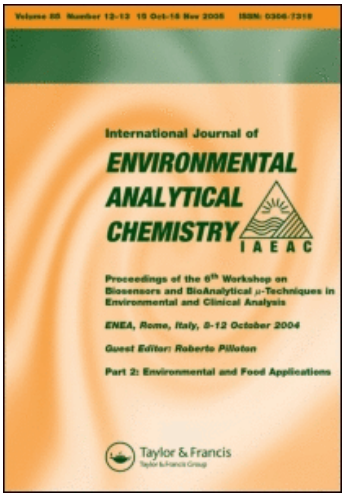

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[a] Institute for Electronic Devices and Circuits, University of Ulm, Germany [b] Department of Neurosciences, NIS Center, CNISM Unit, University of Torino, Italy

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[a]*Institute for Electronic Devices and Circuits, University of Ulm, Germany;* [b]*Department of Neurosciences, NIS Center, CNISM Unit, University of Torino, Italy*

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Diamond applications potential for biosensing devices have been highlighted by several authors, especially concerning the long-term stability of covalent functionalisations on its surface. Additionally, in electrochemistry boron doped diamond electrodes ($N_A \sim 10^{20}\,cm^3$) show high corrosion resistance and a large hydrolysis window. These features, recognised and exploited in industrial applications, have up to now found little resonance in the life-sciences. Here we present diamond microelectrode arrays based on (1) nanocrystalline diamond (NCD) thin films and (2) single crystal diamond (SCD). NCD is necessary for large area applications like arrays, but graphitic grain boundaries may influence its behaviour. The ideal case SCD is covered here for comparison. The array design consists of four electrodes whose sensitive area is delimited by means of a patterned photoresist. Two different patterns were used to realise a layout with four independent openings (15 μm diameter) for simultaneous detection on multiple cells and a layout with one single window (25 μm diameter) intersecting all four electrodes to create a quadrupolar detector suitable for mapping the activity of single cells. Early results validated the suitability of both NCD and SCD devices: (1) cyclic-voltammetry measurements confirmed the adrenaline oxidation potential on the presented microelectrodes around 650 mV; (2) alternating applications of 1 mM adrenaline and saline rinsing solutions showed negligible electrode fouling; and (3) interfaced to single adrenal chromaffin cells, the devices clearly detected sustained sequences of quantal events (10–100 pA amplitude, 50–100 ms duration) associated to the vesicular release of adrenaline and noradrenaline during exocytosis induced by cell-depolarisation.

Keywords: redox; catecholamines; amperometry

1. Introduction

Diamond is a material with outstanding properties: it is a wide band-gap semiconductor, i.e. the intrinsic (undoped) crystals are electrically highly insulating, but they show quasi-metallic conductivity when doped. Diamond is optically transparent over a very wide range from the far infrared to deep-UV (225 nm), it is chemically inert, remains stable in a range of pH 0 to pH 14 and is etch-resistant to nearly all wet-chemical processes. It also exhibits a

*Corresponding author. Email: alberto.pasquarelli@uni-ulm.de

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large potential window for water dissociation, approximately 3 Volts (Figure 1), and as a consequence thereof it is the only semiconductor material used in heavy duty electrochemistry such as purification of wastewater and electrochemical analysis of hazardous substances [1,2]. All these excellent properties are referred to single crystal diamonds (SCDs), i.e. macroscopic crystals having a single lattice, which extends over the whole sample volume and has a negligible amount of defects. For industrial applications, nanocrystalline diamond (NCD) deposited onto silicon substrates by chemical vapour deposition (CVD) is the configuration of choice, due to the possibility of manufacturing large area devices at an affordable price. NCD films consist in a multitude of very small diamond crystals having dimensions ranging between tens and hundreds of nanometers. Depending on the parameters used during the growth process, these crystals can either show a quite uniform orientation (so-called two dimensional NCD or highly oriented NCD) or a random orientation (three dimensional NCD). In all cases, the polycrystalline structure implies two major problems: (1) at the grain boundaries there is a certain amount of graphitic phase (sp^2), which can degrade the electrochemical properties, and (2) any other polycrystalline semiconductor NCD shows a lower mobility of the charge carriers when compared to SCD. For this reason NCD materials cannot be used for high-frequency applications, but they can perfectly fit the bandwidth requested for most bio/electrochemical needs. This technology has indeed gained strong interest as functional material for biological and biochemical applications, especially in the field of biosensors [3].

In electrochemistry, doped diamond devices belong, like gold and platinum, to the family of redox electrodes. Therefore they are primarily suitable for amperometric applications. Due to the much wider hydrolysis potential window when compared to competitor materials, diamond electrodes allow the detection of substances which undergo electrochemical reduction or oxidation well beyond the water dissociation potential (1.23 V). Its high electrochemical activity makes diamond also very sensitive and fast in temporal response.

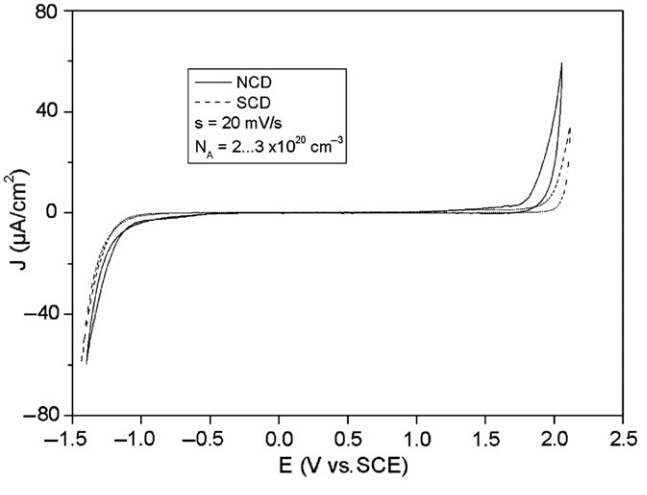

Figure 1. Cyclic voltammetry scan showing the hydrolysis potential window of SCD and NDC electrodes.

Under these premises the amperometric detection of neurotransmitter release appears as a straightforward application for diamond microelectrodes. This method is highly relevant for analysing the behaviour and determining the state of neurons and neuroendocrine cells [4].

This work was focused on the quantal detection of catecholamines (mainly adrenalin and noradrenalin) released from adrenal chromaffin cells. At present, the 'gold standard' to resolve single secretory events is the use of carbon-fibre microelectrodes polarised to +650–800 mV to detect the target molecules by electrochemical oxidation [5,6].

Amperometric burst-signals can be recorded for seconds or minutes depending on the applied stimulus. Each spike is related to the fusion of a single vesicle, i.e. a quantal release of catecholamines. Carbon fibres work with high time resolution, high-sensitivity and are not invasive, but they also show serious drawbacks, being limited in use with one cell at a time and due to the difficult concurrent utilisation of a lateral glass-pipette microelectrode, for amperometric detection with simultaneous patch-clamp recording.

Considering all these issues we have been working on the development of transparent planar diamond microelectrodes able to keep the entire working field obstacles free, thus allowing amperometric, patch-clamp and even fluorescence investigations from the same cell at the same time. After producing and testing a device on a single diamond crystal, in order to determine the reference performances achievable with this almost ideal material, the activities moved to the fabrication of NCD-on-silicon devices which are more convenient for technological processing, but may have lower performances. During an intense experimental activity the process parameters could be fine tuned in order to progressively achieve nearly identical performances as with the SCD device. As a further improvement, we developed a technology to produce NCD thin film devices on sapphire wafers, commonly used as substrates for GaN based electronics. In this way, this approach opens the potential perspective of integrating an electrode array with GaN-based field effect transistor electronics as already demonstrated in a first proof-of-concept experiment [7].

2. Experimental

2.1 *Reference device on single crystalline diamond*

This device was fabricated starting from a Sumitomo HTHP synthetic Ib diamond substrate of 4 mm × 4 mm in size, onto which a mono-epitaxial layer of boron doped diamond 180 nm thick with an acceptor-atoms concentration of $\sim 10^{20}\,cm^{-3}$ was deposited by Microwave Plasma Enhanced CVD (MW-PECVD). This yielded a quasi-metallic conducting layer with a sheet resistivity of ~600 Ohm/□, which was processed by means of lithographic and dry chemical techniques to obtain four convergent independent planar conducting stripes. These structures where insulated by means of a 5 μm thick epoxy-based photoresist film (insensitive to most cleaners and sterilisers used in biology), where four circular openings with a diameter of 16 μm and a pitch of 50 μm (Figure 2a) were obtained by means of a usual lithographic method in order to define the arrangement and the sensitive area of the microelectrodes. The surface was then O-terminated in oxygen plasma to enhance the cell adhesion without the use of biological substrates (laminin, polylysine, collagen, etc.), which adversely affect the amperometric operation of the planar electrodes. The efficacy of this termination method was independently confirmed by means of wetting-angle and XPS measurements.

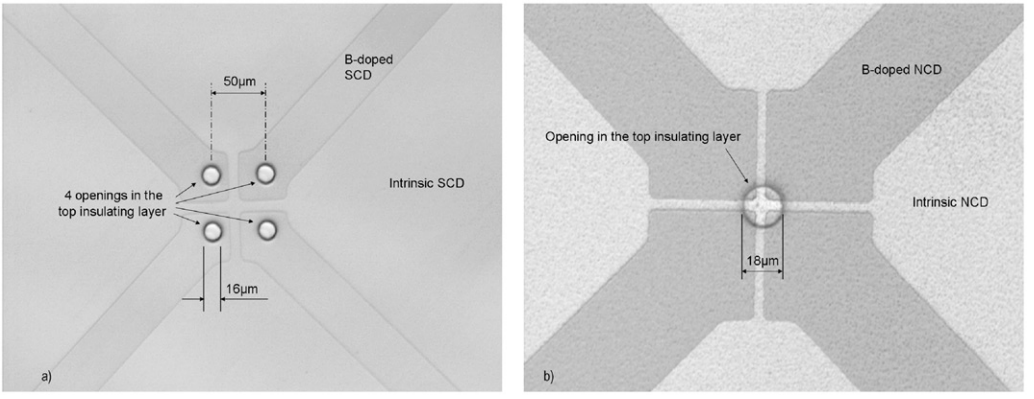

Figure 2. (a) SCD device with four separated circular apertures, having 16 µm diameter and 50 µm pitch. (b) NCD quadrupole on sapphire substrate with a single aperture 18 µm in diameter. Due to limited precision in the alignment of the lithographic mask, the contact area is slightly asymmetric, leading to different signal-to-noise ratios among the electrodes.

Finally the 4 mm large array was packaged and bonded onto a custom-made ceramic chip carrier, designed to provide very low leakage currents, and fitting onto the device a glass ring to provide an adequate perfusion chamber.

2.2 *Thin-film devices on nanocrystalline diamond*

The above-described electrode array process has been transferred to two NCD configurations, the first being a standard diamond-on-Si MEMS process by using a Hot-filament CVD (HF-CVD) process on 4 inch diameter substrates [8]. Here first, after nucleation by bias enhancement (BEN), a 1 µm buffer layer was grown followed by a 200 nm thick quasi-metallically boron doped electrode layer. Obviously in these cases the conducting layer was not mono-epitaxial because of the NCD nature of the underlying insulating layer. However, depending on the outgrowth conditions and doping profiles employed, the influence of the grain boundaries on the electrochemical characteristics (mainly the background current in the absence of redox reactions and water dissociation potential window, as shown in Figure 1) could be widely suppressed. Nevertheless, a wet-chemical process had been used to remove any graphitic clusters.

To enable fluorescence labelling and optical detection, a free suspended NCD membrane of 3 mm in diameter was obtained by removing the corresponding part of the silicon substrate by an inductively coupled plasma etching process (ICP). On these NCD samples different lithographic patterns for the photoresist insulation layer were used to realise devices with a layout having four independent openings (16 µm diameter and 50 µm pitch) for simultaneous detection on multiple cells, and other devices having a layout consisting of one single window (18 µm diameter) intersecting all four electrodes to create a quadrupolar detector suitable for mapping the activity of single cells.

We carried out several experiments and obtained important results regarding the suitability of the electrodes for the target application (as discussed later on), but at the same time it turned out that the mechanical stability of the 1.2 µm thick membranes was a

critical issue, especially in the case of ultrasonic cleaning. For higher mechanical stability essentially thicker membranes and thus diamond films would be needed. However, due to the nanocrystalline nature of the material, a significantly thicker diamond film could appreciably reduce the transparency of the membrane due to increased light scattering at the grain boundary network.

Thus as alternative technology, the thin NCD films have been deposited onto 330 μm thick sapphire wafers. Sapphire is highly transparent, electrically highly insulating and is the standard substrate for GaN based optoelectronics and electronics. In fact sapphire wafers with high crystalline quality are commonly available for diameters up to 150 mm and more. Onto such substrates, diamond films consisting of a 400 nm thick intrinsic buffer capped with a 300 nm thick quasi-metallically boron doped layer with high electrochemical quality could be successfully deposited using our standard HF-CVD process [8].

We then manufactured NCD on sapphire devices with the same quadrupolar layout (Figure 2b) used for the NCD on silicon microarray. Since sapphire is perfectly transparent in the spectral range from 0.3 μm (near UV, the region of interest for fluorescence) to 5 μm (IR), this new chip fits the needs of biological fluorescence investigations and is at the same time very robust, i.e. it can be cleaned in an ultrasound bath without problems.

2.3 *Instrumentation*

We built a custom front-end with a four channel voltage-biased current amplifier having a gain of $10^8\,\mathrm{V\,A^{-1}}$ and a background spectral noise density of 50 fA/sqrt(Hz). All signals are low-pass filtered by means of a 4th order Bessel-filter at a corner frequency (−3dB) of 1 kHz and then acquired using a USB-6216 unit from National Instruments, sampling at rate of 4 kHz per channel with 16 bits resolution. A LabView application developed by the authors was used to control the acquisition. The overall background noise of the measurement system is lower than 10 pA peak-to-peak, when working with a Tyrode solution as electrolyte and an Ag/AgCl ground-electrode.

3. Results and discussion

3.1 *Characterisation*

First the basic electrochemical behaviour was examined, showing negligible dark current in absence of redox species. Cyclic-voltammetry measurements were used to investigate (a) possible artefacts due to inorganic and organic substances present in the solution used for cell manipulation and (b) the oxidation activity of adrenaline on the microelectrodes. Those measurements showed that a standard Tyrode buffered solution (pH 7.4), produces no artefacts; a DMEM culture solution gives rise to little oxidation artefacts at ~900 mV, but poly-L-lysine (PLL) resulted in a complete loss of redox properties of the diamond electrodes, turning them into a nearly ohmic solid/liquid interface. The latter is due to adsorption of PLL to the diamond surface, which results in the modification of the electrode properties. In fact the presence of PLL does not change significantly the conductivity in the bulk of the solution (as can be measured with a pair of non-polarisable Ag/AgCl electrodes), but introduces, with its polyelectrolyte structure, different properties in the solid/liquid junction. Using a 1 mM adrenaline solution, oxidation activity in

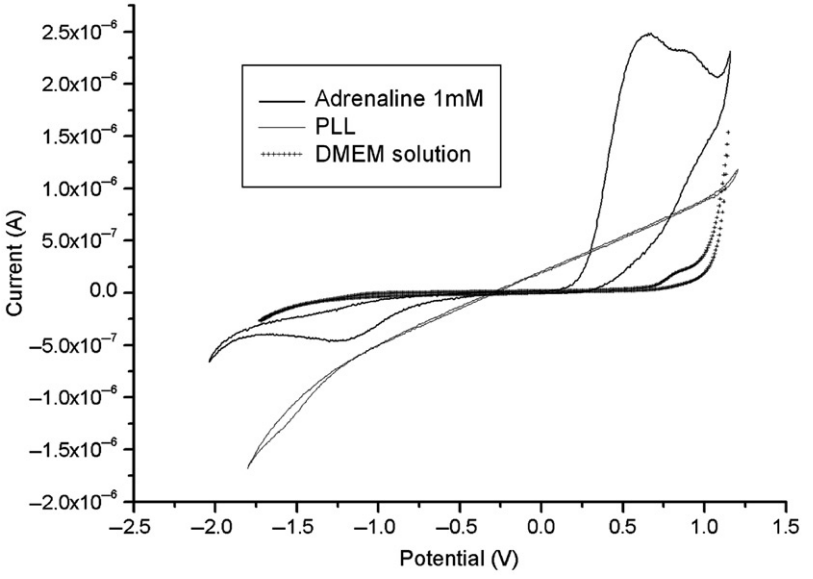

Figure 3. Cyclic voltammetry of 1 mM Adrenaline in Tyrode buffer solution with NCD working-electrodes against Ag/AgCl reference electrode. Scan speed is 50 mV s^{-1}.

Tyrode buffer could be detected starting at ~400 mV and peaking between 650 and 700 mV, confirming the suitability of the described microelectrodes for the target application. These results are shown in Figure 3.

In addition the optical transparency of the NCD-electrodes on sapphire was tested in the spectrum range of interest. Experimental results showed some relevant absorption at short wavelengths. Since crystallites in the analysed NCD film have an average size of 70 nm, this behaviour cannot be simply explained as scattering phenomenon at the grain boundaries. In fact high transmittance up to the near UV has been reported for NCD thin films with comparable grain size, deposited onto transparent substrates [9]. To better understand the origin of this absorption the measurements were repeated after the main layers deposition (Figure 4) and the most suitable explanation for the observed spectral behaviour appears to be linked to the silicon interlayer used to ease the nucleation of diamond. The increase of transmittance at the short wavelengths after the growth of the intrinsic NCD film could be explained with a partial etching of the Si-buffer during the diamond nucleation at the beginning of the deposition process. It is evident that the present technology needs further improvements, e.g. an optimal thickness minimisation of the Si-based interlayer, to exploit the full dynamic range (~four orders of magnitude) of the fluorescence detection.

3.2 *Cell preparation*

We adopted isolated chromaffin cells prepared from mouse adrenal glands. Immediately after explant, the glands were placed in Ca^{2+} and Mg^{2+} free Locke's buffer containing (in mM): 154 NaCl, 3.6 KCl, 5.6 $NaHCO_3$, 5.6 glucose, and 10 HEPES, pH 7.2, at room temperature. Soon after the glands were decapsulated, to precisely separate the medulla from the cortical tissue. The medulla was digested in the Locke's buffer mentioned before,

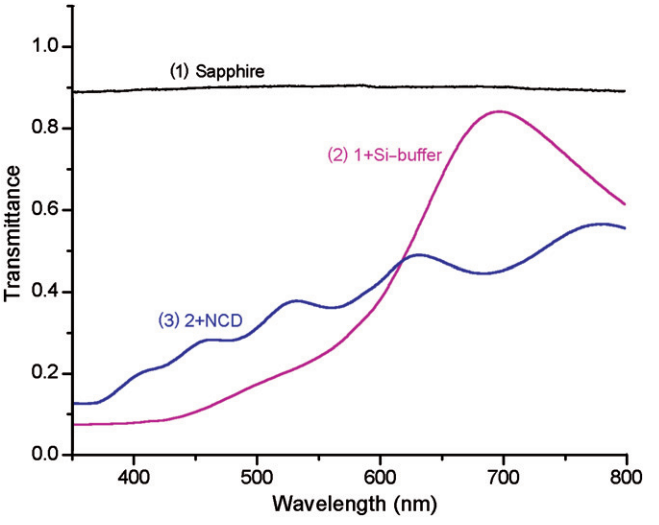

Figure 4. Transmittance measurements at progressive steps: (1) the sapphire substrate alone; (2) sapphire with the silicon buffer layer; (3) final device. The measured data shows significant absorption at shorter wavelengths, which appears to be mainly linked to the silicon-buffer.

containing 20 U ml^{-1} of papain (Worthington Biochemical Corp., Lakewood, NJ) for 60 min at 37°C. Finally the cell suspension was centrifuged for 5 min at 900 rpmi, washed two times, and resuspended in 2 mL DMEM supplemented with 15% foetal calf serum (FCS).

3.3 *Cell measurements*

The typical experimental workflow consisted in placing an isolated cell onto one electrode and setting the biasing voltage between 650 and 800 mV. The cell was then depolarised by dispensing an external solution containing 135 mM TEACl and 10 mM $CaCl_2$. This depolarisation yields a Ca^{2+} influx through voltage-dependent Ca^{2+}-channels, causing exocytosis by vesicle fusion and consequent release of catecholamines. All SCD- and NCD-electrodes could clearly detect persistent quantal release activity lasting two minutes or more, after which exocytosis was limited by Ca^{2+}-channel inactivation. The detected amperometric spikes had peak amplitude of 5 to 80 pA and duration spanning between few milliseconds and several tens of milliseconds, accounting for different vesicles sizes and/or different location on the cell membrane of the exocytotic events (Figure 5a).

More detailed information can be delivered by measuring the cells secretory activity onto the quadrupolar structure with a single opening. In fact it was possible to carry out a few preliminary experiments using the latest NCD on sapphire devices and obtain recordings of simultaneous detection of release events, with different intensities on the individual electrodes (Figure 6).

Under the present experimental conditions the different device technologies did not show significant performance differences. The 'dark current' was in all cases negligible (~10 pA) and also the background noise was practically invariant (Figure 5b). This can be explained by considering (a) the frequency range of the detected signal (DC ~ 1 kHz) is

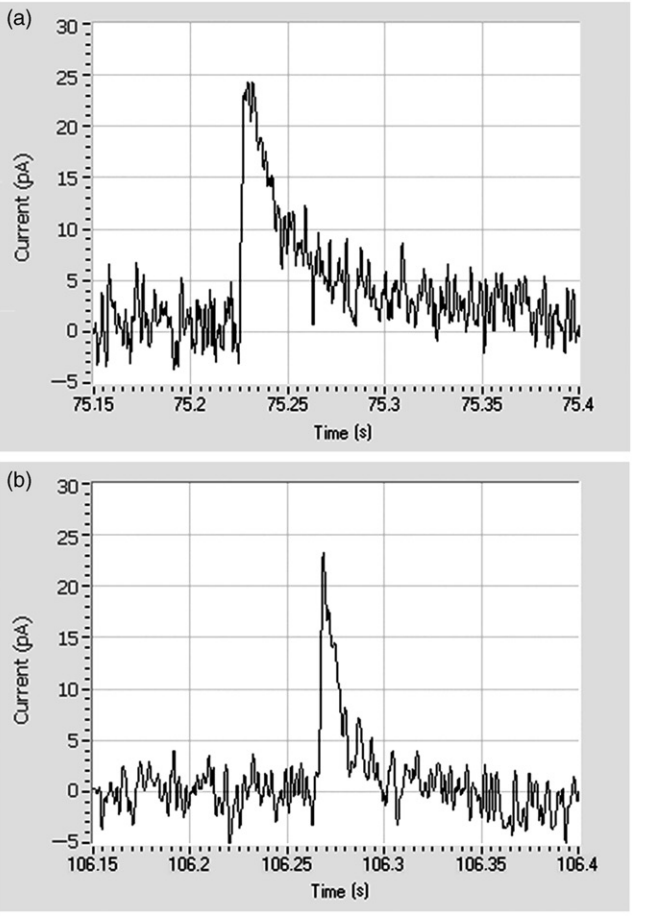

Figure 5. Single quantal release event recorded (a) with an SCD microelectrode and (b) with an NCD on silicon device. Noise level, reaction time and sensitivity are very similar. Since the cell is steadily depolarised, the release events appear randomly. The time scale indicates the time from the start of recording.

perfectly suitable for materials with reduced carrier mobility like nanocrystalline semiconductors and (b) the significant efforts put in optimising the electrochemical properties of the NCD surface, with special emphasis in the reduction of the graphitic phase at the grain boundaries, which is usually responsible for large dark currents.

3.4 *Data analysis*

We analysed the amperometric spikes by means of specific macros developed for the scientific software environment IGOR Pro. As shown in Figure 7, the analysis consisted in measuring for every individual exocytotic event the following parameters: maximum oxidation current (I_{max}), full width at half maximum ($t_{1/2}$), total charge collected (Q), rising slope (m) and time to reach the peak maximum (t_P). Here the goal was to compare time course and amplitude of the spikes recorded by NCD devices

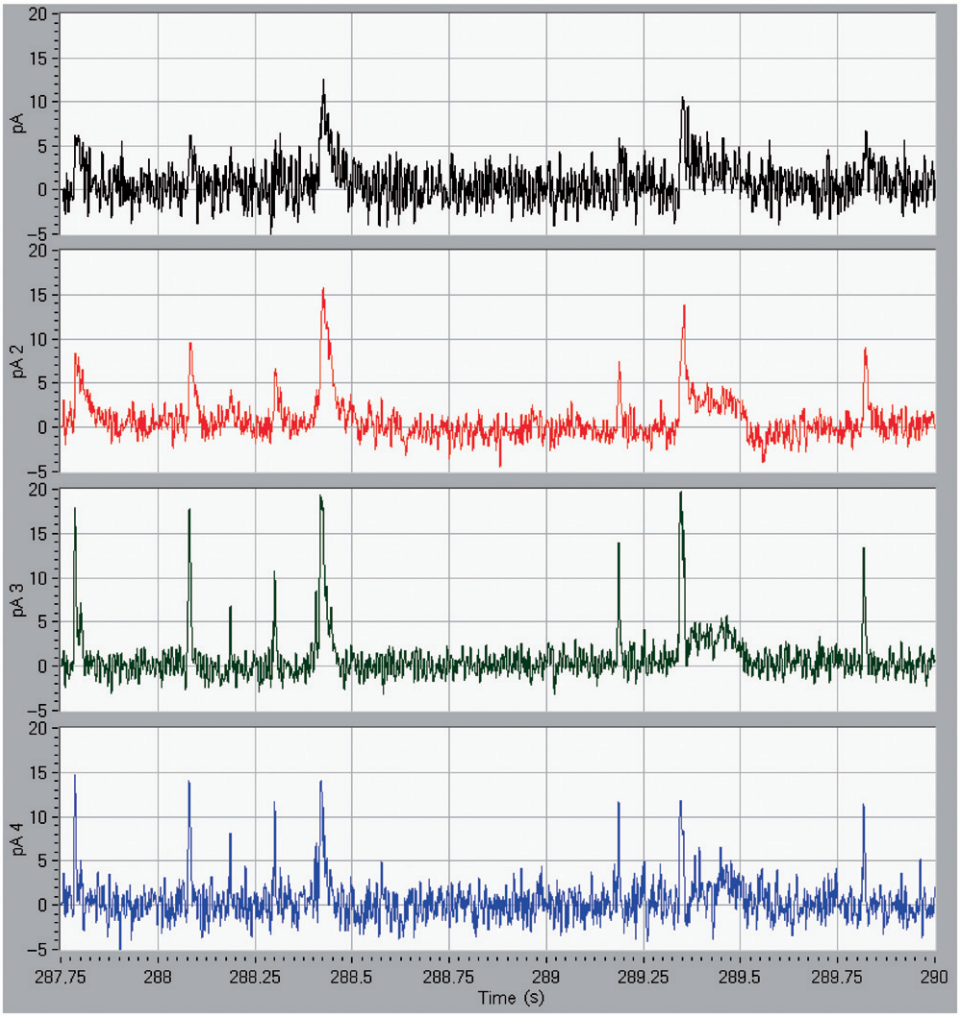

Figure 6. Quadrupolar detection of an exocytosis. The amplitude of the current signals detected on the four electrodes depends on the location of the exocytotic place on the cell membrane with respect to the electrodes. A proper analysis algorithm will allow the localisation of this activity place for every single vesicle fusion event.

with data obtained using standard carbon fibres. The average results obtained after analysing a random collection of 116 spikes extracted from 29 cell recordings are reported in Table 1 together with data from 269 spikes detected with carbon fibre out of 11 experiments. These two datasets show a very close match indeed between the two methods.

4. Conclusion

These results suggest that the presented planar diamond-based devices allow accurate measurements of single exocytotic events, with high time resolution, comparable to those

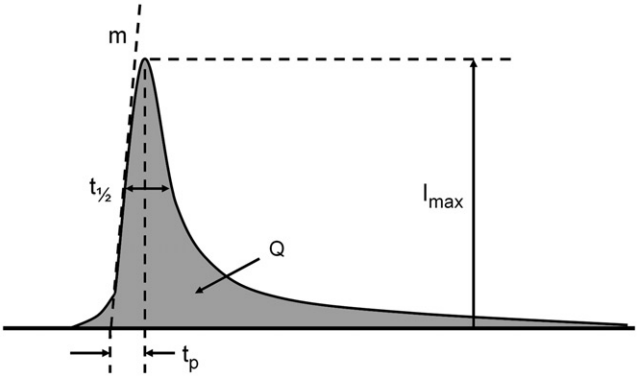

Figure 7. Parameters definition for the analysis of the spike signals.

Table 1. Results of spikes analysis. Comparison between data from NCD planar electrodes and data from carbon fibre microelectrodes. The additional parameter $Q^{1/3}$ is the mean cube-root of the collected charge Q and is related to the diameter of the fused vesicle.

	Imax (pA)	Q (pC)	$Q^{1/3}$ ($pC^{1/3}$)	$t_{1/2}$ (ms)	m (pA/ms)	t_p (ms)
Results of 116 spikes from 29 different cells recorded with NCD devices						
Mean value	31.54	0.37	0.68	9.28	11.49	7.23
Standard error	2.14	0.03	0.015	0.68	1.28	0.73
Results of 269 spikes from 11 different cells recorded with carbon fibre electrodes						
Mean value	33.43	0.35	0.61	7.11	15.43	9.06
Standard error	5.72	0.07	0.05	0.77	3.07	3.18

of classical carbon fibres. However, full advantage of measuring secretion from a planar detecting area will be achieved by exploiting the spatial resolution of the quadrupolar layout and its potential ability to localise, for every single exocytosis, the membrane areas of the cell where vesicle fusion events take place. To the purpose our preliminary experiments with the quadrupolar array show good sensitivity and appear able to furnish relevant topographic information once an adequate data processing algorithm becomes available. We are working to achieve this goal.

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