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Carabelli, Valentina; Marcantoni, Andrea; Picollo, Federico; Battiato, Alfio; Bernardi, Ettore; Pasquarelli, Alberto; Olivero, Paolo; Carbone, Emilio. Planar Diamond-Based Multiarrays to Monitor Neurotransmitter Release and Action Potential Firing: New Perspectives in Cellular Neuroscience. ACS CHEMICAL NEUROSCIENCE. 8 (2) pp: 252-264.
DOI: 10.1021/acschemneuro.6b00328

Planar diamond-based multiarrays to monitor neurotransmitter release and action potential firing: new perspectives in cellular neuroscience

Valentina Carabelli[1,2,3,*], Andrea Marcantoni[1,2,3], Federico Picollo[4,5,2,3], Alfio Battiato[4,5,2,3], Ettore Bernardi[4,5,2,3], Alberto Pasquarelli[6], Paolo Olivero[4,5,2,3], Emilio Carbone[1,2,3]

[1]*Department of Drug Science and Technology, University of Torino, Torino, Italy*

[2] *Nanostructured Interfaces and Surfaces Inter-departmental Centre, University of Torino, Torino, Italy*

[3]*Consorzio Nazionale Interuniversitario per le Scienze fisiche della Materia (CNISM), Torino Unit, Italy*

[4] *Physics Department, University of Torino, Torino, Italy*

[5]*Istituto Nazionale di Fisica Nucleare (INFN), sez. Torino, Italy*

[6]*Institute of Electron Devices and Circuits, Ulm University, Ulm, Germany*

* Corresponding author: valentina.carabelli@unito.it

Keywords: diamond, multiarrays, chromaffin cells, quantal release, action potential firing

Key concepts: amperometry, cell firing, electrochemical imaging, exocytosis

ABSTRACT

High biocompatibility, outstanding electrochemical responsiveness, inertness and transparency make diamond-based multiarrays (DBMs) first-rate biosensors for *in vitro* detection of electrochemical and electrical signals from excitable cells together, with potential for *in vivo* applications as neural interfaces and prostheses. Here, we will review the electrochemical and physical properties of various DBMs and how these devices have been employed for recording released neurotransmitter molecules and all-or-none action potentials from living cells. Specifically, we will overview how DBMs can resolve localized exocytotic events from subcellular compartments using high-density microelectrode arrays (MEAs), or monitoring oxidizable neurotransmitter release from populations of cells in culture and tissue slices using low-density MEAs. Interfacing DBMs with excitable cells is currently leading to the promising opportunity of recording electrical signals as well as creating neuronal interfaces through the same device. Given the recent increasingly growing development of newly available DBMs of various geometries to monitor electrical activity and neurotransmitter release in a variety of excitable and neuronal tissues, the discussion will be limited to planar DBMs.

INTRODUCTION

Diamond neurobiosensors to "view" brain functions

Electrical stimulation and recording of brain neurons activity is widely used to understand the molecular and cellular basis of health problems related to chronic pain, paralysis, hearing loss, retinal degeneration, as well as neuropsychiatric and neurological disorders [1,2]. In *in-vitro* systems the stimulation and recording of neuronal networks is achieved through extracellular microelectrodes, which require an effective miniaturization to address single neurons and induce minimal tissue damage. Neural microelectrodes work by delivering electrical pulses to neurons and transduce biological events (action potentials and post-synaptic activity) in voltage or current signals to be acquired and stored for continuous on-line and off-line analysis. An ideal microelectrode should provide safe levels of stimulation and record small (μV to mV, pA to nA) and fast electrical signals (milliseconds) without introducing size and time distortion and induction of any chemical reactions on the electrode or in the tissue.

Furthermore, simultaneous recording of action potential waveforms (AP firing) and release of neurotransmitters (pre/post synaptic activity) is highly desirable in living neurons as they provide a complete framework of the functional or pathological state of the cells. Many neuropsychiatric and neurological diseases derive from defects or alterations of the molecular components regulating vesicle fusion and neurotransmitter release at the *presynaptic terminals* and signals summation and integration at the *post-synaptic site,* where tonic or burst AP firing are generated and propagated [3,4,5]. Thus, there is an urgent need for innovative microelectrode arrays (MEAs) to achieve selective stimulation and recording of central neuron excitability as well as precise monitoring of synaptic activity.

This is highly desirable since it promotes new understandings of neuropathologies, formulation of advanced therapies and construction of novel neuroprosthetic devices.

In addition to the many conductive (gold, platinum, iridium, titanium) and non-reactive materials (stainless steel, iridium oxide, silicon), diamond has recently attracted the interest of numerous research groups as a substrate for constructing MEAs because of its unique mechanical properties, surface characteristics and excellent biocompatibility [6,7]. The increasing interest and the recent development of new techniques for constructing micro/nano-devices [8,9,10,11,12] has rapidly broadened the number of diamond-based MEAs (DBMs) employed for electrical recording and stimulation and for detecting neurotransmitters release. DBMs can now be used to either resolve the electrical activity in complex neuronal networks (low-density MEAs) [13,14], to identify the extension of cell microdomains (active zones) where neurosecretion occurs (high-density MEAs) [15] or to assay the protein content of the physiological liquids that condition the growth, formation and maturation of complex neuronal networks) [16,17].

Despite the promising applications illustrated above, diamond-based biosensors are still facing significant potential limitations that are related with drawbacks in diamond synthesis and microfabrication and electrical functionalization of the devices. Concerning the synthesis of artificial diamond by Chemical Vapour Deposition (CVD), the technology is still not optimal, despite the remarkable improvements over the last few decades. While large-area polycrystalline samples can be easily grown on a variety of substrates with different shapes at relatively low costs, the production of single crystal samples is still largely limited to homoepitaxial processes. This is due to the high atomic density of the diamond lattice that causes significant mechanical stresses when

grown on non-diamond substrates. This significantly limits the size of CVD-grown single crystal diamonds to areas of few square millimeters. Concerning the microfabrication, despite the significant progresses obtained recently [18], the electrical doping of diamond is still not fully developed and understood. This is not the case for the p-type doping that is now well-established, but refers to the n-type doping that still poses significant challenges due to the unsuitability of most of group V elements to this purpose [19]. Finally, it is worth remarking that the main properties (mechanical hardness, chemical inertness, optical transparency) that make diamond particularly attractive in device applications also have significant limiting factors in the implementation of efficient and scalable lithographic processes. For instance, as extensively described below, high-energy ion-beams are an efficient tool to fabricate diamond microarrays, but their availability is limited to large-scale accelerator facilities and thus, of not easy use compared to conventional lithographic processes. Similarly, plasma etching in diamond is not as efficient as in conventional materials and thus requires the development of specific Reactive Ion Etching setups [20].

Here, we will overview works related to the construction, test and application of DBMs in cellular neuroscience and related fields, underlying the effective technical and functional improvements that each device have brought in the understanding of neuronal and excitable cell function.

What makes diamond attractive for cellular neuroscience?

In the field of cellular sensing, diamond-based substrates offer unique advantages in comparison with conventional materials (silicon, glass, metals and polymers) [6], which directly derive from the extreme physical properties of this material, i.e.: mechanical robustness, wide optical transparency, thermal conductivity, etc. [7]. Most importantly for the applications under consideration, diamond-based substrates offer the specific advantages of being biocompatible, chemically and electrochemically stable, resistant to fouling over multiple acquisition protocols and non-immunogenic [16,11].

The absorption of organic materials on the diamond surface is significantly lower compared to noble metals [21]. For instance, the strong affinity between gold and sulphur promotes the absorption of substances containing sulphur moieties such as cysteine residues. This is significantly less critical in diamond substrates. The kinetics of this

semiconductor material combined with its chemical inertness leads to an electrochemical potential window significantly larger than in the metals. In particular, p-type boron-doped diamond shows a significant extension of the useful cathodic range due to depletion of holes at the interface when the electrode is negatively biased. In the anodic regime, the width of the potential window is related to the energy states available at the electrode surface, which in turn are a consequence of the doping density, the resulting Fermi-level and the kind of surface termination. These parameters set the value of the surface potential and the bending of the energy bands at the interface with the electrolyte. In addition, chemical inertness prevents undesired chemical reactions during the sensing process [22, 23] and the wide band gap (5.5 eV) leads to transparency in the NIR-VIS-NUV spectral range (down to ~ 250 nm wavelength) as well as lower electrical noise.

In vitro tests conclusively demonstrated that diamond-based substrates are non-cytotoxic and display a significantly better cell adhesion and growth in comparison with standard substrates [24, 25]. It is worth noting that biocompatibility is a property of paramount importance not only because it has beneficial effects on cell survival rates, but also because it promotes cell adhesion, which in turn improves signal transduction [26]. Furthermore, the wide optical transparency of the material (ranging from the near UV to the far IR) enables the use of inverted transmission microscopes for imaging a broad range of optical markers, a feature that is highly desirable in most experimental setups.

Remarkably, the chemical inertness of the pristine diamond surface does not prevent its efficient chemical functionalization upon the termination with specific covalent bonds that allows the attachment of a broad variety of molecules, including DNA strands [27,28,29]. Even when not functionalized with specific molecules, the diamond surface can be easily functionalized by hydrogen or oxygen termination. These processes are functional in creating stable surface layers which are respectively hydrophobic or hydrophilic [30,31], and thus display different suitability to cell attachment [32, 33, 34, 35]. It is worth remarking that, apart from the effect of surface termination, cell adhesion in polycrystalline diamond is strongly dependent upon the surface morphology, thus offering a further degree of control of cell growth patterning [36].

The H-termination of the diamond surface favors the formation of an electrically conductive two-dimensional layer in contrast with the insulating O-terminated surface

[37]. These transparent electrodes were exploited to record the activity of cultured neuronal cells with a single macro-electrode [38], and subsequently to record the activity of cultured cardiomyocyte-like and human embryonic kidney cells using arrays of solution-gated field-effect transistors [8]. In combination with the definition of different surface terminations, micro-contact printing and laser irradiation can be successfully employed to define patterns of adhesion molecules on the diamond surface, thus enabling the ordered growth of cultured cells [31, 39].

Besides surface termination, there are two further strategies employed to fabricate micro/nano-electrodes for biosensing, namely boron doping [40, 41] and local graphitization [42,43]. Two-dimensional [44,11] and high-aspect-ratio [45,12] micro/nano-structures can be effectively patterned into boron-doped polycrystalline diamond, allowing for cell attachment and sensing APs.

What limits diamond-mediated biosensing in neuroscience?

The surface functionalization of diamond for biological applications has made significant progresses in the last decade, but it is still facing significant technological limits. Along with the already-mentioned chemical inertness, the CVD growth process produces diamond films that are H-terminated and thus exhibit hydrophobic behavior. This native feature poses a challenge in engineering the attachment of different types of biomolecules that are inherently hydrophilic. Therefore, it is necessary to covalently link biomolecules to such surfaces in a controlled and reproducible way. Different approaches have been adopted to the purpose based on either chemical, photochemical and electrochemical strategies. Such functionalization routes have generally proved to be efficient and versatile in increasing the compatibility of diamond substrates with biological media and the conjugation to antibodies or drug delivery [46, 47]. Nonetheless, it is worth remarking that nanodiamonds functionalization is still far from being fully effective. The surface chemistry of nanodiamonds is significantly more complex with respect to bulk samples. Surface functionalization is often inhomogeneous and partially uncontrolled. Significant progresses have been made in surface homogeneization of nanoparticles [48] as well as in the development of new approaches such as their glycan-functionalization by means of “click” chemistry [49], but many challenges in the

biofunctionalization of nanostructured diamond are still withstanding. An extensive review on the subject is reported in [50].

A main limitation of DBMs when used to determine the electroactive species in biological fluids, is the electrode fouling (*biofouling*) that occurs when macromolecules (proteins and DNA) are adsorpted at the electrode surface. Usually, biofouling of DBM does not affect *in vitro* recordings performed within some days after plating [51], Biofouling affects eletrode performance specifically when used in blood, urine or water samples. Surface contamination of BDDs or biofouling allow stable responses for 3 days of continuous use [52]. However, it is important to notice that fouled electrodes can be cleaned and the initial electrode reactivity restored by applying short cathodic and/or anodic pulses [53].

DBMs biofouling in long-term *in vivo* recordings is more severe than in *in vitro* conditions and markedly affects electrodes functionality. For instance, in the case of boron-doped polycrystalline diamonds implanted in freely behaving Aplysia californica, the functionality of the device is limited to some days after implantation [54]. More details on DBMs biofouling are given in the section on diamonds as neural probes and prosthesis.

Diamond based multiarrays for detecting quantal exocytotic events

Spatial monitoring of exocytosis on single or population of cells started nearly 25 years ago [55,56,57,58] and has been progressively improved using different materials and microelectrode geometries. Here we overview the realization of increasingly efficient planar microarrays trying to emphasize the different strategies adopted to improve the spatial resolution, to couple amperometric recording with other techniques, and to use the same bio-sensitive electrode to monitor and stimulate the secretion.
To this purpose, Table 1 compares the different geometries of planar multiarrays.

Early attempts to monitor subcellular exocytosis

Carbon fiber amperometry has been exploited for real-time monitoring the quantal release of oxidizable neurotransmitters since the 1990s [55,56,57,58]. This standard approach is characterized by sub-millisecond time resolution [59,60,61] and can finely resolve both the kinetics of oxidizable transmitter discharge from the vesicle into the extracellular

compartment and its further diffusion. Using carbon fiber electrodes (CFEs), oxidizable neurotransmitters such as dopamine, adrenaline, histamine and serotonin (5-hydroxytryptamine), can be detected from single cells either by keeping the CFE at a constant positive potential (amperometry) or by applying at high scan rate (> 100 V/s) a linearly varying potential (fast-scan cyclic voltammetry, FSCV).

The need of improving the spatial resolution of exocytosis to resolve subcellular regions of secretion [15] and the impelling demand of detecting quantal release simultaneously from a number of cells stimulated the development of a variety of planar amperometric devices with sensing electrodes arrayed into either high- or low-density configurations [62]. The starting material in these devices was either platinum, gold, silver, indium-doped tin oxide (ITO) or carbon surfaces such as graphite, nitrogen-doped diamond-like carbon (N-DLC) and boron- or nitrogen-doped nanocrystalline diamond (B-NCD, N-NCD). In addition, boron-doped DBMs were specifically developed because of their higher resistance to fouling compared to CF electrodes when measuring serotonin release [63,64].

The spatial resolution of functionally active secretory zones (hot spots) and, in particular, their subcellular distribution over the cell membrane is a key issue that contributes to a deeper understanding of neuroendocrine cells and central neurons functions [65]. This has been initially approached by using pairs of narrow carbon fiber microelectrodes (1 μm diameter tip) free to independently move on top of the same cell [66] and afterwards by developing complex multi-barreled capillary arrays, (carbon MEA micro disks), with variable number of electrodes and diameter [67,68,69]. This procedure successfully discriminated "hot" and "cold" zones of release from the cell apex with a resolution of approximately 4 μm to 8 μm. Improved spatial resolution was achieved only recently using conically shaped nanometric carbon fibers, with 50-200 nm tip diameter, that allow to resolve the vesicular exocytosis inside the synapses of superior cervical ganglion neurons [70].

High spatial resolution MEAs to resolve subcellular exocytosis

Although having high spatial resolution, multi-barreled capillary arrays impose several constraints on the number of recording electrodes and limit the acquisition to single cells. An obvious alternative are the new planar MEAs, designed to resolve with even

higher resolution the microdomains of neurotransmitter release in neuronal cell models and to limit the need of cell manipulation.

Amperometric spikes originating from different zones of a single secretory cell were first resolved using planar MEAs, by placing a bovine chromaffin cell within a circular area where three to four converging platinum electrodes were patterned on a glass coverslip. Spatiotemporal monitoring of exocytosis was performed amperometrically and confirmed by simultaneous fluorescence imaging of vesicle release [71,72,73], by comparing the signal resulting from the same release event in multiple electrodes. This indicates that electrode arrays can achieve spatial resolution comparable to optical imaging [72]; though this comparison can be done only if the electrodes are arranged such that the catecholamine released at the bottom surface of the cell can diffuse to, and be detected by, at least 3 electrodes.

Concerning DBMs, high spatial resolution was initially achieved initially using 4 [74,75,76] and subsequently 9 boron-doped nanocrystalline diamond ultra-microelectrodes (B-NCD-UMEAs) grown on sapphire substrates [41]. The devices operate in amperometric and voltammetric mode, detecting released adrenaline from single chromaffin cells as well as dopamine and serotonin. In particular, the 9 channels array (9-Ch NCD-UMEA), used in constant-potential amperometry could distinguish zones of variable secretory activity (in mouse and bovine chromaffin cells) with maximal resolution of 12 μm^2, corresponding to the area of the smallest electrode (Fig. 1; see also Fig. 4a[15]). The difference in the secretory responses revealed by each electrode is due to the different frequency of release among microdomains of the same cell, whereas the waveform spike parameters remained unmodified among the subcellular secreting zones.

The 9-Ch NCD-UMEA is the first high-density DBM able to resolve the heterogeneity of release due to the coexistence of regions with high secretion rates and membrane areas where secretion is either absent or low. As an additional feature, the 9-Ch MEA is able to induce exocytosis by electrical stimulation applied through any one of the 9 sensing electrodes by either cell depolarization or plasma membrane electroporation [15].

The need of using the same device for stimulation and recording was also recently addressed by Gillis & collaborators. The authors used the same electrode for cell stimulation and signal detection [77]. The chip is based on gold planar microelectrodes that electro-permeabilize a chromaffin cell through a series of voltage pulses and then detect the related catecholamine release in an amperometric recording mode. The

advantage of this system is that it can simultaneously define the precise timing of single cell stimulation and load the cell with membrane impermeable substances.

Thus, planar MEAs offer several advantages with respect to multibareled microelectrodes that are due to the different experimental approach when using the two devices. Planar MEAs favour cell manipulation and combination of amperometric recordings to different techniques while preserving the same high time-resolution, spatial precision and signal-to-noise ratio of multibareled microelectrodes.

Newly available MEAs to resolve subcellular exocytosis

A new generation of B-NCD-UMEAs with different electrode geometry and increased number of sensing probes is now available [44]. The new DBM is grown on high-temperature glass, possesses 12 round-shaped sensing electrodes of 2 μm diameter equally separated covering a total area of ~300 μm^2 that corresponds to the area of a single chromaffin cell of 20 μm diameter (see Fig. 4b). In this way, the new DBM provides higher spatial resolution suitable to resolve single cell secretory activity.

It is worth noticing that subcellular exocytosis can be resolved also using other materials besides DBMs. Multisite cell recordings with further improved spatial resolution has been recently reported using planar platinum UMEAs on top of which single cells or clusters can be positioned [78,79]. Arrays with 16, 25 and 36 square microelectrodes (respectively of 4, 3 and 2 μm width), concentrated in a square area of 30×30 μm^2, are shown to detect amperometric spikes associated with the quantal release of dopamine from PC12 cells. In this way, subcellular heterogeneity of exocytosis could be monitored with a 2×2 μm^2 resolution by the 6×6 UMEA. Culturing of PC12 cells on top of the arrays was favored by mounting the UMEAs on a poly(dimethylsiloxane) chamber and by coating the electrode surface with a thin layer of collagen IV. The system is reliable and tested for pharmacological trials.. More recently, a new concept of thin film MEA probe with high spatial resolution has been proposed [80] (see Fig. 4c). The array is constructed on a probe tip and can be thus precisely positioned in close proximity of the cell. Individual release events with sub-micrometer resolution are resolved by 16 platinum band electrodes (1.2 μm wide), within a 20×25 μm^2 area. This new 16-electrode UMEA, provides an electrochemical imaging of exocytosis activity concentrated in hot spots, confirming previous observations by TIRF imaging [81]. The chip is undoubtedly a valid tool for multi-parametric sensing and looks promising to

address relevant electrophysiological questions related to the localization of exocytotic active zones confined to varicosities and neurite membrane areas.

Multisite detection of exocytosis from cell populations and tissue slices using DBMs

While high-density arrays aim at providing a detailed electrochemical mapping of exocytosis from a single cell, multi-site detection from multicellular samples is targeted to rapidly screening the secretory activity of neuronal networks, cell populations or tissue slices.

Simultaneous detection of exocytosis from different cells was initially performed using B-NCD quadrupole arrays with microelectrode dimensions comparable to the chromaffin cells size (16÷20 μm diameter) [40]. Despite the quite limited number of detecting electrodes, the device could be easily combined with other techniques thanks to its transparency. In this way, quantal secretory events could be simultaneously detected from both the cell apex and its bottom, by simultaneous employing CFEs and BNCD planar arrays. Subsequently, more complex BNCD ultra-microelectrode arrays (8×8 electrodes UMEA) were constructed either on high-temperature glass substrates, to improve transmittance, or on SiO_2 substrates [44,82].

Multisite detection of exocytosis from cultured chromaffin cells and adrenal slices has been also assessed by a new DBMs generation. In these devices, the sensitive elements are sub-superficial graphitic micro-channels in a single-crystal diamond (μG-SCD) in different geometries [83,84,42,43,85]. Bovine chromaffin cells can be cultured on these devices without protein coating, with a density of 150.000 cells per chip, to ensure that each of the 16 (or 64) microelectrodes contact one cell. Besides resolving quantal release from chromaffin cells cultured for days without altered responsivess of the electrodes, the graphitic UMEAs allow also to achieve three main goals (Fig. 2), namely: *i)* the detection of spontaneous and evoked catecholamine release from isolated cells; *ii)* the separation and analysis of spikes with different kinetics, which can be interpreted as different modes of exocytosis (complete fusion events, kiss and run and stand-alone-foot events), according to previous findings [51,86,87] and *iii)* the detection of robust vesicular release in slices of the adrenal gland. Taking advantage of the high sensitivity of the device, it is also possible to resolve spontaneous catecholamine release in 2 mM external Ca^{2+}. From a physiological point of view, this is a relevant issue, as

suggested by previous patch-clamp experiments demonstrating that sustained spontaneous firing activity of chromaffin cells regulates basal secretion near physiological conditions [88,89,90].

Besides patterning multielectrode arrays with different geometries to match the experimental need, relevant efforts have been made to optimize cell positioning on the electrodes. In some cases, cells are cultured in a not-adherent BSA medium and the final positioning of the cell on the recording electrode is performed using a glass patch-clamp pipette [40]. Alternatively, cells are seeded in N-DLC microchips. In this case, cells are removed from the culture flask and centrifuged, then carefully resuspended in bath solution and settled for few minutes on the sensing electrodes (30×40 μm^2 in size) [91]. In another device from the same group, microfluidic traps are constructed on the Pt electrodes (10×15 μm^2 in size) to keep the cells in docking-sites, thus avoiding pressure control and cell manipulation [92].

Interfacing neurons with DBMs to detect action potential firing

Cell adhesion and biocompatibility

Surface biocompatibility and suitability to preserve cell adhesion, intact synaptic contacts and neuronal functions is a key prerequisite for interfacing DBMs to neuronal networks and for constructing implants or prostheses targeted to *in vivo* applications [93,94]. The use of diamond as a promising material for biological applications was first demonstrated by early findings on H- and O-terminated diamond surfaces [26]. Following surface coating with adhesion molecules (poly-d-lysine, poly-dl-ornithine, laminin), the O-terminated diamond preserves synaptic activity, ion channels availability and Ca^{2+} signals during neuronal stimulation in rat hippocampal neurons and chick ciliary ganglion neurons.

Suitability of DBMs for cell attachment and growth without adhesion molecules has been shown in various experimental models to vary as a function of the biological interface. For instance, bipolar neurons can directly grow on bare NCD surfaces, while glial cells require protein coating to increase their survival [95]. Concerning N-DLC devices, the polylysine pretreatment is not effective in increasing chromaffin cells adhesion, even though the N-DLC substrate is markedly better than ITO, Pt and Au [96].

Thus, concerning the property of the material that can improve cell adhesion, it is worth considering the advantages of using functionalization of the substrate. H-, O- and NH_2-terminations of UNCD films have been shown to provide excellent substrates for insect circadian pacemaker neurons in comparison to untreated surfaces, while human fetal neural stem cells SPC-01 preferentially attach to the O- rather than to the H-terminated NCD surfaces [97,98]. In addition, adhesion of neuroblastoma cells or primary cortical neurons to DLC surfaces can be optimized using phosphorus-doped diamond-like carbon (P-DLC), rather than O-DLC termination or undoped DLC [99].

The roughness of the substrate may be also a relevant parameter. By examining the growth of rat cortical neurons while varying the roughness of N-UNCD surfaces, ideal conditions to improve electrochemical sensitivity and biocompatibility can be identified [100]. Concerning the surface functionalization, biocompatibility of DLC can be improved using UV surface functionalization that increases its hydrophilicity [101]. Interestingly, the treatment of various substrates with a monolayer of monodispersed nanodiamonds can be used as a method to facilitate the formation of functional neuronal networks, even in the absence of extracellular matrix protein coatings [17].

Finally, neuronal growth can be also selectively driven along predetermined pathways, using micro-contact printing of laminin that force mouse cortical neurons to adhere to the laminin-covered areas and grow neurites in the patterned surface [31]. Alternatively, the laser micro-machining technique can provide high-resolution patterning of diamond surface [39]. It is important to note that besides regulating cell adhesion, diamond surface terminations can promote to different extents cell differentiation and growth. H- and O-terminations induce differentiation of neural stem cells into neurons or oligodendrocytes, respectively [102]. For a detailed overview on B-NCD suitability for neurointerfaces and biocompatibility of DLC coating see Refs [52,103] [104,105].

Action potential recordings

Despite the availability of a variety DBMs suitable for electrochemical recordings, there are only few examples of DBMs capable of detecting action potential (AP) firing from excitable cells. Spontaneous electrical activity of cultured GT1-7 neuronal cells was first reported using H-terminated conductive diamond (1 mm^2) [38]. AP burst activity, separated by silent intervals, was similar to the one recorded using conventional MEAs. Single cell activity and serotonin release were also monitored from *Aplysia Californica*

neurons using a boron-doped diamond surface grown on a tungsten microelectrode with a final tip diameter of ~30 μm [106].

BDD allows also the recording of electrical activity from cardiomyocyte-like HL-1cells [107]. An array of 64 sensing electrodes can effectively detect cardiomyocytes action potentials, resolve AP waveform propagation (tens of mm/s) and resist the mechanical stress associated with AP-induced contraction.

Besides passively recording APs, diamond electrodes can also stimulate interfaced neurons. This can be performed using 3D-nanostructured BDD microelectrodes, made of vertically aligned carbon nanotubes (VACNTs) embedded in two BDD nanolayers. The 4×15 array can detect local field potentials from the hindbrain down to the spinal cord and reveals spiking activity of cultured hippocampal neurons with amplitudes as small as 10 μV, comparable to those detected by conventional MEAs [12, 108]. In addition, by applying biphasic currents to one of the 3D-nanostructured BDD microelectrodes, it is possible to evoke neuronal bursts firing. A DBM stimulating device made of thousands of N-UNCD microelectrodes is now also available [109]. With this device it is possible to stimulate retinal ganglion cells through individual electrodes and record evoked responses by means of the patch-clamp technique in current-clamp configuration.

Another potential application of DBMs as effective neural/electrode interfaces has been recently achieved using micro-textured NCD surfaces. The new DBMs consist of micro pillars of 5×5 μm^2 that adhere easily to human and murine inner-ear ganglion neurites without any specific extracellular coating [110]. Regenerating auditory neurons show strong affinity for the NCD pillars and the technique can be exploited for neural guidance and for developing neural networks.

Diamond-based multiparameter sensing

The key advantages of DBMs are their wide electrochemical window and excellent optical transparency. Due to these properties, DBMs are ideal devices to combine amperometric detection of released neurotransmitters with electrophysiological recordings [40,61] and fluorescence imaging.

Transparent materials such ITO, DLC deposited on ITO and thin gold films on glass substrates have been used by Lindau's group to construct MEAs able to simultaneously record amperometric and optical signals related to secretion [111] (Fig. 4d). Vesicle fluorescence was imaged with TIRF microscopy while quantal catecholamine release was detected as amperometric spikes. All three types of devices could clearly detect high resolution amperometric signals from chromaffin cells, even though charge and spike amplitude recorded with ITO microelectrodes were smaller than the ones using DLC or gold electrodes, suggesting lower amperometric sensitivity of ITO with respect to gold and DLC. Detection of single quantal events was integrated with fluorescence imaging through the transparent electrodes by combining amperometry and TIRF microscopy associated with the vesicle release of dye-loaded molecules. Single vesicle exocytosis monitored through the same combined approach (amperometry and TIRF microscopy at ITO surfaces) has been recently reported also by other groups [112,113].

Combined detection of amperometric spikes and fluorescence imaging is now possible using newly available DBMs. With respects to previous devices [40,15], the substrate of the new B-NCD arrays consists of a high temperature glass that drastically enhances the transmittance of the chip in the visible/UV range [44]. Under these conditions, simultaneous detection of KCl-evoked secretion and corresponding intracellular Ca^{2+} increases of Fura-2 loaded bovine chromaffin cells has been possible using a B-NCD array of 16 sensing electrodes (Fig. 3) [114].

Further attempts to construct microchips with multi-task purposes have recently led to promising devices. With the ultimate goal of producing an all-diamond probe, an array was constructed with undoped poly-C film used as substrate and a doped poly-C film used for the electrodes. The device was tested for noradrenaline electrochemical sensing and shown to be also potentially suitable for recording neuronal signals [115]. Finally, the need of coupling electrochemical detection of oxidizable neurotransmitters to electrical sensing stimulated the fabrication of an integrated multifunctional MEA (mMEA) based on interdigitated electrodes [116]. The chip works as a multi-functional dopamine sensor capable also of electrical recordings.

Diamond-based neural probes and prostheses

Given the well-established ability of DBMs to interface neuronal networks and record electrochemical events, fluorescence signals and AP firings, there is now a growing interest to develop innovative neural interfaces, with the dual purpose of investigating the function of neuronal networks and provide treatments for the altered neuronal circuitry.

In this regard, increasing the number and density of stimulating electrodes within a chip will significantly improve the spatial resolution. With the goal of fabricating high-density DBM implants, different procedures have been developed, exploiting the electrical conductivity of B- and N-NCDs. The clinical safety of implanted B- and N-NCD electrodes was first analyzed for several weeks after implantation. Both forms of DBMs exhibited similar or lower chronic inflammatory responses compared to the silicone negative control, suggesting good tolerance for *in vivo* sensing [94,52].

Concerning the B-NCD devices it is worth remarking that they have resistivity of ~ 10 mΩ cm or even lower [22, 117., 118], which does not pose problems as far as the voltage safety limitations are concerned for *in vivo* sensing. As confirmed by Electrochemical Impedance Spectroscopy (EIS) [119], the impedance of NCD microelectrodes is mainly capacitive over the whole range of frequencies used for recordings bioelectrical signals and typically larger than that of planar metallic electrodes [120, 121], due to their surface roughness. This increases their biosensitivity. In addition, in *in vivo* applications, low impedance electrodes are prone to phenomena of surface delamination/corrosion, which can lead to a self-enhanced increase of their conductance. This potentially leads to dangerous effects in *in vivo* applications, for instance, in electro-modulating therapies. To this regard, the electrodes exhibit strong resistance to corrosion and significantly more stable passivation of commonly used polymers.

Particular attention has recently been addressed to interfacing stimulating neural devices with damaged or degenerated tissues of the auditory cortex and the retina. Advantages and details of 3D structures are provided in [52, 122]. Neural probes are usually fabricated from a variety of materials, such as silicon and polymers, while electrical sensing electrodes are made with gold, platinum and iridium. Single-material MEMS technology, using poly-C, UNCD and DLC, is now leading to the development of all-diamond neural probes as multi-functional devices to detect individual neuron activity and released neurotransmitters.

For neural applications, a critical issue is the electrode inertness and chemical stability inside an environment rich of ions and proteins that allows to avoid signal reduction due

to the residues formed or adsorbed at the site surface. CVD has been shown to adsorb relatively small amounts of fibrinogen, which is commonly used as a biocompatibility indicator, without producing any inflammatory reaction, or adverse side effect on living cells [123,124]. Also, BDD microelectrodes have been used to detect serotonin levels in the ileum mucosa as an oxidation current, demonstrating resistance to fouling by adsorbed serotonin oxidation reaction products [125, 126] and also to monitor nitric oxide released by myenteric neurons in isolated segments of guinea pig ileum, using continuous amperometry [127]. Continuous amperometry has also been used on diamond microelectrode and video microscopy to simultaneously measure endogenously released norepinephrine from sympathetic nerves innervating rat mesenteric arteries [128].

However, despite the above mentioned examples, it should be noticed that for long-term *in vivo* purposes, diamond, like other biocompatible materials, may induce gliosis, neuronal inflammation, cell death and neuronal migration [52]. A detailed study of diamond biocompatibility for *in vivo* trials and the induction of gliosis in retina has been reported while examining the retinal tissue in close proximity of the implant and by comparing gliosis among diamond and platinum electrodes [129].

A diamond-based neural probe was first surgically implanted into a live guinea pig's auditory cortex, to provide the recording of electrical activity following audio-pulses stimulation [130,131]. This i*n vivo* acute detection of neuronal activity was achieved by microfabricated boron-doped poly-C diamond probes. The chip was surgically inserted into the auditory cortex area of a guinea pig brain in which stable signals of 30–40 μV amplitude and ms duration were acutely recorded over several seconds. Concerning retinal implants, a flexible microarray (25×25 electrodes) for retinal stimulation has been created by deposition of polyimide film onto 3D BDD electrodes and its biocompatibility positively assessed *in vivo* [129].

The *in vivo* biocompatibility of N-UNCD was also demonstrated by cytotoxicity assays on cortical neurons survival and neurite outgrowth. On this basis, hermetic microarrays have been constructed with the idea of providing tissue interface for a biomedical implant. The device, consisting of conducting N-UNCD channels, exhibited low feedthrough resistance and high insulation resistance as a retinal stimulation array [14]. A high-density all-diamond array, consisting of 256-channel stimulating electrodes and a custom built ASIC (Application Specific Integrated Circuit) chip, was recently designed to be interfaced with damaged retinal tissue [132]. Another in vivo use of BDD electrodes

concerns the detection of dopamine release from the monkey striatum during behavioural tasks [133].

All these findings suggest that, although representing a promising tool for realizing implants, stimulating electrodes and restoring neuronal function, DBMs suffer still some limitations when used for long lasting applications [52, 134].

CONCLUSIONS

The recent intensive work of many research groups has produced compelling evidence that diamond is an excellent material for constructing devices with multiple functions for *in vitro* and *in vivo* neuroscience studies. Future work and the development of new techniques will certainly improve the performance of diamond as a substrate material for the construction of better performing DBMs. In addition to this, diamond is expected to cover also a broad spectrum of further applications that go beyond the cited applications and be more widely used for integrative neuroscience studies. For instance, diamond may represent an excellent material to construct biomedical implants or to be adapted for the diagnosis of wide spread neurodegenerative diseases. B-NCD microelectrodes, that are used for *in vitro* and *in vivo* electrochemical detection of the reduced form of glutathione (GSH), have been now proposed as a tool for the assessment of malignant tumors, being the GSH concentration in cancerous cells much higher than in healthy tissues [135]. In addition, being a biochemically inert and long lasting material, diamond is now employed for hermetic encapsulation of biomedical implants [136], or as scaffold material for bone growth or orthopedic prosthesis [137, 138], particularly in its U-NCD and poly-crystalline forms [139].

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Figure 1

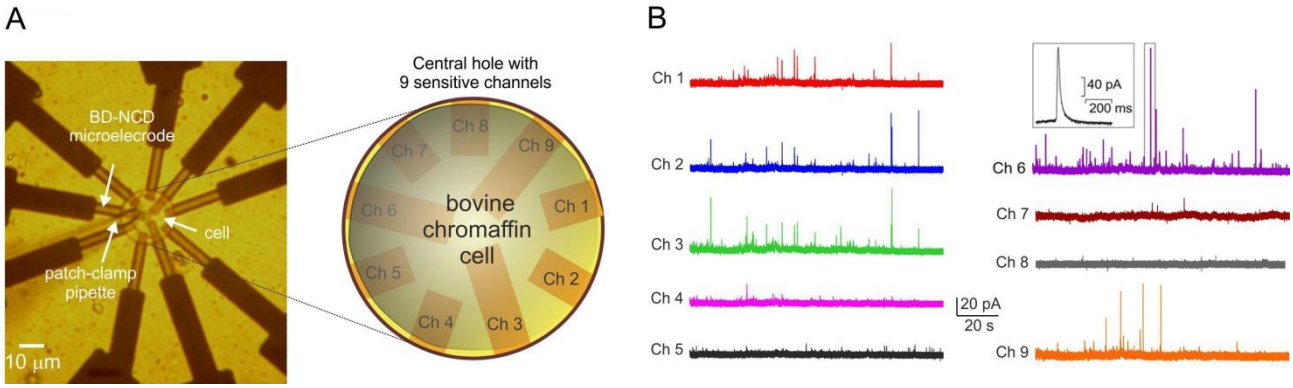

Figure 1. High-density boron-doped diamond arrays for measuring localized secretion

A) Details of a high-density BDD array geometry. The 9 boron-doped electrodes emerge into the central circular area, where a chromaffin cell is positioned by means of a patch-clamp pipette. B) Amperometric spikes detected by the 9 electrodes below the same cell. Inset: a single spike shown on expanded time scale (adapted from [15]).

Figure 2

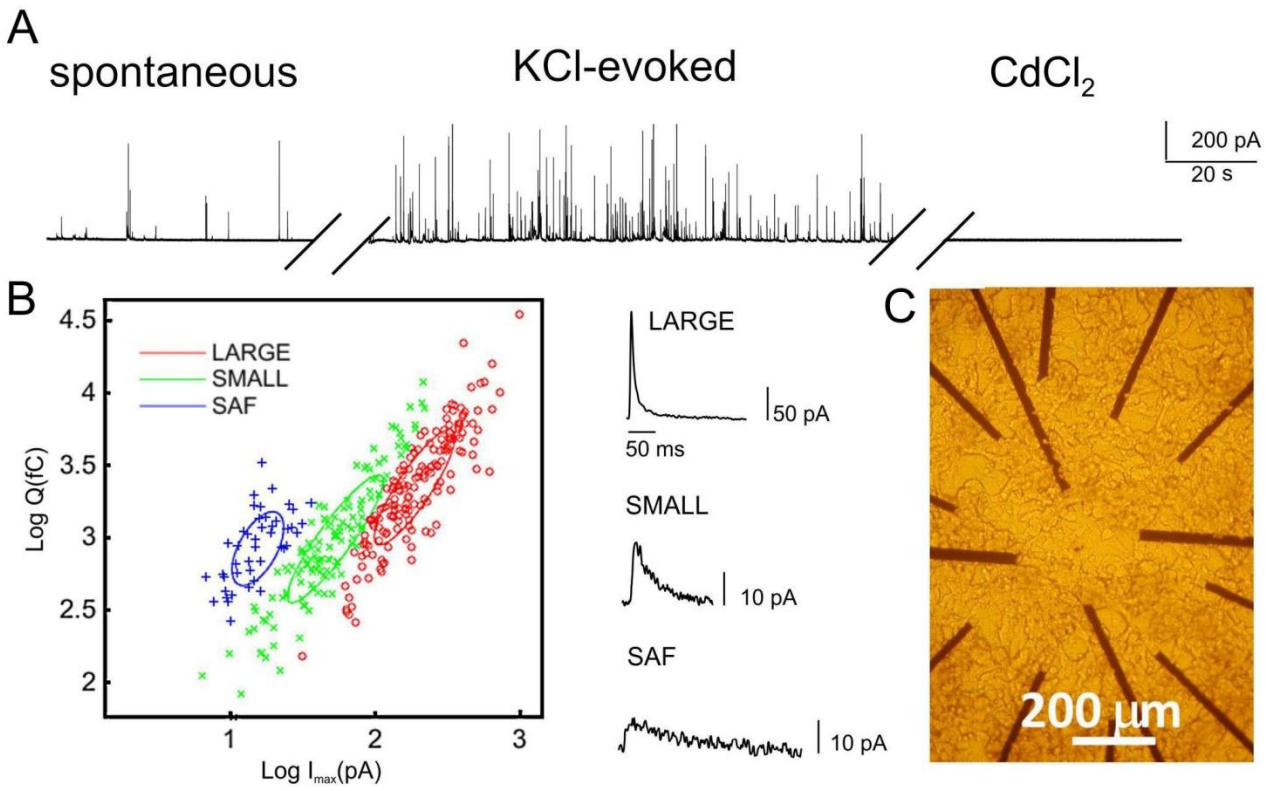

Figure 2. Graphitic micro-channels in a single-crystal diamond to monitor quantal release from cell populations

A) Representative amperometric recordings from one of the 16 electrodes. Spontaneous catecholamine release and KCl-stimulated secretory events from bovine chromaffin cells are blocked by 200 µM $CdCl_2$.

B) Different modes of exocytosis from bovine chromaffin cells are visualized by means of 2D Gaussian mixture analysis. Log(Q(fC)) are plotted versus Log(Imax(pA)) values. Center: Representative events classified as large, small, and stand-alone foot events (SAF). C): Bovine chromaffin cells cultured on the micrographitic UMEA array (adapted from [51]).

Figure 3

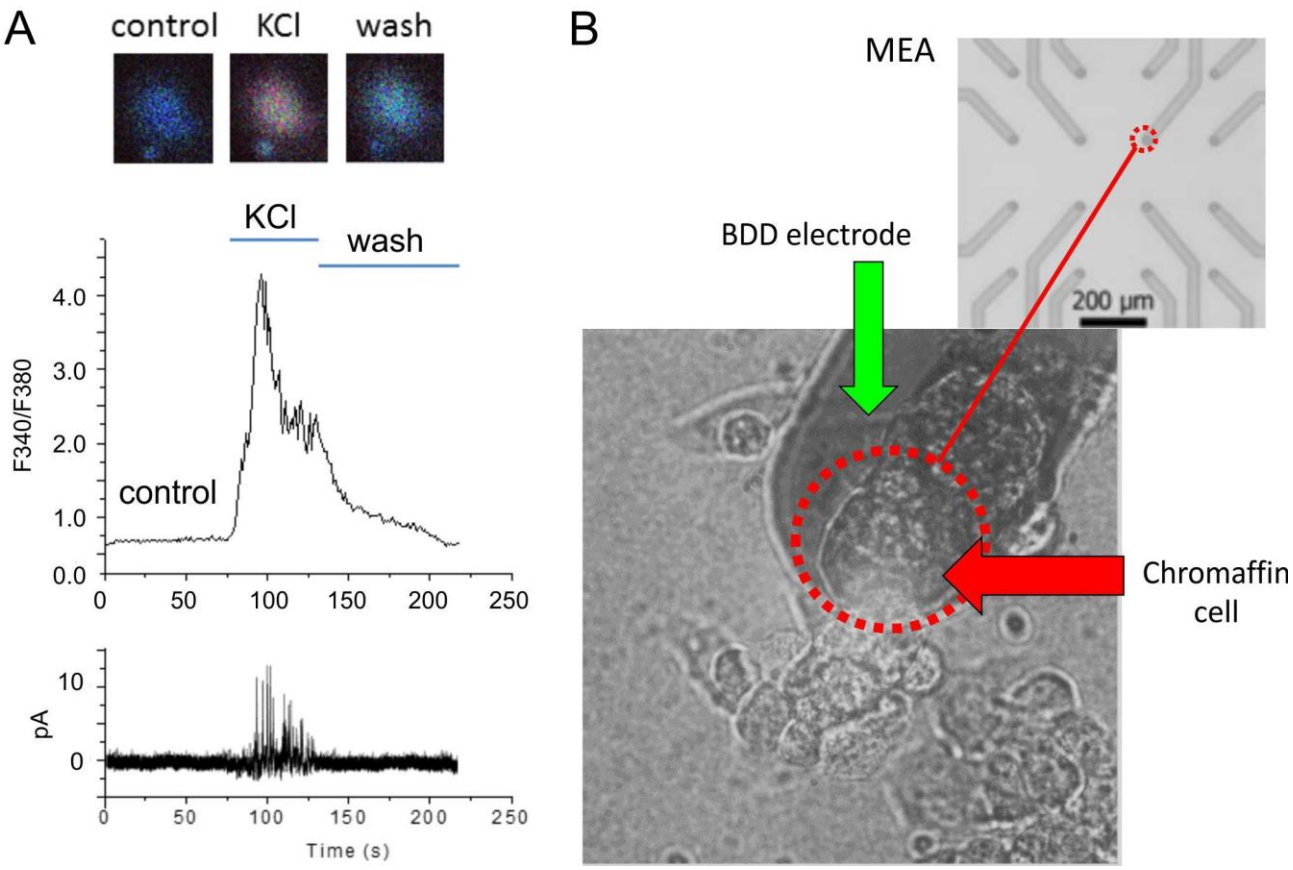

Figure 3. Simultaneous Ca^{2+} imaging and amperometric recordings using boron-doped diamond arrays

Bovine chromaffin cells are cultured on the 16 electrodes array and loaded with Fura-2 AM. A) Simultaneous measurement of intracellular Ca^{2+} increase, estimated as F340/F380 ratio, and quantal release, detected by a sequence of amperometric spikes from a chromaffin cell. B) Details of the multi-array geometry with an enlarged view of one BDD electrode (adapted from [114]).

Figure 4

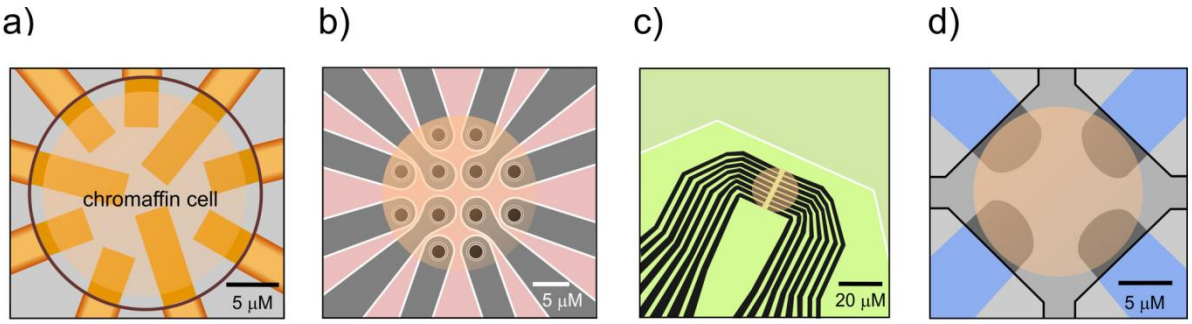

Figure 4. Examples of multiarray geometries used for detecting highly localized neurosecretory events

a) 9 rectangular microelectrodes chip redrawn from [15].

b) 12 round microelectrodes chip redrawn from [44].

c) 16 rectangular microelectrodes chip redrawn from [80].

d) 4 microelectrodes chip redrawn from [111].

The transparent circle drawn in each panel represents a chromaffin cell of 20 μm diameter placed on top of the biosensitive area of the microchip.

TABLE 1- MULTISITE SUBCELLULAR DETECTION OF EXOCYTOSIS

Sensing electrode material	**Sensing electrode array geometry**	**Cellular model**	**Ref**
Carbon fiber MEA	2, 3, 7 carbon microdisks embedded in glass. Tip diameter: 10 ÷ 20 μm	PC12	67-68
Carbon fiber MEA	8÷15 microrings embedded in quartz. Tip diameter: 10 ÷ 50 μm	PC12	69
Pt	4 electrodes, ~3 μm wide, inside 10 x 10 μm square area	Bovine chromaffin	71 72
Pt	3 electrodes, inside a 12 μm diameter circular area	Bovine chromaffin	73
BDD	4 electrodes, inside a 16-18 μm diameter circular area	Mouse chromaffin	74-76
BDD	9-12 electrodes, inside a 22 μm diameter circular area	Mouse and bovine chromaffin	15 44
Pt	16, 25, 36 square electrodes (4, 3, 2 μm width)	PC12	78-79
Pt	16 band electrodes (1.2 μm width) on a movable MEA probe within a 20 μm × 25 μm square area	Bovine chromaffin	80
ITO Gold DLC	4 ITO electrodes inside a 10 x 10 μm square area	Bovine chromaffin	111

BDD: boron-doped diamond, DLC: diamond like carbon, ITO: Indium Tin Oxide.