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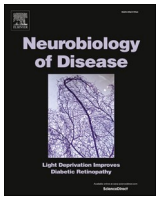

Review

Near-infrared nanoscopy with carbon-based nanoparticles for the exploration of the brain extracellular space

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ABSTRACT

Understanding the physiology and pathology of the brain requires detailed knowledge of its complex structures as well as dynamic internal processes at very different scales from the macro down to the molecular dimensions. A major yet poorly described brain compartment is the brain extracellular space (ECS). Signalling molecules rapidly diffuse through the brain ECS which is complex and dynamic structure at numerous lengths and time scales. In recent years, characterization of the ECS using nanomaterials has made remarkable progress, including local analysis of nanoscopic dimensions and diffusivity as well as local chemical sensing. In particular, carbon nanomaterials combined with advanced optical technologies, biochemical and biophysical analysis, offer novel promises for understanding the ECS morphology as well as neuron connectivity and neurochemistry. In this review, we present the state-of-the-art in this quest, which mainly focuses on a type of carbon nanomaterial, single walled carbon nanotubes, as fluorescent nanoprobes to unveil the ECS features in the nanometre domain.

1. Introduction

Richard Feynman's famous lecture "*There's plenty of room at the bottom*" laid the foundation for a new field in science, nanotechnology, which more than ever in the 21st century holds potential of revolutionizing our approaches to common problems (Toumey, 2009). As of today, investigating, manipulating and controlling the matter at the nanoscale becomes at reach in the field of neuroscience, where the complexities associated with the biological systems are colossal. Applications of nanotechnology in basic and clinical neuroscience has seen a rapid increase only in the last couple of decades, partly because of the complexities associated with neural cells and the central nervous system and the multiscale nature of the interfaced complex 3D brain networks (Silva, 2006). Nanotechnology combined with the latest microscopy techniques have the potential to support this multiscale challenge, where the eventual goal would be to unveil the detailed functioning and pathophysiology of live brains *in vivo*.

A fascinating brain compartment is the extracellular space (ECS) (Nicholson and Hrabětová, 2017). The ECS is a system of interconnected regions limited by neuronal membranes and containing the interstitial fluid and the extracellular matrix (ECM). The interstitial fluid constitutes a key microenvironment for diffusing ions, cellular signalling molecules, homeostasis, and clearance of toxic metabolites, while the ECM serves as a hygroscopic plastic scaffold for cell attachment and biochemical support (Frantz et al., 2010). Despite the importance of the ECS, its structural organization at nanoscale spatial resolution is still unknown in live brains tissues and the local diffusion behaviour of signalling molecules in its volume are poorly understood at such small scales. Recently, unprecedented details on the morphology and dynamics of the brain ECS have been obtained thanks to the technical advances in single-molecule (Cognet et al., 2014; Hrabětová et al., 2018; Soria et al., 2020a) and super-resolution microscopy (Zheng et al., 2017; Tønnesen et al., 2018; Inavalli et al., 2019). However, these methodologies are often limited in penetration depth by the intrinsic photophysics of the fluorescent marker, thus restricting their applications to brain preparations that not entirely preserve the native tissue organization.

Recent developments of carbon nanomaterials have gained much interest in neuroscience applications, such as imaging, drug delivery and electrical sensing of neuronal tissue (Baldrighi et al., 2016). As for many other nanomaterials, carbon-based nanodevices can interact with biological systems at the molecular level, with a high degree of spatial and temporal specificity. They may penetrate the blood-brain barrier and deliver specific therapeutic agents, probes, or biological materials to targeted cells and tissues in living brains (Yang et al., 2007). Carbon nanoparticles exhibit big diversity in structure, morphology, physical properties and chemical reactivity, and have a much lower inherent

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toxic potential than many other kinds of nanomaterials (Simon et al., 2019). Most importantly, the photophysics of these nanoparticles varies from visible to near infrared (NIR) light (Weisman and Bachilo, 2003), therefore matching the transparent window for biological tissues (Jacques, 2013). In particular, NIR luminescent single-walled carbon nanotubes (SWCNTs) have attracted specific attention as molecular nanoprobes for deep-tissue microscopy, due to their unique brightness, photostability, and spectral imaging range (Welsher et al., 2009). Furthermore, SWCNTs demonstrated capability to locally probe chemical species (Barone et al., 2005) leading to novel approaches to access, image and characterize the brain ECS (Godin et al., 2017).

This review article is organized as follows: after a brief introduction of the brain ECS, we will review the use of SWCNTs as fluorescent nanosensors to image, characterize, and chemically probe the brain ECS. This will be followed by an opening to other carbon-based nanomaterials used as imaging agents in neuroscience applications (see Fig. 1).

2. Brain ECS

Even though the narrow space between neuronal cells has been a fascinating topic since the late 80s (Fenstermacher and Kaye, 1988), the brain ECS is still a largely unknown territory, holding potential for the development of novel technologies. The ECS includes all the fluid-filled areas external to the brain cells. One of its main function is to provide a reservoir of ions to support membrane and action potentials. The ECS is therefore key to convey chemical signals and intercellular communication (Syková and Nicholson, 2008). Indeed, in addition to their local journey in the synaptic cleft for fast synaptic response, neurotransmitters can travel up to several microns through the ECS before reaching target cells through a process called volume transmission (Agnati et al., 1995). The ECS has therefore a fundamental role for neural communication, forming together with glial cells a unique and plastic entity that modulates the activity of pre- and post-synaptic receptors and ion channels (Dityatev et al., 2006).

The ECS is a highly dynamic environment: changes in its volume has been proposed to tune neuronal excitability and signal transmission by altering, for instance, the baseline concentration of ions adjacent to individual cells (Colbourn et al., 2019) or to facilitate the metabolic clearance during sleep (Xie et al., 2013). These conformational changes technically limit the investigation of the spatiotemporal molecular mobility profile in relation to the ECS nanoscopic dimensions. Accordingly, historical characterizations of the ECS mainly included volume-averaging techniques, *i.e.* indirect biophysical approaches where the volume fraction and diffusivity of the ECS were extrapolated and averaged from a relatively large volume of tissue and therefore missed the nanoscale resolution (Syková and Nicholson, 2008). Nicholson et al. firstly used an integrative optical imaging approach to measure the effective diffusion coefficients of dextran molecules in the ECS of rat cortical slices (Nicholson and Tao, 1993). This method allowed the detection of fluorescent molecules pressure-ejected from a glass micropipette into the brain. Diffusing fluorescent molecules gave information on global biophysical ECS features, including the quantification of hindrance. Further technical developments included the real time iontophoresis technique, where fluorescent molecules are pulsed from a microelectrode into the brain ECS and their changing concentration can be measured over time using an ion-selective microelectrode. Real time iontophoresis can be used to examine both reversible and irreversible changes of the brain ECS in real time, therefore allowing the biophysical characterization of the brain ECS in both healthy and diseased animals (Syková, 2004; Binder et al., 2004; Slais et al., 2008; Syková and Nicholson, 2008).

Although the biophysical research in the ECS has seen a rapid development in the last decades, the ECS dynamics and structural organization at nanoscale spatial resolution still represent a knowledge frontier in brain research (Nicholson and Hrabětová, 2017). Indeed, our current view of its nanoscale anatomy mainly derives from recent microscopy advances, which suggested that the ECS volume might be larger than initially thought (Korogod et al., 2015) (Tønnesen et al., 2018) (*i.e.* 15–20% of the total brain volume). Individual substructures within the ECS are commonly only nanometers wide, far smaller than most cellular features, making the ECS a very complex environment to visualize and characterize with standard imaging and biological techniques (Soria et al., 2020a). Furthermore, the monitoring of local changes in terms of transient chemical concentrations, including ions or neurotransmitters, is still out of reach in living tissues. To this end, deep-tissue fluorescence microscopy using NIR emitting SWCNTs represents a unique approach to explore and probe the structure and functions of the brain ECS.

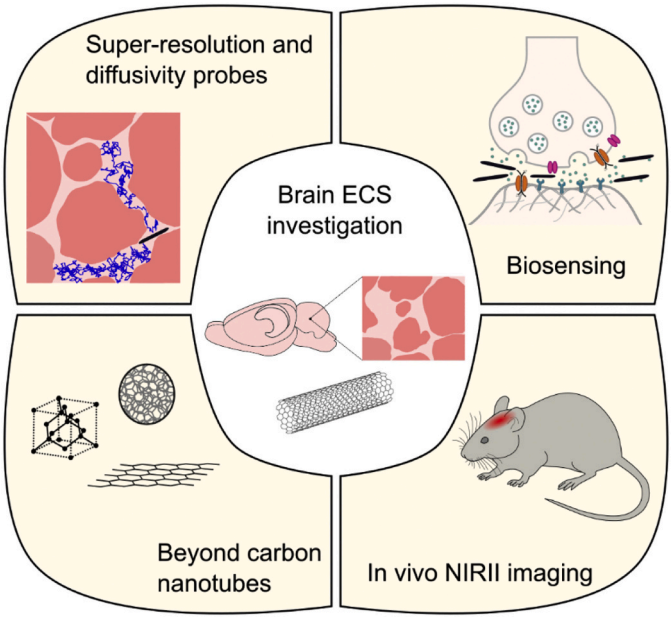

Fig. 1. SWCNT as fluorescent nanosensors to characterize the brain ECS. The optical and physical properties of SWCNTs make these nanomaterials unique to unveil the characteristics of the brain ECS. SWCNTs are currently used in literature as super-resolution and diffusivity probes, as molecular sensors of diffusing molecules, or as *in vivo* NIR-II imaging contrast agents. Other carbon nanomaterials are also currently emerging to characterize the brain ECS.

3. Physical properties of SWCNTs

Discovered in 1991 (Iijima, 1991), SWCNTs consist of a layer of graphene rolled up with a certain direction (*i.e.* chiral vector) to form a cylindrical tube. Compared to double- and multi-walled carbon nanotubes, SWCNTs have a single-layer cylindrical sidewall structure, which provides unique opto-chemical properties for biophysical applications. SWCNT length varies from few tenths of nm (ultra-short) to several hundreds of μm, with a diameter that typically falls in the nm range. This quasi one-dimensional shape confers many exceptional physical properties, including a remarkable accessibility in complex environments. Indeed, long SWCNTs (typically micrometers long) can eventually move by reptation in crowded environments, where their length, diameter, and environment denseness influence their diffusion rates (Fakhri et al., 2009, 2010; Jena et al., 2016). The typical length of SWCNTs currently used to most efficiently explore the brain ECS varies from 400 to 1000 nm. At this length scale, SWCNTs can be mainly considered as rigid rods in the ECS.

The bandgap fluorescence of SWCNTs was first reported in 2002, by isolating the nanomaterials inside surfactant micelles (O'Connell et al., 2002). When individual SWCNTs are illuminated at their optical transition energies, the excitons at the band edge recombine and emit

fluorescence at a specific wavelength. The different chiralities provide distinct spectroscopic signatures for each species (Weisman and Bachilo, 2003). The diameter of the most prevalent semiconducting nanotubes ranges from 0.6 to 2 nm, which corresponds to a band edge transition energy of 0.5 to 1.9 eV. The resulting emission wavelengths range from 700 to 2500 nm, thus covering the transparent windows of biological tissues.

The near infrared (NIR, ≈ 700–1500 nm) photoluminescence of SWCNTs make these nanomaterials particularly attractive for neuroscience applications. This region of NIR fluorescence is optimal for deep-tissue imaging, owing a minimal attenuation in biological tissues *via* reduced scattering and absorption of photons. Indeed, the NIR first region (NIR-I ≈ 650–950 nm) presents a local minimum in the tissue absorption spectrum thus avoiding the absorbance of haemoglobin and water(Jacques, 2013), while the NIR second region (NIR-II ≈ 1000–1350 nm) additionally offers a great optical penetration depth and minimal autofluorescence and tissue scattering (Welsher et al., 2011). This provides clear advantages, such as improved imaging signal-to-noise ratios and resolutions at depths approaching several millimetres (Del Bonis-O'Donnell et al., 2018). Penetration depth could be further improved *via* the non-linear excitation of the fluorophores by using multiphoton imaging strategies. In this scenario, SWCNTs offer the unprecedented advantage of both excitation and fluorescence emission in the NIR window, making them particularly well suited for *in vivo* applications (Bonis-O'Donnell et al., 2017). In the context of single nanotube detection, one drawback of employing laser scanning multiphoton excitation would be to allow significantly lower imaging rates than wide-field one-photon excitation.

SWCNTs do not blink and their NIR emission remains stable to permanent photobleaching even after 10 h of continuous recording, superior to most other fluorescent nanoprobes used in neurological research (Heller et al., 2005). Moreover, encapsulated SWCNTs can be made biocompatible and colloidally stable in complex biological tissues, a key feature for long-term functional imaging *in vivo* (Gao et al., 2017). Most interestingly, their surface chemistry can also be modified to bind specific molecules triggering NIR fluorescence to detect endogenous chemical concentrations(Del Bonis-O'Donnell et al., 2018). In a recent study, Danné et al. presented a thorough investigation at the single nanotube level ((6,5) chirality) on the potential effects of different illumination wavelengths on brain tissue (Danné et al., 2018a). The effects of tissue scattering, absorption, autofluorescence, and tissue temperature increase were systematically examined on continuous-wave excitations of 568 nm, 845 nm, and 1064 nm. They found that 845 nm is the optimal excitation to image (6,5) nanotubes showing negligible tissue autofluorescence or laser absorption that may heat the sample. Using this strategy, SWCNTs were successfully applied as single molecule nanoprobes to characterize the brain ECS. This is also owing to their unusual aspect ratio that can moderate their diffusion rates by their length, making them ideal for single-particle recordings in the intricate ECS network.

4. Biological impact of SWCNTs in the central nervous system

The important question of SWCNT toxicity has often been unfortunately associated to that of multiwalled carbon nanotubes which encompasses a myriad of different structures distinct from SWCNTs; in the case of SWCNTs, their biocompatibility has been mainly established (Heller et al., 2020). More precisely, amongst all the available studies, few of them have been devoted to the interfacing of SWCNTs with the central nervous system. SWCNTs may enter the organism through three different pathways: injection, ingestion and inhalation. Importantly, intravenous administration of ^{13}C-enriched SWCNTs in mice demonstrated that these nanomaterials could cross the blood-brain barrier without inducing acute toxicity, paving the way for imaging of the whole intact brain (Hong et al., 2014). In a previous study, SWCNTs incorporated into feed stock found to distribute throughout different tissues, including the brain, with no observed short-term toxicity in *Drosophila* (Leeuw et al., 2007). *In vitro*, SWCNT treatment has been shown to mitigate autophagic and lysosomal defects in primary glial cells from a mouse model of Alzheimer's disease (Xue et al., 2014), while amine-modified SWCNTs appeared as neuroprotective in an *in vivo* rat stroke model (Lee et al., 2011). More recently, intravenous administration of a single-chirality DNA-encapsulated SWCNTs showed minimal accumulation in brain tissues without any sign of injury or other abnormalities on the short- and long-term (Galassi et al., 2020).

To be applied as single molecule nanoprobes in biological tissues, encapsulated SWCNTs should not only display low cytotoxicity, but also minimal unspecific interactions with cells and molecules while still being highly luminescent over long periods of time. Indeed, unspecific molecule adsorption onto the SWCNT surface could potential interfere with tissue penetration, targeting capabilities or sensing of specific molecules. Gao et al. screened several common coatings for SWCNT encapsulation and established that phospholipid-polyethylene glycol (PL-PEG) is the best coating for single nanotube tracking experiments in live biological samples and better minimise unspecific interactions with cells (Gao et al., 2017). In addition, following intra-ventricular injection, PL-PEG encapsulated SWCNTs revealed undetectable tissue inflammation when freely circulating in the brain ECS, as demonstrated by the gross morphology and density of microglia (Godin et al., 2017).

5. SWCNTs as super-resolution probes of the brain ECS

Single particle tracking of SWCNTs and super-resolution microscopy approaches have been recently combined to allow ECS molecular diffusivity and nanoscale dimensions to be measured for the first time deep into live brain tissues (Godin et al., 2017; Paviolo et al., 2020; Soria et al., 2020b). SWCNTs can be injected intraventricularly in live animals (Fig. 2A) or can passively penetrate organotypic brain slices, in order be individually tracked in organotypic or acute brain slices as they journeyed in the ECS environment. The approach showed that single diffusing nanotubes could be tracked up to 100 μm deep in different live brain tissues using a widefield microscopy approach at millisecond timescale, opening up novel possibilities for studying intact brain tissue dynamics at the nanoscale. In rat pups, nanotubes were found to spread through diverse areas of the brain (*e.g.*, the neocortex, hippocampus, striatum) minutes after the injection, confirming the minimal uptake by neuronal cells and the particularly efficient dissemination of SWCNTs in live brains. Yet the precise distribution of SWCNTs in the whole brain volumes still needs to be evaluated quantitatively, probably at lower resolutions than in super-resolution approaches as described next. For each recorded trajectory, super-resolved images of the ECS can be computed by cumulating the SWCNT localizations (Fig. 2B). Super-localization analysis shows that the brain ECS is a maze of polymorphic channels with widths in the order of 50–500 nm (see an example in Fig. 2C), in excellent agreement with the dimensions obtained by cryo-electron microscopy (Korogod et al., 2015). Importantly, tracking of individual SWCNTs can also provide simultaneous measurements of local ECS diffusivity environment (Fig. 2D). Compared with diffusion in a free medium where molecules move randomly, SWCNT diffusion in the ECS is critically dependent on the physical and chemical structure of the local microenvironment (Fakhri et al., 2010). Accordingly, dense assemblies of matrix proteins in the ECS impact the nanotube movements in certain areas and might eventually restrict their accessibility in the smallest compartments (Soria et al., 2020b). Diffusivity results from different brain models showed specific local rheological properties of the space, ranging from low to high local diffusivity.

Tracking of individual SWCNTs while freely moving in the brain ECS was validated for different animal and brain tissues, including rat pups (Godin et al., 2017), adult mice (Paviolo et al., 2020), and a mouse model of α-synuclein-induced neurodegeneration (Soria et al., 2020b). Overall, different models showed a wide range of dimensions and spatially heterogeneous diffusion patterns. Comparison of young rat

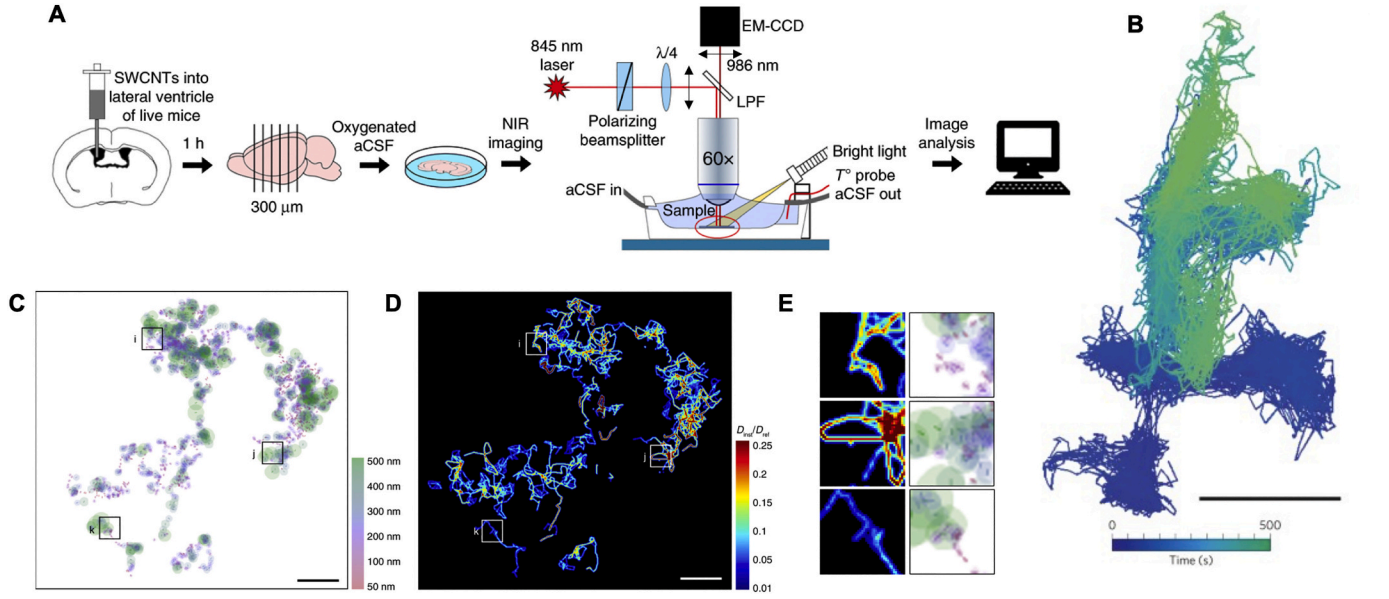

Fig. 2. SWCNTs as super-resolution probes.
A) Schematic setup of SWCNT imaging and tracking in live brain tissues. Fluorescent SWCNTs are injected *in vivo* into the lateral ventricle of adult mice and allowed to diffuse into the brain for 1 h. Acute brain slices are then prepared and kept in oxygenated artificial cerebrospinal fluid (aCSF) before imaging at 37 °C using a NIR fluorescence microscope. Bright light under oblique illumination can be used to obtain a visible image of the slice and to discern the cell bodies in the field of view. Temperature is controlled with a thermal ($T°$) probe. B) Colour-coded trajectory of a single SWCNT diffusing in live ECS (20,000 data points). Scale bar, 1 µm. C) Example of local dimensions of a brain ECS portion and simultaneous D) instantaneous relative diffusivity map. Scale bars are 2 µm. E) Insets from (C, D) showing that diffusive inhomogeneities were not only driven by the geometrical factors of the space. (A, C-E) reproduced from (Soria et al., 2020b)with permission. Copyright 2020, (B) reproduced from (Godin et al., 2017)with permission. Copyright 2016, Nature Publishing Group.

organotypic and mouse acute slices revealed higher ECS local dimensions and diffusivity values in organotypic slices, potentially due to the immaturity of the extracellular matrix in the young rat organotypic tissues. Importantly, results on neurodegenerated tissues showed poor correlation between local ECS width and nanoscale diffusion upon extracellular microenvironment remodelling, suggesting that the diffusive inhomogeneities were not only driven by geometrical factors but also by the molecular composition of the space (Fig. 2E). Indeed, hyaluronan was found to be the major actor for the local variations in ECS diffusivity properties, opening up new hypothesis for the connection between ECS and brain pathologies. Further investigations could include the characterization of the ECS in aging and age-related disorders and for different brain compartments. It would also be interesting to locally monitor inflammatory responses at the mRNA or protein level. Overall, this methodology paved the way towards (challenging) *in vivo* investigations, that should provide the best conditions to address physiological questions that acute slices can only partially address.

Significant challenges in tracking methodologies are still to be addressed to achieve NIR single-molecule localization in 3D (*i.e.* not only in 2D) using a standard fluorescence localization microscopy. An appealing approach would be to adapt self-interference super-resolution microscopy in the NIR. The technique was initially developed in the visible range for 3D super-localization of single molecules and quantum dots at depths of at least 50 µm in uncleared tissue (Bon et al., 2018; Linarès-Loyez et al., 2019). Moreover, recordings at faster acquisition times combined with advanced chemical processing to efficiently brightened the SWCNT emission (see below) could unveil novel sub-diffusive regimes at smaller scales that at present are inaccessible. On a technological side, the quality of measurements will be potentially improved by using predefined homogeneous SWCNT solution in terms of chirality, diameter, and length.

6. SWCNTs as biosensors in the brain ECS

A key point to understand signalling transmission through the ECS would be to individually probe neurotransmitters as they diffuse towards the cell target. While recent advances beyond the scope of this review have been made to shed light on the development of functional fluorescent probes for directly probing the chemical signalling in the brain ECS -including protein based fluorescent sensors, genetic engineering tools, quantum dots, and other synthetic probes-, there remains a lack of *in vivo* applicability (Beyene et al., 2019b). Indeed, a successful neurotransmitter biosensor should satisfy a wide range of physical and optical parameters, including high signal-to-noise ratio, optimal binding kinetics, photostability, and low cytotoxicity. Currently, carbon is one of the most frequently used elements in the fabrication of electrochemical biosensors, due to its chemical inertness, its excellent electrical conductivity, and its versatile surface chemistry (Gilmartin and Hart, 1995). Indeed, carbon can form two stable bonding configurations (sp^2 and sp^3) bearing different spatial geometry. The ultimate sensitivity of SWCNT photoluminescence to chemical interactors propelled them as promising biomolecular tool for neuro-sensing (Boghossian et al., 2011; Cognet et al., 2007). This popularity mainly relies on the advanced and well-controlled bio-functionalization methods, which allows the immobilization of a high number of accessible binding units (Bardhan, 2017). Moreover, the development of large-scale production methods (Pirard et al., 2017) and the availability of low-cost SWCNTs with good qualities lead to reproducible and uniform results, pushing this research area to a higher level (Bianco et al., 2020). The mechanism of sensing using SWCNTs relies on the NIR fluorescence modulation upon selective binding of bio-analytes, such as glucose (Barone et al., 2005), insulin (Bisker et al., 2018), reactive oxygen species (Rawson et al., 2015), hormones (Zhang et al., 2013), and proteins (Bisker et al., 2016; Landry et al., 2017; Chio et al., 2019).

The surface modification of SWCNTs can be achieved by covalent or non-covalent reactions. Covalent chemical functionalization has been mainly used to add synthetic handles for molecular recognition and targeted drug delivery (Chen et al., 2008; Bartholomeusz et al., 2009; Kwon et al., 2010). These types of applications are not deeply influenced by the attenuation of fluorescence introduced by the covalent SWCNT

sidewall functionalization. Recent advances in SWCNT chemistry have now designed a covalent functionalization reaction that re-aromatizes defect sites and restores the original SWCNT lattice and intrinsic fluorescence (Setaro et al., 2017). Noncovalent surface chemistries have the advantage of improving the solubility of the nanomaterials while avoiding the modification of the sidewall composition. This is a preferred approach for sensing and imaging applications, as it maintains the intrinsic opto-electronic properties of the nanomaterials without altering the sp^2-hybridized carbon lattice organization (Dinarvand et al., 2020). Non-covalent processes have been mainly used to attach small molecules surfactants (Moore et al., 2003), poly(ethylene glycol) and its derivative molecules (Gao et al., 2017), bacteriophages (Dang et al., 2011), and for DNA wrapping (Zheng et al., 2003).

Biosensing applications of SWCNTs in neuroscience are at present mainly centred on neurotransmitter recognition. The first reported nanosensor was based on single stranded DNA-wrapped SWCNTs for dopamine recognition (Fig. 3A) (Kruss et al., 2014). In that work, the authors used the corona phase molecular recognition technique to identify adsorbed amphiphilic polymers on fluorescent SWCNTs that allow for selective detection of the specific neurotransmitter. The molecular selectivity of the analyte was imparted *via* the self-reorganization of the polymer adsorbate upon interaction with the analyte itself. The sensor proved a fluorescence increase of about 60–80% upon addition of dopamine. Advances in chemical processes for SWCNT functionalization have improved the sensitivity of detection, and also showed selectivity towards a new neuromodulator target, norepinephrine (Beyene et al., 2018). The fluorescence modulation values of this neuro-sensor (relative change in fluorescence intensity upon detection of up to 3500%) point towards a strong potential for *in vivo* neuroimaging. The suitability of SWCNTs to probe dopamine release has also been extended *in vitro* by generating nanoarray supports for cell culture (see the nanosensor response in Fig. 3B and the proposed sensing mechanism in Fig. 3C) (Kruss et al., 2017). This array of nanosenors allowed mapping hotspots of dopamine release on cell surfaces and assessed the release anisotropy. Recent efforts have been made on monitoring neurotransmitter reuptake mediated by endogenous transporter proteins (Beyene et al., 2019a). In that work, Beyene et al. introduced a novel synthetic optical nanosensor (nIRCat) with high spatial and temporal molecular recognition within a unique NIR spectral profile (1000–1300 nm), demonstrating imaging of synaptic and extrasynaptic catecholamines release and reuptake in brain tissue (see Fig. 3D–E and the response of the nanonsensor to different Ca^{2+} concentrations in Fig. 3F). Importantly, these results open up novel applications for *in vivo* sensing and real-time brain neurochemistry in deep tissues. Although the performances of carbon nanosensors have already exceeded the state-of-the-art for neurotransmitter sensing, there are still some challenges for improving the quality of measurements which mainly include the synthesis of SWCNTs with unique chirality, uniform diameter, and precise length (Bianco et al., 2020).

7. Perspectives based on the chemical functionalisation of fluorescent SWCNTs

A key point to understand properties and functions of neural circuits would be to image the intact brain through optically dense biological tissues, *i.e.* scalp, skull, and neuronal cells. *In vivo* deep-tissue imaging could be accomplished in the NIR-II optical regime, especially for those applications where the biologically relevant milieu is in awake and behaving animals. A pioneer study in 2014 compared the penetration depth and fluorescence imaging in different NIR regions (until 1.7 μm) using SWCNTs as contrast agents (Hong et al., 2014). Importantly, the Dai group demonstrated transcranial imaging of the mouse vasculature with sub-10-μm resolution and without the need of a cranial imaging

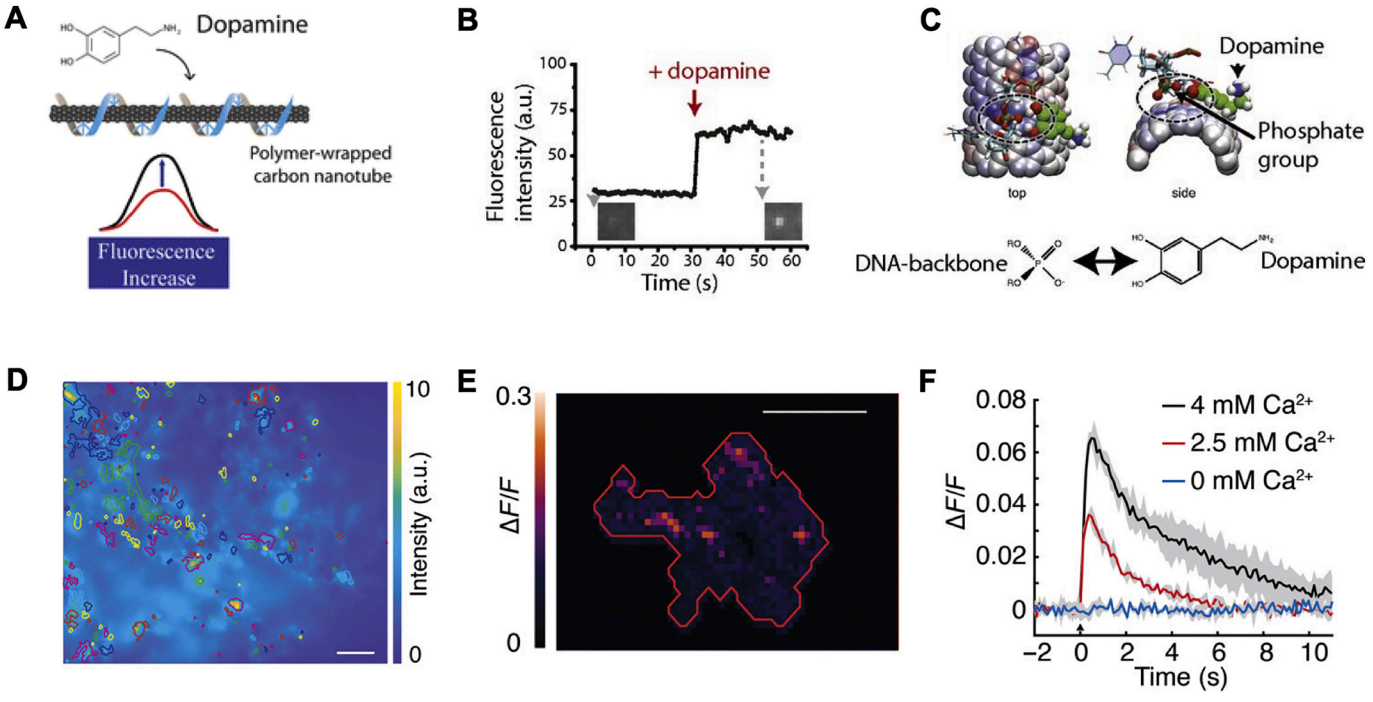

Fig. 3. Chemical imaging of neurotransmitters using SWCNTs.
A) Schematic of the fluorescent turn-on sensor for dopamine. B) Fluorescence intensity trace of a single $(GA)_{15}$-ssDNA/SWCNT imaged on a surface while adding dopamine (10 μM). C) Proposed sensing mechanism: dopamine pulls phosphate groups to the SWCNT surface, which removes quenching sites and increases SWCNT fluorescence quantum yield. D) Imaging of dopamine release evoked by electrical stimulation in striatal tissue overlaid with regions of interest (ROIs) identified using the fluorescence modulation of the nIRCat nanosensor. E) A higher magnification view of an ROI showing the presence of smaller fluorescence hotspots. Scale bar, 5 μm. F) nIRCat with different Ca^{2+} concentrations. (A) reproduced from (Kruss et al., 2014)with permission. Copyright 2014, American Chemical Society, (B—C) reproduced from (Kruss et al., 2017)with permission. Copyright 2017, National Academy of Sciences, (D—F) reproduced from (Beyene et al., 2019a)with permission. Creative Commons Attribution-Non Commercial license, 2019.

window. This significant result confirmed that image and probe the ECS *in vivo* using the photophysical properties of SWCNTs can indeed be possible. However, achieving a stable, biocompatible, and bright SWCNT suspension in the NIR-II has proved to be a challenging quest.

Recent advances in SWCNT chemistry have made possible the creation of controlled sp^3 defects on the nanotube backbones that modulate their bandgaps and result in a red-shifted and brighter emission as compared to the intrinsic NIR fluorescence (Miyauchi et al., 2013; Ma et al., 2014; Hartmann et al., 2016; Ghosh et al., 2010). Sp^3 defects were also proposed as anchor points for conjugation of visible fluorophores to create multicolour SWCNTs and *in situ* peptide synthesis (Mann et al., 2020). A novel NIR-SWCNT sensor with a simultaneous covalent and noncovalent functionalization of the SWCNT surface has also been engineered for sensing applications of dopamine, fibrinogen, and insulin, and for analyte-specific imaging applications (Chio et al., 2020).

Recently, the sp^3 quantum defects concept has been employed to restore the photoluminescence of ultra-short SWCNTs (us-SWCNTS, length < 50 nm) (Danné et al., 2018b). More precisely, sp^3 defects were used to intentionally trap excitons at emissive defect sites that were intentionally incorporated into us-SWCNTS. This process efficiently restored their emission in the NIR window. Super-resolution imaging further demonstrated the preferential localization of excitons at the nanotube ends and their behaviour as independent emitters (Fig. 4A). These results paved the way to the synthesis of engineered us-SWCNTs for bioimaging applications, where bright NIR emission and small size are highly desirable. In another work, Godin et al. showed the conception of a new hybrid nanomaterial consisting of SWCNTs covalently functionalized with photo-switching molecules owing remote control of nanotube blinking properties in the NIR-II window (1065 nm, Fig. 4B) (Godin et al., 2019). In that study, the covalent functionalization preserved the conjugation of the sp^2-hybrid network, thus ensuring the maintenance of the photoluminescent properties without losing excessive fluorescence. These results opened up new directions in super-resolution microscopy of intact brain tissues. Finally, sp^3 –SWCNTs were also applied for live brain imaging of the ECS in organotypic slices using an ultra-low excitation dose in the NIR window (Mandal et al., 2020). Sp^3-defect tailored PL-PEG SWCNTs enabled high signal-to-noise ratio imaging in the first order excitonic transition (emission at 1160 nm), using excitation intensities one order of magnitude lower than the ones used for imaging the intact brain.

8. Other carbon nanomaterials used for imaging in neuroscience

SWCNTs are not the exclusive carbon-based nanomaterials to characterize the brain ECS. In the last decade, other classes of all-carbon materials have been investigated as potential nanoprobes for neuroimaging and sensing, but only few examples demonstrated potential for *in vivo* brain imaging. In this section, we present recent advances in some of the most studied fluorescent carbon nanomaterials -other than SWCNTs- and discuss their application in visualizing brain structure and function. (See Table 1)

Multi-walled carbon nanotubes (MWNTs) are concentric cylinders of graphene with diameter of several tenths of nm. MWNTs functionalized with amine groups may exhibit inherent photoluminescence, where photon emission falls in the visible region of the electromagnetic spectrum (450–650 nm). Interestingly, MWCNT fluorescence can be also stimulated using NIR excitation *via* a two-photon absorption process (Rubio et al., 2015). The main proposed application of MWCNTs in neuroscience is for the development of new diagnostic and therapeutic agents for targeted drug delivery (Kafa et al., 2015; Wang et al., 2016). MWCNTs delivered intravenously could also be imaged using multi-photon fluorescent techniques in brain tissues, showing parenchyma accumulation within 5 min after injection (Wang et al., 2016). While these results show promise for *in vivo* brain imaging, the high absorption and scattering of the visible fluorescence limits potential deep imaging applications.

Carbon nanodots (CDs) are quasi-spherical carbon-based nanomaterials, combining the presence of an amorphous or nanocrystalline core and a graphitic or turbostratic shell (Baldrighi et al., 2016). CDs have generated much excitement because of their tunable fluorescent properties in the VIS range, their good biocompatibility, their chemical stability both in water and in biological fluids, and their intrinsic low photo-bleaching (Cao et al., 2013). Additionally, the large surface area to volume ratio and abundance of sites for covalent modification make CDs an ideal platform for chemical modification and functionalization (Del Bonis-O'Donnell et al., 2018). Indeed, silica-coated CDs combined

Table 1
Summary of carbon nanomaterials for imaging and sensing in neuroscience.

Carbon nanoparticles	Emission wavelength	Applications
Single-walled carbon nanotubes	700–1500 nm	Super-resolution microscopy, sensing of dopamine, norepinephrine and extrasynaptic catecholamines
Multi-walled carbon nanotubes	450–650 nm	Drug delivery, imaging
Carbon nanodots	450–650 nm	Detection of copper ions, imaging
Graphene oxide nanoparticles	425–550 nm	Imaging
Nanodiamonds	600–750 nm	Imaging
Single-walled carbon nanohorns	–	Electrochemical detection of epinephrine and glutamate

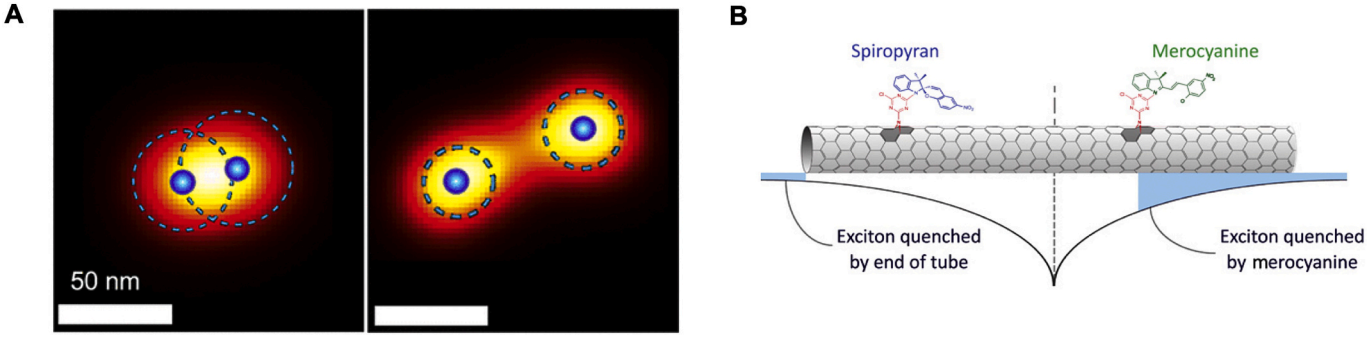

Fig. 4. Advances in sp^3 SWCNT functionalization.
A) Super-resolved NIR emission of exciton localization in two different fluorescent ultrashort nanotubes. Each localization is displayed as a two-dimensional Gaussian of unit amplitude and width equal to the localization precision as commonly used in localization microscopy. Super-resolution imaging demonstrated the preferential localization of excitons at the nanotube ends and their behaviour as independent emitters. B) Schematic representation of a nanotube functionalized with photo-switchable molecules. (A) reproduced from (Danné et al., 2018b)with permission. Copyright 2018, American Chemical Society, (B) reproduced from (Godin et al., 2019)with permission. Creative Commons Attribution-Non Commercial license, 2019.

with self-assembly strategies proved selective detection of copper ions in the striatum of rat brains, showing potential to be used for *in vivo* fluorescent sensing in neurochemistry (Lin et al., 2015). CDs could be also imaged after systemic injection using standard epifluorescence techniques in *ex vivo* brain tissues, showing predominant accumulation at the cortex surface, in the hippocampus and in the ventricles (Qian et al., 2014). Unfortunately, the excitation wavelength did not match the NIR region, limiting the penetration depth of the imaging.

Graphene oxide nanoparticles (GOs) are formed by stacks of graphite layers to form nanoparticles of 1 nm thickness and several tens of nm of lateral size (Baldrighi et al., 2016). Similar to CDs, GOs exhibit tuneable photoluminesce and good photo-physical properties (Cao et al., 2013). Qian et al. used two-photon excitation microscopy to image micro-injected GO particles conjugated with polyethylene glycol into the brains of mice up to 300 μm deep (Qian et al., 2012). Reduced-GOs were also imaged in rat brains using the matrix-assisted laser desorption/ionization mass spectrometry imaging technique (Mendonça et al., 2015). These results show potential for *in vivo* brain imaging.

Nanodiamonds (NDs) are another class of carbaceous nanomaterials that can be potentially used as contrast agents and fluorescent probes for brain imaging and neurochemical sensing. NDs are crystalline nanoparticles, formed by sp^3 carbon atoms arranged in a diamond-like cubic lattice. The introduction of nitrogen-vacancy centres into the ND lattice produces stable luminescence at 600–750 nm range and a long spin-coherence time, making NDs a remarkable contrast agent for high-resolution magnetic imaging (Waddington et al., 2017). In addition to the nanometre-size dimensions (diameter of few tens of nm), NDs possess very interesting properties for bioimaging applications, including tuneable surface chemistry, excellent mechanical properties, and low cellular toxicity (Turcheniuk and Mochalin, 2017). NDs surface is highly reactive and can be functionalized or passivated. Despite these impeccable characteristics, the use on NDs for brain imaging are mainly restricted to intracellular tracking and sensing (Huang et al., 2014; Haziza et al., 2017; Moscariello et al., 2019). However, novel surface modification strategies may help to adapt these technologies for imaging and sensing the brain ECS.

Single-walled carbon nanohorns (SWCNHs) are relatively unexplored carbon nanomaterials, especially for biomedical applications. They are structurally similar to SWCNTs; however, the continuous graphitic surface is wrapped in a conical shape with a closed tip. They are usually 40–50 nm long and 2–3 nm wide, and they commonly assembly into 50–100 nm spherical aggregates (Voiry et al., 2015). SWCNHs demonstrated electrochemical detection of epinephrine with low detection limit, high sensitivity and a wide linear range of concentrations (Valentini et al., 2014). More recently, a similar approach was developed to detect glutamate, achieving a sensor response in the sub-second time-scale (Ford et al., 2019). These results may demonstrate the potential use of SWCNHs for the detection of neurochemicals in biological environments.

9. Conclusions

Optical imaging of the brain ECS is a vivid field of research and continues to reveal details of ECS structure, connectivity, and function. In order to further improve our understanding of the ECS and its interplay with neuronal circuits, the main challenge will be to directly visualize the relationship between brain structure, neuron activity, neuromodulation, and neurochemistry. Research on carbon nanomaterials is going towards this direction, mainly focusing on the development of new sensing probes and imaging techniques for *in vivo* deep brain experiments with minimally invasive surgical procedures. In this review, SWCNTs are proposed as the workhorse candidate for imaging and sensing the ECS, owing to their physical and chemical properties including morphology, intrinsic fluorescence, low toxicity, and available surface modification strategies. SWCNT sensors also hold great potential for the diagnosis and treatment of brain diseases (Komane et al., 2016). Advances in surface chemistry functionalization may also enable the implementation of bright SWCNTs for super-resolution imaging bearing specific chemical functionality for sensing of targeted molecules. This, coupled with novel advances in 3D *in vivo* imaging (Bon et al., 2018), would pave the way to target specific unexplored brain compartments, such as the synaptic cleft or the perineuronal milieu.

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