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Spatially Resolved Detection of Neurotransmitter Secretion from Individual Cells by Means of Scanning Electrochemical Microscopy

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Microelectrochemical methods, especially constant-potential amperometry and fast-scan cyclic voltammetry,[1] provide possibilities to investigate biological systems with cellular or subcellular spatial resolution determined mainly by the size of the available microelectrodes.[2] Among the biological phenomena studied so far at single cells or substructures of single cells are individual exocytosis events,[3] oxygen consumption,[4] photosynthetic activity,[5] and ion channel distribution.[6] In these experiments, the electrochemical sensor—usually a microdisk electrode with a tip diameter of a few micrometers—has to be positioned in close proximity to the object. The approach of the microelectrode to the biological object is, in general, performed under control of an optical microscope using manual- or piezo-actuated micromanipulators for positioning of the sensor tip. The electrode is slowly moved towards the cell until it slightly touches the cell membrane, which is visualized by its bending. The tip is then retracted for a defined distance allowing, in principal, adjustment of the membrane-to-electrode distance within a range of several hundred nanometers to two micrometers. Although this approach has been successfully used in recent years,[3] there are significant drawbacks, such as a) possible contamination of the electrode surface due to its contact with the cell membrane, b) insufficient reproducibility of the tip-to-cell distance, which becomes even more difficult using smaller microelectrodes, that are hardly visible in the optical microscope, c) eventual deterioration of the cell at the contact point or mechanical depolarization of the cell, and d) the impossibility to investigate sequentially different spots on the same cell.

After initial investigations of biological samples by means of scanning electrochemical microscopy (SECM),[7] attempts have been recently undertaken to visualize the metabolism and the redox activity of individual cells.[8] However, one of the major problems in conventional SECM experiments is the constant z-height of the microelectrode, which does not allow differentiation between variations in the tip-to-sample distance and changes of the local electrochemical activity. In order to overcome these limitations, we have introduced a shear-force based constant-distance control into the SECM.[9] The benefits and limitations had been described in detail recently.[10] In short, the microelectrode vibrates at its resonance frequency with typical amplitudes of only a few nanometers with use of a piezo-pusher. Simultaneously, a laser beam is focused onto the very end of the vibrating electrode and the resulting Fresnel diffraction pattern is projected onto a split photodiode. Amplitude and phase information about the vibrating tip is obtained by the amplification of the difference signal from the split photodiode with respect to the agitation signal using a lock-in amplifier. With decreasing tip-to-sample distance, increasing shear forces between tip and sample surface lead to a damping of the vibration amplitude and to a phase shift, which can be used to continuously keep a predefined damping value related to a constant distance of about 50 – 100 nm by means of a software-controlled feedback loop.

Thus, adaptation of the shear-force based constant-distance control of the tip-to-sample gap to biological preparations at the single-cell level should, on the one hand, allow the problems occurring with manual microelectrode positioning to be overcome and, on the other hand, enable detectable variations of chemical species at different sites of a biological preparation.

Using platinum microelectrodes sealed in glass capillaries, which can be easily positioned over hard sample surfaces using the shear-force positioning mode, no satisfying results could be obtained. In preliminary experiments using adher-

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ently growing cultures of living Retzius cells (from the medicinal leech with a typical diameter of about 80 μm) and PC12 cells (a pheochromocytoma tumor cell line with a typical diameter of 12 μm and a height of about 10 μm), the cells were cut into pieces by the vibrating microelectrode or peeled off the surface and locally displaced.

Thus, a successful adaptation of the shear-force based distance-control to soft biological samples is only possible if the force of interaction between the vibrating tip and the biological sample can be minimized. In addition, the shear-force detection has to be optimized with the vibrating microelectrode immersed into liquids and, concomitantly, the overall dimensions of the microelectrode tip have to be kept as small as possible in order to limit the distortions arising from mismatch between tip size and topography of the sample.[11] In order to evaluate the necessary stiffness, resonance frequency, and vibration amplitude, which allows visualization of the topography of a living cell using the shear-force mode, glass capillaries have been drawn to provide overall tip diameters in the range of 5–50 μm. Glass capillaries with tip dimensions of about 5–15 μm and resonance frequencies in the range 1–5 kHz showed a response to topographical changes while scanning over adherently growing Retzius cells (Figure 1) without any deterioration of the cells.

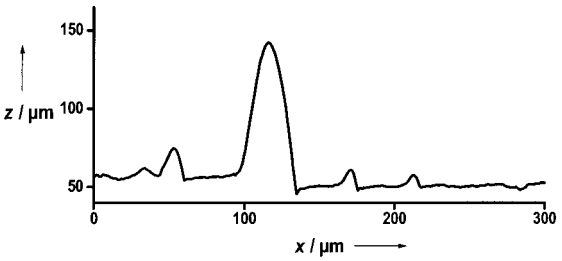

Figure 1. Shear-force based topographical imaging of fragile biological samples: Line scan across a neuron of the medicinal leech (Retzius cell) using a glass capillary with a tip diameter of about 5 μm.

Based on these results, microelectrodes had to be developed showing similar stiffness and vibration characteristics, in order to implement electrochemical detection into shear-force based imaging. Carbon fibers, which exhibit mechanical properties similar to pulled-glass capillaries, have been widely used as microelectrodes in manual approach experiments and several methods have been described to apply a thin insulating layer onto carbon fibers, either using electrochemically deposited polymers (for example polyphenol)[12] or the pH-induced electrodeposition of a lacquer paint.[13] In contrast to the self-limiting film growth of polyphenol, the pH-induced electrodeposition of a paint is followed by a heat-curing process to crosslink the initially deposited prepolymer to an insulating film. A disk-shaped electrode surface is exposed by cutting the tip with a scalpel. For the proper adaptation of the stiffness of carbon-fiber microelectrodes, the electrodeposition of an insulating paint has the advantage that the films can be grown to any suitable thickness by varying the deposition voltage and deposition time. In addition, suitable vibration properties of the carbon-fiber electrodes were obtained after dip coating the fiber in insulating varnish,[14] which resulted in an increase of the resonance frequency with the number of coating layers. The best results with regard to electrochemical quality and stability of the vibration properties were achieved by combining the electrodeposition and the dip-coating method. Parts of the carbon fiber and the connecting copper wire were stabilized by gluing them into a pulled-glass capillary before the electrode was cut with a scalpel to expose the electroactive disc at the tip. The resonance frequencies of the obtained electrodes varied in the range 1–5 kHz according to the length of the fiber protruding from the encasing glass capillary.

The electrochemical quality of the obtained carbon-fiber microelectrodes has been evaluated by cyclic voltammetry and by comparing theory versus their *z*-approach characteristics towards a nonconducting surface in the presence of a freely diffusing redox species, $[Ru(NH_3)_6]^{2+/3+}$ (Figure 2).[15] The approach characteristics are consistent with a disk-shaped electrode surrounded by a thin insulating sheath.

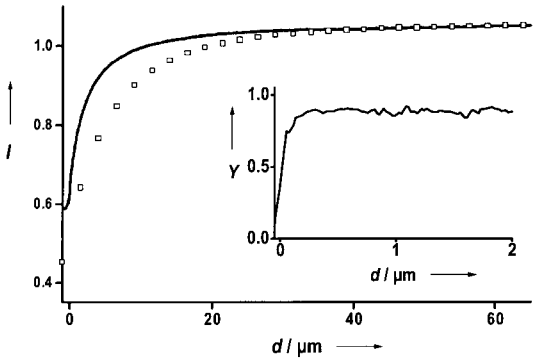

Figure 2. Approach characteristics of a carbon-fiber microelectrode to a glass surface. The obtained current response is compared against theory (rectangles) calculated for an electrode with a thick insulating sheath (tenfold radius of the conducting core). The inset displays the damping of the vibration amplitude multiplied by the sine of the phase shift (*Y* signal) caused by shear-force interaction with the glass surface. Redox mediator: 5 mM $[Ru(NH_3)_6]Cl_3$; electrode potential: −300 mV versus Ag/AgCl; electrode radius: 3.5 μm.

These optimized carbon-fiber microelectrodes have been applied to the visualization of the topography of adherently growing PC12 cells. Using the shear forces occurring between the vibrating carbon-fiber electrode and PC12 cells, reposition the tip is possible and a topographic image is obtained by drawing the *z*-displacement over the *x*,*y*-position of the tip (Figure 3).

Evidently, the interaction forces between the carbon-fiber tip and the sample are sufficiently small to allow the shear-force mode to be applied for visualizing the topography of individual living cells. For this, the tip is kept at a constant small distance over the cell membrane during scanning and, simultaneously, the microdisk surface can be used to detect changes in the concentration of redox-active compounds invoked by the metabolic and secretory action of the cell. Moreover, one may select specific sites at a single cell for perpendicular positioning of the microelectrode in order to monitor changes of the concentration of redox-active species

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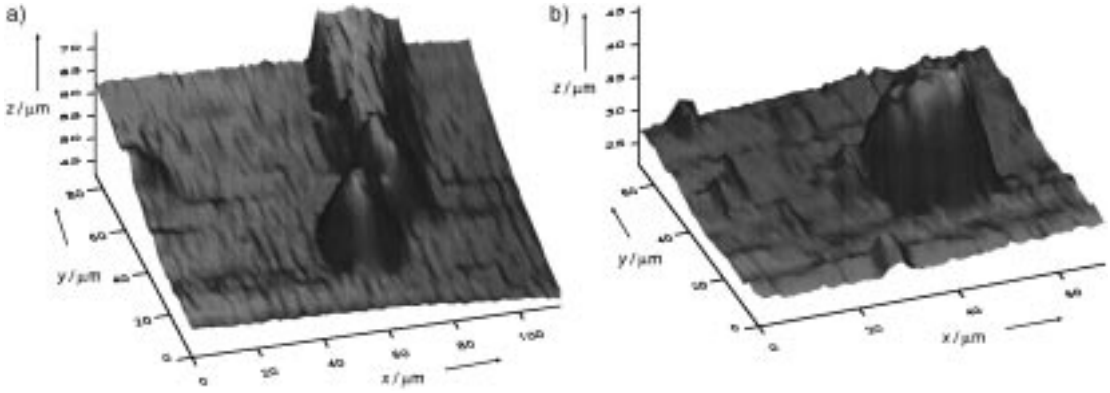

Figure 3. Shear-force based imaging of biological samples with carbon fiber electrodes with optimal stiffness (carbon-fiber microelectrode of diameter about 10–12 µm). a) Topography of a group of PC12 cells with an individual cell in the front. b) Topographic image of an individual PC12 cell.

with time. The discrete release of hormones and neurotransmitters from cells during exocytosis events is of especial interest to gain increased insights into cell–cell communication processes.

In a typical experiment, an adherently growing single cell is identified by means of a high resolution video microscope integrated within the SECM setup. After a coarse approach of the carbon-fiber microelectrode to the cell, the shear-force positioning starts and the topography of the cell is visualized (Figure 4a). The scanning procedure is then stopped at a defined site of interest (such as at the center of the cell). A small glass capillary, which is connected to a liquid-dispensing system, is positioned in close proximity to the selected cell and exocytosis is invoked by application of elevated K^+ concentrations through the positioned glass capillary. The increase of the K^+ concentration leads to a depolarization of the cell membrane causing an influx of Ca^{2+} ions followed by a reaction sequence which finally leads to the fusion of neurotransmitter-containing vesicles with the cell membrane, thus triggering the discrete release of the neurotransmitter molecules into the extracellular space. Due to the small gap between cell membrane and microelectrode tip, the released neurotransmitter molecules are consumed at the electrode which is poised to a constant potential of 700 mV versus Ag/AgCl using a high-sensitive potentiostat. This electrode potential is sufficiently high to oxidize the released neurotransmitter (in the case of PC12 cells, a mixture of the catecholamines adrenaline, noradrenaline, and dopamine). The oxidation current recorded during the single vesicle exocytosis is monitored using fast data-acquisition software (Figure 4b).

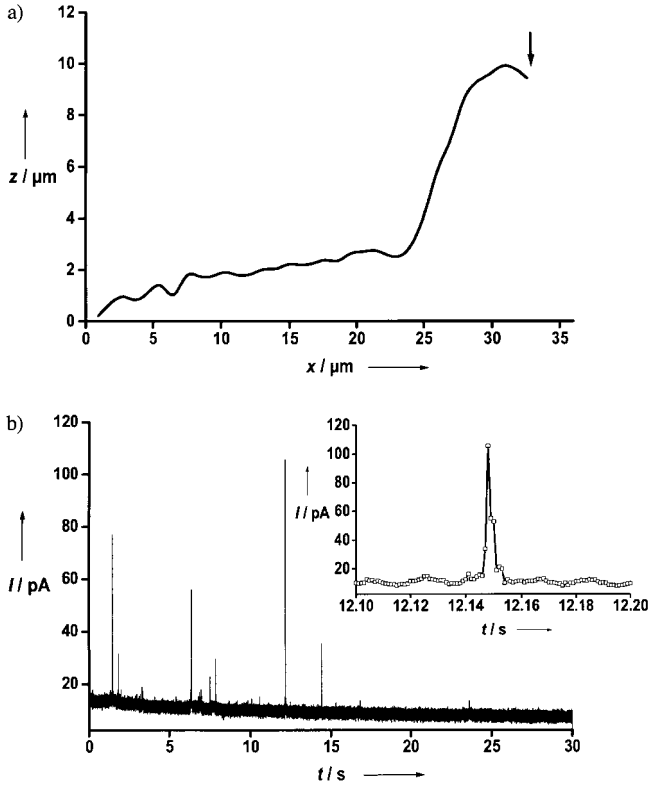

Figure 4. a) Adjusting the position of the microelectrode during scanning over a single PC12 cell. The arrow marks the final electrode position prior to the detection of discrete neurotransmitter release. b) Recording of single exocytotic events at a PC12 cell after application of K^+ ions. The inset shows the time course of the oxidation current with an expanded time scale of a selected single event. Electrode potential: +700 mV versus Ag/AgCl; data acquisition rate: 1000 Hz; tip diameter 10–12 µm.

Due to the size of the electrode disk and the very short gap between the cell membrane and the microelectrode, a 100% collection efficiency can be assumed if the release site at the cell is positioned opposite the microelectrode center. Although electrode fouling may occur due to subsequent reactions of the primarily oxidized neurotransmitter molecules, the charge transferred during the exocytotic event is related to the number of molecules released by the cell. Either a sequence of events can be recorded with the microelectrode kept at the same position or, after a rest period and repositioning of the microelectrode over a different spot of the cell, exocytosis can be invoked again providing a sufficiently small increase of the K^+ concentration after the first depolarization process within the electrolyte solution.

The implementation of carbon-fiber based electrodes in a SECM in combination with a shear-force based distance regulation represents a significant advance for the application of electrochemical scanning probe techniques to fragile biological systems with complex three-dimensional structures. The optimized microelectrodes are suitable for combined topographical and electrochemical studies of soft biological

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preparations using a shear-force dependent height-control system. Based on the topographical data, regions of interest on the cell membrane can be identified and spatially addressed with the microelectrode. Current work is directed towards a further decrease of the diameter of the used microelectrodes to submicrometer dimensions and the precise positioning of these fragile microelectrodes in close proximity to individual cells using shear-force interactions. In principle, the application of such miniaturized microelectrodes should allow elucidation of metabolic reactions even at substructures of cells.

Experimental Section

SECM: The SECM with integrated height control and video microscope has been described elsewhere.[10] All electrochemical measurements were carried out using a VA-10 potentiostat (npi electronic GmbH, Tamm, Germany) and an AgCl-coated silver wire as pseudo reference electrode.

Polymer-insulated carbon-fiber electrodes: A single carbon fiber (SGL Technik GmbH, Meitingen, Germany) of diameter 7 μm and length 15 mm was connected to a copper wire by means of silver epoxy glue (H20, EpoTek, Waldbronn, Germany). Parts of the copper wire and the fiber were inserted into a glass capillary and fixed with glue. The capillary was pulled around the fiber with a standard pulling apparatus (Narishige Model PP 830, Science Products, Hofheim, Germany). The electrodeposition was performed as described elsewhere.[13] The fiber was dipped into insulating paint (RS Components, Corby, Northants, UK) and allowed to dry at least 1 h. The ratio of insulator thickness to the fiber radius varied between 0.25 – 1 depending on the amount of coating cycles. Cyclic voltammetry was used to assure the quality of the obtained insulation. A carbon disc was exposed by cutting the insulated fiber with a scalpel. The microelectrode was inspected under the microscope for a smooth cutting and further characterized by means of cyclic voltammetry.

Retzius cells from medicinal leeches were dissected and cultured as described previously.[16] PC12 cells were grown on poly(ornithine)-covered glass slides following a procedure described elsewhere.[17]

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"Supramolecular" Solid-State Chemistry: Interpenetrating Diamond-Type Frameworks of U^{4+} Ions Linked by *S,S'*-Bidentate $P_2S_6^{2-}$ Molecular Rods in UP_4S_{12}**

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Organic compounds can self-assemble into ordered arrays at low temperature that are capable of molecular recognition, and that can act as biomimetic systems.[1] As a result, organic supramolecular chemistry has afforded many intriguing results,[2] including various three-dimensional (3D) hydrogen-bonded frames of super-diamond-type[3] and super-wurtzite-type structures.[4] In the field of inorganic and coordination polymers, Hoskins and Robson[5] have proposed a strategy for the design of new 3D phases, referred to as

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