Correlation between cell growth rate and glucose consumption determined by electrochemical monitoring

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1. Introduction

Glucose consumption is one of the key indicators of metabolic activities of cells so that it can be tightly correlated to the cell growth rate or the cell population dynamics. Previously, different techniques including electrochemistry, infrared adsorption spectroscopy, Raman spectroscopy, fluorescence, impedance spectroscopy, etc., have been used for the glucose concentration monitoring [1–9]. Among them, electrochemical methods have shown clear advantages such as high sensitivity and high selectivity by proper choice of detection potential and/or electrode material [10–15]. Moreover, the electrochemical sensing elements can be miniaturized for portable devices and they are now routinely used for blood and food sample analyses and bioreactor control. Finally, the electrochemical methods are also useful for analysing many other biological and biochemical factors [16–21] or multiple analytes in real-time [22–25].

The electrochemical methods are based on rather complex reactions at the electrode surfaces which can be changed rapidly due to adsorption or desorption of reagents, surface catalyzing, diffusion and migration as well as convection of species. Accordingly, a large amount of investigations have been developed by using different detection methods (amperometry, cyclic voltammetry (CV), impedance techniques and chrono-amperometry, etc.), different electrode geometry, different material types (Pt, Au, etc.), and different surface coating techniques (self-assembled monolayer, mediators, enzyme, etc.). In the case of high precision glucose detection, both glucose oxidase (GOx) and electrocatalytic molecules based enzyme electrodes are now widely used. With GOx coated electrodes, the detection relies on the quantity of H\textsubscript{2}O\textsubscript{2} generated by the GOx-catalyzed reaction when oxygen is the electron mediator between GOx and electrode surface. Since the detection of H\textsubscript{2}O\textsubscript{2} requires a relatively high oxidation potential, it may cause severe interference from readily oxidizable species presented in physiological samples. In addition, Prussian-Blue [26,27] can be added to reduce the applied potentials (ca. 0.0 V versus Ag/AgCl). Moreover, when electrocatalytic molecules such as benzoquinone, ferric hexacyanoferrate, are used as mediators, the over-potential can be reduced which is favorable for fast electron transfer kinetics [28–30]. Consequently, the measurements will be independent of the oxygen concentration and the reaction interference between coexisting electroactive species can be minimized.

The electrochemical monitoring of glucose consumption is relevant for cell biology studies because of its wide detection range, high sensitivity and easy implementation. In general, the cell...
growth rates are determined by direct methods such as hemacytometer cell counting, whereas indirect methods based on the measurement of nutrient consumption offer complementary information on the cell culture processes. The combination of the two allows a better understanding of metabolic activities of cells so that the cell culture processes can be controlled more accurately. In this work, we are particularly interested in correlation between population dynamics and glucose consumption of cells at different cell growth stages. We firstly describe the fabrication method of a mediator-based glucose micro-sensor and show its linear electrochemical response in the range of 0.3–60 mM with a monitoring sensitivity of $0.383 \pm 0.025 \mu A/mM$ ($n=5$) and a low detection limit of 80 $\mu M$. Then, we compare the results obtained by counting cell number and glucose consumption measurements on cell population increases over a period of 7 days and deduce the glucose consumption of single cell. We believe that our method is robust and versatile for the determination cell metabolic activities on chip.

2. Materials and methods

2.1. Materials

Glucose oxidase (GOx, 100,000 units/g, EC 1.1.3.4, Type VII, from Aspergillus Niger), $\alpha$-glucose, bovine serum albumin (BSA), glutaraldehyde (GA, 25% aqueous solution), sodium chloride (NaCl), sodium phosphate monobasic (NaH$_2$PO$_4$), sodium phosphate dibasic (Na$_2$HPO$_4$), sulfuric acid (H$_2$SO$_4$, 98%), hydrogen peroxide (H$_2$O$_2$), potassium iodide (KI), iodine (I$_2$), n-hexane, trypsin-blue, penicillin/streptomycin (P/S) and trypsin–EDTA were purchased from Sigma–Aldrich and used without further purification. p-Benzquinone (PBQ) was purchased from Fluka and recrystallized from n-hexane. 2-Mercaptoethanol (ME) was purchased from Acros Organics. AZ5214E photoresist and AZ726MIF developer were obtained from AZ Electronic Materials. Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), l-glutamine and fungizone were purchased from Gibco. Base polymer and crosslinker (methylhydrosiloxane dimethylsiloxane copolymers) were purchased from GE RTV 615 kit. Glasses, 24-well plates and hemacytometer were purchased from Fisher Scientific.

All solutions were prepared with deionized water (DI, 18.2 M$\Omega$ cm$^{-1}$). 0.1 M phosphate buffer saline (PBS) with two pH values (pH 7.3 and pH 5.6) was prepared by mixing 0.05 M NaCl with 0.05 M NaH$_2$PO$_4$/Na$_2$HPO$_4$. 0.1 M stock glucose solution was prepared in 0.1 M PBS (pH 7.3) and stored at 4 °C to allow an overnight equilibration before use.

2.2. Device design and fabrication

The operation process and electrochemical micro-sensor are schematically shown in Fig. 1. NIH 3T3 cells were cultured in 24-well plate and 50 $\mu$L of culture medium was taken out every 24 h (A). After culture medium was mixed with 50 $\mu$L of 10 mM PBQ, the glucose concentrations in culture media were measured in the electrochemical micro-sensor (B). We have fabricated different types of electrochemical micro-sensors by using conventional photolithography, lift-off and soft lithography techniques, including open and close chambers as well as microfluidic circuits made of poly(dimethylsiloxane) (PDMS). For the sake of simplicity, only sensor device with open PDMS chamber were used in this work with 0.24 mm$^2$ active area on working electrode.

The glass substrates were cleaned in the piranha solution (H$_2$SO$_4$:$H_2$O$_2$ = 3:1). Firstly, 8 nm titanium (Ti) adhesion layer and 30 nm gold (Au) layer were deposited on the clear glass substrates by an electron beam evaporator (Edward Auto 500). Then, a thin layer of AZ5214E photoresist was spin-coated at 3000 rpm for 30 s. After soft baking for 1 min 45 s at 125 °C on the hotplate, it was exposed to ultraviolet light (Hamamatsu Lighting Cure LC5) at exposure energy of 75 mJ/cm$^2$ for 6 s and developed in AZ726MIF developer. Afterward, the open area of Ti/Au film was removed by...
wet etching of Au in a KI/I2 solution (KI:I2:H2O = 115 g:65 g:100 ml) and reactive ion etch of Ti in a plasma chamber (Nextral N100) of SF6 (30 mTorr, 10.0 sccm and 50 W).

To reduce the interfering signals between oxygen species in particular, a self-assembled monolayer of ME was formed on the fabricated working electrode by liquid phase deposition in a ME aqueous solution (20 mM) over night at room temperature. Then, the ME modified electrodes were rinsed with DI water and dried with nitrogen stream. A 5 μl mixture of 15 mg/ml GOx, 15 mg/ml BSA and 0.1 M PBS (pH 5.6) was finally dropped onto the surface of working electrode. After evaporation, a yellow film appeared on the surface of the electrode. For GOx cross-linking, about 1 ml of 25 wt.% GA was dropped on a glass slide and the sample with electrodes was flipped 1 cm above the glass slide for evaporation. To ensure a saturated GA atmosphere, both glass slide and sample were placed in a sealed device. After 1 h exposed to GA, the electrodes were dried at room temperature for 15 min and then immersed into 0.1 M PBS (pH 7.3) for 30 min for the equilibration of system. Finally, the fabricated glucose micro-sensors were immersed into 0.1 M PBS solution and stored at 4 °C.

PDMS chambers were prepared by mixing PDMS base polymer and cross-linker (methylhydrosiloxane dimethylsiloxane copolymers) at the ratio of 10:1 with a mixer (UNIX Cyclone MM-103S). After removing all bubbles, the mixture was poured onto the silicon wafer and cured thermally for 2 h at 80 °C. Then, the PDMS was peeled off and drilled with punch.

### Table 1

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Mediator</th>
<th>Immobilization method of GOx</th>
<th>Sensitivity (μA mM⁻¹ mm⁻²)</th>
<th>Linear range (mM)</th>
<th>Limit of detection (mM)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Pt</td>
<td>O₂</td>
<td>Entrapment</td>
<td>0.008</td>
<td>1–20</td>
<td>0.8</td>
<td>[34]</td>
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<tr>
<td>Pt</td>
<td>O₂</td>
<td>Entrapment</td>
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<td>0.1–35</td>
<td>–</td>
<td>[35]</td>
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<tr>
<td>Pt</td>
<td>O₂</td>
<td>Crosslinking</td>
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<td>0.1–1</td>
<td>–</td>
<td>[36]</td>
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<td>K₃Fe(CN)₆</td>
<td>Adsorption</td>
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<td>0–44.5</td>
<td>–</td>
<td>[29]</td>
</tr>
<tr>
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<td>2–10</td>
<td>0.12</td>
<td>[38]</td>
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<tr>
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<td>Adsorption</td>
<td>0.049</td>
<td>0–100</td>
<td>0.21</td>
<td>[37]</td>
</tr>
<tr>
<td>Au</td>
<td>PBQ</td>
<td>Crosslinking</td>
<td>1.61</td>
<td>0.3–60</td>
<td>0.08</td>
<td>This work</td>
</tr>
</tbody>
</table>

To illustrate the glucose reaction on the micro-sensor electrode, a schematic representation is included in Fig. 3, showing enzyme reactions and electrochemical reactions at the working electrode. Cyclic voltammograms with varying glucose concentrations from 0.3 mM to 60 mM are displayed in Fig. 4, along with the corresponding calibration graph deduced from these measurements in (B).
2.3. Cell culture and growth rate monitoring

NIH 3T3 cells were cultured at 37°C in 5% CO₂ incubator (Sanyo, Japan) with a culture medium of 88% DMEM (containing 4.5 g/l glucose), 10% FBS, 1% L-glutamine, 1% P/S, 0.01% fungizone until confluence. After dissociation in a 0.25% trypsin–EDTA solution and centrifugation, cells were resuspended at a density of 1 × 10⁶ cells/cm² and seeded into 24-well plate with 1 ml culture medium in each well. The media were renewed everyday. Three wells were assayed every 24 h. Cell numbers were determined by the trypan blue using a hemacytometer. The photos of NIH 3T3 were imaged using microscope (Zeiss Axiovert 200) with CCD camera.

Glucose solutions for micro-sensor calibrations and glucose concentrations in the culture media (diluted by 10 mM PBQ in 0.1 M PBS (pH 7.3) with the same volume) were measured with the electrochemical micro-sensor in the presence of 5 mM PBQ as a mediator at room temperature. CVs were proceeded in the potential range of −0.4 V to 0.6 V (vs. Au pseudo-reference electrode) with scan rate of 0.1 V/s (CH Instrument 660C).

3. Results and discussion

3.1. Calibration of glucose micro-sensor

It is well known that the working electrodes modified with self-assembled monolayer of appropriate molecules can be used to reduce both non-Faradaic background currents and interfering Faradaic currents [31,32]. In our case, the working electrodes were treated in ME solution for the reduction of the background current and the inhibition of the electrochemical reactivity of O₂ due to its potential barrier. Whereas PBQ is an effective mediator, its redox reaction is much less affected by the presence of the ME monolayers [33]. It allows shuttling charges back and forth for a more efficient electric communication between the immobilized GOx and the electrode. Fig. 2 shows the cyclic voltammograms acquired with (black line) and without (red line) the addition of 5 mM glucose. Clearly, the presence of glucose leads to a much increased oxidative current and a decreased reduction current. Such a difference can be understood by considering the fact that the electro-generated hydroquinone was consumed in the reaction that regenerates PBQ, i.e.,

\[
\text{Glucose} + \text{GOx(FAD)} \rightarrow \text{Gluconolactone} + \text{GOx(FADH}_2\text{)} \quad (1)
\]

\[
\text{GOx(FADH}_2\text{)} + \text{PBQ} \rightarrow \text{GOx(FAD)} + \text{Hydroquinone} + 2\text{H}^+ \quad (2)
\]

for the enzymatic reaction (FAD: Flavin Adenine Dinucleotide) and

\[
\text{Hydroquinone} \rightarrow \text{PBQ} + 2\text{e}^- \quad (3)
\]

for the electrochemical reaction at working electrode, respectively. Therefore, the glucose detection of the present work is based on the electrochemical oxidation of hydroquinone generated by the GOx catalyzed oxidation of glucose at anodic potential. These reactions are schematically illustrated in Fig. 3. Glucose is oxidized to gluconolactone. Simultaneously, PBQ, as the electronic mediator,
receives electrons to become hydroquinone. The redox reaction is catalyzed by GOx. Ultimately, the current is detected by the Au electrode and hydroquinone changed to PBQ.

Fig. 4(A) displays a set of cyclic voltammograms for different glucose concentrations ranging from 0.3 mM to 60 mM. This wide concentration range could be adapted for the study of glucose consumption during the whole cell culture processes. The commonly used cell culture media include DMEM, roswell park memorial institute 1640 (RPMI 1640), DMEM/F12 and minimum essential medium (MEM). The concentration levels of glucose in these media are 25 mM, 11.1 mM, 17.5 mM and 5.56 mM, respectively. Obviously, the normal levels of glucose in culture media are well within our linear detection range. We then obtained a calibration curve, as shown in Fig. 4(B), with a linear regression coefficient $r = 0.9978$. Accordingly, we deduced a detection sensitivity of $1.61 \pm 0.10 \mu$A mM$^{-1}$ mm$^{-2}$ ($n = 5$) and a detection limit of about $80 \mu$M. Table 1 shows the comparison of our results with the previous reports on electrochemical monitoring of glucose, including electrode material, mediator, immobilization method of enzyme, sensitivity, linear range and limit of detection [29,34–38]. Clearly, our method has the advantage of a wider linear range, a higher sensitivity and a lower detection limit, indicating a better suitability for the monitoring of glucose consumption in cell culture systems.

We also studied the long-term stability of the fabricated micro-sensors by measuring the peak current of 1 mM glucose containing 5 mM PBQ everyday over 30 days, the sensor being stored in 0.1 M PBS (pH 7.3) at 4°C after each measurement. As shown in Fig. 5, the monitoring current slightly decreased over time but the sensitivity of the sensor still remained about 94% of the initial value after 30 days, indicating this micro-sensor has high stability.

Finally, we checked the suitability of our sensors for the glucose consumption monitoring by using diluted culture medium (containing 12.5 mM glucose and 5 mM PBQ). No significant difference could be observed in CV response curves comparing to the case of pure glucose solution (data not shown). Apparently, the presence of other cell factors in culture medium does not disturb the detection of glucose concentration.

### 3.2. Glucose consumption monitoring of NIH 3T3 cells

The cultured NIH 3T3 cell samples were taken daily for both cell number counting and glucose consumption analyses. Fig. 6(A) shows the attachment and the growth behaviors of NIH 3T3 cells between day 0 (1 h after cell seeding) and day 7. Clearly, after 1 h of cell seeding, most cells adhered but only a few of them began to spread. From day 1, the cell number increases quickly until its confluence stage on day 4. Subsequently, the cell growth slowed down with a little change in cell numbers. Fig. 6(B) shows the growth curve of NIH 3T3 cells cultured in 24-well plate, demonstrating a perfect logistic population growth: exponential growth phase (days 1–4) and stagnating phase (days 4–7). The observed cell growth curve could then be fitted by using well-known logistic growth function in Bartlett form, i.e.

$$N = \frac{\tilde{N}}{1 + e^{-r(t-t_0)}}$$

where $N$ is the cell density, $\tilde{N}$ is the saturation density and $r$ is a rate parameter. When $t = t_0$, $N = \tilde{N}/2$. The best fitting curve was obtained with the following parameters $\tilde{N} = 0.54$, $t_0 = 3.2$ and $r = 1.4$.

At the same time, the daily glucose consumption of NIH 3T3 cells in culture medium has been determined by measuring CV with our micro-sensor. The results obtained over 7 days are reported in Fig. 7(A), indicating the suitability of our sensors for the cell culture monitoring in terms of both detection range and detection sensitivity. Referring the calibration curve Fig. 4(B), the daily glucose consumption could be deduced (Fig. 7(B)). At the beginning of the culture, the daily glucose consumption increased fast, indicating an exponential growth phase. Then, the increase of the daily glucose consumption was slowed when cell growth entered a stagnating phase. In Fig. 7(B), a fitting curve was added by using the same logistic growth function in Bartlett form (Eq. (4)) but different fitting parameters ($\tilde{N} = 5.6$, $t_0 = 2.8$ and $r = 1.2$, $\tilde{N}$ being now the saturated glucose consumption). Obviously, the increase of the glucose consumption does not follow exactly the cell behavior, but roughly speaking they have a quite similar behavior with limited differences for the most important two parameters $t_0$ and $r$. However, the measurable time delay between the point at half of the maximum consumption ($t_0 = 2.8$) and half of the maximum cell density ($t_0 = 3.2$) is meaningful which reflects a phase shift between the glucose consumption and the cell growth regimes.

In general, the glucose consumption of a cell population increases with the increase of the cell number. But this does not mean that the consumption of individual cells of the population also increases. In fact, when the cell density becomes sufficiently high, the available habitat becomes small so that the increase of the population size will slow down and the cell uptaking rate will be reduced (Fig. 8). When the cell population increase rapidly (till day 3), a large portion of glucose was consumed for cell proliferation. From day 4, the cell population came into stagnating phase so that...
the glucose was mainly consumed for cell survival. Accordingly, the glucose consumption per cell remains almost the same as shown by the dotted line (c = 10.2).

4. Conclusions

We fabricated a mediator-based glucose micro-sensor for electrochemical analyses of cell growth rate and metabolic activities. The device showed a linear response for the glucose concentrations in the range of 0.3–60 mM, a higher sensitivity and a lower detection limit comparing to the previously studied systems. It was then applied for the determination of daily glucose consumption of NIH 3T3 cells cultured in 24-well plate during 7 days. The results obtained in this way could be compared with cell population growth curve, showing a close and logical correlation between the cell growth and glucose consumption behaviors. Although the daily glucose consumption of cells showed a similar increase in cell numbers, the increase of glucose consumption should take place prior of cell number increase. Moreover, we deduced a linear decrease of the glucose consumption per cell during the active growing phase but a constant consumption when the culture entered into the stagnating phase. To conclude this work, we have significantly improved the performance of electrochemical micro-sensors which are suited for real-time monitoring of glucose consumption of cells as well as for the systematic study of metabolic activities of cells and bacteria under different stresses or perturbations. Finally, we would like to mention that our method is fully compatible to the microfluidics and ANR-06-PCVI-0006-03 157 (DynaCell 3D). F.Z. and L.W. are grateful to Prof. C. Amatore of Ecole Normale Supérieure and ANR-156-06-BLAN-0029-02 (mPhyChemBio) and for use-real-time monitoring and control of glucose and lactate concentrations in a mammalian cell perfusion reactor, Biotechnol. Bioeng. 53 (1997) 372–378.


Biographies

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