers of amino acids (D-AAs) have been thought to have a minor role in the regulation of biological processes, with L-AAs being the ones that predominate in nature. However, it was reviewed that D-AAs can appear naturally in antibiotics, immunosuppressive drugs, and antitumor agents.[1] As selected significant examples, they prevent biofilm formation and can break down those already formed,[2] may regulate adult neurogenesis[3] (i.e., D-aspartate), and present a role as a neurotransmitter[4] (D-serine) with its availability altered in the nervous system of schizophrenic patients.[5]

Recently, relevant publications highlight that some bacteria produce types of D-AAs, which were not previously known to be synthesized and are present in the supernatants of the stationary phase cultures.[6,7] These works have revealed that different bacteria produce diverse D-AAs: *Vibrio cholerae* generates mainly D-methionine (D-Met) and D-leucine (D-Leu); whereas *Bacillus subtilis* produces principally D-phenylalanine (D-Phe) and D-tyrosine (D-Tyr), among others. The particular D-AAs identified in stationary phase supernatants varied among bacterial species,[6] being the proportion and amount of D-AAs characteristic of each bacteria. Consequently, these D-AAs biomarkers might become a molecular fingerprint. Some of these bacteria present clinical importance, for example, *V. cholerae* is the etiologic agent of the pandemic cholera disease. Hence, this information, based on the identification and detection of D-AAs is highly important and is expected to be extremely relevant in the foreseeable future.

Because of these biological and clinical relevancies, the development of screening methods for detecting different D-AAs in a biological sample has become a must. Since enantiomers present the same physical and chemical properties but react very differently, scientists have attempted for many years to find a simple and efficient method to identify and separate the enantiomers present in a mixture. Several methodologies have been reported to achieve this purpose. One of the most common methods is the chiral derivatization in combination with reversed-phase HPLC methods, using different reagents for the derivatization;[8,9] however, this methodology presents several disadvantages derived from the derivatization step. Hence, other HPLC strategies as chiral stationary phase and 2D-HPLC systems have been reported.[10,11] Capillary electrophoresis has been another technique used in the chiral separation of AAs using both direct and indirect approaches, with different chiral selectors and chiral reagents for diastereomers formation, respectively.[12]

Microfluidic chips (MC) offer excellent opportunities to carry out novel and creative analytical works. One of the most important applications of MCs is their role as point-of-care (POC) devices for clinical diagnosis as analytical screening tools because of inherent miniaturization, portability, and extremely low biological sample required.[13] Some related works for detecting AAs have employed fluorescence detection;[14,15] however, this detection is sophisticated and expensive, making it difficult to design a portable device for in situ monitoring of D-AAs. In this way, electrochemical detection in MC has been proved as an ideal and valuable analytical technique due to its

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inherent facility for miniaturization without loss of performance, high sensitivity, and extremely high compatibility with the micro and nanotechnologies.[16,17] Carbon nanomaterials have been explored as electrochemical detectors in MC since they enhance the analytical performance in terms of selectivity, sensitivity, and reproducibility. These nanomaterials offer low detection potentials, high amperometric currents, resistance to fouling, and good stability versus usage time.[18−22] However, while carbon nanotubes have been previously coupled to these devices, there are only a few examples with graphene.[17,23]

Another valuable merit of MC is its high compatibility with biological molecules for selective and sensitive biosensing.[24−26] In this sense, the use of D-amino acid oxidase (DAAO), which oxidatively deaminates D-AAs to the corresponding alpha-keto acid, generating 1 mol of H_2O_2 per mol of deaminated D-AA has been previously used for D-AA detection using a postcolumn approach.[27]

Here, we propose an electrochemical microfluidic strategy on a single MC allowing the separation and detection of D-AAs, D-Met and D-Leu biomarkers of *V. cholerae* as future portable devices (POCs) for bacterial screening in biological samples, where miniaturization and extremely low biological sample consumption are highly required. The strategy allows the controlled microfluidic manipulation of the D-AAs and the class-enzyme DAAO to govern both the D-AA separation and the specific reaction between DAAO and each D-AA biomarker avoiding any covalent immobilization of the enzyme into the wall channels or on the electrode surface such as in the biosensor-based approaches.[28] Hybrid polymer/graphene-based electrodes are end-channel coupled to the microfluidic system to improve the analytical performance.

EXPERIMENTAL SECTION

Apparatus and Measurements. All electrochemical measurements were performed at room temperature, using a Potentiostat Autolab PGSTAT101 from Eco Chemie. The high-voltage source was a LabSmith model HVS448 high-voltage sequencer with eight independent high-voltage channels and programmable sequencing (LabSmith, Livermore, CA).

The used analytical microchip was previously reported,[29] and some design adaptations were performed.[30] The microchip fabricated by Micralyne (model MC-BF4-001, Edmond, Canada) consisted of a glass plate (88 mm × 16 mm) with a four-way injection cross, a 74 mm long separation channel with a semicircular section of 50 μm width and 20 μm depth. The side arms, measuring 5 mm long, and separation channels have a double-T shaped. The amperometric detector (end-channel detection) consisted of an Ag/AgCl wire as a reference electrode, a platinum wire as a counter electrode, and a carbon screen-printed electrode (CSPE) as a working electrode of 0.3 mm × 2.5 mm (Dropsens, Oviedo, Spain). A spacer (easily removable adhesive tape, 60 μm thick) was placed between the surface of the electrode and the channel outlet to reproducibly control the distance between the electrode and the separation channel and to avoid interference from the electrical field applied for the separation in the detector.

Preparation of Graphene Modified Electrode. Carbon screen-printed electrodes (CSPE) for microchip coupling (0.30 mm × 2.50 mm) were modified following a two-step drop-casting protocol. First, 3 μL of a 0.50 mg mL^{-1} graphene reduced nanoribbons (GRNs) dispersion in H_2O/NH_3 (1%) were deposited (previously optimized, results are not shown) along the electrode. Second, a solution of 100 mg mL^{-1} polyethileneimine (PEI) or polyallylamine (PAA) was prepared in 5 mM sodium phosphate buffer pH 8.0. After 1 h, when the GRN modified electrode was totally dried, the second step of the deposition procedure took place. Then, 3 μL of the polymer preparation was deposited onto the electrode, covering all the length of the electrode with the solution. The modified electrode rested during 1 h until all the electrode was dried, before using it.

Estimation of the Electrochemical Effective Surface Area. To characterize the electrodes and in order to find the electrochemical effective surface area, the slope of the plot of Q vs $t^{1/2}$ obtained by chronocoulometry using 0.50 mM $K_3[Fe(CN)_6]$ in 0.1 M KCl have been calculated. This methodology is based on eq 1, given by Anson:[31]

$$Q(t) = \frac{2nFAcD^{1/2}t^{1/2}}{\pi^{1/2}} + \mathrm{Qdl} + \mathrm{Qads}$$

The different parameters of this equation are the following ones: A is the effective electrochemical surface area of the working electrode (cm^2), c is the concentration of the electroactive species (mol/cm^3), n is the number of transfer electron that is 1, and D is the diffusion coefficient, 7.6×10^{-6} cm^2 s^{-1},[32] Qdl is the double layer charge which could be eliminated by background subtraction, and Qads is Faradaic charge.

Electrophoretic Procedure and Amperometric Detection. Before the application of the microfluidic voltages as it will be described in the Results and Discussion, the channels of the glass microchip were treated before their use with 0.50 M NaOH for 40 min, being rinsed afterward with deionized water for 10 min. The running buffer, the enzymatic, the amino acid, and the detection reservoirs were suitably filled accordingly with the buffer media.

A detection potential of +0.70 V was applied to the working electrode placed in the detection reservoir, to analyze the hydrogen peroxide generated in the enzymatic reaction. All experiments were performed at room temperature.

RESULTS AND DISCUSSION

The optimized electrokinetic microfluidic protocol proposed in Figure 1 consists in (i) the simultaneous electrokinetic injection of DAAO (from enzymatic reservoir, ER) and AAs (from amino acid reservoir, AA) (A), (ii) the electro-focusing of the

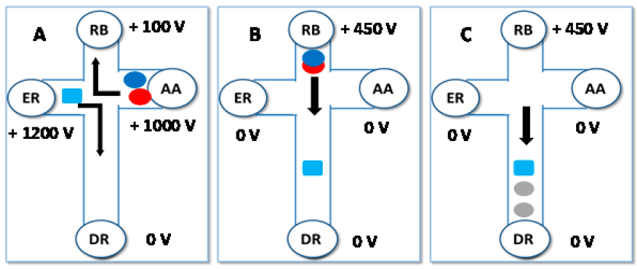

Figure 1. Electrochemical microfluidic strategy: enzyme and D-AAs electrokinetic injection for 5 s (A), enzyme and D-AAs electro-focusing (B), and D-AAs separation and in-channel enzymatic reaction and hydrogen peroxide detection (C). RB, running buffer reservoir; ER, enzymatic buffer reservoir; AA, amino acid reservoir; DR, detection reservoir. Color symbols: DAAO (blue square); D-Met (dark blue); D-Leu (red); H_2O_2 (gray). Conditions: 20 mM borate buffer pH 10.0; 0.50 mg mL^{-1} DAAO; 0.50 mM D-Leu; and 0.30 mM D-Met.

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DAAO and AAs (in downstream and in the upstream of the microchannel, respectively) (B), and (iii) the AAs separation, the selective in-channel reaction, and the hydrogen peroxide detection produced from each D-AA enantiomer after enzymatic reaction (in detection reservoir, DR) (C).

A simultaneous injection of the enzyme (from ER) and the AAs (Figure 1A) was developed by applying +1200 V in ER, +1000 V in AA, and +100 V in RB (running buffer reservoir) during 5 s with the DR grounded at 0 V. Then, for the AAs separation and selective in-channel reaction, RB was pumped at +450 V with all reservoirs grounded at 0 V (Figure 1B,C), until complete detection of hydrogen peroxide from each enantiomer was achieved.

The difference between the high voltages applied between ER (+ 1200 V) and DR (0 V) and between AA (+ 1000 V) and RB (+450 V) reservoirs was essential in order to electro-focus the AA in the upstream and the DAAO in the downstream of the channel. Indeed, applying the same high voltages (+1200 V) from both ER and AA did not give satisfactory results. In addition, the separation voltage applied from RB was optimized to +450 V for achieving the AA separation.

Second, enzyme concentration and in channel enzymatic reaction between DAAO and the target D-AA were carefully assayed in phosphate buffer at pH 8.0 since at this target pH the enzymatic reaction is favored.[33] From Figure 2A can be noticed

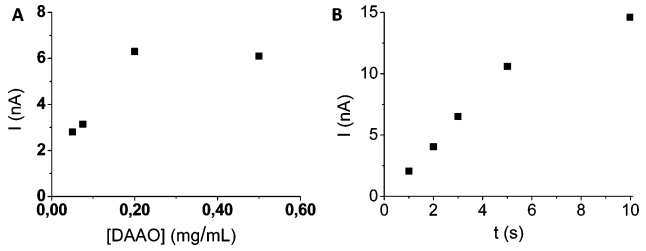

Figure 2. Effect of DAAO concentration on 1 mM D-Met amperometric signal. Conditions: 5 mM sodium phosphate buffer pH 8.0, detection potential +0.70 V (A). Effect of injection time on 0.50 mM D-Met amperometric signal. Conditions: 5 mM sodium phosphate buffer pH 8.0, 0.50 mg mL^{-1} DAAO, detection potential +0.70 V (B). Electrokinetic conditions as in Figure 1

that enzymatic activity increased with the enzyme concentration, revealing 0.50 mg mL^{-1} as the optimum value. The amount of DAAO electrokinetically injected was controlled by the high-voltage and injection time with high reproducibility. Figure 2B illustrates the influence of the injection time of the D-Met as the selected example on the hydrogen peroxide production. It was observed that increasing injection time; the hydrogen peroxide also increases revealing the suitability of the strategy. An optimum time of 5 s was chosen, since with longer injection times, sensitivity was not very enhanced and a well-defined peak shape was not obtained either.

Third, electrochemical detection of hydrogen peroxide, as product of the in-channel enzymatic reaction, was also carefully evaluated. In order to improve the signal obtained with bare CSPE, this electrode was modified with nanomaterials. GRNs previously characterized[34] (1.2 nm height, 3−4 layers, 120 nm × 50 nm, 47% Csp^2, 10% Csp^3 functionalities, and 14% total oxygen content) were evaluated following an off chip approach drop-casting the CSPEs. The edge chemistry is an important characteristic of these GRNs, because the edges present chemical functionalities such as carboxylic acid or carbonyl, among others. Different volumes of GRNs were deposited onto the CSPE for setting the optimal volume of graphene, detecting an improvement in the signal when the amount of GRNs increased. The optimal GRN volume was fixed as 3 μL because higher amounts raised the background signal.

Since the GRNs edge chemistry facilitates the interaction of the negatively charged carboxylic acid moieties of the GRNs with cationic amine groups of some polymers such as PAA and PEI, the influence of these polymers was also assayed. Indeed, the presence of the polymer points out an increase in the electrode effective electrochemical area[35] and provides the conducting bridges for the electron-transfer process. The modification with PEI always showed more electrochemical effective area than the ones modified with PAA (data not shown). So, the chosen electrode for the end-channel microchip detection was the CSPE modified with GRN and PEI, which henceforth will be named as GRN/PEI electrode. Figure 3 shows the impressive enhancement of the electro-

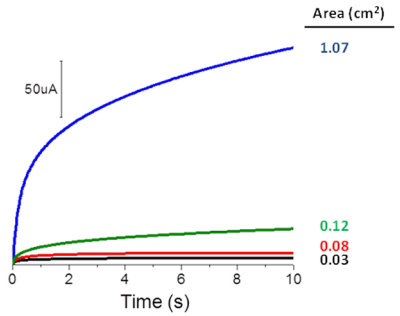

Figure 3. Estimation of electrochemical surface area of the different electrodes (0.30 mm × 2.50 mm): black (CSPE), red (GRN), green (CSPE modified with PEI), blue (GRN/PEI). Conditions: 3 μL of a dispersion of 0.50 mg mL^{-1} GRN plus 3 μL of a 100 mg mL^{-1} polymer preparation.

chemical area of this electrode more than 30 times bigger than the area of the bare CSPE when GRN/PEI was used as electrode modifiers.

Under optimized electrokinetic conditions, the detection potential was also carefully studied. Figure 4 shows the

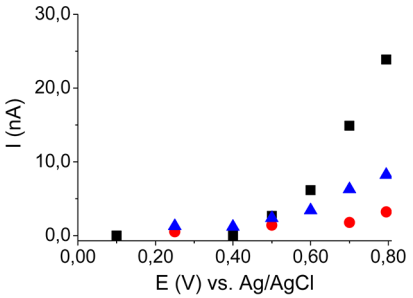

Figure 4. HDVs for 1 mM H_2O_2 on GRN/PEI (black) and CSPE (blue); 2 mM D,L-Met on GRN/PEI (red). Other conditions as in Figure 1.

hydrodynamic voltammograms (HDVs) for the involved molecules: H_2O_2, DAAO, and the moderate electroactive D,L-Met.[36] The optimal detection potential was found to be +0.70 V where H_2O_2 detection showed the best analytical sensitivity on the GRN/PEI electrode in comparison with bare electrode (as control), while D,L-Met did not exhibit electroactivity.

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Under these conditions, a complete isolation of the detection potential from the electrokinetic protocol was also noticed, exhibiting H_2O_2 detection excellent S/N characteristics. Also, as expected, DAAO did not exhibited electroactivity at any assayed potential.

Finally, separation of both AAs was carefully optimized using a 20 mM borate buffer at pH 10.0. Figure 5 illustrates the separation and D-enantiomeric detection of Met and Leu at +0.70 V accordingly to a capillary zone electrophoresis mechanism where both D-AAs remains negatively charged. The detection of D-Leu (pK_{a1} = 2.32, pK_{a2} = 9.58; Mr = 131.17 g mol^{-1}) was achieved through the detection of H_2O_2 with a well-defined peak that migrated at 568.5 ± 6.8 s. The H_2O_2 peak corresponding to D-Met (pK_{a1} = 2.16, pK_{a2} = 9.08; Mr = 149.21 g mol^{-1}) presented higher migration time of 727.8 ± 9.8 s. The sensitivity of the system allowed the detection of the D-AAs concentration indicated in the relevant literature (1 mM).[6] An excellent precision in migration times (RSD ≤ 2%; n = 5) and in peak heights (RSD ≤ 10%; n = 5) were also achieved indicating a very good precision and a very good electroosmotic flow stability. In contrast, when only L-amino acids (L-Met and L-Leu) were injected, no peaks were detected; as the enzyme does not react with L-enantiomers (Figure 5, control).

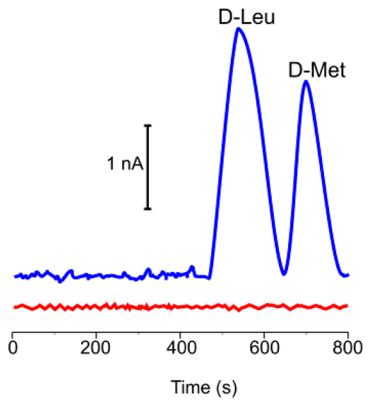

Figure 5. Microchip electropherograms corresponding to the reaction of 0.50 mg mL^{-1} DAAO with a mixture of 0.50 mM D-Leu and 0.30 mM D-Met (blue) and 0.50 mg mL^{-1} DAAO with a mixture of 0.50 mM L-Leu and 0.30 mM L-Met (red). Conditions: borate buffer (20 mM pH 10.0), detection potential +0.70 V. Electrokinetic conditions as in Figure 1.

CONCLUSION

We have demonstrated a simple and creative strategy which allows both the racemic resolution and detection of D-AAs involved in high significance diseases, such as those produced by *V. cholerae*, on a single layout MC, highly compatible with extremely low biological sample consumption. This approach improves on previous literature since it uses a simpler and commercial MC layout and geometry, extremely low amount of enzyme and offers a high versatility allowing the electro-focus of the enzyme along the main channel avoiding the use of additives (i.e., cyclodextrins) for enantiomeric separation as well as any covalent immobilization of the enzyme into the wall channels or on the electrode surface such as in the biosensor-based approaches.

This proof of the concept approach opens novel avenues for the detection of AA enantiomers on microfluidic systems, not only for clinical but also for agro-food relevant applications using commercial and available microtechnology, becoming a breakthrough for future development of POC devices applied to the detection of bacteria.

ASSOCIATED CONTENT

Supporting Information

Scheme showing the core of the strategy in the microfluidic chip (Scheme S-1). This material is available free of charge via the Internet at http://pubs.acs.org.

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All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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