Highly Sensitive Carbon Nanotube-Based Sensing for Lactate and Glucose Monitoring in Cell Culture

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***Abstract*—Monitoring of metabolic compounds in cell cultures can provide real-time information of cell line status. This is particularly important in those lines not fully known, as the case of embryonic and mesenchymal cells. On the other hand, such approach can pave the way to fully automated systems for growing cell cultures, when integrated in Petri dishes. To date, the main efforts emphasize the monitoring of few process variables, like pH, pO_2, electronic impedance, and temperature in bioreactors. Among different presented strategies to develop biosensors, carbon nanotubes exhibit great properties, particularly suitable for high-sensitive detection. In this work, nanostructured electrodes by using multiwalled carbon nanotubes are presented for the detection of lactate and glucose. Some results from simulations are illustrated in order to foresee the behavior of carbon nanotubes depending on their orientation, when they are randomly dispersed onto the electrode surface. A comparison between nonnanostructured and nanostructured electrodes is considered, showing that direct electron-transfer between the protein and the electrode is not possible without nanostructuration. Such developed biosensors are characterized in terms of sensitivity and detection limit, and are compared to previously published results. Lactate production is monitored in a cell culture by using the developed biosensor, and glucose detection is also performed to validate lactate behavior.**

***Index Terms*—Carbon nanotubes, cell culture, electrochemical biosensors, electron transfer, metabolite monitoring.**

I. Introduction

GLUCOSE AND lactate biosensors have been largely reported in literature for clinical purposes. A lot of solutions have been exploited to optimize the response of the sensor. For both the metabolites, electrodes have been structured with polymeric matrices, sol-gel, cross-linker, and mediators [1]. Recently, nanomaterials has been considered as possible electrical connectors to directly link the redox site to the electrode surface, because of the similar dimensions of nanoparticles and redox proteins. Various nanomaterials have been studied since the last ten years, including nanoparticles, nanowires and nanotubes [2].

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Carbon nanotubes (CNTs) have aroused increasing interest for their electrical [3] and electrochemical properties [4] with special focus on biosensor applications [5]. It is possible to distinguish two large categories of CNTs: *single-walled carbon nanotubes* (SWCNT) are a rolled-up graphene single sheet with a diameter of typically about 2 nm, which can behave either as a metal or a semiconductor; *multiwalled carbon nanotubes* (MWCNT) are graphene sheets rolled-up into concentric cylinders with external diameter in the range of 10–100 nm. Due to the small diameter of these nanomaterials, quantum-effects dominate, and therefore these nanomaterials are efficiently coupled with proteins [6]. Studies have demonstrated that CNTs promote the electron-transfer between the enzyme redox active site and the transducing electrode [7].

Regarding oxidase-based biosensors, numerous studies were focused on the detection of metabolites like glucose, lactate, cholesterol, and glutamate. In the case of glucose the main application has been primarily related to diabetes pathology, in order to develop disposable devices for self-monitoring of blood glucose. Glucose detection was also interesting for other applications, like food industry for quality control purposes, or for keeping fermentation under control. Another metabolite of interest is lactate, which is related to anaerobic metabolism and it has been extensively studied in sport medicine, since it is correlated with muscle contraction, or pathological conditions which brings cell to suffer. In fact, elevated levels of lactate are mainly related to lack of feeding or hypoxia, and these levels can have toxic effects on cells. One of the purposes of the present study is to monitor lactate production over the time on a cell culture, and comparing it with the behavior of glucose uptake, to verify that lactate is secreted when there is a lack of feeding.

One possible strategy to develop biosensors for the aforementioned compounds is to employ amperometric enzyme-based biosensors. Oxidases belong to a large family of enzymes, which are able to transform such metabolites in other compounds with the production of *hydrogen peroxide* (H_2O_2). H_2O_2 is an electrochemical specie oxidizable by means of a constant voltage. For glucose and lactate the enzymatic reaction is the following (X = L or G):

$$XOD/FAD + substrate \rightarrow XOD/FADH_2 + product$$
$$XOD/FADH_2 + O_2 \rightarrow H_2O_2 + XOD/FAD$$

where gluconic acid and pyruvate are the enzymatic products for glucose and lactate, respectively, and FAD (*flavin adenine dinucleotide*) is the redox active site, where the exchange of electrons takes place. Since the stoichiometry of the reaction foresees one molecule of H_2O_2 for one molecule of substrate, it is possible to directly measure the concentration of hydrogen peroxide to obtain the concentration of glucose or lactate. Due

to electrochemical properties of H_2O_2, the application of a potential of +650 mV causes the oxidation of hydrogen peroxide, according to the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2^+ + 4e^-.$$

The four e^- released to the electrode generates a current, which intensity is proportional to H_2O_2 concentration. Both the reaction and the application of a potential can be carried out into an electrochemical cell. The cell consists typically of three electrodes connected with a potentiostat, which is able to record the current between the working electrode and the counter electrode, by fixing a potential between the working electrode and the reference electrode. The reaction of oxidation or reduction occurs onto the working electrode.

About the applications of such biosensors to cell cultures, to date the effort in this sense was concentrated to monitor process variables, like pH, pO_2, electronic impedance, and temperature in bioreactors [8]. Recently, Pemberton *et al.* [9] developed an amperometric biosensor to monitor glucose in a liver cell line. The authors report concentration values for different densities of seeded cells after 24 h of culture. Therefore, it is possible to envisage new emerging application areas for metabolite biosensors in cell monitoring. These biosensors can be instrumental in investigating unknown, or non-well-known cell lines, as embryonic or mesenchymal stem cells, also during proliferation or switching state, adding important information to the state-of-the-art of these cell families. Alternatively, such biosensors can be used to monitor cell cultures and to develop automated systems to manage cell cultures, saving time to human work.

The objective of the present work is the development of nanostructured electrodes by using MWCNT for the detection of lactate and glucose. Results from simulations regarding CNTs behavior are presented to demonstrate why this type of nanomaterials is suitable for biosensing. A comparison between nonnanostructured and nanostructured electrodes is investigated to demonstrate that detection is not possible without carbon nanotubes. Then, a fully characterization of the developed biosensors is reported, and performance of the proposed biosensors are compared with literature. Finally, nanostructured electrodes are functionalized with the enzyme probe and employed in a cell culture to monitor lactate production and glucose uptake. For this study the SN56 cell line is used, which derives from fusion of septal neurons of postnatal mice with murine neuroblastoma cells and it can be consider a suitable model to study the effects of hypoxia on brain cells.

II. Materials and Methods

A. Chemicals

Carbon paste screen-printed electrodes (SPE—model DRP-110) and multiwalled carbon nanotubes were purchased from Dropsens (Spain). The electrodes are made of a graphite working electrode, which presents an active area equal to 13 mm^2, a counter electrode, also made of graphite, and a reference electrode, which is made of Ag/AgCl. The total area of the cell is 22 mm^2. Multiwalled carbon nanotubes (diameter 10 nm, length 1–2 μm) were purchased in powder (90% purity), and subsequently diluted in chloroform to the concentration of 1 mg ml^{-1} [2]. Samples were then sonicated in order to obtain an homogeneous solution.

Glucose oxidase from *Aspergillus Niger* (GOD, EC 1.1.3.4, 129.9 units/mg solid), lactate oxidase from *Pediococcus species* (LOD, EC 1.13.12.4, $\geq$20 units/mg solid), D-(+)-glucose, and lithium L-lactate were purchased from Sigma-Aldrich (Switzerland) in lyophilized powder. All the proteins were dissolved in phosphate buffer solution (PBS) 0.01 M at pH 7.4, while glucose and lactate were dissolved in Milli-Q.

B. Cell Cultures

SN56 cell line (clone SN56.B5.G4), derived from the fusion of septal neurons of postnatal day 21 mice with N18TG2 murine neuroblastoma cells [10], was a generous gift from Prof. Wainer (Emory University, Atlanta, GA). Proliferating cells were maintained in Dulbecco's modified Eagle's medium (DMEM, from Sigma-Aldrich, Switzerland), supplemented with 10% fetal bovine serum (FBS, Gibco/BRL, Rockville, MD), 2 mM l-glutamine (from Sigma-Aldrich), and 40 U ml^{-1} penicillin/streptomycin (from Gibco), in 25 cm^2 culture flasks (Corning, New York, NY) in a 5% CO_2 atmosphere at 37 °C. Medium in the stock flasks was changed every 48 h and the cells were subcultured when they reached 80%–90% of confluence.

For experiments, cells were seeded at 5×10^3, 25×10^3, and 203×10^3 cells cm^{-2}, after four passages from thawing. Then, cells were cultured onto uncoated wells in proliferating conditions for 48 h. The surnatant medium was collected from the flasks at different cell densities after 0, 4, 24, and 48 h after seeding. Each sample was then diluted 1:10 in PBS for electrochemical measurements. Then, samples were frozen, and thawed when measurements were performed.

C. Preparation of Electrodes

Nanostructured SPEs were prepared by using MWCNT and the probe enzymes. To prepare the MWCNT modified SPE, 40 μl of the MWCNT-chloroform solution wad deposited by drop casting (5 μl each time) onto the working electrode and it was allowed to dry. Then, 20 μl of glucose or lactate oxidase (15 mg ml^{-1} and 125 mg ml^{-1}, respectively) were dropped onto the working electrode and stored overnight at +4°C, in order to allow the adsorption of the proteins onto the electrode surface. Then, the drop was rinsed out with Milli-Q and the electrode was conditioned for 10 min at constant potential (+550 mV) before the first use. All the functionalized electrodes were stored at +4°C and covered with PBS, when not used.

D. Apparatus

The electrochemical response of electrodes is investigated by chronoamperometries under aerobic conditions. Electrochemical measurements were acquired by using Versastat 3 potentiostat (Princeton Applied Technologies). For calibration and investigation of the detection limit, the electrode was dipped into the PBS solution with a volume of 25 ml under stirring conditions. A volume of 25 μl per step of the target molecule was successively added into the solution with a time-step of 2 min. In

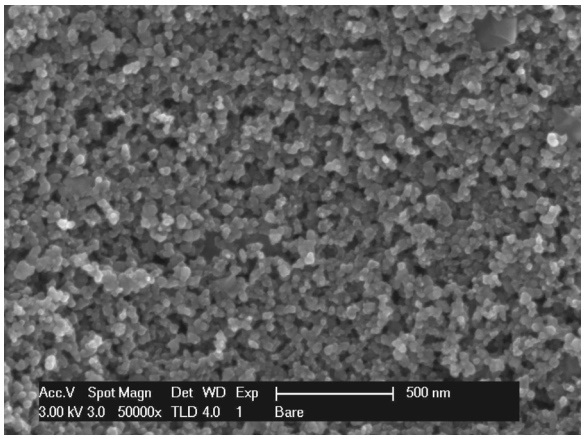

Fig. 1. SEM image of the surface of a bare electrode.

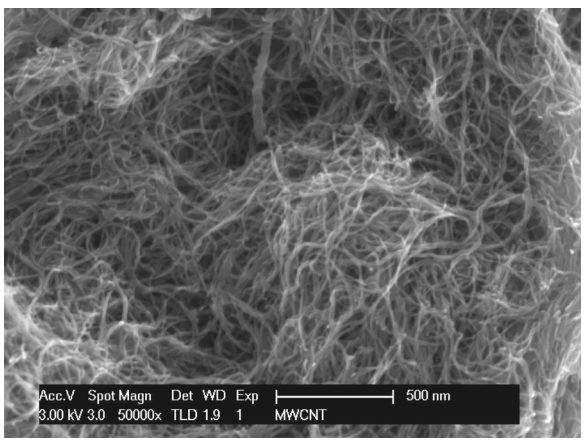

Fig. 2. SEM image of the surface of a nanostructured electrode.

the case of measurements of DMEM, the electrodes were covered with a drop of 100 μl of the diluted medium. In both the cases, the applied potential was +550 mV vs Ag/AgCl.

A Philips/FEI XL-30 F microscope (Netherlands) was used to acquire scanning electron microscopic (SEM) images. The resolution in UHR mode is 2.5 nm at 1 kV.

E. SEM Images

Figs. 1 and 2 depict the acquired pictures for the bare electrode and for CNTs drop cast onto the electrode surface, respectively. As it is possible to see from the picture related to bare electrode, the surface has an important corrugation and graphite is characterized by small aggregates. On the other hand, when carbon nanotubes are dropped onto the surface, they form like "wrapped balls" of thin wires spread onto the surface. This behavior is due to the fact that the ends of carbon nanotubes, which terminate with a carboxylic group, are quite hydrophilic, but the walls, which comprise the majority of the tube, are highly hydrophobic. Therefore, they have the tendency to rapidly coagulate. Consequently, carbon nanotubes are dispersed in chloroform, that is a nonpolar organic solvent, but for the same reason, they also tend to be strongly adsorbed onto the electrode surface when chloroform evaporates [11].

F. Theory of Carbon Nanotube Emissions

It is already known that carbon nanotubes have considerable electron field emission properties. They are used to enhance the emissivity of electrodes made of various materials [11]. CNTs are also used for enhancing sensitivity of biosensors [2], [11]. Many theoretical works have been published related to the field emission at the tip of individual capped CNTs, based both on the density functional theory or the Green's function theory [12], [13], or even using the symmetry properties of CNTs for solving the Schroedinger equation [14], [15]. On the other hand, experimental works have been made to investigate the sidewall field emission properties of CNTs aligned in bundle [16], [17]. Two conclusions were drawn in these works: the sidewall field emission obeys the Fowler–Nordheim equation [16], [17], and field emission performance increases with decreasing bundle diameter [17]. This property suggests that the best emission performance is from the body (the side wall) of individual CNTs. Our aim is to derive an equation, which governs the full emission of an individual CNT including electron emission from both sidewall and tip. The general equation needs to consider the arbitrarily oriented carbon nanotubes with respect to the field, while using a simple model to investigate nanostructured electrodes. Toward this goal, we investigate the situation in which there is a CNT with the axis parallel to the substrate and, therefore, perpendicular to field E. The developed model is based on the following three assumptions:

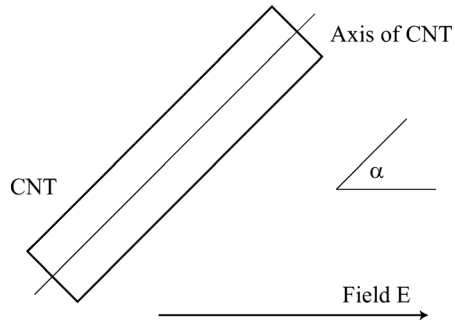

Fig. 3. Model for a linear segment of CNT.

1) the electron emission occurs through the CNT half surface facing the anode (referring to Fig. 3);
2) the field E is uniform both in direction and intensity in the neighborhood of the substrate and of the CNT;
3) the current emitted across the surface σ obeys the Fowler–Nordheim equation considering the projection of E on the normal to σ. In other words, assuming σ as a flat surface, the Fowler–Nordheim equation is

$$I = K_1 \sigma E_\perp^2 exp\left(-\frac{K_2}{E_\perp}\right), \tag{1}$$

where I is the current emitted across σ, $E_\perp$ is the projection of E on the normal to σ, and K_1 and K_2 are suitable constants. Then, if we consider an infinitesimal portion of the CNT surface, $d\sigma$, we have for the current

$$di = K_1 d\sigma E_\perp^2 exp\left(-\frac{K_2}{E_\perp}\right). \tag{2}$$

Assuming a cylindrical coordinate system with the axis of CNT as z-axis, whose origin is at one end of CNT, and the direction of E as origin of coordinate ϑ, we have

$$d\sigma = \rho d\vartheta dz; \qquad E_\perp = E\cos\vartheta, \tag{3}$$

where ρ is the radius of the carbon nanotube. By substituting (3) into (2), we obtain

$$di_S(E) = K_1 \rho d\vartheta dz (E\cos\vartheta)^2 exp\left(-\frac{K_2}{E\cos\vartheta}\right). \quad (4)$$

The total current emitted across the side surface of the CNT is obtained by integrating on the portion of surface of CNT facing the anode

$$i_S(E) = K_1\rho E^2 \int_0^L dz \int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} \cos^2\vartheta exp\left(-\frac{K_2}{E\cos\vartheta}\right) d\vartheta$$

$$= K_1\rho E^2 L \int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} \cos^2\vartheta exp\left(-\frac{K_2}{E\cos\vartheta}\right) d\vartheta. \quad (5)$$

If the axis of CNT is not perpendicular to the field but forms an angle α (like in Fig. 3), (5) changes as follows:

$$i_S(E,\alpha) = K_1\rho E^2 L \int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} (\cos\vartheta\sin\alpha)^2 exp\left(-\frac{K_2}{E\cos\vartheta\sin\alpha}\right) d\vartheta. \quad (6)$$

In this case the emission from the CNT tip facing the anode should be considered. The current from the tip obeys the Fowler–Nordheim equation [14], which is written as

$$i_T(E,\alpha) = K_1' A (E\cos\alpha)^2 exp\left(-\frac{K_2'}{E\cos\alpha}\right). \quad (7)$$

The entire current emitted by an oriented CNT forming an angle α with respect to the field is

$$i(E,\alpha) = i_S(E,\alpha) + i_T(E,\alpha). \quad (8)$$

In the experimental activity on biosensors, possible parameter values may be $\rho = 10$ nm and $L = 100$ nm, where L is the linear shape of CNTs, confirmed by SEM image in Fig. 2, the applied field E may range from 100 V to 1000 V and the emitted current from 0 μA to about hundred of μA. Therefore, suitable values for the constants that appear in Fowler–Nordheim equations were considered $K_1 = 10^{12}$, $K_2 = 35$, $K_1' = (5/2)10^{12}$ and $K_2' = 20$ by comparison with experiments performed in vacuum [16]. In case of a water environment, such as experiments with biosensors during the detection of glucose and lactate in the cell medium, the emitted current is further enhanced by the presence of water molecules close to nanotube surface [12].

G. Monte Carlo Simulations

In order to investigate the role of carbon nanotubes in our structured electrodes, numerical simulations are implemented considering (8). Specifically, Monte Carlo simulations are performed to obtain the final distribution of the carbon nanotubes onto our screen-printed electrodes. In fact, it is possible to imagine a situation such as the one shown in Fig. 4, where active redox species are in contact with all the available surface of a carbon nanotube drop cast onto the surface of a screen-printed electrode. In the figure, electron emission

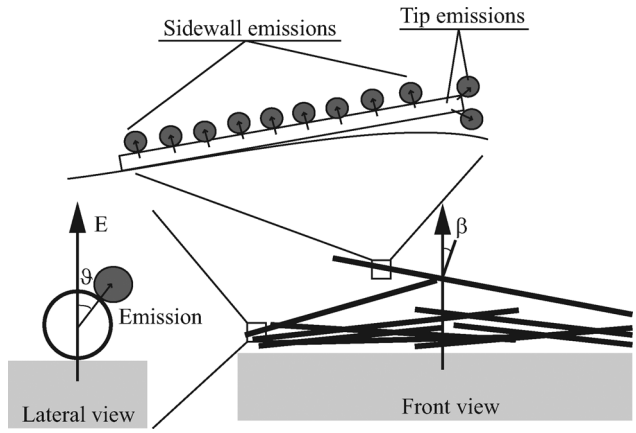

Fig. 4. Carbon nanotubes randomly organized onto the electrode surface and covered with proteins.

toward redox species would happen both from the sidewall and the tip. The electric field is typically normal to the baseline of the electrode. However, a screen-printed electrode has a mean corrugation in the 10-nm scale, as shown in Fig. 1. Then, the single carbon nanotube is positioned forming a certain angle with respect to the electric field, which would be typically normal to the electrode baseline, depending on the region where it was landing, as shown by Fig. 4. Therefore, Monte Carlo simulations are performed to simulate the landing of each single carbon nanotube onto the electrode surface. The final situation reached by each simulation is similar to the drawing in Fig. 4. The figure shows some carbon nanotubes differently organized onto the electrode surface, each of those presenting a different angle with respect to the electric field, which is orthogonal to the electrode surface.

III. Results and Discussion

A. Electron Emission From Carbon Nanotubes

Initial results obtained from Monte Carlo simulations include the angle distribution of CNTs longitudinal axis with respect to the electrode surface. Fig. 5 illustrates such a distribution for one of the simulations. The large majority of carbon nanotubes is distributed to lower angles and the number of nanotubes with angles larger than 10° decays very rapidly. Consequently, the sidewall emission decreases with the increasing angle of the nanotubes. Fig. 6 shows that the decay in the sidewall current is almost linear in a logarithmic scale, confirming first-order approximation of the sinus in (6), while the tip current follows a quadratic shape coherent with first approximation of the cosinus in (7). It is interesting to note that sidewall contribution to the total current is larger than tip contribution by orders of magnitude, even if current density is higher from the tip. Simulations shown in Fig. 6 allow to conclude that the tip emissions in drop cast CNTs are not negligible although the large majority of carbon nanotubes presents angles below 10° with respect to the substrate.

B. Improvements With Nanostructured Electrodes

The improvements of nanostructured electrodes by using MWCNT were previously demonstrated in the case of hydrogen

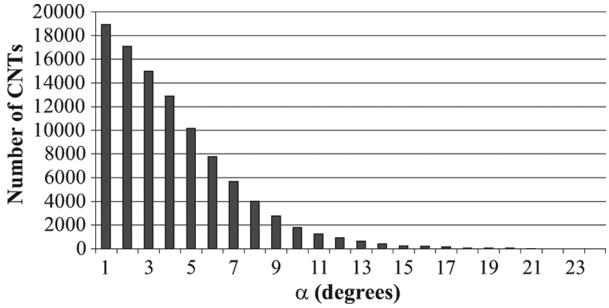

Fig. 5. Results from Monte Carlo simulation for the distribution of carbon nanotubes onto a flat surface.

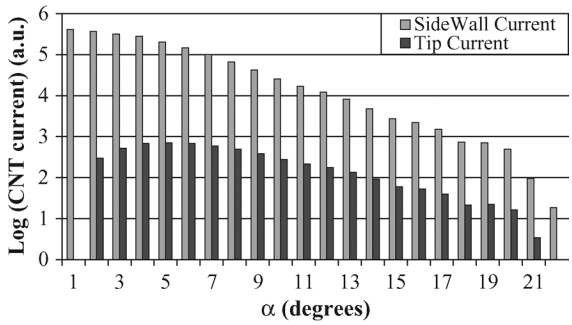

Fig. 6. Results from simulation regarding emission current from carbon nanotubes comparing the sidewall and the tip components, from (6) and (7).

peroxide [18]. The enhancement in terms of sensitivity between the case of nonnanostructured and nanostructured electrodes is about 7 times, also suggesting a similar improvement in terms of performance for oxidase-based biosensors.

Detection of lactate and glucose in the range of mM are performed for electrodes without and with nanostructuration, to evaluate the performance of the two strategies. The results are illustrated in Figs. 7 and 8. In the case of bare electrode, the detection is not possible within the range of interest. The detected current is in the order of nA for bare electrode, and signal to noise ratio is too high to sufficiently distinguish different concentrations of the substrate. On the other hand, the electrodes with carbon nanotubes show good sensitivity and a current range in the order of μA. These results are coherent with literature [2]. Second-generation biosensors [1] overcame the difficulty to directly couple the enzyme with the electrode by using a mediator. The role of the mediator is shuttling electrons from the protein to the electrode surface, despite of the complexity of the system. From these results it is possible to assert that nanomaterials are good candidates to replace the role of mediators, since they allow the direct electron-transfer from the active site of the enzyme to the electrode surface. Moreover, chemical mediators are often bounded successively to the electrode surface, with problems related to mediator concentration and chemical bounding. On the contrary, nanomaterials can be grown onto the electrode surface during microfabrication, enabling integration with VLSI chips [19].

The range of detection is chosen considering that Hwang *et al.* [20] reported a mean value for lactate of 10.9 mM in the case of murine embryonic stem cells. For the case of glucose, instead, the concentration in DMEM is at maximum 22.4 mM (4.5 g l^{-1} of glucose). Since it is not possible to perform measurements in pure DMEM, due to the interferences arising from

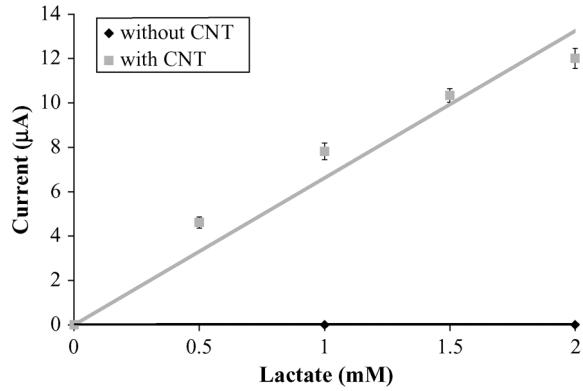

Fig. 7. Lactate detection for nonnanostructured and nanostructured electrodes.

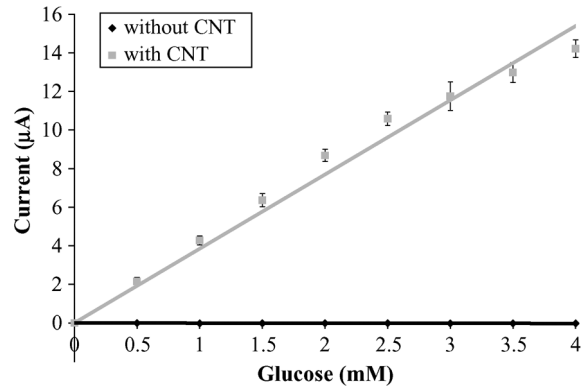

Fig. 8. Glucose detection for nonnanostructured and nanostructured electrodes.

easily electro-oxidizable substances at the same oxidizing potential, all the measurements are performed in diluted DMEM (dilution 1:10 in PBS), so that the maximum concentration of interest is 1 mM for the case of lactate, and 2.24 mM for the case of glucose. Moreover, linear range of the developed biosensor is not able to cover all the range of interest, so dilution is a proper solution. The range from 0.5 to 2.0 mM for lactate and from 0.5 to 4.0 mM for glucose are considered as the most suitable for the objectives of the present research, by considering the dilution of the DMEM.

C. Calibration

Calibration lines are worked out from chronoamperometries within the concentration range of interest (from 0.5 to 2.0 mM for lactate and from 0.5 to 4.0 mM for glucose, see Fig. 9). Both for lactate and glucose, substrates were dissolved in Milli-Q and added into the PBS solution every 120 s, to allow the system to reach the steady-state. The response time of the system after each addition is around 30 s (data not shown). The calibration curve in the case of lactate detection shows a sensitivity of 40.1 μA $\mathrm{mM}^{-1}\mathrm{cm}^{-2}$, while in the case of glucose detection the sensitivity is of 27.7 μA $\mathrm{mM}^{-1}\mathrm{cm}^{-2}$. The detection limit for lactate biosensor is 28 μM, while it is 73 μM for glucose, with a S/N = 3 (see also Fig. 10). It is possible to compare the obtained sensitivities and detection limit with what was found in literature for similar nanostructured biosensors. Table I lists previous works regarding lactate detection. Note that the obtained sensitivity is twice the highest value reported in literature, as another indication that nanomaterials, such as carbon nanotubes, functionalized with oxidases are greatest substrate for biosensors.

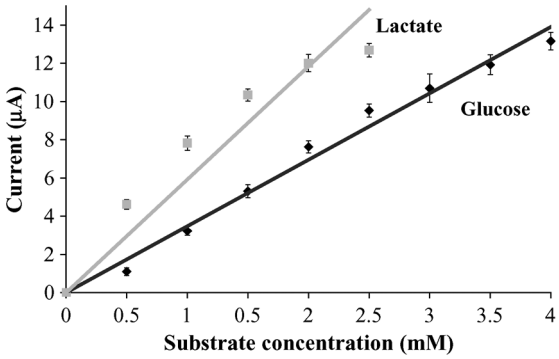

Fig. 9. Calibration lines for lactate and glucose in the concentration range of interest.

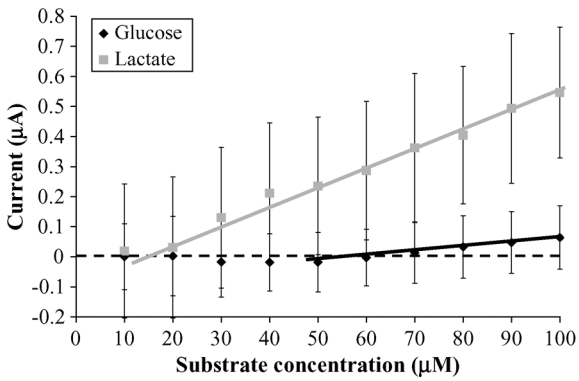

Fig. 10. Detection limit for lactate and glucose biosensors.

TABLE I
PERFORMANCES OF NANOSTRUCTURED-BASED BIOSENSORS FOR LACTATE DETECTION

Method for lactate detection	Sensitivity (μA mM^{-1} cm^{-2})	Linear range (mM)
LDH - MWCNT - CS [21]	8.3	0.005 - 1.2
MWCNT - PVI - CS [22]	19.7	0.005 - 1
MWCNT - sol-gel [23]	2.097	0.3 - 2
Present work	40.1	0.028 - 2

TABLE II
PERFORMANCES OF NANOSTRUCTURED-BASED BIOSENSORS FOR GLUCOSE DETECTION

Method for glucose detection	Sensitivity (μA mM^{-1} cm^{-2})	Linear range (mM)
MWCNT - Nafion® [24]	171.2	0.005 - 0.5
MWCNT - PtNP - CS/MTOS [25]	69.9	0.012 - 6
MWCNT - Nafion® [26]	4.7	0.025 - 2
Present work	27.7	0.073 - 4

Moreover, it is possible to assert that a further structuration of the electrode with polymers, as in the case of Cui *et al.* [22], does not exhibit better performance in terms of detection.

Regarding glucose detection, Table II shows a comparison among the sensitivities found in literature and in the present research. Rahman *et al.* [24] obtained the best sensitivity among the cited examples, but for a narrower detection range (from 1 to 500 μM), not useful for the purpose of the present work. Kang *et al.* [25] obtained a higher value of sensitivity, but they employed a sol-gel matrix combined with carbon nanotubes and nanoparticles, which can enhance the efficiency, despite of the cost and complexity of the sensor.

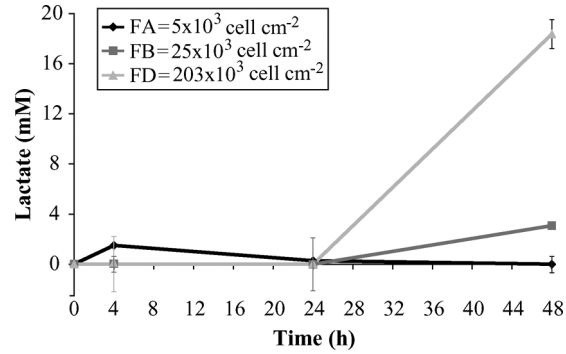

Fig. 11. Lactate production in cell line SN56 after 4, 24, and 48 h.

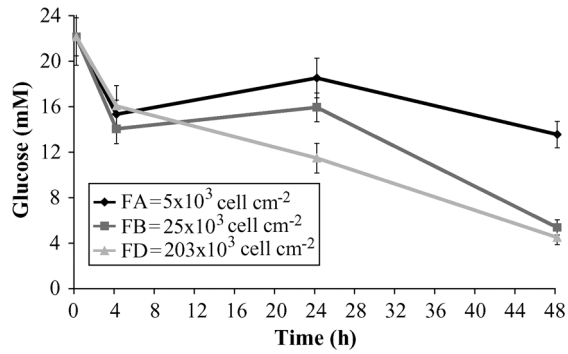

Fig. 12. Glucose uptake in cell line SN56 after 4, 24, and 48 h.

These results also validate the phenomenon reported from Carrara *et al.* [6] regarding the inverse linear correlation between sensitivity and detection limit when using nanomaterials for biosensing. Comparing the obtained values in this work with nonnanostructured biosensors [27], [28], an improvement of performance with respect to the case of mediated electron-transfer is demonstrated. The advantages brought from nanomaterials are really evident, especially in ranges of concentration which are of interest for a large pool of applications, and they make carbon nanotubes the key-element for the future of nanobiosensing.

D. Detection of Metabolites in Cell Cultures

As mentioned above, DMEM is diluted with PBS in a rate of 1 to 10, to be in the linear range of biosensors. Measurements are collected at the time of the seeding, and 4, 24, and 48 h later. The same diluted medium is used for lactate and glucose detection: the probe enzyme onto the electrode is different, but no cross talk or interferences are noticed from the other substrate during measurements. The behavior of lactate production and glucose uptake is depicted in Figs. 11 and 12 along 48 h of cell culture for different densities of cells.

Note that lactate production and glucose uptake are different for various cell densities. It means that with the developed biosensors is possible to recognize different cell densities and behaviors are clearly distinguishable. Another important point is that lactate production and glucose uptake shapes are quite symmetric. The lack of feeding, i.e., glucose, induces the production of lactate, which is a mark of cell suffering. Consequently, the symmetry of shapes is a further confirmation of this phenomena. On the other hand, in the lowest cell density the developed biosensor recorded an uptake of glucose in

the first 4 h, but afterwards the cells have enough feeding to survive more than 48 h without changing the medium. It is also confirmed in two ways by the fact that lactate production is almost zero. On the contrary, for the highest cell density, glucose uptake increases along time, while lactate production reached the maximum after 48 h. Cells are still living, but the change of medium is required. From this perspective, the developed biosensors can be an useful instrument to investigate mechanisms occurring during cell proliferation, while simultaneously provide information to the operators about cell culture conditions and right timing for further cell feeding.

Moreover, observation of changes in the cell culture leads to develop skillful technologies for unknown cell systems. Biosensors developed in this study were tested and validated in a standard cell line, but such emerging devices can be applied to more complex cell lines, like stem cells, where there is a lack of knowledge about the mechanisms that unfold when cells switch from the stem state to the differentiate state.

It is necessary to improve the developed biosensors in order to make their results independent from system variables, like pH, temperature, etc. For this reason, next work will be focused on enhancing accuracy of our sensors, in order to reduce variability among measurements.

IV. Conclusion

This work presents the development of amperometric enzyme-based biosensors nanostructured by using multiwalled carbon nanotubes for metabolite detection in cell culture medium. In particular, biosensors are functionalized for the detection of lactate and glucose by means of oxidases. Results from simulations have demonstrated the role of carbon nanotubes in enhancing biosensing. Monte Carlo simulations are used to model the distribution of CNTs onto the electrode surface. Sidewall current and tip current are computed from the equation developed in the present research, based on the Fowler–Nordheim equation. Results show that there is an almost linear inverse relationship between the logarithm of the sidewall current and the angle formed by CNTs with the substrate, while the tip current follows a quasi-quadratic shape. From the point-of-view of simulation, tip emissions of randomly dispersed carbon nanotubes are not negligible even if the large majority of CNTs presents angles below 10° with respect to the substrate.

Electrochemical measurements are performed for both metabolites by means of chronoamperometries. Sensitivity of 40.1 μA $\mathrm{mM}^{-1}\mathrm{cm}^{-2}$ is reached in the case of lactate, while a value of 27.7 μA $\mathrm{mM}^{-1}\mathrm{cm}^{-2}$ is obtained in the case of glucose detection. Detection limit is also investigated: a value of 28 μM is obtained for lactate, while the detection limit for glucose is 73 μM. From a comparison with nanostructured biosensors by using carbon nanotubes in literature, the presented sensitivities are the highest value obtained among the previous researches in the case of lactate, while it is in the same order of magnitude of what was previously found in the case of glucose. These results are a further confirmation of how carbon nanotubes can improve the performance of biosensors. Once calibration is performed, detection of both these metabolites is performed in cell medium. Cells from SN56 line are seeded in three different densities and allow to proliferate for 48 h. Then, a sample of the medium is collected at 0, 4, 24, and 48 h. Measurements are exploited with nanostructured electrodes functionalized with one of the two oxidases. Medium are diluted 1:10 in PBS to evaluate the concentration. Two behaviors are obtained, one for lactate production and one for glucose consumption. Lactate concentration increases along time, more in the case of higher cell density. Consequently, glucose level decreases in time, with a symmetric behavior, as assumed at the beginning. Such results illustrate the possibility to monitor metabolites in cell cultures by means of amperometric biosensors, enabling to develop fully integrated Petri dishes able to detect metabolites, targeting on automated system. On the other hand, the developed biosensors can add useful information to the knowledge of cell line, such embryonic or mesenchymal stem cells, which are not completely well-known.

Future work will focus on microfabrication of nanostructured electrodes and their functionalization with other oxidases, to detect other metabolite molecules.

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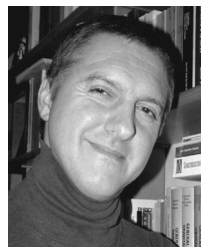

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