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Superior sensing performance of multi-walled carbon nanotube-based electrodes to detect unconjugated bilirubin

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ABSTRACT

The direct electrochemical behaviour of bilirubin in the physio-pathological concentration range and at physiological pH was investigated by cyclic voltammetry. Nanostructured electrodes with a thin film of multi-walled carbon nanotubes exhibited a higher sensing performance than bare electrodes. The detection limit obtained with nanostructured electrodes (4.2 ± 0.1 μM) allows the detection of both normal and pathological levels of bilirubin. Due to its sparse solubility in aqueous solvents, in human fluids bilirubin is found in the form of soluble complex with albumin. Therefore, the nanostructured-sensor response was studied in presence of different concentrations of this protein. A signal weakening was observed with increasing concentrations of albumin due to the decrease of free bilirubin. Finally, bilirubin detection was tested at concentrations typical of newborn jaundice (200–500 μM) and in the presence of normal albumin levels. A detection limit of 9.4 ± 0.3 μM was identified. Since this value is below the minimum critical bilirubin concentration for newborns, our sensor, modified with a thin film of carbon nanotubes, could potentially be used for bilirubin detection in cases of newborn jaundice.

1. Introduction

Bilirubin (BR) is a tethrapyrrole compound of bile. It is a brownish yellow pigment, produced when the liver breaks down old red blood cells. BR is a natural anti-oxidant in human blood [1]. Two main types of BR are present in human fluids: conjugated and unconjugated BR. Conjugated BR forms a complex with gluconic acid, which renders it water soluble. Unconjugated BR, instead, tends to bind to albumin [2]. Therefore, the amount of free unconjugated BR in serum depends on the concentration of albumin and on the intrinsic ability of albumin to bind BR. This binding is very important for the neutralisation of the neurotoxic effect of free unconjugated BR.

An accurate quantification of BR in body fluids is important for diagnostic and therapeutic purposes. The normal level of total BR in the serum of adults ranges from 3.5 to 22.6 μM (0.2–1.3 mg/dl) [3]. Higher and lower concentrations are associated with certain diseases. For instance, jaundice, caused by high BR levels in the blood, is associated with gallbladder and liver diseases (e.g., cirrhosis, hepatitis), blood infection, transfusion reaction, or haemolytic diseases of the newborn (cell destruction) [4]. Conversely, low levels of BR are associated with anaemia and coronary artery diseases [4]. If untreated, high concentrations of unconjugated free BR in neonates can lead to brain damage (hearing loss and "Kernicterus", a potentially lethal syndrome [5]). Very often pathological levels of BR are associated with the accumulation of its oxidised form, a pigment called biliverdin (BV) [6]. Therefore, considering the diagnostic significance of BR, the development of an inexpensive device for its detection is of great interest.

There are several methods to determine the concentration of BR. It can be measured by direct spectrophotometry [7] or by diazo reaction [8]. However, the former method may be affected by the interference of other proteins, and the pH-dependence of the latter could partially compromise the measurement [9]. Colorimetric [10] and fluorometric analysis [11] can also be used to quantify this metabolite. Amperometric detection is simpler and more convenient if compared to the above-mentioned techniques. It can be performed by using the *BR oxidase* (BODx) immobilised onto the electrode surface. BODx catalyses the oxidation of BR to BV. Unfortunately this enzyme is highly unstable. Some strategies have been employed to overcome this problem (conductive polymers [12], mediators [13] and cross-linking agents [14,15] as well as multilayer enzyme networks [13]). Alternatively, the instability related to the enzymatic detection could be solved by exploiting the spontaneous conversion of BR to BV, occurring once suitable potentials are applied.

BR is liposoluble at physiological conditions but poorly soluble in laboratory aqueous solvents. Therefore, the electrochemistry of BR has been investigated in detergents such as Tris Buffer [13,16] or in organic solvents such as dimethyl sulfoxide (DMSO) [17], dimethyl formaldehyde [18] and room temperature ionic liquids [19]. To simulate the physiological environment, some authors have also investigated the

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electrochemistry of BR in aqueous solutions [12,14,15,20]. In all these works, a BR stock solution was prepared by dissolving it in a basic solvent. Lower BR concentrations were subsequently obtained by dilution in aqueous buffers.

Even if BR is an electroactive compound, the possibility of detecting this molecule within its normal concentration range and in the presence of albumin is a problematic aspect of the development of a BR biosensor. To this end, the modification of electrodes with metal nanoparticles and/or nanotubes has proven to be a powerful route to tune the sensor performance [21]. Indeed, nanomaterials improve the electrodes' electrocatalytic properties by increasing their active surface area and thanks to their quantum size effect [21]. Among them, *carbon nanotubes* (CNTs) are interesting components for the electrochemical transducers. CNTs are graphene cylinders formed by rolling one or more graphene sheets, resulting in *single-walled* (SW) or *multi-walled* (MW) CNTs. SWCNTs are found to exhibit metallic, semi-conducting or small-band-gap semiconducting properties, depending on their diameter and chirality. MWCNTs instead are mostly metallic since a single metallic layer results in an entire tube displaying a metallic behaviour. Thus, MWCNTs are better candidates for electrochemical applications [22]. The electrochemical detection of BR with MWCNTs has been carried out in previous works, but ferrocene was used as a mediator [16] and the experiments were performed in the absence of albumin [16].

In the present work, we compare the electrochemical behaviour of bare *screen-printed electrodes* (SPEs) in the presence or absence of a film of MWCNTs in order to detect physio-pathological concentrations of unconjugated BR by *cyclic voltammetry* (CV). Furthermore, the response of modified-SPE was studied in the presence of albumin up to its normal level, and concentrations of BR complexed to albumin were measured in the range typical of newborn jaundice.

2. Materials and methods

2.1. Chemicals

A dispersion of carboxyl group (−COOH) functionalized MWCNTs (DropSens, Spain) was prepared as previously described [23]. The dispersion was sonicated before use to guarantee homogeneity. Stock solutions of BR (Sigma) were prepared in DMSO solvent (10 mM). *Phosphate buffered saline* (PBS 10 mM pH 7.4) was used for dilutions in the presence or absence of fixed bovine albumin at concentrations ranging from 0 to 30 mg/ml. PBS and albumin were purchased from Sigma. Since BR is photosensitive, measurements were carried out in a dark room.

2.2. Electrode preparation and electrochemical apparatus

Electrodes were nanostructured with MWCNT films by casting 30.00 ± 0.12 μl of MWCNT-chloroform solution (six times 5.00 ± 0.02 μl) onto the working electrode of carbon paste SPEs (model DRP-110) purchased from DropSens (Spain). Chloroform was allowed to evaporate from this electrode in between two subsequent deposition steps. The surface area of the working electrode was equal to 12.54 mm^2. The counter electrode was also made of graphite, while the reference electrode was made of Ag AgCl. The electrochemistry of BR was investigated by CV with a Versastat 3 potentiostat (Princeton Applied Technologies). All the experiments were carried out under aerobic conditions at room temperature. During the measurements, the three electrodes were covered with 100 μl of BR-containing solutions. Multiple CVs were acquired using a potential window of −0.4 /+ 0.8 V at a scan rate of 20 mV/s. BR concentrations ranging from 50 to 150 μM by 25 μM steps were used. Electrodes with and without a MWCNT layer were tested. Five multiple CVs were applied, alternating them until two subsequent voltammograms overlapped. The adsorption of BR onto a large variety of carbon nanomaterials has been reported [24]. In addition, the well-known adsorption of albumin to the electrodes could also affect the electrochemical measurements [25]. For these two reasons, in between two subsequent measurements a cleaning procedure was performed by applying potential pulses as reported in [26]. Briefly, we used 3000 fast potential pulses between −0.4 and +0.8 V, repeated 3 times, and 10 multiple CVs (potential window −0.4 /+ 0.8 V and scan rate 100 mV/s).

The two anodic peak currents (Peak I and II) identified in the V cycle of multiple CVs were used to calculate the sensing parameters. A cubic baseline was subtracted to the voltammogram part (positive scan) between 0 and 700 mV. The Igor Pro (Wavemetrics,Lake Oswego, OR, USA) software was employed to fit the two peaks using Gaussians [27] that well described their shapes. The peak positions and heights were optimally fitted by minimising the chi-square, as described in the program package. A flowchart of the adopted procedure is depicted in Fig. 1. The sensitivity was calculated from the slope of the straight line obtained from peak currents *vs.* BR concentration plot. Sensitivity was normalised to the electrode area. The standard deviation $\left(\delta\bar{i}\right)$ of a voltammogram portion in PBS was taken as black signal. The *detection limit* (LOD) was calculated according to the expression $LOD = 3\delta\bar{i}/S$ [28] where S is the sensitivity in μA/mM.

3. Results and discussion

3.1. Electrolyte optimization

Among several possible strategies, the preparation of the BR stock solution using NaOH was deemed unsuitable because of the rapid precipitation [29] and oxidation of BR. Solutions prepared with Tris Buffer (0.05 M, pH 8.0, albumin 30 mg/ml) were also excluded since no peak current could be measured with either bare or nanostructured electrodes. DMSO, instead, was proved to be an efficient solvent for BR. Dilutions with PBS 0.01 M allowed BR detection. They were stable for hours with regard to both oxidation and precipitation processes. All the solutions were prepared daily because of the characteristic BR instability.

3.2. Cyclic voltammetry with bare and nanostructured SPEs

Cyclic voltammetry of BR by using bare SPEs and SPEs nanostructured with MWCNT thin films revealed three oxidation processes. In the investigated BR concentration range (50–150 μM), the first oxidation process (Peak I) appears at an average potential of 252.1 ± 3.3 mV and the second (Peak II) at 481.7 ± 2.0 mV. The positions of the two peaks vary slightly in the presence or absence of a layer of MWCNTs. The third process occurs at higher potentials and only with MWCNT film-based SPEs. All these oxidation processes were found to

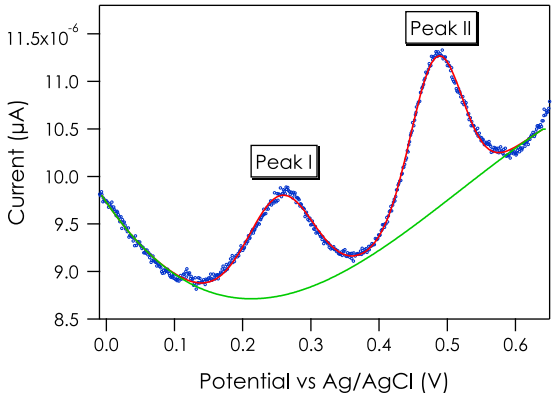

Fig. 1. Fitting example of the two voltammetric peaks by using Gaussian curves and a cubic baseline (BR concentration: 150 μM).