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Short communication

Efficient voltammetric discrimination of free bilirubin from uric acid and ascorbic acid by a CVD nanographite-based microelectrode

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ABSTRACT

We report a novel electrochemical sensor based on nanographite grown on platinum microelectrodes for the determination of bilirubin in the presence of normal concentrations of albumin. The albumin is a protein with an intrinsic ability to bind the bilirubin therefore reducing the concentration of the free electroactive metabolite in human fluids. In addition, the proposed device permits the discrimination of free bilirubin from two interferents, uric acid and ascorbic acid, by the separation of their oxidation peaks in voltammetry. Preliminary measurements in human serum prove that the proposed nanostructured platform can be used to detect bilirubin.

1. Introduction

Bilirubin (BR), a yellow pigment resulting from the breakdown of old red blood cells, is normally present in low concentrations in human plasma. It is conjugated with glucuronic acid in the liver and is removed from the body via the bile in the stool. BR is poorly soluble in water at physiological pH [1]. Free water-insoluble BR is associated with its characteristic toxic effects (destruction of nerve cells in the basal ganglia of the brain, also called "Kernicterus"). Under normal conditions, albumin neutralises the neurotoxic effects of BR by forming a water-soluble complex BR-albumin. In neonates, the BR level is higher because, after birth, red cells are removed from the circulation with degradation of haem to BR, while the normal liver mechanisms, whereby BR is conjugated to glucuronic acid, are underdeveloped, even in a term infant [2]. Consequently, a severe jaundice occurs more easily leading to brain damage and cerebral palsy [3]. In recent years, the high probability of brain injury from hyperbilirubinemia has led to an increased research of suitable devices for an early tracking of the BR-induced neurotoxicity. Therefore, the determination of free BR is an urgent need [4].

Electrochemical sensors are simple and rapid tools to detect free BR in human serum and aqueous solutions. This is due to the intrinsic electroactivity of BR. Unfortunately, the electrochemical determination of BR is challenging. The most part of BR is attached to albumin so that the free BR concentration is very low (some hundreds of nanomolars). Moreover, other electroactive metabolites such as uric acid (UA) and ascorbic acid (AA) are normally present in human serum. By using common electrodes, these three compounds oxidize at almost the same potential resulting in the overlapping of the voltammetric response.

To decrease the detection limit, two strategies are currently employed. The miniaturisation of electrodes increases the mass transport rates, reduces the ohmic drop, significantly enhances the *S*/*N* ratio and allows us to measure in extremely small environments and in highly resistive media [5]. The use of nanomaterials offers unique advantages related to the increase of surface area and to their excellent electric properties [6]. Moreover, a voltammetric discrimination of electroactive metabolites that oxidise at almost the same voltage has been recently achieved by modifying electrodes with carbon nanomaterials [7].

In this study, we voltammetrically detected free BR in the critical concentration range for newborns and in the presence of

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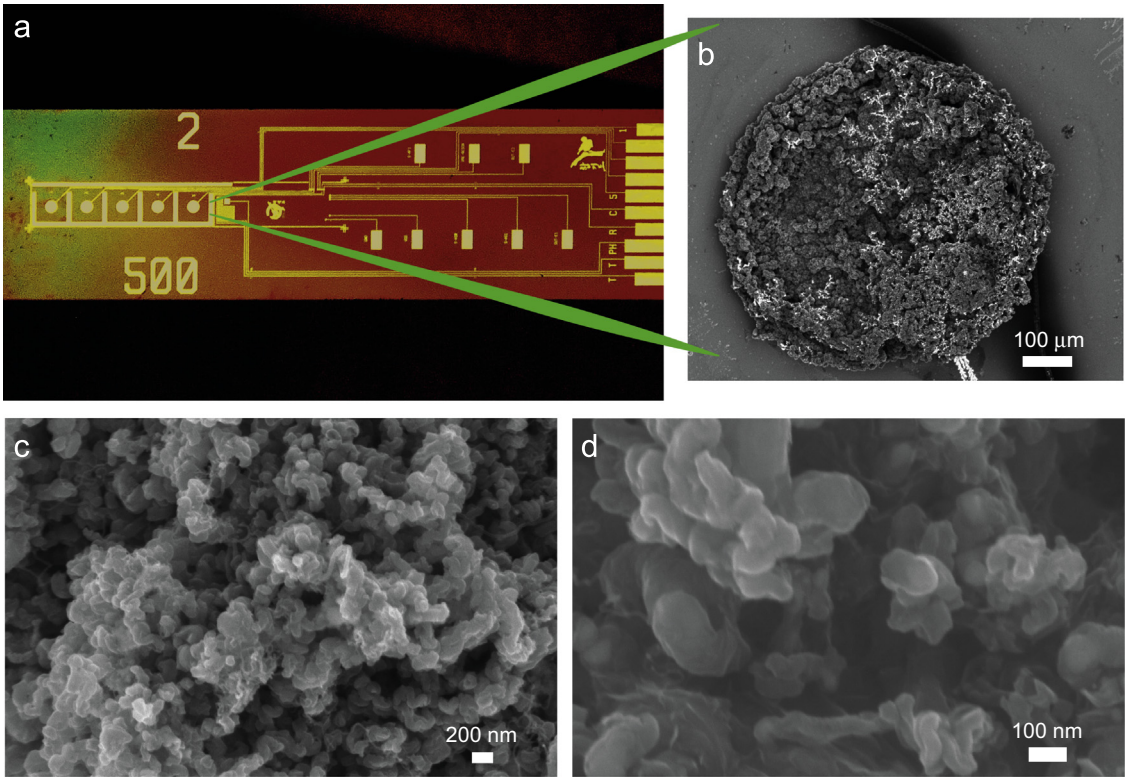

Fig. 1. (a) Optical image of the electrochemical device with five sensing sites; (b) SEM image of nanographite selectively grown on one Pt working microelectrode of the device; SEM image of nanographite at lower (c) and higher (d) magnification.

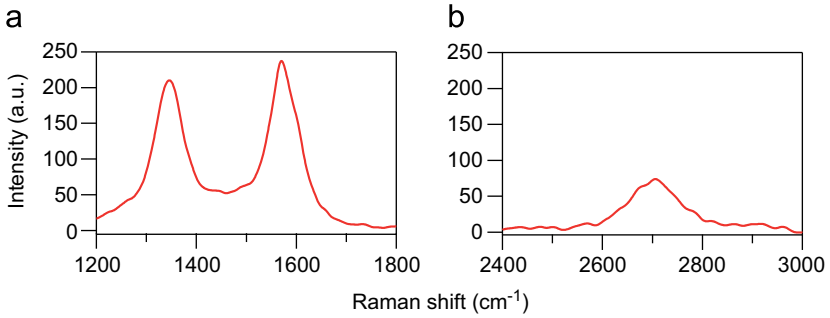

Fig. 2. Raman spectra for carbon nanomaterials grown from 15 s electrodeposited Fe_2Co at 600 °C in the ranges (a) 1200–1800 cm^{-1} and (b) 2400–3000 cm^{-1}.

the normal level of albumin. A platinum microelectrode, nanostructured with directly CVD grown nanographite, was used as a working electrode. Counter and reference electrodes were in the electrochemical platform, both made of Pt. The sensing performance did not vary in the presence of two major BR interferents: UA and AA. The sensor efficiently discriminates the oxidation peaks of the three metabolites. Preliminary studies show the efficiency of this nanostructuration approach to measure BR in human serum.

2. Materials and methods

The device was microfabricated as previously described [8]. Then, a Fe_2Co coating was electrodeposited on the working microelectrodes from iron and cobalt sulphate-based solutions (molar ratio 2:1) by applying −1.4 V [9]. Nanographite grew on a 30 s electrodeposited layer in a CCVD quartz tube at ambient pressure. The synthesis steps were 10 min of annealing under H_2 and Ar flow (60 l/h) and introduction of C_2H_2 and CO_2 (ratio 1:1; flow 0.25 l/h) for 5 min (Ar flow 45 l/h). The furnace temperature was fixed at 600 °C [9]. A Zeiss MERLIN Scanning Electron Microscope was used to investigate the morphology of the carbon materials. Raman spectra were acquired using a homemade micro-Raman microscope as reported in [9]. BR (Sigma) was dissolved in DMSO solvent (10 mM). Stock solution of UA (Sigma) was prepared in boric acid (20 mM, pH 9, BioChemica, AppliChem). AA (Sigma) was dissolved in distilled water obtained with Eau ultra pure, type Ultra Clear™ (BLANCLABO, Switzerland). Dilutions were made in Phosphate Buffered Saline (PBS 10 mM, pH 7.4, Sigma) containing bovine albumin (30 mg/ml, Sigma) or in human serum (VWR). Measurements were carried out in a dark room by using an Autolab potentiostat under aerobic conditions. Cyclic and Square Wave Voltammograms (CVs and SWVs) were registered at scan rates of 10 mV/s and 15 mV/s, respectively, for concentrations of BR ranging from 100 µM to 500 µM by steps of 100 µM. The sensitivity was calculated from the slope of the straight line obtained from the plot peak currents normalized to the electrode area *vs* BR concentration. The peak positions and the heights were computed by curve fitting with Gaussians. A cubic

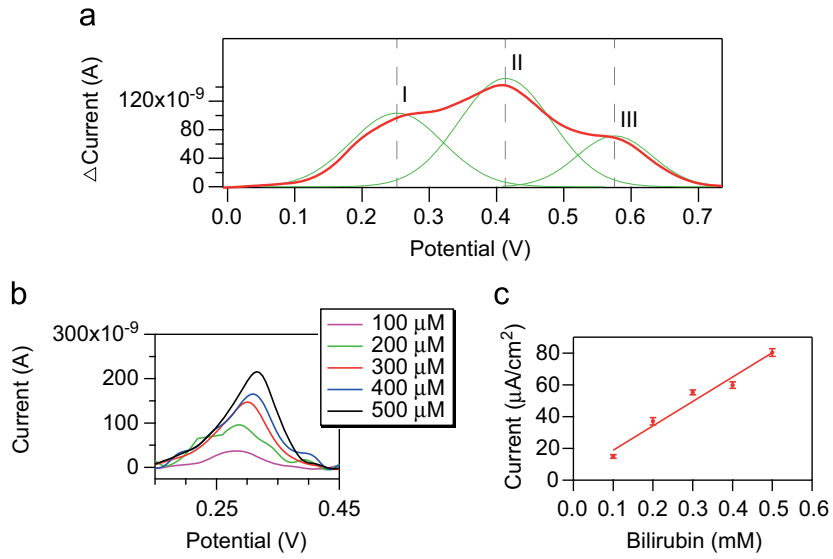

Fig. 3. (a) Background subtracted voltammogram portion comprising the three oxidation peaks of BR at the nanostructured electrode (BR: 600 μM; albumin: 30 mg/ml). Peak I: BR oxidation to biliverdin. Peak II: biliverdin oxidation to purpurine. Peak III: purpurine oxidation to choletelin. (b) SWVs in PBS solutions containing 100, 200, 300, 400 and 500 μM BR and a normal level of albumin. (c) Calibration curve for BR.

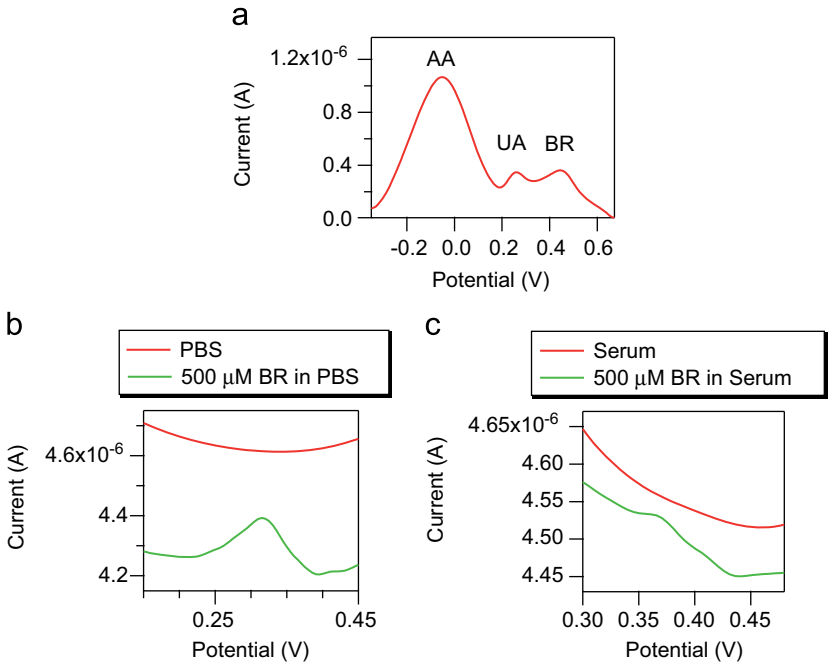

Fig. 4. (a) Electrochemical oxidation of AA (200 μM), UA (100 μM) and BR (400 μM) by SWV in PBS solution containing 30 mg/ml of albumin. (b) Peak II in PBS solution containing albumin (red line) and in PBS solution containing albumin and 500 μM of BR (green line). (c) Peak II in serum (red line) and in serum containing 500 μM of BR (green line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

baseline was subtracted before each fitting. The detection limit was calculated according to the expression $LOD = 3\delta\bar{i}/S$, where S is the sensitivity in μA/mM and $\delta\bar{i}$ the average standard deviation of three overlapped black measurements [10].

3. Results and discussion

In previous works, we reported a voltammetric detection of free BR in synthetic medium either without or with normal albumin concentrations. In the presence of albumin, BR was measured in the critical concentration range for newborns. In this case BR detection was possible only with commercial macroelectrodes modified with multi-walled carbon nanotubes [11]. Scaling down of analytical devices gives undisputed sensing advantages [12]. In addition, the need of miniaturised portable medical sensors is particularly urgent for clinical test and home use [12]. Here, an electrochemical device was microfabricated. Multiple working microelectrodes of the sensor were modified by a direct and selective growth of nanographite [9]. Fig. 1(a) shows the electrochemical multi-sensing platform before the nanomaterial integration. Nanostructured microelectrode and

nanographite at two different magnifications are shown in Fig. 1 (b) and (c, d), respectively.

The carbon grown was very selective (clearly evident in Fig. 1 (b)) and the yield was optimised to prevent the material detachment. The growth was uniform thanks to the homogeneity of the catalyst coating that was electrodeposited by using a counter electrode kept in parallel to the array of working electrodes. The as-produced nanostructures were characterised by a Raman spectroscope. The spectra showed three characteristic peaks: the disorder induced D peak ($\approx 1349\ cm^{-1}$), the G peak that relates to the perfection of the crystal lattice ($\approx 1586\ cm^{-1}$) and the second order of the D peak (2D, $\approx 2698\ cm^{-1}$). An example of Raman spectra is shown in Fig. 2.

Fig. 3(a) shows the CV of nanographite-modified electrode in solution containing 600 μM of BR and a normal concentration of albumin (30 mg/ml). The three peaks related to the oxidation of BR (I) and of the BR products (II and III) are clearly noticeable [11] thanks to the use of a microelectrode modified with CVD nanographite. SWV was employed as a more sensitive analytical technique to detect concentrations of BR in the relative physiopathological range. Fig. 3(b) depicts the current peaks of the sensor measured for an increased metabolite concentration. The sensing parameters were evaluated by looking at the increase of Peak II as previously reported [11] and from a linear regression of the plot peak currents *vs* concentrations of BR (Fig. 3(c)). The sensitivity and the detection limit were $154 \pm 17\ \mu A/(mM\ cm^2)$ and $56 \pm 33\ \mu M$, respectively. The peak potential positively shifted by increasing the concentration of BR going from 280 mV to 306 mV.

Other biomolecules could interfere with the BR determination in human fluid such as ascorbic acid (AA) and uric acid (UA). These biocompounds oxidise at the same potential of BR by using metal electrodes. The use of carbon nanomaterials has been already proven to be a powerful tool for the discrimination of the oxidation peaks of different metabolites in voltammetry [7]. The present study also checked the detection of BR in the presence of both AA and UA even in solutions with physiological levels of albumin (30 mg/ml). Fig. 4(a) shows the SWV of 200 μM AA, 100 μM UA and 400 μM BR in a PBS solution-containing albumin. In the presence of AA and UA, the oxidation peak of BR shifted towards more positive potentials of ≈ 150 mV (average potential of five concentrations of BR is 461 ± 8 mV) due to the change of the pH in solution similar to what is reported in the literature [13]. Average peak potentials of AA and UA were -67 ± 13 mV and 27 ± 8 mV, respectively. Sensing parameters did not vary even in the presence of the two interferents (sensitivity $151 \pm 29\ \mu A/(mM\ cm^2)$; detection limit $56 \pm 32\ \mu M$). Experiments were also carried out in serum containing 500 μM of BR. The height of peak II decreases of five-fold with respect to that obtained by using a synthetic buffer (Fig. 4(b) and (c)).

4. Conclusions

A Pt microelectrode of a biosensor was selectively nanostructured with nanographite by a direct CVD growth. The efficacy of this nanointegration approach for the determination of free BR in the presence of normal levels of albumin was first demonstrated. The sensitivity of the sensor was $154 \pm 17\ \mu A/(mM\ cm^2)$ and the detection limit was $56 \pm 33\ \mu M$. The presence of the two interferents, ascorbic acid and uric acid, does not affect the values of sensitivity and detection limit towards the detection of BR. The nanographite-modified sensor determines the selective discrimination of voltammetric peaks related to the oxidation of the three biomolecules. By using the nanographite-modified microelectrode, the upper limit of the pathological concentration range of BR has been detected in human serum.

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