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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 ($4.2 \pm 0.1$ 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 $9.4 \pm 0.3$ 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

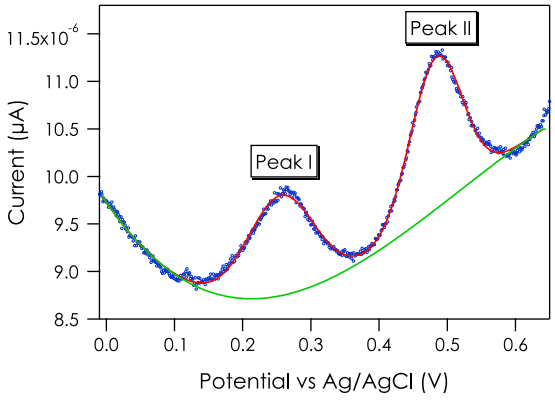


**Fig. 1.** Fitting example of the two voltammetric peaks by using Gaussian curves and a cubic baseline (BR concentration: 150 μM).

be irreversible under our experimental conditions, as some authors have already shown [16,19]. Peaks I and II are attributed to the oxidation of BR and of BV, respectively (Reactions 1 and 2).

$$bilirubin \xrightarrow[-2e^-]{\approx +300\ mV} biliverdin \quad (1)$$

$$biliverdin \xrightarrow[-2e^-]{\approx +500\ mV} purpurine \quad (2)$$

This assumption is supported by the disappearance of Peak I when a BR solution exposed to light for a week is used. Fig. 2 shows the change in colour from orange to green after this period of light exposure. Peak III instead is still present in these working conditions. Indeed, the third oxidation process is related to the purpurine oxidation to choletelin (Reaction 3).

$$purpurine \xrightarrow[-2e^-]{\approx +700\ mV} choletelin \quad (3)$$

Figs. 3 and 4 show the increase of the first and of the second oxidation peak currents with increasing BR concentration. The BR concentration range was selected between 50 and 150 μM because it covers the human plasma levels of BR in many patho-/physiological conditions [3,4]. The best electrochemical response was found when MWCNT film-cast electrodes, rather than bare SPEs, were used. The highest peak currents were registered in the presence of MWCNTs due to the larger electroactive area that derived from the introduction of nanostructures as well as to the excellent catalytic activity of MWCNTs [30]. When bare electrodes were used, the sensitivity and the LOD, calculated with respect to either Peak I or II, remained almost unvaried (Peak I: 6.6 ± 0.3 μA/(mM cm$^2$) and 54.4 ± 2.7 μM; Peak II: 6.4 ± 0.1 μA/(mM cm$^2$) and 56.1 ± 0.8 μM). Conversely, the sensing values change with MWCNT film-cast SPEs depending on whether Peak I or II is used to calculate them. In addition, an improvement of one order of magnitude for both sensitivity and LOD was registered when nanostructures were used. The sensitivity and the LOD calculated by using Peak I were 52.2 ± 2.4 μA/(mM cm$^2$) and 6.9 ±0.3 μM, respectively. The highest sensitivity (86.2 ± 2.4 μA/(mM cm$^2$)), the greatest linearity ($R^2 = 0.99$) and the lowest LOD (4.2 ± 0.1 μM) were obtained by monitoring the increase of Peak II. The lowest LOD value obtained in this case makes it possible to detect BR at concentrations within the physiological [3] as well as the pathological range, such as in cases of acute and chronic diseases [4]. Considering these findings, the following calculations were made with respect to Peak II, and nanostructured electrodes were used for the measurements.

Reproducibility of the MWCNT film-based SPEs was studied. Five SPEs were modified with MWCNT coatings by using the same procedure.

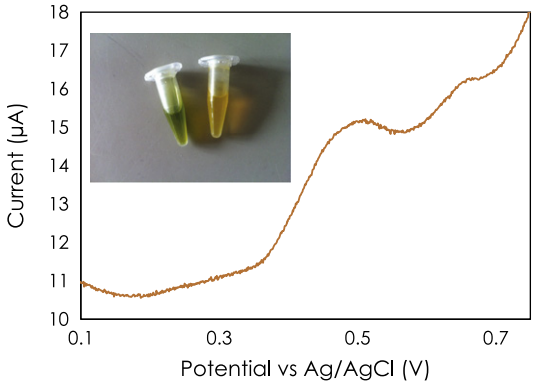


**Fig. 2.** Disappearance of Peak I after a week of light exposure (BR concentration: 500 μM; scan rate: 20 mV/s). In the top left picture change in colour from orange to green of the BR solution after one week of light exposure due to the BR oxidation in BV.

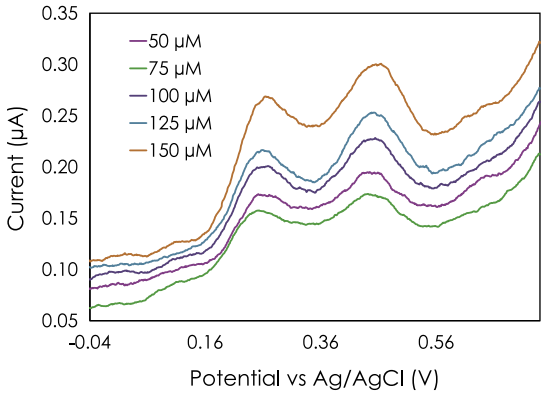


**Fig. 3.** Current Peaks I and II at various BR concentrations by using bare SPEs (50, 75, 100, 125, 150 μM).

Peak potentials and currents (Peak II) remained approximately unchanged. Indeed, for 150 μM BR, the average peak potential and the average peak current from five measurements are 462.6 ± 9.3 mV and 1.8 ± 0.1 μA, respectively. Satisfactory results were obtained by calculating the average sensitivity and LOD with the relative standard error. Values of 97.2 ± 4.8 μA/(mM cm$^2$) and 3.7 ± 0.2 μM were obtained for sensitivity and LOD, respectively.

### 3.3. Measurements in the presence of albumin

BR is present in body fluids in two forms, water-soluble and water-insoluble. Water-soluble BR is called conjugated BR, and water-insoluble BR is called unconjugated BR. In human plasma, unconjugated BR is transported bound to a protein carrier, i.e. albumin. The association mechanism of BR with albumin can be described by the following expression (Reaction 4).

$$A_{free} + BR_{free}\,K \rightarrow A/BR_{complex} \quad (4)$$

The relation between free albumin, free BR and their complex in terms of concentrations can be expressed by the equilibrium constant (Eq. (5)) [2].

$$K = \frac{\left[A/BR_{complex}\right]}{\left[A_{free}\right]\left[BR_{free}\right]} \quad (5)$$

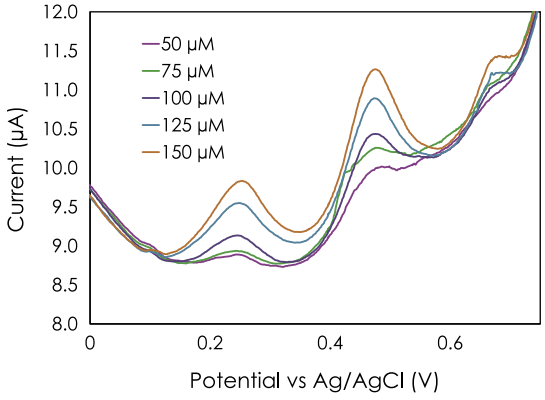


**Fig. 4.** Current Peaks I and II at various BR concentrations by using MWCNT film-cast SPEs (50, 75, 100, 125, 150 μM).

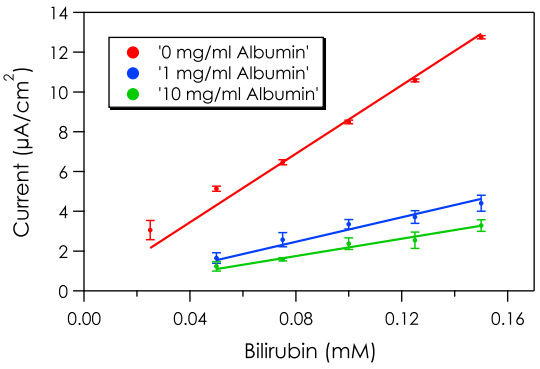


**Fig. 5.** Calibration plots for the BR detection in the presence of the following albumin concentrations: 0, 1 (albumin–BR molar ratio range: 0.1–0.3) and 10 mg/ml (albumin–BR molar ratio range: 1–3).

Because free BR is present in a small amount relative to the albumin–BR complex, the concentration of the latter is almost equal to that of the unconjugated BR. Therefore, Eq. (5) can be written as Eq. (6).

$$\left[BR_{free}\right] = \frac{\left[BR_{unconjugated}\right]}{K\left(\left[A_{free}\right] - \left[BR_{unconjugated}\right]\right)} \tag{6}$$

We measured the peak current response by increasing the concentration of albumin in solution. With a concentration of albumin of 1 mg/ml, the sensitivity decreases almost three fold (30.8 ± 1.0 μA/(mM cm$^2$)) and the LOD increases almost four fold (12.4 ± 0.4 μM). Hypoalbuminemia characterises certain pathologies such as renal disease, chronic infections and inflammation. In these cases, patients show plasma albumin levels as low as 10 mg/ml [31]. By using this concentration of albumin the sensing performance declines further (sensitivity: 21.8 ± 0.6 μA/(mM cm$^2$); LOD: 23.3 ± 0.7 μM). Calibration curves for albumin levels of 0, 1 and 10 mg/ml are shown in Fig. 5. The voltammetric response of the MWCNT film-sensor becomes negligible for normal albumin concentration (30 mg/ml). However, the signal is still present even if the molar ratio between BR and albumin is equal to 0.33. Other authors [32] were unable to measure BR for BR/albumin molar ratios below 1. The reduction of the peak current as a function of increasing albumin concentrations at a fixed BR amount (150 μM) is shown in Fig. 6. The most evident drop in current occurs when passing from 0 mg/ml to 1 mg/ml of albumin concentration. When passing from 1 to 30 mg/ml of albumin in solution, instead, the current decreases only slightly. These findings indicate that the majority of BR binds to albumin when 1 mg/ml of the protein is added into the solution. By increasing the protein concentration from 1 to 30 mg/ml, the amount of free BR remains quite the same. This electrode behaviour is in agreement with Eq. (6). However, the adsorption of serum albumin onto the different electrode materials [26] also plays a role in the decrease of the BR current peaks.

**Table 1**
Peak potential shift by increasing the albumin concentration and using a MWCNT film-based SPE.

| Albumin concentration (mg/ml) | Potential (mV) |
|---|---|
| 0 | 483.7 ± 0.2 |
| 1 | 504.2 ± 0.7 |
| 5 | 520.7 ± 1.8 |
| 10 | 530.5 ± 1.3 |
| 30 | 545.1 ± 1.8 |

Moreover, we noticed that the highest oxidation potentials were registered when the albumin concentration was highest. This potential shift is due to the fact that the more BR–albumin complex is present in solution, the more the electron transfer kinetics decreases. Furthermore, the highest potential shift was registered passing from an albumin concentration of 0 to 1 mg/ml (Table 1). This corresponds to the albumin addition step at which the majority of the BR–albumin complex forms.

### 3.4. Detection in newborn jaundice

An excessive amount of BR in the human body (jaundice) is recognised in various diseases. In particular, the highest levels of BR are found in babies with jaundice. Newborns usually have higher red blood cell breakdown and their immature livers are not efficiently conjugating BR, and thus removal of BR from the bloodstream is delayed. Clinically, untreated jaundice in neonates can lead to mental retardation, cerebral palsy, deafness and even death, due to the passage of free BR through the blood–brain barrier [5]. Critical levels of unconjugated BR for healthy newborns are greater than 15 mg/dl (≈250μM), and up to 30 mg/dl (≈500 μM), but are lower in premature babies. Fig. 7 shows the experimental calibration curve registered with MWCNT film-SPEs in the BR concentration range critical for newborns and in the presence of normal levels of albumin (30 mg/ml). A linear relation between peak current and BR concentration was found in the investigated range with the following regression equation: $I_p$ (μA/cm$^2$) = 3.79 [BR](mM), with a correlation coefficient of 0.99. The sensitivity and the LOD were determined to be 30.2 ± 0.8 μA/(mM cm$^2$) and 9.4 ± 0.3 μM, respectively. It should be noted that the LOD is below the minimum critical BR value for newborns. As a consequence, the sensor could be used to monitor the BR level in babies affected by both acute and chronic newborn jaundice.

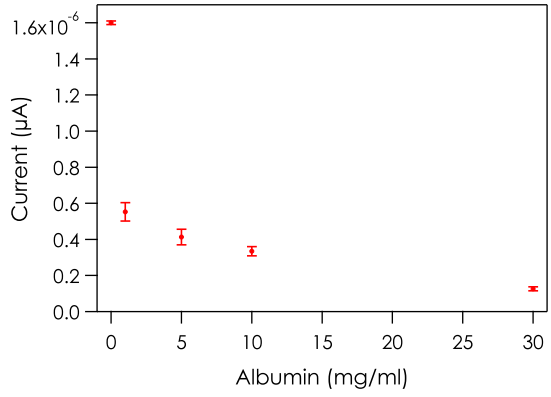


**Fig. 6.** Current Peak II versus albumin concentration (albumin: 0, 1, 5, 10, 30 mg/ml; BR concentration: 150 μM; albumin–BR molar ratios: 0.1, 0.5, 1, 3).

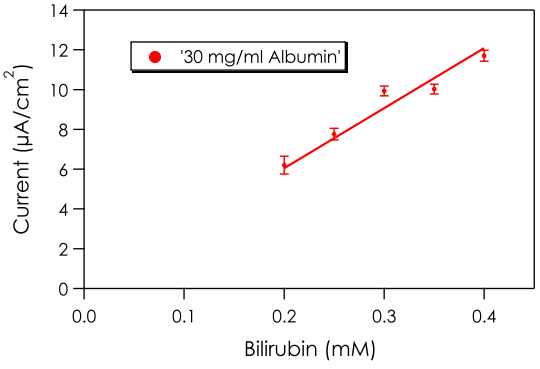


**Fig. 7.** Calibration plot for the BR detection in the range 200–400 μM critical for neonatal children (albumin concentration: 30 mg/ml).

## 4. Conclusions

The BR electrochemistry has been investigated with SPEs in the presence and absence of MWCNT layers in the BR physio-pathological range (up to 150 μM). Three oxidation processes were recognised by cyclic voltammetry. The first two occur at ≈300 mV and ≈500 mV, respectively. The last process appears at ≈700 mV only for high BR concentrations in solution. All these reactions were found to be highly irreversible.

Comparing the sensing parameters, namely sensitivity and LOD, by using both nanostructured and non-nanostructured SPEs, we found a marked enhancement in the case of MWCNT film-based electrodes. In the range 0–150 μM of BR, monitoring the increase of Peak II gave the highest sensitivity (86.2 ± 2.4 μA/(mM $cm^2$)), the greatest linearity and the lowest LOD (4.2 ± 0.1 μM). Interestingly, the LOD was found to be low enough to detect BR down to the relative physiological range. The increase of Peak II detected using MWCNT film-based SPEs was used for the following investigations.

The effect of albumin on the BR voltammetric response was also studied. Mixtures of a fixed BR concentration (150 μM) with increasing albumin concentrations up to the relative normal values were prepared. A reduction of the current signal was registered as well as a worsening of the sensitivity and the LOD. This behaviour can be explained by the decline of free BR as the albumin level increases. To a certain extent, this phenomenon is influenced by the adsorption of albumin onto the electrode surface [26]. However, BR has been detected at a BR/albumin molar ratio smaller than 1.

The measurement of BR is of great interest for clinicians for the diagnosis and treatment of certain diseases. In particular, the highest BR levels have been registered in neonatal jaundice (critical range: 200–500 μM) and are associated to permanent brain damages. Therefore, experiments in this concentration range were performed with solutions containing relative physiological levels (30 mg/ml) of albumin. A LOD below the minimum critical BR concentration for newborns was obtained (9.4 ± 0.3 μM). The experiments performed in the presence of various albumin concentrations indicate the possibility that only free BR, the neurotoxic form of BR, is detectable by using the modified sensor. Future developments of this work could be the electrochemical study of a panel of redox probes (cationic, anionic and neutral) under various concentrations of albumin for an accurate electrochemical characterisation of the modified electrodes.

Free unconjugated BR is an electroactive biocompound. In human fluids, BR is found in a water soluble complex with albumin. The BR–albumin complex is not electroactive, therefore in the presence of albumin only the BR that remains dissociated from albumin is oxidised and reduced spontaneously at the electrode. This study proves the possibility of detecting free unconjugated BR only by nanostructuring electrodes with MWCNT thin layers. The proposed sensor was used to detect BR in the relative critical concentration range for newborn jaundice, in the presence of albumin at levels typically found in human serum. The adsorption of lipids and other proteins onto the electrode will occur if serum is used as electrolyte. The decrease of the sensor response in these experimental conditions is well-documented in the literature [33]. Thereby, in order to gain fundamental insight about the detection of BR with the proposed modified electrodes, future measurements will be carried out by using serum as the support electrolyte.

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