P450-Mediated Electrochemical Sensing of Drugs in Human Plasma for Personalized Therapy

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Abstract— **Nowadays, the concept of personalized therapy gains momentum. Pharmacogenomics, which represents a first answer to these needs, has the drawback of neglecting some variations of therapy response due to non-genetic factors. The aim of this paper is to investigate the feasibility of a non-genetic approach to personalized therapy, via the point-of-care drug monitoring in biological fluids with electrochemical biosensors. The proposed biosensor is based on the use of P450 enzymes as probe molecules, thanks to their key role in human metabolism. Multiwalled carbon nanotubes are used to enhance biosensor sensitivity. Results show how the proposed system is capable to detect drug amounts within the corresponding pharmacological ranges in human serum.**

Keywords – P450, personalized therapy, electrochemistry, human serum.

I. INTRODUCTION

At present, the most common clinical practice is the so called *trial and error medicine* [1]: according to the symptoms, a drug therapy is prescribed, and if it is not effective, or has significant side effects, it is modified. This approach, which is time consuming and expensive for both the patient and the health care system, is beneficial only to 20-50% of individuals while 7% experience severe adverse drug reactions [2]. Breakthrough targeted therapies could save many lives and a great deal of money. Pharmacogenomics, which considers the unique patient's genetic polymorphism, represents a first step towards personalized medicine. However, lifestyle and drug interactions, which cannot be predicted with genetics, also play an important role in influencing the drug response.

A system based on the direct drug monitoring in the patient's blood represents a more accurate alternative to the pharmacogenomics. P450 proteins represent good candidates for the realization of biosensors for drug detection, since they account for the 75% of body metabolism [3], and often mediate the rate-limiting steps in the biotransformation of xenobiotics [4]. Biosensors based on P450s have already been proposed [4-6]; however, they were always tested in artificial buffers. In this work we propose a biosensor based on *carbon nanotubes* (CNT) and two different cytochrome P450 isoforms, 3A4 and 2C9. These protein isoforms can detect drugs in undiluted human serum. The integration of carbon nanotubes greatly improves the sensor performance [7-9], while CYP 3A4 and 2C9 are chosen because they represent the two most important isoforms involved in drug metabolism [4, 10]. This sensor is an alternative as compared to traditional drug essay techniques, such as spectrophotometry and chromatography, which usually require sample dilution.

II. MATERIAL AND METHODS

A. Reagents

Carbon paste screen-printed electrodes (model DRP-110 and DRP-110 CNT) were purchased from Dropsens. Cytochrome P450 3A4 and 2C9 microsomes were purchased from Sigma-Aldrich and used without further purification. Cyclophosphamide (CP) was purchased from Sigma and diluted in PBS 100mM pH 7.4 to the working concentrations. Naproxen (NAP) was purchased from sigma and diluted in ethanol to the working concentrations. Multi walled carbon nanotubes (MWCNT - diameter 10 nm, length 1-2 µm, COOH content 5 %) were bought in powder (95% purity) from DropSens (Spain), diluted in chloroform to the concentration of 1 mg/ml [4] and then sonicated for 20 minutes in order to break macro-aggregates. Human serum was purchased from Lonza and used without any dilution.

B. Electrodes preparation

The electrodes were made of a graphite working electrode (area, 12.56 mm^2), a graphite counter electrode and an Ag/AgCl reference electrode. The working electrode area was 12.56 mm^2 while the total area of the cell was 22 mm^2. CNT nano-structuring was obtained by gradually dropping 30 µl of CNT solution onto the working electrode and waiting until complete evaporation of the chloroform. Electrode functionalization was obtained by drop cast of P450 solutions onto the working electrode and incubation at 4ºC overnight. The excess of cytochrome was then removed by washing with milliQ water.

C. SEM measurement

Morphological analysis of the structured and functionalized electrodes was carried out with Scanning Electron Microscopy (SEM). A Zeiss SUPRA 40 SEM instrument was used to acquire images for bare electrodes, electrodes structured with carbon nanotubes, and electrodes functionalized with the cytochrome P450. Images were acquired in 5-20 kV range.

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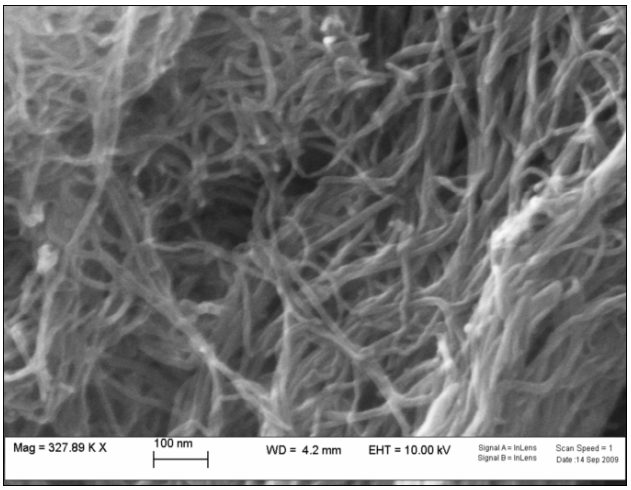

Figure 1. SEM image drop cast MWCNT onto the working electrode.

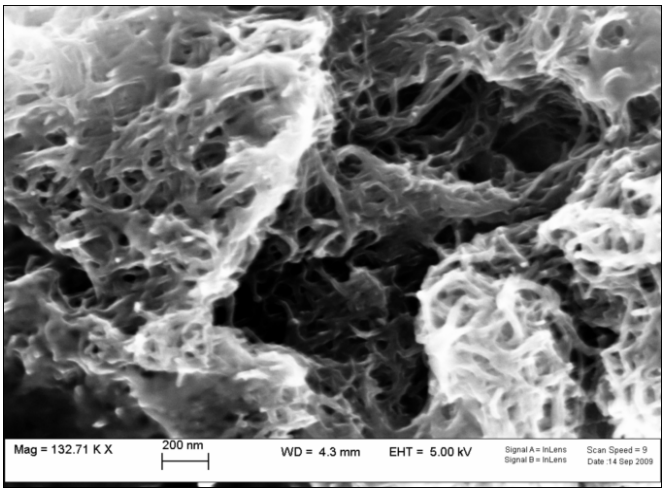

Figure 2. SEM image drop cast MWCNT and P450 microsomes onto the working electrode.

D. Electrochemical measurement

The electrochemical response of the electrodes was investigated by cyclic voltammetry under aerobic conditions. Voltammograms were acquired using a Versastat 3 potentiostat (Princeton Applied Technologies). The electrode was covered with 100 µl of PBS 100 mM at pH 7.4 or serum. Drug samples were added in drops of 1 µl. The cyclic voltammograms were acquired with potential sweeps between -600 and +300 mV vs Ag/AgCl at a scan rate of 20 mV/sec. Peak current values were then extracted according to the procedure reported in [8].

III. RESULTS

A. Electrodes Nano-Structuring and Functionalization

The SEM image analysis proved that the screen-printed electrode surface is highly-corrugated at the nano-scale (data not shown). Surface corrugation widely changed after the structuring with carbon nanotubes. Fig. 1 shows carbon fibers randomly organized onto the screen-printed carbon paste surface, as also shown in other works [11,12] . The fiber has thicknesses ranging from 8 nm up to 20 nm. These values are coherent with the average carbon nanotube diameter declared by the producer, 10nm. After bio-functionalization with the P450 microsomes, fiber size increased to 25 - 48 nm, as shown in Fig. 2.

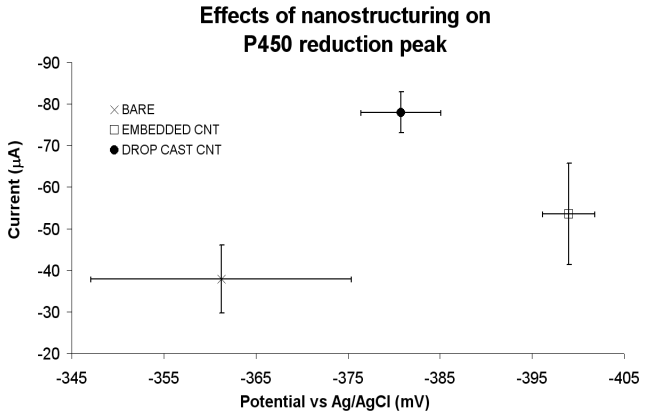

Figure 3. Current and potential variation of P450 versus different electrode nano-structuring.

B. Sensitivity improvement by Electrode Nano-Structuring

The data obtained from SEM images lead to investigations of the most appropriate CNT nanostructuration. We compared the current peaks obtained from cytochrome P450 drop cast onto bare graphite, a commercial screen-printed with CNT embedded in the graphite paste, and an electrode structured by us with 30 µl of drop cast CNT. Fig. 3 shows the average values obtained from the three different electrodes. Drop cast CNT gave the best results in terms of current intensity and reproducibility of results. Peak current increased of 116% compared to the bare electrode and 50% respect to the embedded CNT. Standard deviations in current and potential are also lower, which demonstrate increased reproducibility of measurements.

C. CYP3A4 response to Cyclophosphamide

The biosensor was tested in sensing Cyclophosphamide (CP), a chemotherapy drug used for treatment of lymphomas, some forms of leukemia and some solid tumors. P450 peak current was first acquired in absence of drugs (blank), and then, in presence of CP in concentrations of 100, 200, 300, 400 and 500 µM. The related current values were subtracted to the blank, in order to monitor the effect of the drug addiction. Fig. 4 shows the average current values obtained in serum and PBS. Error bars represent the standard deviation between the electrodes. The sensor performance is very close for the two buffers: the measured sensitivity resulted in 0.06 nA/µM mm^{-2} in case of PBS and 0.04 nA/µM mm^{-2} in case of serum. For both media, the average current increase between blank and CP 100 µM was 0.13 µA, a value 40% higher than that between two subsequent drug concentrations. Peak increase becomes smaller for higher concentrations of CP.

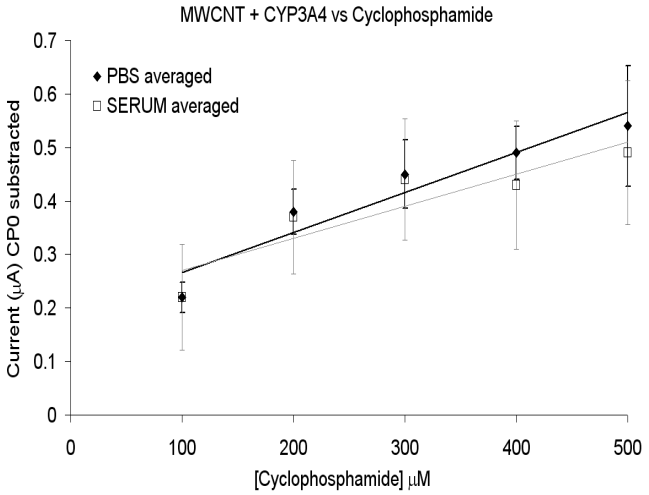

Figure 4. Variation of CYP3A4 peak current at different concentrations of cyclophosphamide. The plot shows the difference between the current in presence of the drug and the blank.

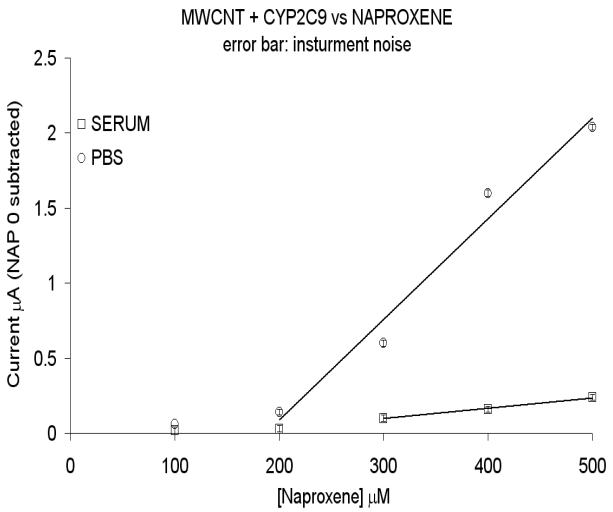

Figure 5. Variation of CYP2C9 peak current at different concentrations of Naproxen. The plot shows the difference between the current in presence of the drug and the blank.

D. CYP2C9 response to Naproxen

The biosensor was tested in sensing Naproxen (NAP), a non-steroidal anti-inflammatory drug commonly used for pain reduction, fever, inflammation and stiffness. P450 peak current was first acquired in absence of the drug (blank) and then, in presence of NAP in concentrations of 100, 200, 300, 400 and 500 μM. The resultant current values were subtracted from the blank, in order to monitor the effect of the drug addiction. Fig. 5 shows the current values obtained in serum and PBS from a single electrode. Error bars represent the standard deviation in measurements with the same electrode. Measured sensitivities differ of one order of magnitude in the different buffers: 0.53 nA/ μM mm^{-2} in case of PBS and 0.05 nA/ μM mm^{-2} in case of serum. In the latter case, the value is comparable with sensitivity obtained with the CYP3A4. The sensor detection limit was 200 μM for both media.

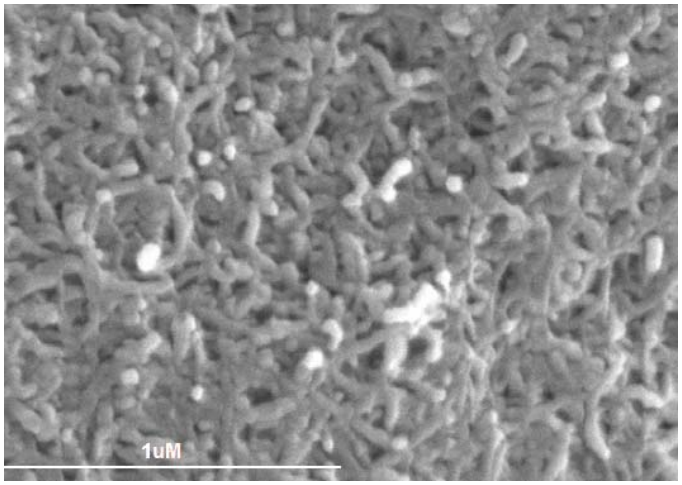

Figure 6. SEM image of screen printed electrode DRP110-CNT, reprinted form the constructor's technical data-sheet [12]

IV. DISCUSSION

A. Electrode nanostructuring

SEM images showed an average increase of 20 nm after the drop cast of P450 microsomes. Taking into account that the cytochrome P450 is a globular protein with size below 10 nm, this means that a single protein layer is surrounding each single carbon nanotube fiber. Therefore, each probe protein is in direct contact with the nanotube surface accounting for a direct electron-transfer between CNT and proteins. Increased sensitivity of drop-casted CNT can be due to the three-dimensional architecture of the nano-structuring. SEM images reported from electrode constructor [13] and from [14] show that CNT embedded in carbon paste change the texture of the carbon surface, allowing a deeper penetration of the electrolytes and a larger amount of probe proteins, as shown in Fig. 6. Our data shows how the drop cast nanotubes present a less compact texture, which allows us to increase the amount of probes adsorbed.

B. Drug detection

In case of CYP 3A4, the optimal concentration window for cyclophosphamide sensing, can be placed at lower concentrations as compared to the maximum one. This is due to the diminishing of peak increase with higher drug amounts and to the current gap between the blank and the first drug concentration. On the other hand, the CP pharmacokinetic range recorded in the blood during therapies is typically between 0 and 77 μM [15]. Luckily, Fig. 4 does not show a detection limit. Therefore, further refining the proposed biosensor is possible for smaller drug concentrations. An additional reason for lowering the operating window is related to the enzyme kinetics. If the drug amount is larger than the enzyme concentration on the electrode, the rate of catalysis approaches asymptotically to a limit value. Since peak current depends by the rate at which cytochrome and electrode are exchanging electrons, a constant speed of catalysis means

constant electrons exchange and, therefore, constant current. Achieving a response proportional to the drug addiction could be possible by lowering the substrate concentration or augmenting the quantity of the enzyme drop cast on the electrode surface. Data from CYP2C9 reported in Fig. 5 is a characterisic example of current response proportional to the drug concentration. Results demonstrate that detection limit for Naproxen sensing is determined at 200 μM since current variations at lower concentration are smaller than the acquisition noise. The lower sensitivity achieved in serum is attributed to plasma proteins, which can affect the P450 performance onto the electrodes and can retain drug molecules. In case of naproxen for example, it was demonstrated that 99.7% of compound in the blood is bound to albumin [16], therefore effective naproxen concentration at the electrode can be lowered due the presence of those complexes. These two results show the possibility to accomplish real-time electrochemical drug detection in biological buffer due to the intrinsic nature of the drug/P450 interactions. However, the advantage of a wide substrate range in using P450 proteins is also a problem for the unequivocal identification of compounds when more than one drug is present in the patient's blood. Different P450 isoforms can have the same drug compound as substrate and different drugs can be metabolized by the same protein. For example, naproxen is metabolized both by CYP2C9 and CYP1A2 [17], or dextromethorphan is a common substrate of CYP2D6 and CYP3A families [18]. A general approach to this issue is the design of an electrode array containing different P450 isoforms, which may be selectively queried by choosing the protein combinations that can grant the unequivocal identification of a single compound. This principle related to *irredundant cover problem,* has been explained more in detail in our previous work [8].

V. CONCLUSIONS

In this paper, we demonstrated the feasibility of P450-based technology for drug monitoring in human serum for personalized therapy. The sensor, based on drop-cast MWCNT and two different cytochrome P450 isoforms is capable to detect drugs in micromolar concentrations. The amounts measured were close to therapeutic range of the considered drugs. Applications in drug therapy are therefore feasible. The proposed approach has the potential to represent a valid alternative to pharmacogenomics methods, since it can account for factors that cannot be predicted on the sole genome knowledge, such as lifestyle and drugs interactions. We claim that such technology can be used to develop point-of-care and low cost devices for personalized therapy. The potential socio-economical relevance of the technology proposed here is high, since the possibility to overcome the traditional trial and error drug therapy can allow a huge decrease in the medical care costs, and reduce the time needed to find the right therapeutic regime, improving the quality of the life of the patients.

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