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An Innovative System of Membranes for the Monitoring of Endogenous and Exogenous Metabolites

C. Baj-Rossi[1] · S. S. Ghoreishizadeh[2] · G. De Micheli[3] · S. Carrara[3]

Abstract The development of a low-cost, robust, and versatile biosensors for the rapid detection of endogenous and exogenous metabolites in small animals is of great interest for health-care, pharmaceuticals, and in translational medicine. This work presents a complete in vitro characterization of a system of membranes for the development of a biosensor that will be integrated with the dedicated electronics into an implantable device for small animals. The system of membrane consists of an "inner" permselective layer, designed to filter the signal generated by the oxidation of interfering substances present in biological fluids; an "outer" layer made by an epoxy-enhanced polyurethane film, that regulates the passage of glucose and oxygen to the electrode surface and provides a biocompatible layer for a correct integration with the surrounding tissue. This system of membrane is employed in a glucose sensor that successfully monitors glucose in both the human and mouse physiological range, in PBS and in human serum at 37 °C. The sensor showed good stability for 30 days, and the permselective membrane effectively filtered out ascorbic acid and uric acid. Moreover, with the same system of membrane, we developed a biosensor for detection of the anti-inflammatory drug acetaminophen. The integration with the dedicated electronics is successfully used to measure glucose in the physiological range.

Keywords Biosensor · Permselective membrane · Analyte-diffusion membrane · Implantable · Interferences

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✉ C. Baj-Rossi
camilla.bajrossi@gmail.com

S. S. Ghoreishizadeh
s.ghoreishizadeh14@imperial.ac.uk

G. De Micheli
giovanni.demicheli@epfl.ch

S. Carrara
sandro.carrara@epfl.ch

[1] Microsystems for Space Technologies Laboratory, École Polytechnique Fédérale de Lausanne, Neuchâtel, Switzerland

[2] Department of Electrical Engineering, Centre for Bioinspired Technology, Imperial College, London, UK

[3] Laboratory of Integrated Systems, École Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

1 Introduction

Nowadays, the health-care system and the pharmaceutical research are demanding high sensitive implantable devices for monitoring endogenous and exogenous metabolites to improve both personalized medicine and translational medicine [1]. Small rodents are commonly used animals for developing new treatments and research applications in translational medicine, because they save costs and time [2]. However, since the reduced size of mice, the implantable system has to be small, light-weight, and biocompatible, thus posing some technological challenges [3].

Another technological challenge is the realization of an implantable device useful for personalized medicine, because, up to date in the market, few prototypes of commercial fully implantable biosensors for continuous glucose monitoring only have been developed [4–7], although not yet approved for commercialization.

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The only available drug sensors in the market are screening tests for drug abuse, which can analyze both blood and urine samples [8, 9]. The research community is currently interested in developing sensors for drug monitoring, and new sensors would represent a new interesting market opportunity.

The objective of the work presented in this paper is the characterization and testing of a system of membrane on the sensing platform reported in [10] that in future developments will be implemented in an autonomous implantable device [11] for the real-time and continuous monitoring of glucose, drugs, and other parameters, such as pH and temperature, with fully electronics that can actuate the device, collect the data, and transmit the data to an external user [12, 13]. The device will be implanted subcutaneously in mice used in research on animals.

The design of an effective membrane system and an external packaging must promote the biocompatibility and biostability of the implantable device. However, the same system of membranes should meet other requirements: (i) filtering the interference from interfering compounds present in biological samples, e.g., uric acid (UA) and ascorbic acid (AA); (ii) obtaining current steps that are higher than 10nA upon glucose injection that can be detected by the front-end electronics; and (iii) extending the linear range of the glucose sensor up to 15 mM, because the device will be implanted in the peritoneum of mice to monitor for for 30 days drugs and glucose in the physiological ranges, which is equal to 6–9 mM, and 12–15 mM for glucose concentration in normal and diabetic mice, respectively [14].

This paper shows the in-vitro characterization of a system of membranes composed by a permselective layer, normally used to eliminate interferences, which defines the selectivity of the sensor; an intermediate enzyme layer used to specifically detect glucose; and an outer layer that controls the glucose and oxygen fluxes and that provides at the same time a biocompatible interface, necessary for any implantable devices [15–17]. The complete system is shown to be (i) effective for detection of glucose and the drug acetaminophen in the physiological ranges, (ii) stable for 30 days, (iii) effective in filtering the interfering compounds, and (iv) able to generate current levels compatible with the dedicated electronics.

2 Materials and Methods

2.1 Microfabrication of the Sensing Platform

The microfabrication of the sensing platform was realized at the EPFL Center of Micronano Technology (CMI). Silicon wafers with 500 nm of native oxide were chosen as substrate. Chip metalization was realized by evaporation of 10 nm of Ti, followed by 100 nm of Pt. Metal passivation was made via atomic layer deposition of Al_2O_3, followed by dry etching with argon ion milling. Details on the microfabrication can be found in [18].

A photograph of the sensing platform is depicted in Fig. 1. The platform measures 31 x 12 mm and hosts an array of four independent cells in the three-electrode configuration: a working electrode (WE), a counter electrode (CE), and a reference electrode (RE), all made in Pt. The WEs are surrounded by a semicircular CE and a small RE. This design was chosen to grant that each cell presented the same structure and distance between electrodes. The platform also contains a fifth cell, which is employed as pH sensor. It has a diameter of 300 µm and it is based on the deposition of an anodic Iridium oxide film. The RTD used as a temperature sensor consists of a Pt wire, 4 nm wide and 93 mm long. The characterization and calibration of the pH and temperature sensors is reported in [10].

2.2 Electrode Preparation

Multi-walled carbon nanotubes (MWCNTs, ∼10 nm diameter and ∼1–2 µm length) with 5 % –COOH groups content, were purchased as a powder (90 % purity) from Metrohm (USA). For drop-cast deposition, a 1 mg/ml solution of MWCNTs, prepared in chloroform, was sonicated for 3 h to obtain a homogeneous suspension. A 0.7 % *w*/*v* chitosan solution (pH 5) was prepared as described in [19]. MWCNTs (0.2 µl) were transferred on the electrode surface by direct drop-cast of MWCNTs dissolved in chloroform and then of MWCNTs dissolved in chitosan.

Glucose oxidase (GOx) from *Aspergillus niger* and bovine serum albumin (BSA) were purchased from Roche and Sigma-Aldrich, respectively, in powder and dissolved in a 100 mM PBS (pH 7.4). D-(+)-glucose was purchased from Sigma-Aldrich (Switzerland) in powder and dissolved in PBS, pH 7.4.

The drugs acetaminophen and etoposide, and the interfering substances ascorbic acid (AA) and uric acid (UA) were purchased as a powder from Sigma-Aldrich. Due to

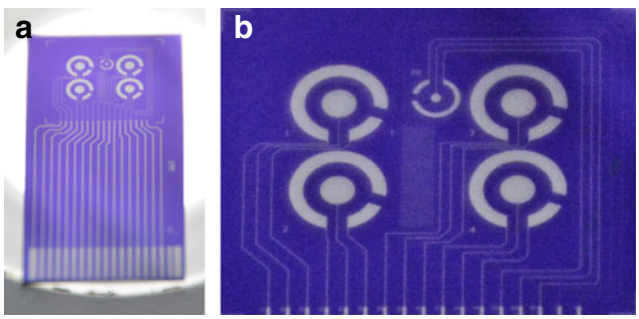

Fig. 1 Photograph of the sensing platform in the version for in vitro tests (**a**), and for implantable applications (**b**)

its low solubility in water, acetaminophen was dissolved in ethanol, while etoposide in DMSO. A H_3BO_3 buffer solution (0.02 M, pH 9.0 at 25 °C) was employed to prepare 3.57 mM UA stock solution. Solutions of AA, UA, and acetaminophen were freshly prepared before each measurement. All the dilutions were carried out in a PBS (10 mM, pH 7.4) solution. H_2O_2 was purchased from Reactolab SA, Switzerland, and diluted in DI water. In all measurements presented in this study, the interference of AA and UA were tested at the highest concentration in their physiological ranges: 85 and 500 µM for AA and UA, respectively [1].

For the measurements with the permselective membrane, cellulose acetate (CAc) (average Mn ~50,000) was purchased from Sigma-Aldrich in powder. A solution 5 % (*w*/*v*) was obtained by dissolving 500 mg of CAc in 50 % ethanol and 50 % acetone (5 ml each). The solution was stirred for 15′ with a speed of 250 rpm, to obtain a homogeneous solution. The CAc membrane was transferred on the electrode by dip-coating [16]. A layer of Nafion 0.5 or 5 % was added by drop-casting of 1 µl of Nafion solution on the top of the cellulose acetate membrane, and then dried for 10′ in air at room temperature [17, 20]. After this step, the enzyme layer was deposited, first by drop-casting the double layer of MWCNTs (in chloroform and in chitosan), and the solution of the enzyme. For the glucose and acetaminophen detection, 2 µl of a solution 15 mg/ml of GOx and 20 mg/ml of BSA, respectively, were dropped on the WEs. Electrodes were stored overnight at 4 °C for protein adsorption. Before drying the enzyme, a 1 µl drop of glutaraldehyde (from Sigma-Aldrich) 0.25 % is added on the enzyme solution and left at room temperature for drying. The preparation and deposition of the biocompatible outer membrane is described in [10].

2.3 Electrochemical Measurement Procedure

All experiments were carried out in a PBS 1X, pH 7.4, as supporting electrolyte. For measurements in biological fluids, human serum from human male AB plasma, USA origin, sterile-filtered was purchased from Sigma-Aldrich. It was kept at 37 °C until use. Electrochemical measurements were performed using an Autolab electrochemical workstation (Metrohm, Switzerland). Electrodes were tested for glucose sensitivity in chronoamperometry at +650 mV. The sensors were first dipped in PBS under stirring conditions for 30′ stabilization at +650 mV and then tested against repeated injections of glucose.

Chronoamperometry measurements were also performed with the autonomous integrated circuit (IC), as it will be shown the last section.

Sensitivity and limit of detection (LOD) are the key parameter used to evaluate the sensing performances. Sensitivity per unit area was computed from the slope of the calibration line. The LOD was computed as three times the signal-to-noise ratio, according to [21, 22].

3 Results and Discussion

Without any membrane to cover the electrode, the calibration of glucose presents a quite linear response upon glucose injection, but with a clear saturation after a concentration of 8–9 mM, as shown in Fig. S1a. From the regression analysis of the calibration line a sensitivity of 47 ± 2 µA/mM cm^2 and a LOD of 0.6 ± 0.1 mM were obtained. As the device will be employed for in vivo measurements in mice, an analysis of the selectivity for glucose over interfering species during characterization is therefore necessary. Figure S1b shows how relevant the interference of UA, AA, and acetaminophen is, if compared to the current signal obtained upon the injection of glucose 1 mM on a GOx-MWCNT-modified electrode. However, in the present application, the drug acetaminophen is not considered an interfering substance in mice, unless it is administered on purpose for pharmacological or toxicological studies [23, 24].

The characterization of the inner permselective membrane, to reduce the problem of the interfering substances, is presented in the next section, followed by the characterization of the combination of the inner and outer membrane. Finally, a long-term study in 30 and 50 days proves the stability of the sensor.

3.1 Permselective Membrane

One of the simplest strategies to reduce the problem of interfering substances is the use of a permselective membrane [1]. Different polymeric materials, also in multi-layers, have been used for blocking interfering electroactive compounds based on their charge or size: electro-polymerized films [25–28], plasma-polymerized films [29], CAc membranes [30], and Nafion, a negatively-charged perfluorinated ionomer able to effectively repel the negatively-charged AA and UA [31]. Polymeric multi-layers, as a combination of Nafion and CAc, have been used to combine the properties of different films with different anti-interference properties [16, 17, 20]. For the present application, a combination of CAc and Nafion as permselective inner membrane was selected as fast and effective method to block interfering substances with the additional advantage that it requires a simple preparation and deposition by dip or spin coating.

An initial membrane characterization of the inner membrane layer was performed with a bare electrode, in presence of H_2O_2. As shown in Fig. 2a, the CAc/Nafion 5 % membrane resulted in an excellent permselectivity, respect to the bare electrode but also respect to the use of the CAc membrane alone. With the CAc/Nafion 5 % membrane, the

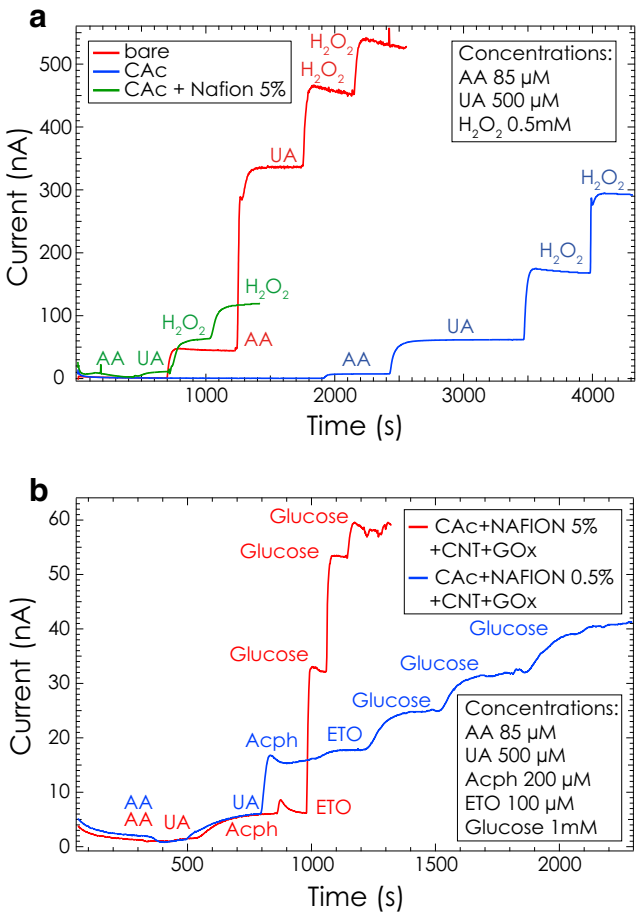

Fig. 2 Comparison in chronoamperometry at 650mV, between a bare electrode, an electrode with the CAc membrane, and an electrode with a CAc/Nafion (5 %) membrane, towards the screening of the interferents AA and UA, and in presence of H_2O_2 (**a**). Comparison in chronoamperometry at 650mV between the use of a layer of Nafion 0.5 % and Nafion 5 % with a CAc-membrane modified MWCNT/GOx electrode, in the presence of glucose and in presence of the interfering substances AA, UA, and the drugs acetaminophen and etoposide (**b**)

interference from AA is completely eliminated and the signal generated by the oxidation of UA is drastically reduced. As expected, the addition of the CAc membrane, and later of the Nafion layer, results in a significant reduction of the current response.

MWCNTs and GOx were immobilized on the top of the CAc/Nafion permselective membrane (Fig. 2b). In this work, glutaraldehyde was selected to stabilize and cross-link the immobilized enzyme [1, 16, 17, 32]. Figure 2b also reports the comparison between the use of a layer of Nafion 0.5 % (blue plot) and Nafion 5 % (red plot). A high Nafion concentration (5 % instead of 0.5 %) is necessary for a complete filtering of UA and AA. The ability of the membrane to filter the presence of another electroactive drug, etoposide, was also tested: Fig. 2b shows that etoposide does not give any response with the CAc/Nafion 5 % membrane.

A lower Nafion concentration (0.5 %) mitigates the saturation of the response upon glucose injection, but it is less efficient in screening the interfering substances, as shown in Fig. 2b. With Nafion 5 %, the current response upon glucose injection is significantly higher, but it saturates after the second injection of glucose 1 mM. From these results, it is reasonable to assume that the enzyme quickly saturates due to the lack of oxygen, thus resulting in an extreme non-linearity in the sensor response. With an outer membrane (normally realized in polyurethane (PU) [33], Nafion [34], chitosan [34], silicone elastomer [35], polycarbonate [36], silicone [15], and layer-by-layer assembled polyelectrolytes [37]), even if the income of glucose to the enzyme layer is limited, the enzyme layer is thin enough that the hydrogen peroxide produced from the reaction between GOx and glucose can be efficiently re-converted to oxygen at the electrode surface [26], thus resulting in both a rapid sensor response and an extended linear range.

3.2 In Vitro Characterization of the Complete System of Membranes

In a previous work [10], an epoxy-enhanced PU membrane first developed by Yu et al. [33] was developed as outer membrane for a glucose biosensor based on MWCNT and GOx. Moreover, in vivo tests were also performed by subcutaneously implanting four prototypes in mice for 30 days. The tests proved that the material used for the fabrication of the device and the external epoxy-enhanced PU membrane generated an inflammation level comparable to a commercial implantable chip, thus proving that the device, after 30 days, is well tolerated by the host.

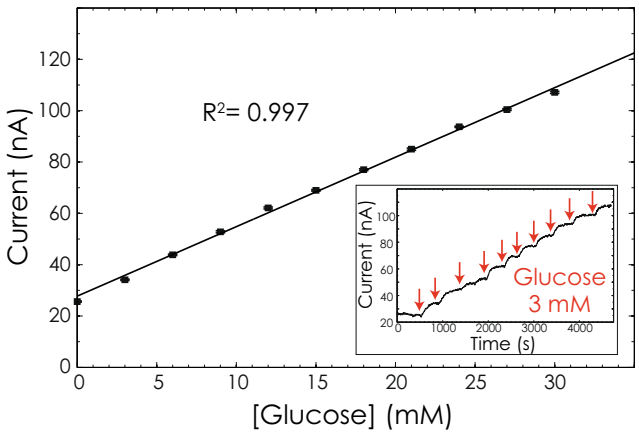

Fig. 3 Calibration in chronoamperometry (650 mV) with injection of glucose 3 mM (up to 30 mM), obtained with electrodes with the complete membrane system: CAc/Nafion 5 % membrane on a MWCNT/GOx 1 mm-electrode, with the outer epoxy-enhanced PU membrane

Figure 3 shows the calibration in chronoamperometry (650 mV) upon injection of glucose 3 mM obtained with electrodes with the complete system of membrane: the CAc/Nafion 5 % membrane with the layer composed by MWCNT/GOx, and the outer epoxy-enhanced PU membrane. Figure S2 shows the calibration in chronoamperometry of the same electrode-membrane system, with injection of glucose 7 mM (up to 42 mM). From the calibration reported in Fig. 3, a sensitivity of 0.4 ± 0.1 µA/mM · cm^2 and a LOD of 0.6 ± 0.2 mM were obtained. As expected, by adding the outer PU membrane, a vast linear range, quick sensor response, higher signal stability, and an optimum screening of the interfering substances were obtained: AA is completely screened (0 %) and UA is detected as 2 % of the current step due to the glucose injection, which are acceptable values for in vivo applications [1]. Moreover, the calibration showed the same LOD as for glucose detection without any membrane (0.6 ± 0.1 mM), with a wider linear range and with a current range compatible with the constraints given by the IC that will be integrated with the sensing platform in the implantable device.

3.3 Long-Term Stability

Finally, the most important problem of the long-term stability of the biosensor was addressed. Many studies reported the performance of glucose sensors after storage in PBS at 4 °C, and by measuring the response of the sensors to a single glucose concentration [38, 39]. In order to be coherent with real applications, the enzyme electrode was stored in pH 7.4, at room temperature instead of 4 °C and its stability over 50 days was tested by performing a complete calibration on the extended human physiological range (up to 30 mM). A complete calibration instead of a single glucose measurement increases the stress for the enzyme, thus increasing the possibility of loosing enzyme activity, but it represents a more realistic experiment [40].

Figure 4 reports the results of 50-day stability tests in terms of (a) LOD (mM), (b) sensitivity (µM/mA · cm^2), and (c) % of the signal due to the interferents respect to the average current step obtained with addition of glucose 3 mM. Figure 4a, b shows an oscillation of the values of the LOD and sensitivity and that the sensor performances started degrading after 45 days. Figure 4c shows an optimal filtering of AA and UA until 20 days. At 25 and 30 days, UA gives an interference of about 15 % respect to the response upon injection of glucose 3 mM. After 30 days, the membrane starts loosing its functionality, as proved by the gradual increase of the interference from UA. AA is completely filtered until 45 days. It is important to note that the real function of a permselective membrane is to decrease the permeability of the interfering substance, and not to completely filter out the interference [1, 26, 38, 39]. The decrease of the sensor sensitivity is mainly due to the partial degradation of the enzyme activity over the time, and it represents the major challenge in the development of a long-term implantable sensor.

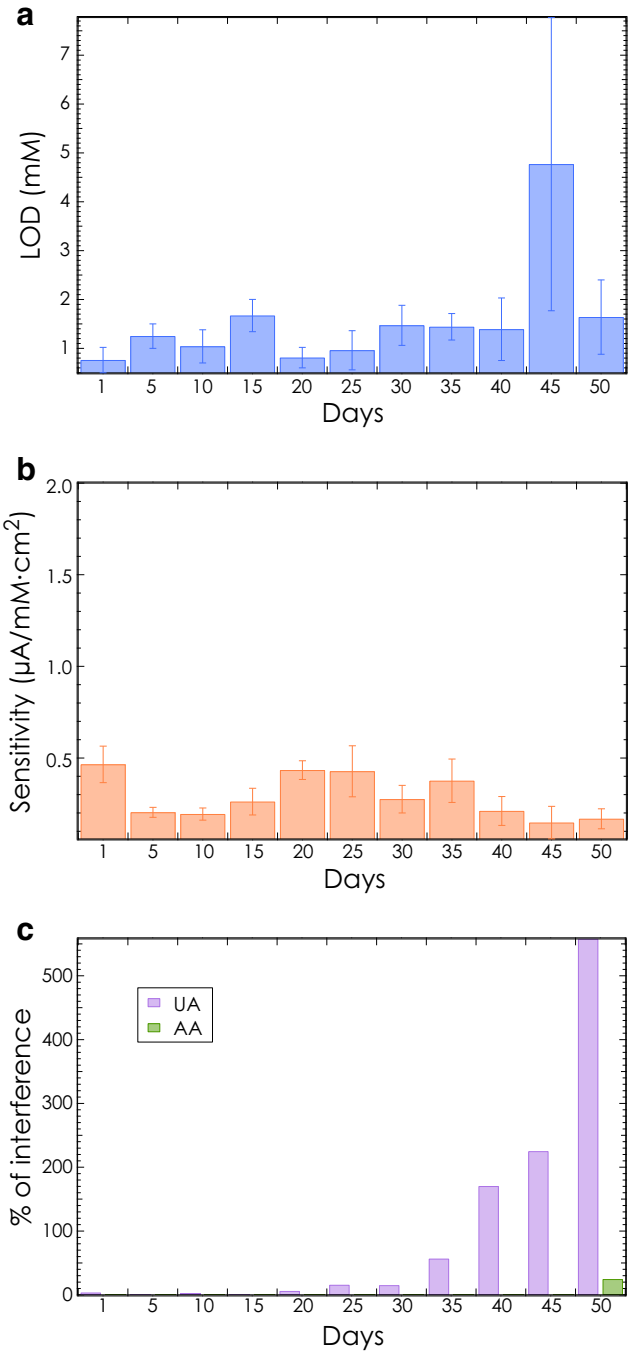

Fig. 4 Long-term stability in 50 days of biosensors with the CAc/Nafion membrane, CNTs, GOx, and the PU-epoxy membrane, toward the detection of glucose in the range 3–30 mM: LOD (**a**), sensitivity (**b**), and in presence of the interfering substances AA and UA (**c**)

In general, a fluctuation in the value of LOD and sensitivity was observed, but the LOD falls in both human and mouse physiological range.

3.4 Application to Biological Samples

The biosensors were also applied to detect variations in glucose concentration in human serum at 37 °C. Figure S3 shows the calibration curve and the plot of the current vs. time (in the inset). The average values of sensitivity and LOD obtained from three different biosensors were 0.42 ± 0.02 µA/mM · cm^2 and 0.6 ± 0.2 mM, respectively, which are very close to the values obtained for the first day in PBS (sensitivity of 0.4 ± 0.1 µA/mM · cm^2 and LOD of 0.6 ± 0.2 mM). These values of sensitivity and LOD proved that the biosensors are capable of measuring glucose in human serum without losing sensitivity. The main differences are that in serum the blank current value is higher due to the presence of traces of glucose and that the current steps are about 1 nA lower than the current steps registered in PBS.

It is thus concluded that the biosensors reported in this study were stable and reproducible for the determination of glucose in biological samples. Overall, high sensitivity, excellent selectivity, linearity, and long-time stability exhibited by the biosensors presented in this study make them good candidates for in vivo detection of glucose in mice.

3.5 Monitoring Acetaminophen and Glucose with the Same Sensing Platform

The possibility to measure a drug, acetaminophen, and a metabolite, glucose, with the same sensing platform was also investigated. Initially, measurements in chronoamperometry of acetaminophen were performed with different systems, reported in Fig. S4. BSA was used to create a space between the inner and outer layer of membranes to let the drug diffuse to the electrode (Fig. S4c). The sensor with the complete system of membranes was calibrated towards addition of acetaminophen (Fig. S4c), obtaining a sensitivity of 2.4 ± 0.6 µA/mM · cm^2 and a LOD of 152 ± 17 µM, which is still compatible with the physiological range in mice (0.3–1.6 mM [41]).

Figure 5 shows the detection of glucose 6 mM and acetaminophen 0.6 mM from the same sample. The measurements were performed with a single platform, where one electrode was functionalized with MWCNTs and GOx (PAD 4), to specifically detect glucose, and the second electrode (PAD 1) was functionalized with BSA only. Both electrodes have the inner membrane (CAc/Nafion 5 %) and the outer layer (epoxy-enhanced PU). Figure 5 shows that only the PAD 4 is selective for glucose, and that acetaminophen

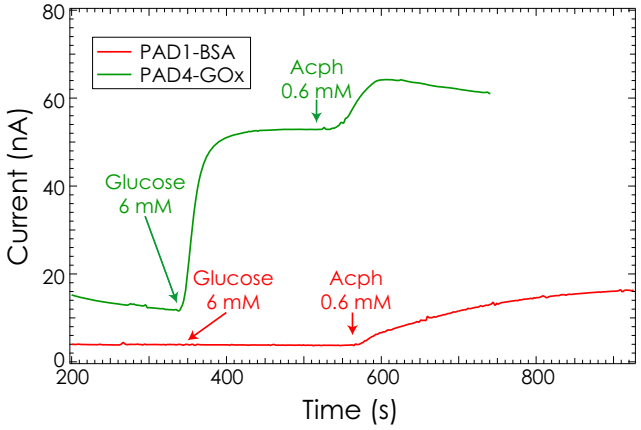

Fig. 5 Detection of glucose 6 mM and acetaminophen 0.6 mM added in the same sample. One electrode is functionalized with MWCNTs and GOx (PAD 4), to be specific toward glucose, while on the second electrode (PAD 1), BSA is immobilized. Both electrodes have the complete system of membranes

creates interference on the PAD 4, but it can be monitored with the PAD 1.

3.6 Measurements with the Dedicated Electronic System

The sensing platform was finally tested for glucose detection in chronoamperometry with an autonomous and reconfigurable integrated CMOS circuit (ARIC) [12], which provides control and read out four the biosensors. A single fully on-chip waveform generator on ARIC generates different voltage profiles to control the sensor in chronoamperometry and cyclic voltammetry. ARIC reads out the electrochemical sensors and streams out the digitized measured data. The streamed out data is given to the other IC to be transmitted. ARIC is implemented in 0.18 µm technology and consumes 0.93 mW from 1.8 V supply voltage. More details on the design and its testing are reported in [42]. The output serial data of ARIC is given directly to a

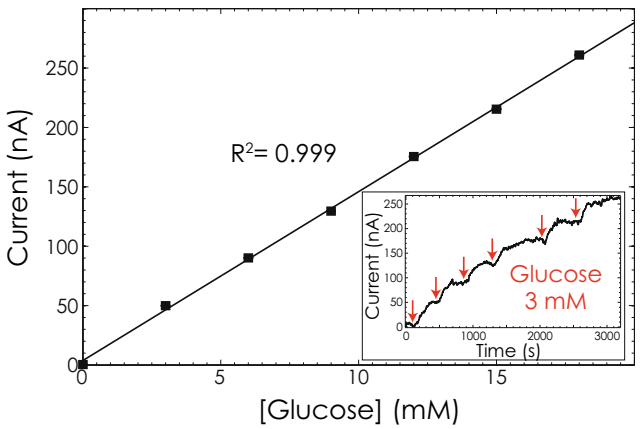

Fig. 6 Measurements acquired with the ARIC on the sensing platform

blue-tooth transceiver that send them to an android device, which shows the received data in real-time after filtering it. The Android interface and the receiver block are described in [11].

The measurements reported in Fig. 6 showed that the average sensitivity and LOD obtained from three different biosensors were 0.7 ± 0.2 μA/mM · cm^2 and 0.18 ± 0.06 mM, respectively, which are very close to the values obtained for the first day in PBS with the commercial potentiostat.

4 Conclusions

This paper presents a complete in vitro characterization of a system of membranes that consist of an "inner" permselective layer, designed to filter the signal generated from the oxidation of interfering substances present in biological fluids, such as AA and UA; an "outer" layer made by an epoxy-enhanced PU film, that regulates the passage of glucose and oxygen to the electrode surface and provides a biocompatible layer for a correct integration with the surrounding tissue. This system of membrane was employed in a glucose sensor and it successfully monitored glucose in both the human and mouse physiological range, in PBS and in human serum at 37 °C, and by using the dedicated IC. The sensor showed good stability for 30 days, and the permselective membrane effectively filtered out AA and UA. Moreover, with the same system of membrane, we developed a biosensor for detection of the anti-inflammatory drug acetaminophen.

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