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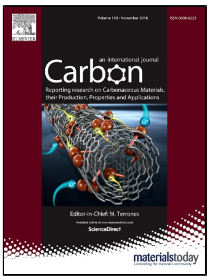

Properties and behavior of carbon nanomaterials when interfacing neuronal cells: how far have we come?

Rossana Rauti, Mattia Musto, Susanna Bosi, Maurizio Prato, Laura Ballerini

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Properties and behavior of carbon nanomaterials when interfacing neuronal cells: how far have we come?

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Abstract

In the last two decades, an increasing amount of studies have investigated the use of components based on carbon-(nano)materials in the engineering of neural interfaces, to improve the performance of current state of the art devices. Carbon is an extremely versatile element, characterized by a variety of allotropes and structures with different properties due to their sp, sp^2 or sp^3 hybridization. Among the diverse carbon nanomaterials, carbon nanotubes and graphene are naturally excellent electrical conductors, thus representing ideal candidates for interfacing electrical-excitable tissues. In addition, their dimensional range holds the potential to enhance the material interactions with bio-systems. Successful interfacing of the nervous system with devices that record or modulate neuronal electrical activity requires their stable electrical coupling with neurons. The efficiency of this coupling can be improved significantly by the use of conductive, *ad hoc* designed, nanomaterials. Here we review different carbon-based nanomaterials currently under investigation in basic and applied neuroscience, and the recent developments in this research field, with a special focus on *in vitro* studies.

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1. Introduction.

Nanotechnology ability to control or assemble materials at the nanoscale has fostered the development of diverse nanomaterials and nanostructures, including quantum dots [1], nanofibers, nanotubes [2] and nanowires [3,4]. These nanomaterials are of particular interest for biomedical applications in neurology, where conductive materials may promote electrical and chemical communication within the nervous system at the micro- and nano-scale levels. Applications of nanostructures to neuroscience have rapidly expanded from molecular imaging [5], to neuro-regenerative scaffolds [6] and neural interfaces [7-9].

In this framework, carbon-based nanomaterials (CBNs) and in particular nanotubes deserve particular attention, due to the exponential increase in neuroscience applications of materials composed mainly by carbon with different hybridization or structures [10].

In this review we focus on the applications of CBNs-based technology in vitro and, in part, in vivo to provide a picture of past and ongoing research in this field, highlighting the goals that have been achieved and the insights reached in understanding CBNs interactions with neural tissues.

2. Carbon and carbon-based nanomaterials

Carbon is the most versatile element in the periodic table [11], owing to the large number of bonds of different type and strength that can form with it or with many other elements. Moreover, the ability of carbon orbitals to hybridize in sp, sp^2 and sp^3 configurations paves the way to the existence of a number of allotropes. To date, the three naturally occurring allotropes of carbon (diamond, amorphous carbon and graphite), have been joined by additional ones deriving from synthetic processes (such as graphene, carbon nanotubes, fullerenes, carbon nanohorns, nanodiamonds) [12; Figure 1].

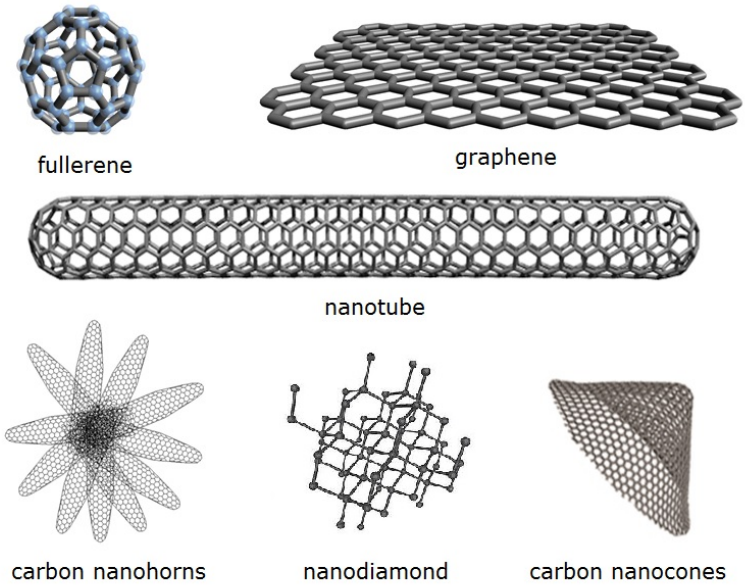

Figure 1. *Carbon allotropes derived from synthetic process.*

The interest in CBNs has increased exponentially in the last decades, first with the discovery of fullerenes (1985), then with that of carbon nanotubes (CNTs; 1991) and finally with the synthesis of graphene (GR) (2004).

The properties of these CBNs make them widely used in many fields ranging from material science [13], energy production and storage [14], environmental sciences [15,16], biology [17-19] and medicine [20,21]. Table 1 summarizes the main properties of the most common CBNs [22-25]:

Carbon Material	Dimensions	Hybridization	Electrical conductivity (S cm^{-1})	Young modulus (GPa)
Graphite	3	sp^2	~4000 p, 3.3c	--
Graphene	2	sp^2	~2000	856.4 ± 0.7 (z) 964.0 ± 0.68 (a)
SWCNTs	1	Mostly sp^2	10^6-10^7	1000
MWCNTs	1	Mostly sp^2	10^3-10^5	1000
Fullerene C60	0	sp^2	10^{-5}	--
Diamond	0	sp^3	10^{-2}-10^{-15}	
Carbon nanohorns	3	Mostly sp^2	10^{-1}	240-730

Table 1 Comparison of some properties of various carbon nanomaterials

Among the many carbon nanomaterials, CNTs and GR are currently the most popular representatives and have been extensively studied for their excellent mechanical strength, electrical and thermal conductivity and optical properties. The Young's modulus and tensile strength of CNTs and GR can reach 1 TPa and 130 GPa respectively [20,21]. Carrier mobility of graphene is around 860 $cm^2 \times V^{-1} \times s^{-1}$ (hole mobility of 844 $cm^2\ V^{-1}\ s^{-1}$ and carrier mobility of 866 $cm^2\ V^{-1}\ s^{-1}$), and the current density of metallic CNTs is orders of magnitude higher than those of metals such as copper [26,27]. Thermal conductivities of CNTs and GR are about 3000-3500 W/mK and 5000 W/mK respectively [28]. The light absorption ratio of single-layer graphene is limited to 2.5 % [29]. A large amount of the research efforts were focused on exploiting these properties for various applications including electronics, biological engineering, filtration, lightweight/strong composite materials, photovoltaic and energy storage [30-32]. CNTs and GR are naturally good electrical conductors and their biocompatibility can be modulated [33], making them good candidates for improving electrodes for neural interfaces. Electrical recording or stimulation of nerve cells is widely employed in neural prostheses (for hearing, vision, and limb-movement recovery), in clinical therapies (treating Parkinson's disease, dystonia, and chronic pain), as well as in basic neuroscience studies. In all these applications, electrodes of various shapes and dimensions stimulate and/or record neuronal activity to directly modulate behavior or to interface machine. The performance of the electrodes can be significantly improved by implementing the device with nanomaterial-based coatings (such as CBNs), since their high surface area can drastically increase charge injection capacity and decrease the interfacial impedance with neurons [34].

Signal transmission in neuronal systems results from ionic currents passing through specific ion channels across the cell membrane. Extracellular recordings monitor the electrical field associated with this dynamic. The time course of the extracellular action potential is typically ~1 ms and the amplitude is in the range of a few tens to a few hundreds of microvolts [35-37]. This amplitude is significantly smaller than the corresponding intracellular spike, which is in the tens of millivolt range. A reverse process takes place during stimulation where charges are delivered from the electrode to affect membrane potential [37-39]. Stimulating neurons and recording extracellular signals can be achieved using a conducting electrode placed close to the cell or its processes [37]. Clearly, an effective interface is a prerequisite for both stimulation and recording.

A successful brain interface will record neuronal signals with a stable ≥ 5:1 signal-to-noise ratio [35]. The electrode's impedance contributes to the noise, with higher impedance providing a lower signal-to-noise ratio [35].

Brain electrodes have been manufactured with a variety of different materials, including tungsten, platinum, iridium oxide, titanium nitride, poly(ethylenedioxythiophene) (PEDOT). The quality of

the recording electrodes depends on their impedance at 1 kHz, in vivo usually ranging between 50 kΩ□ and 1 MΩ [35]. In stimulating electrodes, the amount of charge required for stimulation is orders of magnitude higher than the recorded one [37,40], thus delivering the appropriate charge to the tissue without causing electrode or tissue damage is the main aim in any electrode design [35,37,41]. An additional important parameter related to stimulation electrodes is the reversible charge storage capacity (CSC), also known as the reversible charge injection limit [37,42], that is the total amount of charge that may be reversibly stored, including storage in the double layer capacitance or any reversible Faradaic reaction [37].

In general, electrodes used in neural stimulation can be divided into two major categories. Macroelectrodes which exhibit high-charge/phase and low-charge density thresholds; they are typically placed on the surface of the target tissue and have a geometric surface area (GSA) larger than 100.000 μm^2 [35]. Conversely, microelectrodes exhibit low-charge/phase thresholds and high-charge density thresholds, and they are typically penetrating electrodes with GSA smaller than 10.000 μm^2 [35]. The Huntington Medical Research Institutes, upon an extensive study, has suggested the GSA safe window for penetrating microelectrodes (GSA $\leq$ 2000 μm^2) in the brain with charge/phase thresholds of $\sim$1 nC ph^{-1} [43,44]. Similarly, Kuncel and Grill [45] identified GSA for safe macroelectrodes used in clinical studies (GSA = 0.06 cm^2) with estimated charge density <10 $\mu C\ cm^{-2}$ ($\sim$0.5 $\mu C\ ph^{-1}$) to avoid tissue damage [45].

The material used, the size and the shape of the electrode, together with the electrolyte composition, and the electrical stimulation waveform, will thus influence the CSC. We refer the reader to specialized reviews for a detailed description of the electrochemical electrode-electrolyte interface of recording and stimulation neuronal electrodes [35,37,42,46].

The peculiar physical features of certain classes of CBNs, very high mechanical strength and electrical conductivity, combined with the low dimensions favoring tissue adhesion, suggested the potential engineering of artificial scaffolds composed by CBNs to interface neuronal activity and to promote neuroregeneration, e.g., after spinal cord injuries [47-50].

CNTs are among the most studied carbon nanomaterials for biomedical applications [51-53] in particular in neuroscience, due to their privileged interactions with neuronal cells [53-60], which make them potential components of innovative diagnostic and therapeutic systems for brain pathologies. More recently, we have witnessed a growing interest also in graphene [61,62], nanodiamonds [63,64] and carbon dots [53,65,66]. Conversely, fullerenes are now experiencing a

gradual loss of interest due to increasing concerns regarding their toxicity [67-69]. A detailed analysis of the diverse CBNs is reported in the following paragraphs.

3. Carbon nanotubes (CNTs)

CNTs have been observed by Ijima in 1991 [70] and exhibit outstanding mechanical, thermal, and conductive properties. They are unique nano-objects made of one-atom-thick sheets of sp^2-hybridized carbon (graphene) rolled in a cylindrical shape.

Two major forms of CNTs have been used in biological applications: single walled CNTs (SWCNTs) and multi walled CNTs (MWCNTs). SWCNTs are made of a single layer of graphene and their diameter ranges from 0.7 to 1.4 nm, while their length can vary from few hundreds of nm up to many μm. MWCNTs consist of multiple concentric cylinders of rolled-up graphene sheets that form tubes with diameters up to 100 nm.

CNTs possess high surface area, high mechanical strength, accompanied by ultralight weight, electron-rich properties, and excellent chemical and thermal stability [71]. These properties make CNTs very promising in different fields: they have been used in conductive composites, for energy storage and energy conversion devices, sensors, field emission displays and radiation sources, hydrogen storage media and nanometer-sized semiconductor devices, probes, and interconnects [72]. Their poor solubility and their potential toxicity have been discussed and partially alleviated in the past decade through the functionalization of the CNTs surface by means of many different approaches, aimed at increasing their solubility and safe by design features, to promote biomedical applications [73]. CNTs have been proposed as biosensors [74], ion channel blockers [75], biocatalysts [76], tools in cancer diagnosis and therapy [77] and nanovectors [78].

Among the number of possible biological applications of CNTs, tissue interfacing and engineering are the most intriguing ones [79]. Due to their peculiar features, CNTs appear to be suitable for the interaction with electrically active tissues, such as neuronal and cardiac tissues. In particular, many studies have demonstrated that CNTs substrates are able to sustain neuronal survival and to promote neuronal process outgrowth [54,57,73,74].

Most of our knowledge on neural interfaces has been gained by studying 2D structures/devices, more recently biologists have explored the use of 3D topographical complexes reminiscent of the physiological extracellular environment in which cells routinely operate in vivo [80]. In 2009, Ghibaudo and colleagues [81] have reported differences in cellular interactions between 2D and 3D substrates. Cells interfaced to 3D microenviroment showed more elongated and branched shapes [81].

Gui et al., [82] have molded CNTs into a 3D porous sponge with a very high porosity while retaining the desired mechanical properties. The sponge structure obtained was very stable, showing excellent compressibility and ability to recover volume by free expansion [82]. Bosi et al. were able to fabricate 3D PDMS scaffolds with pores layered by an irregular CNTs carpet stably entrapped in the PDMS matrix (Figure 2) [83]. These 3D scaffolds made of polymer-CNT and of pure CNT were applied not only to study the activity of primary hippocampal neurons in vitro (Figure 2) [83], but, in the form of pure CNTs 3D scaffolds, for the growth and functional reconnection of spinal cord organotypic slices (Figure 3) [50].

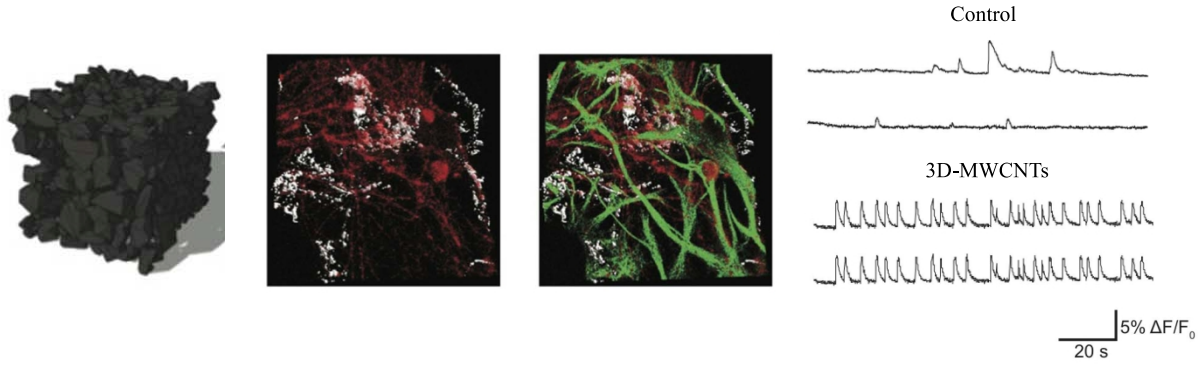

Figure 2. *On the left, sketch of the PDMS_MWCNTs scaffold. In the middle, confocal micrographs show hippocampal cultures grown (9 DIV) on 2D-PDMS (left) and 3D-MWCNTS (right) immune-stained for β-tubulin III (in red), GFAP (green) and DAPI (blue). Scale bar: 100 μm. Repetitive Ca^{2+} activities spontaneously recorded in 2D- and 3D-MWCNTs. (Modified with the permission from Bosi et al. 2015 [83]).*

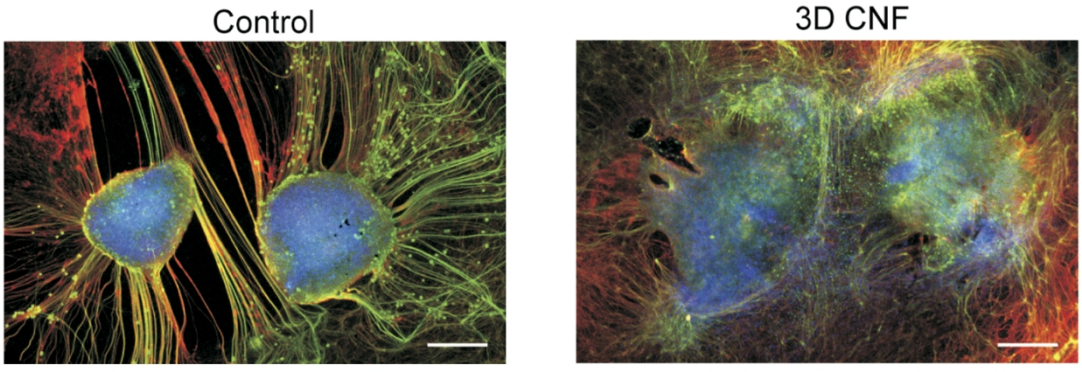

Figure 3. *Spinal organotypic slices cocultured in Control and in 3D CNTs after 14 days of growth. Immunofluorescence is for neuron-specific microtubules (β-tubulin III; red), neurofilament H (SMI-32; green), and nuclei (DAPI; blue). (Modified with permission from Usmani et al. 2016 [50])*

CNTs based components may contribute to the development of robust and biocompatible neuroprosthetic devices, with the aim of restoring abilities to patients who have lost sensory or motor function because of disease or injury.

Indeed, CNTs, beyond their being manufactured in 3D structures, display desirable properties for use in stimulation/recording electrodes: (i) CNT-based electrodes have been successfully miniaturized and do not seem to inflict tissue damage; (ii) CNTs have the ability to operate as ballistic conductors which aids in lowering electrode impedance and increasing charge transfer; (iii) CNTs display exceptional flexibility and they can be twisted and bent to a large degree, although they are five times mechanically stronger than steel [84].

In addition, CNTs are attractive as neural electrodes both in vitro and in vivo because of the high electrochemical surface area (ESA)/ GSA ratio inherent in the nanotube geometry, which gives rise to a large double-layer charge capacity. For neural stimulation, Wang et al. [85] have found charge-injection capacities of 1–1.6 μ C cm^{-2} with vertically aligned nanotube electrodes, and work on the development of nanotube and nanofiber neural interfaces has been reported [35,85].

Such properties allowed for engineering CNT-based electrodes used in interfacing neuronal activity in vitro and in vivo [84; see also below]: (i) stimulation of action potentials/ Ca^{2+} excitability in a small group of neurons in culture via multi-electrode arrays, (ii) stimulating and recording from neurons in hippocampal organotypic slice cultures as well and in the whole mount mouse retina, (iii) stimulation of and recording from rat and monkey cortices, and (iv) recording human electroencephalogram (EEG) [84,87].

Historically, the first experiment reporting neuronal electrical stimulation through CNTs, was performed by Liopo and colleagues [88]. CNTs were deposited onto polyethylene terephthalate films and a separated stimulation chamber was created putting a ring in the middle of the film to contain dorsal root ganglion neurons. The stimulating electrodes were attached to the CNTs substrate outside of the ring. It has been found that a current step of 1 μ A amplitude, applied directly to the CNTs substrate, elicited a neuronal response, monitored as inward trans-membrane current by whole-cell patch recordings.

Similarly, Gheith and coworkers [89] showed that neurons were activated by steps of electrical stimulation delivered through SWCNTs films, made by the layer- by-layer method, which consists in alternate layering with a negatively charged polyacrylic acid polymer and positive charged SWCNTs.

New insights were obtained by Mazzatenta and coauthors [90]: by using an experimental setting similar to that reported by Liopo, these authors found that neuronal circuits, chronically grown on

SWCNTs substrates, could be effectively stimulated via the SWCNTs-layers. In fact, they observed that the delivery of voltage steps via silver wire-SWCNTs layer induced the appearance of fast inward currents in hippocampal cultured neurons, monitored in voltage-clamp mode, which were abolished by tetrodotoxin (TTX), a selective blocker of voltage gated fast sodium channels. When recording in current clamp, supra-threshold stimulations elicited repetitive action potentials (APs). However the effective stimulation of neural network via SWCNTs was proved by monitoring the emergence of synaptic responses in neurons due to action potentials elicited by CNTs electrical stimulations of the pre-synaptic cells.

In addition, the presence of tight contacts between neuronal membranes and CNTs was imaged by SEM [90], indicating, together with electrophysiological experiments, the presence of a tight electrical coupling between CNTs and neuronal membranes.

The next advances in CNT-based neuronal interfacing were provided by Wang and collaborators [85]. These authors designed a prototype of neural interface, using vertically aligned MWCNTs pillars as microelectrodes (VACNF), which offered a high charge injection limit (1-1.6 mC/cm^2) without faradic reactions. Rat hippocampal primary cultures were grown on these devices and, while neurons were stimulated via CNTs electrodes, neuronal activity was optically monitored by live calcium imaging, highlighting that the use of CNTs as safer and more efficient neural prostheses electrodes when compared to metal ones [85].

In a different study, CNTs were layered by electrically conductive polymers, such as polypyrrole, to improve the mechanical properties of the substrate and the efficacy of the electrical stimulation, improving also CNT biocompatibility [86]; potentially, this might provide an additional strategy enabling controlled and localized drug release [91]. Carbon nanofibers electrode architectures have been further employed to provide long-term, neuron-electro-analytical measurements of the dynamic processes of intercellular communication between excitable cells. Multi-element electrode arrays composed of individually addressed VACNF have been used as growth substrates of neuronal-like cell lines (PC12) and primary neurons (rat hippocampus) over extended periods (days to weeks) [92]. Neuronal activity was indirectly monitored at the electrode site via detection of oxidized species generated by the cultured cells, i.e. neurotransmitters. Preliminary data suggested that quantal release (in vesicular quanta) of easily oxidized transmitters could be observed at the nanofiber electrode upon at least 16 days of culturing [92].

Nowadays, in vivo recordings by CNTs-coated sharp electrodes have been reported in the motor cortex of anesthetized rats and in the visual cortex of monkeys [37]. Compared with bare metal electrodes, CNTs coated ones reduced the noise and improved the resolution of the detected spontaneous activity [93]. CNTs-coated sharp electrodes were tested in the anesthetized rat motor

cortex (controlling limb movement) and in awaken trained monkey V4 visual cortex (involved in perception of form-with-color) [37,84]. In these diverse in vivo experimental models, CNTs-coated electrodes outperformed their paired control electrodes in terms of reduced noise (~17 dB) and increased sensitivity of detection (on average 7.4 dB more power) of spontaneous electrical neuronal activity throughout various ranges of acquisition frequencies (1–1000 Hz) relevant to brain (patho)physiology [37]. Due to their mechanical strength, CNTs endured the advancement of electrodes through the dura mater and remained intact even after recordings were completed, as assessed by electron microscopic investigation of the used electrodes, thus planar and 3D electrodes coated by CNTs enhanced the interface performance in in vitro and in vivo models [37,84].

More recently, CNT-fiber's performance and biocompatibility were further tested in vivo for neural stimulation and recording by Vitale and collaborators [94]. In vivo chronic studies in Parkinsonian rodents showed that CNTs microelectrodes stimulate neurons as metal electrodes with 10 times larger surface area, while eliciting a significantly reduced inflammatory response [94], with the very same CNTs microelectrode that can record neural activity for weeks. These authors thus conclude that CNTs fibers are the ideal candidate material for the development of small, high charge density, low impedance, flexible microelectrodes capable of stable interfacing of neural ensembles [94].

Another issue addressed by several studies is related to the optimization of the production process to obtain CNT-based Multielectrode Array (MEA) systems more easily, cost-effectively and with a high degree of reproducibility. Shein and collaborators [95] prepared CNTs-MEA systems by means of a conventional micro-fabrication technique, where CNTs were deposited through a chemical vapor deposition growth procedure utilizing metal electrodes as catalyst. The authors seeded rat cortical neurons on these chips: after several days in culture, neurons and glial cells aggregated and accumulated on CNT covered regions allowing the detection of neuronal activity via CNT electrodes up to 60 days *in-vitro* with high stability. Adjacent electrodes were used to stimulate and to record evoked neuronal responses. In this work, CNTs were exploited to design biocompatible, long lasting stimulation/recording systems, where micro-fabrication technique allowed the design of patterned network.

Shoval and coauthors [96] employed a similar procedure to develop CNT-MEA devices, which were exploited to record the activity of whole-mount neonatal mouse retinas. After minutes from the placement of retinas on electrodes, the authors could monitor neural spontaneous activity as typical bursting and propagating waves with a higher signal-to-noise ratio in comparison with commercially available electrodes. Interestingly, the recorded signals underwent over a period of minutes to hours to a gradual increase in the signal amplitude, suggesting a dynamic interaction between CNTs and neurons, which resulted in enhanced cell electrode coupling.

Chen and collaborators [97] developed a flexible CNT-MEA, with an improved electrode impedance and charge-transfer capacity by more than six times, thanks to the presence of CNTs. CNT-MEA was used to record electrocorticograms from the rat cortex in vivo, again showing improved signal-to-noise ratio [97].

Very recent developments are pointing to the potential use of SWCNTs in manufacturing multifunctional human-machine interfaces of the future [98]. Proof of principle experiments in humans demonstrating the crucial role of SWCNTs in obtaining effective prototypes for wearable or patchable smart systems [98] clearly indicate the future potentials of these materials.

In more visionary developments, CNTs might not only improve electrodes' quality, but might also support and direct axons regrowth and functions, thanks to CNT intrinsic properties. The electrical activity of rat hippocampal neuronal networks developed on CNTs microelectrodes is characterized by earlier onset (4 days after seeding) in comparison to the ones of cultures grown on control electrodes. The authors suggested that the increase in surface roughness in CNT immobilized microelectrodes provides cells with a larger surface area to adhere with, boosting the activation of integrins, and promoting a faster neuronal differentiation [99].

Under controlled experimental conditions, CNTs showed also a good biocompatibility in the brain in vivo. Intravenous (i.v.) administration of ^{13}C- enriched SWCNTs in mice [53,100] demonstrates that these nanomaterials (10–30 nm × 2–3 μm bundles) are able to cross the Blood Brain Barrier (BBB) and accumulate inside the brain tissue, yet only to a limited extent. This study suggested that SWCNTs did not show acute toxicity despite their accumulation in several organs (especially liver, lungs, and spleen) and despite their slow clearance [53].

Aurand and co-authors [101] implanted PDMS-CNTs scaffolds into the adult rat visual cortex for 2, 4 and 8 weeks showing minimal immune response following their implantation into the CNS and confirming the biocompatibility of CNTs-scaffolds and supporting their application as neural interfaces.

CNTs were also probed for neuroregenerative applications in spinal cord injury (SCI) model rats. Post-injury administration of PEG-functionalized SWCNTs (PEG-SWCNTs) in the lesion site was found to promote axonal survival and repair, while delayed administration was able to achieve a dose-dependent reduction in the lesion volume in both gray and white matter, and an increase in the number of neuronal fibers in the lesion epicenter with a modest sprouting of corticospinal tract axons into this region [47,53]. Neither alterations in reactive astrogliosis at the lesion site nor toxicity or neuropathic pain were detected. As outcome, a dose-dependent moderate recovery of motility in treated rats was achieved.

In alternative approaches, CNTs based systems have been rigorously investigated in cancer therapy to carry and deliver drugs, and assessed for potential gene, thermal, photodynamic and lymphatic targeted therapy [102,103]. Current treatments for brain cancer and other CNS diseases are of limited success, partly due to the difficulties posed by the drugs insolubility and poor distribution, lack of selectivity and the inability to cross the cellular barrier and the BBB. Ad hoc engineered CNTs (shape, dimensions, functionalizations with different molecular moieties) may show, together with good electronic properties, a remarkable cell membrane penetrating capability, high drug-loading and pH-dependent therapeutic unloading capacities, thermal properties, large surface area and easy modification with molecules, which render them suitable candidates as drug delivery nano-vectors [104].

Functionalized CNTs may show good pharmacokinetic profile, the ability to make complexes with a desired selectivity and specificity allowing safe, effective and target delivery of therapeutic agents to the tumor cells [103,105,106].

The large amount of CNTs applications in biomedicine and the ongoing developments mentioned above, have prompted since decades multiple studies addressing their potential toxicity. Yet, toxicity of CNTs is still a matter of debate, indeed a number of investigations highlighted toxic effects in cells upon CNTs exposure [53,107-109]. The danger of CNTs is lower by their being engineered and immobilized in platforms, substrates or electrodes or higher when used as free, unbound particles. In fact, when used as substrates for in vitro studies, CNTs substrates were shown to have no toxic effects on cell lines, dissociated primary cultures, or organotypic slice cultures [23,57,110], accordingly all studies reporting the use of CNT to implement in vivo electrodes did not observed nano-tube related toxicity [37,84,93,94,98]. Different and more complex is the case of unbound particles in fact both MWCNTs and SWCNTs may have toxic effects in their soluble forms, when not properly functionalized. The reported cytotoxicity is mainly due to the capacity of CNTs and nanoparticles in general to enter into cells and disperse in the cytoplasm as demonstrated by Simon-Deckers and collegues in 2008 in human pneumocytes [111].

Several studies have been conducted to understand the risks related to CNTs exposure also in the perspective of biomedical applications. Contaminants, such as Fe, Ni, Co, and Y nanoparticles deriving from CNTs synthesis processes, may significantly contribute to the material toxicity [53,112]. Pulmonary exposure and ingestion represent the major issues for workers involved in the manufacturing of CNTs [113]. Another important factor that has been the focus of many studies is the potential of CNTs to induce DNA damage and mutation, possibly leading to the onset of cancer, the so-called genotoxicity [114]. MWCNTs for example are able to enter and accumulate in mouse

embryonic stem cells inducing oxidative damage of DNA [115,116]. Additional determinants of CNTs toxicity, are their size and surface functionalization together with the way and dose of administration. By optimizing these features CNTs were further developed towards clinical applications [117], for example CNTs were functionalized enabling biomacromolecules translocation inside cells and thus used as proteins and nucleic acids nano-vectors [118,119]. Many studies showed that MWCNTs can induce inflammation, fibrosis, angiogenesis and cytotoxicity to macrophages [120] dependent upon MWCNTs length, iron content or crystal structure [120,121]. Conversely, no toxicity has been observed in SWCNTs in a study on mice over period of three months [122]. Yang et al., showed that higher molecular weight PEG chain attachment to CNTs allowed for a safe elimination from the body with no residual toxicity [123]. Yang et al. [124] noted that PEGylated CNTs has lower reticuloendothelial system (RES) uptake, prolonged circulating time and reduced deposition in liver and spleen [120].

Pondman et al. [125] overcome the activation of classical inflammatory pathway, thus reducing CNTs overall toxicity, by coating CNTs with recombinant globular heads. Coated CNTs lack the collagen region of human C1q that will help escaping phagocytosis [120,125,126]. Silva et al. [127] showed that purified or functionalized MWCNTs induced smaller or negligible inflammation at pulmonary level. Selecting the right forms of SWCNTS is another strategy to reduce toxicity [119,128]. In experiments involving neuronal cells, which are commonly considered particularly sensitive to toxicants and to inflammation, high purity and functionalized CNTs rarely show toxicity [53,129-131]. Importantly, CNTs can be enzymatically degraded by peroxidases [132] in macrophages [133], eosinophils [134] and microglia [135], thus mitigating the concerns regarding possible toxic effects due to their accumulation inside the body [53].

In summary, we believe that this large amount of studies testifies how CNTs still represent cutting-edge nanomaterials for biomedical applications in neurology.

4. Graphene

Among the new generations of carbon based nanomaterials, graphene (GR) is definitely the most recently developed and engineered in many fields of applications: this carbon allotrope consists of a single layer of carbon atoms arranged in a hexagonal honeycomb lattice and can be considered the founder of many other allotropes of carbon, such as graphite, carbon nanotubes and carbon nanohorns. GR is the thinnest compound known to man at one atom thick, the lightest material known (with 1 square meter coming in at around 0.77 milligrams), the strongest compound discovered (between 100-300 times stronger than steel and with a tensile stiffness of 150,000,000 psi), the best conductor of heat at room temperature (around 5000 W/mK) and also the best

conductor of electricity known (with a reported carrier mobility of more than 15,000 $cm^2 \times V^{-1} \times s^{-1}$ [20]). The excellent electrical and chemical properties of GR combined with its biocompatibility provide opportunities for new biomedical applications. After the groundbreaking experiments of Geim and Novoselov [136] on GR, research on this carbon allotrope has grown exponentially with more than 30000 publications in the last decade. Its simple molecular architecture and GR ability to combine with other existing nano- and biomaterials make it suitable for a variety of purposes and it has been developed in a wide variety of GR-based materials. Single layer graphene, bi-layer graphene, multilayer graphene, graphene oxide (GO), reduced graphene oxide (rGO) and chemically modified GR are the members of the GR-based nanomaterial family: each member of this family possesses its own features in terms of oxygen content, number of layers, surface chemistry, purity, lateral dimensions, defect density and composition. Due to its highly reactive surface, single layer defect-free GR production is challenging and it is also difficult to suspend in water solutions. These are the main reasons why GO and rGO are usually preferred for biological applications.

Nonetheless, GR has already been engineered for several biomedical applications, including cellular imaging and drug delivery [137], bio-analysis [138], stem cell research [139,140] and even photothermal therapy for tumors [141].

GR films were shown to have excellent biocompatibility supporting the growth of primary cultures of mouse hippocampal neurons and promoting neurite sprouting and outgrowth, especially during hippocampal early developmental phases [142]. Fabbro et al. observed that GR-based materials are inert neuron-interfacing materials, able to preserve the basal physiological level of neuronal activity [143]. They noticed the uncommon ability of GR-based substrates (GBSs) to support neuronal development (in terms of neuronal passive properties, spontaneous synaptic activity, synaptogenesis, and short-term synaptic plasticity) without pre-coating with adhesion-promoting peptides (e.g., polylysine or polyornithine). More recently, GR was reported to tune the extracellular ion distribution at the interface with hippocampal neurons, a key regulator of neuronal excitability. The ability of GR to trap ions is maximized when a single layer GR is deposited on electrically insulated substrates. These biophysical changes caused a significant shift in neuronal firing phenotypes and affected network activity [144].

One of the first observations related to the possible use of GR in the brain environment was that the biocompatibility and broad-spectrum transparency, flexibility and mass-producibility makes GR an ideal candidate for replacement of commonly used indium-tin oxide in neural interfacing devices. Indeed, there are several examples of effective GR-based electrode devices in the recent literature. A GR-based, carbon-layered electrode array (CLEAR) device was implanted on the brain surface in

rodents for high-resolution neurophysiological recording. The optical transparency of the device at > 90 % transmission over the ultraviolet to infrared spectrum demonstrated its utility through optical interface experiments using this broad-spectrum light wavelength transparency. These experiments included optogenetic activation of focal cortical areas directly beneath electrodes, in vivo imaging of the cortical vasculature via fluorescence microscopy and 3D optical coherence tomography [145; Figure 4].

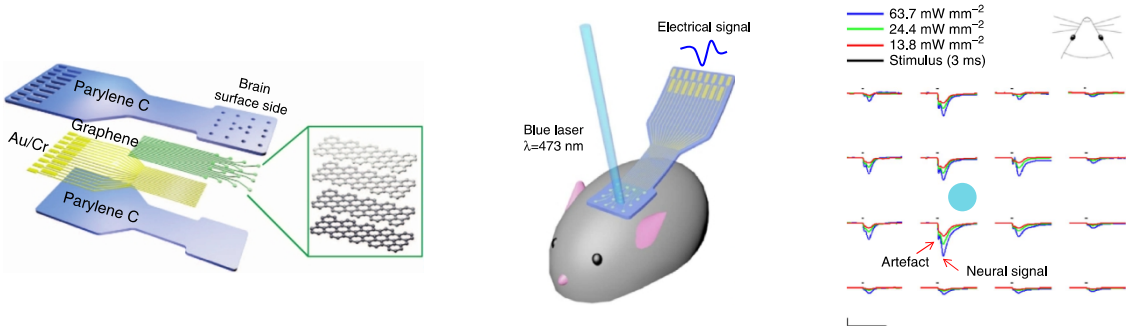

***Figure 4**. Left panel: diagram of carbon-layered electrode array (CLEAR) device construction showing the layered structures; middle panel: schematic drawing of opto-experimental setup, showing the CLEAR device implanted on the cerebral cortex of a mouse, with an optical fibre delivering blue light stimuli to the neural cells; right panel: optical evoked potentials recorded by the CLEAR device. X-scale bars represent 50 ms, y-scale bars represent 100 mV (Modified with the permission from Park et al. 2014 [145]).*

GR and GR related materials (GRMs) offer several benefits as novel components for the engineering of neural interfaces, including multi-functionality and biocompatibility. Kostarelos et al., [146] reported the manufacturing of flexible neural implants characterized by very low noise levels. Using a flexible array of GR field-effect transistors, the implants successfully detected slow-wave activity, synchronous epileptic activity and audio-visual responses in rats, matching the performance of state-of-the-art platinum electrode implants [147].

Recently, Thunemann and collaborators [148] explored transparent graphene array technology integrated with 2-photon imaging and single-photon optogenetic photostimulation. With this approach, these authors obtained simultaneous mapping of surface local field potentials and high-resolution 2-photon imaging of neuronal calcium transients in in vivo animal models [148].

The merging of integrated in vivo optical imaging and stimulation methods by engineering graphene-based electrodes proves that transparent graphene technology is a versatile platform applicable to numerous different experimental settings. Whenever depth-resolved electrical recordings are not required, optically transparent graphene technology allows seamless integration

with depth-resolved optical imaging and stimulation, circumventing the need for more invasive brain probes [148].

This combination of measurements holds the potential to bridge research models (cell cultures, brain slices, in vivo mouse recordings, etc.), to human non- invasive electro-/magnetoencephalography measurements [148,149].

GR is also explored as a novel platform for the local delivery of therapeutic molecules, and the preliminary results are encouraging. Functionalization of GR and GO can tailor their properties and enable their use as carriers of therapeutic molecules, while their biosensing, optical and photothermal properties are also being exploited for combinatory interventions [150]. As an electroactive material, GR is considered emerging as a next-generation neuronal tissue engineering scaffolds to enhance neuronal regeneration and functional recovery after brain injury. Electrospun microfiber scaffolds coated with self-assembled colloidal graphene were implanted into the striatum or into the subventricular zone of adult rats [151], while microglia and astrocytes activation levels were suppressed with GR functionalization. In addition, self-assembled GR implants prevented glial scarring in the brain 7 weeks following implantation. Astrocytes guidance within the scaffold and redirection of neuroblasts from the subventricular zone along the implants was also demonstrated. Song et al. observed [152] that 3D GR supported the growth of microglia and showed good biocompatibility. Microglia is a macrophage like phagocytic cell normally inactive unless provoked by damaging xenobiotics. These cells are derived from myeloid cells and constitute 12 % of brain cells [153]. The observations indicated that 3D GR offered milder neuroinflammation on microglial cells compared to 2D GR, which further suggested that the topographical features could affect inflammatory behaviors. Additionally, the 3D GR foams facilitated the growth of neural stem cells and PC-12 cells (originated from neural crest) and proved that they can be used for neural repairing and neurogenesis.

Additional researches supported the ability of GR substrates to promote neurites sprouting and outgrowth [142], to enhance neuron electrical signaling [154] and to reduce tissue inflammatory response [152]. In neurology, GR represents a promising tool for neuronal implants or bio-devices, with potential applications that range from neuro-oncology to neuro-regeneration [117,155]. Recently, it was reported that small graphene oxide nanosheets (s-GO) interfere specifically with neuronal synapses, without affecting cell viability. In particular, in cultured neuronal networks, upon chronic s-GO exposure, glutamatergic release sites were sized down [155]. Different studies reported the use of GBSs at the CNS level for cell labeling and real-time live-cell monitoring [157,158]; delivery to the brain of molecules that are usually rejected by the BBB [160,161], and

cell analysis based on GR-electrodes [93,94]. In addition, interfacing GR with neuronal cells might be of help in promoting neuronal regeneration [142,143,161,162, Figure 5].

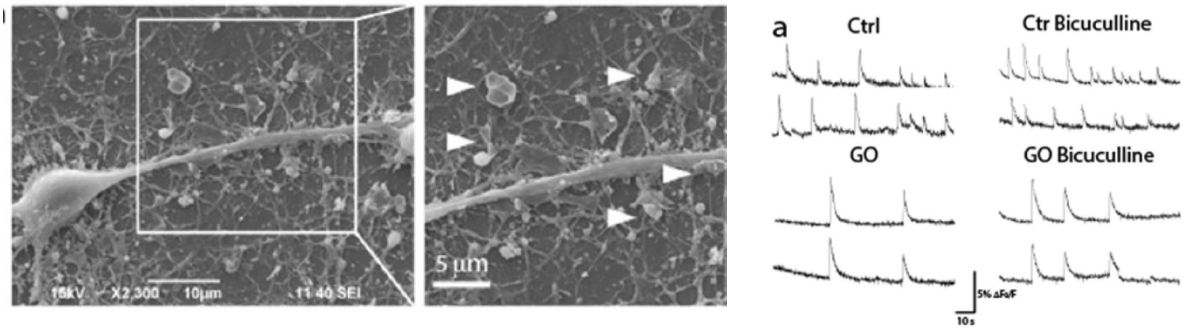

Figure 5. *SEM images showing large number of graphene oxide flakes (white arrowheads) in contact with the neuronal cortical cell membrane, exposed to GO flakes for 14 days. On the right panel, representative spontaneous (left panels) or bicuculline-evoked (right panels) Ca^{2+} oscillations recorded in 14 DIV cortical cultures in control or GO conditions. (Reprinted with the permission from Bramini et al. 2016 [162], American Chemical Society).*

Among the different possible implementations of GBSs, the production of GR-based scaffolds for cell growth and differentiation is particularly promising. 3D GR foams (3D-GF) can be obtained using nickel foam template for chemical vapor deposition of GR. Neural stem cells growth on these substrates allows their electrical stimulation in more physiological 3D geometries [163; Figure 6].

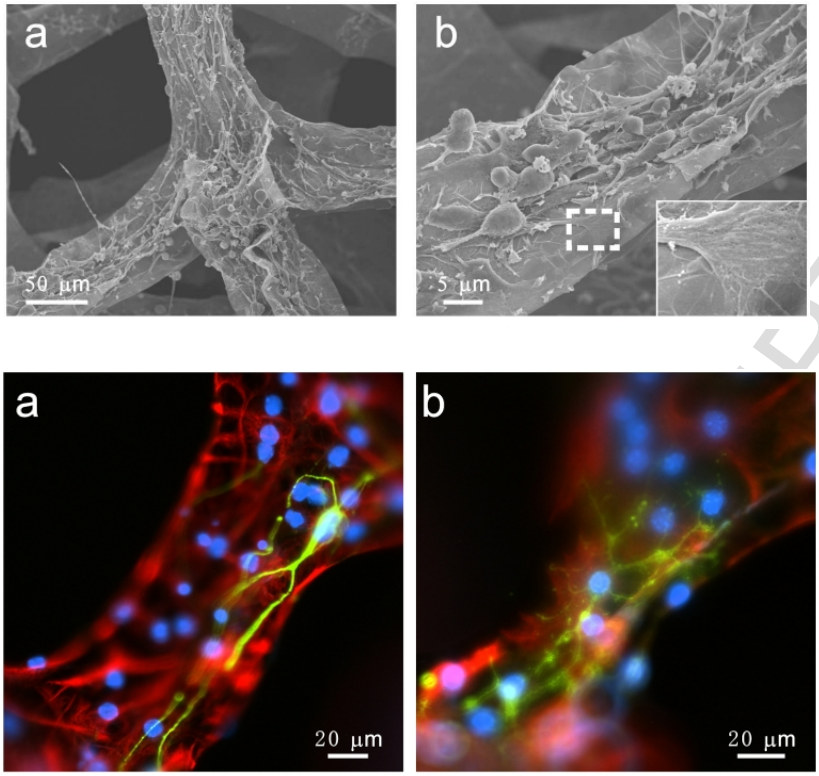

***Figure 6**. Top panel: low- (a) and high- (b) magnified SEM images of NSCs cultured on 3D-GFs. The inset illustrates the interaction between the cell filopodia and 3D-GF surface. Bottom panel: Representative fluorescence images of differentiated NSCs under differentiation conditions, the cells were immunostained with Tuj-1 for neuron (green, a), GFAP for astrocyte (red, a&b), O4 for oligodendrocyte (green, b) and DAPI for nuclei (blue, a&b) (Modified from Li et al., 2013 [163])*

Neuronal dissociated hippocampal cultures, grown on 3D-GFs built as previously described, were also able to recapitulate two basic properties of the complexity of the brain: firstly, the coexistence of local and global electrical activity, and secondly, the existence of neuronal assembly with a degree of correlated electrical activity varying in space and time [164]. With a different strategy Martìn et al. built hybrid hydrogels with polyacrylamide and graphene and showed that GR improves the neuronal biocompatibility of the 3D scaffold [165].

López-Dolado et al. [49,166] were the first to study the in vivo tissue response in the injured rat spinal cord to the implantation of flexible and porous 3D scaffolds composed of rGO. These scaffolds were fabricated by using the ice segregation-induced self-assembly (ISISA) technique. The results revealed that these substrates allowed the formation of a soft interface at the injury site, with no significant differences in the fibroglial scar features with respect to lesions without scaffolds. Due to its porous structure, extracellular matrix molecules (e.g., collagen) and different cell phenotypes were able to infiltrate and migrate to the inner parts of the scaffolds contributing to

the stabilization of both the scaffold and the lesion site [166]. In the brain, Defterali et al. [167] explored the biocompatibility of rGO and its influence on neurogenesis in the adult mice olfactory bulb (OB) in vivo. Major findings revealed that rGO had no deleterious effects on the survival of the resident populations of neurons and astrocytes and of the newly generated neurons. Recent studies by Mendonça et al. [168] focused on the effects of rGO on the blood-brain barrier (BBB) components in vivo. rGO and rGO-PEG were injected intravenously and their toxic effects on BBB integrity analyzed. Both materials caused a notable downregulation of astrocyte markers (GFAP and connexin-43), endothelial tight (occludin) and adherens (β -catenin) junctions and basal lamina (laminin) at 3 h after administration. Interestingly, this effect disappeared after 7 days of exposure to rGO, while in the rGO-PEG group it was permanent and increased over time [168].

The studies reported above suggest that GR and its derivatives are suitable candidates for biomedical applications in the CNS. For this reason, it is expected that the high attention given nowadays to graphene will stimulate rapid improvements both in GR engineering for medical applications, including brain interfaces, and in the understanding of its eventual toxic effects.

5. Diamond

Diamond, a natural as well as a synthetic material, is currently under investigation in several fields of applications. In any list summarizing the specific material properties, diamond is often at the extreme [168]: crystalline diamond shows the highest atomic density of any bulk crystal, the highest bulk modulus and highest thermal conductivity. Diamond, a wide band gap semiconductor, is optically transparent from the far infrared to the ultraviolet, making it an ideal candidate for optical applications [170]. The attractiveness of diamond is that different morphologies and forms can be obtained from this sp^3-hybridized material. Indeed, modulation of the growth parameters results in microcrystalline to ultra-nanocrystalline CVD diamond films. Ultra-nanocrystalline films have the advantage of possessing smooth surfaces, lower strain and improved fracture resistance. Such films are characterized by diamond domains that are ≈10 nm or less in size, with thin sp^2 boundaries.

Nanoscale diamond particles (also termed nanodiamonds, NDs) and diamond nano-films represent the most interesting forms of diamond explored for applications in drug delivery or medical diagnostics.

Due to its chemical and biochemical inertness, diamond is generally considered as a biocompatible material, meaning that it is chemically non-cytotoxic when in contact with biological cells [171]. This makes diamond a material of interest for coating medical devices, building artificial organs, and as a growth support for biological cells. ND particles and thin films have been used as substrates for cultivation of different cell phenotypes including neurons [172], fibroblasts [173],

osteoblasts [174] and many other cell lines [175]. Guarina and collaborators [176] used fluorescent nanodiamonds (FND) to evaluate their functional implications on hippocampal neurons, using MEA recordings. The firing frequency of neurons was differently affected depending on the developmental stage of incubation with FNDs (7 versus 14). When FNDs were applied at 14 days in vitro they drastically reduced the neuronal firing frequency (Figure 7).

In all cases diamond exhibited no measurable cytotoxicity and, in some cases, appeared to promote cell adhesion and proliferation over conventional materials such as glass or tissue culture polystyrene.

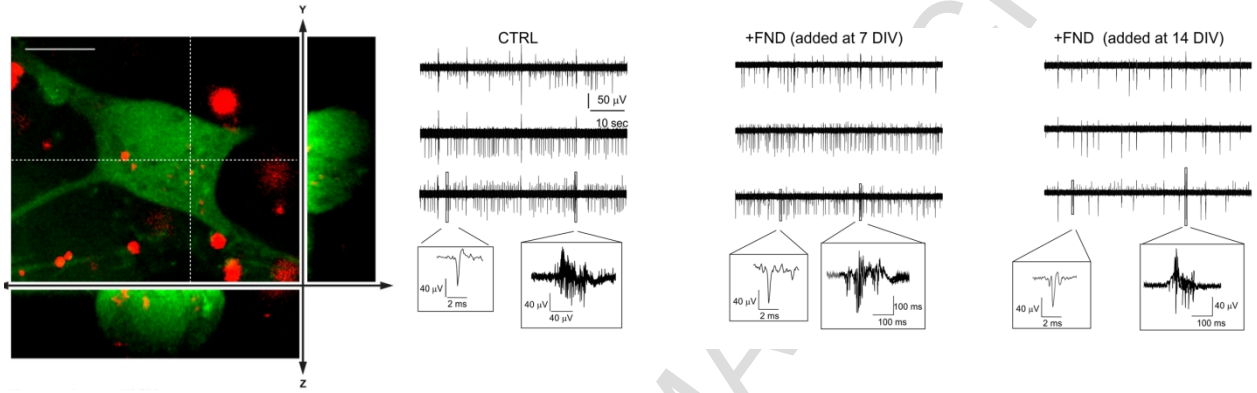

***Figure** 7. Confocal fluorescence micrograph of cultured hippocampal neurons (14 DIV), exposed to 40 µg/ml FND for 2 days, and stained in green with the cytoplasmic labelling dye (CellTracker™ Green CMFDA). Red emission is from FNDs. The entire field and cross-sections (XZ and YZ) were shown. Representative traces of spontaneous firing at 18 DIV (data from 3 representative MEA channels) under control conditions (CTRL), without FNDs, with FNDs seeded at 7 DIV and at 14 DIV. Insets: higher magnification of single spikes and bursts. (Modified with the permission from Guarina et al. 2018 [176]).*

In neuroscience, in addition to the employment as growing substrate, NDs were applied in the development of biosensors for recording neuronal activity, thanks to their peculiar electrical and chemical properties and stability [177,178].

Despite the encouraging results in vitro for biological applications, in vivo applications of NDs in the CNS are still in their early days [53]. To date only one report suggests the possible use of NDs for therapeutic applications in the CNS: CED (convection-enhanced delivery, an experimental high efficiency intracranial delivery system) of DOX-loaded NDs (4-8 nm) is found to provide efficient treatment of different aggressiveness gliomas in mice striatum [53,179]. The treatment positively impact mice survival (with respect to DOX treatment) of 1.4 times in the case of the most aggressive tumor and 1.8 times in the case of the less aggressive one. Notably, in the latter case tumor is eradicated in 3 out of 5 mice, while all mice treated with non-conjugated DOX die [53].

Diamond thin films have been proposed in vivo, as coatings for implants and prostheses [180].

The increasing interest and the recent development of new techniques for constructing micro/nanodevices [181-185] has rapidly broadened the number of diamond-based MEAs (DBMs) employed for electrical recording and stimulation and for detecting neurotransmitter release [186]. DBMs can now be used to either resolve the electrical activity in complex neuronal networks (low-density MEAs) [187], to identify the extension of cell microdomains (active zones) where neurosecretion occurs (high-density MEAs) [188] or to assay the protein content of the physiological liquids that condition the growth, formation, and maturation of complex neuronal networks [189,190].

Ariano and colleagues fabricated a device to record extracellular activity of cultured neurons, based on hydrogen terminated (H-terminated) conductive diamonds. The device allows recording the entire activity of the network in a way similar to conventional microelectrode array (MEA) and with comparable neuronal activity signals [177]. 2D and 3D MEA systems based on diamond and consisting of 256 electrodes on a surface of 28.8 mm^2 have also been developed with the purpose of studying ex-vivo models, in order to obtain more information from a more complex neuronal network [191]. Finally, Halpern and colleagues successfully implanted diamond electrodes in *Aplysia californica* attaching it on the buccal nerve 2, a primary nerve involved in the feeding behavior of Aplysia and recording extracellular electrical activity for up to 9 days after the implantation [192].

Diamond in the form of nanowires should also be considered. The use of diamond nanowires is believed to address positively issues related to improving the overall performance of sensors, including sensitivity and selectivity [193-196].

In the field of cellular sensing, diamond-based substrates offer unique advantages in comparison to conventional materials (silicon, glass, metals, and polymers), [197] which directly derive from the extreme physical properties of this material, i.e., mechanical robustness, wide optical transparency and thermal conductivity [198].

In vitro tests demonstrated that diamond-based substrates are non-cytotoxic and support significantly better adhesion and growth of cells in comparison with standard substrates [199,200].

Furthermore, 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 [201-203].

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 [177]. These transparent electrodes have been exploited to record the activity of cultured neuronal cells with a single

macroelectrode [177] and subsequently to record the activity of cultured cardiomyocyte-like and human embryonic kidney cells using arrays of solution-gated field-effect transistors.

One of the main challenges when dealing with nanodiamond for brain interfacing is represented by the difficulties in integrating diamond on flexible substrates and this difficulty has been faced by the retinal implant community. The Diamond to Retina Artificial Microinterface Structures (DREAMS) project, funded by the European Commission, utilized an array of boron doped diamond (BDD) microelectrodes transferred to a flexible substrate [204] and have successfully developed this general strategy to fabricate a range of MEA types [205,206]. Reporting the MEA from a sacrificial layer to a flexible substrate also enables the fabrication of flexible implants for retinal stimulation. Here the ultimate challenge was to make the fabrication of diamond compatible with a soft substrate material, and it was achieved using a sacrificial substrate lift-off technique, enabling the preparation of such implants on polyimide as well as on parylene [204].

The future for diamond-based substrates looks promising; however, it should be noted that the nanomaterial is still of limited use when developing biomedical applications, especially in the neurosciences field. May be in the future, as in the case of GO, suitable tailoring of the nanomaterial chemical, morphological and physical properties will help to overcome its current strong limitations [53].

6. Carbon nanofibers

Carbon nanofibers (CNFs) have been classified as linear, sp^2-based discontinuous filaments, where the aspect ratio is greater than 100 [207]. Depending on the angle of the graphene layers that compose the filament, CNFs have even been classified as stacked (graphene layers stacked perpendicularly to the fiber axis) or herringbone/cup-stacked (graphene layers stacked at an angle between parallel and perpendicular to the fiber axis) [208].

The typical lengths and diameters of carbon nanofibers are in the ranges of 5-100 mm and 5-500 nm, respectively [209]. CNTs and GR are the most studied carbon nanomaterials for neural interfaces, however CNFs are also attractive in bio-interfacing developments due to their chemical and physical properties [1]: CNFs are chemically stable and inert in physiological environment [2], they are biocompatible for long-term implantation due to CNFs solid carbon skeleton [3], they are electrically robust and conductive for signal detection [4], they can be manufactured into 3D structures allowing intra-tissue and intracellular penetration [210], CNFs possess high surface-to-volume ratio, which greatly reduces electrical impedance, and [5] ultra-micro scale sizes that

provide high spatial resolution. CNFs have been applied as promising materials in many fields, such as energy conversion and storage, reinforcement of composites and self-sensing devices.

In addition, CNF based materials have been developed as electroconductive scaffolds for neural tissues to facilitate communication through neural interfaces. Electrical fields are able to enhance and direct nerve growth [211], therefore electroconductive scaffolds have been applied to enhance the nerve regeneration process, not only providing physical support for cell growth but also delivering the functional stimulus. CNFs may represent novel, versatile neural interfaces, being capable of dual-mode operation by detecting electrophysiological and neurochemical signals, not only at the extracellular level with high spatial resolution, but also at the intracellular level by penetrating into single neurons [9].

Despite the longstanding experience on these nanomaterials and the deep knowledge of the CNFs-neuron interface in vitro, in vivo experiments on their possible application for the treatment of brain and spinal cord injuries or diseases are still limited to few examples [53,212,213]. In the first report CNFs impregnated with subventricular stem cells were employed to promote neuroregeneration after experimental stroke [53]. The animals receiving the CNF-based treatment show reduction of the infarcted volume as well as recovery of motor and somatosensory activity. These data indicate that CNFs are optimal support material for neuronal tissue regeneration [53].

Recently, Guo and collaborators [104] developed a polymer-based neural probe with CNFs composites as recording electrodes *via* the thermal drawing process [213]. They demonstrated that *in situ* CNFs alignment was achieved during the thermal drawing, which contributes to a drastic improvement of electrical conductivity by 2 orders of magnitude compared to a conventional polymer electrode. The resulting neural probe has a miniature footprint, with a recording site reduced in size to match single neuron, yet maintaining impedance value able to capture neural signals. In chronic settings, long-term reliable electrophysiological recordings with single-spike resolution and minimal tissue response over extended period of implantation in wild-type mice were shown [213].

A future development might lead to a smart system able to diagnose and treat neurological diseases (e.g. by local drug delivery) responding to real-time detection of electrical and chemical information from the target nervous tissue.

7. Fullerenes

The first fullerene C60 came to life in 1985 [214] but the family of fullerenes includes a wide range of carbon-based molecules with different number of carbon atoms and symmetries. The most common fullerene is also called buckyball and consists of 60 carbon atoms arranged into 12

pentagons and 20 hexagons to create a structure with the geometry of a hollow sphere [214-216]. C_{60} attracted great attention because of its very stable and symmetric structure [217].

Fullerenes are considered zero-dimensional materials, which possess very interesting physical and chemical properties [218-222] for medicine and technology.

The main issue of C_{60} in the biomedical field is represented by its natural water repulsion and its resulting hydrophobicity. This insolubility in aqueous media induces fullerenes to aggregate [223] and this pushed the research to develop several strategies to overcome the problem. Hydroxyl and malonic acid functionalized fullerenes found important applications in neuroprotection against free radicals generated by fatty acid aerobic metabolism, which neurons are rich of [224], after brain injury or inflammatory response to diseases. These derivatives of fullerene can interrupt chain reactions, generating the radicals by removing intermediate peroxyl radicals and showing robust neuroprotection activity in several *in vitro* models of CNS injury and neurological disease including Parkinson's disease [225]. For this ability fullerenes can prevent excitotoxicity produced by the leakage of neurotransmitters and excitatory ions that results from the free radical damage consequent to a neuroprosthetic surgery and this effect could probably be due in part to their capacity of inhibiting glutamate channels [226].

Fullerenes have been extensively studied in a number of applications such as organic photovoltaics [53,219,227], gas storage [228], and molecular sensing [229]. In the last 30 years fullerenes were considered among the cutting-edge nanomaterials for biomedical applications: they were proposed as oxidative damage protecting agents, photosensitizers for photodynamic therapy of cancer, antiretroviral agents and as drugs and gene delivery vectors [230,231]. Fullerenes also were the pioneering carbon nanomaterials investigated in vivo for their potential applications in the therapy of brain diseases. However, the raising concerns of their toxicity have reduced significantly the interest in developments from these materials in the biomedical scientific community [53].

Fullerene-based therapeutics can significantly ameliorate experimental allergic encephalomyelitis (EAE), a rodent model of human multiple sclerosis [MS] characterized by inflammation in the CNS [232]. Fullerene derivatives have demonstrated to protect neurons from oxidative and glutamate-induced injury, and restore glutamine synthesis and glutamate transporter expression in astrocytes under inflammatory insult. The *in vitro* efficacy translated into *in vivo* efficacy, as treatment initiated after disease onset reduced the clinical progression of chronic EAE in mice, suggesting this may be useful in the treatment of progressive MS and other neurodegenerative diseases. Oxidative stress, through the generation of radical oxygen species, is an underlying mechanism that mediates mast cells signaling and MS pathology [233]. Indeed, several antioxidants are currently in various phases of human clinical trials (i.e., lipoic acid, inosine and Triomar® [Pronova Biocare, Oslo,

Norway], see [ClinicalTrials.gov. http://clinicaltrials.gov/]). Since fullerene derivatives can stabilize MCs [234], are potent antioxidants [230,235] and are anti-inflammatory agents [236], if rationally designed these compounds may be used as a platform for new areas of therapeutic research for MS.

In vivo, fullerenes are the first carbon nanomaterials found to distribute in the brain after systemic administration. Biodistribution studies using a 14C-radiolabeled carboxylated C60 derivative (14 C-C60) in rats after i.v. administration [237] reveal that the nanomaterial rapidly spreads in several organs including brain, indicating that it is able to cross the BBB despite its high molecular weight (995 Da). No toxic effects are observed after i.v. administration, while toxicity is observed after intraperitoneal injection [53]. This raises concerns about the possible occurrence of long-term toxicity or toxicity after chronic administration since the fullerene can reach with time toxic concentrations inside specific sites. Although extensive researches have been conducted to address the intrinsic neuroprotective properties of fullerenes, there are very few reports regarding in vivo drug delivery and imaging applications within the CNS [53].

Despite some good results achieved, fullerenes represent the "past" of carbon nanomaterials research [53]. This is mostly due to concerns related to their accumulation in several organs, their long persistency in the body and their-in general-unpredictable toxicity. With all these serious impairments, it is not easy to say if the risk-benefit ratio will still provide opportunities for the development of these nanomaterials in biomedical applications.

8. Other carbon nanomaterials

Single-wall carbon nanohorns (SWCNHs), reported by Ijima in 1999, are tiny graphene sheets, wrapped up to form horn-shaped cones with a half fullerene cap, having 30-50 nm length and 2-5 nm diameter. They have the tendency to group together and form aggregates (spherical clusters or bundles) like "dahlia" flowers or buds, with an overall diameter of 80-100 nanometers.

Being their structure similar to tiny carbon nanotubes, SWCNHs maintain most of the typical properties of nanotubes: high electrical conductivity, high thermal conductivity and possibility of functionalization. SWCNHs peapods (functionalized with CdSe/ZnSe QDs), encapsulating Gd3N@C80 fullerenes and delivered to U87 tumor bearing mice by convection-enhanced delivery intratumoral infusion [238], enabled tumor imaging either *in vivo* by MRI (thanks to $Gd3^{+}$) and *ex vivo* by confocal microscopy (owing to the presence of QDs). SWCNHs showed to be retained inside the tumor for at least 3 days. Although this study indicates SWCNHs as a possible brain drug delivery nano-platform, other reports on the in vivo bio-distribution of SWCNHs have demonstrated that they could not cross the BBB [239,240]. This precludes the SWCNHs to be

delivered i.v. to the brain, leaving the more dangerous and complicated intracranial administration as the only feasible option available at the moment.

Carbon dots (CDs) are a recently discovered class of discrete, quasi-spherical CBNs [241], which essentially combine the presence of an amorphous core and a graphitic shell. CDs are expected to have a huge impact in biotechnological and environmental applications, based on their high potential as a nontoxic, fluorescent alternative to the popular semiconductor-based quantum dots (QDs). Their peculiar properties have been exploited in photocatalysis [242], electrocatalysis [243], as sensitizers for solar cells [244], as well as for sensing applications [245]. Due to their high intrinsic fluorescence that can span from the visible to the near infrared [246,247], CDs were considered particularly appealing for bioimaging applications (for a review see Peng Z. et al. [248]). Depending on the synthetic strategy adopted, they might expose functional groups on their surface, allowing surface passivation with biocompatible polymers or grafting additional biomolecules [249,250]. Finally, molecules like anticancer drugs and nucleic acids can be non-covalently loaded on their surface, allowing the use of these nanomaterials for delivery purposes [251,252]. CDs seem to display a very good biocompatibility [253], probably resulting from the high density of charged groups on their surface, which confers high stability to their suspensions in water and biological fluids. Several authors have reported that CDs penetrate cell lines in vitro [254-258]. No toxicity was observed in various studies conducted on cell lines [253,256] and on animals [259]. However, Borisova et al. reported that these nanoparticles could interfere with exocytotic mechanisms, and therefore hamper the normal neuronal and brain functions [260]. However, the effect of CDs on cellular biochemistry has not been thoroughly explored.

Given their recent discovery, only a few studies have applied CDs to the CNS with the aim of diagnosis and therapy. Interestingly, the CDs used in *in vivo* biodistribution studies exhibited very good BBB crossing capabilities and a strong tendency to accumulate in the brain even if they were not specifically functionalized: 100 nm fluorescent CDs, prepared via the inexpensive and efficient pyrolysis of a glucose and glutamic acid mixture, were taken up by the brain tissues after i.v. administration in mice [261]. Epifluorescence imaging, made possible thanks to the CDs bright fluorescence emission, revealed that they readily crossed the BBB after systemic injection and diffused in the brain tissues, where they reached the highest concentration within 1 h. *Ex vivo* imaging of brain slices indicated that CDs were mostly accumulated in the cortex, in the hippocampus and in the ventricles. The authors hypothesized that the presence of still intact glucose and glutamine molecules on the CDs surface endowed the nanoparticles of "CNS-targeting" capabilities. From the available epifluorescence images, the nanomaterial did not show diffusion in other specific body regions apart from the brain and the blood. Interestingly, the nanomaterial was

795 also rapidly cleared from the CNS. *In vitro* studies [262,263] have demonstrated that CDs
796 dispersions in plasma had high stability, and good hemocompatibility with moderate cytotoxicity
797 for brain endothelial cells, detected only at very high concentrations. In summary, they provided *in*
798 *vivo* data, although referring only to early time-points, suggested that the nanomaterial had an
799 adequate safety profile for biomedical applications in the CNS.
800 Also 3-4 nm glycine-derived CDs were able to cross very efficiently the BBB and accumulate in the
801 brain. Moreover, they were able to target a human glioma tumor xenographted in mice brain [264].
802 Epifluorescence imaging indicated that they displayed a maximum brain uptake just 5 min after tail
803 vein injection, and strongly localized inside the tumor mass to be then rapidly cleared. Systemically,
804 CDs distributed in the liver, kidneys and hearth. In vitro hemolysis, plasma stability and
805 cytotoxicity studies indicated a high biocompatibility of this nanomaterials [259,265]. Although
806 these CDs displayed fast and consistent accumulation inside the tumor, their potential use as vectors
807 for delivering antitumor drugs in the CNS is not suggested at the moment because of their fast
808 excretion from the tumor lesion and their accumulation in the heart, which is a known target of
809 anticancer drugs toxicity.
810 Also these nanomaterials are in their early stages of development for biomedical applications:
811 suitable chemical modification with molecules able to increase their plasma circulation time and/or
812 with targeting moieties might improve their retention in the brain allowing future applications in
813 tumor therapy. A deep toxicological evaluation of their effects in the CNS in particular but also in
814 the whole body is needed since current available data, albeit very promising, are not sufficient to
815 draw clear conclusions.
816

817 **9. Conclusions**

818 CBNs have been studied in a plethora of technological fields, including biomedical applications.
819 Many CBNs showed unexpected and outstanding interactions when interfacing electrically active
820 tissues, such as the neuronal and the cardiac ones. In particular, CNTs are in the spotlight for their
821 powerful influence on the physiology of neuronal cells and axons. The precise biophysical
822 mechanisms of these special interactions are not completely understood, but the features and the
823 remarkable applications of such materials, together with their ability to manipulate neural activity,
824 still hold strong promises in manufacturing interfaces enriched by artificial cues that can improve
825 the interfacing electrode performance and guide tissue reconstruction. The ability of CNT-based 3D
826 structures to dictate neurite web morphology toward successful reconnection of segregated spinal
827 explants has been explored in vitro [81] and the same material has been implanted in vivo in the rat
828 brain with a limited tissue reaction surrounding the implants [83]. The new player among CBNs,

GR, has also displayed interesting features that can be exploited to interface neurons and other CBNs are under investigation for their own peculiar properties.

In this review we have reported some of the more recent CBN applications related to engineering brain interfaces. We have discussed their properties and their performances in improving and boosting neuronal growth, in developing new research lines in neurophysiology and neurobiology and in providing novel methods to explore brain functions. For their peculiarities CNTs and GR seem to be the most promising materials for the future development of innovative human interfaces or sensors. Hundreds of researchers are exploring their potentialities and several international projects are involving their usage in multiple biological fields of application. We strongly believe that a great future awaits CBNs particularly for the production of multifunctional human (brain) interfaces and in tissue engineering to support neuronal regeneration.

10. Author Contributions

RR, MM, SB, MP and LB conceived, structured and participated in writing the review.

11. Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be constructed as a potential conflict of interest.

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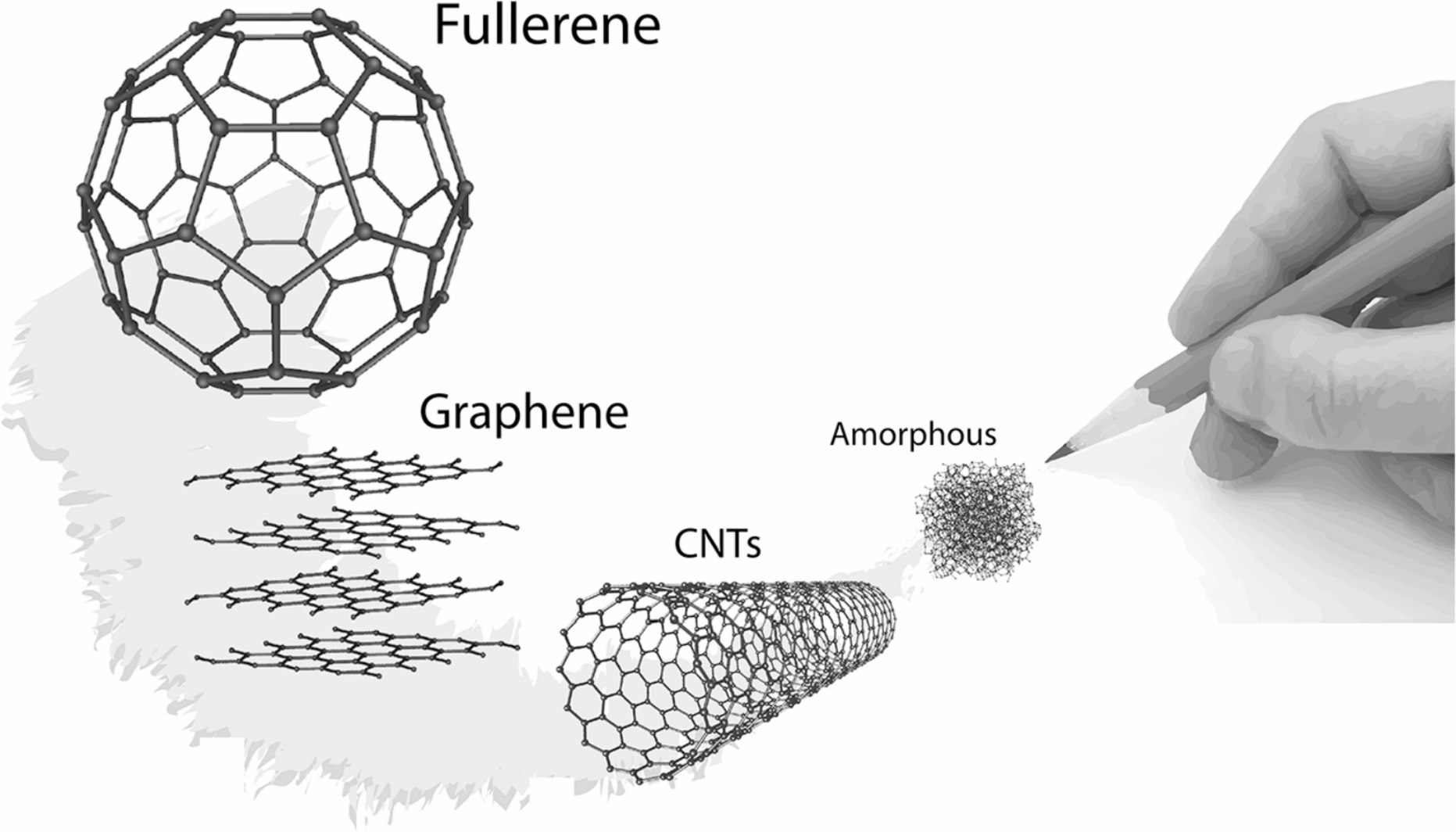
Fullerene
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