Biosensors and Bioelectronics 96 (2017) 275–280

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Class enzyme-based motors for "on the fly" enantiomer analysis of amino acids

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ARTICLE INFO

Keywords:
Class-enzyme motors
Millimeter motors
Enantiomers
Phenylketonuria
Vibrio Cholerae

ABSTRACT

Here, two class-enzyme motors are properly designed allowing the rapid dispersion of the class-enzyme D-amino acid oxidase (DAO) and L-amino acid oxidase (LAO) for selective *"on the fly"* biodetection of D and L-amino acids (AAs), respectively. The efficient movement together with the continuous release of fresh class-enzyme leads to a greatly accelerated enzymatic reaction processes without the need of external stirring or chemical and physical attachment of the enzyme.

Ultra-fast detection (< 2 min) and accurate quantifications of L-phenylalanine (L-Phe) in plasma and whole-blood newborns samples diagnosed with *Phenylketonuria* and total D-AAs in *Vibrio cholera* cultures are pioneer illustrated as relevant examples of each enantiomer determination.

These results opens clearly novel avenues in biosensing for fast screening diagnostics, decentralized monitoring and design of future points of care.

1. Introduction

Analysis of L-amino acids (L-AAs) enantiomers is very important in metabolic rare diseases termed aminoacidopathies. One relevant example is *Phenylketonuria* which is an autosomal recessive metabolic disorder characterized by a dysfunctional or absent enzyme phenylalanine hydroxylase (PAH), which is responsible for the metabolism of the amino acid L-phenylalanine (L-Phe). With little to no functional PAH, Phe is accumulated in blood and brain of people who suffer *Phenylketonuria* (De Groot et al., 2010). Nowadays, there are no treatment for this disease, so dietary restriction of Phe is essential. Furthermore, people who suffer this disease have to carry out periodic control of their Phe levels.

Current diagnosis of *Phenylketonuria* is carried out by sophisticated laboratory methods, measuring of Phe level and its ratio with other amino acids using tandem mass spectrometry (MS/MS). This method is included in newborn screening program; however, other methods are still utilized because it is necessary to confirm the positive Phe value. Thus, bacterial inhibition assay, fluorimetric test, high performance liquid chromatography (HPLC), gas chromatography - mass spectrometry (GC/MS) or genetics test are also used (Blau et al., 2014). However, they are extremely time consuming, the complete process takes several days, and requires qualified staff. Therefore, novel screening strategies for rapid diagnosis and further control by the patient are highly needed.

Moreover, until 30 years ago, it had been considered that D-amino acids (D-AAs) enantiomers were excluded from most of living systems, and it were only in the cell wall of microorganisms. However, D-AAs, in the form of free amino acids, peptides and proteins, have been identified in various living organisms from bacteria to mammals (Yang et al., 2003). One of these works have revealed that *V. cholerae* (the etiologic agent of the pandemic cholera disease) generates mainly D-Met and L-Leu (Lam et al., 2009).

Various techniques, such as spectrophotometry (Sarkar et al., 1999; Sacchi et al., 1998), HPLC (Visser et al., 2011; Hamase et al., 2007) and electrophoresis (Huang et al., 2009), have also been employed for determination of these D-AAs amino acids in food and biological samples. Despite being highly sensitive, these methods have some limitations, such as labor-intensive, time-consuming, sample pretreatment, expensive equipment and require a skilled person to operate.

Hence, identification and detection of D-AAs is also highly important and is expected to be extremely relevant in the near future. Because of these biological and clinical relevancies, the development of screening methods for detecting different D-AAs in a biological sample has become necessary, although rarely they have been reported (Batalla et al., 2015).

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http://dx.doi.org/10.1016/j.bios.2017.04.051
Received 17 March 2017; Received in revised form 26 April 2017; Accepted 29 April 2017
Available online 06 May 2017
0956-5663/

On the other hand, self-propelled motion of miniaturized objects through fluid environments constitutes nowadays an exciting field (Wang, 2014, 2016; Maria-Hormigos et al., 2016; Jaideep et al., 2017; Kherzi and Pumera, 2016). One relevant example constitutes the Marangoni effect-based motors which describes the movement of the object based on surface tension gradient. Objects move from lower surface tension to higher surface tension areas in an attempt by the system to attain the desirable lowest-free-energy state (Zhao and Pumera, 2012). The surface tension gradient can be generated by asymmetric release of the chemical from the object or by modification of the surface the object moves on (Sharma et al., 2012).

Some relevant examples about the development of this kind of motors have been recently presented for environmental remediation, (Zhao et al., 2011; Seah et al., 2013; Moo and Pumera, 2015) efficient biocatalytic degradation of chemical pollutants (Orozco et al., 2014) and more recently for visual hydrogen peroxide detection (Moreno-Guzman et al., 2015). However, the use of these motors are still underexploited in other complex analytical applications involving tedious and high consuming time protocols, qualified users and expensive instrumentation (Jurado-Sánchez and Escarpa, 2016).

Here, we are proposing the use of conical motors in millimeter size for analysis of amino acids enantiomers in highly relevant clinical samples. To this end, two class-enzyme motors are proposed. The concept relies on the self-propelled motion by the Marangoni effect, where the asymmetric release of surfactant induces fluid convection and rapid dispersion of the class-enzyme D-amino acid oxidase (DAO) and L-amino acid oxidase (LAO) for each selective detection of D-AAs and L-AAs, respectively. The efficient movement together with the continuous release of fresh class-enzyme leads to a greatly accelerated enzymatic reaction processes without the need of external stirring or chemical and physical attachment of the enzyme as in common classical biosensing approaches.

By the first time, detection of L-Phe in plasma and even whole blood newborn samples with diagnosed *Phenylketonuria* and the detection of total D-AAs in cultures *of Vibrio cholerae* were chosen as relevant examples of each enantiomer determination.

2. Experimental

2.1. Reagents and solutions

Polycarbonate membranes were purchased from Whatman, Maidstone, UK (25 mm diameter and 0.3 μm pore diameter). $CuSO_4 \cdot 5H_2O$ (Sigma) (7791-20-0 Sigma-Aldrich) solution of 1 M in 0.3 M H_3BO_3 (456671 Fluka) was used to fabricate the copper microwires electrodes. CH_2Cl_2 (75-09-2 Sigma-Aldrich), C_3H_8O (67-63-0 Sigma-Aldrich) and C_2H_6O (64-17-5 Sigma-Aldrich) were also use to cut the membrane with the desired size and shape, biopsy punch of 4 mm diameter was used (Biopunch).

A 10 mM stock solution of D-Val, D-Met, D-Leu, D-Phe and its L-AAs (Sigma-Aldrich) were prepared by suitable dilution with a 0.1 M phosphate buffer solution pH =7.0. Sodium dodecyl sulfate (SDS) was acquired from Merck (Darmstadt, Germany), SDS solution was prepared in Milli-Q water.

Graphene oxide (GO) 2 mg/mL (dispersion in H_2O) was purchased from Sigma-Aldrich (St. Louis, MO, USA). GO was dispersed to obtain a 0.1 mg/mL sample in H_2SO_4 (Sigma) 0.1 M and Na_2SO_4 (Sigma) 0.5 M by ultrasonication in a bath for 30 min, followed by tip sonication using a VCX130 (Sonics, Newtown, USA) for 2 min at 130 W.

DAO from porcine kidney, ≥1.5 units/mg solid and LAO from *Crotalus adamanteus* Type I, ≥0.3 unit/mg were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme solution was prepared in 0.1 M phosphate buffer solution pH =7.0. All other reagents were of the highest available grade.

2.2. Samples

Whole blood and plasma samples were collected from healthy and ill newborns of *Phenylketonuria*. The samples were provided and diagnosed by Centro de Diagnóstico de Enfermedades Moleculares (CEDEM, Madrid, Spain).

For *Phenylketonuria* analysis, 50 μL of newborns blood samples (healthy and diagnosed with *Phenylketonuria*) on 6 mm disk of Whatman 903 paper were placed at the bottom of a tube using tweezers. Then, 1 mL of milli-Q water was added, sonicated for 15 min and an aliquot of 300 μL was diluted in a final volume of 3.0 mL in the Petri dish releasing motor navigation. Analysis of newborn plasma samples was carried out directly without pretreatment taking 30 μL of sample and then diluted as above.

D-AAs containing supernatants from stationary phase (18 h) cultures of *V. cholerae* grown in LB (Luria-Bertani) liquid medium. Releasing of D-AAs, mainly D-Met and D-Leu, in the stationary phase of these bacteria cultures are known to have concentrations around 1 mM (Lam et al., 2009). Before their analysis, samples were pretreated by boiling at 100 °C during 30 min, followed by centrifugation at 15.500 rpm for 15 min, and subsequent filtration by a hydrophilic filter. These solutions were diluted 1:10, 1:20 and 1:100 of the raw sample were analyzed by triplicate and the presented D-AAs were determined.

2.3. Apparatus and electrodes

All electrochemical measurements were carried out using an Autolab PGSTAT 12 potentiostat from Metrohm (Utrecht, The Netherlands). The electrochemical software was the general-purpose electrochemical system (GPES) (EcoChemie B.V.). Autolab type II potenciostat (Eco Chemie, Utrecht, The Netherlands) to achieve microwires electrodeposition. All experiments were performed at room temperature.

The Electrochemical Impedance Spectroscopy (EIS) measurements were performed, at room temperature, on an electrochemical station PGSTAT-204 (Autolab, Utrecht, Holland).

Carbon screen printed electrodes (DS 110, Dropsens, Oviedo, Spain) were used to be modify by GO by casting 10 μL on the carbon working electrode. Copper thick-film electrodes were also purchased of Dropsens (DRP-Cu10, Dropsens, Oviedo, Spain). These electrodes include a silver pseudo-reference electrode and a carbon counter electrode.

Copper microwires electrodes were fabricated by template electrodeposition protocol using a cyclopore polycarbonate membrane (0.3 μm pore diameter). To this aim, one side of the membrane was sputtered with a gold film (75 nm), which was used as the working electrode during the electrodeposition. Copper microwires electrodes were electrodeposited at −1.0 V for −30 C on the pores of the membrane. The membrane was, next, cut with the desired shape (circle of 4 mm diameter) with a biopsy punch (Biopunch, Redding, US) and stuck in double-sided adhesive tape (Fixo, Spain). After that, the membrane is removed to obtain the copper microwires by immersing in dichloromethane 30 min and then, subsequently, gently rinsed in isopropanol, ethanol and water. The adhesive tape, non-conductive substrate, with orientated microwires was directly stuck as working electrode. This fact, provide stability to the final electrode and then it is pasted into a ceramic substrate with two additional connections for reference and counter electrode. Conductive silver paint (Electrolube, UK) for create the electric contact and epoxy protective overcoat (242-SB, ESL Europe) for the isolation were used.

To generate copper oxides, copper microwires and copper thick-film electrodes were activated using 0.1 M NaOH (+0.7 V, 150 s) before use.