Supporting information

Simultaneous Quantification of Vesicle Size and Catecholamine Content by Resistive Pulses in Nanopores and Vesicle Impact Electrochemical Cytometry

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Chemicals and Solutions

All chemicals were of analytical grade and obtained from Sigma-Aldrich, Sweden, unless otherwise stated. The chemicals were used as received without other treatments. All solutions were prepared using 18 MΩcm-1 water from Purelab Classic purification system (ELGA, Sweden).

Lock's buffer contained 1.54 M NaCl, 56 mM KCl, 36 mM NaHCO₃, 56 mM glucose and 50 mM HEPES with pH 7.4 was diluted 10 times with distilled water.

Homogenizing buffer (HO) contained 0.3 M sucrose, 1 mM EDTA, 1 mM MgSO₄, 10 mM HEPES, 10 mM KCl and Complete Protease Inhibitor obtained from Roche was used for tissue homogenization and following amperometric recording. The solution should be adjusted to pH 7.4 and its osmolality ~320 mOsm/kg.

Bath solution (LO) consisted of 3 mM D-glucose, 20 mM HEPES, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.4 mM CaCl₂, 58 mM NaCl, 60 mM KCl. The osmolality was ~150 mOsm/kg and 1% v/v DMSO was added to improve the vesicles breaking efficiency.

Chromaffin Vesicle Preparation

Chromaffin vesicles were isolated according to our previously reported procedure which was developed by the Borges laboratory (Universidad de La Laguna, Tenerife, Spain). Bovine adrenal glands were obtained from a local slaughterhouse.

Briefly, after cutting off the outer fat and cleaning internal blood of adrenal glands with Lock’s buffer, we separated the medulla with a surgical scissor and mechanical homogenized in ice-cold homogenizing buffer. Then the dispersion was centrifuged at 1 000 x g for 10 min at 4°C, the supernatant collected, and centrifuged again at 10 000 x g for 20 min at 4°C to obtain pellet chromaffin vesicles. The pellet was re-suspended in 1 mL homogenizing buffer and used as vesicle stock solution.

Carbon Fiber Microdisk Electrode Fabrication

The microdisk electrode for VIEC was fabricated as previously described. Briefly, a 33-μm diameter carbon fiber was aspirated into a borosilicate capillary (1.2 mm O.D., 0.69 mm I.D., Sutter Instrument Co., Novato, CA, U.S.A.). The capillaries were subsequently pulled with a vertical puller (Model PE-21, NARISHGE, Japan). The fiber was fixedated and the electrode was sealed by dipping the pulled tip in epoxy (Epoxy Technology, Billerica, MA, U.S.A.). The glued electrodes were cured in an oven at 100°C overnight and subsequently cut at the glass junction and beveled at 45° angle (EG-400, Narishige Inc., London, UK). Prior to use for VIEC, the electrode responses were tested by performing cyclic voltammetry (-0.2 to 0.8 V vs. Ag/AgCl, at 100 mV/s) in a solution of 100 μM dopamine in PBS (pH 7.4).

Nanopipette Fabrication

The nanopipette for RP recording was fabricated with a laser puller with filament (Model P-2000, Sutter Instruments Co., Novato, CA, U.S.A.) to pull borosilicate glass capillaries (1.0 mm O.D., 0.78 mm I.D., Sutter Instrument Co., Novato, CA, U.S.A.). The geometry of the nanopore was characterized by scanning electron microscopy (JEOL.
The radii varied from 250–500 nm, and half cone angle was 2.83° based on the mean value measured from 10 pipettes.

**RP-VIEC Recording Configuration**

The RP-VIEC recording was synchronously performed by two sets of amplifiers (Axopatch 200B, Molecular Devices, Sunnyvale, CA, U.S.A.). They shared the same reference electrode (Ag/AgCl). See the scheme in Figure 1A.

RP recording: 0.1 mL vesicle suspension was diluted into 1.0 mL homogenizing buffer (HO), filtered by 0.45 μm filter (Cellulose acetate membrane, VWR, Sweden) and injected into the nanopipette. Then the nanopipette was connected to a microinjector system (Femtojet, Eppendorf Co., Germany) with a holder, in which a platinum wire was placed. Then the holder was connected to an amplifier (Amplifier 1 in Figure 1, the work potential was set at +13 mV). The microinjector conducted the pressure which was set at 0.5–1 s, 50–100 hPa, without compensation pressure. The pressure value and time were adjusted based on the RP amount during each pushing stage. The nanopipette was placed at ~45° to the carbon fiber microdisk electrode surface plane in bath solution (LO), and the distance was ~10 μm. Based on a finite simulation via COMSOL multiphysics (data not shown), the time between the vesicle exiting the pore to impacting on the electrode is ~20 ms, which is significantly smaller than all the time differences between the chosen pairs of RP and VIEC signals.

VIEC recording: The carbon fiber microdisk electrode was placed in bath solution and connected to another amplifier (Amplifier 2 in Figure 1, the work potential was set at +700 mV).

The recorded RP and VIEC signals were filtered at 2 kHz using a 4-pole Bessel filter and digitized at 10 kHz using a Digidata model 1440A with Axoscope 10.3 software.

**Data Processing and Statistics**

All RP and VIEC data were analyzed by a series of home-built programs in MATLAB (MathWorks Inc., Natick, MA, U.S.A.). Due to the periodic pulse, the baseline of RP and VIEC trace both would go up and down in cycles.

RP signal: the conversion from RP signal to size of vesicle was proceeded based on the algorithm reported by Prof. Róbert E. Gyurcsányi.14 Briefly, the RP raw traces were normalized based on the nearby baseline and converted into percentage ratio (ΔI/I). Then RP signals were searched and counted only in the pushing stage in each cycle, and the detection limit was 5 times the standard deviation of the baseline noise in the pushing stage. Then, the normalized RP signals (ΔI/I) were converted into normalized resistance changes (ΔR*, see the Equations 4/5 in Ref [4]) and the radius ratio of vesicle to nanopore (Rves/Rnop, see the curve in Figure S4). After we measured the size of nanopore by SEM, the radius of vesicle was obtained. A discussion of the error rate and comparison of accuracy for the RP method to other methods, including SEM, NTA, and DLS, for nanoparticles is given in Ref 4. We used the same method here.

VIEC signal: after subtracting the baseline of a VIEC raw trace, the VIEC current spikes were searched and counted in each cycle, and the detection limit used was 6 times the standard deviation of the baseline noise in whole cycle. Then, we calculated the area of each spike which was the total charge transferred from the released catecholamine to the electrode. The molecule amount in moles was obtained by use of Faraday’s Law, in which the charge transfer number of electrons is 2.

All traces were visually inspected and false positives were removed.
Figure S1. The scatters plot showing the time when VIEC current spikes (n=340) appear after applying pressure, and the their histogram distribution and cumulative curve.

Figure S2. Examples showing the different combinations of RP signals and VIEC current spikes. (A) 1 RP vs. 1 spike; (B) 2 RPs vs. 1 spike; (C) 2 RPs vs. 0 spike; (D) 2 RPs vs. 2 spikes; (E) 4 RPs vs. 1 spike; (F) 4 RPs vs. 4 spikes. The signals are indicated by an asterix. Among those situations, the pair of RP signal and spike in (A) and (B) were chosen. *Some zones in VIEC trace are shown in flat curve, they result from the data process, and no spikes were found in those zones by manually checking the raw traces.

Discussion of Figure S2: Because these examples happened under different conditions related to the vesicle concentration and purity or outflow solution volume of which it is hard to be clear exactly, we adjusted the vesicle concentration during each experiment based on the frequency and number of spikes. Empirically, the situations for (C) and (D) are quite common (~30%, respectively); for situation (E) these are relatively rare (~20%) and usually this is related to the impurity of the vesicle solution; for situation (F) these are relatively common in the beginning but are minimized by diluting the vesicle solution leading to fewer events (~10%).
Figure S3. An example showing the raw traces of RP and VIEC recording as shown in Figure 2. The insets are magnifications of both signals. The pink zones indicate when the pressure is applied, and white zones indicate when it is stopped. The RP signal comes from the nanopore blocked by the passing particle which induces the current decrease. While the VIEC results from the electrooxidation of vesicle contents whose current signal is positive.

Figure S4. The numerical relationship between the ratio of the vesicle to nanopore radii and normalized resistance change ($\Delta R^*$), when the half cone angle is 2.83°.
**Figure S5.** The SEM image of typical nanopore used in RP recording.

**Figure S6.** Histogram of catecholamine concentration. Dotted line indicates the median.
Figure S7. The statistical distribution of vesicle content versus radius of each individual recorded chromaffin vesicle. The relationship between content concentration versus radius is shown in a scatter plot in part 1; the normalized frequency histograms of content are depicted in part 2 and vesicle radius in part 3. The insets in 2 and 3 compares the percentage column of DCV versus NDCV group. Part 1 and 2 share the Y-axis, and part 1 and 3 share the X-axis. The significant differences are evaluated via two-tailed Mann-Whitney Test.

Figure S8. The normalized frequency histograms (A) and percentage column of content amounts (B) of DCVs vs. NDCVs group. The significant differences were evaluated via a two-tailed Mann-Whitney Test.
Figure S9. The scheme depicts the maturation of exocytotic vesicles. The NDCVs, or called immature vesicles (IMVs), are formed from the trans-Golgi network (TGN) and experienced budding to sort out the unnecessary protein, in which step their sizes decrease. Then they become mature vesicles (MCVs) with condensation of lumen (so also called DCV) and acidification which strengthens the ability to transport catecholamine inwards.

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