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A diamond-based biosensor for the recording of neuronal activity

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ABSTRACT

We have developed a device for recording the extracellular electrical activity of cultured neuronal networks based on a hydrogen terminated (H-terminated) conductive diamond. GT1-7 cells, a neuronal cell line showing spontaneous action potentials firing, could maintain their functional properties for days in culture when plated on the H-terminated diamond surface. The recorded extracellular electrical activity appeared in the form of well-resolved bursts of fast and slow biphasic signals with a mean duration of about 8 ms for the fast and 60 ms for the slow events. The time courses of these signals were in good agreement with those recorded by means of conventional microelectrode array (MEAs) and with the negative derivative of the action potentials intracellularly recorded with the patch clamp technique from single cells. Thus, although hydrophobic in nature, the conductive H-terminated diamond surface is able to reveal the spontaneous electrical activity of neurons mainly by capacitative coupling to the cell membrane. Having previously shown that the optical properties of H-terminated diamond allow to record cellular activity by means of fluorescent probes (Ariano, P., Baldelli, P., Carbone, E., Giardino, A., Lo Giudice, A., Lovisolo, D., Manfredotti, C., Novara, M., Sternschulte, H., Vittone, E., 2005. Diam. Relat. Mater. 14, 669–674), we now provide evidence for the feasibility of using diamond-based cellular biosensors for multiparametrical recordings of electrical activity from living cells.

1. Introduction

The recording of electrical activity from neuronal networks, both *in vitro* and *in vivo*, has experienced substantial advances in the last few years, owing to the design and fabrication of improved microelectrode arrays and to the development of suitable software tools (Le Van Quyen and Bragin, 2007; Hofmann and Bading, 2006). By these means, long time recordings of electrical activity (see e.g. Potter, 2001) and of its modulation by agonists and pharmacological agents from single elements of a neuronal population have seen widespread applications (see e.g. Stett et al., 2003). Most systems presently available suffer, however, from some limitations, particularly regarding surface reactivity and optical transparency, critical for obtaining long term recordings of multiple parameters (e.g. optical and electrical).

Among the materials investigated as potential substrates for the fabrication of biosensor devices, diamond is a relatively new entry; however its peculiar properties, such as chemical inertness, biocompatibility, optical transparency, high conductivity when the surface is properly functionalized, have attracted increasing interest from biochemists, biophysicists and material scientists (see e.g. Martinez-Huitle, 2007; Carlisle, 2004). Relevant results are represented by the development of diamond-enzyme interfaces exploiting electron transfer mechanisms (Härtl et al., 2004; Zhao et al., 2006) and of microchambers for DNA synthesis (Adamschik et al., 2001). These examples highlight the feasibility of building diamond-based molecular biosensors: the main biological achievement would be however to develop cellular sensors to record electrical and optical activity from cultured cells and by means of implanted devices. Some preliminary steps have been accomplished: one group has described the ordered growth of neurons and of their processes over single, oxidised diamond crystals on which grids of adhesion molecules were drawn (Specht et al., 2004); others (Chong et al., 2007) have described how adhesion and survival of fibroblasts and non-differentiated PC12 neuronal cells (both electrically unexcitable) can be modulated by surface topography and functionalization. In a previous paper (Ariano et al., 2005), employing more physiologically relevant models (primary cultures

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of chick ciliary ganglia and rat hippocampal neurons), we have shown that H-terminated epitaxial diamond can be used as a convenient substrate to grow viable neuronal cultures and that its optical properties allow neuronal activity by means of fluorescent probes to be recorded.

Up to now, however, the last key step was missing, *i.e.* the evidence that conductive H-terminated diamonds can be successfully employed in the fabrication of electrodes for recording electrical signals from excitable cells. This task has been now accomplished: in the present paper we provide evidence that by using a single macroelectrode of about 1 mm^2 surface it is possible to record electrical activity from a population of spontaneously firing neurons.

2. Materials and methods

2.1. Hydrogen terminated diamond electrode

We used a commercially available (Sumitomo Electric Industries, Japan) high purity (IIa type) 5 μm thick diamond layer epitaxially grown onto a Ib type HPHT (high pressure high temperature) diamond substrate, with (1 0 0) orientation and $(3 \times 3 \times 0.5)\,mm^3$ dimensions. The homoepitaxial layer showed a surface roughness at the nanoscale (root mean square roughness below 3 nm), as observed by $20 \times 20\,\mu m^2$ non-contact AFM maps (Manfredotti et al., 2003).

The as-purchased sample was first oxidised in a sulfochromic mixture at 170 °C and in a boiling solution of H_2O_2:NH_4OH, in order to remove contaminants and to oxygen terminate the surface. After this treatment the surface was hydrophilic and highly electrically resistive; the resistance between the two inner tips of a four-point probe with a distance 0.635 mm was higher than $10^9\,\Omega$.

The oxidised sample was annealed for 1 h at 900 °C in high vacuum conditions (10^{-5} Pa) to induce desorption of oxygen and residual adsorbates. After 2 h in high vacuum conditions, surface hydrogenation was carried out in a hot filament CVD reactor, locating the samples for 1 h at a distance of 2 cm from two Ta hot (2100 °C) filaments and using purified hydrogen gas (90 sccm) at a pressure of about 3000 Pa (Fizzotti et al., 2007).

The HTD surface was hydrophobic (wetting angle around 90°) and, after a transient of a few hours in humid ambient air, the resistance measurements showed a stable value of stable resistance between the two inner tips of the four-point probe of $(58 \pm 2)\,k\Omega$, corresponding to a sheet resistance of $(23 \pm 1)\,k\Omega$/sq. The need for air exposure to recover a high surface conductivity is suitably interpreted by the electrochemical transfer-doping model: the hydrogen termination lowers the ionization energy of diamond and a quasi two dimensional hole accumulation layer is induced by the electron transfer which takes place between diamond valence band and a thin aqueous wetting layer as it forms spontaneously on all surfaces in air (Maier et al., 2000). Assuming a hole mobility of $60\,cm^2\,V^{-1}\,s^{-1}$, the sheet hole concentration relevant to a sheet resistance of 23 kΩ/sq of the hydrogen terminated surface, is about $5 \times 10^{12}\,cm^{-2}$, in good agreement with experimental data available in literature (Ristein, 2006).

The optical transmittance in the visible range, not influenced by the surface termination, is shown in Fig. 1, inset. The transparency of the substrate allowed the experiments to be carried out in a conventional electrophysiological set-up, using an inverted microscope for cell imaging and cytofluorimetric recordings.

2.2. Electrode mounting

The diamond was attached to a high resistance printed circuit board shaped to fit the inverted microscope stage (Fig. 1). Two

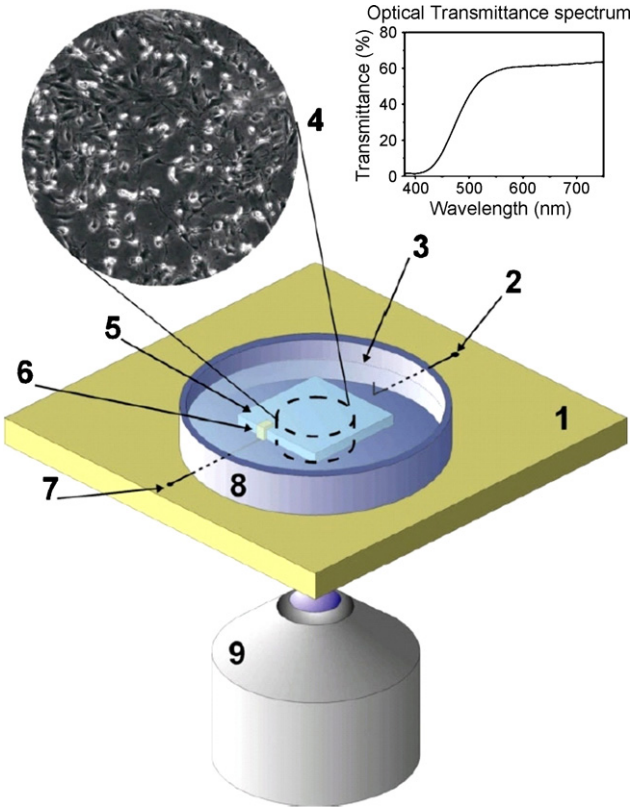

Fig. 1. Scheme of the experimental set-up (1) high resistance board; (2) silver/silver chloride reference electrode; (3) extracellular medium; (4) cell culture; (5) hydrogen terminated diamond; (6) silver paste bond; (7) connection to the amplifier circuit; (8) housing; (9) microscope objective. The dashed lines indicate the hole drilled in the board for optical observation and corresponds to the area on which cells are plated. A sylicone elastometer passivates bondwires, interconnects and the diamond surface except for the area inside the dashed line. Inset: optical transmittance spectrum of the diamond substrate.

conductive copper pathways were connected respectively to the diamond surface by means of a 20 μm gold wire and a silver paste bond and to a silver chloride reference electrode placed in the extracellular medium. The electrical insulation of conductors, interconnects and bondwires from the electrolyte was ensured by a silicone elastomer. The fraction of the diamond surface acting as recording area was about 1 mm^2.

2.3. Signal recording

Electrical signals from cells cultured on the diamond surface were fed into a low noise (<5 μV_{RMS}) amplification ($G = 10^4$) and filtering (bandwidth: 0.02–24 kHz) custom stage before signal digitalization and recording (Obeid et al., 2004). Signal acquisition and analysis were performed by means of a Digidata 1440 board and pClamp10 software (Molecular Devices, Toronto, Canada).

2.4. Recording from MEAs

For simultaneous recording of extracellular action potentials from 60 metallic microelectrodes (diameter: 30 μm) we used an array of 60 Titanium nitride (TiN) microelectrodes (MEA1060, Multichannel System GmbH, Reutlingen, Germany) already successfully used with other excitable cells (see e.g. Martinoia et al., 2005; Marcantoni et al., 2007). Data from the 60 channels were acquired at 10 kHz and not low-pass filtered.

2.5. Cell cultures

GT1-7 cells (generously donated by Prof. P.L. Mellon) were grown in monolayer at 37 °C in a humidified atmosphere of 5% CO_2/air, in DMEM (4500 mg/l glucose) containing 2 mM glutamine, 50 μ/ml gentamycin, supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA). GT1-7 cells were chosen after having performed extensive trials with several other neuronal models, both primary neurons and neuronal lines, and having experienced significant difficulties in obtaining cultures with: (i) good adhesion properties in the absence of massive substrate functionalization with extracellular matrix proteins that could have hindered the physical interaction between the diamond surface and the neuronal membrane; (ii) an optimal balance between the expression of differentiated properties of excitable cells and a proliferative rate sufficient to achieve a coverage of a significant percentage of the electrode surface in a few days of culture; (iii) a neuronal population endowed with autorhytmic properties and thus amenable to the recording of its electrical activity in the absence of exogenous stimulation (Funabashi et al., 2001). On the basis of these requirements, GT1-7 cells represent an appropriate model of differentiated, electrically excitable and autorhytmic neuronal cells that can be grown at high density.

For the recordings, cells were plated on poly-L-lisin (PL) coated hydrogen terminated diamond in the presence of B27 medium supplement (Invitrogen) and cultured for 5–7 days (Funabashi et al., 2001). During the recordings, the medium was replaced with standard Tyrode solution of the following composition (in mM): 130 NaCl, 4 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4, unless otherwise specified.

3. Results

In five experiments, electrical activity could be recorded from GT1-7 cells plated at high density on the fraction (1 mm^2) of the HTD surface free of the silicone insulating layer covering interconnects and bond pads. Consistently with previous observations by other groups (Funabashi et al., 2001; Pimpinelli et al., 2003), electrical activity was episodic, with bursts separated by silent intervals.

Fig. 2A shows typical recordings from GT1-7 cells plated at high density on HTD. In order to check that the signals recorded from HTD were due to the electrical activity of the cells, we changed the external bathing solution from the Tyrode solution to one containing 0 mM Ca^{2+} and 300 nM tetrodotoxin (TTX), a selective blocker of voltage dependent Na^+ channels, thus inhibiting any voltage-dependent Na^+ or Ca^{2+} influx and as a consequence the generation of APs: in these conditions, the activity was completely and reversibly abolished (Fig. 2C, the insets show two stretches of the traces at a more expanded time scale).

As for the duration of the signals, trains of fast (a few ms) spikes could be observed (Fig. 2B, lower trace), as well as more frequent slower signals, lasting some tens of ms (Fig. 2B, upper trace). The fast signals from the HTD electrode could be ascribed to the firing of isolated neurons. The slow signals were likely the result of the synchronized activity of clusters of cells. Since in GT1-7 cells clusterization is a prerequisite for the emergence of synchronized electrical activity (Funabashi et al., 2001), and since we were recording the collective behavior of a population of densely packed cells plated on a single macroelectrode, it is quite reasonable that slow signals were the most common.

Fig. 2D shows the histograms of the rise times (i.e. the time interval between 10% and 90% of peak amplitude) of 206 signals from three experiments. A major peak is at 2.5 ms; the distribution is quite scattered, in accordance with the hypothesis that the recorded activity originates from multiple clusters with several synchronously firing units.

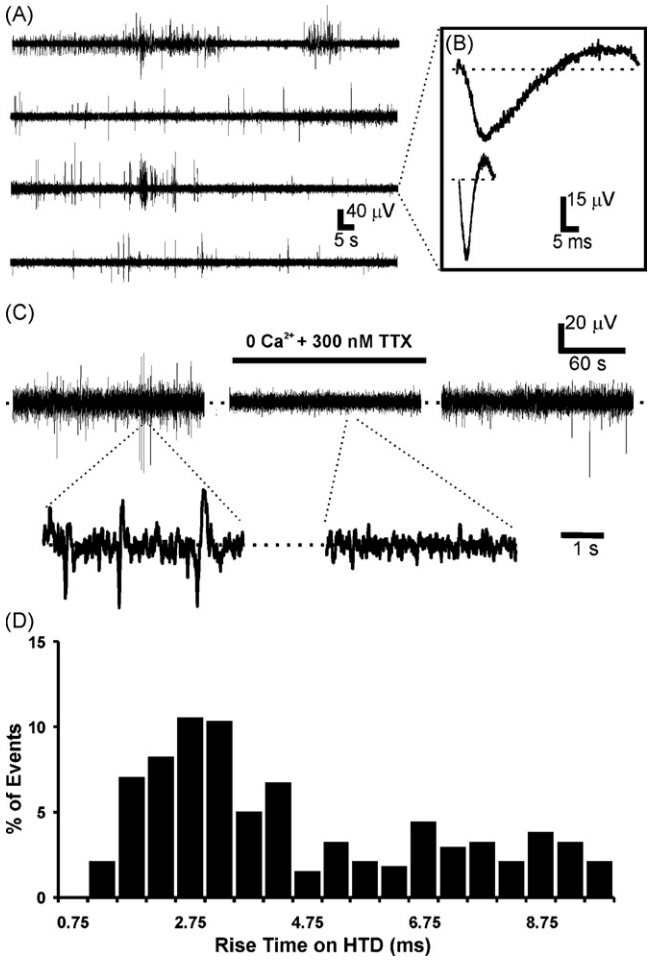

Fig. 2. Electrical activity form GT1-7 cells recorded by means of HTD electrode. (A) Three traces showing episodic bursts of extracellular signals. (B) Top trace: average of a sample of $n = 10$ slow signals; lower trace: average of a sample of $n = 30$ fast signals. (C) Signal recording after the substitution of the standard Tyrode solution with a similar one containing 0 Ca^{2+} and 300 nM of the voltage dependent Na^+ channel blocker tetrodotoxin (TTX). The insets show stretches of the traces on a expanded time scale. (D) Histogram of the rise times of 206 extracellular signals from three experiments.

These signals, and their time courses, were compared with those recorded from single electrodes on TiN microelectrode array. They consisted in most cases of asynchronous trains of fast spikes of 20–30 μV (Fig. 3A and B), characterized by an early inward (negative) deflection lasting 2–4 ms and a late outward (positive) phase lasting 5–7 ms. On average, a fast spike was complete within 8–10 ms (Fig. 3C, upper trace).

Recordings from MEAs showed also slower signals, which occurred synchronously at different electrodes (arrows in Fig. 3A and B) covering a relatively large area of the recording system (0.8×0.2 mm^2 for seven microelectrodes). These slow signals (Fig. 3C, lower trace), strongly resembling those recorded with the HTD macroelectrode (Fig. 2B, upper trace), are most likely derived from the synchronized activity of a cluster of neurons simultaneously active around a single microelectrode. This complex activity gives rise to a series of APs whose capacitative effects sum up to originate broad extracellular recordings with slow negative and positive voltage deflections lasting 40–100 ms. The correspondence of MEAs recordings with those using HTD electrodes is strengthened by the rising time distribution of slow events shown in Fig. 3D.

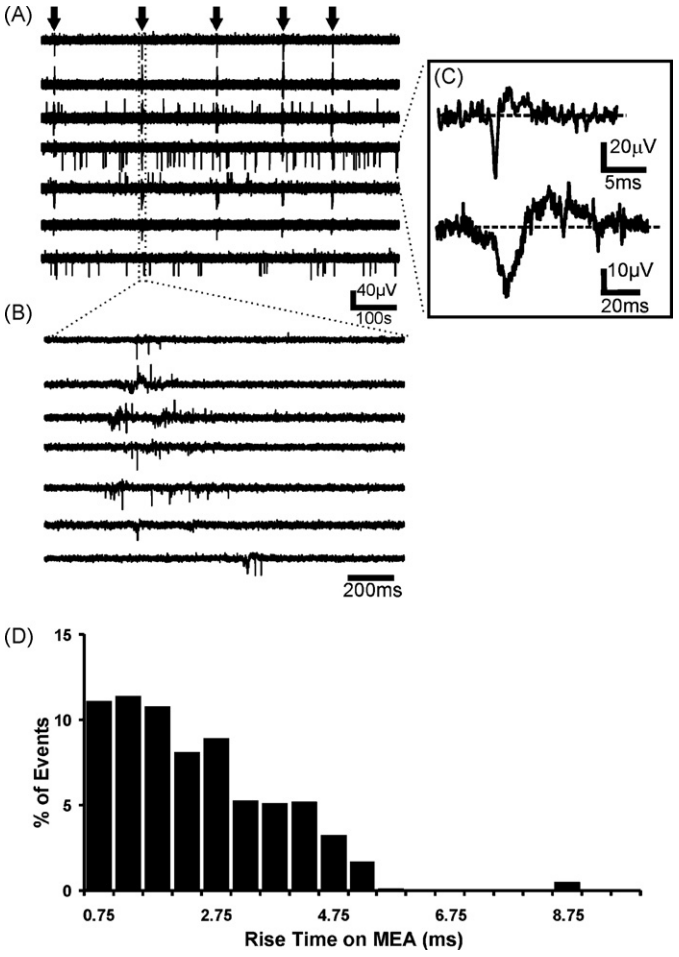

Fig. 3. Spontaneous extracellular action potentials (APs) recorded with MEAs. (A) Simultaneous AP recordings at room temperature from seven microelectrodes covering an area of $0.8 \times 0.2\,mm^2$. GT1-7 cells were kept at room temperature and superfused with the Tyrode standard solution. Notice the predominance of spontaneous inward deflections in all traces and the existence of action potentials synchronism (top arrows) at intervals of 100–200 s. (B) Same recordings at a more expanded time scale to highlight extracellular APs (mainly inward deflections) of different duration. (C) Top: average of $n = 51$ APs of short duration on an expanded time scale; bottom: average of six compound APs of longer duration. (D) Histogram of the rise times of 741 extracellular signals associated to the slow events obtained from eight neurons plated on two different MEAs.

The histogram obtained from $n = 741$ events peaks at around 2 ms and shows a broad distribution, similar to that of Fig. 2D up to 8 ms.

The signals recorded from both MEAs and the HTD electrode, showed a strong similarity with the waveform and duration of the negative first derivative of the AP recorded intracellularly by means of the perforated patch clamp technique (Cesetti et al., 2003), as shown in S.I. Fig. 1, thus providing further evidence for a capacitative-coupled extracellular membrane potential recording by the HTD set-ups.

4. Discussion

In this paper, we provide the first report of electrical activity from living neurons recorded by a H-terminated diamond electrode. The device is able to detect the spontaneous extracellular electrical activity of GT1-7 cells, and can be used to monitor the effects of either changes in the extracellular medium such as ion substitution or addition of pharmacological agents, e.g. channel blockers. The recording of optical signals from cells cultured on the same device has been described in a previous paper (Ariano et al., 2005).

In order to confirm that the signals derived from the diamond electrode were related to the intracellular electrical activity of the neuronal cells, parallel recordings were performed on neuronal populations with commercially available MEAs and on single cells with the patch clamp technique. The time courses of the extracellular signals recorded from diamond are in good agreement with those obtained by means of the other two approaches, thus providing solid support to the physiological relevance of our observations. Signal-to-noise ratios are comparable to those obtained with the MEAs system we used (Fig. 3) and are in fairly good agreement with the recordings of other groups on GT1-7 cells using MEAs of different shape and material (Nunemaker et al., 2001). Part of the difference in the signal-to-noise ratio may derive from the different MEA construction. Our system uses circular disks of 30 μm diameter made of columnary deposited TiN which favours more specific capacitative couplings with neurons while that used by Nunemaker et al. (2001) is based on gold electrodes of 10 μm diameter which favour more specific resistive coupling with neurons.

Diamond is one of the most interesting and promising materials developed in recent years in the biosensors field, for both *in vitro* (Härtl et al., 2004; Zhao et al., 2006; Martinez-Huitle, 2007) and *in vivo* (Zhou and Greenberg, 2005) applications. For molecular, enzyme based biosensors, one of the tasks has been to functionalize the diamond surface with linker molecules; in this paper we show that efficient interfacing of hydrogen terminated diamond with electrically excitable cells can be obtained by means of a simple coating with a thin electrostatic layer of poly-L-lysine, thus avoiding complex functionalization procedures and improving the cell-to-substrate contact (Sorribas et al., 2001). As compared to commercially available, optically opaque systems, diamond-based electrodes allow the exact localization and number of cells covering the electrode and the simultaneous recording of electrical activity and optical signals generated by fluorescent intracellular probes sensitive to Ca^{2+} and second messengers. From this point of view, diamond-based minielectrode arrays can be the optimal answer to the demand for multiparametrical recordings from cell populations.

While the present prototype consists of a single macroelectrode, it is conceptually feasible to design microelectrode diamond arrays on a single chip, by modifying its electronic and hydrophilic/hydrophobic properties at the nanoscale, thus opening a whole new field of applications, in parallel with the increasing use of this material for the development of molecular biosensors.

5. Conclusions

The chemical inertness of diamond, its optical transparency, mechanical robustness and stable electrical conductivity, when hydrogen terminated, make it a suitable material for the fabrication of stable MEAs that can be employed for long-lasting extracellular recordings of electrical and optical signals from living neurons.

The evidence that a hydrogenated diamond electrode can be used to reliably record electrical activity from a neuronal network, together with the previous description of the recording of optical signals from cells cultured on the same device (Ariano et al., 2005), could be of great relevance for the development of a new generation of multipurpose biosensors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2008.10.017.

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