Microelectrode array biochip: a novel tool for in vitro drug screening based on the detection of drug effect on dopamine release from PC12 cells

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Abstract

This material contains the experimental details of solution preparation, HPLC analysis of the K\textsuperscript{+}-stimulated exocytosis from PC12 cells, and the steps involved in the fabrication of microelectrode array (MEA) biochip. In addition, this material includes the results of the calibration curve of DA concentration vs. DA oxidation current, and
the results in evaluation of drug toxicity by using trypan blue staining and hemocytometer.

**Solution preparation**

The preparations of a balanced salt solution (BSS), a salt solution containing either 40 mM KCl or 120 mM KCl, a phosphate buffer solution (PBS), and a catecholamine stabilizing mixture are detailed as follows:

The BSS solution was composed of 138 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose, and 10 mM HEPES. A balanced salt solution containing either 40 mM KCl or 120 mM KCl was composed of 40 mM KCl, 100.5 mM NaCl, or 120 mM KCl, 20.5 mM NaCl, respectively, with other components same as those in BSS. The PBS contained 0.14 M NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄, and 1.76 mM KH₂PO₄. All these solutions were at the pH of 7.4. The catecholamine stabilizing mixture contained 0.1 M HAc, 0.1% Na₂EDTA, and 0.12% glutathione (pH=4.9). Deionized water obtained from a Millipore water system was used throughout the experiment.

**HPLC analysis of the K⁺-stimulated exocytosis from PC12 cells**

For HPLC analysis, a K⁺-stimulated exocytosis sample from PC12 cells in 2 plates of Petri dishes (100 mm × 20 mm style, Corning Incorporated, USA) with 80-90% of the cell surface coverage was collected. The sample collection procedures were described as followed. After cultured in Ham’s F12 medium, PC12 cells in each Petri
dish were washed 3 times with blank BSS, followed by incubation in 2.5 ml of 40 mM KCl solution for 3 min. The K⁺-stimulated exocytotic solution was collected and the cells were washed and incubated in another 2.5 ml of 40 mM KCl solution for 3 min. After collecting the exocytotic solution, the procedures of washing, KCl stimulating, and solution collecting were repeated twice more. All the collected solution, totally amounted to 20 ml, was the K⁺-stimulated exocytosis sample from PC12 cells. The whole sample collection procedures were performed at room temperature (~25 °C). A 20 ml of 40 mM KCl solution was used as the negative control sample. Immediately after collection, the exocytosis sample and the negative control sample were each mixed with 5 ml of the catecholamine stabilizing mixture and freeze dried. The freeze dried samples were dissolved in 1.5 ml of water and filtered. A 100 µl of a filtered sample was injected into an embedded reversed phase column (Vydac, 218TP54, C-18, 5 µm, 4.6 mm i.d. × 250 mm) in a HPLC/UV (AKTA Puriﬁer, Amersham Pharmacia Biotech., Sweden) detection system. The mobile phase A was water, and B was a buffer containing 90 mM NaH₂PO₄, 50 mM citric acid, 0.05 mM Na₂EDTA, 1.7 mM 1-octanesulfonic acid, and 7.5% (v/v) acetonitrile (pH = 3.1). Gradient from 0% to 100% B was eluted in 85.1 min (from elution time of 8.9 min to 94 min) at a flow rate of 1.0 ml min⁻¹.

External standard samples containing DA, L-dopa, NE, serotonin, epinephrine, HVA, and DOPAC were injected into the column and eluted at the same condition.

Fabrication of MEA biochip
MEA biochips were fabricated from two 6” silicon wafers of about 670 µm thick by using standard semiconductor processes. The size of the integrated biochip is 2 cm × 2 cm with a chamber volume of 1.4 cm × 1.4 cm × 0.7 cm (width × length × height). The steps involved in the fabrication are briefly outlined as followed. Firstly, the silicon base plate was passivated with a layer of thermally grown SiO₂, followed by evaporation of a Ti layer on the silicon base plate to increase the adhesion of the subsequent evaporated gold layer to the silicon substrate. The gold layer and the underlying Ti layer were patterned using standard photoresist and wet etching, forming a 5 × 5 array of planar Au disk microelectrodes (10, 30, 60, or 90 µm in diameter) on the 1 mm center of the silicon base plate. The distance between neighbor electrodes is 250 µm. After removal of the photoresist, an insulating layer with a SiO₂-Si₃N₄-SiO₂ sandwich structure was evaporated to the whole surface of the substrate by plasma enhanced chemical vapor deposition (PECVD) process. Subsequently, the SiO₂-Si₃N₄-SiO₂ insulating layer was patterned using standard photoresist and plasma dry etching to create openings above the microelectrode recording sites and above the connector pads. Thereafter, both sides of the silicon cover plate were passivated by a layer of thermally grown SiO₂ and a followed layer of Si₃N₄ evaporated by PECVD process. The SiO₂-Si₃N₄ insulating layers were patterned using standard photoresist and plasma dry etching, exposing the silicon surface at the areas of chip chamber and connector pads. After removal of the photoresist, a KOH etching was performed to etch the exposed silicon through the cover plate. The cover plate was then aligned with the base plate by an aligner and bound to the base plate with polydimethylsiloxane at room temperature. Finally, the bonded
wafer was diced and wire bound with a printing circuit board to form a biochip.

Calibration curve at collagen coated biochip microelectrodes

Figure S-1 illustrates a calibration curve of DA concentration vs. DA oxidation current, derived from an amperometric curve in responding to the injections of concentrated DA standards at a collagen coated microelectrode (30 µm in diameter), at the applied potential of +0.3 V vs. a biochip microelectrode. The amperometric detection limit (low limit) of DA at this collagen coated microelectrode was 0.14 µM, and the detection sensitivity was 0.082 nA µM⁻¹. The calibration condition performed did not reach the DA detection high limit, and the linearity was up to over 7.3 µM.

![Calibration Curve](image)

**Figure S-1.** Calibration curve of DA concentration versus DA oxidation current derived.
from an amperometric curve in responding to injections of concentrated DA standards at a biochip microelectrode of 30 µm in diameter (+0.3 V vs. biochip microelectrode)

Evaluation of drug toxicity by counting the percentage of viable cells

The toxicity of reserpine and nomifensine on the viability of PC12 cells were evaluated in one way by counting the percentage of viable cells. For measurement, after incubation in drug solutions, PC12 cells cultured in a 6-well cell culture plate incubated were detached from the culture plate by trypsinizing and then centrifuged and washed twice with PBS in a microcentrifuge tube. After the supernatant was pipetted out, 80-120 µl of PBS was added into the microcentrifuge tube and the tube was stirred until the cells were well suspended. The cell suspension was then stained with trypan blue (cell suspension: trypan blue sol = 1:1) and the number of viable and dead cells was counted by using a hemocytometer. The percentage of viable cells after incubation with drugs was compared to that without drugs (Table S-1).

Table S-1. The effect of reserpine and nomifensine on percentage of viable cells, counted by using trypan blue staining and hemocytometer (n=2)

<table>
<thead>
<tr>
<th>Test</th>
<th>Reserpine (1 µM for 30 min)</th>
<th>Nomifensine (80 µM for 20 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Blank</td>
<td>Blank</td>
</tr>
<tr>
<td>Viable cells (%)</td>
<td>90.6±1.3</td>
<td>81.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>89.7 ± 0.6</td>
<td>86.4 ± 0.3</td>
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</tbody>
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Table S-1 shows that incubating PC12 cells in 80 µM nomifensine for 20 min or in 1 µM reserpine for 30 min was nontoxic to PC12 cells.