

Quantitative Measurement of Transmitters in Individual Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes

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Abstract: The quantification of vesicular transmitter content is important for studying the mechanisms of neurotransmission and malfunction in disease, and yet it is incredibly difficult to measure the tiny amounts 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 nanotip conical carbon-fiber microelectrode was used to electrochemically measure the total content of electroactive neurotransmitters in individual nanoscale vesicles in single PC12 cells as these vesicles lysed on the electrode inside the living cell. The results demonstrate that only a fraction of the quantal neurotransmitter content is released during exocytosis. These data support the intriguing hypothesis that the vesicle does not open all the way during the normal exocytosis process, thus resulting in incomplete expulsion of the vesicular contents.

During neuronal transmission, vesicles are the major organelles involved in the storage and release of chemical messengers, such as 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: the electrophoretic separation of vesicles, lysis, and the 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 amount in single artificial vesicles in a high-throughput manner.^[2] With this method, we then successfully quantified the vesicular contents of individual vesicles isolated from pheochromocytoma (PC12) cells and mouse brain tissue.^[3] Recently, Cheng and Compton also investigated the electrochemistry of liposomes, which in this case expelled encapsulated ascorbic acid when they impacted electrodes.^[4] We have since characterized the contents of mammalian vesicles isolated from adrenal glands by single adsorption and rupture events at electrodes without separation.^[5] This approach

enables nanoscale mammalian vesicles to adsorb to carbon microdisk electrodes and subsequently rupture and expel their contents (mainly catecholamines, such as norepinephrine, epinephrine, and dopamine), thus eliciting an oxidation current that can be used to quantify the catecholamine content of the vesicles. These methods are effective for quantification of the content of individual isolated vesicles; however, it is an exciting prospect to measure the content of vesicles directly in the intracellular environment, where vesicle isolation is not a concern and 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 to enable insertion into the cell.^[6] They also show increased sensitivity, kinetics, and signal-to-noise ratio and a faster time response for many neurotransmitters as 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 measurements with high spatial and temporal resolution and minimal disturbance to the cells.^[7] These advantageous properties make flame-etched carbon-fiber microelectrodes particularly attractive for effective detection of the contents of individual vesicles in the cell-cytoplasm environment.

Herein, we describe the fabrication of flame-etched carbon-fiber microelectrodes to obtain conical nanotips that could be adapted for the intracellular detection of the catecholamine content of individual nanoscale vesicles in PC12 cells. These electrodes were fabricated by careful flame etching of the cylindrical carbon fibers to create sharp tips (diameter at the tip: 50–100 nm, length: 30–100 μ m; Figure 1a,b; see also Figure S1 in the Supporting Information). The nanotip conical carbon-fiber microelectrodes have good electrochemical characteristics and show high sensitivity to dopamine. Well-defined, nearly sigmoidal shaped voltammograms were observed, thus demonstrating that diffusion-limited mass transport is involved in the electrochemical process, as expected for conical electrodes with micrometer dimensions. Limiting current plateaus of approximately 1.5–2.5 nA were observed in 0.10 mM dopamine (Figure 1c). These values are within the theoretically expected range for electrodes with a long conical taper and a small tip.^[8]

For intracellular measurements, the nanotip conical carbon-fiber microelectrodes are pushed through the cell membrane without significant damage to the membrane. In this way, the active conical electrode surface was exposed to catecholamine-containing vesicles in the cell interior (Figure 2a). We observed the process with a 40 $\times$ objective, as the electrode was carefully pushed through a PC12 cell membrane (Figure 2b; see also Movie S1 in the Supporting

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Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201504839>.

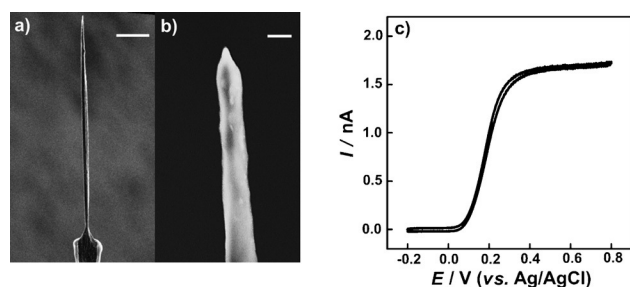


Figure 1. Characterization of the electrode used for intracellular vesicle electrochemical cytometry. a) Global view (scanning electron microscopy) of a nanotip conical carbon-fiber microelectrode (scale bar: 5 μm). b) Amplified view of the tip of another nanotip conical carbon-fiber microelectrode (scale bar: 400 nm). c) Representative cyclic voltammogram (CV) of 0.10 mM dopamine at a nanotip conical carbon-fiber microelectrode (scan rate: 0.10 V s^{-1}).

Information) for subsequent amperometric recording. To provide evidence that the electrode was placed inside the cell, we carried out a series of experiments at different insertion depths of the nanotip conical carbon-fiber microelectrodes. The limiting reduction currents of the biocompatible redox probe $[\text{Ru}(\text{NH}_3)_6]^{3+}$ dropped to 75 and 50% of their primary value outside the cell after the insertion of approximately 25 and 50%, respectively, of the active electrode tip into the cell (Figure 2c). This result indicates that the cell membrane seals around the electrode satisfactorily after insertion. Also, the limiting current was restored to 95% of the original value after withdrawing the electrode from the cell, and the baseline and noise levels did not change when the electrode was inserted into the cell.

Representative traces from the amperometric recording of the content of 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, we carried out two sets of control experiments. Without chemical stimulation, when the electrode was 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 (see Figure S2), no current transients were detected. More importantly, when the electrode was strongly pushed against the PC12 cell membrane, but without breaking into the cytosol, exocytotic events were still not observed (Figure 3a). Thus, unstimulated exocytosis of the PC12 cells were not recorded on the nanotip conical carbon-fiber microelectrodes when the electrode came in contact with or entered the cell. After the tip of the electrode had penetrated through the cell membrane, again without any chemical stimulation, well-defined amperometric spikes were recorded continuously in approximately 80% of cases (Figure 3b). A 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, we also carried out the same experiments in Ca^{2+} -free physiological saline to more completely examine the origin of the transients (see Figure S3). Under these conditions, no significant difference in shape or the amount of catecholamine detected was observed. Considered in the context of our vesicle-impact experiments recently

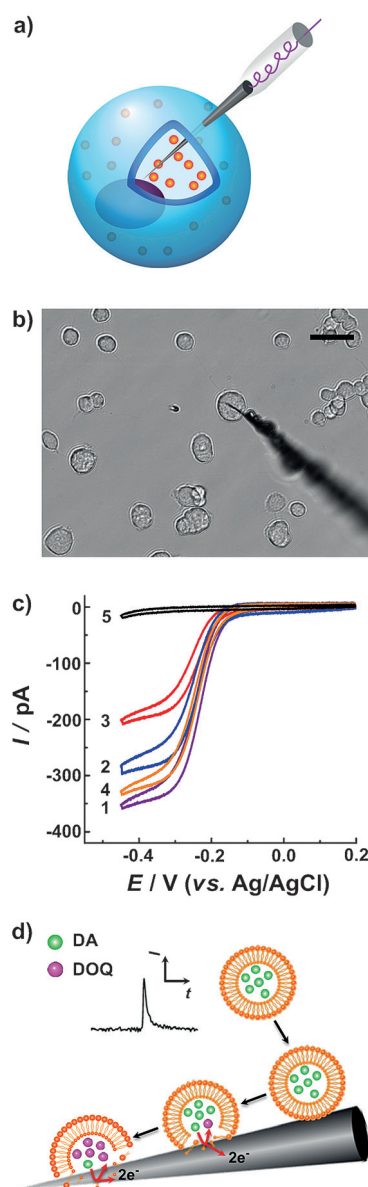


Figure 2. a) Schematic illustration (small orange circles represent vesicles) and b) bright-field photomicrograph (electrode approaching a cell from the lower right) of a nanotip conical carbon-fiber microelectrode placed in the cytoplasm of a single PC12 cell (scale bar: 20 μm). c) CVs recorded at a nanotip electrode at different positions from outside the cell to inside the cell with a surrounding solution of 0.10 mM $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (scan rate: 0.10 V s^{-1}). Curve 1: outside the cell; curve 2: approximately 25% inside; curve 3: 50% inside; curve 4: outside after withdrawal from the cell; curve 5: background CV in physiological saline. d) Mechanism of the adsorption and opening of vesicles on the in situ electrode. DA = dopamine, DOQ = dopamine orthoquinone.

reported for isolated vesicles,^[5] this result 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 catecholamines in each vesicle, we considered several issues to develop a simple mechanistic concept. Vesicles diffuse and possibly electro-migrate (owing to their net negative charge) to the electrode surface.^[9] According to our proposed mechanism for the

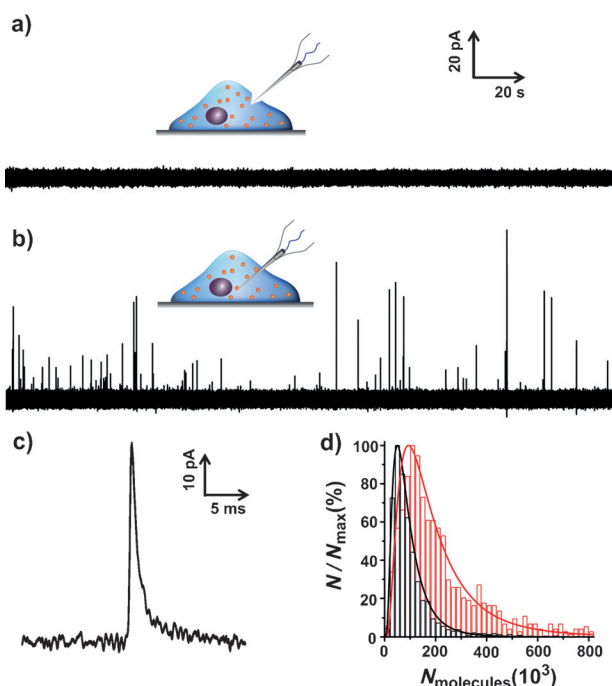


Figure 3. a,b) Amperometric traces for a nanotip conical carbon-fiber microelectrode pushed against a PC12 cell without breaking into the cytoplasm (a) or placed inside a PC12 cell (b). c) Amplified amperometric current trace. d) Normalized frequency histograms describing the distribution of the vesicular catecholamine amount as quantified from untreated PC12 cells by intracellular vesicle electrochemical cytometry (red, $n=1017$ events from 17 cells) and by K^+ -stimulated exocytosis at the same electrode (black, $n=1128$ events from 17 cells). Bin size: 2×10^4 molecules. Fits were obtained from a log normal distribution of the data.

measurement of transmitters in individual vesicles with nanotip conical carbon-fiber microelectrodes inside the cell (Figure 2d), the vesicles move to the electrode and then adsorb onto the carbon surface, and the membrane subsequently opens towards the electrode. Studies of vesicle adsorption and subsequent opening toward the surface support this mechanism,^[10] and in our previous study we demonstrated vesicle adsorption and rupture with quartz-crystal microbalance experiments.^[5] To further investigate this process, we used scanning electron microscopy to examine vesicle adsorption on a carbon microelectrode and found a large number of vesicles adsorbed to the surface after it was dipped in the vesicle suspension (see Figure S4).

The oxidation of catecholamines expelled from individual vesicles results in a current transient at the electrode. Thus, oxidizable material was measured coulometrically, and the area under each current transient appeared to represent complete oxidation of the catecholamines in each vesicle. This relationship can be quantified by Faraday's law, $N = Q/nF$, in which N is the mole amount of catecholamines oxidized from each individual vesicle, Q is the charge calculated from 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 (96485 C mol^{-1}). The number of dopamine molecules corresponding to each individual current transient was

quantified, and the results are presented in a normalized frequency histogram (Figure 3d).

We carried out a set of experiments in which the electrode used for intracellular vesicle electrochemical cytometry was placed on top of a neighboring cell. Subsequent high K^+ stimulation of the cell resulted in amperometric current transients resulting from exocytosis (see Figure S5). Thus, we could directly measure the amount released from each vesicle, again by the simple application of the Faraday equation. We used the part of each electrode close to the base to increase the collection efficiency for exocytosis. Furthermore, the electrode was pressed gently onto the cell to force the membrane to partially surround it, thus ensuring that the surface area covered was considerably larger than a vesicle and that nearly 100% of the catecholamines released would be oxidized. To test this hypothesis, we compared the exocytosis results at the conical electrode to those at a $5 \mu\text{m}$ microdisk electrode, as regularly used in release experiments. The distributions and catecholamine amounts released were nearly identical (see Figure S6). We then compared the intracellular vesicle electrochemical cytometry and extracellular exocytosis measurements at the same electrode and found that the total catecholamine content in each vesicle as measured by intracellular vesicle electrochemical cytometry was clearly higher than the amount released during stimulated exocytosis (see Table S1 in the Supporting Information for peak parameters). Fitting of the data clearly shows a negative shift in the amount released during stimulated exocytosis (black) as compared to that observed by intracellular vesicle electrochemical cytometry (red; Figure 3d). Thus, as we and others have reported recently, only part of the quantal content is released during exocytosis, which is consistent with our results from the cell-free model.^[3b,5,11]

The distributions of the number of molecules from the intracellular vesicle electrochemical cytometry measurements and exocytotic measurements are asymmetric and deviate from normality (Figure 3d), hence motivating the use of the median as a 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 the catecholamine amount in individual vesicles from single cells, which helps to minimize the impact of the cell-to-cell variation. The total vesicular catecholamine content measured by intracellular vesicle electrochemical cytometry ((114500 ± 15300) molecules) is significantly different from the amount detected for stimulated exocytosis in single-cell amperometry experiments with the same electrodes ((73200 ± 5820) 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 content of 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 characterized by an open/closed mechanism.^[12] This result is also consistent with studies that suggest that most exocytosis events are followed by rapid endocytosis in PC12 cells.^[13] Interestingly, the different distributions found

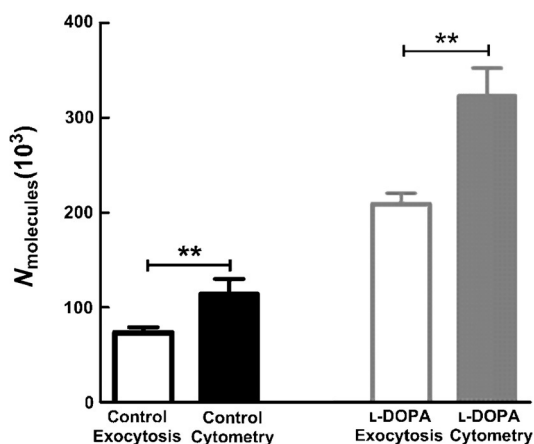


Figure 4. Average number of catecholamine molecules per vesicle as determined by stimulated exocytosis (blank) versus intracellular vesicle electrochemical cytometry (solid) with (19 cells) or without treatment (17 cells) with L-DOPA (100 μ M, 2 h). Error is the standard error of the mean. **Two-tailed Mann–Whitney rank-sum test, $p < 0.01$.

for vesicular content versus exocytosis release (Figure 3d) might indicate that the fraction of catecholamines released during exocytosis varies for each vesicle.

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 compared the vesicular content detected by intracellular vesicle impact cytometry ((114 500 $\pm$ 15 300) molecules) to that detected by vesicle impact cytometry^[5] in a suspension of PC12 vesicles ((112 500 $\pm$ 2500) molecules) and obtained nearly identical results, thus suggesting that we are detecting 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-3,4-dihydroxyphenylalanine (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 was carried out following treatment with L-DOPA (see Figure S7). As expected, exposure to L-DOPA increased the number of catecholamine molecules present ((323 100 $\pm$ 29 000) molecules) as compared to the values from control cells (Figure 4). The total vesicular catecholamine content was significantly higher in the L-DOPA treated cells (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 $\pm$ 11 800) molecules). Interestingly, this ratio indicates that approximately 65 % of the catecholamine molecules in a vesicle are released during exocytosis from L-DOPA-treated PC12 cells: an amount very close to that observed for the control cells. This result 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 have developed an amperometric method (intracellular vesicle electrochemical cytometry) capable of

directly measuring the catecholamine content of single vesicles in living PC12 cells with high spatiotemporal resolution by the use of nanotip conical carbon-fiber micro-electrodes. Although this process is invasive, the cells survive and direct measurement is possible. By comparing the results from intracellular vesicle electrochemical cytometry with those from chemically stimulated exocytosis, we found that approximately 64 % of the catecholamine molecules in each vesicle were released during exocytosis, thus supporting the concept of open and closed exocytosis. These measurements were made with the same electrode for each pair of intracellular vesicle electrochemical cytometry and release experiments; thus, the signal-to-noise ratio and detection limit were the same in each case for purposes of comparison.

Acknowledgements

Funding for this project was provided by the European Research Council (Advanced Grant), the Knut and Alice Wallenberg Foundation, the Swedish Research Council (VR), and the National Institutes of Health. We thank Joakim Wigström for excellent technical assistance with the scanning electron microscopy experiments.

Keywords: catecholamines · electrochemistry · exocytosis · nanoelectrodes · vesicles

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 11978–11982
Angew. Chem. **2015**, *127*, 12146–12150

- a) S. O. Rizzoli, W. J. Betz, *Nat. Rev. Neurosci.* **2005**, *6*, 57–69; b) R. Jahn, D. Fasshauer, *Nature* **2012**, *490*, 201–207; c) H. Park, Y. Li, R. W. Tsien, *Science* **2012**, *335*, 1362–1366.
- D. M. Omiatek, M. F. Santillo, M. L. Heien, A. G. Ewing, *Anal. Chem.* **2009**, *81*, 2294–2302.
- a) D. M. Omiatek, A. J. Bressler, A.-S. Cans, A. M. Andrews, M. L. Heien, A. G. Ewing, *Sci. Rep.* **2013**, *3*, 1447; b) D. M. Omiatek, Y. Dong, M. L. Heien, A. G. Ewing, *ACS Chem. Neurosci.* **2010**, *1*, 234–245.
- W. Cheng, R. G. Compton, *Angew. Chem. Int. Ed.* **2014**, *53*, 13928–13930; *Angew. Chem.* **2014**, *126*, 14148–14150.
- J. Dunevall, H. Fathali, N. Najafinobar, J. Lovric, J. Wigström, A.-S. Cans, A. G. Ewing, *J. Am. Chem. Soc.* **2015**, *137*, 4344–4346.
- a) T. G. Strein, A. G. Ewing, *Anal. Chem.* **1992**, *64*, 1368–1373; b) A. M. Strand, B. J. Venton, *Anal. Chem.* **2008**, *80*, 3708–3715; c) K. T. Kawagoe, J. A. Jankowski, R. M. Wightman, *Anal. Chem.* **1991**, *63*, 1589–1594; d) Y.-T. Li, S.-H. Zhang, L. Wang, R.-R. Xiao, W. Liu, X.-W. Zhang, Z. Zhou, C. Amatore, W.-H. Huang, *Angew. Chem. Int. Ed.* **2014**, *53*, 12456–12460; *Angew. Chem.* **2014**, *126*, 12664–12668.
- a) B. Tian, T. Cohen-Karni, Q. Qing, X. Duan, P. Xie, C. M. Lieber, *Science* **2010**, *329*, 830–834; b) K. Yum, N. Wang, M.-F. Yu, *Nanoscale* **2010**, *2*, 363–372; c) P. Actis, S. Tokar, J. Clausmeyer, B. Babakinejad, S. Mikhaleva, R. Cornut, Y. Takahashi, A. López Córdoba, P. Novak, A. I. Shevchuck, J. A. Dougan, S. G. Kazarian, P. V. Gorelkin, A. S. Erofeev, I. V. Yaminsky, P. R. Unwin, W. Schuhmann, D. Klenerman, D. A. Rusakov, E. V. Sviderskaya, Y. E. Korchev, *ACS Nano* **2014**, *8*, 875–884.
- C. G. Zoski, M. V. Mirkin, *Anal. Chem.* **2002**, *74*, 1986–1992.

- [9] a) B. M. Quinn, P. G. van't Ho, S. G. Lemay, *J. Am. Chem. Soc.* **2004**, *126*, 8360–8361; b) A. Boika, S. N. Thorgaard, A. J. Bard, *J. Phys. Chem. B* **2012**, *117*, 4371–4380.
- [10] a) E. Reimhult, B. Kasemo, F. Höök, *Int. J. Mol. Sci.* **2009**, *10*, 1683–1696; b) K. Dimitrievski, B. Kasemo, *Langmuir* **2008**, *24*, 4077–4091.
- [11] a) C. Amatore, A. I. Oleinick, I. Svir, *ChemPhysChem* **2010**, *11*, 159–174; b) L. J. Mellander, R. Trouillon, M. I. Svensson, A. G. Ewing, *Sci. Rep.* **2012**, *2*, 907; c) A. Oleinick, F. Lemaitre, M. G. Collignon, I. Svir, C. Amatore, *Faraday Discuss.* **2013**, *164*, 33–55.
- [12] a) R. G. W. Staal, E. V. Mosharov, D. Sulzer, *Nat. Neurosci.* **2004**, *7*, 341–346; b) A. M. Aravanis, J. L. Pyle, R. W. Tsien, *Nature* **2003**, *423*, 643–647; c) F. Pinaud, S. Clarke, A. Sittner, M. Dahan, *Nat. Methods* **2010**, *7*, 275–285; d) Q. Zhang, Y. Li, R. W. Tsien, *Science* **2009**, *323*, 1448–1453.
- [13] a) J. Klingauf, E. T. Kavalali, R. W. Tsien, *Nature* **1998**, *394*, 581–585; b) S. P. Gandhi, C. F. Stevens, *Nature* **2003**, *423*, 607–613.
- [14] a) T. L. Colliver, S. J. Pyott, M. Achalabun, A. G. Ewing, *J. Neurosci.* **2000**, *20*, 5276–5282; b) J. Wang, R. Trouillon, Y. Lin, M. I. Svensson, A. G. Ewing, *Anal. Chem.* **2013**, *85*, 5600–5608.

Received: May 28, 2015

Revised: July 15, 2015

Published online: August 12, 2015