A Self-Contained System With CNTs-Based Biosensors for Cell Culture Monitoring

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***Abstract*—Biosensors have been applied to disparate fields, especially for endogenous compounds such as glucose and lactate. The main areas of application are certainly related to medical and diagnostic purposes. However, metabolic monitoring can be also of interest in cell analysis. Cells can be cultivated for several purposes, such as understanding and modeling some biological mechanisms, the development of new drugs and therapies, or in the field of regenerative medicine. All the aforementioned applications require a thorough knowledge of the biological system under study. In this paper, we propose the development of a self-contained system based on electrochemical biosensors for cell culture monitoring. The detection is based on oxidases immobilized onto carbon nanotubes. We also develop an architecture to record the signal generated by the biosensor and transmit it to a remote station by means of a Bluetooth module. We calibrate the system for glucose and lactate detection in phosphate buffer solution. We achieve a sensitivity of 55.5 $\mu\mathrm{A}/\mathrm{mM\ cm}^{-2}$ and a detection limit of 2 μM for glucose, as well as a sensitivity of 25.0 $\mu\mathrm{A}/\mathrm{mM\ cm}^{-2}$ and a detection limit of 11 μM for lactate. We finally validate the two biosensors for metabolic monitoring in culture medium and we detect lactate production in neuroblastoma cells after 72 h of cultivation. The integrated system proposed in the present work opens new opportunities towards the development of novel tools for cell analysis.**

***Index Terms*—Amperometric biosensor, carbon nanotube, cell culture, metabolic monitoring, potentiostat.**

I. Introduction

BIOSENSING has been widely described in literature in the last two decades. Applications of biosensors related to disparate fields have been presented, although medicine and diagnostics remain the main driving force. However, several works have also been published on the application of such biosensors to cell analysis [1], [2]. Cells are cultured for several purposes, including protein expression, drug testing, tissue engineering, etc. As an example, stem cells have recently attracted the attention for their really promising features and they potentially represent novel treatments for uncured diseases. Indeed, they possess the prolonged, even unlimited, ability of self-renewal that can generate at least one type of highly-differentiated progeny. Their capability to differentiate in diverse cell types, such as epithelial cells, muscle, skin, neural cells, bone, cartilage, makes them perfect to be employed in several medical fields, like tissue engineering, drug screening, and cellular therapies. Moreover, their application in a wide variety of diseases envisages new successful cures for the treatment of Parkinson's disease, muscular dystrophy, cardiovascular disease, and even cancer [3]–[5].

The lack of real-time and quantitative information with respect to cellular behavior in cultures still remains the fundamental bottleneck of any bioprocess. Consequently, the control, the optimization and the scale-up of bioprocesses are essentially manual and result in poor productivity and low product quality. So far, cell analysis still demands reliable instruments to handle this information and optimize the whole process. Biosensors are definitely good candidates to fulfil these requirements, when combined with other strategies. The detection is typically based on a biological recognition element to ensure selectivity of the analyte. Antibodies and enzymes are usually exploited for this purpose, immobilized on the bare electrode by chemical modification of the surface or adsorbed onto nanomaterials. Amperometric measurements, such as those described in the present article, can be optimally performed under flow conditions. The chemical reaction taking place in the electrochemical cell needs to be transduced into a readable signal. Therefore, it is necessary to integrate methods from different domains to achieve self-contained systems for this purpose. Lab-on-chip technology is already at a commercial level, but it needs to be adapted to the particular constrains required by the cell cultures. Bioprocess monitoring demands a continuous measurement to follow cell evolution, despite the fact that online sensors can be highly invasive for the sterile environment of incubators.

In this paper, we describe a self-contained platform for the detection of glucose and lactate for the monitoring of cell cultures. We microfabricate an integrated electrochemical cell for multiple detection and we present a novel strategy for the immobilization of different enzymes onto the electrodes by means of carbon nanotubes. We realize a wireless potentiostat to record the signal generated in the electrochemical cell. The developed system is optimized for the detection of glucose and lactate in cell culture medium.

The paper is organized as follows. In Section II, we will give an overview about requirements and strategies developed in the last two decades for bioprocess monitoring. Then, in Section III we will focus on our method and we propose a strategy for the development of integrated biosensors by using complementary

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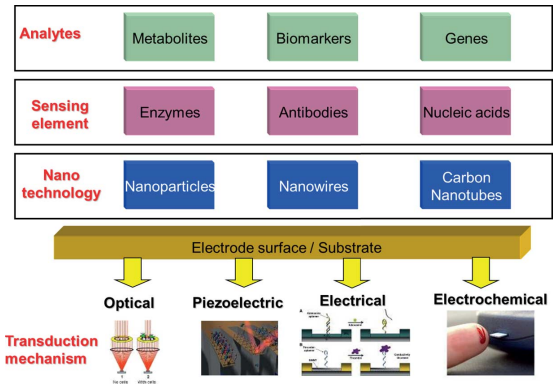

Fig. 1. Resume of the main features characterizing biosensors for the monitoring of several analytes interesting in cell culture monitoring.

metal–oxide–semiconductor (CMOS) compatible technology. We will move on with Section IV by describing an architecture adapted for electrochemical measurements, minimizing the invasivity of *online* measurements. All the blocks considered previously will be then integrated in a self-contained system and results related to calibration will be reported in Section V. Finally, in the same section we will also demonstrate that our system is suitable for the monitoring of neuroblastoma cell line.

II. State-of-the-Art

There is a lack of quantitative information with respect to cell behavior and poor automated control and optimization of bioprocesses. Quantitative measurements of nutrient levels, toxic molecules, and growth factor concentrations should be performed *online* and in real-time to achieve high-quality products and massive yield. Physicochemical parameters such as pH, temperature, dissolved oxygen, and CO_2 concentration, instead, can affect cell expansion rate and cell population. Hence, multisensor systems are required to control all those parameters simultaneously. Hereunder, we want to propose a general overview on sensors developed for the monitoring of biotechnological processes, with a particular focus on stem cell culture monitoring. Fig. 1 resumes the following classification.

A. Analytes

When talking about analytes in cell culture, we generally refer to those compounds involved in cell proliferation, differentiation, and death. **Nutrients** and **metabolites** are by far the most important analytes to keep under control. Limiting glucose levels, which is the most important carbon source for cells, leads to a more efficient metabolism and less accumulation of lactate, for example. Numerous *offline* glucose biosensors have been developed, and some of them have been also commercialized, like the Yellow Springs electrochemical analyzer (Yellow Springs, OH). Real-time monitoring have been also proposed in flow-injection-analysis (FIA) systems [2], in microflow systems [6], [7], and in quiescent conditions (CITSens Bio, Switzerland). Glutamine is an important energy and nitrogen source. However, the catabolism of glutamine produces ammonia, which becomes toxic to the culture in high concentration, inhibiting its growth and glycolysation. Lactate is another common product of anaerobic metabolism and it is toxic for cells, as in the case of ammonia. *Offline* detection of lactate is normally carried out by high-pressure liquid chromatography (HPLC) or enzymatic colorimetric methods. The functionalization of amperometric biosensors with oxidases specific for different compounds makes possible to develop platforms for multiple detection by using the same detection principles [8].

The phenotype of cells can be evaluated by the expression of **biomarkers**. Embryonic stem cells, for example, can express biomarkers such as POU transcription factor octamer-4 (Oct-4), placental alkalin phosphatase (AP), several stage-specific embryonic antigene (SSEA), etc., [9]. Cytokines are also interesting biomarkers, because they are involved in intracellular communication and help cells toward proliferation and differentiation into specific lineage. ELISA (Enzyme-Linked ImmunoSorbent Assay) test and immunoflorescence analysis are the most common methods of detection. At last, supplied or released hormones, such as insulin-like growth factor (IGF), are typically bound to a fluorescent molecule to be detectable by simple microscopy.

Gene expression can be inferred by genome analysis, which requires DNA extraction in high concentrations. DNA strains are extracted by cells lysis and purified. DNA analysis can be determined by spectrophotometric methods at 254 or 256 nm, electrophoresis, or fluorescence methods [10]. However, microarray methods are by far the most common strategies for gene expression analysis [9].

B. Sensing Element

The choice of the sensing element is closely related to the target of interest. Biological compounds are naturally selective and they can confer high specificity for the detection. Several biosensors are based on **enzymes**, characterized by a protein structure and an active site. When the active site binds the target molecule, the chemical reaction is catalyzed and transduced in a signal, typically electrical or luminescent, that can be measured and correlated to the analyte quantity. Denaturation and chemical stability are the two main concerns regarding enzymes. Oxidases are a large family of enzymes and they are frequently used for the development of electrochemical biosensors and in ELISA test. For the specific application of cell culture monitoring, examples of glucose oxidase [6], lactate oxidase [7], glutamate oxidase [11], amino acid oxidase [12] are largely reported in literature.

Antibodies are another common sensing element, able to specifically bind the corresponding antigen. Differently from enzymes, antibodies do not promote or catalyze any chemical reaction, but they are typically coupled with a label molecule that can generate an electrical or fluorescent signal when the binding takes place [13]. The antigen can be a molecule or a cell, such as a bacteria. ELISA test is by far the most popular analytical assay based on the complex antibody-antigen.

Nucleic acids are normally used for the monitoring of gene expression. The sensing mechanism, called hybridization, is based on the specific base-pairing of complementary nucleotides leading to double-stranded sequences of nucleic acids. Microarrays can be used to distinguish relevant modification in gene expression in different states of the stem cells, for example. Electrochemical detection has been also used alternatively to optical techniques, such as the direct electrochemical

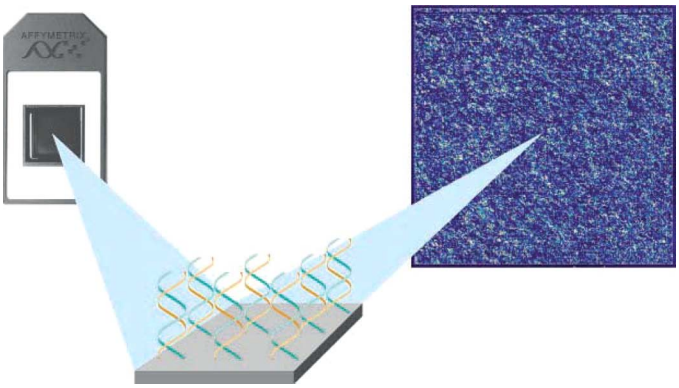

Fig. 2. Commercial DNA microarray from Affymetrix for gene expression analysis. Picture reprinted from http://www.affymetrix.com.

oxidation of guanine, redox-active markers, and by exploiting the electron transfer properties of DNA [14], [15].

C. Transduction Mechanism

Once we have defined the target molecule and the sensing element, the next step is the definition of the transduction mechanism toward the development of the biosensor. **Optical methods** are by far the most common strategies, because of the numerous advantages. They allow *in situ* monitoring, due to their minimal invasivity. *Fiber-optic sensors*, also known as optodes, are based on the change in the optical properties of a particular indicator, such as a dye. Biological compounds can be coupled to the optode for the detection of targets, like glucose and hydrogen peroxide [16]. *Near-infrared spectroscopy* can quantify the concentration of certain organic species by the analysis of the adsorption bands in the spectrum [17]. *Fluorescence sensors* are based on the response of particular molecules which emit light after an excitation at particular wavelength. DNA microarray are often developed by exploiting fluorophores and chemiluminescent compounds bound to the probe or to the target. Fig. 2 depicts a commercial DNA Microarray from Affymetrix for gene expression.

Impedimetric and **capacitive methods** are often used for biomass and viability measurements, and they will be discussed in Section II-D.

Electrochemical biosensors are the easiest and cheapest solution for bioprocess monitoring and they are the most widely described [1], [2], [7]. They allow *in situ* and *online* measurements, since they need to be in contact with the cell medium. However, they suffer from some drawbacks, such as thermal stability of the immobilized enzyme during sterilization process, electrode fouling due to the continuous contact with the solution, consumption of the analyte and bioprocess interference. Different strategies have been proposed to overcome these disadvantages. The biological element can be contained in a reservoir, separated from the bulk by a sterilized membrane. Therefore, the analyte can diffuse through the membrane and interact with the enzyme, although the measurement is no more *in situ*. Flow-injection analysis systems are often coupled with enzymatic biosensors. The culture sample is peristaltically withdrawn from a bioreactor, prior to cell filtration step. In this way it is possible to avoid contamination problems and sterilization issues, since the biosensor is not in direct contact with the cell culture. On the other hand, clogging and fouling of the microfilter are quite common, due to cell precipitation [18]. *Amperometry* is by far the most common transduction mechanism when dealing with oxidative and reductive reactions. The sensor typically consists of three electrodes: the working electrode (WE), which contains the biological recognition element, the counter electrode (CE), which collect the current generated from the reaction, and the reference electrode (RE), which is used to keep the potential constant without current flowing (refer to Section IV). Two techniques are typically used in electrochemistry for the detection and quantification of compounds. *Cyclic voltammetry* applies a linear-sweep potential between the working and the reference electrodes, forward and backward within a potential window. The generated current from the enzymatic reaction is then monitored continuously. The hysteresis plot gives qualitative and quantitative information about the detected target. In particular, the peak hight is proportional to the analyte concentration, whereas the position with respect to the potential gives qualitative information, since compounds have different oxidation and reduction potentials. *Chronoamperometry* is another well-established technique where a constant potential (typically corresponding to the oxidation or the reduction potential of the analyte) is applied between WE and RE, and the generated current is monitored in function of time. Current generally decays over the time and it reaches an approximative steady-state after a certain time, which intensity is proportional to the analyte concentration.

D. Monitoring Physicochemical Parameters

Small changes in medium composition, pH, temperature, and dissolved gases pressure can alter cell metabolism, especially in the case of stem cells. Moreover, strategies for monitoring cell density and quantify viable cells have been also proposed.

Temperature and cell growth rate are strictly connected and should be kept constant over the cell culture. Sensors based on platinum resistance temperature detector (RTD) can be easily developed by thin film technology or purchased from the market [19]. The electrical properties of RTD varies according to the temperature. Temperature can also be measured by using a diode-based proportional-to-absolute temperature (PTAT) thermometer, which generates an output voltage proportional to the temperature [20].

pH is by far the most widely and controlled parameter in cell culture, even if pH sensors are difficult to integrate. Ammonia is the primary nitrogen source and its uptake causes acidification of the medium due to the release of NH_4^+. The measurement is often performed by standard potentiometric ion selective glass electrode. Ion selective field effect transistors (ISFET) can be also used to monitor small pH variations. The gate is realized by the deposition of Si_3N_4, which plays the role of the chemical sensing layer. Iridium oxide-based sensors are another approach for pH measurement, since oxide properties confer a direct correspondence between the open-circuit potential and the pH. The advantage is that they can be easily realized with thin film technology. An example of temperature and pH sensors integrated on the same platform are showed in Fig. 3.

Partial pressure of dissolved gases like O_2 and CO_2 are other important parameters to control. Oxygen is hardly soluble in aqueous solutions and cells can suffer from oxygen limitation. Clark amperometric electrodes [21] are by far the

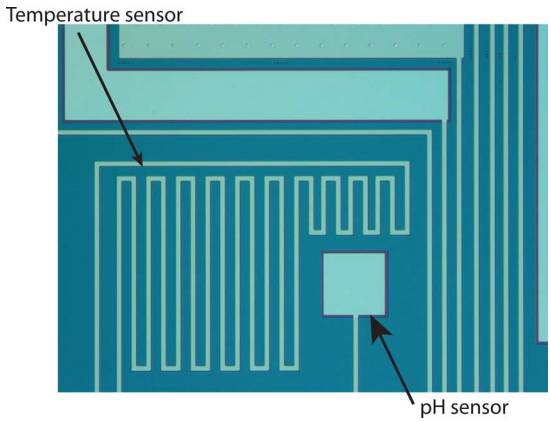

Fig. 3. Magnified image of RDT and pH sensors realized with thin film technology. Pt metal layer is deposited onto Si substrate. RDT has a width of 20μm, while pH sensor has an area of 0.04 mm^2.

most common strategy to measure dissolved oxygen. The drawback of this method concerns the consumption of the dissolved oxygen, that can become a problem when handling low volume bioreactors. Carbon dioxide can also affect growth of animal cells, which often requires its supplementation. Severinghaus electrodes consist of a glass electrode separated from a bicarbonate solution by a CO_2 permeable membrane. Partial pressure of CO_2 can be measured by pH variation of the bicarbonate solution. As in the case of dissolved O_2, carbon dioxide can also be sensed by ISFET.

Cell density, **cell viability**, and **adhesion** can also be quantified by optical, electrical, and calorimetric-based methods. Optical methods are typically noninvasive and nondestructive, highly desirable when dealing with cell cultures, and they do not interfere with the culture environment, reducing contamination risks. Optical density has been proposed for measuring cell growth and biomass concentration and commercial *online* and *in situ* biosensors are typically based on light absorption (Optek-Danulat, Essen, Germany) or scattering (Cerex MAX, USA). Major problems concern turbidity of the medium, which can change over the cell culture, gas bubbles formation along the light path, light dispersion on cell debris, and noisy measurements. *In situ* microscopy is another well-establish technique and it can be coupled with image analysis to automate the monitoring [22].

Impedimetric and capacitive methods, instead, can be used to sense biomass, cell viability and adhesion. Undamaged cell membrane exhibits dielectric properties, so cell viability and adhesion can be detected by measuring the effective electrode impedance at low frequencies [23]. Capacitance spectroscopy over a wide range of electric field can be also used to determine the average cell size and the number of cells [24]. Finally, heat generated during growth have been also presented in the past to measure the amount of active cells in the bioreactor [25].

E. Nanomaterial-Based Biosensors

According to many researches [26], [27], nanomaterials are the new frontier of biosensing. They exhibit many interesting properties from the biosensing point-of-view, including comparable dimension with biological recognition elements, high electron transfer rate [28], considerable electronic emission [29], and high surface area [30], due to their 3-D structure. Nanomaterial-based biosensors have been widely presented in literature and their application have been mainly focused on diagnostic and therapeutical purposes. To date, the use of nanomaterials to enhance the sensitivity of detectors is still in the domain of research. Hereunder, we will give a general overview about nanomaterials used for biosensor development toward general applications.

Nanoparticles (NPs) are typically metallic, exhibiting interesting electrical, magnetic, and optical properties for biosensing applications. Among the interesting electrical features, there are high sensitivity and improved limit of detection in voltammetry [31]. Quantum dots are semiconductor crystals whose size is within 10 nm. Quantum confinement confers different properties to quantum dots with respect to larger particles. Thereby, they have remarkable optical properties, suitable to be used as labels for sensing elements [32].

With the improvement of micro- and nanofabrication techniques, **nanowires (NWs)** have been arising more interest in biomedical applications, since they can interact with biomolecules at the nanoscale. Metallic and semiconductive NWs can be used, and they provide different transduction mechanisms. NWs can be functionalized with proteins, enzymes or antibodies in conductive measurements or in field-effect transistors [33].

Carbon nanotubes (CNTs) exhibit interesting electrochemical properties. The electron current through the nanotube is based on ballistic conductivity, so the measured mean-free path results to be two orders of magnitude higher then the best macroscale conductor [34]. Electron transfer depends on atomic arrangement, so emission properties are different for tips and walls of CNTs [35], [36]. Moreover, they have been largely reported for their ability to adsorb proteins onto their walls, resulting in an excellent immobilization method [37]. Surface modification by using carbon nanotubes can be accomplished in different manner and for several purposes. Directly growing aligned carbon nanotubes has been proposed on different substrates [27]. Self-assembly is another way to obtain aligned CNTs by modifying surfaces with thiols or other functional groups [38]. Carbon nanotubes can be also randomly dispersed on the electrode surface and many efforts have been addressed to obtain well-dispersed CNT solutions. Carbon nanotubes can be used as a forest of nanomaterials, patterned arrays or as single-sensors. NWs and CNTs can be used to replace the channel in field-effect transistors for biosensing purposes. As in the case of electrochemical biosensors, the biological recognition element can be either adsorbed on NWs or CNTs surface and modify the conductivity of the channel [39].

III. System Microfabrication and Functionalization

The monitoring of cell cultures should be noninvasive to prevent contamination as well as not to interfere with the biological processes. Cells are typically cultured in plastic flasks, called Petri dishes, filled with the culture media, a mixture of nutrients, hormones, and salts developed for an optimal growth and proliferation. Flasks are mainly stored in the incubator, which provides constant temperature of 37 °C to reproduce physiological conditions, constant relative humidity (RH) of 45% to prevent water evaporation from the medium, and dissolved CO_2 of

5% to maintain the proper pH. The incubator is sterile, to avoid any type of contamination from fungi and bacteria to the cells. The presence of cables connected to the monitoring station are highly undesirable, because they should pass through the door of the incubator and they could represent a source of contamination for the sterile environment. Thereby, a wireless connection is strongly preferable and power supply should be guaranteed over the culture time by a standard battery. Taking into account all these considerations, we need to design our system to be as small as possible, easy to interface to the electronic part, and suitable to survive to an hostile environment such as the one in the incubator.

In this paper, we will focus on the steps needed to develop the biosensor, intended as the place where the analytes and the recognition element react together. Following the classification presented in Section II, our biosensor can be described as following.

- *Analyte*: Metabolites (glucose and lactate).
- *Sensing element*: Enzymes (glucose oxidase and lactate oxidase).
- *Transduction mechanism*: Electrochemical (amperometric).
- *Nanomaterial*: Carbon nanotubes.

We describe in this section the microfabrication of the electrochemical cell. Afterwards, we present an innovative strategy to selectively drop cast carbon nanotubes and enzymes onto the WEs. Due to the size of the developed electrodes, an automatic system with precise movement control is required. We also describe the realization of the fluidics to optimally measure the metabolites and we conclude the section with the integration of those blocks together. In Section IV, we will describe the data acquisition electronics.

A. Microfabrication

The detection of multiple targets needs multiple working electrodes to detect the metabolites with the same system. The biosensor consists of five gold WEs, a gold CE, and a platinum RE. The microfabrication process is conceived in four steps: gold evaporation, platinum evaporation, SiO_2 passivation, electrode and pads opening. Metal layers are evaporated by physical vapor deposition (PVD) onto 525 μm $\langle 100 \rangle$ silicon substrate with 500 nm of SiO_2. Au and Pt thin films (200 nm) are deposited to form the electrodes and the connections by a liftoff process. Ti is put on top and under both Au and Pt (20 nm) as stick layer for metals and passivation. All working electrodes are identical and they have an area around $0.25 mm^2$. Connector insulation is achieved by radio-frequency (RF) sputtering of SiO_2 and successive wet-etching of Ti and SiO_2 is performed by BHF (7:1) to open the passivation next to the electrodes and the pads and guarantee electronic connections. The process flow and a magnified image of the integrated electrochemical cell are shown in Fig. 4. The interface between the chip and the potentiostat is achieved by using a commercial edge card socket (Mini Edge-Card for Transceiver, MECT by Samtec).

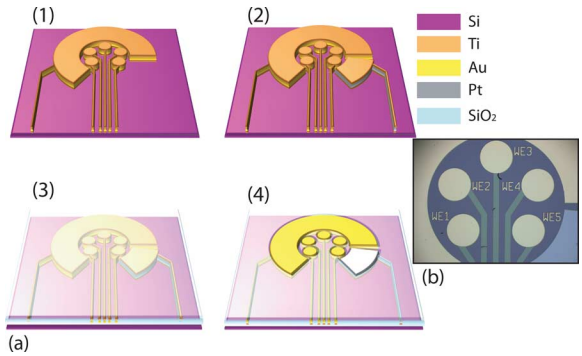

Fig. 4. (a) Process flow for fabrication of the electrochemical cell with multiple WEs: (1) Ti/Au/Ti deposition; (2) Ti/Pt/Ti deposition; (3) SiO_2 sputtering; (4) SiO_2 and Ti etching. (b) Optical image of the microfabricated integrated electrochemical cell.

B. CNT Spotting

Multi-walled carbon nanotubes (MWCNTs—diameter 10 nm, length 1–2 μm) are purchased in powder (90% purity) from Dropsens (Asturias, Spain). Then, they are dispersed in a solution of distilled water 50 vv% and ethanol 50 vv%, with 0.5 wt% of Nafion (purchased from Sigma-Aldrich, Switzerland). Final concentration of MWCNTs is 1 mg/ml. MWCNTs are dispersed in Nafion, according to the results reported from Wang *et al.* [40]. The solution appears well-dispersed and suitable for ultra-low volume deposition. Automatic spotting of CNTs is performed with a commercially available noncontact spotter (sciFLEXARRAYER DW by Scienion, Dortmund, Germany). The minimum dispensed volume is around 400 pl and can be increased by increasing the number of drops per spot. According to our previous results obtained with macroelectrodes [37], we deposit 600 ng of CNTs, corresponding to a volume of 600 nl of MWCNTs/Nafion solution. The single drop has a diameter around 50–80 μm in average, so we pattern the electrode area as a matrix of 5×5 spots to uniformly cover all the exposed area. Then, we dispense 10 drops for each spot (around 4 nl per spot) for five cycles. The dimension and volume of the drops can be monitored at the end of each spotting by using the camera installed on the instrument.

C. Functionalization With Enzymatic Probes

Glucose oxidase from Aspergillus Niger (GOD, EC 1.1.3.4, 129.9 units/mg solid), D-(+)-glucose, lithium L-lactate, and L-glutamic acid were purchased from Sigma-Aldrich (Switzerland) as lyophilized powders. Lactate oxidase from Pediococcus species (LOD, EC 1.13.12.4, $\geq$ 20 units/mg solid) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All the enzymes are dissolved in phosphate buffer saline (PBS) solution 0.01 M at pH 7.4 in order to have 1 unit/mm^2 spotted onto the electrode. So, GOD is dissolved in a concentration of 50 mg/ml and LOD in 66 mg/ml. Metabolites are also dissolved in pure PBS. The concentration of GOD and LOD is enough to spot 100 drops for each electrode (5×5 matrix, 4 drops per spot).

The success of the functionalization can be checked by the acquisition of SEM images. SEM images are acquired by using

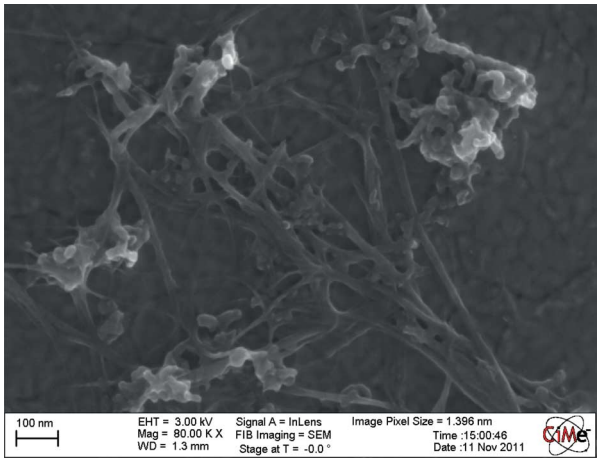

Fig. 5. SEM image of the developed biochip after the spotting of lactate oxidase onto randomly dispersed carbon nanotubes.

a Philips/FEI XL-30 F (The Netherlands) scanning electron microscope. The resolution in UHR mode is 2.5 nm at 1 kV. Fig. 5 shows carbon nanotubes with an adsorbed layer of enzyme (lactate oxidase in this case). The morphology of modified electrodes is clearly related to carbon nanotubes properties and their arrangement on the electrode surface. On the background of the image it is possible to note the flatness of the electrode surface. Carbon nanotubes are randomly dispersed, forming tangled bundles. Protein layer entirely wraps each carbon nanotube and results in diameter enlargement, as already discussed in our previous work [41] for screen-printed electrodes. Moreover, it is possible to also distinguish Nafion nanoparticles, which are solvent residues.

D. Fluidic System

The electrochemical response of the developed biosensor is investigated by chronoamperometry under aerobic conditions. Chronoamperometry requires a continuous transport of new solution to compensate for the depletion of electrochemical active species at the interface of the electrode. Mechanical agitation facilitates the diffusive mechanism promoted by the concentration gradient existing between the bulk and the interface. Fresh solution on the electrodes can be guaranteed by connecting the electrochemical cell to a fluidic system. We conceive the fluidics with an inlet channel, a chamber, and an outlet channel molded in PDMS (polydimethylsiloxane). Silicon elastomer base and curing agent are purchased from Dow Corning GmbH (Wiesbaden , Germany). Base and curing agent for PDMS are mixed in a rate of 1–10 and the solution is put in the desiccation chamber for about 30 min to pull out all the air bubbles. Then, the mixture is poured in a PMMA (polymethilmethacrylate) mold and let dry in the oven at 80 °C for 1 h. When the PDMS is solidified, it is kept in plasma oxygen (Femto by Diener Electronic GmbH, Germany) for 70 s with a power of 20 W to increase the hydrophilicity before using it. The PDMS chamber is then fixed inside an in-house chip holder made of polymethylmethacrylate (PMMA) to provide easy connections with the rest of the fluidic system. The inlet and outlet channels are connected to a peristaltic pump (Gilson SA, Mettmenstetten, Switzerland) by means of Tygon tubes of 0.25 mm of internal diameter. The sample is circulated with a velocity of around 13 $\mu\mathrm{l/min}$. Once the measurement is run, the solution at the inlet of the fluidic circuit is changed around every 5 min.

IV. Architecture for Electrochemical Measurements

The previous section was dedicated to describe the realization of the integrated electrochemical cell. Moving further on the system, now we need a proper electronics to quantify the chemical signal generated by the recognition element in a readable measurement. In this section, we present an architecture for the amperometric detection of metabolites by using oxidases as biological recognition element. We first describe the general building blocks required to perform amperometric measurements. We will then enter into details about constrains related to the particular application, such as current range, sampling frequency for real-time measurements, and detection with multiple working electrodes. We eventually show the realization of the prototype on a printed circuit board (PCB) by using off-the-shelf components. The proposed solution can be used inside the incubator, prior sterilization step, and it can communicate with a remote station via Bluetooth connection.

A. General Considerations on the Potentiostat

The potentiostat is an electronic instrument used in electrochemistry. Its main task lies in controlling the voltage difference between reference and working electrodes by sinking or giving electrons from or into an electrochemical cell through the counter electrode. The general reactions promoted by an oxidase (XOD) are the following:

$$\mathrm{metabolite} + \mathrm{XOD} \rightarrow \mathrm{product} + \mathrm{XOD_{reduced}}$$
$$\mathrm{XOD_{reduced}} + \mathrm{O_2} \rightarrow \mathrm{H_2O_2} + \mathrm{XOD}.$$

Therefore, the common product of these reactions is the $\mathrm{H_2O_2}$ that is an electroactive species. Indeed, the hydrogen peroxide electrolyzes as follows:

$$2\mathrm{H_2O_2} \rightarrow 2\mathrm{H_2O} + \mathrm{O_2^+} + 4\mathrm{e^-}$$

and the four $\mathrm{e^-}$ released to the electrode are optimally detected at a certain potential, specific for hydrogen peroxide.

Different approaches can be exploited for the design of the voltage generator and the current detector. Regarding the application of the potential, it can be controlled in three configurations: grounded working electrode (GWE), grounded reference electrode (GRE), and grounded counter electrode (GCE). In the GWE configuration, the desired voltage is applied to the positive input of an operational amplifier, whereas the negative input is connected to the reference electrode. The output of the amplifier is then connected to the counter. The operational amplifier controls the current passing through the cell such that the cell potential is kept at the desired voltage. The input bias current of the operational amplifier can affect the potential of the reference electrode, unless it is really small (in the range of the pA or even less) and the input resistance of the operational amplifier is very high (in the range of GΩ). Usually another operational amplifier is connected at the reference, acting as voltage buffer

to minimize current flowing in the RE [42]. The GRE configuration is similar to the GWE configuration, except the fact that WE and RE are swapped.

In the GCE configuration, two operational amplifiers are used to set the voltage between WE and RE. One operational amplifier is directly connected to the WE, configured as a summing amplifier. A voltage follower is instead connected to the RE. The output potential of the summing amplifier is added to the RE potential, so that the desired voltage can be applied between the WE and the RE. The voltage follower connected to the reference avoids current flowing through it.

Regarding the readout circuit, many strategies have been presented as well [42]. The most straightforward approach is to insert a resistor in the current path of the counter electrode and measure the voltage drop across the resistor. Thus, the voltage drop can be measured with an instrumentation amplifier. This configuration is less sensitive to noise and has better stability with respect to other configurations. However, it needs more components and suffers more from mismatches. In more complicated realizations, the current can be injected directly into a current-input A/D converter [43] or it can be converted to frequency [44] or time [45]. Nevertheless, the use of transimpedance amplifier is by far the most common approach. In this case, the current flows through a resistor placed between the output and the negative input of an operational amplifier, while the positive input is grounded. Ideally, no current is adsorbed by the input pin of the operational amplifier and the counter is directly grounded, due to the virtual short-circuit at the inputs of the operational amplifier. However, this approach requires high values of resistance to measure low currents. Alternatively, high value resistors can be replaced by capacitors. Thus, the output voltage is proportional to the current charging the capacitors. According to these arguments, we choose to base our potentiostat on GCE configuration for the potential control and on transimpedance amplifier for the current detector.

B. System Requirements

Hydrogen peroxide is an electrochemical species that can be optimally detected when a potential of 650 mV drops between working and reference electrodes. An error of 10 mV on that voltage value is tolerated. The current generated by the redox reaction strongly depends on the electron transfer efficiency of the transducer and on the area of the working electrode. The microfabricated electrodes described in Section III-A have an area of 0.2 mm^2 and CNT structuring for each working electrode. Both these features affect the current intensity in the electrochemical cell. From previous measurements performed by similar platforms, we infer that the expected range of current goes from 1 nA up to around 100 nA [46] in a first approximation. Due to such small range, we use a transimpedance amplifier based on switched capacitors. The current flowing through the cell charges the capacitor and the generated output voltage is proportional to the input current. Once the measurement is performed, the capacitors can be discharged by closing the switch, resetting the system for the successive measurement. For different current ranges, it is possible to switch *on* or *off* different capacitors in parallel to adjust the range of detectable currents.

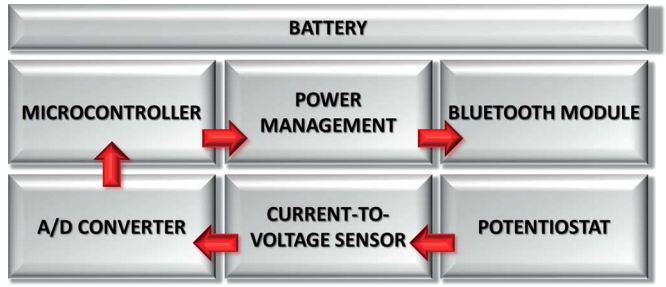

Fig. 6. General building block diagram for a biosensor-based platform.

The analog voltage needs then to be converted to digital prior to be acquired by an analog-to-digital converter (ADC) with appropriate accuracy and transmitted to the remote station. Metabolic changes in cell cultures can be sampled slowly in the order of some hertz for the single continuous measurement, while each electrode can be sequentially activated every 10 min to detect one metabolite at the time.

The microcontroller coordinates all the blocks of the system, receiving and transmitting signals from/to the other blocks. The user can select a specific metabolite from the remote station or run a bench of sequential measurements scanning all the working electrodes. Indeed, the microcontroller can drive a multiplexer to select the proper WE. Once digitized, the current value is sent by the microcontroller to the remote station by means of the Bluetooth module. Finally, the power management block supplies the voltages required by the other blocks. A schematic of the described building blocks is illustrated in Fig. 6.

C. Architectural Solution

Based on the requirements discussed in the previous section, we designed the architecture showed in Fig. 7. The potentiostat consists of two MAX4039 (Maxim). Each MAX4039 includes two operational amplifiers and has a buffered reference of 1.2 V. The chosen ICs consume 800 nA of supply current per amplifier and have low input bias current (typically lower than 1 pA) to avoid reference polarization. The input offset voltage is generally below 200 μV. Amplifier O_1 acts as a voltage buffer to read the voltage V_{RE} of the reference electrode without polarizing it. Amplifier O_2 is configured as a control amplifier and it fixes the voltage V_{WE} of the working electrode to the value $V_{RE}+0.65$ V. Thus, a constant voltage of 0.65 V is set between the working and the reference electrodes. The analog switch ADG758 (Analog Devices) selects the physical electrode of the cell to be connected to V_{WE}. That switch has low ON resistance (typically 3 Ω) and low leakage current (100 pA).

The current generated in the electrochemical cell is read on the counter electrode by means of a current-to-voltage converter IVC102 (Burr–Brown). Current-to-voltage converters can use either feedback resistors (R_F) or feedback capacitors (C_F). When a feedback resistor is used, the sensor gain is directly determined by the value of R_F. This approach is not practical to sense low current values, requiring extremely high feedback resistance. This issue can be overcome by using a capacitance as feedback element. Indeed, the cell current is used to charge the capacitor C_F, while the switch S_1 is kept open. If the switch

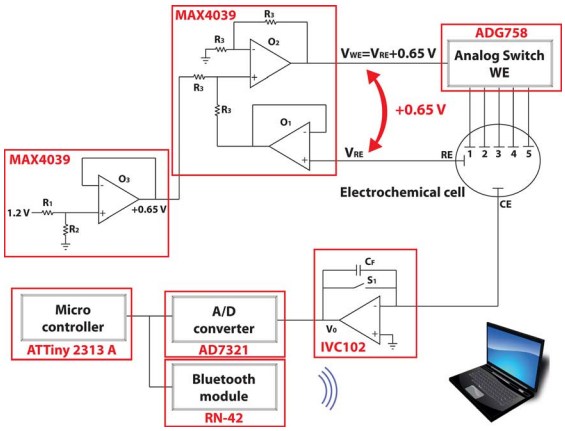

Fig. 7. Schematic of the architecture conceived for the electrochemical measurements of metabolites in cell culture monitoring. The different blocks are detailed with the components used in our implementation.

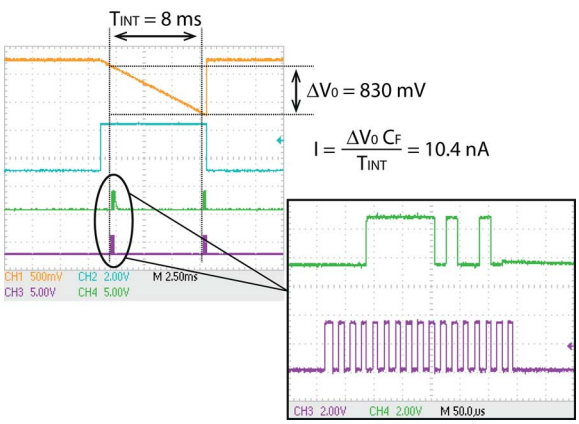

Fig. 8. Clock signal of the ADC (SCLK) and the output of the ADC.

is opened at time $t = 0$, the voltage difference ΔV_O across the capacitor between time $t = t_1$ and time $t = t_2$ is proportional to the charging current I

$$I = -\frac{\Delta V_O C_F}{T_{\mathrm{INT}}} \tag{1}$$

where the integration time to charge the capacitor is $T_{\mathrm{INT}} = t_2 - t_1$. In Fig. 8 signals V_O and S_1 are shown during a measurement. High gain can be achieved by using small capacitance values C_F and long integration time T_{INT}. In this design, C_F is chosen equal to 100 pF and T_{INT} is chosen equal to 8 ms ($t_1 = 1$ ms, $t_2 = 9$ ms). Thus, since the maximum output of the converter is $V_{\mathrm{SAT}} = -9$ V, the voltage ramp across the capacitor saturates at $t_2 = 9$ ms for a maximum current I_{MAX} equal to

$$I_{\mathrm{MAX}} = -\frac{V_{\mathrm{SAT}} C_F}{t_2} = 100\ \mathrm{nA}. \tag{2}$$

The output voltage V_O of the current-to-voltage converter is digitized by a 12-bit plus sign serial ADC (AD7321 from Analog Devices). The input range is set to ± 10 V. By using that configuration, the least significant bit (LSB) is equal to 2.441 mV.

A microcontroller is used to set the analog switch, drive the current-to-voltage converter, and read the ADC. The communication between the microcontroller and the user is achieved by means of a Bluetooth module emulating the serial protocol RS232. Bluetooth was chosen to simplify the communication between the system and the remote station, i.e., a laptop. Moreover, class-2 Bluetooth protocol can ensure a communication range up to 15 m, compatible with the typical dimensions of a standard biological laboratory. By means of the LabVIEW interface, which communicates with the microcontroller via Bluetooth, the user can set the parameters of the measurement, such as the working electrode connected to the switch, the integration time T_{INT} of the current-to-voltage converter, the sample rate, and the ADC input range. For every sample the output of the ADC is read by the microcontroller and displayed at the remote station. Data are not stored in the internal memory of the microcontroller. The clock signal of the ADC (SCLK) and the output of the ADC are reported in Fig. 8. The microcontroller used in this design is a low-power 8-bit (ATTIny 2313A by Atmel). The bluetooth module is an RN-42 by Roving Networks with on board chip antenna, UART connection interface and a power consumption of 26 μA in sleep mode, 3 mA in connection mode, and 30 mA in transmission mode.

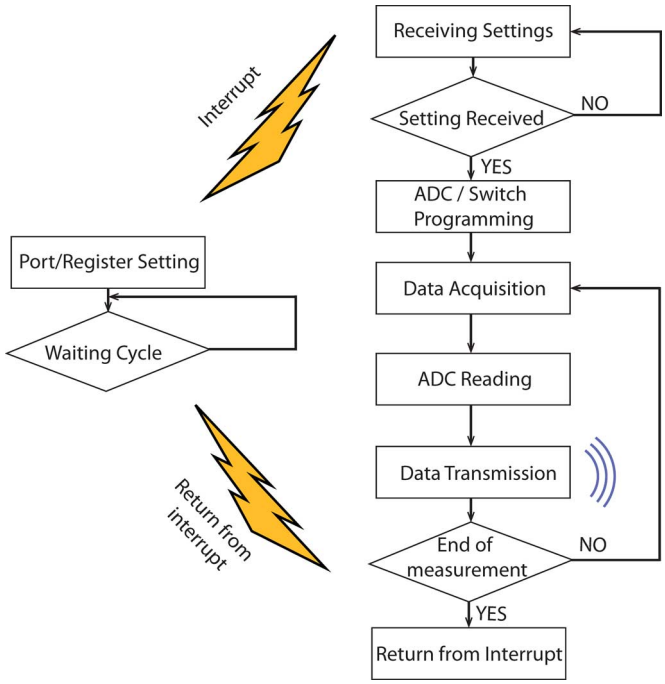

Fig. 9. Flowchart of the microcontroller code.

Finally, power supply is generated by using a single 9 V battery. Most components need stable voltage supply of different values. Several voltages are generated on board for the different circuits: 3.3 V and 5 V (LP38693), 10 V (LMC7660), and -10 V (LM2662). The chosen approach for both the potential control and the current-to-voltage sensor can be integrated in a chip, similarly to what was described in [47].

D. Microcontroller Code

The flowchart of the microcontroller code is reported in Fig. 9. When powered, the microcontroller initializes the registers and the I/O ports; then, it waits for a request of measurement

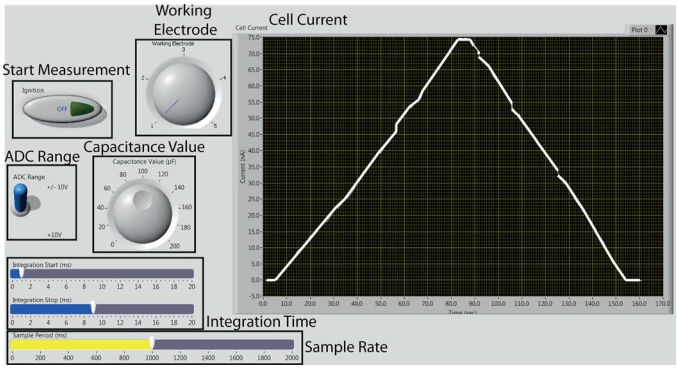

Fig. 10. LabVIEW interface to control the electronic circuit form a remote station.

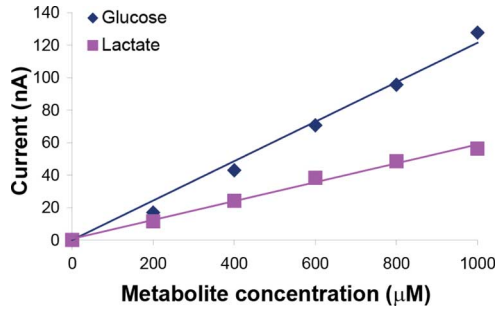

Fig. 11. Calibration lines for glucose and lactate detection in phosphate buffer solution. Measurements are acquired by using the commercial system.

from the user. An interrupt is generated at microcontroller level when the user starts a new measurement; the parameters of the measurement are set by the microcontroller, according to the user request. Output values read by the ADC are immediately sent to the remote station via Bluetooth. Once the measurement is stopped, the microcontroller returns from the interrupt to the initial loop, waiting for a new measurement.

E. LabVIEW Interface

A user friendly LabVIEW interface is designed to communicate with the microcontroller and plot the received data. A screen-shot of the interface is shown in Fig. 10. The user can directly set the parameters of the measurement from the main panel. At the end of the measurement, the cell current is directly plotted on the screen. Data collected from the measurement can be postprocessed directly with LabVIEW or be exported and stored in other formats (such as a spreadsheet).

V. Results and Discussion

In Section III-A, we described the fabrication and functionalization of the biochip, while in Section IV we realized a potentiostat to carry out electrochemical measurements with the develop electrodes. In this section, we are going to present the measurements performed with the developed biosensors. The first step is the integration of the fluidic system with the functionalized biochip. A series of measurements is carried out to prove the ability of enzymes immobilized onto carbon nanotubes in selectively detecting glucose and lactate in phosphate buffer solution. We successively move on in monitoring glucose and lactate also in cell culture medium in the physiological range. This set of experiments is useful to point out that measurements in pure medium cannot be performed and *online* sampling strategies are required. Then, as an example, we will propose the validation of the whole system for the monitoring of the lactate production in neuroblastoma cell line after 72 h of cultivation. We will show that the integration of the biosensors with the fluidics allows us to precisely detect the metabolite levels in undiluted medium. Finally, we will test the system with the developed architecture and we will present some measurements acquired by our potentiostat. We will also compare the results with those obtained by a commercial potentiostat (Autolab by Metrohm).

A. Biosensor Calibration for Glucose and Lactate in PBS

Glucose and lactate oxidases are spotted onto two different electrodes and performance of the two biosensors are evaluated. Both the metabolites are detected in a range between 0 and 1000 μM with steps of 200 μM in PBS, to evaluate the sensitivity and the detection limit of the two biosensors. For all measurements the integrated platform is hosted in an in-house chip holder and connected to the peristaltic pump. The flow is adjusted at $13\mu\mathrm{l/min}$ and the measurement starts with PBS flowing through the camber. The signal recorded for PBS is considered as the baseline, so the successive values of currents are referred to the baseline value. Once the measurement is run, we change the solution at the inlet of the fluidic system every 3 min. Calibration lines for glucose and lactate are depicted in Fig. 11. For glucose the sensitivity results in a value of $55.5\mu\mathrm{A/mM\,cm^{-2}}$ and a detection limit of 2 μM ($\mathrm{S/N} = 3\sigma$), whereas for lactate the sensitivity is 25.0 $\mu\mathrm{A/mM\,cm^{-2}}$ with a detection limit of 11 μM. Table I compares the state-of-the-art of CNT-based biosensors for both glucose and lactate detection. In the case of glucose monitoring, our biosensor shows the best performance for both sensitivity and detection limit, as illustrated in Table I. All previous works reported in the comparison foresee to randomly drop cast CNTs solution onto the working electrode. Glucose oxidase can be directly mix with CNTs, as proposed by Tsai *et al.* [26], or CNTs can even be functionalized with butyric acid, for example, to increase their immobilization prior to deposition, as described in [48]. The last approach reported in Table I obtains the best sensitivity, even if the value is half of what we achieve with our biosensor. Wang *et al.* [40] proposed to directly grow CNTs onto a silicon substrate and evaporated afterwards a thin gold film on the top. GOD was then drop cast on the top of carbon nanotubes after their detachment from the substrate. Also in this case the sensitivity of the biosensor results to be lower than in our case. Finally, an approach slightly different foresees to press synthesized MWCNTs to form a sheet-like structure. Then, GOD can be covalently bound to the CNTs of the so-formed "paper" [49]. Also this approach does not give sensitivity as high as in our case.

The literature on lactate biosensors is smaller as compared to the case of glucose detection. However, several modification and functionalization have been proposed as well. CNTs can be incorporated in mineral oil to form a paste [50] or into sol-gel film to form a matrix [51]. The obtained mixture can then be

TABLE I
COMPARISON OF SIMILAR SURFACE MODIFICATION BASED ON CNT AND OXIDASES FOR THE DETECTION OF GLUCOSE AND LACTATE

	Modification	Sensitivity	Linear range	Limit of detection
GLUCOSE	CNT mat + GOD [49]	4.05 μA mM^{-1} cm^{-2}	0.2 - 2.18 mM	–
	MWCNT/Nafion + GOD [26]	4.7 μA mM^{-1} cm^{-2}	0.025 - 2 mM	4 μM
	MWCNT + GOD [40]	14.2 μA mM^{-1} cm^{-2}	0.05 - 13 mM	10 μM
	MWCNT-BA + GOD [48]	23.5 μA mM^{-1} cm^{-2}	0.01 - 2.5 mM	10 μM
	MWCNT/Nafion + GOD (present work)	55.5 μA mM^{-1} cm^{-2}	0 - 1 mM	2 μM
LACTATE	MWCNT/mineral oil + LOD [50]	0.204 μA mM^{-1} cm^{-2}	0 - 7 mM	300 μM
	Titanate NT + LOD [53]	0.24 μA mM^{-1} cm^{-2}	0.5 - 14 mM	200 μM
	MWCNT + sol-gel/LOD [51]	2.1 μA mM^{-1} cm^{-2}	0.3 - 1.5 mM	0.3 μM
	N-doped CNT/Nafion + LOD [52]	40.0 μA mM^{-1} cm^{-2}	0.014 - 0.325 mM	4 μM
	MWCNT/Nafion + LOD (present work)	25.0 μA mM^{-1} cm^{-2}	0 - 1 mM	11 μM

used as electrodes after the oxidase immobilization. However, the resulting sensitivities are lower than the value obtained from our biosensor of two and one orders of magnitude, respectively. Carbon nanotubes can be also doped with nitrogen to enhance the sensor response, resulting in a really high sensitivity of 40.0 $\mu\mathrm{A}/\mathrm{mM\ cm}^{-2}$, although the detection range is indeed narrow (from 14 to 325 μM) [52]. Yang *et al.* [53] proposed titanate nanotubes for the detection of lactate. The obtained sensitivity is again two orders of magnitude lower than our case, suggesting that carbon gives better performance as material used for detection of hydrogen peroxide and not only for the structure in the nanoscale range. Sensitivities and detection limits of our biosensor are better as compared with previous results presented in literature, as showed in Table I. Thus, in the next section we proceed with the calibration of our biosensor in cell culture medium, with a particular attention on measuring the metabolites in the physiological range.

B. Biosensor Calibration for Glucose and Lactate in Cell Culture Medium

Cell culture medium contains several compounds for cell proliferation and growth. It is a complex mixture enriched with hormones, nutrients, and antibiotics for an optimal development of the cells. Due to all those salts and components, pure medium is not suitable to perform electrochemical measurements, due to the numerous electroactive species that can interfere with the detection. Some works [54] propose the dilution of cell culture medium prior to perform the detection. However, detection limit is affected by the dilution rate and sensor calibration has to be tuned according to the dilution step. Another approach alternative to dilution is the employment of dialysis membranes. They allow the *online* monitoring of the cell culture, while minimizing the sample volume required to perform the measurement. Working principles and the integration of the microdialysis probe in the fluidic system are described in our previous work [55]. Briefly, the microdialysis probe is immersed in the culture medium and connected to the inlet of the PDMS chamber. The solution exits from the outlet channel and is then wasted away by the peristaltic pumps. For glucose detection, we use glucose-free medium (D5030) and D5030 enriched with glucose in concentrations of 5, 10, 15, and 20 mM. The use of the membrane contained in the microdialysis probe allows to significantly extend the linear range of the biosensor playing the role of a diffusion barrier. In this way, we can easily measure glucose uptake in our cell culture medium without any dilution step. Nominal glucose concentration in commercial DMEM is of 3.15 g/l, equal to around 17.5 mM. We also prepare DMEM enriched with lactate, similarly to what we have done for glucose. Lactate concentrations span between 0 and 20 mM, as in the case of glucose. The range is chosen accordingly to data reported previously in literature for NG-108 neural cells [56] and murine embryonic cells [57]. Calibration lines for glucose and lactate detection in cell culture medium are depicted in Fig. 12. Glucose biosensor shows a sensitivity of 1.9 $\mu\mathrm{A}/\mathrm{mM\ cm}^{-2}$ and a detection limit of 1 mM. Although the sensitivity is one order of magnitude lower than in PBS measurements, the linear range spans up to 20 mM, which enables to measure glucose in cell culture medium. Lactate biosensor is also less sensitive respect to the case of pure PBS, with a sensitivity of 0.4 $\mu\mathrm{A}/\mathrm{mM\ cm}^{-2}$ and a detection limit of 1.1 mM. However, even in this case we fit the concentration range of interest. The system is now ready to be validated in conditioned cell culture medium.

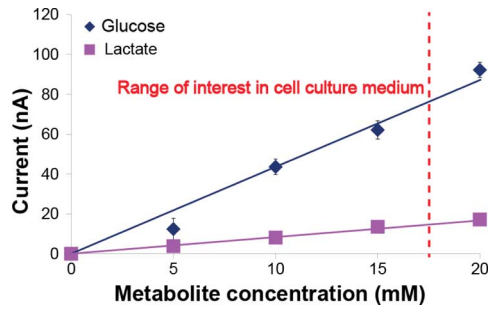

Fig. 12. Calibration lines for glucose and lactate detection in cell culture medium. Measurements are acquired by using the commercial system.

C. Metabolic Monitoring in a Cell Line: An Example

Glucose and lactate biosensors have shown to be able to detect physiological concentrations of such metabolites in cell culture medium. Lactate production, in particular, is interesting from a biological point-of-view. Cells metabolize glucose mostly via the glycolytic pathway and they produce lactate, which is toxic to animal cells in high concentrations and can inhibit cellular metabolism. For this reason, monitoring lactate production enables a more efficient glucose metabolism and limits toxic accumulation in culture medium [18].

Neuroblastoma cells are derived from animal solid cancers. They can show an immature appearance, possessing a proliferative behavior, and then evolve in a differentiation state by

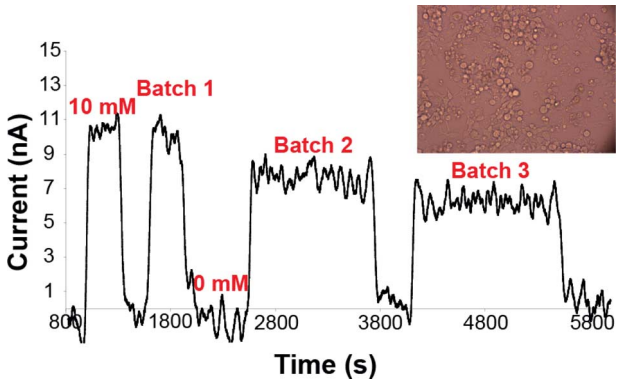

Fig. 13. Measurements relative to the three batches of neuroblastoma cells and a reference of 10 mM of lactate. Measurements are acquired by using the commercial system. Inset: neuroblastoma cells after three days of cultivation.

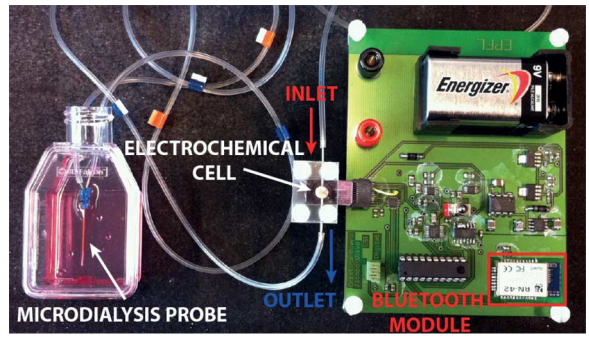

Fig. 14. The developed potentiostat realized with off-the-shelf components. The potentiostat is connected to the biosensor, while the fluidic system enable the *ex situ* sampling of the medium.

elaborating long neurite-like processes. These properties make them also interesting from the metabolic point-of-view.

We performed lactate detection in surnatant conditioned medium collected from three different batches of neuroblastoma cells (NG-108 cell line). We first dip the microdialysis probe in fresh DMEM (without lactate) to measure the baseline. The fresh medium is then changed with medium containing a known lactate concentration (10 mM) without stopping the peristaltic pump and the electrochemical measurement. The two obtained current values are used as a further check on the calibration of the biosensor. Successively, the surnatant coming from one of the three batches is measured for 5 min before rinsing the biochip. The second and the third batches are measured subsequently. The current measured for the three batches and for a reference of 10 mM of lactate are depicted in Fig. 13. The inset of the image shows how neuroblastoma cells look after three days of cultivation. Indeed, they appear almost at confluence, i.e., covering most of the Petri dish. Some of them have already developed their processes as neurons, so their are at a cell state almost differentiated. However, others still show the characteristic round shape, that is typical for proliferative state. Lactate concentration results in a value of 10.0 ± 2.2 mM for the first batch, 7.8 ± 2.1 mM for the second batch, and 6.4 ± 2.0 mM for the third batch after 72 h of cultivation. The difference in the three batches is most probably due to different cell concentration. In the present experiment cells are always seeded with the same rate for the three batches, but changes in the number of cells are intrinsically subjected to statistical fluctuation. However, lactate values measured in the three samples are similar to those reported in [56] for NG-108 cell line. The increase of lactate levels from 0 mM (no lactate in the initial medium) to an average concentration of 8 mM are a confirmation that cells proliferate. This evidence is fundamental to distinguish different cellular state, since proliferation and differentiation have different metabolic profiles [41], and their recognition can be automated.

D. Measurements With Our Architecture: Towards a Fully Self-Contained System

In the previous sections, we have shown an exhaustive characterization of the biosensors, by presenting their calibration both in PBS and undiluted medium. They have been also validated for the detection of lactate in a cell culture, so we have proved that our biosensors are suitable for cell monitoring. Since the final goal of the present research is the development of a self-contained device for the monitoring of the metabolic activity of cells, we now test the developed architecture together with the rest of the system characterized so far. As already mentioned in Section II-D, hydrogen peroxide is the common product of all the reactions promoted by the oxidases. So, its detection is fundamental for the purpose of our research. We start by detecting H_2O_2 using our architecture and we perform a comparison with analogous measurements acquired with a commercial potentiostat. All measurements are performed as described in Section V-A. For measurements with the commercial potentiostat, a knob attached to a proper connector allows us to mechanically switch among the different working electrodes. In this case the electrode is connected to the instrument through cables, one for each electrode of the cell. Such configuration is not suitable for the application of the system in cell culture monitoring. Differently from the previous case, our circuit communicates with the remote laptop via Bluetooth, enabling measurements inside the flask and close to the incubator. An image of the system is showed in Fig. 14. The integrated platform is fixed in the in-house chip holder and connected to our potentiostat.

We first measure different concentrations of hydrogen peroxide (40, 80, 100, 150, and 200 μM) on one bare working electrode. The two recorded signals are superimposed and depicted in Fig. 15. Even thought raw data show statistical fluctuations, after a moving average filtering the result compares well with the typical staircase of chronoamperometry and the signal recorded by the commercial potentiostat. Our potentiostat is able to follow the successive injections of more concentrated H_2O_2 and the current increases more for higher concentration (for example between 100 and 150 μM). Moreover, it fully meets the range of response without any saturation effect. Currents span from 1 nA up to about 65 nA and our circuit perfectly acquires the entire signal.

We also repeat the same measurements in the case of CNT-enhanced electrodes. The calibration curves obtained by the two instruments are depicted in Fig. 16. Also in the case of CNT modification, our circuit shows the typical staircase graph and records signals up to 80 μM. As in the previous case, to higher concentrations of hydrogen peroxide correspond higher currents

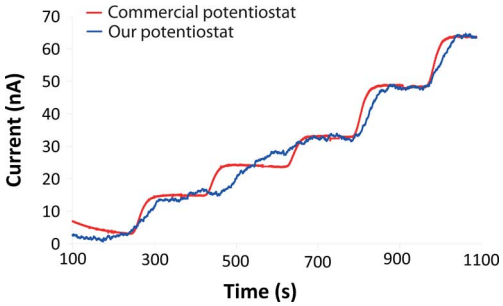

Fig. 15. Superimposed chronoamperometries acquired with the commercial potentiostat and our developed wireless system.

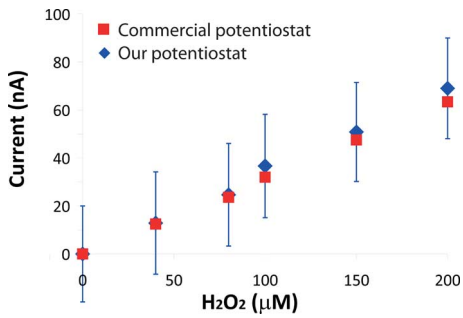

Fig. 16. Calibration lines for H_2O_2 detection measured with the developed circuit and a commercial potentiostat.

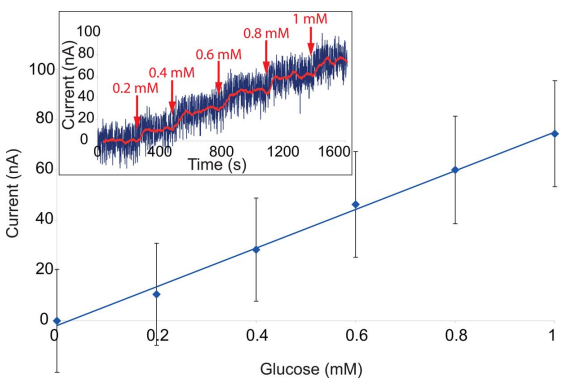

Fig. 17. Calibration line for glucose in PBS in the range from 0 to 1 mM measured with our potentiostat. Inset: raw data of the real-time measurement. The red line is an average filtering of the raw data.

and the stair amplitude is more marked for 150 and 200 μM. As we already showed in Section III-C, carbon nanotubes enable an efficient immobilization of the enzyme, that is not possible on bare electrodes. Graphite is well known as a proper electrode material for the detection of hydrogen peroxide. However, CMOS compatible technologies do not allow carbon as a source material for fabrication. The proposed approach, instead, enables the development of H_2O_2-sensitive sensors on gold electrodes successively modified with nanomaterials. CNTs permit the immobilization of the enzyme onto the electrode surface and, more important, a favorable electron transfer between the active site of the protein and the electrode surface.

Based on the results obtained for hydrogen peroxide detection, we also test our potentiostat with functionalized electrodes. As an example, Fig. 17 illustrates the calibration line in PBS for glucose biosensor acquired with our potentiostat. The inset shows the raw data of the real-time measurement. The acquired measurement is quite noisy, so postprocessing filtering is required to extract data for the calibration (red line superimposed to raw data in the inset of Fig. 17). However, our architecture is able to follow the increase of glucose concentration and the current perfectly fits the input range of the transimpedance amplifier without any saturation effect.

VI. Conclusion

The lack of real-time and quantitative information with respect to cellular behavior in cultures does not enable the optimization and scale-up of bioprocesses, which still remain manual and with low product quality. On the contrary, the development of reliable measurement instruments can be fundamental for the optimization of the whole process. The present paper shows the realization of a self-contained system based on electrochemical biosensors for cell culture monitoring. The electrochemical biosensors are based on carbon nanotubes, ensuring a robust immobilization of the enzymes onto gold electrodes. The described platform is developed for the detection of different metabolites, like glucose and lactate, in cell culture medium. We have fabricated the electrochemical cell by using CMOS compatible technology. We achieved an integrated electrochemical cell with five working electrodes to detect multiple metabolites with the same platform. We showed that spotting tools are suitable for nanostructuring and functionalization of the electrodes, although they were originally developed for microarrays and DNA printing. We have presented the design and a first realization of the wireless potentiostat. We achieved the selective functionalization of the electrodes with glucose and lactate oxidase and we calibrated the developed biosensor under flow conditions in phosphate buffer solution. We obtained a sensitivity of $55.5 \mu\mathrm{A}/\mathrm{mM}\,\mathrm{cm}^{-2}$ and a detection limit of 2 μM for glucose, whereas a sensitivity of $25.0 \mu\mathrm{A}/\mathrm{mM}\ \mathrm{cm}^{-2}$ and a detection limit of 11 μM for lactate. We tested our carbon nanotube-based biosensor in cell culture medium without any dilution, by integrating a microdialysis probe to our fluidic system. Glucose biosensor results in a sensitivity of 1.9 $\mu\mathrm{A}/\mathrm{mM}\ \mathrm{cm}^{-2}$ and a detection limit of 1 mM in the culture medium. Lactate detection, instead, is performed with a sensitivity of 0.4 $\mu\mathrm{A}/\mathrm{mM}\ \mathrm{cm}^{-2}$ and a detection limit of 1.1 mM in the same condition. We performed lactate detection in surnatant conditioned medium collected from three different batches of neuroblastoma cells (NG-108 cell line). The measured values are similar to those reported in literature for the same cell line. The increase of lactate levels in DMEM confirms that cells proliferate and this evidence can be the distinguishing key for different cellular states. Finally, we performed measurements of hydrogen peroxide and glucose with the developed architecture. The results show that our potentiostat is suitable for the online monitoring of cell cultures. The comparison between our circuit and a commercial instrument demonstrates that our system is able to follow current changes. Glucose biosensor calibration, instead, demonstrates that the input range is suitable for recording currents generated in the electrochemical cell. The present results show that our approach is suitable for the development of biosensors for the

online monitoring of cell culture and represent a novelty with respect to the state-of-the-art in cell culture monitoring.

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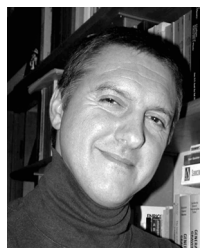

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