Analytical Techniques

DOI: 10.1002/ange.200600510

Coupling of Electrochemistry and Fluorescence Microscopy at Indium Tin Oxide Microelectrodes for the Analysis of Single Exocytotic Events**

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Exocytosis of secretory vesicles is an essential process for communication between cells and organs in complex living systems. Following appropriate stimulation, vesicles containing bioactive molecules (neurotransmitters, peptides, hormones, etc.) at high concentration are transported via the cytoskeleton to eventually dock and fuse with the cell membrane, thus resulting in the release of their contents into the extracellular medium.[1] The four main steps of exocytosis,[2,3] particularly the intermediate phase of the fusion pore, are controlled by multiple biological[1] and physicochemical factors.[4,5] Over the past 20 years, several important methods have been developed for the investigation of exocytotic processes in single living cells with individual resolution of single secretory events. These methods are based either on electrical measurements[6,7] (that is, electrophysiological or electrochemical) or on optical detection.[8] Among them, amperometry at microelectrodes[3,4,9] and fluorescence microscopy (including observation in an evanescent wave field)[8,10] appear to be currently two of the most powerful techniques. They are in fact complementary, since each one provides an extremely precise way to monitor specific steps of

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[**] This work was financially supported by the ENS, CNRS, and Université Pierre et Marie Curie, Paris 6, as well as by the French Ministry of Research (MRT). The MRT (Y.V.) and DGA (C.C.) are thanked for supporting PhD grants.

the whole event. The first method enables accurate investigation of the dynamics of release (kinetics and quantum of charge) from individual vesicles once fusion begins. Conversely, the second technique provides precious data concerning the status of the vesicles before and during fusion (displacements, docking location, geometric area of fusion). Consequently, combining both techniques should afford a mighty tool for studies of single secretory events, with the aim of correlating both sources of information and enabling a complete analysis in situ of each event from predocking to full release.

We report here the analysis of exocytosis at single adrenal chromaffin cells on transparent indium tin oxide (ITO) microelectrodes that allow a simultaneous observation of fluorescence and amperometric measurements on stained cells (Figure 1). The methodology was applied to neuroendocrine cells but may be transposed to any adherent or immobilized secretory cell (neurons, granulocytes, lymphocytes, pancreatic β cells, etc.) that emits electroactive compounds through vesicular release. This study is aimed at demonstrating that both techniques may be coupled without introducing severe difficulties to each other. Coupling with evanescent wave measurements will require implementation of the current device onto adequate ITO-coated optical prisms rather than onto optical flat windows as documented here, and access to an evanescent wave microscope rather than a epifluorescent microscope as used here. However, we believe that the results presented here establish that the optical quality of the present device will enable such work to be implemented in further work.

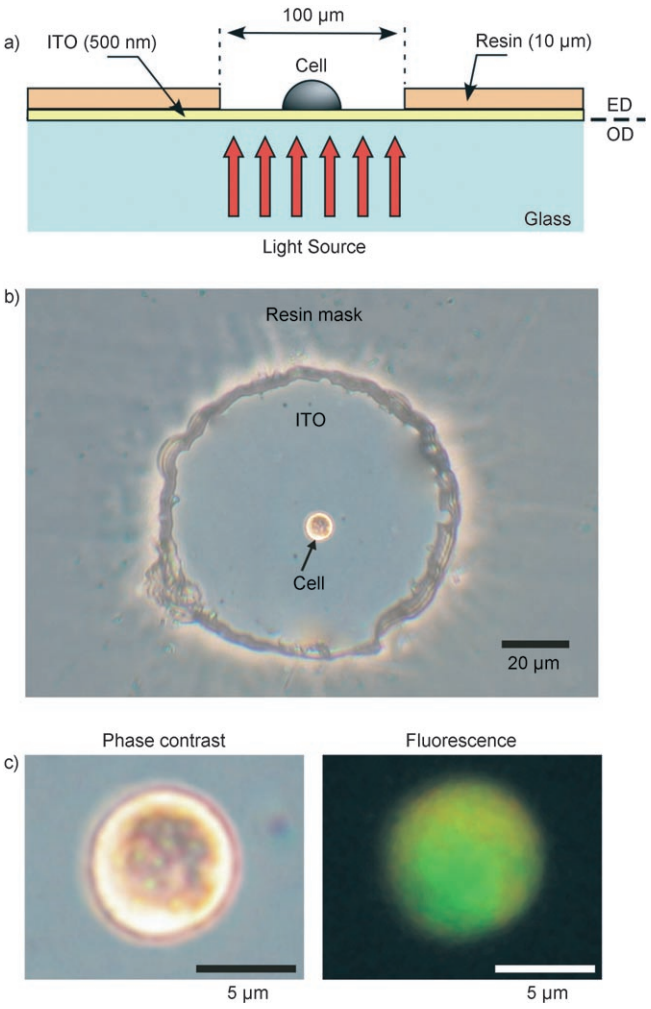

Figure 1. a) Schematic vertical cross-section of an ITO microelectrode. The electrochemical detection (ED) is performed on the ITO surface (upper part of the device) while the optical detection (OD) is obtained through the glass and ITO (lower part). b) Photograph (inverted microscopy) of a chromaffin cell adhering to an ITO microelectrode (100 µm diameter); the insulating resin appears as the dark gray external area. c) Close-up image obtained using phase contrast (left) or fluorescence (right) microscopy of the same cell preincubated with acridine orange and adhering onto the ITO surface.

Optical (for example, fluorescence) detection requires coverslips or slides with excellent optical properties (low refraction index), and a surface suitable for cell adhesion or immobilization through appropriate chemical modification. Conversely, amperometry requires an excellent electrical conductivity for a direct electrochemical detection of electroactive species secreted by the cell. ITO-coated glass slides possessed this set of requirements.[11] For example, thin films of ITO (typically 500 nm thickness) offer an excellent active surface for the electrochemical oxidation of epinephrine (Figure 2a), the main catecholamine released by chromaffin cells. Fluxes of catecholamines emitted during exocytotic events are in the range of 1000 molecules per ms.[4] A precise amperometric monitoring thus requires detection with a very high signal/noise ratio. This is achieved by restricting the active electrochemical surface to at most a few times the surface area of a single cell. This ensures the minimization of non-Faradaic information, such as noise of capacitive origin, while allowing a quantitative collection of released molecules. ITO-coated slides were thus insulated by a thin photoresist layer (10 µm thickness) through which circular openings (40–300-µm diameter wells) were created by photolithography (Figure 1a).

The root-mean-square (rms) noise detected on such micrometric surfaces of ITO by amperometry under experimental conditions appropriate for the analysis of living cells was typically 0.5 pA[12] and reached 0.3 pA for the smallest electrode diameters.

An aliquot of chromaffin cells in culture medium was deposited into a well of poly(dimethylsiloxane) (PDMS) positioned over an ITO microelectrode. The reduced aliquot volume and microelectrode dimensions meant that only one to two individual cells generally adhered to the free ITO surface (Figure 1b). Such isolated cells were left to adhere for at least 20 minutes before being stimulated by injecting a 2 mM barium solution in its vicinity with a micropipette. The ensuing secretion (Figure 2b) of catecholamines was followed by amperometry at the oxidation potential of +650 mV versus Ag/AgCl (Figure 2c). It is noteworthy that the data were obtained for secretion at the basal pole of each single cell, namely, under usual conditions for observation of cell fluorescence and for working with evanescent waves techniques.

The frequency of exocytosis was 3 Hz on average (Figure 2b), thus allowing the monitoring of numerous ampero-

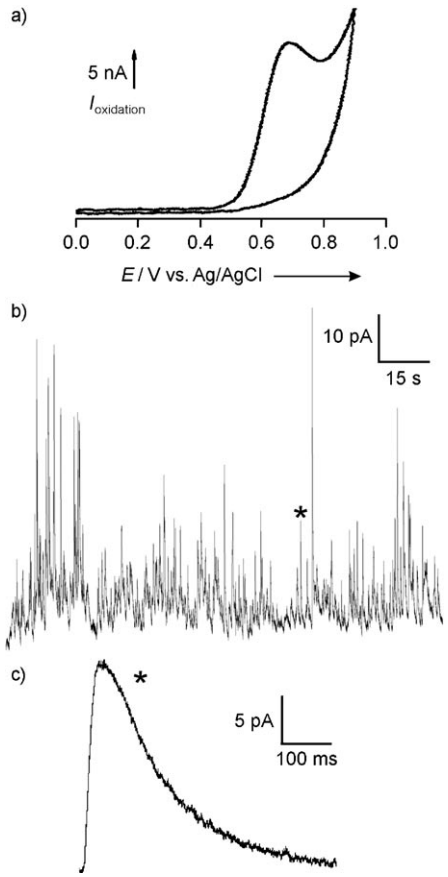

Figure 2. a) Cyclic voltammogram (initial potential: 0 V versus Ag/AgCl; sweep rate = 20 mVs^{-1}) of epinephrine (50 μM) in vitro in phosphate-buffered saline (PBS) buffer/$HClO_4$ (0.1 M) solution on an ITO microelectrode (well diameter = 40 μm) at 25 °C. b) Amperometric trace (E = 650 mV versus Ag/AgCl) corresponding to the exocytosis of a single chromaffin cell on an ITO microelectrode. Secretion was triggered by injection of a barium solution (2 mM). c) A representative spike from the trace (see labeling by asterisk in (b)) corresponding to a single vesicular event.

metric spikes. Each isolated spike (Figure 2c) was analyzed according to the method we and others have described previously.[12,13] The mean values of the main quantitative and kinetic parameters (Q, I_{max}, t_{20-90}, $t_{1/2}$) are summarized in Table 1. Clearly, the charges and maximum currents measured herein as well as the experimental reproducibility are in good agreement with those obtained at carbon microelectrodes in previous studies on cells from the same origin. Interestingly, it appears however that the kinetics of vesicle fusion are slower under the present conditions of analysis at the basal pole of the cells than at the apical one. This difference does not represent intrinsic differences in the kinetics of the electrochemical oxidation of catecholamines as demonstrated by in vitro experiments performed at each type of electrode (that is, carbon and ITO) but probably stems from specific molecular and physicochemical conditions at different exocytosis sites.[8] Thus, these studies show unambiguously that the electrochemical monitoring of exocytosis is feasible and reproducible on ITO microelectrodes with a similar accuracy as obtained with carbon microelectrodes.

Table 1: Mean values (± S.D.) of quantitative and kinetic parameters of amperometric spikes (n = 245).[a]

Q [fC]	I_{max} [pA]	t_{20-90} [ms]	$t_{1/2}$ [ms]
1745 ± 106	19.8 ± 1.2	19.3 ± 0.9	72.6 ± 2.5

[a] Detected during exocytosis of chromaffin cells (n = 5) on the surface of ITO microelectrodes.

Furthermore, fluorescent microscopy may be achieved at these same ITO microelectrodes simultaneously to amperometry. Prior to experiments, chromaffin cells were thus stained with acridine orange, a classical fluorescent probe known to accumulate into acidic compartments such as the secretion granules.[14,15] As evidenced in Figure 1c, the green fluorescence was easily observed through the ITO surface. Some granules appeared as green spots while other substructures appeared as orange areas (less acidic compartments), thereby establishing that the optical spectrum on ITO is large enough to record subtle differences in the granules. These studies will be extended to other fluorescent probes such as FM1-43 or green fluorescent proteins (GFPs)[14] and to different ITO surfaces.[11]

In this study, we have established the validity of the coupling between two essential analytical techniques that are among the most efficient for the direct and real-time analysis of biological exocytotic phenomena. The simultaneous optical and electrochemical detection of exocytotic events was shown to be feasible on the same transparent ITO material. The present procedure displays all the required qualities for the association of electrochemical techniques with total internal reflection fluorescence microscopy (TIRFM). This coupling should afford a comprehensive view about the "life" of a secretory vesicle before and after its fusion with the cell membrane together with a precise kinetic monitoring of the released material. Potential applications of this methodology include studies of other key biological processes involving secretion (cell communication, immune defense, oxidative stress, etc.) and especially its integration into microfluidic systems for parallel analyses on multiple cells.

Experimental Section

Bovine chromaffin cells were prepared by collagenase digestion of the medulla of adrenal glands obtained from a local slaughterhouse (Meaux, France). Cells were purified and isolated by using previously described methods.[12,16] Cells were used from the third to the eighth day after preparation.

Classical glass slides (75 mm × 25 mm × 1 mm) for microscopic observation were coated with a film of ITO (500 nm thickness; ACM, Villiers Saint Frédéric, France) to afford a material of low electrical resistance (20 ohms per square) and low refraction index (1.52). Micrometric disk surfaces of ITO were delimited by photolithography by two steps: first, an insulating resin (SPR220, MicroChem Corp., Newton, MA) was spin coated onto the whole ITO surface to form a film with an average thickness of 10 μm. Then, the film was exposed to a UV light through a mask made of a laser-printed transparency. After development of the resin, patterns of circular openings of 40 to 300 μm were obtained in the photoresist. Slabs of poly(dimethylsiloxane) (PDMS, 5 mm thickness) with holes punched through (10 mm diameter) were used as wells positioned over the ITO microelectrodes for solution, microcapillary, and reference electrode positioning.

The PDMS slabs were obtained by heating a mixture (weight ratio 10:1) of PDMS prepolymer (RTV 615 A) and its curing agent (RTV 615B, General Electric Silicones, Waterford, NY) for 1 h at 80 °C. The PDMS slab offers the advantage of immediately adhering and of being easily removable from the insulating resin delimiting ITO microelectrodes.

About 1 mL of an isotonic physiological saline solution (154 mM NaCl, 4.2 mM KCl, 0.7 mM $MgCl_2$, 11.2 mM glucose, 10 mM HEPES, pH 7.4) containing cells (at most 3×10^5) was injected into a well of PDMS lying on an ITO microelectrode. The cells were allowed to seed for 20 to 30 min at room temperature. In general, only one to two cells adhered to the surface of each ITO disk. A glass microcapillary (10–20 μm diameter) was then positioned with a micromanipulator (MHW-103, Narishige, Tokyo, Japan) at a distance of 20 to 30 μm from the single cell located in the well and used to inject (Femtojet injector, Eppendorf, Hamburg, Germany) a stimulating solution (2 mM $BaCl_2$ in Locke buffer supplemented with 0.7 mM $MgCl_2$, without carbonates) towards the cell surface for 10 s. This configuration allowed the stimulation of one cell at a time and to study its response. All chemicals were from Sigma–Aldrich (St-Louis, MO). Experiments were conducted at room temperature (22 ± 1 °C).

Amperometric detection of exocytosis at ITO microelectrodes was achieved at +0.65 V versus an Ag/AgCl reference electrode using a commercially available picoamperometer (model AMU-130, Radiometer Analytical Instruments, Copenhagen, DK), for which the adjustable time response was set at 1 ms. The output was digitized at 10 kHz, displayed in real-time, and stored on a computer (Powerlab-4SP A/D converter and software Chart 5.0, ADinstruments, Colorado Springs, CO) without any subsequent digital filtering. Each amperometric trace obtained during cell secretion was analyzed according to previously described methods.[12] The characteristics of each spike, that is, the maximum oxidation current I_{max} (pA), the total electrical charge Q (fC), the half-width $t_{1/2}$ (ms), and the rise time t_{20-90} (the delay between $I = 20\%$ of I_{max} and $I = 90\%$ of I_{max} in ms), were determined using home-made software.[12] All values are reported as the mean ± SD considering Gaussian-type distributions of the data.

Voltammograms (sweep rate 20 mV s^{-1}) of 50 μM epinephrine in a phosphate-buffered saline/$HClO_4$ (0.1M) solution were recorded in vitro with the same ITO microelectrodes with a different potentiostat (EI400, Ensman Instruments, Bloomington, IN).

For the fluorescence experiments, chromaffin cells were preincubated for 30 to 60 min in culture flasks containing DMEM/F12 medium supplemented with 10 mM acridine orange (Molecular Probes Inc., Eugene, OR). The cells were then washed, resuspended in isotonic saline solution, and allowed to seed on the ITO microelectrode surface. Acridine orange labeling was detected on an inverted microscope (Axiovert-135, Carl Zeiss, Germany) equipped with a Zeiss filter set 10 (excitation filter BP 450-490, BS 510, emission filter BP 515-565). Pictures of the fluorescent cells were taken with a 4 mega pixels camera (power shot A80, Canon, Japan) directly connected to the microscope body.

Received: February 7, 2006
Published online: May 9, 2006

Keywords: analytical methods · electrochemistry · exocytosis · fluorescence · indium–tin oxide

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