Multiplexing pH and Temperature in a Molecular Biosensor

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***Abstract*—Robust and reliable measurements in electrochemical biosensing of molecules are crucial for personalized medicine. Electrochemical sensors based on cytochrome P450 can detect the large majority of drugs commonly used in pharmacological treatments. The same cytochrome can detect different substrates; each of them changes the electrochemical response of the enzyme in a specific manner. Our system exploits the measure of electrical potential to identify the drug type, while current measurements decode the drug concentration. Since potential and current are affected by pH and temperature, and since variations occur in the patient samples, we propose a novel design for multiplexing biosensing with pH and temperature control, which ensures more precise measurements for drugs identification and their quantification.**

I. INTRODUCTION

The cytochromes P450 are the major enzymes involved in drug detoxification, and account for the 75% of the total metabolism [1]. This feature makes them ideal candidates to develop biosensors for drug monitoring in personalized therapy. These cytochromes have a central role because they are suitable to metabolize a wide range of substrates [2]. Recently, several works have shown the possibility to detect endogenous compounds [3], single drugs [4], or drug mixtures [5], using different isoforms. P450-mediated detection is mainly based on the measurement of current peaks, which originate from cyclic voltammetry. In presence of a substrate, this peak is affected by a potential shift, which varies according to the used drug [6]. This feature, called *drug electrochemical signature,* allow to distinguish different compounds in presence of a solution with multiple P450 substrates [5]. According to the electrochemical theory [7 ,8], pH and temperature variations change the peak position and, if not properly considered, can twist the results obtained with the peak analysis leading to false-positives in case of a solution with unknown substrates. Living matter is very sensitive to pH and temperature levels: outside the acceptable ranges, proteins are denatured, enzymes lose their ability to function and cells apoptosis may occur. For this reason, pH and temperature dependence of biological molecules became extremely important when working with biosensors, since a variation of these parameters can influence the output response [9, 10]. In this work, we are showing a prototype of biosensor for drug detection based on P450 3A4, which supports pH and temperature measurement. The proposed system and the related circuits represents a valid candidate for realization of devices for multiple drug monitoring in personalized therapy: P450 3A4 is involved in the metabolism of 36% of all known drugs [11], opening the possibility to use the sensor for a wide range of compounds. Temperature and pH monitoring ensures strict and precise control of the electrochemical detection when multiple drugs are present in the sample, and extend the applicability of the sensor to different biological fluids, with different pH and temperature levels, like blood, urine, and interstitial liquid.

II. MOLECULAR BIOSENSING

A. Reagents

Carbon paste screen-printed electrodes (model DRP-110) were purchased from Dropsens. Cytochrome P450 3A4 microsomes and PBS solution were purchased from Sigma-Aldrich, and used without further purification. Multi walled carbon nanotubes (MWCNT - diameter 10 nm, length 1-2 μm, COOH content 5%) were purchased in powder (95% purity) from DropSens, diluted in chloroform to the concentration of 1 mg/ml [2] and then sonicated for 20 minutes in order to break macro-aggregates.

B. Electrode nanostructuration

Commercially available screen-printed electrodes (Dropsense, Spain) were used for electrochemical investigations. The electrochemical cell was composed of a graphite *working electrode* (WE), a graphite *counter electrode* (CE) and an Ag/AgCl *reference electrode* (RE). The WE area was 12.56 mm^2 while the total area of the cell was 22 mm^2. CNT nano-structuring was obtained gradually dropping 30 μl of CNT solution onto the WE and waiting until complete evaporation of the chloroform. Sensor functionalization was obtained by drop cast of P450 solutions onto the WE and incubation at 4ºC overnight [3]. The excess of cytochrome was then removed by washing with milliQ water.

C. Electrochemical Measurements

The electrochemical response was investigated by cyclic voltammetry under aerobic conditions. Drops of samples were spread onto screen-printed electrodes to form the electrochemical cell.

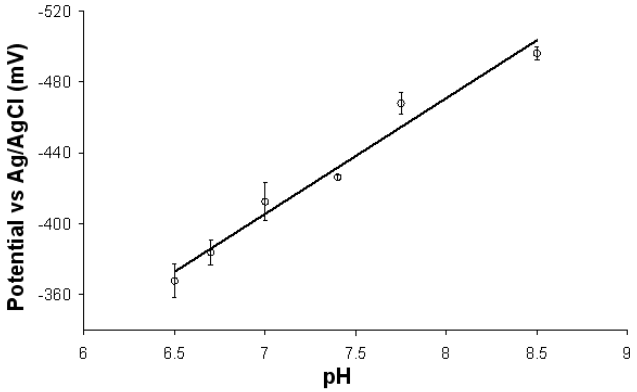

Figure 1. Peak potential shift as due to pH. Error bars represents\ the inter-electrode variation.

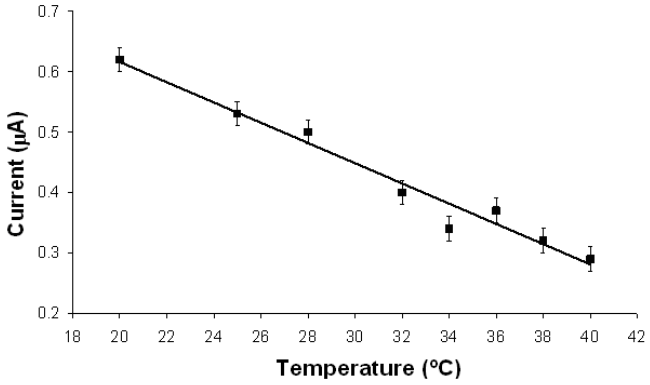

Figure 2. Peak current shift as due to temperature. Error bars represent the inter-electrode variation.

Thus, these measurements were close to quasi-static conditions we may reach in working with the auto-monitoring strips. Voltammograms were acquired using a Versastat 3 potentiostat (Princeton Applied Technologies). For pH measurements, the electrodes were covered with 100 µl of PBS (phosphate buffer solution) 100 mM at various pH. For temperature measurement, the electrodes were covered with a 100 µl of PBS 100 mM at pH 7.4, pre-heated at different temperatures. Peak current and potential values were then extracted according to the procedure reported in [5]. Fig. 1 confirms the shift in potential of the P450 peak current induced by different pH values. Acidic values of pH, correspond to less negative values of potential, while basic pH move the potential towards more negative values. The pH variation resulted to be reproducible and linear. We obtained a shift of 65.3 mV/pH from the regression equation. The Nernst equation explains this linear behavior [8]:

$$E = E^0 - \frac{RT}{nF}\ln\left(\frac{C_r}{C_o}\right) - \frac{RT}{F}pH\,. \qquad (1)$$

In the equation, E is the potential position in the voltammogram of the current peak, E^0 is the standard potential, R is the gas constant, F is the Faraday constant, n is the number of electrons involved in the redox, C_r and C_o are the concentrations of the reduced and oxidized species at the interface.

Equation (1) also shows that the peak potential depends on temperature. Thus, variation in temperature affects the peak position, too. Moreover, the temperature also affects peak current, as shown by figure 2. Current is decreasing regularly when the sample is heated, showing a relationship between the two parameters. From the regression equation, we obtained a variation of -16.7 nA/°C.

The Randles-Sevchick equation links the current peak to temperature [7]:

$$i \propto nFAD\left(\frac{nFvD}{RT}\right)^{1/2} C_r\,, \qquad (2)$$

introducing the scanning velocity v, the working electrode area A, and the diffusion coefficient D. The equation (2) shows an inverse relationship with the square of temperature that is approximated by a linear curve for small windows in temperature, as shown by figure 2.

III. Sensing Design

To address the need for pH and T measure and control, the multiplexing between a temperature sensor, a pH sensor, and a potentiostat-based sensor for electrochemical detection based on P450 cytochromes was designed and simulated for future development of VLSI biochips. The circuitry was designed in standard 0.18 µm UMC CMOS technology. The developed architectures were simulated using Cadence® Virtuoso® circuit simulator. The power consumption of the whole circuit was of 48 µW for voltage supply V_{dd} of 1.5 V.

A. The Molecular Biosensing

The electrochemical sensor is shown in Fig. 3. The potentiostat configuration was used to stabilize the voltage at the working electrode and nullify the current in the reference electrode accordingly with literature for three-electrode cell configuration [12]. The potential on the RE must be stable for reducing fluctuations in current measurements [13]. In the chosen topology, based on reference [12], the WE was kept to a fixed potential, making it insensitive to noise and interference pickup [13].

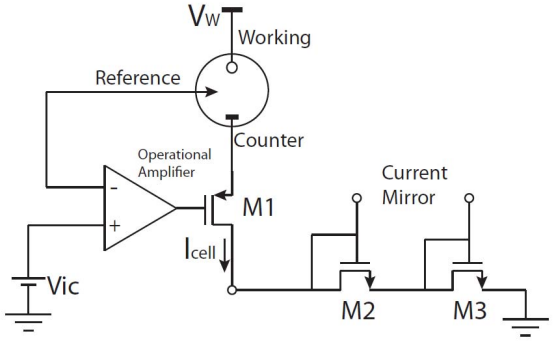

Figure 3. The voltage-follower to polarized the reference electrode and the two transistors to amplifier current emerging from the counter electrode.

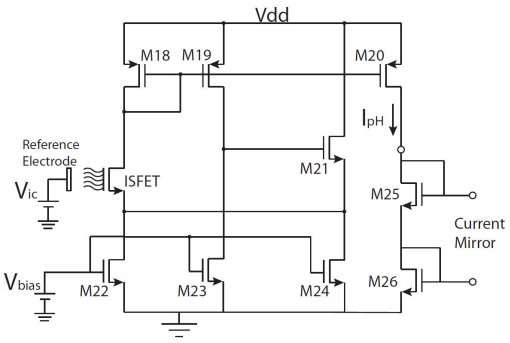

Figure 4. The ISFET sensors proposed by Premanode [15] with a current feedback for the pH measure.

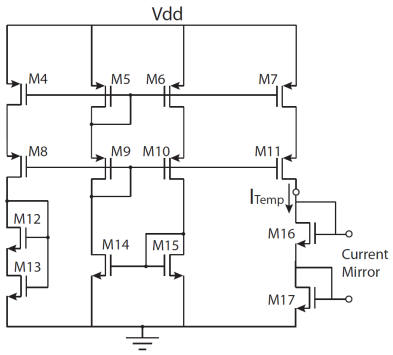

Figure 5. The circuit for temperature measures proposed by Rossi [16].

In Fig. 3, the operational amplifier (called control amplifier) keeps the cell potential on the RE at a desired value (V_{ic}) by following the current cell through the PMOS transistor M1. The PMOS M1 modulates the current in the counter electrode until the inputs potentials of the control amplifier are the same. Because a very low current comes from the CE, M1 works in *weak inversion* (WI). A current mirror, also working in WI, was used to reduce noise in the current-to-frequency converter and for multiplexing the three sensors of the biochip. The design of amplifier was made considering a two stage configuration with a gain about 70 dB and a phase margin about 80°, which allows low power and low noise solution [14]. A power dissipation of 32 µW is obtained from the simulations. A very low sensitivity to temperature, in the order of 95 fA/°C in the range of biological samples, was verified by simulations.

B. The pH Sensing

The ISFET with the readout circuit proposed by Premanode et al. [15] shown in Fig. 4 was used for pH measure. The voltage follower (M21) keeps constant the voltage V_{gs} at the ISFET by automatic compensation of any changes in the ISFET threshold-voltage due to pH. For the selected readout, a power dissipation of 2.5 µW is obtained from the simulations. Although many papers deal with the problem of temperature sensitivity, light sensitivity and drift, no general solutions for prevention, reduction or compensation of these effects exist. Therefore, the best is to simultaneously measure the temperature and compensate with respect to its variations as well as for offset. Thus, a temperature sensor has been also included in order to control the possible impact on the pH evaluation.

C. Temperature Sensing

Temperature sensors require cells that generate PTAT (Proportional To Absolute Temperature) voltage. Rossi et al [16] had introduced the approach followed in this paper, which is based on a quasi-constant current to obtain this voltage in standard CMOS technology using no resistors. The main goal of this approach is to achieve the minimum possible consumption while low voltage operation is achieved.

The simulations showed that the precision of device shown in Fig. 5 is around 0.5 °C, for a temperature range from -40 °C to 120 °C [16]. In the case of biomedical applications, the temperature range is more limited and the precision is improved to 0.05 °C. In the circuit of Fig. 5, the M12 and the M13 work in weak inversion and they modulate current in the cascode current mirror M4-M11. The circuit has been designed to work with an output current range of 350 nA – 400 nA, following the range of temperature in samples from patients. This solution was chosen for low-power consumption and its high sensitivity to temperature changes. Results from simulations showed sensitivity in the order of 4 nA/°C in T range of biological samples and a power dissipation of 1.4 µW.

IV. MULTIPLEXING

A. Multiplex of three parameters

A multiple configuration was used to select the information from the three sensors. The analog multiplexer works by mirroring the current in the current mirrors, referred as M2, M3 (Fig. 3), M16, M17 (Fig. 5), M25, M26 (Fig. 4) and M27, M28 (Fig. 6).

Every sensor generates a current in its own first mirror branch, while the other branch is common and connected to a current-to-frequency converter. A series of MOS switches, controlled by external output, decides which current is introduced in the current-to-frequency converter.

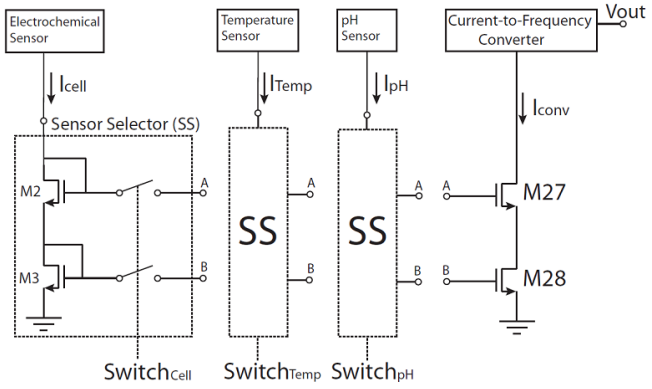

Figure 6. The architecture for multiplexing.

For this reason, it is important that the current range of each sensor belongs to the linearity range of the converter, as been confirmed by simulations. In this way the sensors, which are not subject to current interruption, do not need a transient time before the next acquisition.

B. Current-to-frequency converter

The current-to-frequency converter was realized using the circuit shown in Fig. 7. Different comparators were used for reducing the power consumption. The converter reads the current coming from the current mirror and converts it into a periodic voltage signal with a frequency directly proportional to the input current value. The capacitor Cp is charged and discharged for generating a saw-tooth signal in input of the two comparators. The following equation gives the time required to charge or discharge the capacitor [17]:

$$\Delta T = C_p \frac{\Delta V}{I_{conv}} \tag{4}$$

Consider the capacitor Cp initially charged and the switch MOS M29 open, the current of the mirror discharges the capacitor. When the value of the voltage reaches V_{down}, the high-gain comparator closes the switch M29 (V_c) that enables the charging of Cp. A second low-power comparator was inserted with a S-R flip flop for keeping the MOS M29 closed until the capacitor is fully charged. To minimize the injection of switching noise from the converter in the other circuits, an independent supply voltage DV_{dd} of 1.5 V was used. According to Equation (4), the discharge time will be inversely proportional to the mirror current. A static power dissipation of 12 µW and a good linearity in the range of 350 pA – 600 nA was obtained from simulations. In this range, frequency response of the circuit was between 370 Hz to 590 Khz.

V. CONCLUSIONS

In this paper, we proposed a new architecture for multiplexing molecular biosensing with temperature and pH measures. The sensor was designed to identify drugs by using the P450 3A4 as probe protein. The biosensing was done with cyclic voltammetry, where the drugs are recognized from the peak potential and quantified by the peak current. Since both peak potential and peak current are depending on pH and temperature, their monitoring ensures precise estimations of the analites when multiple drugs are present into the sample. Controlling the smallest variations in current and potential is crucial to reduce the risk of false positives, since differences in drug electrochemical signatures often relate to few mV, and since a variation of few nA is often sufficient to distinguish between different drug concentrations.

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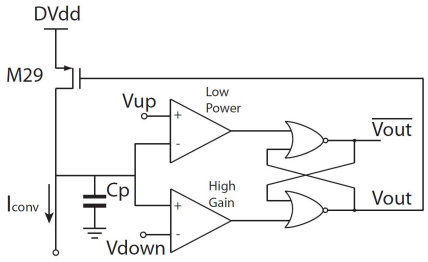

Figure 7. current-to-frequency converter.

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