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Quantitative Measurements of Transmitters in Vesicles One at a Time in Single Cell Cytoplasm with Nano-tip Electrodes**

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Abstract

Quantification of vesicular transmitter contents is important for studying the mechanisms of neurotransmission and malfunction in disease and yet it is incredibly difficult to measure the small contents of neurotransmitters in the attoliter volume of a single vesicle, especially in the cell environment. We introduce a novel method, intracellular vesicle electrochemical cytometry. A nano-tip conical carbon fiber microelectrode is used to electrochemically measure the total contents of electroactive neurotransmitters from individual nanoscale vesicles in single PC12 cells as these vesicles lyse on the electrode inside the living cell. The results demonstrate that only a fraction of quantal contents of neurotransmitter is released during exocytosis. These data support the intriguing hypothesis that the vesicle does not open all the way during the normal exocytosis process, resulting in incomplete expulsion of the vesicular contents.

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Keywords

nanoelectrodes; catecholamine; amperometry; vesicle; exocytosis

During neuronal transmission, vesicles are the major organelles involved in the storage and release of chemical messengers, for example, neurotransmitters; therefore, they have an important role in synaptic signaling.[1] In 2009, we developed a method (termed electrochemical cytometry), based on three major steps: electrophoretic separation of vesicles, lysis and electrochemical detection of expelled neurotransmitters by a normal cylindrical carbon fiber microelectrode in a microfluidic device at the end of the capillary. We demonstrated quantification of the total transmitter in single artificial vesicles in a high throughput manner.[2] With this method, we then successfully quantified the vesicular contents in individual vesicles isolated from pheochromocytoma (PC12) cells and mouse brain tissue.[3] Recently, Compton and coworkers have also investigated the electrochemistry of liposomes, in this case encapsulated with ascorbic acid as they impact electrodes.[4] We have now characterized the contents of mammalian vesicles isolated from adrenal chromaffin cells by single adsorption and rupture events at electrodes without separation.[5] This approach allows nanoscale mammalian vesicles to adsorb to carbon micro-disk electrodes and subsequently rupture and expel their contents (mainly catecholamines such as norepinephrine, epinephrine and dopamine) eliciting an oxidation current that can be used to quantify the catecholamine contents of the vesicles. These methods are effective to quantify the contents of individual isolated vesicles; however, it is an exciting prospect to measure the contents of vesicles directly in the intracellular environment where the vesicle isolation procedure is not a concern, but also direct comparison can be made to exocytotic release.

Flame-etched carbon fiber microelectrodes can be fabricated with a sharp tip and somewhat larger effective surface allowing insertion into the cell.[6] Also, they have increased sensitivity, kinetics, signal to noise ratios and a faster time response for many neurotransmitters compared to cylindrical carbon fiber microelectrodes and electrochemically etched carbon fiber microelectrodes.[6b] The nanoscale tips make these electrodes nearly ideal single cell surgical tools for high spatial and temporal resolution measurements with minimal disturbance to the cells.[7] These advantageous properties make flame-etched carbon fiber microelectrodes particularly attractive for the effective detection of individual vesicular contents in the cell cytoplasm environment.

Herein, we have fabricated flame-etched carbon fiber microelectrodes to obtain conical nano-tips that could be adapted for intracellular detection of catecholamine contents in individual nanoscale vesicles in PC12 cells. These electrodes are fabricated by carefully flame-etching the cylindrical carbon fibers to sharp tips (50–100 nm diameter at the tip, 30–100 μm length) (Figure 1a, 1b, S1). The nano-tip conical carbon fiber microelectrodes have good electrochemical characteristics and high sensitivity to dopamine. Well-defined, nearly sigmoidal-shaped voltammograms have been achieved, demonstrating that diffusion limited mass transport is involved in the electrochemical process as expected for conical electrodes with micrometer dimension. Limiting current plateaus of approximately 1.5–2.5 nA have

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been observed in 0.10 mM dopamine (Figure 1c). These values are within the range theoretically described for electrodes with a long conical taper and a small tip.[8]

For intracellular measurements, the nano-tip conical carbon fiber microelectrodes are pushed through the cell membrane without significant damage. This then leads to the active conical electrode surface being exposed in the presence of the catecholamine containing vesicles in the cell interior (Figure 2a). We observed the process with a 40× objective, as the electrode is carefully pushed through PC12 cell membrane (Figure 2b and Movie S1) for subsequent amperometric recording. To provide evidence that the electrode is placed inside the cell, a series of experiments have been carried out at different insertion depths of the nano-tip conical carbon fiber microelectrodes. The limiting reduction currents of the biocompatible redox probe, $[Ru(NH_3)_6]^{3+}$, drop to 75% and 50% of their primary value outside the cell after approximately 25% and 50% insertion of the active electrode tip into the cell, respectively (Figure 2c). This indicates that the cell membrane seals around the electrode satisfactorily after insertion. Also, the limiting current is restored to 95% of the original value after withdrawing the electrode from the cell, and the baseline and noise levels do not change when the electrode is inserted into the cell.

Representative traces from the amperometric recordings of the content from individual PC12 cell vesicles are presented in Figure 3. To show that random exocytosis events at the electrode portion outside the cell do not occur, two sets of control experiments have been carried out. Figure S2 shows the electrode placed on top of a PC12 cell, in a similar manner to when exocytosis is monitored, but with the edge of the conical electrode placed along the cell. Without chemical stimulation, no current transients are detected. More importantly, when the electrode is strongly pushed against the PC12 cell membrane, but without breaking into the cytosol, exocytotic events are still not observed (Figure 3a). Thus unstimulated exocytosis is not occurring at the PC12 cells during manipulation of the nano-tip conical carbon fiber microelectrodes at and into the cell. After the tip of the electrode is pushed through the cell membrane, again without any chemical stimulation, well-defined amperometric spikes are recorded continuously in ca. 80% of cases (Figure 3b). Typical amperometric spike displays a single event with well-defined rising and decaying phases (Figure 3c). Since Ca^{2+} entry is a critical step to stimulate exocytosis, the same experiments have also been carried out in Ca^{2+} free physiological saline to more completely examine the origin of the transients (Figure S3) and no significant difference in shape or amount detected is observed. Considered in the context with our vesicle impact experiments recently published for isolated vesicles,[5] this strongly suggests that the transients are from the impact and collapse of vesicles at the electrode surface inside the cell.

In an attempt to quantify the amount of catecholamine in each vesicle we have considered several issues to develop a simple mechanistic concept. Vesicles diffuse and possibly electromigrate (owing to their net negative charge) to the electrode surface.[9] Figure 2d presents the proposed mechanism of measurements of individual vesicular transmitters with nano-tip conical carbon fiber microelectrodes inside of the cell. It appears that the vesicles move to the electrode and then adsorb onto the carbon surface followed by opening of the membrane towards the electrode. Studies of vesicle adsorption and subsequent opening toward the surface support this mechanism,[10] and in our previous work we demonstrated

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vesicle adsorption and rupture with quartz crystal microbalance experiments.[5] To follow up, we have used scanning electron microscopy to examine vesicle adsorption on a carbon microelectrode showing a large number of vesicles adsorbed to the surface after dipping in the sample solution (Figure S4).

Oxidation of catecholamine expelled from individual vesicles results in a current transient at the electrode. Thus oxidizable material is coulometrically measured and the area under each current transient appears to represent complete oxidation of catecholamine in each vesicle. This can be quantified with Faraday's law (N = Q/nF), where N is the mole amount of catecholamine oxidized from each individual vesicle, Q is the charge calculated by the time integral of current transients from the amperometric trace, n is the number of electrons exchanged in the oxidation reaction ($2e^-$ for catecholamines) and F is the Faraday constant (96 485 C/mol). The number of dopamine molecules from each individual current transient has been quantified and the normalized frequency histogram is shown in Figure 3d.

We have carried out a set of experiments where the same electrode used for intracellular vesicle electrochemical cytometry, is placed on the top of a neighboring cell. Subsequent high K^+ stimulation of the cell results in amperometric current transients resulting from exocytosis (Figure S5). Thus we can directly measure the amount released from each vesicle, again with the simple application of the Faraday equation. We used the part of each electrode close to the base in order to increase the collection efficiency for exocytosis. In addition, the electrode was pressed gently onto the cell to force membrane to partially surround it ensuring a surface area considerable larger then a vesicle to be covered and to ensure nearly 100% of the catecholamine released would be oxidized. To test this, we compared the exocytosis results at the conical electrode to those at a 5 μm microdisk electrode as regularly used in release experiments. Figure S6 shows the distributions of $N_{molecule}$ in single release events detected at the two electrodes. The distributions and amounts of release are nearly identical. Comparing the intracellular cytometry and extracellular exocytosis measurements at the same electrode, the total contents of catecholamine in each vesicle measured by intracellular vesicle electrochemical cytometry is clearly higher than the amount released during stimulated exocytosis. Fitting the data clearly shows a negative shift in the amount released during stimulated exocytosis (black) compared to that observed by intracellular vesicle electrochemical cytometry (red) (Figure 3d). Thus, as we and others have published recently, only part of the quantal contents is released during exocytosis, which is consistent with our results from the cell-free model.[3b, 5, 11] The peak parameters for these data are summarized in Table S1.

The distributions of the number of molecule from intracellular vesicle electrochemical cytometry and exocytotic measurements are asymmetric and deviate from normality (Fig. 3d), hence motivating the use of the median as statistical analysis tool as it is less sensitive to extremes. Additionally, intracellular vesicle electrochemical cytometry transients are grouped by cells instead of pooling all vesicle data from a population of cells and we compare the mean of the medians of catecholamine amount in individual vesicles from single cells, which helps to minimize the impact of the cell-to-cell variation. The total vesicular catecholamine contents measured by intracellular vesicle electrochemical cytometry (114 500 ± 15 300 molecules) is significantly different from the amount detected

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in stimulated exocytosis measured from single cell amperometry experiments with the same electrodes (73 200 ± 5 820 molecules) (two-tailed Mann-Whitney rank-sum test, $p<0.01$) (Figure 4). According to these data, we estimate that approximately 64% of the total catecholamine inside the vesicles is released during stimulated exocytosis in these PC12 cells. Although somewhat higher than measured previously, this result again supports the hypothesis this time observed in a living cell, that exocytosis is open and closed.[12] This result is also consistent with the studies which suggest most of the exocytosis events are followed by rapid endocytosis in PC12 cells.[13] Interestingly, the different distributions for vesicular content versus exocytosis release (Figure 3d) might mean that the fraction of catecholamine released during exocytosis for each vesicle varies.

Although highly unlikely, it is possible that under the conditions we used the vesicle membrane fraction facing the cytoplasm solution may keep a partial structure. We have compared the vesicular content detected with intracellular vesicle impact cytometry (114500 ± 15300 molecules) to that with vesicle impact cytometry[5] in solution containing PC12 vesicles (112500 ± 2500 molecules) and the results are nearly identical suggesting that we are measuring all the molecules in the vesicles.

In another series of experiments, we compared stimulated exocytosis with intracellular vesicle electrochemical cytometry in PC12 cells after treating them with L-DOPA. L-DOPA is the direct biochemical precursor to dopamine, and it is the main drug used to treat Parkinson's disease.[14] Intracellular vesicle electrochemical cytometry of single vesicles in single PC12 cells following treatment with L-DOPA is shown in Figure S7. As expected, the L-DOPA exposure increases the number of molecules (323 100 ± 29 000 molecules) and this is compared to the values from control cells (Figure 4). Comparing the L-DOPA treated cells, the total vesicular catecholamine content is significantly higher (two-tailed Mann-Whitney rank-sum test, $p<0.01$) than the amount released (single cell amperometry with the same electrode in each case, 209 000 ± 11 800 molecules). Interestingly, this ratio indicates that approximately 65% of the catecholamine in a vesicle is released during exocytosis from L-DOPA treated PC12 cells, very close to the control cells. This suggests that the catecholamine content in the vesicle does not affect the mechanism of cell exocytosis in terms of the open and closed process.

In summary, we present an amperometric method (intracellular vesicle electrochemical cytometry) capable of directly measuring the vesicular catecholamine contents of single vesicles in living PC12 cells with high spatiotemporal resolution by use of nano-tip conical carbon fiber microelectrodes. Although this process is invasive, the cells survive and direct measurement can be accomplished. By comparing the results from intracellular vesicle electrochemical cytometry with those from chemically stimulated exocytosis, we find that ~64% of the catecholamine in each vesicle is released during exocytosis, supporting the concept of open and closed exocytosis. These measurements were done with the same electrode for each pair of intracellular vesicle electrochemical cytometry and release experiments, thus having the same signal to noise and detection limit for the comparison.

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Supplementary Material

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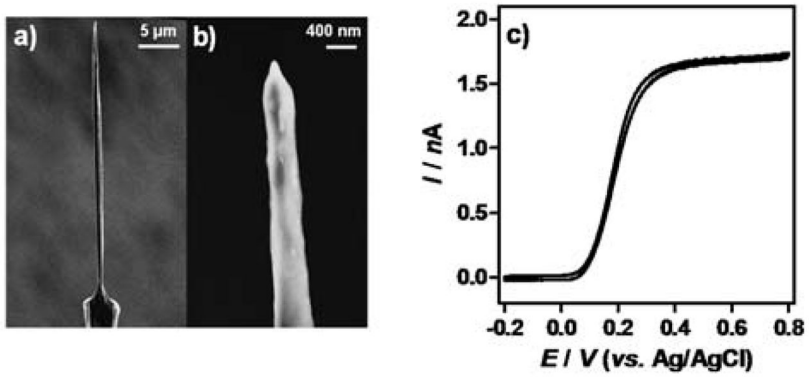

Figure 1.

Characterization of the electrode used for intracellular vesicle electrochemical cytometry, a) global view (scanning electron microscopy) of a nano-tip conical carbon fiber microelectrode showing its shape, scale bar, 5 μm; b) an amplified view of the tip of another nano-tip conical carbon fiber microelectrode, scale bar, 400 nm; c) representative cyclic voltammogram (CV) of 0.10 mM dopamine at a nano-tip conical carbon fiber microelectrode, scan rate, 0.10 V s^{-1}.

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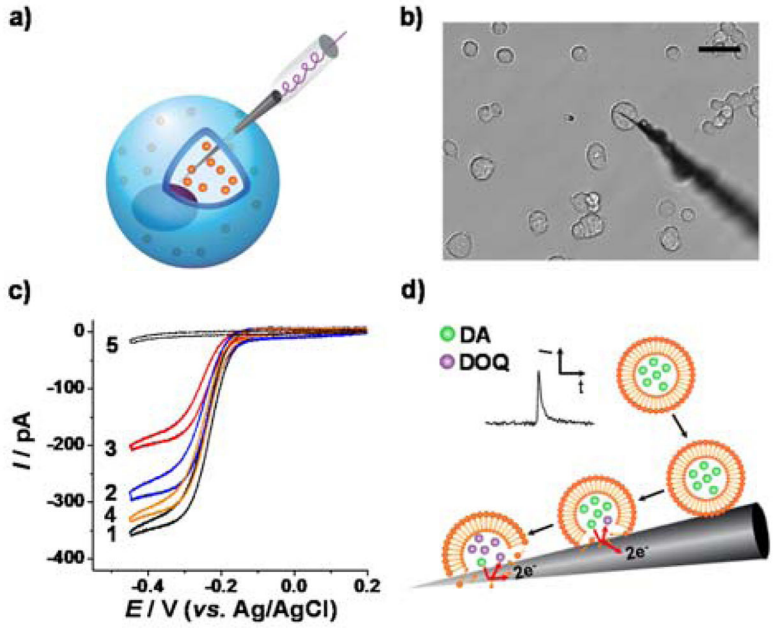

Figure 2.

a) Schematic (small orange circles represent vesicles) and b) bright-field photomicrograph (electrode approaching a cell from the lower right) of a nano-tip conical carbon fiber microelectrode placed in the cytoplasm of a single PC12 cell, scale bar, 20 μm. c) CVs recorded at a nano-tip electrode at different positions from outside the cell to inside with a surrounding solution of 0.10 mM $[Ru(NH_3)_6]^{3+}$, outside the cell (curve 1), approx. 25% inside (curve 2), 50% inside (curve 3), and outside after withdrawal from the cell (curve 4). Curve 5, background CV in physiological saline. Scan rate, 0.10 V s^{-1}. d) Representation of the mechanism of vesicles adsorbing and opening on the in situ electrode. DA, dopamine; DOQ, dopamine orthoquinone.

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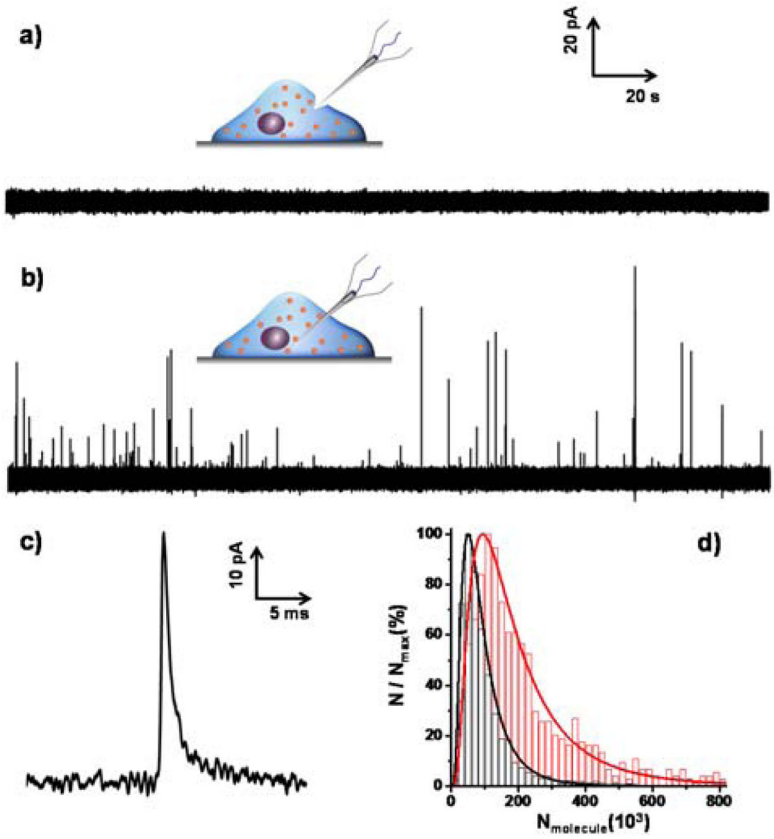

Figure 3.

Amperometric trace for a nano-tip conical carbon fiber microelectrode pushed against a PC12 cell without breaking into the cytoplasm (a) or placed inside a PC12 cell (b); c) amplified amperometric current trace; d) normalized frequency histograms describing the distributions of vesicular catecholamine amount quantified from untreated PC12 cells by intracellular vesicle electrochemical cytometry (red, n=1017 events from 17 cells) and that from K^+-stimulated exocytosis at the same electrode (black, n=1128 events from 17 cells). Bin size = 2 x 10^4 molecules. Fits were obtained from a lognormal distribution of the data.

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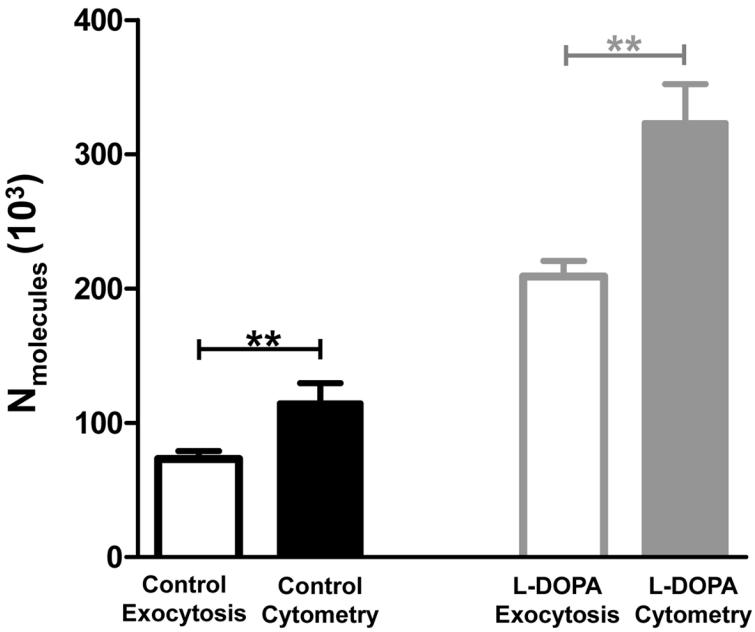

Figure 4.

Average number of catecholamine molecules per vesicle from stimulated exocytosis (blank) versus intracellular vesicle electrochemical cytometry (solid) treated with (19 cells) or without (17 cells) L-DOPA (100 μM, 2 h). Error is SEM. Two-tailed Mann-Whitney rank-sum test. **, $p < 0.01$.