Recent Advances in the Characterized Identification of Mono-to-Multi-Layer Graphene and Its Biomedical Applications: A Review

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Abstract: The remarkable mechanical, electrical, and thermal capabilities of monolayer graphene make it a wonder substance. As the number of layers in graphene flakes increases to few-layer graphene (number of layers ≤ 5) and multi-layer graphene (number of layers ≤ 10), its properties are affected. In order to obtain the necessary qualities, it is crucial to manage the number of layers in the graphene flake. Therefore, in the current review, we discuss the various processes for producing mono- and few-/multi-layer graphene. The impact of mono-/few-/multi-layer graphene is then assessed with regard to its qualities (including mechanical, thermal, and optical properties). Graphene possesses unique electrical features, such as good carrier mobility, typical ambipolar behaviour, and a unique energy band structure, which might be employed in field effect transistors (FETs) and utilized in radio frequency (RF) circuits, sensors, memory, and other applications. In this review, we cover graphene’s integration into devices for biomolecule detection as well as biomedical applications. The advantages of using graphene in each situation are explored, and samples of the most cutting-edge solutions for biomedical devices and other applications are documented and reviewed.

Keywords: 2D nanosheet; nanomaterial; layered material; monolayer; medical imaging

1. Introduction

The sixth element is truly fascinating due to its allotropic forms [1,2]. Its allotropes are as soft as graphite [3,4] and as hard as diamond [5]. Graphite is a three-dimensional structure that is made up of stacking multiple one-atom-thick layers, which are formed from strong sp² hybridized carbon atoms arranged in a hexagonal lattice [6,7]. This two-dimensional monatomic thick crystal structure consists of single atomic sheet of graphite, and is called “graphene” [8]. Recently, graphene, an allotrope of carbon, has become a hot topic of research due to its good physico-chemical properties [9,10]. When stacked, graphene forms different types of graphene, such as monolayer graphene [11], few-layer graphene [12] and multilayer graphene [13]. The properties of graphene are dependent on the number of stacked layers; for example, monolayer graphene has better properties than few-layer graphene and multilayer graphene [14,15].

Scientists described the theoretical existence of single layer graphene more than 80 years ago [16]. Then, the practical existence of two-dimensional graphene was considered physically impossible [17]. However, in 2004, Geim et al. isolated single-sheet graphene via the scotch tape method and demonstrated its properties experimentally [18]. This was the first time researchers came to know about the remarkable properties of graphene [19]. Since then, there has been an exponential rise in studies of graphene-based materials to determine the various applications of their properties, such as in biomedical applications [20,21].

Due to the expert attention paid to graphene-based materials, high-performance materials were successfully produced. However, achieving these high performance levels.
involves various challenges, especially in relation to monolayer graphene [22,23]. One of these challenges is the synthesis of monolayer or few-layer graphene in bulk with high purity, which is an extremely difficult process [24]. Other challenges include the restacking of monolayer graphene in few-layer and multi-layer graphene [25,26]. This restacking results in the decreased performance of the graphene-based devices. Therefore, the control of lateral size and aggregation states, in addition to the process of the oxidation of graphene in graphene oxide, is essential for developing graphene-based high-performance devices [27,28].

After achieving the synthesis of monolayer graphene, which exhibits good characteristics for a roadmap of graphene-based devices, the characterization of the order of stacking in graphene became a subject of interest for researchers [29,30]. These characterization techniques include atomic force microscopy (AFM) [31], Raman spectra [32], Raman mapping [33], and Transmission electron microscopy (TEM) [34]. Among these techniques, Raman spectra and TEM are methods frequently used to determine whether the synthesized material is monolayer graphene, few-layer graphene, or multi-layer graphene [32,34]. After characterization, the graphene material can be used for different applications, such as biosensors [35], tissue engineering [36], drug carriers [37], and other biomedical applications. In this review, we discuss the characterized differentiations of graphene and their particular advantages in biomedical applications based on the number of layers.

2. Production of Mono-, Few-, and Multi-Layer Graphene

Graphene, especially monolayer graphene, has received a great deal of attention since 2004 due to its good mechanical, electrical, and thermal properties [18]. However, the synthesis of monolayer graphene is extremely difficult and expensive [38,39]. Therefore, synthesizing few-layer graphene or multi-layer graphene is also a subject of interest [40]. A general scheme represents monolayer, few-layer, and multi-layer graphene (Figure 1). Mono-, few-, and multi-layer graphene can be synthesized using various methods, including micromechanical exfoliation [41], chemical vapor deposition [42], and chemical methods such as oxidizing graphene into graphene oxide [43] and then reducing it chemically [44] or thermally [45]. A few methods provide high-quality, large-scale few-layer and multi-layer graphene but small amounts of monolayer graphene, such as the chemical method; however, the purity and defect density remain matters of concern [46,47]. Similarly, large-scale monolayer graphene can be synthesized by chemical vapor deposition, but purity still is a topic of concern [48]. The various methods for producing mono-, few-, and multi-layer graphene are as follows.

![Figure 1. Schematic overview of monolayer, few-layer and multi-layer graphene.](image)

**2.1. Synthesis of Few- to Multi-Layer Graphene**

2.1.1. Exfoliation of Graphite

Graphite is an abundant material and well known for its lubricating properties [49,50]. However, it has poor mechanical, electrical, and thermal properties. Graphite can be exfoliated into different types of graphene, but we must first overcome the weak van der Waals forces which hold the different types of graphene into graphite [51]. A general scheme of the process for obtaining different layers of graphene is shown in Figure 2.
There are several ways to overcome these weak interactions, and the most promising among them is the sonication of graphite in different solvents; however, the yields of multi-layer and monolayer graphene are very poor in this process.

Another promising strategy to obtain graphene from graphite involves oxidizing graphite by various methods, such as Hummer’s method, and reducing it chemically or thermally to obtain a large-scale yield. However, the redox results of graphite into graphene mostly provide high yields of few-layer graphene or multi-layer graphene.

Another method of producing few- and multi-layer graphene involves the exfoliation of graphite via graphite intercalation. Different types of chemicals can be inserted to graphite interlayer space, thereby increasing the interlayer distance of adjacent graphene sheets in graphite. This phenomenon also changes the properties of graphene, since the increase in interlayer spacing affects electronic coupling between adjacent graphene sheets in graphite.

Another way to exfoliate graphite into few- and multi-layer graphene is via ball milling. This is a way of exfoliating graphite via mechanical exfoliation. Ball milling has been extensively used in the past to reduce the particle size of a material. Scientists thus propose ball milling as a way to mechanically exfoliate graphite in small-size nano-graphite, increasing the mixing time to obtain few- or multi-layer graphene. Thus, ball milling is a promising technique for exfoliating graphite into graphene. The advantage of using ball milling to produce graphene is its low production cost, its easy handling, and its ability to produce graphene at large scale.

The plasma synthesis method is another significant way to produce graphene with few-to-multiple layers. Microwave plasmas produced by surface waves at a stimulation frequency of 2.45 GHz and under atmospheric pressure conditions were successfully used to produce highly structured and stable self-standing graphene sheets.
successfully used to produce highly structured and stable self-standing graphene sheets [16]. There were also investigations into how the addition of hydrogen affects the density of the carbon precursor (C2, C) and the structural soundness of synthetic graphene sheets. Changes in the sp3/sp2 ratio and the C2 and C number densities were shown to be correlated [59]. Microwave-driven plasmas were used to control oxygen functions and the sp2/sp3 carbon ratio (~15) to a high degree [60].

2.1.2. Synthesis of Monolayer Graphene

(a) In 2004, for the first time, Geim and Novoselov developed a method of synthesizing graphene using micromechanical cleavage as “scotch tape” via mechanical exfoliation [18]. This was for the first time in history that any scientist experimentally synthesized monolayer graphene. After synthesizing the monolayer graphene, these scientists further demonstrated its outstanding properties [61]. However, due to the uneven thickness of the graphene flakes and its high production costs, the mechanical exfoliation method was not suitable for the mass production of graphene that might be used to study graphene-based devices.

(b) Another method of producing monolayer graphene is the chemical vapour deposition method [62]. Monolayer graphene can be grown epitaxially on a silicon carbide substrate, and can be used for various applications, such as transistors. The size of the monolayer graphene grown depends on the size of the silicon wafer. The surface of the silicon wafer also influences the properties of the synthesized monolayer graphene.


The properties of graphene depend on a number of internal factors (such as the number of graphene layers stacked in a graphene flake) and external factors (such as temperature); these factors affect the final performance of devices based on graphene flakes. It is well known that monolayer graphene exhibits extremely good mechanical, thermal, and electrical properties, and that these properties decrease as the number of layers increases—such as from monolayer graphene flakes to few-layer and multi-layer graphene flakes. The sections below describe the effects of these factors on the mechanical, thermal, and optical properties of mono-, few-, and multi-layer graphene.

3.1. Mechanical Properties of Mono-, Few-, and Multi-Layer Graphene

Figure 3 shows the effect of the number of layers in graphene flake on mechanical properties (such as fracture stress and fracture strain). As expected, it was found that the mechanical properties decrease as the number of layer increases from monolayer to few-layer and then to multi-layer graphene. However, these changes in mechanical properties are not significant, as shown by Zhang et al. [14]. It is clear that the fracture strain and tensile strength are not significantly affected by the transition from few-layer graphene to multi-layer graphene. This is due to the dominance of the “nano effect” of graphite as the number of layers increases from few-layer to multi-layer graphene. Zhang et al. [14] further report that the mechanical properties are significantly affected by temperature, which is an external factor. The studies show that, as the temperature increases from 300 K to 2000 K, the tensile strength decreases significantly from 125 GPa to around 43 GPa. This fall in tensile strength is due to the softening of the structures. In addition, it is well known, and was demonstrated by Zhang et al [14], that the atoms of graphene undergo severe movement at higher temperatures, leading to such reductions in the mechanical properties. Similarly, fracture strain (67%) and Young’s modulus (23%) drop significantly as the temperature increases from 300 K to 2000 K [14].
3.2. Thermal Properties of Mono-, Few-, and Multi-Layer Graphene

The coefficient of thermal expansion (CTE) was studied as a function of temperature and the number of layers stacked in graphene flakes. It was found and demonstrated by Mag-isa et al. [15] that thermal properties such as CTE are highly dependent on internal factors (the number of layers in the graphene flake) and external factors (temperature) (Figure 4). For example, it was noted that, as the temperature increases from 20 °C to 140 °C, the COE is affected. This was expected, due to increase in the thermal kinetics of the processes as the temperature increases. Similarly, the thermal properties are affected by increasing the number of layers, as the thermal properties of monolayer graphene differ from those of few- and multi-layer graphene [12,15]. It is interesting to note that the results for few-layer graphene are placed between those of monolayer graphene and multi-layer graphene, as expected. Moreover, all of the values of CTE studies reported by Mag-isa et al. [15] and other researchers are negative. A negative CTE is generally regarded as a non-close-packed system with directional interactions, such as ice or graphene.

Figure 3. (a) The fracture stress and (b) fracture strain of mono- to few- to multi-layer graphene. Reproduced from [14] with the permission of Elsevier.

Figure 4. The thermal properties of the graphene-based flakes: (a) coefficient of thermal expansion against temperature; (b) coefficient of thermal expansion against mono-, few-, and multi-layer graphene. Reproduced from [15] with the permission of Elsevier.
3.3. Optical Properties of Mono-, Few-, and Multi-Layer Graphene

By taking into account the surface conductivity ($\sigma$), the refractive index ($n$) and the extinction coefficient ($k$) of graphene can be determined. Knowledge of the frequency-dependent surface conductivity $s_G$, which can be determined from either a microscopic model or from experiments, is necessary for understanding the optical characteristics of graphene [63]. The surface conductivity ($\sigma_G$) of graphene is dictated by the high-frequency expression acquired from the Kubo model [64].

4. Determination of Mono-, Few-, and Multi-Layer Graphene

The determination of the number of graphene layers and defects is critical for tailoring its properties for intended applications, and involves microscopic as well as spectroscopic measurements.

4.1. Atomic Force Microscopy (AFM)

AFM is defined as high resolution microscopy with a resolution at the atomic scale. AFM can determine various features of a material, such as thickness, grain height, topological features, phase diagrams, and roughness. These features are then correlated with the properties and target applications of the material, such as graphene. Xu et al. [65] studied and employed the AFM technique to determine thickness of graphene flake. The corresponding histogram shows that the flake thickness ranges from 1.1 to 1.6 nm. By considering an interlayer distance 0.33 nm, a total of 4–5 layers can be estimated to be present in the graphene flake. It therefore falls into the category of few-layer graphene [65].

Chen et al. [66] demonstrated the use of AFM for determining the roughness of graphene flakes. The authors found that the pristine polymer forms a homogenous film with a mean roughness in the range of 2.2 nm. As the graphene is added to the polymer, the composites made of polymer-graphene also exhibit good film-formation properties, with a mean roughness of 3.1 nm [66]. Zhang et al. [67] also studied the early stages of atomic layer deposition (ALD) on epitaxial graphene (EG) via AFM (Figure 5). It can be seen that the EG-based sample shows flat topological features. The small surface roughness and very narrow distribution of height values can also be noticed. Fitted with a single, the Gaussian peak shows a full-width half-maximum (FWHM) of 0.2 nm. Similarly, different topological images of the specimen were shown as a function of different ALD cycles, increasing from 10 to 80 [68]. No significant differences can be seen in the topology images; the height values and Gaussian fit of FWHM remained practically the same as the number of ALD cycles increases. Gao et al. [69] also studied the thickness of graphene flakes with the help of the AFM technique. The authors state that the thickness of the graphene flake used in the work was in the range of 0.4–1 nm. This shows that the graphene flake was monolayer-to-few-layer graphene.

Yuan et al. [70] investigated the thickness of the graphene flake with the help of the AFM tool. The authors demonstrated the sheet-like topological feature of the graphene flake. They further investigated the flake thickness and its lateral dimension. It was found from the histogram that the graphene flake consisted mostly of monolayer graphene, while a low percentage of few-layer graphene and multi-layer graphene was witnessed. The lateral dimension was in the range of 2 to 10 µm, and therefore had very high shape anisotropy [70]. Finally, Bhuiyan et al. [71] investigated the graphene flake thickness with the help of AFM microscopy. From this study, the authors demonstrate the smooth, sheet-like topology of the graphene flake, which had a thickness of around 1 nm; this shows that the graphene flake used in the work is few-layer graphene [71].
The HRTEM images provided by the authors show exfoliated graphene sheets in which TEM and HRTEM. The TEM images show the wrinkled morphology of the graphene via HRTEM. This technique shows that, in the graphene flakes, the distribution of few-layer graphene [74]. Ding et al. [75] studied the number of graphene layers per graphene, the type of graphene obtained using an indium catalyst at 150 °C. On the other hand, Long et al. [73] examined the morphology of graphene flakes via TEM and HRTEM. The TEM images show the wrinkled morphology of the graphene flakes, indicating a stable structure. The HRTEM images of the graphene flakes show the stacking morphology of borophene on the reduced graphene oxide. Moreover, the investigation shows a good interaction between borophene and the reduced graphene oxide [73]. Navik et al. [74] investigated the morphology of few-to-multi-layer graphene via HRTEM. This technique shows that, in the graphene flakes, the distribution of few-layer graphene is around 70% and the remaining 30% belongs to multi-layer graphene. The HRTEM images provided by the authors show exfoliated graphene sheets in which the exfoliation process produces large transparent graphene flakes, which mostly belong to few-layer graphene [74]. Ding et al. [75] studied the number of graphene layers per graphene flake via HRTEM. The authors demonstrated that the graphene flake used in the work contains mono- and bi-layer graphene. Thus, a high degree of exfoliation was achieved in the work and the remarkable properties of the related materials were studied.
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Figure 6. (a–d) Typical TEMs of graphene nano flakes in different forms, synthesized by an indium catalyst at 150 °C. Reproduced from [72] (CC BY).

4.3. Raman Spectra

Raman spectra (named after Sir C.V. Raman from India) is a famous spectroscopy technique that provides information about the vibrational modes of molecules, etc. It is most prominently used in determining the number of graphene layers in a graphene flake. The graphene flake generally shows three characteristic bands, namely, the D-band, G-band, and 2D band [76]. The position of the 2D band is different for different types of graphene flake. It is around 2702 cm\(^{-1}\) for tri-layer graphene, and increases with the increase in the number of layers to as much as 2720 cm\(^{-1}\) for multi-layer graphene and 2725 cm\(^{-1}\) for bulk graphite [14]. Campanelli et al. [77] used Raman spectra to demonstrate the interaction of Yb\(^{3+}\) with monolayer and bi-layer graphene. Their paper demonstrates that there is a good interaction between Yb\(^{3+}\) and graphene flakes, as confirmed using Raman spectra. A shift of ~5 cm\(^{-1}\) to ~7 cm\(^{-1}\) was observed at different Raman bands in the presence of Yb\(^{3+}\), indicating a good interaction [77]. Wang et al. [78] demonstrated the use of the Raman spectra technique for identifying monolayer and bi-layer graphene. The authors reported on systematic and statistical tests of the signatures of the so-called buffer layer and its coupling with the epitaxial graphene layer via Raman spectroscopy. The findings show a coupling between graphene and buffer layer [78]. Silva et al. [79] demonstrated the different number of graphene flakes with mono-, few-, and multi-layer graphene via
Raman spectra (Figure 7). A statistical analysis of the different types of graphene flake with mono-, few-, and multi-layer graphene was presented. The protocol is based on the position of the 2D band, which is different for different type of graphene layers, as discussed above. The authors demonstrated that the Raman spectra is suitable for the statistical analysis of mono-, few-, and multi-layer graphene with relatively low costs [79].

Figure 7. (a) Representative Raman spectra of different graphene flakes varying from monolayer to few-layer to multi-layer graphene; (b) a plot of the experimental values obtained from the ratio intensity of the G-band to Raman signals from the Si substrate; (c) a magnified view of the (b) graph from monolayer to multi-layer graphene [79].

4.4. Raman Mapping

Raman mapping is known as a laser-based microscopic technique which is obtained via Raman spectra. Recently, Raman mapping has played a significant role in determining the homogeneity of the graphene flakes used for different applications. Huang et al. [80] studied monolayer graphene through Raman mapping at different temperatures of over 150 °C and 250 °C. The Raman signatures indicate the formation of defects in graphene, even at low temperatures. The Raman maps show that the ratio of intensity of the D to G bands was 1.85 and 4.01 for 150 °C and 250 °C, respectively, indicating higher defect density for the 250 °C sample [80]. Bouhafs et al. showed [81] the synthesis and characterization of large-area few-layer graphene by chemical vapor deposition. The Raman map used in this study shows clear ABA and ABC stacking in the studied few-layer graphene. Bleu et al. [82] studied few-layer graphene via Raman spectra and Raman mapping. The most significant peaks for the few-layer graphene studied through Raman spectra are the D, G, and 2D bands that are located around 1350, 1580, and 2700 cm⁻¹, respectively (Figure 8).
In addition, the intensity ratio of the D to G band can be used to estimate the defect density, and the intensity ratio of 2D to G reveals the number of graphene layers stacked. The histogram was estimated via Raman maps and few-layer graphene was found to be dominant in the sample [82]. Moreover, the Raman maps show the good homogeneity of the synthesized few-layer graphene, with low defect density.

Figure 8. Raman analysis of the synthesized few-layer graphene; (a) a Raman map of the ratio of intensity of the D-band to the G-band; (b) a Raman map of ratio of the intensity of the 2D band to the G-band; (c) Raman spectra of few-layer graphene; (d) the distribution of the number of graphene layers in few-layer graphene. Reproduced from [82] with the permission of Elsevier.

5. Biomedical Devices and Other Biomedical Applications

5.1. Use as a Biomedical Device

The anatomical characteristics of different layers of graphene are different. Mono-to-multi-layer graphene is highly sensitive and is used in sensor applications, including biomolecules, elements, gas, pressure, and electrochemical detection [83,84]. Due to its large surface area, electrical conductivity, biocompatibility, easy operation, tunable structural transformation, and excellent mechanical strength, layered graphene is used as a biomedical device for regenerative drugs [85,86], bio-mining [87] detection [88], and cancer cures [89].

On the other hand, there is a basic restriction on the use of monolayer graphene in device applications: that is, the light–graphene interaction is weak due to the atomic thickness of graphene. In particular, graphene exhibits a wavelength-independent absorption of 2.3% in the visible and near-infrared regions, which limit its further application in areas such as sensors and photodetectors [90]. In recent years, several approaches have been proposed to solve these problems, such as the angle-insensitive broadband absorption enhancement of graphene [91], and electrically modulating and switching the infrared absorption of monolayer graphene [92].
5.2. Use as a Sensor Device

Additionally, biomolecules with hydrophobic domains or systems, such as DNA strands or proteins, have a tendency to naturally adsorb on graphene [93]. Another simple way to modify graphene for the targeted immobilization of bio-receptor units is to functionalize it [94].

5.2.1. Nucleic Acids Sensor Device

Compared with devices made using bilayer and few-layer graphene, sensor devices made with functionalized monolayer graphene exhibit superior sensitivity, better repeatability, and stronger construction [95]. Monolayer-graphene-based FET devices are used as nucleic acid sensors with high selectivity and low detection limits [96,97]. In this device, the fixed graphene layers are determined by using 1-pyrenebutanoic acid succinimidyl ester (PBASE) as a link chemical and acquired a 10 pM detection limit (Figure 9). To recognize pesticides, more GFET detectors based on microfluidics were created [98].

Figure 9. Characteristics of G-FETs. (a) The relationship between PBASE’s molecular structure and the probe DNA. (b) PBASE is used to functionalize the graphene surface. A probe DNA (shown in orange) is immobilized by interacting with the PBASE. The probe DNA is then hybridized with the target DNA (shown in blue) and unrelated control DNAs (in green). (c) Graphs of the source drain current versus the constant source drain voltage (0.1 V) for different conditions of graphene. (d) A schematic showing the equivalent circuit made up of four parallel plate capacitors and a resistance (RL) linked in series and the sensing model of a G-FET. Reproduced from [97] (CC BY).

5.2.2. Mammalian Cell Sensor Device

For cell-sensing applications, layered graphene is used as an immobilizer for detecting cells or various pathogens. Recently, scientists developed few-layer graphene-based FETs with HER2-specific aptamers and the ability to detect very low concentrations of HER2-cells [98]. Moreover, the devices exhibited highly sensitive detection against breast cancer cells and SK-BK-3 cells at the single-cell level.

5.2.3. Monolayer Graphene and Immune Sensor Devices

The surface modifications of functionalized specific polymers improve the interfacial interactions between them when used as high-performing medical devices [99,100]. As evidence, when the monolayers of graphene are mixed with a bioactive immobilizer (biotinylated cholera toxin), they showed a remarkable response to the anti-CT test [101]. Additionally, pyrene-NTA-conjugated graphene-monolayer-based sensor devices can detect up to 4 pL−1 of anti-CT, which is also a very significant result for anti-CT approaches [102].
A high-sensitivity surface plasmon resonance (SPR) biosensor made of a monolayer of graphene, a four-layer MoS2, and a layer of gold was studied [103]. The maximal sensitivity of the sensor, 282°/RIU, was shown by computational models using the transfer matrix method (TMM), which was about two times more sensitive than the typical Au-based SPR sensor. Additionally, the Kretschmann-configured manufactured sensors were employed to detect okadaic acid (OA). To create a competitive inhibitory immunoassay, the okadaic acid-bovine serum albumin bioconjugate (OA-BSA) was mounted on the graphene layer of the sensors. According to the findings, the sensor had a very low sensitivity limit (LOD) for OA of 1.18 ng/mL, which is roughly 22.6 times lower than that of a traditional Au immunoassay. Figure 10 depicts the schematic diagram of the OA detection process (Figure 10).

![Figure 10. The flowchart for the method of detecting okadaic acid (OA). Reproduced from [103] (CC BY).](image)

5.2.4. The Development of Imaging Devices for Biomedical Purposes

Bio-imaging is an important aspect of regenerative medicine [104]. Imaging techniques use different layers of graphene (a single layer to multiple layers) for imaging contrast agents such as fluorescence/confocal imaging [105,106], Forster resonance energy transfer (FRET) imaging [106], coherent anti-Stokes Raman scattering imaging (CARS) [107–109], magnetic resonance imaging (MRI) [110], surface-enhanced Raman scattering (SERS) [111], ultrasound imaging [112,113], photoacoustic imaging [114], electron paramagnetic resonance imaging (EPRI) [115,116], and positron-emission tomography (PET) [117,118]. Among these techniques, a few, such as computer tomography (CT), PET, MRI, and ultrasound imaging, are well-established and for use in humans. These imaging techniques used to better understand and monitor the affected site of the body for therapeutic purposes. Additionally, there are two fundamental necessities for bio-imaging: (1) rapid, selective, and sensitive instruments, and (2) good, non-toxic, biocompatible, biodegradable imaging contrast agents that have good bio clearance and are capable of crossing the physiological body membrane barrier.

Currently, the most developed EPRI technique is used to detect and quantify various physiological parameters in a cancer-effected microenvironment in in vivo models, such as pO2 [119–121], pH [116,122], and oxidation situations [116,123]. Normally, for this method, TAM (triphenylmethyl radical) [124] or other compound chemicals [125] are used. In EPRI for localized oxygenation sensing, carbon-based ink is used [126]. CARS imaging technology is highly sensitive in providing signals to process images in in vivo, ex vivo, and in cell cultures, up to micrometer-level penetrations [127,128].
Mono-to-multi-layered graphene-based materials are used in the biomedical field as contrast agents [129,130]. The key benefit of this is that the processes are biocompatible, less toxic, and metal-free [131]. Mono-to-multi-layered graphene-based materials are mainly used in fluorescence imaging [132] where they are used in modified form, display layer, and surface-chemistry-dependent fluorescence [133]. To enhance the biocompatibility of mono-to-multi-layered graphene, a few common polymers are used, such as polyethylene mine [134], PEG (polyethylene glycol) [135–138], polystyrene [139], and polypeptides [140]. Recently, scientists shortened the varied imaging techniques of different layers of graphene used, and determined that various imaging techniques can be performed on one platform [141]. Therefore, we assume that, in the coming decade, different layers of graphene-based materials and their diverse morphology will play a significant role in biomedical applications and in determining the future of therapeutic approaches.

5.3. Use in Other Biomedical Applications

5.3.1. As a Delivery Carrier and in Treatment

Ligand-Based Drug Delivery

Most interferences with drug-delivery systems are due to the presence of many proteolytic enzymes in the cytoplasm of a cell. Graphene and its derivatives are used as drug carriers or carriers that bypass those interfering enzymes. Its properties make it a promising carrier for biomedical applications; for example, COOH and OH are the main group ligands of GO that are acceptable for binding to various organic molecules, such as proteins, DNA, and many polymers. This ligand makes it biocompatible, cell selectable, and efficient. Some reported polymer binding ligands are used to deliver anti-cancer drugs such as doxorubicin (DOX) [142]. Other reports noted that the drug Ibuprofen has been delivered using chitosan-containing GO [138].

Graphene’s unmodified basal plane site with open surface π electrons is hydrophobic, creating π-π interactions for loading drugs [143,144]. GO has been transformed into a carrier for the supply of water-soluble cancer drugs. The solubility of physiological and aqueous solutions can be increased by functional NGO with PEG [145]. Figure 11 shows a scheme of monolayer, few-layer, and multi-layer graphene applications.

Figure 11. Cartoon illustrating the applications of monolayer, few-layer, and multi-layer graphene.
Stimuli Response Delivery

Some drug delivery occurs due to changes in temperature, pH, light, and salt concentrations, whereby the polymer ligand can detect the particular physiological change and deliver the drug. A few reports show that the biopolymers bound with graphene materials are capable of pH-responsive drug delivery or of being used as transporters [146]. For example, DOX is an anticancer drug that is easily released from the GO-Dox complex in a low pH body environment due to its high solubility at a low pH or in acidic conditions [147]. On the other hand, pH-sensitive delivery has been shown to be effective in the treatment of tumors using folic acid with DOX and camptothecin [148]. A photochemically regulated gene-delivery carrier was also reported where the low molecular weight PEI and rGO were combined with polyethylene glycol (PEG) and delivered the plasmid DNA by physiochemical assays [149].

Gene Delivery

Gene delivery is a good alternative way to cure many genetic diseases, as genes can be delivered to a cell through a vehicle or carrier. Many scientific works have reported gene delivery through GO or GO-based materials. To prepare for gene delivery, polyethyleneimine (PEI) was adhered to the surface of the GO sheet via covalent coupling and electrostatic contact with stacking plasmid DNA (PDNA) [150]. The long chain [138] and the branching chain are parallel to the GO [151]. In contrast to other complex compounds, PEI has high transfection effectiveness and low cytotoxicity. Additionally, chitosan complex GO has been used for the delivery of anti-cancer medication drugs and plasmid DNA [152].

5.3.2. Therapeutic Applications

Many results report that the different layers of graphene or graphene oxide can inhibit the growth of tumor spheres in various cell lines, including breast, lung, ovarian, pancreatic cancers, and glioblastoma [153]. Few-layer GO not only inhibits tumor development when injected into mice as an immunocompetent containing CT26 colon cancer cells [154], but also inhibits autophagy [155], immunity, and cell death [156]. In xenograft models, the conjugate PEG-GO exhibits malignancy and thus has a high therapeutic value [157]. An example of this is an rGO/iron oxide NP coated with PEG (rGO-IONP-PEG), which can be used for photothermal treatment (PTT) in vivo and as a triple-mode sensor for fluorescence, photoacoustic tomography (PAT), and magnetic resonance imaging (MRI) [158,159]. Another example is fluorinated GO, which can be used as a magnetically sensitive drug carrier and can be imaged via MRI and photoacoustic tomography [160].

5.3.3. Tissue Engineering for Cell Growth

Monolayer Graphene and Scaffold Formation for Fibroblasts

In a recent study, monolayer graphene’s cytotoxicity and usefulness as a scaffold for murine fibroblast L929 cell lines were assessed. The authors demonstrated the impact of monolayer graphene toxicity through tests on cell viability, morphology, cytoskeleton architecture (microfilaments and microtubules), cell adhesion, and migration. They discovered that the fibroblasts grown on a monolayer of graphene showed modifications in cell attachment, motility, and cytoskeleton organization [161]. Within 24 h of culture, monolayer graphene was discovered to show no cytotoxicity toward L929 fibroblasts and to improve cell growth and adhesion. Additionally, as shown by the results, the monolayer graphene aided in the migration of cells. In the end, it was shown that monolayer graphene is not hazardous to murine subcutaneous connective tissue fibroblasts, and may even help injured tissues heal [161].

Few-Layer and Multi-Layer Graphene and Scaffold Formation

Graphene with different numbers of layers has different effects on different cell line cultures. A mammalian cell culture was tested using a few-layer graphene sheet platform. They described an investigation on the behavior of NIH-3T3 fibroblasts on several carbon
nano-materials layers, including carbon nanotubes, RGO, and GEO. Layers treated with carbon nanomaterials demonstrated excellent compatibility and improved gene transfection efficiency [162]. When compared to conventional polystyrene tissue culture plates, graphene-chitosan hybrid films have demonstrated positive outcomes in tissue engineering to repair and enhance tissue function [163].

Differentiation of Stem Cells

Stem cells are important for living organisms due to their continuous regeneration powers and ability to convert into any kind of cell type where required. In regenerative medicine, stem cells are used as a therapeutic agent. Different layers of graphene are used in stem cell cultures for different purposes. The different layers of graphene showed various types of toxicity effects, electrical conductivity behaviors, biocompatibility, etc. A recent study showed that human mesenchymal stem cells (hMSCs) could differentiate into neurons very well on a surface made of few-layer graphene because the graphene served as an electrically coupled cell-adhesion layer for the hMSCs [164]. In another study, multi-layer graphene also improved the osteogenic differentiation to a greater extent than the general growth factor [165]. A comparative study found that osteogenic differentiation was stimulated but adipogenic differentiation was impeded on graphene as compared to the GO. This occurred due to the different surface characteristics of different layers of graphene or graphene derivatives which come from chemical functionalization [166,167].

5.3.4. Anti-Microbial Effects

The different layers of graphene and their derivatives showed various diversified antimicrobial effects on both Gram-positive and Gram-negative bacteria [168,169]. Few-layer graphene oxide and reduced graphene oxide both showed antibacterial effects on their surfaces [168]. Meanwhile, some reports showed that the bacterial growth was intensified, rather than inhibited, on the monolayer graphene surface [170]. The monolayer GO is more effective than the few-layer and multi-layer GOs because monolayer GO is more efficient in charge transfer with bacterial cells [169]. This happens due to oxidative stress induced by membrane disruption [170,171].

5.3.5. Digital and Analog Devices and Equipment

Graphene is frequently employed by researchers in the field of digital and analog device and equipment. A novel electro-optical encoder based on a graphene–Al₂O₃ multi-layer stack has been designed. It was demonstrated that employing various chemical opportunities for graphene yields the desired encoding operation for the intended structure [172]. In a different study, a switch made of graphene was proposed as a solution to power and footprint issues. The structure may be a good contender for PIC uses, since adding graphene monolayers improves the interaction between light and the material, and lowers the cutoff intensity for the switching function [173]. By altering the chemical prospects of graphene, a graphene-based plasmonic waveguide was created and studied for switching functions at terahertz frequencies [174]. A graphene layer and a silicon ridge were added above and below the channel, respectively, to create a new plasmonic channel. Low energy consumption and the opportunity for on-chip integration are benefits of the sub-wavelength scale [175]. The graphene waveguides are encased between two SnO₂ layers in a small, graphene-based plasmonic D flip-flop, which is shown. The flip-flop’s compact area is a crucial component for use in optical integrated circuits [176].

6. Conclusions, Current Trends, and Future Prospects

Since 2004, when its remarkable properties were first demonstrated, graphene has attracted a good deal of attention from scientists around the world. These properties change as monolayer graphene is restacked to form few-layer graphene, and few-layer graphene is stacked to form multi-layer graphene. However, the outstanding properties are limited to monolayer graphene, while few-layer and multi-layer graphene only show the “nano
effect” of graphite. However, the frequent use of monolayer graphene is difficult due to the challenges of synthesizing it in bulk and its processing costs. Scientists have come up with different approaches to synthesize graphene, such as the exfoliation of graphite into graphene or the synthesis of graphene from chemical vapor deposition. Chemical vapor deposition is the most suitable technique for synthesizing monolayer graphene, while the exfoliation of graphite can be used to synthesize few-layer and multi-layer graphene. A significant advancement in the characterization of graphene flakes has been witnessed, and various methods for characterizing graphene have been proposed by scientists. Among them, AFM, TEM, and Raman spectra have been found to be the most promising.

The future of graphene is bright. For the last two decades, researchers around the world have demonstrated a great interest in graphene. The increasing number of publications on graphene are evidence of the attention being paid to graphene. In particular, monolayer graphene is of key interest due to its outstanding mechanical, electrical, and thermal properties. Synthesizing monolayer graphene in bulk remains a key challenge, and new methods for synthesizing graphene will be explored in the coming decade.

Its remarkable and easy modification facilities, which include surface alterations using any type of required ligands, render differently layered 2D graphene materials very interesting for biomedical purposes. Monolayer graphene and few-layer graphene have no toxicity, higher biocompatibility, and are highly capable of strong UV absorption, SERS, and fluorescence and fluorescence quenching; this makes them some of the most powerful nanomaterials for biosensors, therapeutics, and tissue engineering, as well as for biomedical electronic devices. Still, a few challenges remain, even though the different layers of graphene show promising results concerning their functionalization and optimizations by fractionation based on the number of layers, size, morphology, and chemical functionalities. Moreover, there are many incomparable benefits and still many opportunities to explore the interesting characteristics of these materials and their possible uses. We presume that interdisciplinary efforts with chemistry, biology, and engineering will stimulate the automatic recognition of graphene-layer-based platforms for biomedical applications, and will lead to various successful and innovative applications. In addition, we conclude that different layers of graphene-based materials and their diverse morphology will perform a significant function in the subsequent decade in biomedical applications, and will shape the future of therapeutics.

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