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Synthesis of Highly Sensitive Molecular Imprinted Sensor for Selective Determination of Doxycycline in Honey Samples

Nadia El Alami El Hassani, Soukaina Motia, Benachir Bouchikhi, Nezha El Bari

***Abstract*—**Doxycycline (DXy) is a cycline antibiotic, most frequently prescribed to treat bacterial infections in veterinary medicine. However, its broad antimicrobial activity and low cost, lead to an intensive use, which can seriously affect human health. Therefore, its spread in the food products has to be monitored. The scope of this work was to synthetize a sensitive and very selective molecularly imprinted polymer (MIP) for DXy detection in honey samples. Firstly, the synthesis of this biosensor was performed by casting a layer of carboxylate polyvinyl chloride (PVC-COOH) on the working surface of a gold screen-printed electrode (Au-SPE) in order to bind covalently the analyte under mild conditions. Secondly, DXy as a template molecule was bounded to the activated carboxylic groups, and the formation of MIP was performed by a biocompatible polymer by the mean of polyacrylamide matrix. Then, DXy was detected by measurements of differential pulse voltammetry (DPV). A non-imprinted polymer (NIP) prepared in the same conditions and without the use of template molecule was also performed. We have noticed that the elaborated biosensor exhibits a high sensitivity and a linear behavior between the regenerated current and the logarithmic concentrations of DXy from 0.1 pg.mL^{-1} to 1000 pg.mL^{-1}. This technic was successfully applied to determine DXy residues in honey samples with a limit of detection (LOD) of 0.1 pg.mL^{-1} and an excellent selectivity when compared to the results of oxytetracycline (OXy) as analogous interfering compound. The proposed method is cheap, sensitive, selective, simple, and is applied successfully to detect DXy in honey with the recoveries of 87% and 95%. Considering these advantages, this system provides a further perspective for food quality control in industrial fields.

***Keywords*—**Electrochemical sensor, molecular imprinted polymer, doxycycline, food control.

I. Introduction

DXy is an antibiotic used as veterinary drugs to treat a large number of bacterial and protozoal infectious diseases as well as feed additives to promote animal growth. Nevertheless, in the case of non-respect of withdrawal periods, these antibiotic residues may remain in animal tissues or by-products, such as honey, milk, edible tissues, or eggs, which can seriously affect human health [1]. According to these health concerns, its use to treat food-producing animals has been restricted within European Union (EU), USA, Canada, and China, and the maximum residue level (MRL) for DXy at 100 µg.kg^{-1} in muscle, 300 µg. kg^{-1} in liver, 600 µg.kg^{-1} in kidney, and 200 µg.kg^{-1} in eggs has been legally set [2]. In the case of beekeeping, DXy is still given to treat the most replied diseases for honeybees such as American foulbrood [3] due to its low cost and high efficiency. For honey samples, the MRL has been fixed at 100 µg.kg^{-1}.

A series of analytical methods have been used for DXy estimation by using high performance liquid chromatography [4], [5], follow injection analysis [6], fluorometry [7], Raman spectroscopy [8], optical sensor [9], infrared spectroscopy [10]. However, most of these methods are time consuming, solvent-usage intensive and expensive, which limit their use in quality control laboratories as pharmaceutical dosage forms. Recently, some studies involving immunochemical determination of DXy have also been reported [11]. The principal advantage of this method is manifested in the high selectivity of the antibodies, but they remain very expensive product and non-reusable after their first contact with the target antigen. Hence, a new cheap material able to mimic the role of antibodies was been created.

These mimic methods by means of MIPs, offer a clear advantages for sensor technology when compared with antibodies: due to their simple achievement, stability, reusability, low-cost, and simple selective binding sites in polymeric matrices [12], which provide a emergent promise for bio-sensing development in food analysis [13]. Until today, some MIPs for DXy detection were reported [14], [15].

In this study, we describe the synthesis of a sensitive and very selective biosensor based MIP for DXy detection in honey samples. Furthermore, this approach based on MIPs as electrochemical sensor has shown a great enhancement in the detection limit of DXy in complex matrices such as honey.

II. Material and Method

A. Materials

DXy, OXy, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), poly(vinyl chloride) carboxylate (PVC-COOH), methanol,

Nadia El Alami El Hassani (PhD student and member of Biotechnology Agroalimentary and Biomedical Analysis Group) is with Department of Biology, Faculty of sciences, Moulay Ismaïl University, B.P. 11201, Zitoune, Meknes, Morocco (e-mail: elalami.iaa@gmail.com).

Soukaina Motia (PhD student and member of Sensor Electronic and Instrumentation Group) is with Department of Physics, Faculty of sciences, Moulay Ismaïl University, B.P. 11201, Zitoune, Meknes, Morocco (e-mail: sokainasafae@hotmail.fr)

Benachir Bouchikhi (Professor and responsible of Sensor Electronic and Instrumentation Group) is with Department of Physics, Faculty of sciences, Moulay Ismaïl University, B.P. 11201, Zitoune, Meknes, Morocco (e-mail: benachir.bouchikhi@gmail.com).

Nezha El Bari (Professor and responsible of Biotechnology Agroalimentary and Biomedical Analysis Group) is with Department of Biology, Faculty of sciences, Moulay Ismaïl University, B.P. 11201, Zitoune, Meknes, Morocco (corresponding author, e-mail: n_elbari@hotmail.com)

ethanol, 1,4 dioxane, phosphate buffered saline (PBS) , potassium hexacyanoferrate (II) $K_4[Fe(CN)_6]$, and (III) $K_3[Fe(CN)_6]$ were all purchased from Sigma-Aldrich (France). Acrylamide (AAM), N,N-methylene-bisacrylamide (NNMBA), tetra-methyl-ethylene-diamine (TEMED) were provided from Fluka (Germany), ammonium persulphate (APS) from Scharlauchemie (Spain), acetic acid from Riedel-dehäen (Germany). A commercial honey sample assured DXy-free was used for recovery study. Screen-printed gold electrodes (Au-SPE) were provided by Dropsens, Spain under the reference DRP-8X220AT.

B. Preparation of DXy Imprinted Polymer

Firstly, the Au-SPE was washed three times with ethanol, and then rinsed with ultra-pure water. Then, a film of PVC-COOH was achieved by deposing 4 µL of completely dissolved PVC-COOH (8.4 $mg.mL^{-1}$) in dioxane, the incubation of this film was permitted to dry for 2h at room temperature. The carboxylic groups were incubated for 2h in an aqueous solution of EDC (50 mM) and NHS (25 mM). Afterword, a solution of 1 $mg.mL^{-1}$ of DXy was casting on the activated carboxylic groups for 3h at 4 °C. Next, the electrode was rinsed with PBS buffer (pH 7.4) to remove any unbound DXy. Polymerization step was carried out by casting on the surface of Au-SPE a mixture of AAM (1 M), NNMBA (0.07 M) as crosslinking agent and APS (0.06 M) as precursor, all prepared in PBS (pH 7.4), in the presence of TEMED (5%). The polymerization reaction was performed overnight in room temperature. After washing with deionized water, the DXy template was extracted from the polymer by a mixture of methanol and acetic acid (7:3 (v/v)) for 10 min. The developed biosensor based MIP was ultimately ready to use for DXy recognition after washing with ultra-pure water.

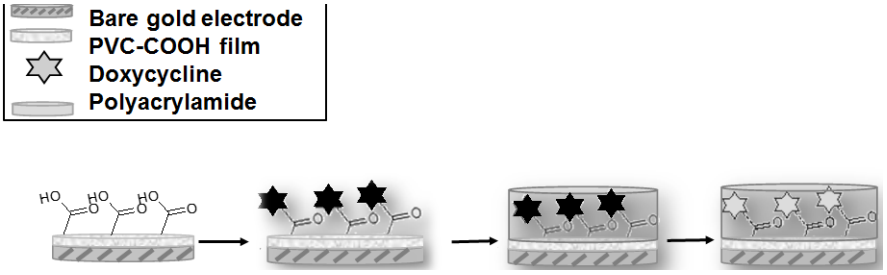

Fig. 1 Synthesis steps of DXy imprinted polymer

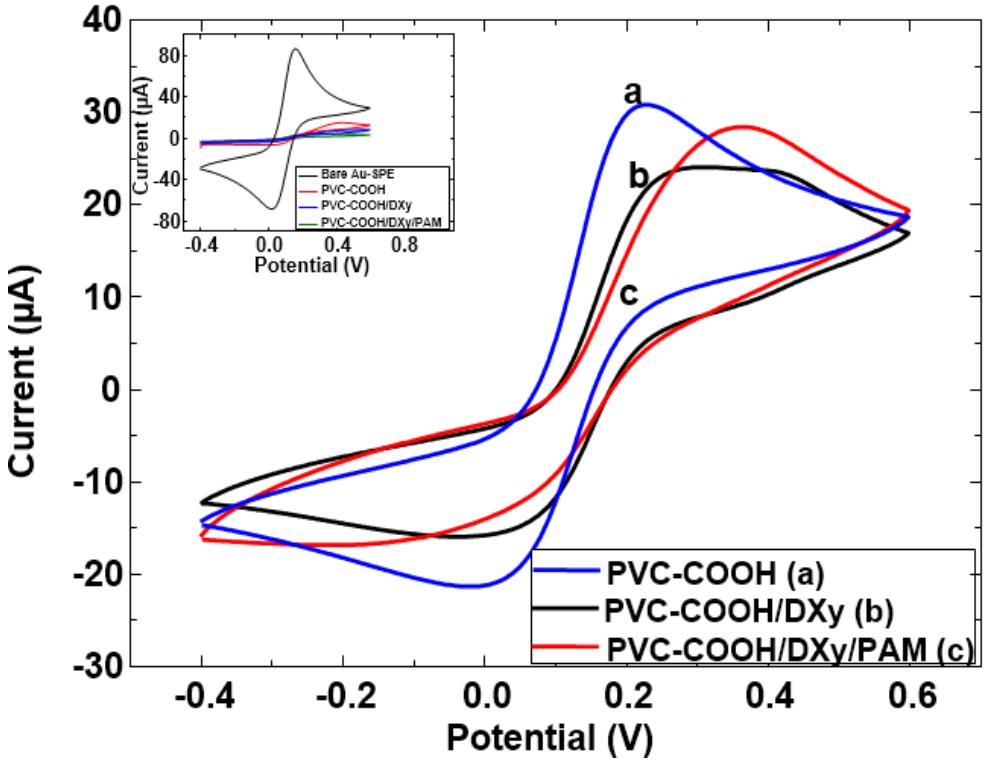

Fig. 2 Cyclic voltammograms of 5 mM $[Fe(CN)_6]^{3-/4-}$ at Au-SPE electrode after: (a) PVC-COOH deposition, (b) DXy- binding, (c) AAM polymerization. Scan rate, 50 mV/s. Inset: Cyclic voltammograms taking into account the CV of the bare Au-SPE

C. Electrochemical Measurements

The electrochemical measurements were performed by cyclic voltammetry (CV) scanned between −0.4 V and +0.6 V at a scan rate of 30 $mV.s^{-1}$ and by differential pulse voltammetry (DPV) in the potential range from −0.1 V to +0.2 V with a scan rate of 10 $mV.s^{-1}$ in a solution of 5.0 mM $[Fe(CN)_6]^{3-/4-}$ prepared in PBS buffer (pH 7.4).

III. Result and Discussion

A. Electrochemical Characterization of the Developed MIP Biosensor

Cyclic voltammetry assay was used in order to confirm the surface modification of electrodes. As can be identified in Fig. 2, there is a relationship between peak current and all steps of Au-SPE surface modification. In the inset of Fig. 2, we can observe that the application of PVC-COOH film on bare gold electrode surface results in a decrease in the electron transfer process noticed by the high peak-to peak potential over the Au-SPE. After DXy binding on the Au-SPE/PVC-COOH surface, a decrease of peak current was also observed. Afterword, the polymerization of AAM toward polyacrylamide (PAM) on the modified electrode surface, shows a small increase of current, which reveals the conducting behavior of the forming DXy-PAM matrix.

B. Detection of DXy by MIP Biosensor

The detection of DXy on the prepared MIP biosensor was performed with different concentrations solutions from 0.1 $pg.mL^{-1}$ to 1000 $pg.mL^{-1}$. The obtained differential pulse voltammograms were presented in Fig. 3 (a). We can observe that the increase of DXy concentrations leads to a decrease of peak current associated to the oxidation potential of $[Fe(CN)_6]^{3-/4-}$, which illustrates the cavity filling by the specific template. An NIP was also performed without use of template molecule, in order to investigate the biosensor sensitivity. Fig. 3 (b) represents the differential pulse voltammograms of the oxidation peaks, revealing the non-sensitivity of NIP material, which explain the non-formation of specific sites capable to recognize DXy. Fig. 3 (c) shows the calibration curves represented by a linear correlation between the variation of peak currents and its related logarithmic concentration for MIP and NIP sensors. The linearity of both plots was explained by the coefficient of determination (R^2) of 0.982 and 0.994 for MIP and NIP, respectively. Furthermore, the high sensitivity of MIP was expressed by its high slope compared to the NIP one, explaining that the electrochemical behavior of MIP sensor is not depending on the non-specific reaction between polyacrylamide and DXy. The LOD for this system was expected to be 0.1 $pg.mL^{-1}$. It is interesting to highlight that this LOD is 106 times lower than the MRPL of DXy fixed by the EU.

C. Selectivity Study

The selectivity of this biosensor was performed by incubating the fabricated DXy biosensor with different concentrations of OXy as interfering molecule with a chemical formula close of that of DXy (Fig. 4).

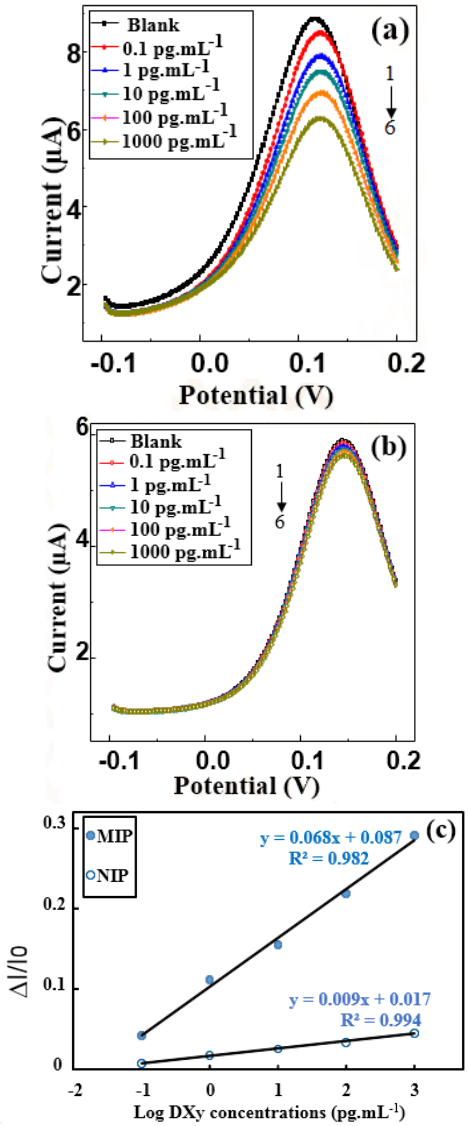

Fig. 3 Differential pulse voltammograms for DXy detection at the five concentrations on: (a) MIP, (b) NIP sensors and (c) Related calibration curves.

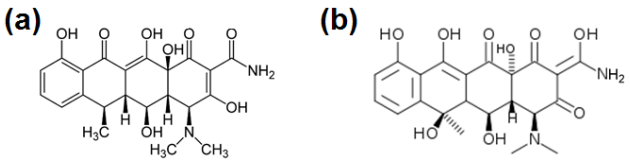

Fig. 4 Chemical structure of (a) DXy, and (b) OXy

As can be seen in Fig. 5, the generated current from this compound was negligible, which explains the ability of this system to reveal the slight variation in the chemical structure of the interfering compounds. This indicates that the synthetized biosensor based MIP represents an excellent selectivity for DXy detection and can offer credible signal in the presence of interfering species.

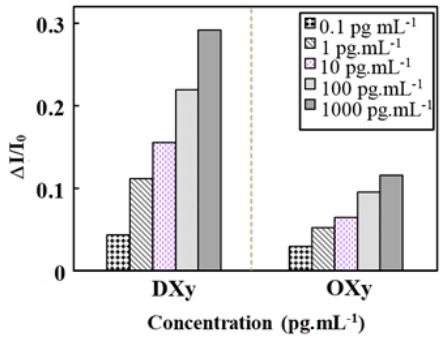

Fig. 5 Selectivity histogram for MIP system in the presence of DXy and its interferent OXy using DPV measurements

D.Recovery Study

The use of the developed biosensor based MIP for honey quality control, was performed by a spiking method realized as follow: 1 g of honey was dissolved in 1 mL of PBS buffer (pH = 7.4) under magnetic stirring. The solution was diluted to a ratio of 1:10 (v/v) and filtered through a 0.8 µm cellulose acetate filter. Next, the sample was spiked with known amounts of DXy standard solutions at: 10 pg.mL^{-1} and 100 pg.mL^{-1}, and measurements were performed by DPV as described before. Table I recapitulates the MIP sensor results of DXy detection in spiked honey sample. Recoveries of 87 % and 95 % were obtained, with a relative standard deviation coefficient (RSD) of 12.2 % and 11.1 %. It was demonstrated that the method was adequate for the total content determination of DXy in real matrix such as honey samples.

TABLE I
DETERMINATION OF DXY IN SPIKED HONEY SAMPLES

Added (pg.mL^{-1})	Found (pg.mL^{-1})	Recovery (%)	RSD (%)
10.0	8.7	87.0	12.2
100.0	95.5	95.0	11.1

IV. CONCLUSION

A molecular imprinted polymer based on Au-SPE was synthetized and used to detect DXy residues in honey samples. This MIP based biosensor presented a high detection ability to DXy when compared to the non-imprinted material with a remarkable selectivity for DXy than the other analogous molecules. The detection limit of the developed technique was found to be 0.1 pg.mL^{-1} with recoveries of 87% and 95%. Therefore, the present biosensor has a good sensitivity, selectivity, and accuracy, which allows it to be used in honey quality control.

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