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Review Article

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Abstract

In this article, we have summarized the recent important results related to the electrochemical detection of vesicular exocytosis by amperometry with microelectrodes over the past three years. In this fascinating scientific field that began 40 years ago, the historical carbon fiber amperometry method still continues to be used to address biological questions by the pioneered groups of the field but also by other research groups thus showing this has become an indispensable routine technique for analyzing exocytosis. Furthermore, new methodologies (coupling with fluorescence, use of nanoelectrodes, microarrays) have blossomed and demonstrated how new analytical methods could be built to push back the limits of the initial technique.

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Amperometry, Carbon fiber microelectrodes, Vesicular exocytosis, Fluorescence, Nanoelectrodes.

Introduction

Chronoamperometry using a single carbon-fiber ultramicroelectrode is nowadays a standard technique for investigating vesicular exocytosis at the single-cell level (see Figure 1). By applying a constant potential at the electrode surface, the released neurotransmitters (e.g. catecholamines) by the emitting cell are oxidized. As a consequence, each vesicular event is displayed as an amperometric spike whose shape features the dynamics and the number of electroactive species released (see Figure 2).

This basic tool from an electroanalytical point of view offers many advantages beyond the monitoring of exocytotic activity. First, ultramicroelectrodes and isolated single cells are globally the same sizes (~μm). Second, ultramicroelectrodes have a fast time response (~ms) and a direct current−concentration relationship ($i = 4nFDrC^*$ where $F = 96{,}485$ C mol^{-1}; n = number of electrons, r = electrode radius, C* = electroactive species concentration) that allows one to monitor individual vesicular events in terms of real time neurotransmitter fluxes within the electrode-cell cleft. Eventually, the low measured currents (~pA) and ohmic drop enable to only work with two electrodes (working and reference) which results in easier manipulations at a micrometric level.

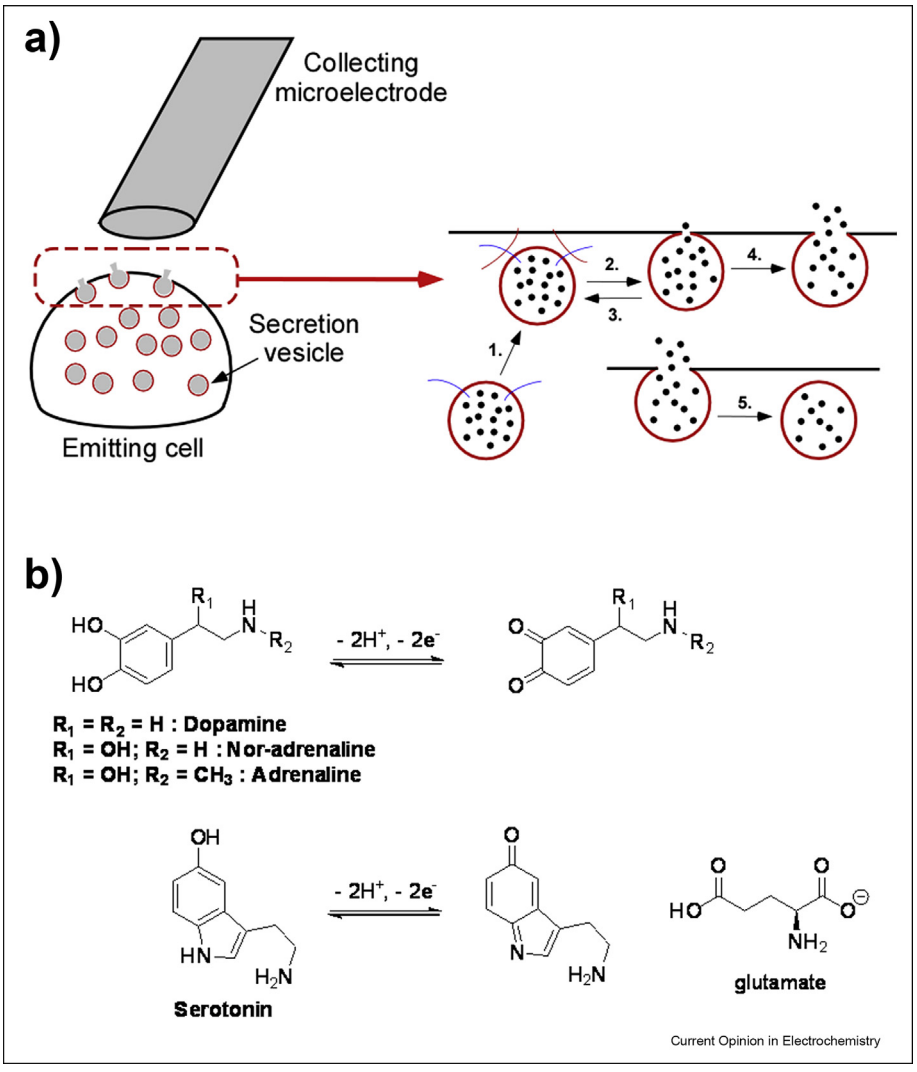
Over the past forty years, carbon fiber amperometry (CFA) has significantly contributed to questions related to the exocytotic mechanism [1,2]. First of all, many parameters that govern this important mechanism of neurotransmission were addressed (plasticity, fusion pore, physicochemical and biological assistances) [3]. Second, CFA analytically evolved to be more informative either by means of analytical couplings (fluorescence, electrophysiology) or by being involved at a new scale (arrays, nanoelectrodes) [4–7]. As a consequence, this short review is aimed at giving the very recent state of the art of this important field. More particularly, only the three past years will be globally considered to 'take a photograph' of the field. A broader vision is given by the cited reviews within this article.

Contributions of the historical CFA technique

CFA as a usual technique to investigate exocytosis

CFA is nowadays used as a standard and compulsory technique to address several issues of exocytosis by biologists. *A remaining challenge is to understand how exocytosis is governed by biological processes.* First of all, a very interesting result is the unexpected role of endophilins. These proteins usually act as endocytotic adapters, but amperometry and microscopy measurements at chromaffin cells

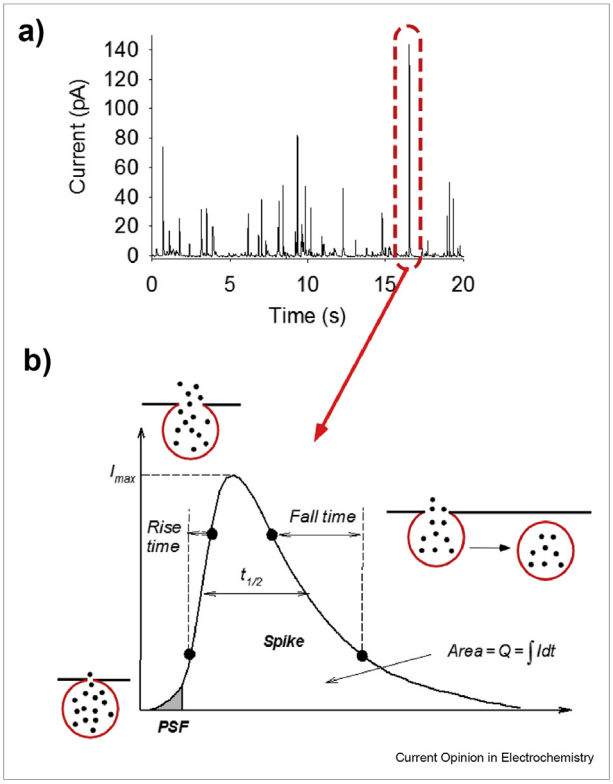
Figure 1



(a) Left: Simplified scheme of amperometric detection of exocytosis at the single-cell level. A collecting electrode (i.e. a carbon fiber ultramicroelectrode) is positioned at the top of the cell. The electrode potential is held at a sufficient value to oxidize the released neurotransmitters. Right: Simplified scheme of neurotransmitters released by vesicular exocytosis. Neurotransmitters (dopamine, adrenaline, nor-adrenaline, serotonin are displayed as black points) are stored within vesicles (20–500 nm radius depending on the cell model). After an appropriate stimulation, secretion vesicles reach and then dock at the cell membrane (step 1.). SNARE (soluble N-ethylmaleimide sensitive fusion protein attachment receptors; red and blue lines) complexes favor the docking and subsequent mixing of the cell and vesicular membranes. The formation of a nanometric fusion pore thus ensues through which the release starts (step 2.). Such a release depends on the fusion pore stability. Closure of the fusion pore corresponds to a 'kiss and run' release (step 3.). Expansion of the fusion pore leads to a more massive release (step 4.) that is predominantly partial (step 5.; see text). **(b)** Electrochemical oxidation of the main investigated neurotransmitters (dopamine, nor-adrenaline, adrenaline, and serotonin).

evidence their action as an indispensable vesicular partner to stimulate the priming/fusion of docked secretory vesicles [8]. With the same cell model, the central role of extracellular and intracellular sphingosine-1-phosphate, a complex signaling lipid, is demonstrated for the first time by using CFA and especially by analyzing the PSF properties (that reflects the fusion pore stability; see Figure 2) [9]. CFA also contributes to important findings related to the interconnected role between pannexin-1 channels and some purinergic and nicotinic acetylcholine receptors to trigger exocytosis [10]. In the same way, the effects of some signaling pathways from a receptor tyrosine kinase-like EPHB6 on catecholamines secretion are undoubtedly proven with CFA at chromaffin cells with important consequences on the relationship between effect on catecholamine secretion and blood pressure regulation [11]. The issues concerning SNAREs (Soluble NSF Attachment proteins Receptors) during the docking of vesicles with the cell membrane are still addressed with CFA (and complementary measurements, including fluorescence microscopy or electrophysiology). Notably, the fundamental questions are related to cell membrane integrated

Figure 2



(a) Typical amperogram recorded during exocytotic releases. (b) Each spike of current corresponds to an individual vesicle whose release can be analyzed in different ways (maximum current, half time, rise and fall times, area proportional to the number of detected neurotransmitters by means of Faraday's law: N = Q/nF). PSF (prespike feature) can also be analyzed with similar parameters (time length, current, and area) and corresponds to the fusion pore step. The increase and decrease in current can be correlated to the fusion opening and the end of the release, respectively.

proteins like syntaxin 1A [12], vesicular membrane proteins like synaptobrevin-2 [13], or cytoplasmic proteins like dynamin-1 [14]. Furthermore, CFA evidences that an excess of nitric oxide during the NO-mediated production of cyclic guanosine 3′,5′-monophosphate (cGMP) could lead to a derivative, 8-nitro-cGMP, that modulates the exocytotic kinetics and corresponds to a novel regulatory pathway of exocytosis [15]. *Other biological challenges also need to be addressed, like the relationship between exocytosis and calcium.* In this way, a new Ca^{2+}-independent and voltage-dependent pathway of exocytosis is also demonstrated with CFA that is correlated to electrophysiological and fluorescence techniques in chromaffin cells [16]. *The above results were obtained by biologists who appropriated the electroanalytical technique*.

Historical researchers still contribute to CFA

Herein, it has to be emphasized that some past high contributing groups in the field still investigate exocytosis by means of CFA at the single-cell level. *They contribute to many challenges related to the physicochemical effects of exogenous molecules/ions, the role of calcium or the relationship between exocytosis and diseases.* For instance, C. L. Haynes and coworkers have shown how the *Plasmodium chabaudi* parasite or antimalarial drugs (chloroquine, quinine) affect the serotonin secretion by blood platelets [17,18]. The group of R. Borges unravels the different mechanisms triggered by some secretagogues (Ca^{2+}, Ba^{2+}, Sr^{2+}) in chromaffin cells by combining CFA and electrophysiological or fluorimetric techniques [19]. The group of A. Albillos takes benefits from the real-time analysis with CFA to address important issues by showing that chromaffin cells may be involved in deleterious effects in cirrhosis due to an increased exocytosis activity [20]. In addition to morphological analyses and electrophysiological monitoring, CFA allows the same group to contribute to key issues related to the catecholamine storage. Hence, they show that adrenergic chromaffin cells are not adapted to nor-adrenaline accumulation and remember their specificity for adrenaline storage (vesicular shape and matrix) even in case of adrenaline deficiency [21]. Furthermore, J. Rettig et al. use CFA as a complementary technique of fluorescence microscopy and electrophysiology to address the multifaced role of calcium. They demonstrate that some CAPS (calcium-dependent activator proteins for secretion) modulates the catecholamine loading and release from vesicles in chromaffin cells [22]. Furthermore, A. G. Ewing and colleagues show that some counter-anions (Cl^-, Br^-, NO_3^-) in the stimulation solution for triggering exocytosis may enter the lipid bilayer and act as chaotropic agents whose alter the dynamics of the exocytotic release [23].

Another important challenge is the theoretical treatment of amperometric spikes, i.e. how to extract physicochemical information by modeling amperometric spikes. In this way, A. G. Ewing, C. Amatore, and coworkers have given evidence of how mixing single-cell experiments (transmission electron microscopy and CFA) and ingenious modeling of amperometric spikes help to investigate the effect of zinc treatment on the neurotransmitter storage and release within PC12 cells [24]. Finally, M. B. Jackson and colleagues suggest a new and accurate model of release where the fusion pore is not static but dynamically changes during the release [25].

All these recent results confirm the importance of amperometry at the single-cell level, which is used by physical chemists and biologists and possibly in combination with other analytical tools to address biological issues related to regulatory pathways of the exocytotic release.

New analytical tools as an evolution of the CFA technique

Over the past three years, enhancements of the historical CFA configuration were proposed and included

many challenges related to the automatic procedure analysis of the amperometric signals, the combination of amperometry and TIRFM (total internal reflection fluorescence microscopy) associated with the search of ideal dual convenient probes, nanoelectrodes, glutamate sensors, microarrays, and electrochemical cytometry (see Figure 3).

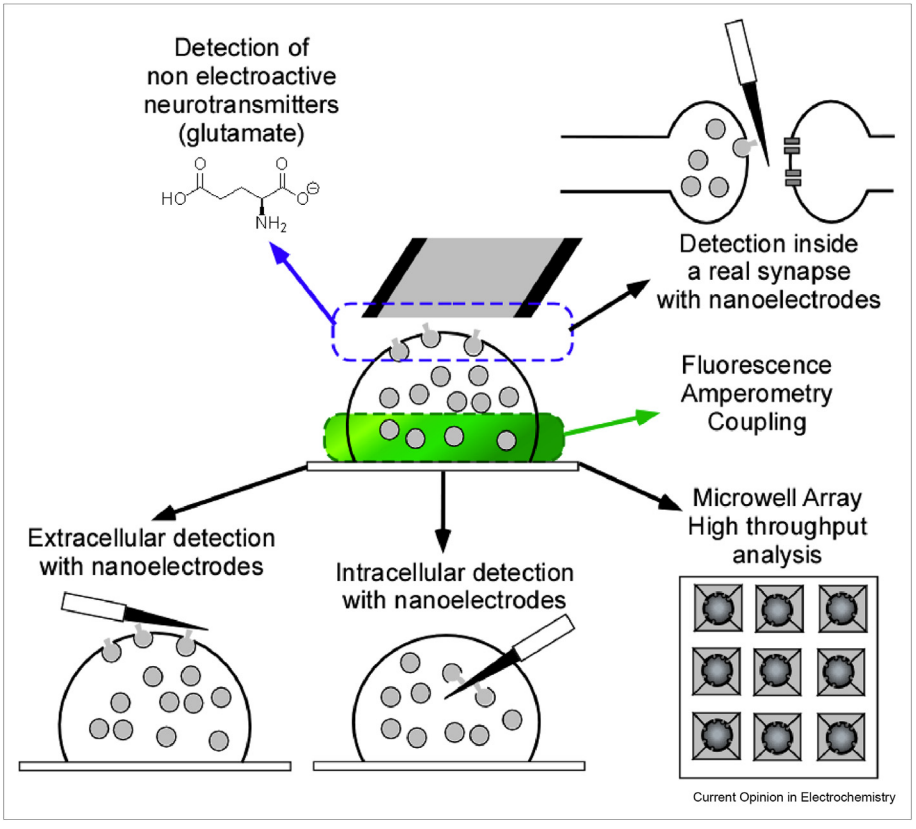
Data treatment for amperometric spikes analysis

An interesting challenge is the data treatment of amperometric spikes. Indeed, depending on the cell model, about up to five hundred spikes can be extracted from the amperometric traces. For avoiding a time-consuming process, their extraction and analyses are often achieved by means of appropriate semi-automatic treatments that can lead to misleading conclusions (selected overlapped spikes, wrong spike parameters …). This is why important tools still need to be built. As such, a matched-filtering approach (M-F A) can be associated with a two thresholds procedure to reduce false positives and the use of a template library to cover the morphological zoology of amperometric spikes. This outperforms the usual derivative-threshold approach or some commercial applications (ClampFit, MiniAnalysis) and is validated in terms of performance by Receiver Operating Characteristics plots (detection by the algorithms of 95% of manually identified spikes and a rate of 2% of false positives) [26]. Furthermore, this M-F A added to an estimation approach that fits the data with a multiexponential function shows overlap rejection, a two-step simulation procedure, and a novel baseline estimate. It also estimates spike and foot signal features automatically [27].

Fluorescent coupling for exocytosis analysis

The fluorescence-electrochemistry coupling for analyzing exocytosis is a remaining analytical challenge since it would allow one to "see" an entire exocytotic event in terms of vesicular motions/release sites (fluorescent vesicles) and secretion kinetics (amperometric spikes) [6]. In this context, recent interesting advances were reported. The coupling method TIRFM-amperometry is successfully performed at the ITO electrode with a unique fluorescent false neurotransmitter FFN102 probe (a pH-dependent fluorescent and electroactive molecule accumulated in N13 cells vesicles). This demonstrates an increased fraction of coupled fluorescence-amperometry events (34%) when compared to similar studies with independent

Figure 3



Main highlights of the recent developments from the historical carbon fiber amperometry for investigating vesicular exocytosis.

electroactive and fluorescent probes [28]. Moreover, FFN102 can be used in PC12 cells to partially replace endogenous vesicular dopamine at the level of 12%. Because FFN102 is probably stored in fast vesicular diffusion compartments, electrofluorescence monitoring of the fusion pore opening is expected in the future [29]. In order to enhance the FFN electrochemical detection, FFN42 is designed and presents an adapted behavior in terms of electroactivity, pH-dependent fluorescence, and uptake in vesicles of N13 and PC12 cells. It thus paves the way for future applications in coupled method detection with FFN probes with lower oxidation potential on ITO and optimal excitation wavelength [30]. In a different but also promising way, Neurosensor 510 (NS510) has a strong affinity with catecholamines and is involved in the 'fluorescence neurotransmitter adduct strategy'. NS510 selectively reacts with nor-epinephrine and therefore labels some secreting vesicles of chromaffin cells. Beyond the interesting discrimination of nor-adrenaline from other neurotransmitters, encouraging TIRFM-amperometry experiments are performed with NS510-loaded chromaffin cells. The optical and electrochemical conditions are favorable to a dual cell analysis [31].

Nanoelectrodes for extracellular detection

CFA was historically achieved with micrometric electrodes. A new challenge is to take benefits from nanoelectrodes for the detection of vesicular exocytosis. The first and important work is to investigate how decreasing the electrode size could modify its electrochemical properties. Comparison of the usual carbon microdisk and cone-shaped nanotip electrodes for measurements of exocytosis (on PC12 cells) shows slight different dynamics of release (measured fluxes and kinetics spikes parameters) and similar values for the released charge detected. This is mainly due to different cell-electrode distances and noise levels for the nanoelectrodes and microelectrodes, whereas the exocytotic release did not intrinsically change [32]. *One of the most important insights of nanoelectrodes is to use them inside real single neuronal synapses.* As an example, cone-shaped carbon fiber nanoelectrodes are used for amperometric monitorings in individual dopaminergic synapses to assess the assumed neuroprotective properties of harpagide, a natural product that restores normal dopamine releases from injured neurons in Parkinson's disease models [33].

Nanoelectrodes for intracellular detection

The carbon nanoelectrodes have a size suitable to be inserted into a single cell without altering its functions. *This is an important opportunity to address a crucial issue related to the electroanalysis of intracellular vesicles by selectively sampling nanometric vesicles within the cell cytoplasm by amperometry.* Adrenaline or nor-adrenaline enriched vesicle types can be figured out by FSCV (fast-scan cyclic voltammetry) on the basis of the curved shape [34]. But the most abounding works devoted to nanoelectrodes are related to intracellular vesicle impact electrochemical cytometry (IVIEC) that corresponds to the amperometric detection of catecholamines within the intracellular vesicles by inserting a nanotip electrode in the cell cytoplasm. Intracellular vesicles thus rupture on the electrode surface that electrochemically measures their released content. Hence, short-interval repetitive stimuli effect on PC12 cells studied by combined IVIEC and CFA suggests a possible link between triggered plasticity and quantal release [35]. The role of ATP on the purinergic autoreceptors in chromaffin cell vesicles as a source of the energy-carrying molecule was also investigated by the same combination [36,37]. CFA and IVIEC methods can also be applied to the effects of Zn^{2+} treatment on PC12 cells. Variations in dynamics and vesicle contents are evidenced and probably result from membrane composition, as suggested by mass spectrometry imaging [38]. CFA and IVIEC can be coupled to study the effects of cognition-changing psychostimulants (cocaine and methylphenidate) with similar effects on the vesicle content and amount of neurotransmitters released, but opposite ones on the fraction released [39]. Finally, the CFA/IVIEC comparisons help to conclude that the serotonin vesicular release is also partial in pancreatic β-cells [40]. In the same idea, carbon fiber nanoelectrodes combined with modeling in Drosophila larval neuromuscular neuron revealed that the vesicular octopamine released is a low fraction (e.g. 5%) of the whole content, thus confirming that vesicular release is partial and a mean of presynaptic plasticity [41]. Of note, both works belong to more important and broader investigations related to *the debate of the vesicular release*. They confidently confirm that the neurotransmitter release in vesicular exocytosis is mainly partial, i.e. the vesicles do not entirely release their catecholamine content [42,43].

Glutamate biosensors

Neurotransmitters like catecholamines can be easily oxidized at a bare carbon electrode surface. *Another analytical challenge is to extend the electrochemical detection to non-electroactive neurotransmitters*. An interesting example is the case of glutamate (Glu). An enzymatic micro-biosensor by co-modification of glutamate oxidase (GluOx) and Pt nanoparticles (Pt NPs) on the surface of a carbon fiber electrode can be performed to overcome the nonelectroactivity of Glu. H_2O_2 is the product of Glu transformation on GluOx and was detected on Pt NPs. This sensor application is operational on single hippocampal varicosities with high sensitivity and spatiotemporal resolution [44]. An enzymatic amperometric biosensor based on GluOx-gold nanoparticle modified surface CF electrode is detailed by the group of A-S Cans. The same sensor can be applied on large unilamellar vesicles (LUVs) for a calibration curve of glutamate released from these prefilled vesicles [45]. While the question of its selectivity could be

raised (the sensor is also able to partially detect interferents like dopamine, glucose, or acetylcholine), this sensor contributes to measurements of random bursts of spontaneous glutamate exocytotic release spikes in the model of the nucleus accumbens of rodent brain slices [46].

Microarrays

A drawback of the single-cell technique is related to the required number of experiments to overcome cell variability. *A last analytical challenge is to implement a high throughput analysis.* This is why an amperometric CMOS (complementary metal-oxide-semiconductor) chip technology (cell culture, microwell traps, and electrochemical recordings) is developed for high-throughput drug testing (bupropion and citalopram) in the treatment of neurodegenerative diseases. It is efficient on chromaffin cells and associated with whole-cell patch-clamp experiments [47]. Furthermore, Micro-Graphitic Single-Crystal Diamond Multi-Electrode Arrays are used as multifunctional biosensors for monitoring in real-time dopamine liberation and spontaneous action potentials firing in networks of neurons cultured *in vitro* [48].

Conclusion

This survey of the very recent works devoted to amperometric recordings of vesicular exocytosis is a 'snapshot' of a broader field which has started in the 90s. However, this reflects, to some extent, the current state of the art of this research domain. First of all, CFA is still a 'routine' technique that is no longer reserved for its only inventors. This positive point means that it is a 'popular' technique in vesicular exocytosis similar to NMR in molecular chemistry. This is why CFA still helps to understand the exocytotic mechanism in terms of biological issues (SNAREs, drug effects …). Second, new analytical tools, essentially based on a coupling of methods with amperometry (microchips, fluorescence, cytometry) or the development of nanoprobes, blossomed and offered encouraging and very exciting results. The main works resulting from the 2018–21 period correspond well to the scientific trends already identified by more comprehensive reviews [1,5]: (1) The use of nanoelectrodes offers many new opportunities related to intracellular analysis or within the synapse. (2) Amperometry needs to be integrated with other techniques, especially fluorescence imaging, to provide a comprehensive view of the entire exocytotic event. (3) Amperometry involving catecholamines needs to be extended to other neurotransmitters (e.g. glutamate). (4) Microelectrode arrays are requested to achieve a combinatory analysis of the release.

In conclusion, the new analytical implementations and genuine innovations somehow contribute to methodological breakthroughs. What is very exciting in this scientific area is that the best is yet to come because the ideal fluorescence-amperometry coupling needs to be built, whereas the proof of concept has been validated. Moreover, the electroanalysis of real neuronal synapses with adapted nanoelectrodes will be an important trend. Indeed, such new methodologies are expected to shine more light on biological processes of secretion without insurmountable analytical difficulties in the medium term.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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