Molecularly imprinted electrochemical sensor prepared on a screen printed carbon electrode for naloxone detection

Frederico Lopes, João G. Pacheco *, Patrícia Rebelo, Cristina Delerue-Matos

REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida 431, 4200-072 Porto, Portugal

ABSTRACT

Naloxone (NLX) is a pharmaceutical used as opioid antagonist. A molecular imprinted polymer electrochemical sensor for simple and rapid detection of NLX was prepared through the modification of commercial available screen printed carbon electrode (SPCE). The SPCE was modified with multi-walled carbon nanotubes (MWCNT) by drop coating to increase the signal response and improve the sensitivity. The MIP preparation was carried out via in situ electropolymerization using 4-aminobenzoic acid (4-ABA) as functional monomer. The morphology of the obtained sensor was characterized by scanning electron microscopy (SEM). Several parameters controlling the preparation and performance of the MIP sensor were studied and optimized. The electrochemical behavior of NLX at MIP and control non-imprinted (NIP) sensor was evaluated by differential pulse voltammetry (DPV), demonstrating a better MIP response and the success of the imprinting. The proposed MIP/MWCNT/SPCE sensor showed a linear relationship between peak current intensity and NLX concentration in the range between 0.25 and 10.0 μM, with limits of detection (LOD) and quantification (LOQ) of 0.20 μM and 0.67 μM respectively. The repeatability and reproducibility were also tested with relative standard deviations (RSD) of 4.6 and 9.6% respectively. Moreover, the applicability of the method was successfully confirmed with detection of NLX in biological samples (urine and human serum). The sensor is promising to be used for screening NLX in point-of-care people with opioid overdose.

1. Introduction

Opioid dependence is a neurobehavioral syndrome, affecting millions of people worldwide, characterized by the repeated, compulsive seeking and use of an opioid despite adverse social, psychological, and/or physical consequences [1]. Naloxone (NLX), is a derivative of morphine and a specific opioid antagonist at mu, kappa, and delta opioid receptors [1,2] witch as high affinity for opiate receptors without activate then [3]. It reverses the action of many narcotic drugs, including heroin, and thus can be used in the treatment of overdoses.

Several analytical methods have been reported for the determination of NLX mainly by high performance liquid chromatography (HPLC) [4] specially coupled with MS detection [1,5–9]. Since NLX molecules can be easily oxidised, liquid chromatography with electrochemical detection was also used [10–12]. However, this methods are costly, time consuming due to extensive sample preparation and producing large amounts of liquid waste witch is not suitable for a green chemistry laboratory. Recently a sensor based on potentiometric measurement was described [13].

Screen-printing technology is a well-established technique that allows the production of disposable biosensors and chemical sensor [14]. The screen printed electrodes (SPE) are simple, low cost, portable, small sized and capable of mass production [15]. The reduced size of the SPEs lowers the sample volume down to 30–40 μL, which is advantageous especially in the analysis of biological samples and can be easily integrate in miniaturized portable devices [16]. The SPEs are very versatile since the composition of the printing inks could be modified with several substances such as metals, enzymes, polymers or complexing agents [17]. Otherwise deposition of different agents on the surfaces of manufactured electrodes could also be easily achieved such as molecular imprinted polymers [17–19].

Molecularly imprinted polymers (MIPs) are synthetic materials with artificially generated recognition sites capable of specifically binding a target molecule, mimicking natural receptor systems including antibodies, hormones and enzymes. MIP are prepared by a process that involves a formation of a complex between the

* Corresponding author.
  E-mail address: jpgpa@isep.ipp.pt (J.G. Pacheco).

functional monomers and the target molecular (template) and followed by a polymerization in the presence of a crosslinking and a solvent (porogen). Finally the template is removed and a polymeric matrix with specific cavities complementary in size, shape and functionality are produced. MIPs are ease to prepare, low cost, and show high chemical and mechanical stability. They have found applications in several different areas, such as purification and separation, catalysis, sensors, drug delivery systems [20–24]. Due to their high affinity toward the template molecule they are excellent tools to be used as selectivity elements with the construction of sensors. The main difficulty consists in the integration of the polymer with the transductor. Initially, MIPs were prepared normally by free radical polymerization and in most of cases a bulk polymer was obtained, which results in poor compatibility with the transductor and limited mass transport and rebinding kinetics. Several approaches have proposed for in situ preparation directly on the transducer surface, such as: drop-coating of a solution of a pre-prepared polymer, spin-coating, electropolymerization, grafting, and layer by layer deposition [25]. Electropolymerization has proved to be an excellent choice especially in the preparation of MIP electrochemical sensors [25–28]. It allows an ease control of polymers thickness and morphology, is highly reproducible and permits polymerization and operation in water solutions. Otherwise the use of initiator and cross-linking is normally dispensed [29].

In the present work, a novel MIP electrochemical sensor for NLX detection was constructed by electropolymerization of 4-aminobenzoic acid (4-ABA) on the surface of SPCE modified with multi-walled carbon nanotubes (MWCNT). To the best of our knowledge this is the first report of the preparation of a MIP toward NLX molecules. The combination of electropolymerization for MIP preparation with SPCE technology can be and excellent choice to ease and low cost fabrication of electrochemical sensors with high potential by applied to commercial analytical solutions.

2. Experimental

2.1. Reagents

Naloxone hydrochloride dehydrate (NLX), 4-aminobenzoic acid (4-ABA), ascorbic acid, glucose, urea, Naltrexone and Noroxymorphone hydrochloride were obtained from Sigma-Aldrich. Multi walled carbon nanotubes (MWCNTs) functionalized with —COOH groups (DropSens) were prepared in N,N-dimethylformamide

(DMF, Sigma-Aldrich). Stock solutions of NLX (50 mM) were prepared in water and stored at 4 °C. Daily working solutions of NLX with different concentrations were prepared. Phosphate buffer solutions were prepared from KH_2PO_4 (Riedel-de Haën) and K_2HPO_4 (Riedel-de Haën) and the pH adjusted with HCl or NaOH 1 M solutions. All commercially available reagents were of analytical grade and were used without further purification. All solutions were prepared using ultrapure water (resistivity = 18.2 MΩ cm), obtained from a Millipore (Simplicity 185) water purification system. Human serum form male AB plasma was obtained from Sigma-Aldrich.

2.2. Apparatus

All electrochemical experiments were carried out with an Autolab PGSTAT 204 potentiostat-galvanostat controlled by Nova 1.10 software (Metrohm Autolab). Screen printed electrodes (Dropsens, DRP-110) with working (d = 4 mm) and auxiliary electrode of carbon, and a reference electrode of Silver were used. The surface of the working electrode was modified to prepare the MIP/MWCNT/SPCE.

2.3. Sensor fabrication

The preparation of MIP on SPCE as obtained by electropolymerization using cyclic voltammetry. The stepwise construction process is illustrate in Fig. 1. Prior to modification a bare SPCE was rinsed with distilled water and then activated in 0.5 M H_2SO_4 by cycling the potential between − +0.2 V and +1.3 V at 100 mV/s during 5 cycles.

A suspension containing 2 mg of MWCNT in 1 mL DMF was prepared, sonicated for 2 h and diluted with an ultrapure water/DMF mixture (50:50), to obtain a final concentration of 1 mg/mL. A MWCNT/SPCE was prepared by deposition of 4 μL on the working electrode's surface of the prepared MWCNT suspension. The electrode was allowed to dry at room temperature and thoroughly washed with ultrapure water. A drop of 40 μL of the polymerization solution (40 mM 4-ABA, 0.5 mM NLX in phosphate buffer solution) was placed in the MWCNT/SPCE and cyclic voltammetry (CV) was performed in the potential range from −0.20 V to +1.00 V at a scan rate of 100 mV/s for 20 cycles. After that, 40 μL of methanol/HCl 0.1 M (50:50) solution were consecutively placed and replaced each 5 min on the surface of the sensor during 40 min. This procedure permits the removal of the entrapped NLX molecules and produces
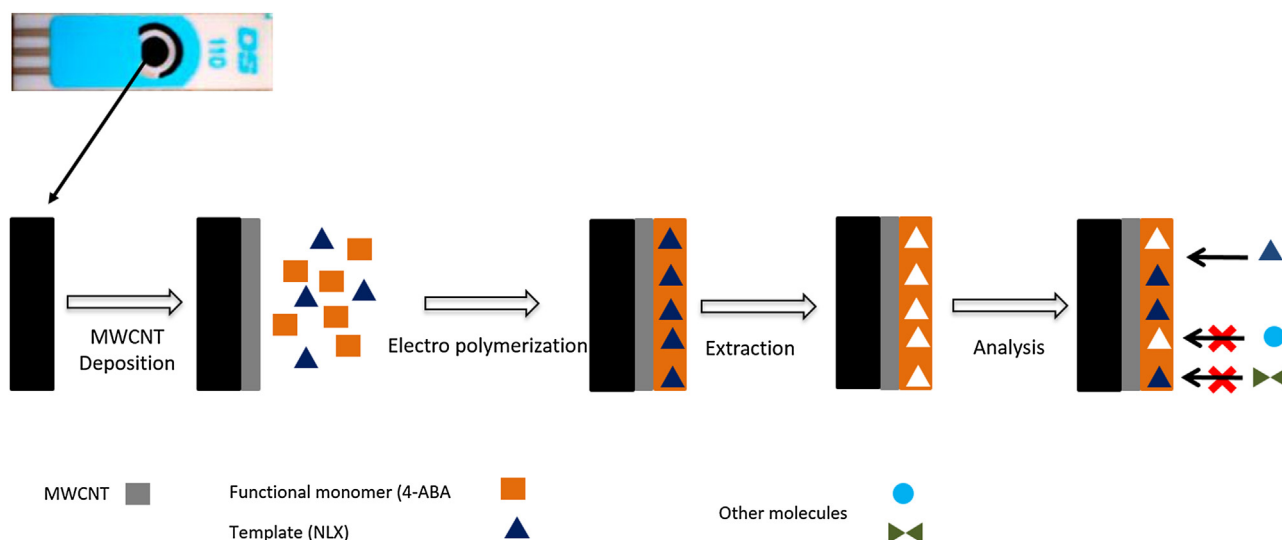

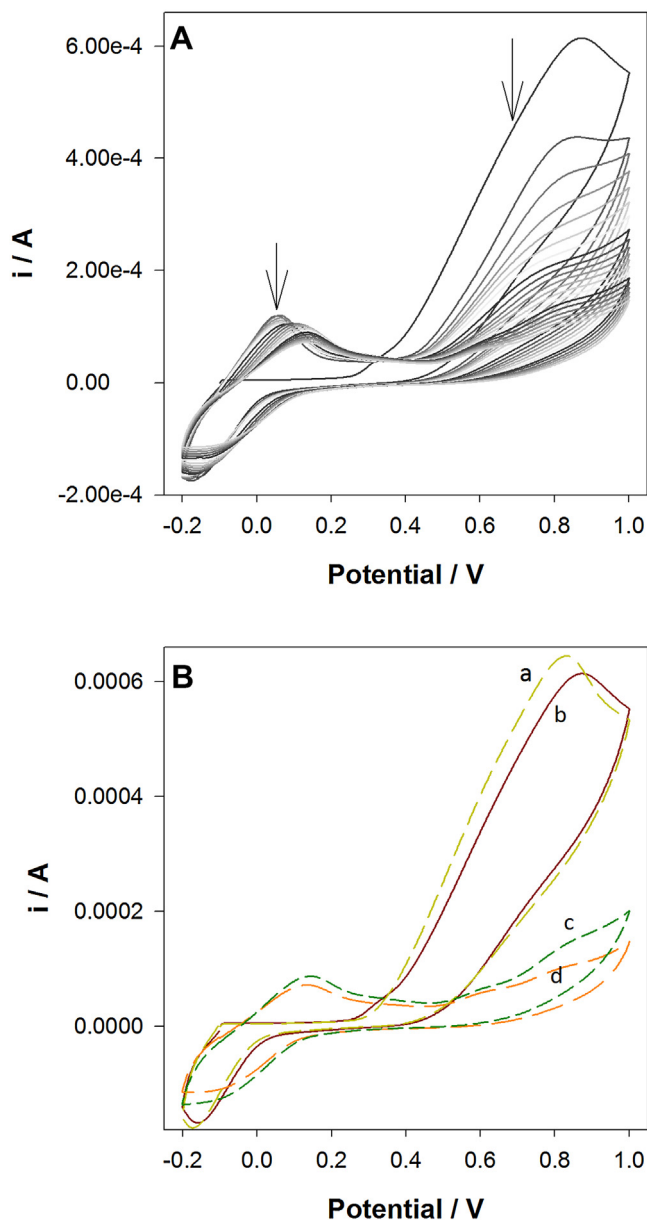Fig. 1. Schematic illustration of the preparation of the MIP/MWCNT/SPCE electrode.
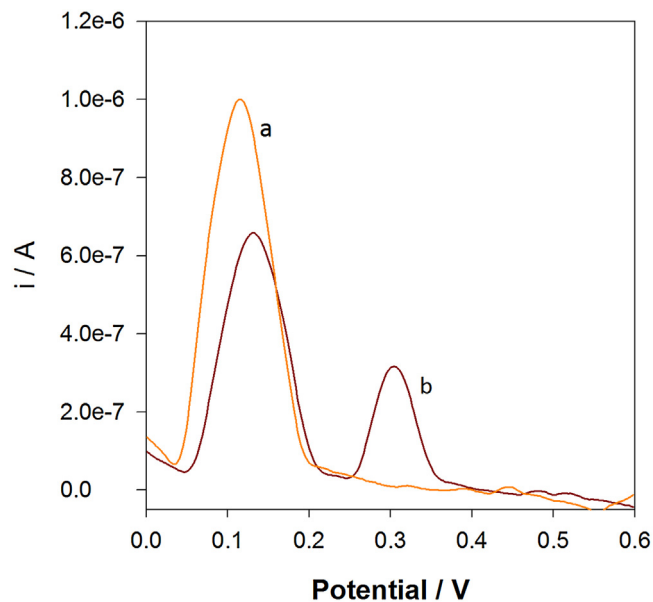
Fig. 3. DPV voltammograms in 0.1 M phosphate buffer pH 7 of a (a) NIP/MWCNT (a) and (b) MIP/MWCNT/SPCE after polymerization.

0.1 M (50:50) for 40 min. All measurements were executed at room temperature. SEM analyses were performed using FEI QUANTA 400 FEG/EDAX Pegasus X4M equipment.

2.5. Sample preparation

Urine samples were collected from healthy individuals who were not taking NLX in sterile bottles, while human serum samples were commercial and stored at $-20\,^{\circ}C$. The samples were spiked with two different concentrations of NLX, diluted to 50% in 0.1 M phosphate buffer solution pH 7 and then analyzed without further treatment, using the conditions described above.

3. Results and discussion

3.1. Imprinting of NLX molecules

The molecular imprinting of NLX molecules was performed by electropolymerization. $40\,\mu L$ of polymerization solution, containing 40 mM 4-ABA and 0.5 mM NLX in phosphate buffer were placed at a MWCNT/SPCE and the imprinting was performed by CV, through 20 cycles in the range between -0.20 and $+1.00$ V at a scan rate of 100 mV/s. In Fig. 2(A) the formation and growth of the poly 4-ABA film in the present of NLX can be observed. During the first cycle an oxidation peak at +0.8 V and a reduction peak around -0.1 V was registered, resulting from the formation of a polymeric film. It is also observed a slight peak around +0.30 V resulting from the oxidation of NLX molecules. After the second cycle an oxidation peak at 0.1 V was also observed. As the number of scans increases, the peaks current intensity decreases indicating a constant grow of the polymeric chain. A control non-imprinted sensor was also prepared in the same conditions without the presence of NLX during the electropolymerization. The formation of the NIP film has a similar behavior in the decreasing of the peaks with the number of cycles. However there are slightly differences between NIP and MIP films grow as it can be observed in Fig. 2(B). As expected in the first cycle of NIP formation no peak around +0.30 V was observed. The peaks related to the 4-ABA in the MIP are smaller than the peaks in NIP, both in first and the last polymerization cycle. This probably occurs due to the presence and incorporation of NLX during
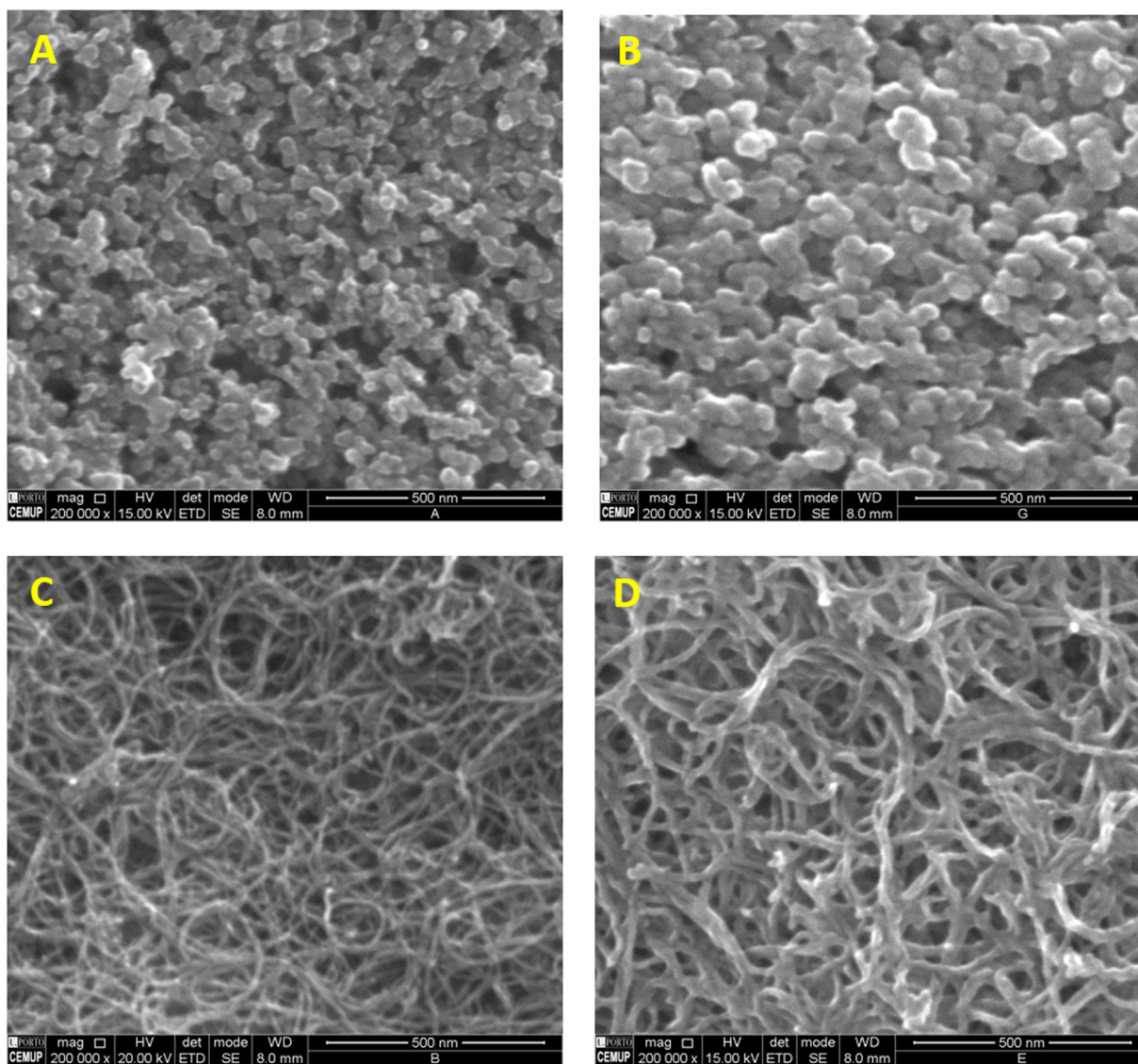

Fig. 2. (A) Electropolymerization of MIP from a solution containing 20 mM 4-ABA and 0.5 mM NLX in 0.1 M phosphate buffer pH 7; (B) comparison between NIP and MIP preparation; (a) first CV of NIP, (b) first CV MIP, (c) 20th CV of NIP and (d) 20th CV of MIP.

a surface that is complimentary in size, shape and functionality to NLX molecules.

A non-imprinted polymer-modified electrode, named NIP/MWCNT/SPCE, was also prepared using the same procedure in the absence of NLX.

2.4. Electrochemical measurements

Electrochemical measurements were performed by differential pulse voltammetry (DPV). A common 3-step procedure was used for the voltammetric detection of NLX at MIP/MWCNT/SPCE. Initially a drop of $10\,\mu L$ of solutions of sample containing NLX was placed at the sensor during 5 min. Then the sensor was washed with water dried, and $40\,\mu L$ of 0.1 M PBS (pH 7) was dropped for analysis of NLX by DPV from +0.15 V to +0.5 V using a pulse amplitude of 50 mV and a step potential of 5.25 mV. Finally, the extraction of NLX was achieved by dropping $40\,\mu L$ of a solution methanol/HCl

Fig. 4. SEM images of de (A) SPCE, (B) MIP/SPCE, (C) MWCNT/SPCE e (D) MIP/MWCNT/SPCE.

the formation and grow of the polymeric film. These results indicates that under these conditions a poly 4-ABA film with entrapped NLX molecules was prepared at the surface of the MWCNT/SPCE. After the electropolymerization and before template removal a DPV analysis of the prepared MIP and NIP was performed in 0.1 M phosphate buffer solution pH 7. The obtained results, expressed in Fig. 3, shows a clear difference between NIP and MIP after polymerization. In the NIP a peak around +0,15 V was registered, related to the poly 4-ABA. In the MIP these peak was also registered, however with less current intensity, and a peak at +0.3 V was observed. These results proves the entrapment of the NLX molecules.

3.2. SEM characterization of the electrodes

The morphologies of electrodes with different modifications were studied by SEM. The obtained images shows that there are clear differences between the morphologies of the electrode's surfaces. Fig. 4(a) shows a rough surface characteristic of the bare SPCE. The successfully deposition of the MWCNTs on the electrodeís surface can be confirmed in Fig. 4(c). The formation of a polymer layer can be confirmed in Fig. 4(b) and (d) in a MIP/SPE and a MIP/MWCNT/SPCE, respectively. These imagens shows a

slightly thickening from SPCE and MWCNT/SPCE to MIP/SPCE and MIP/MWCNT/SPCE. These results indicates that a thin polymer was formed under the used conditions.

3.3. Electrochemical behavior of NLX

After the preparation of the sensor it ability to rebind to NLX molecules was tested. The electrochemical behavior of NLX at different prepared electrodes was evaluated by DPV in 0.1 M phosphate buffer pH 7 after 5 min of incubation in 0.05 mM of NLX. The DPV voltammograms are shown in Fig. 5 for (a)NIP/SPCE, (b) MIP/SPCE, (c) NIP/MWCNT/SPCE and (d) MIP/MWCNT/SPCE. It can be observed a pronounced difference between the obtained peaks for MIPs and NIPs either in the presence or not of MWCNTs. The fact that there are peaks for NIPS indicates that the poly 4-ABA have good interaction and ability to bind to NLX molecules, although these are non-specific binding. In MIPs there are an increase in the peak current, which results from the formation of specific cavities, during the MIP preparation, that are able to selective binding to NLX molecules. These results indicates a better response obtained from MIPs and proved the successfully preparation of a molecularly imprinted sensor under the conditions described. There are also
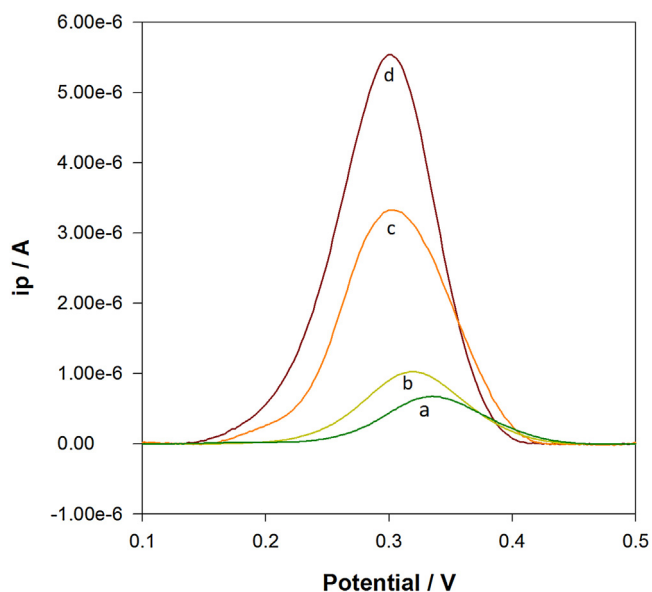
Fig. 5. DPV voltammgrams in 0.1 M phosphate buffer pH 7 after 5 min of incubation in 0.5 mM NTL at (a) NIP/SPCE, (b) MIP/SPCE, (c) NIP/MWCNT/SPCE and (d) MIP/MWCNT/SPCE.

differences on the responses when the sensors where previously modified with MWCNTs. Due to the increase in the surface area and in electric conductivity there is an increase in the peaks current observed. It can also be observed that when MWCNT are present oxidation potential of NLX was singly move to left, which indicates a more favorable oxidation process. The presence of MWCNT can enhance the current response without affect the imprinting and improving the sensitivity of the sensor.

Then a study of the influence of the concentration of MWCNT on the response was performed. Solutions with concentrations of MWCNT between 0.1 and 2 mg/mL were prepared, deposited on different SPCEs and several MIPs were prepared and the peak current measured. The obtained results are represented in Fig. 6. The analysis were performed in triplicate. As it can be seen there is a large increase of the response with the increase of concentration
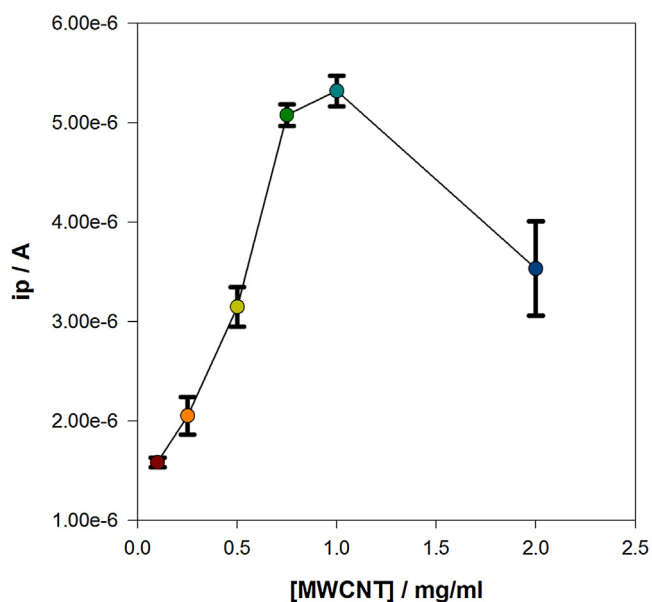

Fig. 6. Variation of the peak current intensity with the concentration of MWCNT deposited onto de SPCE.
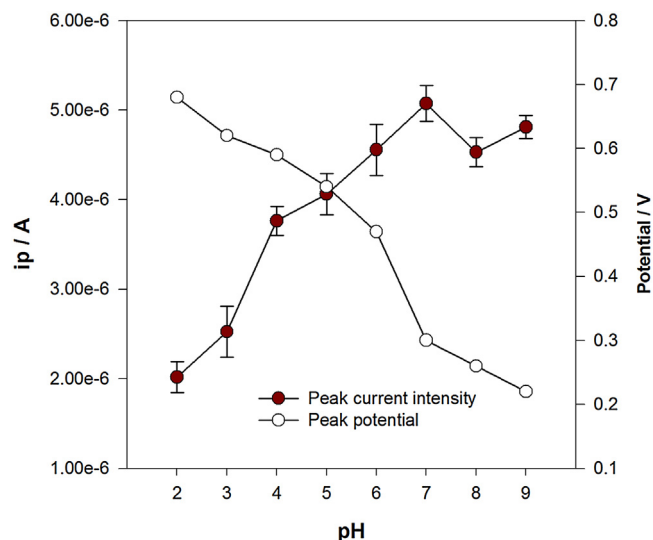

Fig. 7. Variation of the peak current intensity with pH of the measuring solution.

of MWCNTs until 0.75 mg/mL and a small increase from this concentration to 1 mg/mL where the maximum of peak current was registered. It is also important to note that increasing the concentration to 2 mg/mL has a negative effect, with a decrease in the registered peak current. So 1 mg/mL was used in the modification of SPCEs.

The influence of the pH of the supporting electrolyte in the oxidation behavior of NLX at MIP/MWCNT/SPCE was also investigated. Solutions with pH ranging from 2 to 9 were tested and the peak current and the oxidation potential registered (Fig. 7). The peak current increases with the pH until a maximum at pH 7. From 7 to 9 there is a slight decrease and stabilization. The oxidation potential decreases with the increase of pH. The phosphate buffer solution with pH 7 was used because it produces the highest current at a potential around +0.30 V

3.4. Optimization of the preparation conditions

Several parameters of MIP preparation with influence in the efficiency, namely concentration of monomer, concentration of template and number of CV cycles were studied. The results were performed in triplicate and expressed in Fig. 8. It was observed that with the increase of monomer 4-ABA concentration there is an increase in the sensor response with a stabilization around 40 mM (Fig. 8(A)). The optimal value for NLX concentration during the polymerization was found to be 0.5 mM (Fig. 8(B)). Higher values of NLX shows a decrease in the sensor response, probably because too many molecules could be entrapped affecting the extraction and the quality of formed cavities. Finally there is an increase in the response of the MIP sensor with the increase of CV cycles until twenty and then a decrease and stabilization were observed (Fig. 8(C)). With the increase of number of cycles there is an increase in the thickness of the polymeric film. If the film is too thick the NLX molecules imprinted near the surface will be more inaccessible to be extracted and the mass transport to that cavities is difficult.

A critical step in the preparation of a MIP is the extraction after the polymerization to achieved good selectivity. If the template is too entrapped it is difficult to remove the efficiency of the polymer compromised. In the case of electrochemical sensors it is also important to guarantee that after each measurement, the sensor can be regenerated before a new analysis. Normally this process is accomplished by solvent extraction. Several solvents were tested to remove NLX: water, 1 M NaCl solution, methanol, 0.1 M HCl solution
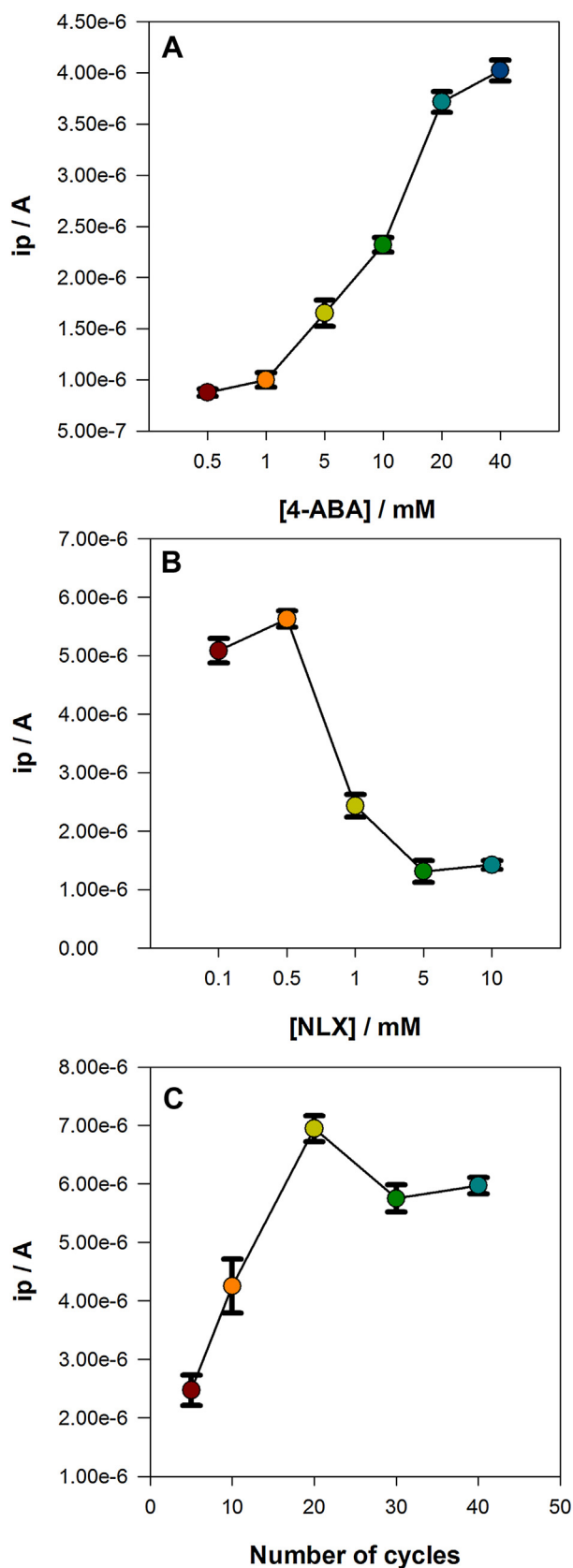
Fig. 8. Optimization of the electropolymerization conditions: (A) Variation of the peak current intensity with the concentration of monomer 4-ABA; (B) Variation of the peak current intensity with the concentration of template NLX and (C) Variation of the peak current intensity with number of CV cycles.
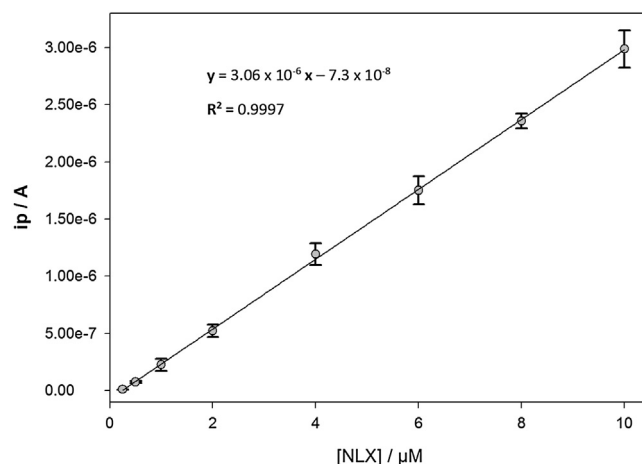

Fig. 9. Relationship between peak current intensity and NLX concentration in the range 0.25–10.0 μM.

and a mixture of methanol and 0.1 M HCl (50:50). The peak current response to NLX was evaluated accordingly the solvent used in the extraction It can be observed that solvent that permits the better sensor response is the mixture methanol and 0.1 M HCl, while the poor extraction as observed with water. Along with the type of solvent, the extraction time as also studied. It was found that the optimal extraction time was 40 min.

3.5. Calibration curve

The MIP/MWCNT/SPCE sensor DPV responses towards NLX at different concentration were registered under the optimized conditions and the correlation between the oxidation peak current and the concentration was established (Fig. 9). The calibration curve shows a linear relationship in the range of between 0.25 and 10 μM (r = 0.9997, n = 3) with a limit of detection (LOD) estimated to be 0.20 and the limit of quantification (LOQ) 0.67 μM. The limits of detection (LOD) and quantification (LOQ) were calculated using the following equations: LOD = 3 s/m and LOQ = 10 s/m, where "s" is the standard deviation of the intercept and "m" is the slope of the calibration plot.

3.6. Repeatability, reproducibility and stability of MIP/MWCNT/SPCE

To estimate the repeatability of the sensor a solution of NLX at a concentration 4 μM was analyzed using the same MIP/MWCNT/GCE under the optimized conditions. The relative standard deviation (RSD) of the measurements was 4.6% for six consecutive assays indicating a good precision for the fabricated sensor. Additionally, the reproducibility of independently prepared MIP/MWCNT/SPCE sensors was also estimated. Five different MIP/MWCNT/GCE electrodes were fabricated and their peak current responses for 4 μM NLX were used to estimate a RSD of 9.6%. These results shows an acceptable reproducibility in the preparation of new sensors. The sensor could be used at least 15 times without significantly loss of the signal. When not in use it was stored in phosphate buffer solution and after 20 days a loss of about 10% was registered.

The proposed sensor is precise, easy to reproduce and has a stable response.
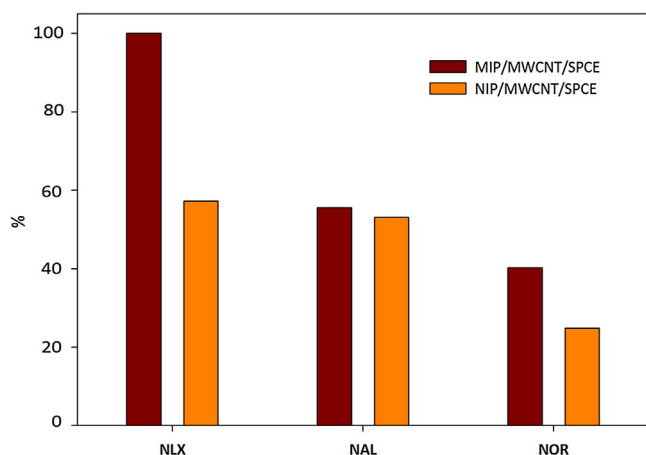
Fig. 10. The oxidation peak current percentage of 10 μM of NLX, NAL and NOR on MIP/MWCNT/SPCE and NIP/MWCNT/SPCE.

3.7. Selectivity of the sensor

Two analogues of NLX, Naltrexone (NAL) and Noroxymorphone (NOR) were prepared at a concentration of 10 μM in phosphate buffer 0.1 M pH 7. These solutions were incubated in MIP/MWCNT/SPCE and NIP/MWCNT and a DPV analysis was performed. The obtained oxidation peaks were compared with the oxidation peak of NLX at the same conditions, as expressed in Fig. 10. The MIP sensor shows a higher value for NLX confirming the imprint effect. The oxidations peaks for NAL and NOR corresponds to 55 and 40% of the NLX peak, respectively. The oxidation peaks of NAL at MIP and NIP sensor are similar.

The response of 10 μM of NLX at MIP/MWCNT/SPCE in the presence of 100 μM of ascorbic acid, urea, and glucose was tested. In the presence of 10 times-fold of ascorbic acid and urea the response of NLX was 95 and 99% of the NLX response alone, respectively. In the presence of glucose the response was 70%, indicating that this compound could interfere in the analysis of NLX.

3.8. Detection of NLX in biological samples

The MIP/MWCNT/GCE sensor was finally used to the detection of NLX in urine and human serum in order to validate the application in complex samples. Samples were spiked with two different naloxone concentration and the analysis performed by standard addition method. The results are summarized in Table 1. Recoveries between 92 and 111% with RSD between 3 and 10% were found. These results proves the ability of the proposed sensor to be used as a fast, cheap, easy to perform method for NLX analysis in biological samples without the need of laborious pre-treatment steps or methods.

Table 1
Determination of NLX in spiked samples using the proposed MIP/MWCNT/SPCE sensor.

Sample	Added/μM	Found/μM	Recovery/%	RSD/%
Serum	0	0	–	–
	2.0	1.96	98	10
	8.0	7.29	91	6
Urine	0	0	–	–
	2.0	2.14	107	8
	8.0	8.9	111	3

4. Conclusions

This work reports for the first time the preparation of a MIP electrochemical sensor for NLX detection. Commercial available SPCE electrodes were used to the fabrication of the sensor, because they requires small amounts of reagents, are small, cheap and disposable. MWCNTs were initially deposited onto de working electrode of SPCE to increase the peak current signal, enhancing the sensitivity. A thin polymeric film of poly 4-ABA providing selective adsorption towards NLX molecules was prepared by electropolymerization. The formation of the film was confirmed by SEM analysis, and DPV was used to show the differences between MIP and NIP sensors. The proposed sensor is easy to prepared and operate, has good analytical performance good precision and stability. It was successfully applied in biological samples.

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Biographies

Frederico Lopes obtained his master degree in computer engineering and medical instrumentation, in 2016 at the School of Engineering of the Polytechnic Institute of Porto (ISEP-IPP, Portugal).

João P. G. Pacheco obtained his PhD in Chemistry in 2010 (Faculty of Sciences, University of Porto, Portugal). Currently he is a postdoctoral research in REQUIMTE and his present research interests include the development of analytical (electrochemical) methodologies based on the use of molecularly imprinted polymers for pharmaceutical, clinical, and food analysis.

Patrícia Rebelo obtained her MSc in Health of Sciences in 2014 (University of Minho). Presently, her work is mainly focused on the development of analytical methodologies using molecular imprinted polymers for detection of biomarkers.

Cristina Delerue-Matos obtained her PhD in Chemical-Physics, specialty in electrochemistry, in 1990. At the moment, she is coordinator professor at the School of Engineering of the Polytechnic Institute of Porto (ISEP-IPP, Portugal) and also coordinates the REQUIMTE/ISEP research group (www.graq.isep.ipp.pt). Her research interests include the development of analytical methodologies for environmental, food, pharmaceutical, biochemical and industrial control. She is co-author of more than 175 publications in scientific journals.