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Graphene-Based Nanotechnology in Neurodegenerative Disorders

Christos Tapeinos

Graphene-based materials (GBMs) demonstrate unique electrochemical, mechanical, thermal, and optical properties rendering them attractive candidates for numerous biomedical applications. Since graphene's discovery, GBMs have been at the forefront of biomedical research offering innovative solutions for numerous diseases, including neurodegenerative disorders (NDs). There are numerous reviews in which synthesis and functionalization methods of GBMs are discussed. However, this review focuses specifically on the recent research advances of GBMs for NDs, and more specifically, on sensing and therapeutic applications. After a short description of NDs' main characteristics, significant attention is given to the functionalization strategies used to improve the biomedical properties of GBMs, and recent applications for Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, and amyotrophic lateral sclerosis. A description of the use of GBMs and neural stem cell technology and known toxicity issues, followed by several limitations that current GBMs need to overcome, completes this review.

1. Introduction

Advances in nanobiomedical research over the years led to the development of numerous systems to treat life-threatening diseases. These systems' role was either diagnostic or therapeutic, or both in the form of theranostics. Different materials (e.g., synthetic and natural) with different architectures (e.g., quantum dots or nanoflakes), sizes (e.g., nano–, micro and macroscale), and physicochemical properties (surface charge and colloidal stability) paved the way for state-of-the-art theranostics in the nanobiomedical field. Among these, an emerging interest has been shown to 2D nanomaterials, and especially graphene and its derivatives. Since its discovery in 2004, graphene and its derivative materials, named graphene-based materials (GBMs), have been robustly studied as drug and gene delivery systems, bioimagers, biosensors, and bioelectronics, both in the fields of cancer therapy and tissue engineering (**Figure 1**).

Graphene, a single layer of atoms arranged in a honeycomb lattice, demonstrates excellent electrochemical, mechanical, and optical properties that render it suitable for various biomedical applications. In addition, physicochemical modifications (e.g., oxidation and reduction) result in the derivative GBMs, named graphene oxide (GO) and reduced GO (rGO). Each of these materials exhibits unique properties that can be tuned depending on their use. For example, the excellent mechanical strength, stiffness, and electrical conductivity make GBMs suitable for bone and neural tissue engineering, whereas their excellent optical properties render them a good fit for optical applications. Moreover, GBMs' large surface area and their ability to adsorb various aromatic biomolecules (e.g., DNA or RNA) through π–π stacking and/or electrostatic interactions make them useful in drug/gene delivery and biosensing applications. One more characteristic that makes GBMs a right candidate for biomedical applications is their good biocompatibility.

Despite all the advantages aforementioned, GBMs also demonstrate several limitations. In particular, graphene's high hydrophobicity results in aggregates in aqueous solutions and consequently reduced stability. Furthermore, the lack of functional groups does not allow surface modification, limiting its use in targeting drug/gene delivery and imaging.[1,2] To overcome these limitations, GO and rGO have been used. The functional groups on the surface of these GBMs led to an easy functionalization with a variety of synthetic and natural materials,[1,3] improving properties such as tissue specificity, colloidal stability, and biocompatibility. Functionalization of GBMs is a requirement for their use as therapeutics and diagnostics in vivo, particularly in neurodegenerative disorders'(NDs) treatment.

Although GBMs are proven a promising approach for treating NDs, they lack characteristics that other systems present. For example, lipid-based nanostructures can be considered more appropriate for treating neurodegenerative diseases due to properties such as inherent ability to cross the blood–brain barrier, high biocompatibility, enhanced colloidal stability, lack of organic solvents during their synthesis, and cost-effective and straightforward scale-up procedures.[4] However, these

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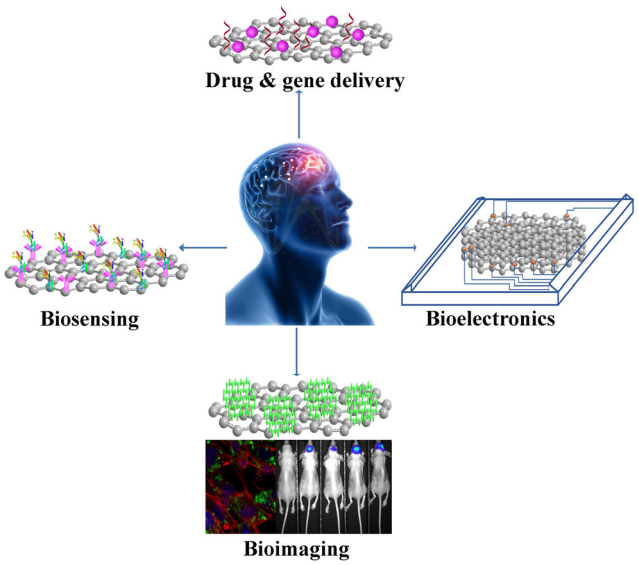

Figure 1. Graphene-based applications for neurodegenerative diseases.

nanostructures are characterized by low encapsulation efficiency, which becomes even lower in hydrophilic molecules, and a burst release of their encapsulated cargo. These drawbacks can be eliminated using GBMs since the various types of bindings (e.g., electrostatic interactions, hydrogen bonding, π–π interactions, and covalent functionalization) can offer enhanced stability and sustained release for hydrophilic components. From these examples, it is evident that each system's limitations can be counteracted by combining the two materials (lipids and GBMs) in hybrid nanocomposites.[5] Another alternate approach in the use of GBMs is the use of polymeric nanostructures. Compared to the lipid-based ones, polymeric nanostructures show high loading capacity with controlled and stimuli-responsive release profiles, good drug bioavailability, enhanced blood circulation, and encapsulation of both hydrophobic and hydrophilic substances. However, their noncontrolled biodistribution, their inability to cross the blood-brain barrier (BBB), and in several cases, the toxic byproducts of their degradation are some of the limitations that also need to be considered.[6] Notably, the combination of polymers with GBMs is one of the most common approaches due to the versatility in functionalization strategies that polymers can offer, resulting in nanocomposites with enhanced properties. Following the same rationale, hydrogels made of extracellular matrix (ECM)-based components are a preferential strategy for ND treatment since they allow the delivery, support, and growth of neural stem cells (SCs).[7] These ECM-based hydrogels present high biocompatibility and low immunogenicity, and to date, they have shown promising results for numerous brain disorders. Nonetheless, these hydrogels lack proper mechanical strength and electrical conductivity. As in lipids and polymers, these properties can be obtained by combining these materials with GBMs such as GO and/or conductive polymers such as polypyrrole/polyaniline,[8] leading to materials with fewer limitations and more appropriate for NDs' treatment. To date, there are several promising approaches for NDs, several of which are as follows: 1) nanoparticles for Alzheimer's disease (AD) treatment, 2) carbon nanotubes and gold nanoparticles for the detection of Parkinson's disease (PD), 3) gold nanoclusters for imaging PD and multiple sclerosis (MS) and ultrasmall superparamagnetic iron oxide nanoparticles for MS imaging.[9] Although these approaches constitute "significant competitors" to the GBM technology, graphene still holds great promise due to its versatile properties. More specifically, the high conductivity, transparency, and flexibility render GBMs the best candidates for the fabrication of functional brain implants and neuromodulation therapies. Furthermore, GBMs' mechanical and conductive properties make these materials suitable for the engineering of scaffolds able to support SCs' survival, proliferation, and differentiation, ultimately promoting neuronal reconstruction.

Hitherto, numerous reviews discussing the various synthesis methods, characterization techniques, and biomedical applications of GBMs have been presented.[1,2,10,11] However, none of them focuses specifically on NDs' diagnosis and treatment. Thus, in this article, the main characteristics for NDs, followed by the various functionalization methods that improve the suitability of GBMs for treating the different NDs, will be discussed. In addition, the latest advances in the field of GBMs diagnostics and therapeutics will be highlighted, aiming at providing useful insights into the designing principles for ND-specific engineered nanomaterials. GBM's role in SC technology and known toxicity issues, followed by the Conclusions and Outlook, will complete the specific review.

2. Main Characteristics of Neurodegenerative Disorders

NDs constitute diseases characterized by the progressive loss of brain neurons leading to vocal, motor, and cognitive dysfunctions. The most common NDs that will also be discussed in this review are AD, PD, Huntington's disease (HD), MS, and amyotrophic lateral sclerosis (ALS). Although the pathogenesis and the degeneration mechanisms are different for each condition, they share common characteristics, such as misfolded protein aggregation, neuroinflammation, autophagy dysregulation, oxidative stress, and neuronal loss.[12] It has to be emphasized that NDs should not be confused with secondary neurodegeneration pathologies (SNDPs) such as ischemic stroke[7,13] and traumatic brain injury[14] since these pathologies have different primary causes.

The diagnosis and treatment of NDs' represent a significant clinical challenge since most of the symptoms become evident at the late stages. Even in the case of successful diagnosis, the current administered medication has a more comforting role than curative. To date, several diagnostic methods for NDs have been reported. These include high-performance liquid chromatography,[15] spectrophotometry,[16] electrochemical detection,[17] enzyme-linked immunosorbent assay (ELISA)-based assays,[18] surface plasmon resonance,[19] and others.[20,21]

AD is a chronic neurodegenerative disease, constituting the most common type (≈60–70%) of dementia. It is characterized by amyloid-beta (Aβ) deposition, the absence of cholinergic neurons, and tauopathy.[22] The causes for AD are poorly understood, with 70% of the risk to be considered inheritable and attributable to genetic factors. More than 50 million people worldwide have

dementia, and there are 10 million new cases every year.[23] Although the onset mechanisms are not well understood, several hypotheses, the most important of which are the amyloid and the tau ones, have been suggested. In the amyloid hypothesis, the deposition of oligomeric or fibrillar amyloid β-peptides (Aβ) and the creation of plaques in the brain are responsible for the disease's pathogenesis.[24] In contrast, the hyperphosphorylation and aggregation of the tau protein lead to the loss of biological activity and subsequent neural toxicity.[25]

After AD, PD constitutes the second most common ND that mainly affects motor functions. As in AD, the disease causes are unknown but hereditary, environmental, and genetic factors are considered responsible for its pathogenesis. PD usually affects older people (>60 years old), but younger people (<50 years old) can also be affected (early-onset PD). The disease is characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of protein aggregates called Lewy bodies. The most frequent protein in these aggregates is α-synuclein, classifying PD as one of the most common synucleopathies.[26]

HD is a progressive ND. Like AD and PD, its pathogenesis has been related to the accumulation and aggregation of misfolded proteins. More specifically, the mutated huntingtin (Htt) gene causes the excessive repetition of the trinucleotide cytosine–adenine–guanine (CAG), leading to the creation of the unstable Htt protein and subsequent cellular toxicity.[27–29] Although there are no commercial diagnostic or therapeutic tools for HD, promising approaches using GO, as later described, have been proposed. To delay or inhibit the disease's progression, synthesis reduction or more effective clearance of the Htt protein needs to be conducted.

MS is a neurodegenerative autoimmune disease affecting the central nervous system. In MS, the immune system attacks the protective sheath (myelin) that covers the nerve cells, causing demyelination, consequently disrupting the nervous system's ability to transmit signals. Like AD, MS is characterized by an excessive tau protein amount and a more specific protein named myelin basic protein (MBP). The excess of these proteins is considered responsible for MS pathogenesis. Their detection and quantification can lead to an early diagnosis and monitoring of the disease.

ALS is a neurodegenerative disease that causes the loss of the upper and lower motor neurons that control voluntary muscles. Akin to other fatal neurodegenerative diseases, there is no cure, and the current treatments focus on alleviating the symptoms and improving the patients' quality of life.

3. GBMs Functionalization Strategies

Graphene exhibits excellent mechanical, thermal, and electrical properties. However, its large surface area and the strong van der Waals forces lead to its aggregation and inability to be further used in biological systems. In addition, although graphene has shown a therapeutic aspect in several pathologies (e.g., AD, PD, HD, and cancer), its biological compatibility has been debated. Thus, to improve the colloidal stability and the biological compatibility of graphene, several functionalization strategies have been proposed. These strategies, described later, are divided into two main categories based on the type of surface binding,

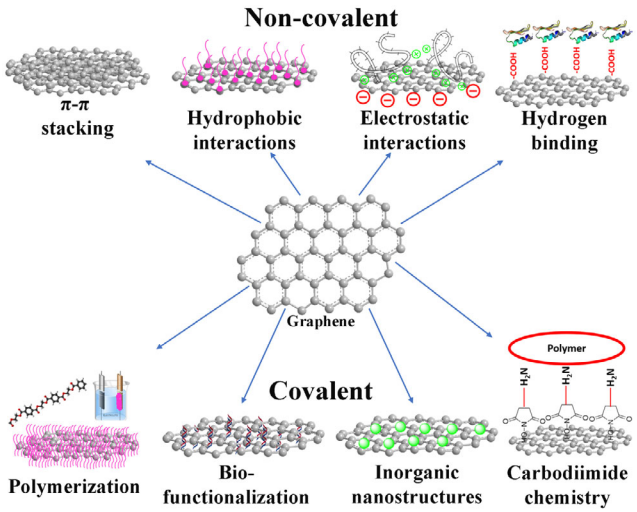

Figure 2. Functionalization strategies of GBMs.

noncovalent and covalent (**Figure 2**). This classification was realized because graphene does not exhibit functional groups on its surface, making its functionalization, based on the covalent binding, unfeasible. In contrast, GBMs such as GO and rGO support covalent binding due to epoxy, hydroxyl, and carboxyl groups on their surface.

3.1. Noncovalent Binding

The noncovalent binding of various molecules and/or structures is the preferred functionalization method for graphene, rGO, and other GBMs, where functional groups are not present. One of the main advantages of this type of binding is that it allows GBMs to keep intact their characteristics. It has already been demonstrated that surface oxidation significantly diminishes their properties. Furthermore, noncovalent binding excludes organic solvents, catalysts, and other hazardous chemicals used during covalent surface functionalization, resulting in cost/eco-friendly and specific products with enhanced biological compatibility. Thus, this functionalization is favorable for biomaterials for central nervous system (CNS) and NDs. Noncovalent binding can be achieved by various methods including, electrostatic interactions, hydrogen bonding, hydrophobic interactions, and π–π stacking.

3.1.1. Electrostatic Interactions

These interactions constitute one of the simplest ways for surface functionalization, coating, or decoration, depending on the different surface charge between the pristine and the coating material. Since graphene and other GBMs have a negative surface charge, a coating material with a positive charge can easily be attached and stabilized on their surface because of these interactions. For example, the negative surface charge of rGO can be easily functionalized with the positively charged polyethyleneimine (PEI), acting as an anchor for the loading of negatively charged DNA molecules.[30] It has to be stressed out that other biomolecules or nanostructures (e.g., nanoparticles)[31] can be

attached to the surface of rGO–PEI providing the system with a high versatility on the type of theranostics that can be used. In another example, the carboxyl groups of graphene quantum dots (GQDs) acted as a negative substrate to attract and detect the positively charged biomarker dopamine.[32] Chitosan is another positively charged biocompatible material used for coating[33] or encapsulation.[34] However, the amine and hydroxyl groups on the surface of chitosan allow additional interactions with GBMs such as GO through hydrogen bonding, further stabilizing the produced composites.

3.1.2. Hydrogen Bonding

Even though several graphene-based drug delivery systems have been presented in the last years,[10] only a few studies where GO's functionalization was based on hydrogen bonding were reported for neurodegenerative diseases. In one of these works, silk fibroin was attached to GO films through hydrogen bonds, creating a scaffold for neuroregeneration.[35] One of the reasons why hydrogen bonding is not seen as the preferred way of interaction is that, in most functionalizations, hydrogen bonding is combined with other noncovalent interactions such as those aforementioned as electrostatic, hydrophobic, and π–π stacking.

3.1.3. Hydrophobic Interactions

As previously said, the lack of functional groups on the surface of graphene, rGO, and other GBMs creates a hydrophobic substrate suitable for the functionalization/loading of other hydrophobic molecules such as proteins.[36] Even though hydrophobic interactions are relatively stronger than hydrogen bonding, they are still not selected as the preferred type of binding for GBMs and neural engineering applications. However, hydrophobicity has been robustly used in cancer, where surface functionalization of GBMs with amphiphilic molecules demonstrated enhanced therapeutic efficacy in various in vitro and in vivo studies.[37] It should be stressed out that a variety of hydrophobic molecules such as surfactants[38] and lipids[39] have been used to functionalize graphene's surface, aiming to improve both its biocompatibility as well as its solubility and its colloidal stability.

3.1.4. π–π Stacking

π–π stacking is the final and the most common type of noncovalent functionalization for GBMs. This strong binding is based on the nonpolar interactions between the aromatic structure of graphene and molecules containing aromatic rings through the overlapping of π orbitals. π–π stacking functionalization is proportional to covalent binding without affecting the graphene's structure, allowing it to keep its properties.[3] Materials that use the π–π stacking binding belong to the polycyclic aromatic hydrocarbons' family and can be natural such as chitosan and hyaluronic acid or synthetic such as pyrene.

Summarizing, several noncovalent binding methods can be used to functionalize graphene and GBMs, allowing them to preserve their properties. Even though only a single binding method is realized during surface functionalization, in most of these coatings and depending on the material, a combination of interactions is observed. For example, in hyaluronic acid, except π–π stacking, hydrogen bonding between some hydroxyl groups in GO or rGO can also occur. In chitosan, it is typical for π–π stacking and electrostatic interactions to commonly act together. In contrast, for curcumin, π–π stacking and hydrophobic interactions dominate. In other natural materials such as collagen, hydrogen bonding and electrostatic interactions are the driving coating interactions. However, the electrostatic interactions depend on the pH, as in chitosan, and can be eliminated at a neutral or basic pH.

3.2. Covalent Binding

In contrast to the noncovalent binding, the covalent one demands extra chemicals and multistep reactions that are time-consuming and sometimes very costly. Nevertheless, covalent binding is used robustly due to its versatility in using materials, functional groups, and tailored-made sensitivities and properties that cannot be achieved with the noncovalent one. Surface functionalization using covalent binding can be achieved through several chemical procedures, including radical, anionic, and cationic polymerizations, as well as cycloaddition reactions based on click chemistry. For a more detailed description of the characteristics, mechanisms, advantages, and drawbacks of each polymerization technique, the readers are directed to the comprehensive work of Punetha et al. [3]

3.2.1. Radical, Anionic, and Cationic Polymerizations

One of the most studied biofunctionalization methods to improve the biocompatibility, and the colloidal stability of GBMs is coating via surface polymerization. The coating can be realized using a variety of methods, including radical polymerizations such as atom transfer radical polymerization (ATRP), ring-opening polymerization (ROP), nitroxide-mediated polymerization (NMRP), and reversible addition-fragmentation chain transfer polymerization (RAFT). In addition, more controllable types, such as the anionic and cationic polymerizations, have also been used. Two main approaches have been reported for the functionalization of GBMs with polymers, the "grafting to," and "grafting from." In the first case, prepared polymers are attached to the surface of GBMs through reactions such as amidation, esterification, and radical coupling. In the latter case, monomers are directly polymerized on the surface of GBMs containing functional groups such as -OH, -NH_2, -COOH, and -COCl, allowing for better control of the coating. To achieve these coatings, the reactions described earlier were combined with click chemistry. Polymers such as polystyrene (PS), poly *N*-isopropyl acrylamide (PNIPAAm), poly-L-lactide (PLLA), and poly-ϵ-caprolactone (PCL) are some of the preferred coating materials that have been reported.[3] The majority of the polymerization methods enable various monomers' polymerization, resulting in specific functionalities and controllable properties, including composition, molecular weight, and molecular architecture. However, the use of catalysts and toxic chemicals, low polymerization efficiency, multistep synthetic procedures, and lack of control in polymer chain length are some drawbacks that forbid the robust use of these techniques. Furthermore, the lack

of functional groups on graphene's surface and some of its derivative materials make the covalent binding even more complicated, suggesting the need for alternative functionalization methods, such as the ones aforementioned.

3.2.2. Electrochemical Polymerization

Electrochemical polymerization (ECP) is another technique that can be used for the surface functionalization of GBMs. Although not all materials are suitable for ECP, a few such as polypyrrole, polyaniline, poly(3,4-ethylene dioxythiophene) (PEDOT), and Nafion chitosan, and cellulose have already been studied.[40] Electropolymerization is typically used for the coating of electrodes or the formation of films because of its ability to easily control the thickness of the coating. Nevertheless, nanosponges and hybrid graphene nanosheet/multiwalled carbon nanotube (MWCNT)/foam using ECP have also been reported.[3,41] In all of the reported studies, the electrical properties of the already electrically conductive materials have been improved, suggesting the potential use of these hybrids for various energy and biomedical applications.

3.3. Functionalization Using Biological Molecules

Biological molecules or biomolecules refer to molecules present in organisms responsible for one or more biological processes. Biomolecules include large macromolecules (e.g., carbohydrates, lipids, proteins, and nucleic acids) and small molecules (e.g., primary and secondary metabolites such as ethanol and antibiotics, respectively, and natural products such as camptothecin and curcumin). From the aforementioned definition, it is clear that the functionalization of GBMs with biomolecules is of great importance in nanomedicine. Among the wide range of biomolecules, nucleic acid (e.g., pDNA, single-stranded DNA, and small interfering RNA),[42] proteins (e.g., lectins),[43] and antibiotics (e.g., levofloxacin)[44] have been used to functionalize the surface of graphene and GBMs. These molecules' binding is achieved by noncovalent interactions such as the ones aforementioned[45] or covalent conjugation through carbodiimide chemistry (Figure 2).[21] The combination of noncovalent and covalent binding using bifunctional linkers has also been reported.[46]

3.4. Functionalization Using Inorganic Nanostructures

As mentioned in the previous paragraphs, GBMs exhibit excellent optical, mechanical, and electrochemical properties that make them attractive candidates in biomedicine. However, the properties of pristine graphene and its derivatives are not always sufficient to provide the best possible therapeutic/diagnostic outcome. Thus, several approaches where GBMs have been combined with inorganic nanostructures (e.g., nanorods and nanoparticles) have been proposed. These nanostructures, mostly metal-based, can have various sizes and morphologies (e.g., nanoflakes or nanowires) and exhibit properties such as large surface areas, magnetism, and high electron transfer rates, resulting in nanocomposites with enhanced/combined properties. These combinations lead to improved therapeutic outcomes[47] and enhanced sensitivity and selectivity[48,49] in the various diagnostic products.

4. GBMs in Neurodegenerative Diseases

4.1. Graphene-Based Sensors for AD

AD is characterized by the formation of Aβ plaques (*amyloid hypothesis*) and misfolded tau proteins (*tau hypothesis*) that lead to neurofibrillary tangles. Thus, the detection of Aβ monomers and tau protein concentration can provide important information about the disease's stage and potential treatment options. Due to the excellent electrical properties of GBMs, their use as electrochemical detectors of the aforementioned biomarkers has been thoroughly studied in the last few years. For example, oxygen-plasma-treated rGO (OPT–rGO) sensors, covalently conjugated with an antibody against the Aβ peptide, were successfully used to detect neural-derived exosomal Aβ peptides from the plasma of healthy and AD patients.[50] The antibody-immobilized OPT–rGO sensors demonstrated higher sensitivity to alterations of their electrical characteristics compared to plain OPT–rGO. In a similar study,[46] an antibody (H31L21)-conjugated dual-layer graphene/GO sensor was also used to detect Aβ peptides. In particular, the sensor demonstrated high specificity toward $A\beta_{1\text{-}42}$ despite the interference of $A\beta_{1\text{-}40}$ and apolipoprotein-E4 (Apo-E4) species present in human and mice plasma. Conjugation of antibodies or other targeting groups such as aptamers toward amyloid peptides is a usual technique for accurate and sensitive detection. Based on this, a fluorescent binding DNA (bDNA) containing an $A\beta_{1\text{-}40}$ oligomer-targeting aptamer ($Apt_{A\beta}$) was covalently conjugated on the surface of GO.[21] The fluorescent-modified $Apt_{A\beta}$ was then combined with complementary DNA (cDNA) to form a double-stranded DNA (dsDNA), the fluorescence of which was linearly decreased in the presence of $A\beta_{1\text{-}40}$ oligomers. In a different approach, GQDs were used as an electrochemical sensor for Aβ detection.[51] The GQDs' photoluminescence's quenching, due to the noncovalent (electrostatic interactions and hydrogen bonds) binding with the Aβ peptide, led to the detection not only of the peptide monomers but also of the fibrillary process, providing information on early-stage aggregation.

On the *tau hypothesis*, modifications (e.g., phosphorylation) on the microtubule-associated protein tau leads to neurofibrillary tangles, impairing neuronal axons and subsequently leading to neurodegeneration.[52,53] These tangles can be detected using graphene-based methods such as immunoassays[54] that measure the fluorescence decrease in fluorescein isothiocyanate-labeled tau proteins[54] or sensors that provide an electrochemical measurement (e.g., current decrease) depending on the concentration of tau proteins.[55] Since both the Aβ peptide and the tau protein have been related to AD pathogenesis, a sensor based on a reduced graphene field-effect transistor (rgFET) where both biomarkers can be detected has also been developed (**Figure 3**).[56] It is noteworthy that the developed rgFET provided a multiplex reading with a well-defined output signal for each of the biomarkers and a femtomolar limit detection.

A different biomarker, except Aβ peptide and tau protein that can help on AD diagnosis, is the Apo-E4 aforementioned.

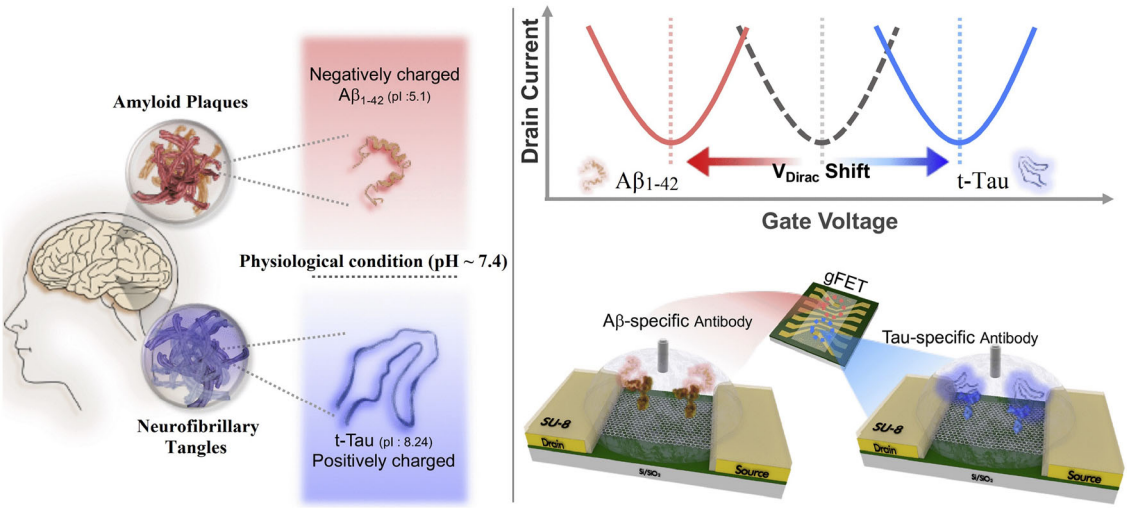

Figure 3. Schematic illustration of the rgFET for multiplex detection of Aβ1-42 and t-Tau. Reproduced with permission.[56] Copyright 2020, Elsevier.

Although apolipoproteins naturally enhance the amyloid peptides' breakdown, some mutations such as the Apo-E4 cannot do that. Given this, two graphene-based systems for the detection and quantification of Apo-E4 have been presented. In the first system,[57] graphene was combined with mesoporous silica hybrids (GSHs). This system was used for the covalent conjugation of ferrocene carboxylic acid with the amine groups of the GSHs (Fc-GSHs) and as a reservoir for loading methylene blue (MB) (π–π stacking/electrostatic interactions). Subsequently, a second covalent conjugation was performed between the Fc-GSHs and single-stranded DNA from sequences related to AD. The derived data from the DNA hybridization resulted in reproducible and accurate detection of Apo-E4. On the second system,[58] a combination of GQDs and curcumin (GQDs–Cur) as a coating material was used. GQDs–Cur was electropolymerized on the surface of an indium tin oxide electrode and was subsequently used for the covalent immobilization of an amino-substituted DNA probe. Quenching on the fluorescence and electrical values of Cur due to a DNA complex hybridization resulted in the accurate detection of Apo-E4.

Other biomarkers used for AD diagnosis are specific metaphorical RNAs (mRNAs), such as the beta-site amyloid precursor protein cleaving enzyme 1 (BACE-1) and microRNAs (miRNAs) such as the noninvasive plasma miRNA-137. For the former case,[59] a sensor based on GO and upconversion nanoparticles (NaYF4: Yb, Er) was fabricated to detect BACE-1. For this sensor, amino-functionalized oligonucleotides were covalently conjugated with the upconversion particles. The dispersion was then mixed with GO and irradiated. Hybridization of the nucleotide chain on the nanoparticles' surface led to reduced absorption of the GO nanoflakes and a reduced fluorescence derived from the irradiation. For the latter case,[60] a screen-printed carbon electrode, the surface of which was coated with a combination of electrochemically reduced GO and gold nanowires, was fabricated. Gold increased the sensor's sensitivity, and along with the use of doxorubicin as an electrochemical label, the sensor can detect the circulating miRNA-137 in the femtomolar range. Additional information concerning the used GBMs, biomarkers, and detection limits are shown in **Table 1**.

4.2. Graphene-Based Therapeutics for AD

Despite the several reported studies on the use of graphene-based nanotechnologies for AD diagnosis, there are only a few studies where GBMs are used as a therapeutic approach. Plain GO nanoflakes, GO quantum dots (GOQDs), GO-coated electrospun nanofibrous scaffolds, and graphene-based nanocomposites such as GO/iron oxide (GOIO) and GO/graphitic carbon nitride (GO/g–C_3N_4) represent some of these approaches. Even though all these strategies try to treat AD by dissociating the amyloid fibrils, each of them follows a different path. Autophagy, one of the primary mechanisms for protein aggregates' clearance, is known to be disrupted in AD patients.[61] Therefore, autophagy regulation is a prominent approach with promising therapeutic potential. One of the reported studies[62] showed that GO nanoflakes can promote microglia-induced autophagy by inhibiting the mammalian target of the rapamycin (mTOR) pathway and leading to Aβ clearance. This clearance subsequently reduced the toxicity in neurons.

Another strategy for Aβ's clearance is the interactions' interference during the fibrils' formation. It has been demonstrated that these interactions, including hydrophobic, electrostatic, hydrogen bonding, and van der Waals, can be easily disrupted when GBMs are in contact with the fibrils. In particular, it was demonstrated that GOQDs reduced the partially unfolded hen egg white lysozyme's (HEWL) hydrophobic interactions, resulting in the inhibition of the fibrillar growth and their corresponding protein molecules responsible for fibrils' formation.[63] GOQDs have also been used as a delivery system for neuroprotective peptides such as the glycine–proline–glutamine one.[64] In this article, the effect of GOQDs-peptide was assessed on APP/PS1 transgenic mice (AD in vivo model) using the Morris water maze. The results showed improved learning

Table 1. GBMs, biomarkers, the limit of detection, and the detection range for the fabricated AD sensors.

Electrode type[a)]	GBMs[a)]	Biomarker[a)]	Detection limit	Detection range	Ref.
–	dsDNA–GO	$A\beta_{1-40}$	0.1 nM	0.1–40 nM	[21]
–	Oxygen-plasma-treated rGO	Aβ-peptides	22.1 fM	22.1 fM–221 pM	[50]
SPE	Ab-conjugated Graphene/rGO	$A\beta_{1-42}$/Apo-E4	2.398 pM	11 pM–55 nM	[46]
–	GQDs	Aβ-peptides	50 μM	0–66.45 μM	[51]
–	Ab-conjugated GO	Tau protein	0.14 nM	0–0.28 nM	[54]
GCE	Ab-conjugated GO	Tau-441	75 fM	0.08–80 pM	[55]
Au	gFET	$A\beta_{1-42}$	222 fM	22.2 fM–22.2 nM	[56]
		t-Tau	21.8 fM	2.18 fM–2.18 nM	
GCE	Graphene–MSH	Apo-E4	10 fM	10 fM–100 nM	[57]
ITO	GQDs–curcumin	Apo-E4	0.36 pM	1.3–14.5 pM	[58]
–	GO–upconversion nanoparticles	BACE-1	500 fM	200 fM–5 nM	[59]
SPE	ERGO–Au nanowires	miRNA-137	1.7 fM	5–750 fM	[60]

[a)]Ab: antibody, Apo-E4: apolipoprotein E4, dsDNA: double-stranded DNA, ERGO: electrochemically reduced graphene oxide, GCE: glass carbon electrode, gFET: graphene field-effect transistor, GQDs: graphene quantum dots, ITO: indium-tin-oxide, MSH: mesoporous silica hybrids, SPE: screen-printed electrode.

and memory capability, attributed to the reduction in the Aβ peptides in the brain and the serum, reduced microglial activation, and a decrease in proinflammatory cytokines. In addition, the increase in the nerve growth factor and brain-derived neurotrophic factor, the increase in the dendritic spines of the mice' brains, and the demonstrated neurogenic effect suggested the potential use of this system for AD therapy. Following the same rationale,[47] it was shown that GOIO nanocomposites reduce the amyloid peptide aggregation due to the reduced hydrophobic interactions among the fibrils that the nanocomposite induces. Moreover, the nanocomposite dissociated preformed $A\beta_{42}$ fibrils, minimizing the induced toxicity to neuroblastoma cells. A different nanocomposite made of GO and graphitic carbon nitride ($GO/g\text{-}C_3N_4$)[65] led to the fibrils' dissociation by destroying the β-sheet secondary structure through the ultraviolet-induced $g\text{-}C_3N_4$ photocatalyst's degradation. This work provided an alternate stimuli-responsive approach for the treatment of AD using heterojunctions. An interesting study on the effect of surface inhomogeneity of GO nanosheets as a potential Aβ dissociation mechanism has also been reported.[66] The authors of the study showed, using molecular dynamics simulation, that the Aβ peptides tend to bind to the scattered surfaces of oxidized and nonoxidized regions of GO, affecting the self-assembly of the Aβ peptides. More specifically, it was suggested that the aromatic peptide residues bind to the sp^2 (nonoxidized) regions, whereas the polar residues demonstrate an affinity for the oxidized ones, resulting in the dissociation of the fibrils.

It should be emphasized that most of the therapeutic approaches to date have been designed to target the amyloid plaques, neglecting the effect of the tau neurofibrillary tangles. However, the failures derived from therapeutic systems in clinical trials[53] showed that Aβ is not the proper AD target, and the tau hypothesis should be considered. In one of the reported studies targeting tauopathy,[25] poly(lactide-*co*-glycolide) (PLGA) scaffolds were coated with GO and loaded with the tau inhibitor MB. The precoating with GO granted efficient MB loading (π–π stacking and electrostatic interactions) and a therapeutic controlled release. The released MB induced the autophagy of the seeded neural progenitor cells (NPCs), which resulted in their inactivity and consequently helped them cope with AD's stressed conditions. Finally, it was shown that MB can reduce NPCs' apoptosis and inhibit tau phosphorylation.

4.3. Graphene-Based Sensors for PD

To date, there are no specific tests or methods for the diagnosis of PD, leaving most of the patients untreated for several years until the symptoms are evident. As a ND, PD worsens over time, resulting in a point where the administered medication fails to treat the symptoms. Therefore, finding ways to detect PD in its early stages is essential for its treatment and for improving the patients' quality of life. Due to the excellent electrical properties and the large surface area of graphene and its derivative materials, numerous graphene-based nanocomposites have been studied as potential diagnostic tools. However, even if graphene is an excellent sensor material for several biomarkers' electrochemical detection (e.g., dopamine, levodopa, and homovanilic acid), its pristine form lacks sensitivity and selectivity.

Therefore, to improve the properties of GBMs, functionalizations of their surface with conducting polymers, metal nanoparticles, carbon nanotubes, GQDs, and others have been used. For example, zinc oxide (ZnO) nanostructures in the form of nanoflowers,[48] nanosheets,[49] and nanoparticles[67] have improved the sensitivity/selectivity of the graphene-based sensors. These nanostructures are n-type semiconductors characterized by good electrochemical activity, large surface areas, and high electron transfer rates that ultimately enhance the coated sensors' performance. Other metal-based nanostructures that have been reported are gold (Au) nanowires[68] and nanoarrays,[69] gold,[70] silver (Ag),[71,72] platinum (Pt),[73,74] and hematite ($\alpha\text{-}Fe_2O_3$),[75] nanoparticles, and finally gold[76] and manganese dioxide (MnO_2) nanorods.[77] Recently, metal dichalcogenides such as

alpha-manganese sulfide (α-MnS) nanoparticles[78] and tin disulfide (SnS_2) nanorods[79] have been reported to improve the sensing ability of the fabricated sensors through enhancement of the electrochemical conductivity and structural stability. Chalcogenides are a promising material for electrode fabrication due to their excellent stability, high surface-to-volume ratio, and low cost. In addition, the graphene-based sensors have been combined with other organic, inorganic, and hybrid components. The organic components such as fullerene,[76] multi MWCNTs,[80] and GQDs[81] increase the surface area and the electrochemical conductivity of the sensors, resulting in lower detection limits of biomarkers such as dopamine. Nitrogen doping through the thermal treatment of rGO with p-phenylamine has also been reported to boost the sensors' electrochemical properties. This improvement can be attributed to the enhanced semiconducting properties and surface-active sites. In additionto the organic components, the sensors' properties can also be improved using inorganic materials such as the layered doubled hydroxides (LDHs). LDHs are layered materials with flexible tunability and the ability to intercalate inorganic and organic anions, biomolecules, and genes[82] that can be used for the detection of various biomarkers in biological fluids. For example, nickel aluminide (NiAl)–LDHs have been combined with graphene, on a layer-by-layer structure, resulting in the detection of dopamine in live human neuroblastoma cells.[83] A worth-mentioning study that was recently published, showed that it was possible to monitor the secretion of dopamine from dopaminergic cells, including neural stem cells (NSCs), at the single-cell level using a graphene substrate on the top of which homogeneous gold nanoarrays with tooth-like structures were fabricated.[69] The system improved significantly the dopamine's detection limit, using surface-enhanced Raman spectroscopy. The detection mechanism was based on Raman-dye labeled aptamers that were bound on the surface of the gold nanoarrays and that were detaching in the presence of dopamine to create a dopamine–aptamer complex. The detachment led to a decrease in the intensity signal that was proportional to the dopamine concentration. Finally, the use of hybrid materials such as metal-organic frameworks (MOFs)[84] on the sensor's surface increases further the surface area and accelerates the electron transfer processes.

It should be emphasized that although the nanostructures aforementioned enhance the electrochemical sensitivity of the sensors, additional coatings are also applied. Polymers such as polyaniline,[48] polydopamine,[80] and combined poly(3,4-ethylene dioxythiophene) polystyrene sulfonate (PEDOT:PSS)[85] allow efficient oxidation of biomarkers such as dopamine and uric acid, resulting in the detection of the former without the latter's interference. Ionic liquids such as 1-butyl-3-methylimidazolium hexafluoro phosphorene[71] and oligosaccharides such as β-cyclodextrin[79] have also been used as binding materials for the various nanostructures, improving their stability. Finally, Dawson's heteropolyacid (DHPA) clusters, which can act as inorganic ligands and coordinate with organic complexes of transition metals such as Au, Pt, and Ag nanoparticles, have also been studied.[72]

One more thing that needs to be clarified for the earlier referenced graphene-based nanocomposites is that they were used either as coatings for already prepared electrodes (e.g., glassy carbon electrode,[48] screen-printed electrode,[68] fluorine-doped tin oxide electrode,[85] carbon paste electrode,[81] organic field-effect transistor,[73] indium tin oxide electrode,[70] rGO paste electrode,[84] and pencil graphite electrode[72]) or directly as they are.

Finally, each of these sensors was able to detect either separately or simultaneously a variety of biomarkers such as microRNA,[68] dopamine,[48,67,70,73–75,77–80,83,85,86] levodopa (L-DOPA) (a precursor of dopamine),[49,72,81,84] homovanilic acid (product of dopamine catabolism),[76] α-synuclein,[20] uric acid,[48,86] and ascorbic acid.[86] Although only the first four are related to PD, the detection of the last two is useful as a control for dopamine and levodopa detection. The similar oxidation potential and the high concentration of uric acid and ascorbic acid in biological fluids such as blood serum and urine make it challenging to detect dopamine and L-DOPA. Therefore, sensors with high selectivity and detectability are needed for the detection of proper markers. Additional information concerning the electrode types, GBMs, biomarkers, and detection limits are shown in **Table 2**.

4.4. Graphene-Based Therapeutics for PD

As in AD, the main therapeutic approaches for PD focus on the dissociation of formed fibrils or the fibrillation inhibition of α-synuclein.[87] To achieve this therapeutic effect, graphene sheets[88,89] and GQDs have been used.[88–90] These reported studies have shown that the electrostatic interactions between the negatively charged surfaces of the GBMs and the positively charged regions of α-synuclein are mainly responsible for the dissociation and the formation inhibition of fibrils. However, the mechanism in which the formation of the fibrils is inhibited differs depending on the GBMs used. More specifically, graphene sheets sequester the α-synuclein monomers, preventing primary nucleation and elongation, whereas GQDs affect the secondary formation processes.[88] Of note, the α-synuclein aggregation inhibition is time- and concentration-dependent[88,90] with low concentration to potentially enhance the aggregation phenomena.[88] In addition to the aggregation inhibition, GQDs have been shown to reduce the number of Lewy bodies and the subsequent Lewy neurite formation, bringing to inhibition of neuronal death and synaptic loss.[90]

Alternative approaches have suggested PD treatment through electrical[91] and photothermal stimulation.[30] In contrast to the methodology followed earlier, where the designed therapeutics target fibril formation/clearance, electrical stimulation blocks the abnormal nerve signals that cause PD symptoms. Given this, minimally invasive graphene-based microelectrode arrays were used to stimulate the diseased tissue to modulate neural circuits and reduce motor symptoms.[91] In contrast, photothermal stimulation has been used to promote or inhibit cellular functions and consequently lead to a therapeutic effect. For example, rGO nanoparticles were combined with poly ethylene imine and pDNA and were successfully functionalized with the specific neuro-peptide, neurotensin (**Figure 4**).[30]

Near-infrared (NIR) irradiation of nanoparticle-treated neurons improved cell permeability and uptake while helping the nanoparticles to escape endo/lysosomes. This subsequently led to improved transfection providing another way to treat PD.

Table 2. GBMs, biomarkers, the limit of detection, and the detection range for the fabricated PD sensors.

Electrode type[a)]	GBMs[a)]	Biomarker[a)]	Detection limit	Detection range	Ref.
GCE	Graphene–CMP	a-synuclein	2.5 fM	7–571 fM	[20]
GCE	rGO–PANI–ZnO	dopamine	0.8 nM	0.001–1 μM and 1–1000 μM	[48]
		Uric acid	42 nM	0.1–100 μM and 100–1000 μM	
		Combination	17 nM for DA	0.1–90 μM and 90–1000 μM for DA	
			120 nM for UA	0.5–90 μM and 100–1000 μM for UA	
–	rGO–ZnO NPs	Dopamine	167 nM	5–70 μM	[67]
SPE	rGO–Au nanowires	miR-195	2.9 pM	10–900 pM	[68]
–	GO–Au nanoarrays	Dopamine	1 nM	1 nM–100 μM	[69]
ITO	GO–Au NPs	Dopamine	1.28 μM	0.1–30 μM	[70]
ITO	GO–Ag NPs	Dopamine	0.2 μM	0.1–100 μM	[71]
PGE	GO–DHPA–Ag NPs	Levodopa	0.76 nM	3–100 nM and 100 nM–10 μM	[72]
–	rGO–Pt–OFET	Dopamine	0.1 fM	0.01–0.1 fM	[73]
4-shank MEA	rGO–Pt NPs	Dopamine	N/A	N/A	[74]
–	Graphene–ZnO	Levodopa	1 μM	1–75 μM	[75]
GCE	ERC60–GO–Ph	HVA	30 nM	0.1–7.2 μM	[76]
GCE	GO–MnO_2 NRs	Dopamine	27 M	0.1–80 μM and 80–410 μM	[77]
GCE	Graphene/β-CD/SnS_2	Dopamine	4 nM	0.01–150.76 μM	[79]
–	Graphene–polydopamine–MWCNTs	Dopamine	1 μM	7.0–297.0 μM	[80]
		Uric acid	15 μM	20.0–320.0 μM	
CPE	GQDs + ionic liquid	Levodopa	10 nM	0.05–250 μM	[81]
–	Graphene LBL–NiAl LDH	Dopamine	2 nM	0.1–97 μM	[83]
rGO paste electrode	MOF	Levodopa	25 nM	0.1–85 μM	[84]
FDTO	Graphene–PEDOT: PSS	Dopamine	105 nM	1–30 μM	[85]
–	Nitrogen-dopped rGO	Dopamine	9.6 μM	1–60 μM	[86]
		Uric acid	0.01 μM	1–30 μM	
		Ascorbic acid	0.2 μM	0.1–4 mM	

[a)]β-CD: β-cyclodextrin, CMP: conjugated microporous polymer, CPE: carbon paste electrode, DHPA: Dawson heteropoly acids, ERC60: electrochemically reduced fullerene, FDTO: fluorine-doped tin oxide, GCE: glass carbon electrode, HVA: homovanilinic acid, ITO: indium tin oxide, LBL: layer-by-layer, LDH: layered double hydroxides, MEA: microelectrode array, MOF: metal-organic framework, NPs: nanoparticles, NRs: nanorods, OFET: organic field-effect transistor, PANI: polyaniline, PCE: pencil graphite electrode, PEDOT/PSS: poly(3,4-ethylene dioxythiophene) polystyrene sulfonate, Ph: phenylamine, SnS_2: tin disulfide, SPE: screen-printed electrode, ZnO: zinc oxide.

4.5. Graphene-Based Approaches in Other Neurodegenerative Disorders

HD, MS, and ALS also belong in the class of neuronal disorders, for which GBMs have been proposed for their diagnosis and treatment. Although not many studies where GBMs are used for HD have been reported, in this article, a few cases will be described.

As described in Section 2, a more effective clearance of the Htt protein or synthesis reduction constitutes potential therapeutic approaches for HD. With this in mind, GO was used as a therapeutic strategy able to enhance autophagy (intracellular clearing mechanism) through autophagosome formation and normal autophagic flux.[27] In this article, it was demonstrated that Htt can be inactivated through enhanced ubiquitination due to ubiquitin's preferential binding on GO's surface, suggesting the potency of GO in HD treatment. The therapeutic effect of GO in HD has also been reported in a more recent study,[28] where Htt's structural transformations upon binding on GO and molybdenum sulfate (MoS_2) were studied. The results showed that typical polyglutamate domains (Q22) unfold and elongate after their surface binding, whereas initially collapsed HD domains (Q46) remain mostly collapsed. This folding/binding behavior was attributed to hydrogen bonds competing with both GO and MoS_2 surface hydrophobic interactions.

As indicated, HD is characterized by excessive repetition of the trinucleotide CAG. With this in mind, a fluorescent platform based on GO and an RNA probe to detect CAG repeated sequences has been reported.[29] The system's particularity was based upon an additional element, ribonuclease H (RNase H), an endonuclease that specifically hydrolyzes the phosphodiester bonds of RNA that is hybridized to DNA. This endonuclease boosted the system's sensitivity, allowing for a detection limit for CAG of 108 pM, which is 18 times lower than standard GO/DNA without DNase use.

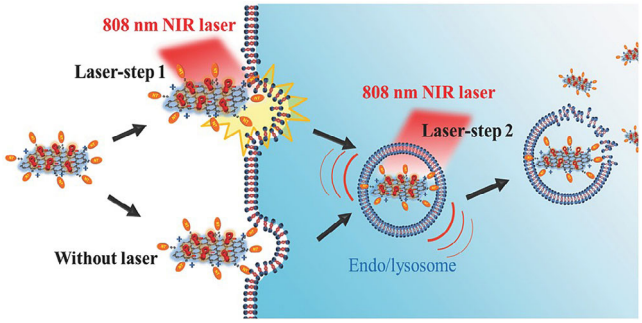

Figure 4. Schematic diagram of two-step NIR laser treatment on gene transfection in neuron cells, using neurotensin-conjugated reduced graphene oxide nanoparticles. Reproduced with permission.[30] Copyright 2016, Wiley.

As previously described, MS is characterized by excess on tau and myelin basic proteins, rendering their detection and quantification a significant aspect for MS treatment. To achieve this detection, in one of the reported studies, an antibody-conjugated GO nanocomposite was proposed.[92] The reported nanocomposite consisted of molybdenum sulfide (PbS)/cadmium sulfide (CdS) nanocrystals, conjugated with anti-tau and anti-MBP antibodies that were subsequently combined with GO. The final nanocomposite was able to detect both biomarkers with high specificity and sensitivity (limit of detection: MBP = 0.30 nM/Tau = 0.15 nM).

In addition to diagnosis, GBMs and more specifically GQDs have been used as therapeutics for CNS demyelination.[93] Using an experimental autoimmune encephalomyelitis model, it was demonstrated that GQDs can reduce immune filtration, demyelination, axonal damage, and apoptotic death in the CNS. Briefly, the study showed that GQDs can access immune and CNS cells during neuroinflammation and alleviate immune-mediated damage through specific pathways. This approach can be used not only for MS but also for other neuroinflammatory and demyelinating diseases as well.

ALS is not a chronic disease but a rapid one and its early detection is of great importance. However, there is still no reliable laboratory test that can successfully detect the progression of ALS. Taking this into consideration, an interesting approach related to graphene phononics was reported.[94] In this article, the shifts in Raman signals of bare graphene substrate and substrate treated with CSF from ALS and healthy patients were analyzed. The study resulted in the use of graphene's phono vibration-energies as sensitive measures of the composite dipole moment of the interfaced CSF, suggesting a potential way for ALS detection.

4.6. GBMs and Neural Stem Cell Technology

One common characteristic of the NDs, as described earlier, is the progressive loss of neurons. Neurons constitute a class of cells that cannot be renewed after injury leading to the severe symptoms that describe NDs. Although the presented sensing technology and the therapeutic approaches can lead to early detection and amelioration of the symptoms, they fail to address the lost neurons problem. Therefore, many studies have focused on alternative approaches, such as SCs, for neuronal regeneration. SCs are undifferentiated or partially differentiated cells with the unique ability to differentiate in various cell types, including brain cells. SCs' differentiation is determined by several factors, among which are the cell–biomaterial interactions.[95] It has been shown that biomaterials with different composition, stiffness, and topography affect SCs' behavior through spatiotemporal dynamics and mechanosensory stimulation.[96] Based on this, GBMs were suggested as a biomaterial-based approach able to provide a tailored microenvironment regulating the adhesion, proliferation, and differentiation of the NSCs. For instance, 3D graphene foams (3D–GFs) have been studied for their effect on the differentiation of neural SCs. The stiffness[97] and the impact of collagen coating[98] were assessed in NSCs' responses using these scaffolds. From a mechanistic point of view, these scaffolds were also used to study the NSCs' proliferation through regulatory metabolic pathways, which revealed pathways related to PD.[99] In another study, 3D porcine acellular dermal matrix, mostly made of collagen type I, was used as a substrate on top of which rGO nanosheets were placed. This combination provided a 3D porous conductive scaffold that promoted the differentiation of mesenchymal SCs (MSCs) into neuronal cells with high protein and gene expression. The enhanced conductivity provided by the rGO layer enabled the attachment of the MSCs, keeping them in an active proliferation and differentiation state.[100] Even without using a substrate, GO nanosheets have also been shown to sustain mouse embryonic SCs' self-renewable properties instead of influencing their pluripotency through downregulation of vinculin.[101] Patterning of GBM substrates has a significant role in the differentiation of SCs. This was shown through a work where hierarchical structures on a GO-patterned substrate generated synergistic topographical stimulation of human NSCs, resulting in enhanced focal adhesion, integrin clustering, and neuronal differentiation. The substrate's effect on the human NSCs was attributed to the microgrooves and the "nanoroughness" of the surface. Notably, the differentiated human NSCs exhibited sodium current channels and action potentials without the use of chemical agents typically required for neurogenesis.[102] Except for the topography, stiffness, and roughness of GBM-based substrates, the electrical properties as well contribute significantly to the survival, proliferation, and differentiation of SCs. This was shown through a study where NSCs were used as a model to explore the alterations in membranes' bioelectrical properties.[103] This study demonstrated that the used graphene film can modulate the cells' membrane properties during critical development stages by increased firing action of potentials. This subsequently led to enhanced NSCs' differentiation, spine density, synapse proteins' expression, and synaptic activity.

Composite hydrogels based on graphene and polyurethane,[104] composite films based on graphene and PLGA,[105] graphene-based electrodes,[106] graphene, and cellulose fibers,[107] 3D brain cortex-mimetic graphene-based scaffolds[108] and bioresorbable elastomeric scaffolds[109] are a few more examples of GBMs were the mechanical properties,[104] topography,[109] and external electrical stimulus[106] were used to control the NSCs' behavior. A more comprehensive discussion on the effect of GBMs in NSCs' behavior can be found in the works of Xia et al.,[95] Akhavan,[110] and Zhang et al.[111]

5. Toxicity of GBMs

GBMs' attractive characteristics do not present themselves without any drawbacks, especially in terms of cytotoxicity. GBMs' toxic character is affected by several factors, among which are hydrophobic interactions, surface charge, surface chemistry, size, and morphology. For example, pure graphene's strong hydrophobic character leads to strong interactions with the phospholipidic cell membrane's lipid tails, resulting in subsequent integrity damage.[112] These interactions can also lead to phospholipids' extraction from the cell membrane, as was demonstrated in vitro using alveolar epithelial cells and macrophages.[113] In contrast, since hydrophobicity strongly affects the cytotoxicity of graphene, it can be assumed that a more hydrophilic derivative such as GO would not lead to a forced disruption of the cell membrane and consequent cell death. However, it was demonstrated that although GO does not interact electrostatically with neutral and negatively charged phospholipids (cell membrane phospholipids), it can still confer cell death, even without cell penetration, again due to hydrophobic interactions.[114] Size and morphology also play a significant role, and/or in combination with surface chemistry and/or other parameters can significantly alter the toxic character of GBMs. For example, the effect of GQDs on SH-Y5Y and primary cortical neurons was compared to reduced GQDs and nanosized GO (nano-GO) (≈5–20 nm).[90] This study showed that the increased carboxylic groups on the surface of GQDs significantly reduced the cytotoxicity of the cells treated with 20 μg/ml (≈100% viability for GQDs vs 35% for nano-GO and 40% for rGQDs) for 72 h. Similar results were also reported for GQDs on OLN-93, PC-12, primary oligodendrocytes/neurons,[93] and human keratinocytes (HaCaT) and 3T3 fibroblasts[63] for a concentration of up to 50 μg ml^{-1}. In contrast to the aforementioned results, two other studies presented contradictory data for the reduced GO and the nanosized GO.[90] In the first case, nanosized GO (10–40 nm) did not present any cytotoxicity on SH-SY5Y after 24 h for a concentration up to 200 μg ml^{-1},[62] whereas rGO nanoflakes of average size 170–220 nm demonstrated low cytotoxicity (up to 20%) on PC-12 cells after the 5th day of treatment.[30] In a different study where GO nanosheets (thickness: 2.23 and 4.20 nm, size: 615–814 nm, $z = -25{,}8$ mV) were used to treat mouse embryonic SCs, the biocompatibility studies demonstrated that the nanosheets are not toxic up to a concentration of 32 μg ml^{-1} after 48 h.[101] Concerning composites such as the presented GO/iron oxide and GO/graphitic carbon nitride, the data showed increased toxicity for concentrations higher than 2.5 μg ml^{-1} on SH-SY5Y,[47] and higher than 100 μg ml^{-1} on PC-12 cells,[65] respectively. In a different approach, a hydrogel-based composite consisting of GO functionalized with acetylcholine and poly (acrylic acid) showed no toxicity on PC-12 derived neurons at a concentration of up to 200 μg ml^{-1} for 7 days and almost 100% viability on primary rat cortical neurons for 14 days.[115] From the studies mentioned earlier and from other reported works,[116,117] it is evident that the biological compatibility data for GBMs are controversial, suggesting that additional parameters need to be considered when assessing the cytotoxic profile of GBMs. A few of these parameters are dose, time, and used cell line/primary cells. The in vivo assessment of GBMs is even more complexed since their intrinsic physicochemical properties are altered after their administration in the human/animal body. The biological media's ionic strength, absorption of various biomolecules (mainly proteins), reticuloendothelial system clearance, biodegradation, biodistribution, anatomical and physical barriers such as the BBB and the administration site (e.g., oral, nasal, and intravenous) are several parameters that significantly affect GBMs biocompatibility. For a more comprehensive review on the safety/cytotoxicity of GBMs, the readers are directed to the work of Fadeel et al.[116]

6. Conclusions and Outlook

GBMs' applications in neurodegenerative diseases have significantly grown in the last few years. The excellent mechanical, electrical, thermal, and chemical properties of this new class of materials gave rise to numerous biomedical applications leading to enhanced therapeutic outcomes. Despite all this progress, unresolved challenges in the synthetic methods, functionalization strategies, and biocompatibility of the GBMs need to be addressed before their clinical translation. More specifically, fabrication methods using green chemistry (excluding hazardous chemicals) that allow the significant scale-up of GBMs for all the needed applications is one of the limitations that need to be mastered. In addition, surface functionalization with simple and cost/eco-friendly procedures that improve GBMs' properties (e.g., colloidal stability) and biocompatibility is also crucial in the biomedical field. Even though several studies have shown the biocompatibility of this class of materials with human cells and tissues further, long-term studies on their in vivo toxicity and fate need to be conducted. In addition to these limitations, several other biological constraints such as the noncontrolled biodistribution, short circulation times, and immunogenicity, which characterize most biomedical nanomaterials, should also be considered. Moreover, anatomical barriers such as the blood–brain barrier and cell internalization mechanisms render even more complicated the use of GBMs in the brain.

As mentioned throughout the article, GBMs are characterized by several intrinsic characteristics that render them attractive candidates for neuromodulatory treatments. However, the controversial data concerning their toxicity presented through the years and the limitations aforementioned make it difficult to use these materials as "an established way" for NDs' treatment. One reason for these contradictory data lies in insufficient knowledge of these materials' interactions with living organisms and how these interactions alter their intrinsic characteristics. Acknowledging these limitations, the graphene flagship project has answered several questions on the safety of GBMs and their interactions with living organisms, carving the way for a most robust use.[116] From a biological point of view, the unknown causes of NDs and the little knowledge in the cellular mechanisms and signaling pathways governing these diseases constitute one more impediment for their diagnosis and treatment. These mechanisms and pathways are even more complicated in the case of NSCs due to the multifactorial dependence on their behavior. Thus, it is evident that the "material's knowledge" is not enough to make GBMs a "first-line" treatment approach. In the next few years, the significant focus should be given to single cells' interactions with GBMs. A pipeline connecting

materials properties (e.g., size, shape, morphology, and charge) and cell responses should be made. Improving the understanding of NDs pathology and mechanisms is also crucial since it will allow a better material design to overcome the current limitations. The development of in vitro models that accurately mimic the in vivo conditions will bridge the gap on the different toxicity data among in vitro and in vivo studies, allowing the further development of GBMs. One more significant aspect that should be taken into consideration while developing GBMs is their functionalization. It has been demonstrated that nonfunctionalized GBMs' limitations overcome their advantages, making functionalization an integral part of their future use. Although many studies focus on GBMs' functionalization, there is still insufficient data on how each functionalization affects GBMs' properties inside a biological microenvironment. This knowledge is of consequential importance, especially for the GBMs' use inside a complex microenvironment like the cerebral one. The earlier described studies show that a lot of work is still needed in the material and the biological microenvironment aspects until an appropriate GBM for NDs is developed.

Summarizing, in this review, several GBMs' functionalization strategies that improve the suitability of GBMs for NDs, followed by specific examples in the five most studied NDs, have been discussed. The notable focus was given in systems both for sensing and treatment that have been presented in the last 5 years. Moreover, the uses of GBM in SC technology and several toxicity issues are also described.

Conflict of Interest

The author declares no conflict of interest.

Keywords

graphene oxide, graphene quantum dots, graphene-based materials, neurodegenerative disorders, stem cell technology

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