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Review

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Emerging Frontiers of Graphene in Biomedicine

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Graphene is a next-generation biomaterial with increasing biomedical applicability. As a new class of one-atom-thick nanosheets, it is a true two-dimensional honeycomb network nanomaterial that attracts interest in various scientific fields and is rapidly becoming the most widely studied carbon-based material. Since its discovery in 2004, its unique optical, mechanical, electronic, thermal, and magnetic properties are the basis of exploration of the potential applicability of graphene. Graphene materials, such as graphene oxide and its reduced form, are studied extensively in the biotechnology arena owing to their multivalent functionalization and efficient surface loading with various biomolecules. This review provides a brief summary of the recent progress in graphene and graphene oxide biological research together with current findings to spark novel applications in biomedicine. Graphene-based applications are progressively developing; hence, the opportunities and challenges of this rapidly growing field are discussed together with the versatility of these multifaceted materials.

Keywords: Graphene, graphene oxide, nanosheet, functionalization, biomedicine, nanobiotechnology

Introduction

Graphene, a single layer of carbon atoms arranged in a hexagonal lattice, has been attracting the attention of scientists since it was first isolated from bulk graphite [37]. It is a true two-dimensional carbon allotrope with a fully π-conjugated network that exhibits many unique physical and chemical properties [50, 54]. Graphene is one of the most promising candidates for biomedical applications owing to its intrinsic properties, simple molecular design and compatibility with other nanomaterials [22]. Based on the fascinating actions of similar carbon-based nanomaterials such as carbon nanotubes and fullerenes, an increasing number of studies have explored the potential of graphene materials for various biomedical applications [3, 15, 26, 54]. In the first biomedical application, graphene oxide (GO) is used as a drug carrier to selectively kill cancer cells [30].

Graphene offers a large thin surface with high mechanical strength and electrical conductivity (Fig. 1), laying the foundation for novel DNA sequencing technology and biosensors for glucose, hemoglobin, and cholesterol levels [42, 48, 53]. Progress in graphene synthesis and functionalization further facilitates exploration the of potential application of graphene in drug and gene delivery and tissue engineering [12]. The versatility of graphene and GO surfaces provides immense possibilities for covalent linkage with chemically diverse biomolecules [11, 28]. The use of functionalized graphene with triggered release by external stimuli, such as pH change, magnetic fields, or near-infrared radiation, is fast emerging as a new drug delivery system [12]. Graphene is also used for biosensing through fluorescence quenching [6], enhancement of cell growth and differentiation [24], and graphene-assisted laser desorption/ionization for mass spectrometry [5]. This review highlights the utility of graphene nanomaterials in biomedicine together with research prospects and challenges in this rapidly developing area.

Graphene and Graphene Oxide (GO)

Graphene is the finest and most durable monolayer that can exist freely [12]. Individual graphene sheets were first separated from graphite when Scotch tape was used to peel

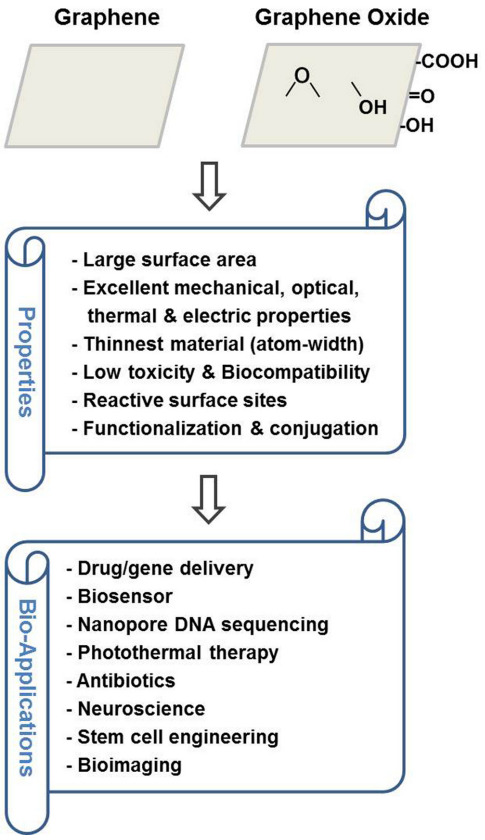

Fig. 1. Graphene and graphene oxide (GO) have excellent physico-chemical properties and huge potentials for bioapplications.
Schematic diagrams of the graphene nanosheet and graphene oxide are shown together with their unique properties suitable for various biomedical applications. Graphene, a single layer of carbon atoms arranged in a honeycombed lattice, is depicted as a planar structure that can accommodate various functionalities on its surfaces. Graphene oxide has uncharged polar groups on the basal surface (outside the plane) and charged hydrophilic peripheral groups (-O- and -OH within the plane). Graphene is hydrophobic, whereas GO and reduced GO are hydrophilic. Graphene family materials have a high surface-to-volume ratio, excellent electrical conductivity, robust mechanical strength and flexibility, unparalleled thermal conductivity, remarkable biocompatibility, and ease of functionalization.

off multiple layers [37]. Through this process known as mechanical exfoliation, the thinnest monolayer of single carbon atom thickness is obtained. The thinness and stability of graphene permits interjection of molecules of interest between the graphene layers, giving rise to different sandwiched molecules. Researchers are developing ways to revolutionize the production of high-quality graphene sheets. The most common way to create graphene is by chemical deposition, in which carbon atoms are extracted from a carbon-rich source by reduction [51]. Other methods for preparing graphene include growth from a solid carbon source, sonication, cutting open carbon nanotubes, and reducing carbon dioxide or graphite oxide [7]. However, the quality of the graphene sheets produced by current methods falls short of the theoretical potential and requires time to perfect. GO is a novel two-dimensional nanomaterial produced by oxidizing natural graphite. GO has good biocompatibility and low toxicity for various biomedical applications, including drug and gene delivery [3]. One of the advantages of GO is its easy dispersability in water and in different matrixes, due to the presence of the oxygen-containing groups at the sheet edges and within the sheet planes (Fig. 1). Functionalizing GO can change its properties; thus, modified GO can be used in many biological applications.

Drug and Gene Delivery

Drug and gene delivery applications exploit such properties as large specific surface area, π–π stacking, and hydrophobic interactions to load small molecule drugs (Fig. 1). The negative surface charge coming from a free π electron cloud is utilized to condense genes and anticancer drugs [52]. Additionally, the large flat surface of graphene allows for high-density biofunctionalization by modifying both the covalent and noncovalent surfaces. GO can be linked to hydrophilic polymers for further improved stability in biological solutions [31]. Various studies on the *in vivo* behavior and bioactivity of graphene demonstrate that it is a promising material that could replace existing materials for drug delivery [5, 50]. In one study, 6-armed polyethylene glycol (PEG) was covalently linked to the surface of GO sheets to form biocompatible GO-PEG. GO-PEG is nontoxic to human lung and breast cancer cells in cell viability assays [49]. In addition, a widely used chemotherapeutic paclitaxel (PTX) linked to GO-PEG through hydrophobic interactions as well as π-π stacking produces a GO-PEG/PTX nanocomplex with a relatively high PTX loading capacity. This complex also exhibits high cellular toxicity to human lung and breast cancer cells over a wide range of PTX concentrations and times, as compared with PTX alone [49]. The PEGylated GO drug delivery system has potential biomedical applicability.

Functionalized GO sheets can also be used for efficient gene delivery. Graphene functionalized with polyethyleneimine (PEI) has been widely used as a nonviral vector owing to its tight electrostatic interactions with the negative charge of nucleic acids [43]. It is also easy to chemically modify PEI to increase transfection efficiency and cell selectivity. Moreover, the relatively high toxicity of PEI in human cells

can be reduced by coupling to GO [8]. In the latest study, a photothermally controlled gene delivery vector was developed by linking reduced graphene oxide (rGO) and low-molecular-weight branched polyethyleneimine (BPEI) *via* a hydrophilic PEG spacer [16]. This rGO-PEG-BPEI nanocomposite shows higher gene transfer efficiency compared with unmodified controls in PC-3 and NIH/3T3 cells, without observable cytotoxicity. Moreover, the rGO-PEG-BPEI nanocomposite demonstrates enhanced gene transfer efficiency upon NIR irradiation. This implies that GO-PEG-BPEI mediates photothermally controlled gene delivery that can achieve spatial and temporal site-specific gene delivery [16]. In another study, amine-terminated PEGylated GO is successfully used to deliver a large amount of proteins, such as ribonuclease A and protein kinase A, to the cell cytoplasm with no loss in biological activity [44]. Moreover, bone morphogenetic protein-2 is successfully loaded onto a Ti substrate using positively ($GO–NH^{3+}$) and negatively ($GO–COO^{-}$) charged GO nanosheets [2]. Most of these studies highlight the potential of graphene as a novel delivery platform for drugs and genes. However, more study is needed to demonstrate its potential *in vivo*.

Sensitive Platform for Biosensing

Coupling graphene with molecular interaction represents a new paradigm in nanobioscience toward the development of novel detection tools. GO can be used as a sensitive and selective DNA detection platform. The interaction between GO and dye-labeled single-stranded DNA quenches dye fluorescence, while the presence of target DNA leads to binding of dye-labeled DNA with the target, which releases the DNA from GO, and restores dye fluorescence [50]. Spectroscopy studies suggest that single-stranded DNA is promptly adsorbed onto graphene and forms strong molecular interactions. In addition, single-stranded DNA constrained on a graphene surface is protected from DNase I [45]. Constraining the DNA probe on the graphene surface improves the specificity of the response to complementary DNA. A fluorescent single-stranded DNA probe exhibits minimal background fluorescence because of the extraordinarily high quenching efficiency of GO, whereas strong emissions are observed when it forms a double helix with specific targets, leading to a high signal-to-background ratio [46]. Importantly, the large planar surface of GO allows simultaneous quenching of multiple DNA probes labeled with different dyes, leading to a multicolor sensor for detecting multiple DNA targets in the same solution [14]. A GO-based multicolor fluorescent DNA nanoprobe allows rapid, sensitive, and selective detection of DNA targets in homogeneous solution, and a GO-based sensing platform is suitable for detecting a range of biomolecules, including proteins [9]. Small molecule-DNA and protein-DNA interactions require similar evaluation to take advantage of the graphene-DNA interactions. Future assays for direct detection of unamplified target molecules with high sensitivity are crucial. A tool that combines DNA hairpin probes with other molecular devices, such as ribozymes and aptamers [17], will lead to robust and sensitive, high-throughput diagnostic methods. An RNA aptamer against a prostate cancer biomarker, such as prostatic acid phosphatase, could be effectively used for this purpose [18].

One of the most ambitious biological applications of graphene is rapid, inexpensive electronic DNA sequencing [10]. Accurate, rapid, and affordable genome sequencing is widely regarded as the next frontier in biomedical science. It will eventually revolutionize personalized medicine, enabling physicians to examine genetic makeup and tailor therapies to an individual's genome. The idea behind graphene-based DNA sequencing is to integrate thin (0.34 nm thick) graphene layers as nano-size electrodes into a nanopore, such that DNA can be drawn through miniscule pores in the graphene (Fig. 2). High-resolution nanopore sequencing allows DNA to be analyzed one nucleotide at a time to more clearly, quickly, and accurately detect repeated, omitted, or mutated nucleotides [40]. Despite the challenges in fabrication and handling, graphene biosensors have a bright future.

New Frontiers for Neuroscience

In a developing nervous system, neuronal self-organization is mediated by orchestration of a multitude of mechanical, chemical, and electrical signals. However, little is known about the spatiotemporal regulation of this developing network, particularly in humans, mainly due to a lack of non-invasive methods to quantify the process [34]. Graphene has recently being used to fabricate a new type of microelectrode to assess the intricate circuitry of the brain [23]. Real-time high-resolution optical imaging as well as electrophysiological recording of neuronal locations and firing patterns are crucial in elucidating how individual neural circuits operate in neurological disorders. To date, either high-resolution optical images or electrophysiological data were obtained separately, because traditional opaque metallic microelectrodes blocked the investigator's view and created shadows. However, a transparent, flexible graphene electrode enables simultaneous optical imaging and

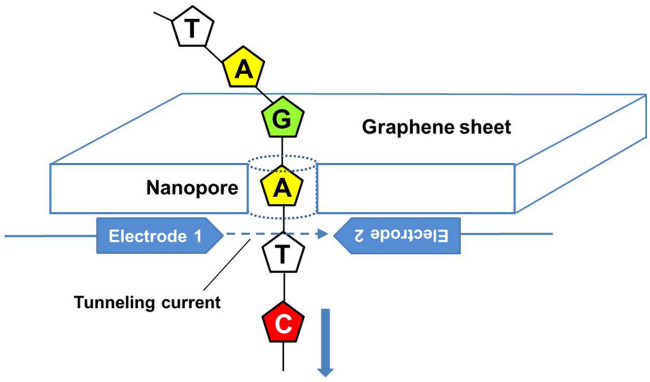

Fig. 2. Graphene-based nanopore sequencing.
Passing a strand of DNA through a tiny hole (~2 nm) in a sheet of graphene generates tunneling current, which is measured for identification of the nucleotide base that happens to be blocking the hole at that moment. Measuring the way the current changes as the DNA strand is pulled through the hole provides a direct reading of the nucleotide sequence. This third-generation sequencing is expected to overcome the limitations of next-generation sequencing, which is prone to random errors during amplification.

electrophysiological recording [23]. This is important because no single technology provides simultaneous high spatial resolution and temporal resolution. In this study, rat hippocampal slices are imaged with calcium using confocal and two-photon microscopy, while simultaneously measuring electrophysiological data. The temporal details of seizures and seizure-like activity were observed at the cell level with very high resolution. Graphene microelectrodes could have wider application as cardiac pacemakers or peripheral nervous system stimulators. These probes could also increase the longevity of neural implants because of the nonmagnetic and anti-corrosive properties of graphene. On the other hand, the nonmagnetic characteristics of graphene allow for safe, artifact-free reading of magnetic resonance images, unlike metallic implants. Graphene is inherently a low-noise material, which is important for high signal-to-noise ratio recording of neural circuit(s). Another advantage of graphene is that it is flexible, and thin flexible electrodes can be manufactured to encompass neural tissue. A graphene electrode array device, which can be implanted on the rodent brain surface for high-resolution neurophysiological recording, was recently developed [38]. Transparent graphene-based neural micro-electrodes could allow for simultaneous optical imaging and electrophysiological recordings together with optogenetic regulation of the underlying neural circuits.

Graphene also exhibits excellent biocompatibility and significantly promotes neurite sprouting and outgrowth of mouse hippocampal cells [27]. Neurite numbers and average neurite length on graphene are significantly enhanced after cell seeding, as compared with polystyrene substrates. Especially on day 2 of the neural development period, graphene substrates efficiently promote neurite sprouting and outgrowth to the maximal extent, suggesting the potential of graphene as a neural interface [27]. The reported protective role of GO sheets and their protein-coated surfaces on amyloid fibrillation suggests that graphene can scavenge amyloid monomers and offer protection from neurodegenerative diseases [32].

Antibacterial Applications

Graphene can prevent the formation of pathogenic and corrosive microorganisms and even kill bacteria, making it a potential antimicrobial coating for surgical equipment or other surfaces [41]. Bacterial viability decreases after exposure to graphene materials [29]. One of the two possible mechanisms is the physical damage that often occurs upon direct contact of bacterial membranes with the sharp edges of graphene sheets. Oxidative stress also seems to be involved [13]. Graphene materials with a small particle size and those in an oxidized state appear to have greater antibacterial effects. Moreover, microbial growth after exposure to the graphene-linked nanocomposite such as poly N-vinyl carbazole (PVK)–G leads to greater bactericidal and bacteriostatic effects, as compared with exposure to pure PVK or pure graphene solutions. Graphene also inhibits buildup of the biofilm. Consistent with the above findings, ~80% inhibition of biofilm formation was observed in the PVK–G thin film group, as compared with almost no inhibition from the PVK and unmodified surfaces [41].

Graphene-based composite materials such as chitosan–polyvinyl alcohol nanofibrous scaffolds containing graphene were explored for wound healing potential [31]. The samples containing graphene accelerated wound healing more rapidly than other groups, and it is thought that the free electrons of graphene inhibit cell division and prevent microbial multiplication. Subsequent antibacterial experiments using *E. coli*, *Agrobacterium*, and yeast confirm that growth of the prokaryotic cells (*E. coli* and *Agrobacterium*) is inhibited by graphene, whereas no growth inhibition occurs in eukaryotic (yeast) cells. Antibacterial activity and no significant cytotoxicity toward eukaryotic cells, including human fibroblasts [33], suggest that graphene composites could potentially be used in clinical applications.

Stem Cell Platform

Graphene and GO can support stem cell growth. They also reportedly act as pre-concentrators for chemicals, proteins and growth factors on their surface to promote cell differentiation [47]. Graphene and GO platforms have been investigated to proliferate and differentiate induced pluripotent stem cells (iPSCs), which hold great promise as a cell source for regenerative medicine [4]. In comparison with the glass surface, iPSCs cultured on the GO surface adhered and proliferated at a faster rate. Moreover, graphene favorably maintained the iPSCs in the undifferentiated state, whereas GO accelerated the differentiation. Therefore, the different surface characteristics of graphene and GO appear to govern the behavior of iPSCs, which can be utilized for iPSC culture and differentiation [4]. The differentiation potentials of graphene and GO were also studied on human mesenchymal stem cells (hMSCs) and pre-osteoblasts that were allowed to differentiate into osteoblasts [35]. Graphene and GO promoted cell adhesion, proliferation, and differentiation of hMSCs compared with other surfaces, including glass. Graphene accelerated differentiation of hMSCs cultured in osteogenic medium at a rate comparable to the presence of BMP-2 on uncoated surfaces. The exact mechanism of graphene in stem cell differentiation remains unclear. However, it is generally hypothesized that the surface characteristics, including mechanical stiffness, nano-topography, and large absorption capacity, influence the molecular or signaling pathways that govern the fate of stem cells [2].

Toxicity and Biocompatibility

One critical issue to be resolved before graphene finds further application in biomedicine is the potential short- and long-term toxicities [36]. Inorganic nanocrystals such as quantum dots usually contain heavy metals and have been extensively studied for their biological toxicities [19, 20]. Although graphene and GO appear to be relatively safe in terms of elementary composition, confirmation of their *in vivo* behaviors as well as *in vitro* cellular toxicity is of great importance. Studies frequently show that mammalian cell viability decreases slightly after exposure to graphene materials, as they induce oxidative stress and apoptosis [39]. The most hydrophilic forms of graphene penetrate cellular membranes but are generally less toxic than that of hydrophobic forms, which accumulate on cell membrane surfaces. Few studies have demonstrated graphene–cell interactions and the *in vivo* efficacy of these carriers in animals. The *in vivo* fate of graphene carriers after local/systemic administration needs further attention. Future emphasis on the mechanisms of clearance and toxicity as well as tissue distribution is required to realize their true potential. Furthermore, different graphene-family materials exhibit markedly different physicochemical and biological properties and surface functionalities. Correlations between these properties and biological function will decide opportunities and limitations for each biomedical application. Clarity in the graphene-based materials and the specific characterization protocols are also needed to avoid generalization of the capabilities and limitations [1]. These may have a significant impact on true safety, toxicity, and clearance after administration. Knowledge of the *in vivo* behavior of different graphene-based materials will eventually expand their biomedical applications.

In conclusion, graphene is a rapidly rising star in nanobiotechnology. It is possibly the most versatile material, and scientific research on its application has grown exponentially in the last decade. Despite its short history, the excellent physicochemical and biological properties are of interest to biomedical scientists in recent years. Progress to date supports the use of graphene for a wide variety of biomedical applications, including neuroscience, stem cells, drug and gene delivery, antibiotics, and biosensing and imaging. Atom-width graphene sensors could provide unprecedented insights into brain structure and function, as well as correlations between different neural circuits and neurological disorders. Graphene also provides therapeutic modes to treat diseases. However, the field of graphene biotechnology is in its infancy, and concerns about the use of carbon nanomaterials such as graphene in humans are justified. Studies on the biocompatibility as well as long-term effects of different graphene forms are required in the near future. Nevertheless, appropriate therapeutic and other uses for graphene materials hold immense promise.

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