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Synthesis of Graphene-based Nanoparticles for Biomedical Applications – a Mini-review

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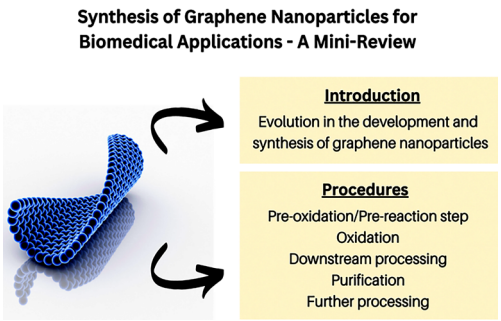
Graphene is a form of carbon allotrope that has received tremendous attention from researchers in various fields. Graphene oxide (GO) has unique characteristics that have proven to be useful in many areas, including biomedical and pharmaceutical applications. Its large surface area and richness in oxygen-containing functional groups enable the loading of large quantities of drug molecules and targeting agents for various diseases such as cancer. This review describes the evolution of developing and synthesizing graphene-based nanoparticles, with a specific interest in biomedical applications. The important procedures involved in the production of GO include pre-oxidation and pre-reaction steps, oxidation, downstream processing, and purification, with further processes like carboxylation and particle size reduction that are needed to obtain the graphene-based nanoparticles suitable for biomedical applications. This review will guide researchers to begin graphene research, understand the important parameters, and ensure the successful production of graphene-based nanoparticles.

Keywords: drug delivery, graphene, graphene oxide, synthesis

GRAPHICAL ABSTRACT



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INTRODUCTION

Graphene is a carbon-based material initially discovered in the early 1990s. In 2004, Andre Geim and Konstantin Novoselov (Gerstner 2010) from the University of Manchester reported their successful attempt to produce graphene sheets, which led to their Nobel Prize in Physics in 2010. Since then, research on graphene-related delivery materials has been escalating and graphene oxide (GO), in particular, has attracted great attention for its potential in various applications including electronic and energy storage (Stoller *et al.* 2008), optics (Robinson *et al.* 2008), chemistry (Zhao *et al.* 2017), medicine (Jain *et al.* 2021), and biology (Priyadarsini *et al.* 2018) GO has a 2D planar hexagonal sp^2- and sp^3-bonded carbon structure with abundant oxygen-containing functional groups such as hydroxyls/epoxides and carboxylic groups, which are located at the basal plane and edge of GO, respectively. These functional groups confer specific properties to GO, including biodegradability (Kotchey *et al.* 2011), water dispersibility (Compton and Nguyen 2010), optical transparency, and photoluminescence suppression. They also enable the conjugation with different materials for nanoscale applications. They may serve as the building block for different hybrid materials with novel properties that are useful in important areas, including drug targeting and drug delivery (Chang *et al.* 2012).

The graphene family of nanomaterials consists of many types of derivatives. Among others includes monolayer graphene, few-layer GO, GO, reduced graphene oxide (rGO), graphene nanosheets, and graphene nanoribbons, with a sheet thickness of between 1–10 nm (Ou *et al.* 2016). The progress of graphene-related research in biomedical studies was rather slow in the first 10 years following its discovery, but it has increased tremendously in the new millennium. The first publication on graphene nanoparticles for drug delivery was reported in 2008, in which Sun *et al.* reported the production of nano-GO for cellular imaging and drug delivery. Since then, the research on graphene-based materials for pharmaceutical and biomedical areas has advanced, which further expanded its potential in targeted drug and gene delivery, antitumor delivery, and controlled stimuli-responsive drug release (Hoseini-Ghahfarokhi *et al.* 2020).

The physical properties of graphene are highly attractive for nanomedicine applications due to its thermal and mechanical properties, which could be tailor-made to suit the delivery of guest molecules into thermally responsive systems such as cancer cells (Jedrzejczak-Silicka *et al.* 2017). Its high surface area, transparency, flexibility, resistance, and tunable hydrophilic-hydrophobic ratios have made it highly promising for different biomedical applications (Lazăr *et al.* 2023). In addition, the functionalized graphene-based carrier is a promising approach to designing a smart drug delivery system for photodynamic therapy that has been shown to have higher efficiency for theranostic applications (Hoseini-Ghahfarokhi *et al.* 2020), as antibacterial agents, and for tissue engineering purposes (Wu *et al.* 2017) (Figure 1).

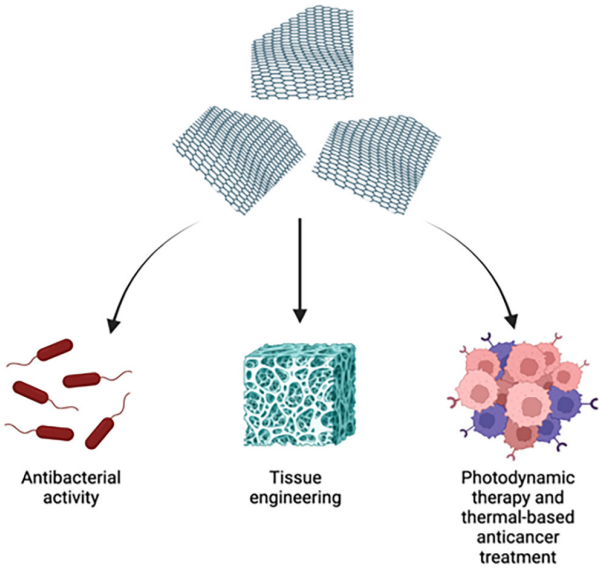

Figure 1. Potential applications of graphene-based delivery systems.

METHODS OF GRAPHENE OXIDE (GO) SYNTHESIS

Background

GO is a derivative of graphene that is typically obtained from oxidation and exfoliation of graphite. GO consists of various oxygen-based functional groups such as epoxy, hydroxyl, and carboxyl groups, whereby the carboxyl groups are commonly present at the edges of GO sheets. In contrast, the faces of the sheets mostly contain epoxy and hydroxy groups (Figure 2) (Abdolhosseinzadeh *et al.* 2015) (Bai *et al.* 2019). Epoxy and hydroxyl groups are also believed to be present on the edges. Further studies also reveal the presence of plane defects and holes on the GO sheet, adding a further layer of complexity in elucidating the true chemical structure of GO. Carbonyl, carboxyl, and even ester groups are also believed to be formed on the defects of the GO sheet (Mohamadi and Hamidi 2017).

The extensive sp2-hybridized graphene network on the carbon sheet is transformed to accommodate aromatic and alkene groups along with simple sp3-hybridized bonded carbons, resulting in a carbon sheet with a combination of sp2-hybridized, as well as sp3-hybridized bonds. The disruption of the delicate sp2-hybridized network, and thus the mobility of delocalized π-electrons, unsurprisingly

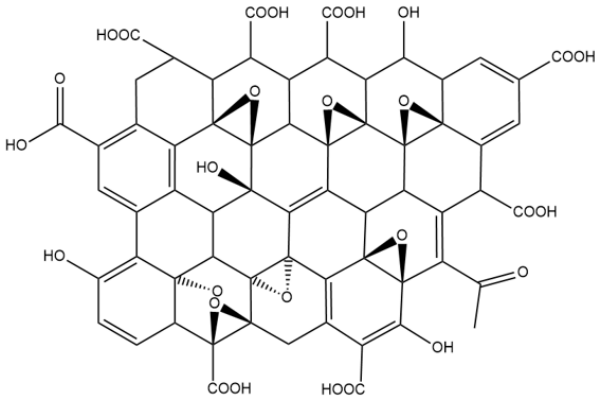

Figure 2. Proposed molecular structure of GO.

disrupts the electrical conductivity of graphene. This electrical conductivity can be partially recovered upon reduction of GO to rGO, the process of which involves partial removal of the oxygen functional groups from GO (Jaafar and Kashif 2018).

Regarding physical appearance, GO is most commonly available in the form of brownish solids or suspensions. GO is one of the more popular derivatives of graphene used in biomedical applications due to its relative hydrophilicity and ability to be functionalized *via* its oxygen-based functional groups (Jain *et al.* 2021; Silva *et al.* 2021). However, this promising stability profile depends on the environment and surrounding conditions, with its aqueous instability in high-salt environments being a well-known drawback to its application in the biomedical field (Hong *et al.* 2012).

The synthesis of GO as conducted today are successor of methods conducted by Brodie (1859), which was subsequently improved upon by Staudenmaier about 40 years later (Staudenmaier 1898). Both methods use potassium perchlorate ($KClO_4$) as the oxidizing agent. Their methods were then improved upon by Hummers and Offeman in 1959 by replacing $KClO_4$ with potassium permanganate ($KMnO_4$). This paved the way for the current-day GO synthesis methods, which are collectively known as "improved Hummers' methods" (Table 1). However, more recent methods advance towards GO synthesis with iron-based oxidation (*e.g.* potassium ferrate, K_2FeO_4), which reduces the production time significantly while maintaining the quality and characteristics of the GO formed (Peng *et al.* 2015b).

The carbon-to-oxygen ratio, or the atomic ratio of carbon to oxygen, is deemed to be a very important parameter in evaluating the properties of GO. The consensus on the completeness of a chemical synthesis method is ascertained when the carbon-to-oxygen (C/O) ratio of GO reaches 2.0 (Kovtyukhova *et al.* 1999). However, the molar ratio of oxygen groups may not strictly represent of the amount of oxygen-based functional groups due to the possibility of the intercalation of water or sulfur and oxygen-based impurities (Kovtyukhova *et al.* 1999). Shin *et al.* (2013) conducted experiments on the effects of strong acids on the structural integrity of graphene by using pyrolytic graphene, which refers to graphene with covalent bonds between the sheets. From their experiment, it can be implied that although concentrated acids by themselves act as an effective medium to exfoliate graphite into graphene, they may also cause structural damage to graphene sheets in addition to introducing sulfur-containing and nitrogen-containing functional groups *via* sulfonation and nitration respectively (Shin *et al.* 2013). Based on such inconsistencies in the C/O ratio, Marcano *et al.* (2010) in their pioneering article on the Tour's method analyzed their samples using X-ray photoelectron

Table 1. Evolution of chemical synthesis methods of graphene oxide.

Name of method and reference(s)	Year	Oxidizing agent(s)	Weight ratio of graphite to oxidizing agent	Acid and/or salt components in media	Reaction time	Carbon-to-oxygen ratio	Characteristics
The "potassium chlorate" era							
Method by Brodie (Mohamadi and Hamidi 2017; Ciszewski and Mianowski 2013)	1859	$KClO_3$ (potassium perchlorate)	1:8.5	Fuming nitric acid	3–4 d [112]	2.16	The use of $KclO_4$ caused the production of chlorine dioxide (ClO_2), which is flammable and toxic; the method produces graphite oxide rather than graphene oxide
Staudenmaier method (Staudenmaier 1898; Ciszewski and Mianowski 2013)	1899	$KclO_3$	1:11	Concentrated sulfuric acid and fuming nitric acid	1–10 d[112]	2.89	Increased efficiency compared to Brodie's method; results in similar harmful gas emissions due to ClO_2 emission

Table 1. Cont.

The 'potassium permanganate' era							
Hummers' method (classical) (Hummers and Offeman 1958)	1957	$KmnO_4$ (potassium permanganate)	1:3	Sodium nitrate and concentrated sulfuric acid	About 1 h	2.1–2.9	Releases harmful gas emissions in the form of nitrogen dioxide (NO_2) and dinitrogen tetroxide (N_2O_4); sodium and nitrate ions are difficult to dispose of in wastewater[29]; introduction of $KmnO_4$ as oxidizing agent results in Mn^{2+} waste, as well as potentially dangerous manganese (VII) oxide (Mn_2O_7) intermediates, which can explode in temperatures above 55 °C[20]
Improved Hummers' method (IHM)							
IHM–Marcano / Tour Method (Marcano *et al.* 2010)	2010	$KmnO_4$	1:6	Concentrated phosphoric and sulfuric acid (1:9 volume ratio, respectively)	About 12 h	N/A	Reduce toxic gas emissions; compared to classical Hummers' method, has increased oxidation efficiency; retains greater structural integrity
Chromate oxidation (Chandra *et al.* 2010)	2010	$K_2Cr_2O_7$ (potassium dichromate)	Approximately 1:7.5	Sodium nitrate and sulfuric acid (5%)	About 5 d	N/A	Safer and simple but takes a longer duration; overall efficiency compared to other methods is yet to be determined as of 2021
IHM (Chen *et al.* 2013)	2013	$KmnO_4$	1:3	Concentrated sulfuric acid	About 1–2 h	2.36	Removal of sodium nitrate reduces waste ions; the authors also proposed a method to remove manganese (Mn^{2+}) ions, making their method eco-friendly
The "iron" era and future research prospects of graphene oxide synthesis							
Iron-based oxidation (Peng *et al.* 2015a)	2015	K_2FeO_4 (potassium ferrate)	1:6	Concentrated sulfuric acid	1 h	2.2	Reduced manganese-based waste products (eco-friendly), much shorter reaction time, sulfuric acid can be re-used, high exfoliation degree; several potassium and iron-based products can be separated using ammonia and can be used as fertilizers
IHM (Yu *et al.* 2016)	2016	$KmnO_4$ and K_2FeO_4 (potassium ferrate)	1:0.6 ($KmnO_4$) 1:0.4 (K_2FeO_4) Overall ratio of 1:1 in a pre-oxidation step, with additional $KmnO_4$, leading to a total GO: $KmnO_4$ ratio of 1:1.1	Concentrated sulfuric acid with trace amounts of boric acid as a stabilizer	About 5 h	2.12	Pre-oxidation step introduced, aimed to increase the utilization of oxidants by increasing penetration of oxidants

spectroscopy (XPS). This was done to estimate the ratio of functional groups to the signature patterns formed by graphene sheets, thus demonstrating a promising method of quality control that may be widely incorporated in the future (Marcano *et al.* 2010).

Graphene Oxide (GO) Synthesis

[i] Pre-oxidation procedures and pre-reaction steps. Several attempts have been made in numerous studies to increase the efficiency of graphene or graphite oxidation to minimize or prevent incomplete oxidation, which may lead to the formation of by-products such as graphite-core/GO-shell particles. Pre-oxidation, being one of these measures, was first reported by Kovtyukhova *et al.* in 1999 and commonly involves the reaction of graphene with potassium persulfate and phosphorus pentoxide ($K_2S_2O_8$ and P_2O_5) under strongly acidic conditions (concentrated sulfuric acid) at elevated temperatures for prolonged durations (typically 80 °C in an oil bath for 5–6 h). The pre-oxidized graphene will then be filtered and dried (Kovtyukhova *et al.* 1999). Variations regarding the pre-oxidation reagent exist such as using a mixture of impure manganese dioxide (MnO_2), sulfuric acid, and phosphorus pentoxide (P_2O_5) (Sun *et al.* 2015).

Grinding of graphite with salt is another method commonly employed to reduce the size of graphite to increase oxidation efficiency (Rasoulzadeh and Namazi 2017). It is suggested that this method could reduce the size of GO to be produced from the micrometer range to 30–50 nm. However, more studies are needed to ascertain this technique's efficiency.

The harshness of the oxidation reaction is known to affect the functional groups of GO. It is suggested that as the degree of oxidation increases, the reaction begins to produce carboxyl and hydroxyl groups, followed by the formation of epoxy groups deep in the surfaces between graphene sheets that had been "shielded" in the period when graphene existed as multi-layer sheets (Krishnamoorthy *et al.* 2013).

In addition to the intensity of oxidation, the type of oxidizing agents also plays a role in the properties of the GO formed. For instance, Poh *et al.* (2012) compared the properties of GO formed by the Staudenmaier, Hofmann, and Hummers' methods. They discovered that the GO formed by the Hummers' method has a significantly improved electrochemical property, which is important, especially in biosensing applications (Poh *et al.* 2012). Several experiments reported applying successive oxidation reactions, whereby GO is oxidized several times. This will result in an increased amount of oxygen-based functional groups, typically observed by a decrease in the C/O ratio (Hong *et al.* 2012).

[ii] Downstream processing. Most GO synthesis methods in drug delivery applications fall into one of two categories: [1] the method that maintains a steady temperature throughout a long duration of time (steady reaction or SR); and [2] the method that standardizes temperatures at specified levels (standard temperature or ST). The SR conducted in drug delivery studies are often replicates or derivatives of the Tour's method (Marcano *et al.* 2010; Saifullah *et al.* 2018). Conventionally, the oxidant (typically potassium permanganate or $KMnO_4$) is added slowly to the reaction mixture, with most experiments utilizing an ice bath while specifying a maximum temperature of 20 °C (Saifullah *et al.* 2018). Upon complete addition of the oxidant, the temperature is maintained at a particular value for a set amount of time (usually 50 °C for 12 h) before the reaction mixture is diluted with iced distilled water and halted with the addition of hydrogen peroxide (H_2O_2) for further processing and purification (Karki *et al.* 2018).

In contrast to SR, the execution of ST follows a much more specified and multi-step approach towards GO production. The reaction mixture is first incubated in an ice bath to effectively equilibrate the reaction mixture at 0 °C until $KmnO_4$ is added, upon which the temperature is maintained at a temperature of about 30 °C for a set duration. In the next step, the temperature is spiked by adding distilled water, causing an exothermic reaction, upon which the system's temperature is controlled at temperatures of about 95–98 °C. The reaction is then cooled as H_2O_2 is added to terminate the reaction. Each step can take approximately 2–4 h (Karki *et al.* 2018). The temperature changes throughout the synthesis reaction in both SR and ST are illustrated in Figures 3 and 4, respectively (Karki *et al.* 2018; Hummers and Offeman 1958).

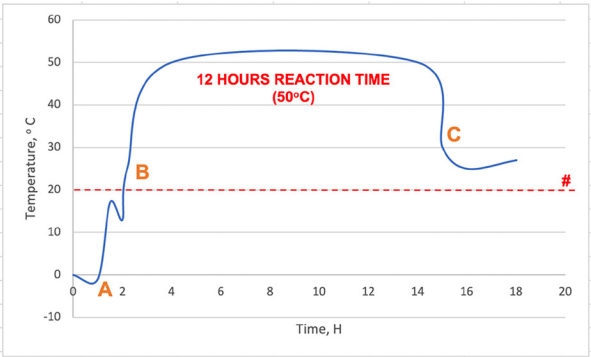

Figure 3. Visual representation of temperature changes throughout GO synthesis reactions through SR method. [A] The beginning of gradual $KmnO_4$ addition (some studies indicate a maximum temperature of 20 °C during the addition of $KmnO_4$). [B] The addition of KmnO4 is completed. [C] The dilution and addition of H_2O_2 to terminate the reaction.

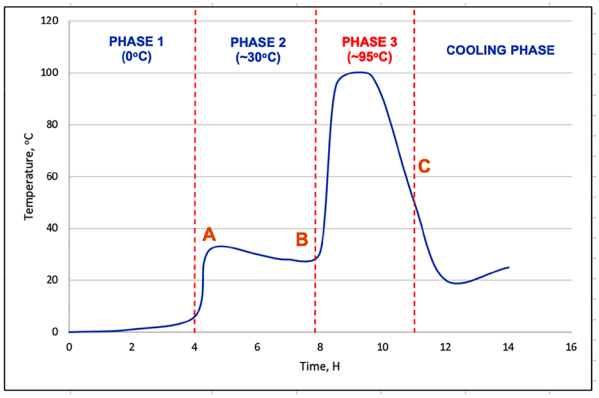

Figure 4. Visual representation of temperature changes throughout GO synthesis reactions through ST method. [A] The beginning of gradual $KmnO_4$ addition. [B] Dilution with distilled water. [C] The addition of H_2O_2 to terminate the reaction (some studies specified a temperature of 60 °C for this process).

Compared to ST, SR has the advantage of simplicity, whereby a single temperature is maintained throughout the key reaction step. However, SR lacks consistency in terms of temperature in certain steps such as the phase involving the addition of distilled water to dilute the reaction mixture. ST solves the issue by specifying a temperature of around 95 °C upon adding distilled water. Still, multiple setups such as water or oil baths will be needed to maintain specific temperatures throughout the reaction process.

Using an ice bath before and during the addition of $KMnO_4$ is important to prevent exothermic reaction that may lead to explosion (Peng *et al.* 2015a), besides preventing the initiation of the oxidation reaction prematurely above the threshold temperature, which in turn may cause unintentional variations in the reaction time. Considering that temperature will impact the GO particle size and its C/O ratio (Zimba *et al.* 2019), temperature variations may affect GO properties, making it difficult to compare two experiments of different GO synthesis methodologies.

After oxidation reactions are completed, several procedures will be followed to remove impurities. At the end of chemical oxidation, the reaction is first halted by adding distilled water, followed by H_2O_2 to reduce excess permanganate (Hummers and Offeman 1958). The next step involves washing with 5–30% HCl (Karki *et al.* 2018) to remove metal ions (Chen *et al.* 2015). This is typically followed by repeated washing with distilled water to neutralize the product. On occasion, ethanol and ether were also reported to be used in the washing step (Marcano *et al.* 2010).

[iii] Purification. There are many ways of purifying GO. For biomedical applications, pH is very important to ensure the GO produced is biocompatible with the targeted biological systems. In addition, pH will also influence the release of drug molecules loaded on GO surfaces. Hence, purification and pH adjustment are important to ensure the resulting GO meets the desired functionality for its intended purposes.

Centrifugation is one such method and is used by the original developers of the Tour's method (Marcano *et al.* 2010). There is no strict rule concerning the precise parameters of centrifugation. Marcano *et al.*, for instance, purified GO with a force of 4,000 rpm, whereas other authors used speeds as high as 10,000 rpm (Hosseinzadeh *et al.* 2018). Dialysis using a dialysis membrane may also be employed for further purification of GO, typically using a membrane of a molecular weight cut-off between 8,000–12,000 g mol^{-1} (Chen *et al.* 2013). The duration of dialysis is usually a week, though occasionally two weeks may be required (Alam *et al.* 2017; Wang *et al.* 2010).

Abdel-Motagaly *et al.* (2018) described a two-step purification of graphene using a hollow-fiber dialyzer that allows ultra-fast purification of the crude GO mixture. This method requires simple setup components and produces a high product yield. The two steps include [1] the crossflow filtration (CFF) process and [2] the dialysis process, and the efficiency of this method was monitored by measuring the electrical conductivity of the GO suspension. They reported that this method is 100 times faster under optimum conditions than conventional purification methods such as using dialysis bags. The energy-dispersive X-ray analysis showed a small amount of impurities such as manganese and sulfur remained in the purified GO, which a second stage of CFF can further eliminate.

Another improved method for GO purification is *via* tangential flow filtration (TFF) which uses an ultrafiltration membrane. This method reduces the purification time significantly compared to the conventional centrifugation method, besides being more ecological as less waste is generated during the process and is less laborious. The product yield obtained was comparable to centrifugation, suggesting TFF potential in future manufacturing of graphene for commercial use (Alhourani *et al.* 2021). Figure 5 summarizes the procedures and factors involved in the production of GO.

Further Processing

[i] Carboxylation of graphene oxide (GO). Given efficient use, the non-covalent functionalization of the large surface area of GO with organic molecules or polymers has been widely explored through weak van der Waals interactions (Mann and Dichtel 2013), hydrogen bonding interaction (Mirhosseini *et al.* 2019), and π–π interactions (Xu *et al.* 2016; Zhou *et al.* 2015; Ghosh *et al.* 2010). However, the resulting non-covalent complexes

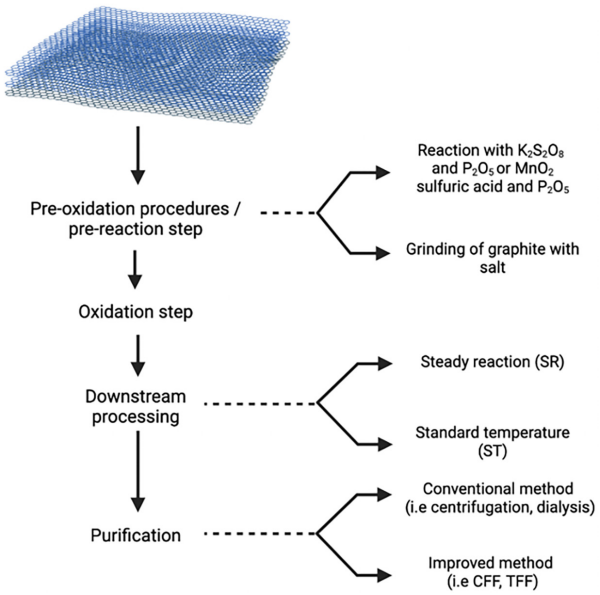

Figure 5. Procedures and factors involved in GO production.

have low stability due to these weak interactions leading to the release of the organic molecules from the GO surface, thereby complicating GO use especially in biological and medical applications.

The opportunity to conjugate various bioactive molecules onto the GO surface by covalent bonds *via* the oxygen-containing functional groups significantly enhances the stability of the resulting complexes and their applications in various areas (Park *et al.* 2021). Several studies have been published showing the selective covalent functionalization of GO using hydroxyl (Vacchi *et al.* 2018, 2020; Guo *et al.* 2020a; He *et al.* 2014), epoxide (Vacchi *et al.* 2016) or carboxylic (Mei *et al.* 2015; Guo *et al.* 2020b) groups paving the way for the elaboration of double GO functionalization strategies (Guo *et al.* 2020a, b; Vacchi *et al.* 2020; Imani *et al.* 2018). The carboxylic groups are typically used for immobilization or conjugation (covalent or electrostatic) of biomolecules (Imani *et al.* 2015a) such as peptides/proteins, enzymes, targeting agents, antibodies, and NH_2-functionalized natural or synthetic polymers. These carboxylic groups considerably widen the possibility to multi-functionalize the GO surface but the limited amount of the carboxylic groups at the edge compared to the hydroxyl and epoxide groups at the basal plane induces a meaningful lowest functionalization efficiency (Vacchi *et al.* 2016). Based on this observation, the scientific community has sought to develop methods to produce carboxylated GO (GO-COOH) and to compare its properties with classical GO. It is most interesting to note that GO-COOH can be produced by converting the hydroxy and epoxide groups on the GO surface into carboxylic ones. The most widely reported method in the literature for this conversion involves the treatment of GO with a halogenoacetic acid (*i.e.* $ClCH_2COOH$ or $BrCH_2COOH$) under strongly basic conditions using an excess amount of sodium hydroxide (NaOH) to afford GO-COOH after HCl neutralization and purification (Figure 6).

The successful functionalization of GO-COOH is supported by structural characterization studies (Imani *et al.* 2015a; Peng *et al.* 2015b; Dai *et al.* 2015; Zhao *et al.* 2017; Ma *et al.* 2019; Xu *et al.* 2019; Zimba *et al.* 2019; Guo *et al.* 2020b; Yao *et al.* 2021; Zhang *et al.* 2021), including several analyses such as spectroscopic (FT-IR, UV-Vis, Raman, ^{1}H and MAS NMR, and XPS), crystallographic structural (XRD), thermogravimetric (TGA), and surface morphology (AFM, SEM, TEM, and FESEM images). The quantification of carboxylic groups on the GO surface has been determined by acid-base titration or colorimetric methylene blue (MB) assay (Imani *et al.* 2015a). As such, in 2015, Imani and co-workers studied the carboxylation process by synthesizing carboxylated nano-GO sheets (NGOS-COOH) using different $ClCH_2COOH$ concentrations (0.5, 1, 2, and 3 M). The quantification of carboxylic groups on NGOS-COOH was determined by MB colorimetric assay. The MB compound is known to chemically react with one COOH unit under acidic media through a bleaching reaction affording a colorless leucomethylene molecule. The molarity of carboxylic groups (M_{COOH}) can be calculated using UV-Vis spectroscopy. The results indicated that the highest conversion of hydroxy and epoxide groups was

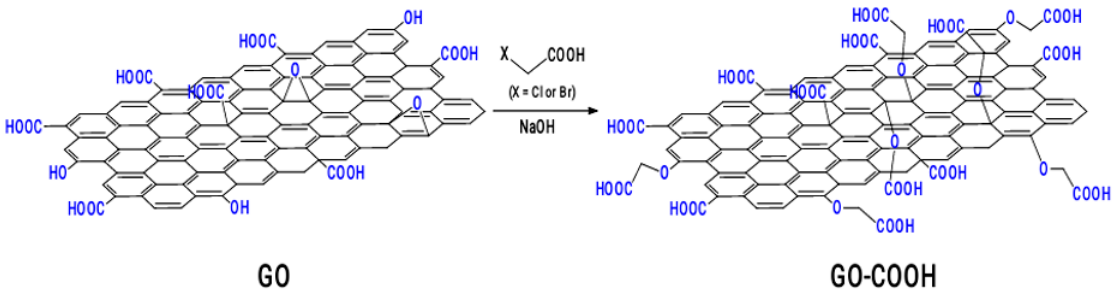

Figure 6. Carboxylation of GO into GO-COOH.

achieved using 2M $ClCH_2COOH$ (*i.e.* M_{COOH} around 0.086 µM/mg) and NGOS-COOH showed a better dispersion and exfoliated level. The conjugation capacity of NGOS-COOH was compared to the nano-GO sheet (NGOS), in which R8 peptide was used as a model. The authors found that the bioconjugation with NGOS-COOH was 2.5 times more efficient than NGOS (13.4% *vs.* 5.3%), highlighting the interest in using NGOS-COOH for bioconjugation applications. In the same year, Peng *et al.* (2015b) synthesized and estimated the content of carboxylic groups on the surfaces of GO and GO-COOH. After one hour of sonication of GO or GO-COOH in 0.1 M aqueous NaOH solution under an inert atmosphere followed by two days of stirring, the resulting mixtures were dialyzed and titrated using a 0.1 M aqueous HCl solution until a neutral pH value was achieved. The amount of HCl used allowed the estimation of the number of carboxylic groups, in which an average amount of carboxylic groups of 0.696 and 1.243 mmol was obtained for GO and GO-COOH, respectively, demonstrating the successful carboxylation of GO using $ClCH_2COOH$ and a large excess of NaOH. The authors highlighted the interest of GO-COOH to covalently immobilize enzymes *via* an amide bond [*i.e.* myoglobin (Mb) used as a model] and showed that around 90% of the Mb catalytic activity was preserved after immobilization. This finding showed that GO-COOH has better water solubility than GO and it attracts interest in the design of materials for different applications.

In 2021, Yao *et al.* studied the effect of various amounts of $BrCH_2COOH$ on the carboxyl group content in GO-COOH. The titration also used an aqueous HCl solution. The authors found that the content of carboxylic groups of 6.48 mmol/g was the best (*i.e.* GO/ $BrCH_2COOH$ of 1/20) because a better content could induce higher hydrophilicity, leading to a decrease of the coating resistance to wet rubbing. It was also observed that the physical and mechanical properties of GO-COOH were improved as the carboxylic group content increased. GO-COOH/chitosan composite was finally designed using chitosan as a matrix and GO-COOH as filler to produce a leather finishing agent (Yao *et al.* 2021). Other publications indicated that carboxylation of GO can increase some properties such as dispersibility (Dai *et al.* 2015; Zhao *et al.* 2017; Zhang *et al.* 2021), high-temperature resistance (Zhang *et al.* 2021), absorption capacity (Zhao *et al.* 2017; Ma *et al.* 2019; Zimba *et al.* 2019), hydrophilicity (Dai *et al.* 2015; Peng *et al.* 2015b), and thermal stability (Xu *et al.* 2016). However, some articles pointed out the reduction of GO during the carboxylation process using an excess amount of NaOH (Dai *et al.* 2015; Xu *et al.* 2016; Guo *et al.* 2020b) that might affect its intrinsic properties and induce a decrease in the conjugation efficiency of carboxylic groups. Based on this observation, various research studies were conducted to design a new protocol for effective GO carboxylation to avoid the use of strongly basic conditions (NaOH). To give just one example, Xu and co-workers (2019) used tris-sodium citrate instead of NaOH and demonstrated that the carboxyl group density on GO-COOH was doubled.

[ii] Exfoliation and particle size reduction. For graphene and its derivative to properly serve their function as drug carriers, their size must be reduced by breaking the particles into single-layered sheets in a process collectively known as "flaking." Further "cracking" processes will break each sheet into smaller sheets (Figure 7).

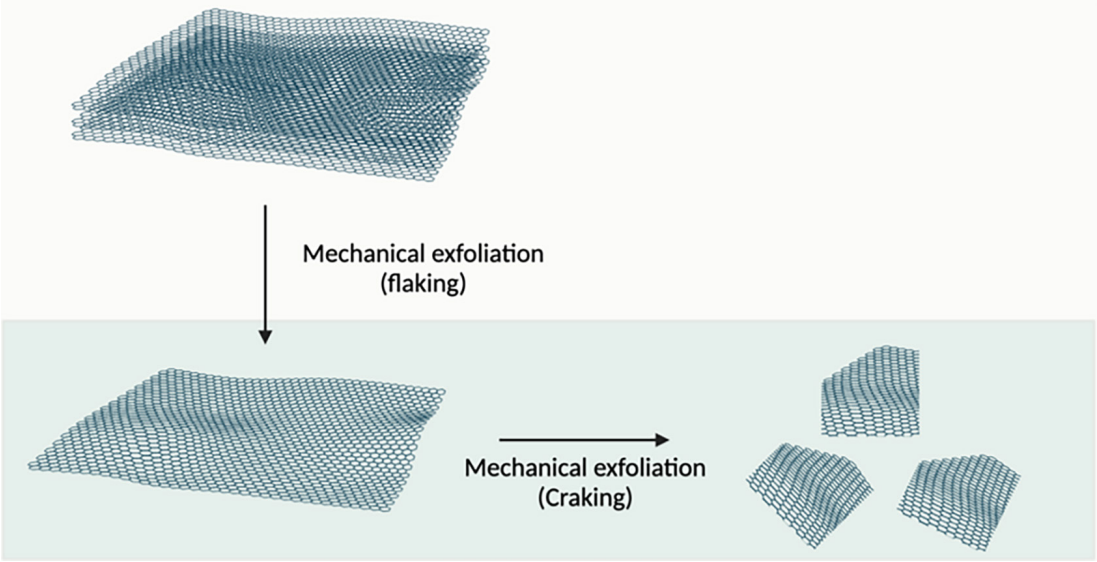

Figure 7. Mechanical exfoliation of graphene.

Ultrasonication, the process of applying ultrasonic waves of frequency beyond 20kHz onto any given substance, is by far the most common method used to exfoliate GO into single-layered sheets while also cracking the sheets under more intense conditions. It is believed that the ultrasonic sound waves could achieve this by producing air bubbles, which will cause several effects including mechanical cavitation of water-dispersed particles, as well as producing free radicals through water hydrolysis amidst the formation of the air bubbles (Majid *et al.* 2015). In the process, a large amount of heat energy should be treated cautiously to not overheat the mixture. As an example, Yuan *et al.* reported that the ultrasonic waves from a bath sonicator with a frequency of 53 kHz increase the oxidation efficiency, suggesting the presence of ultrasonic waves can break the inter-layer Van der Waal's forces of attraction between the graphene flakes to allow reactants and solvents to enter these inter-layer spaces (Yuan *et al.* 2017).

Probe sonication is the most widely used method of producing nano-sized GO dispersions in the experimental setting (Le *et al.* 2019). Table 2 summarizes the examples of probe sonication parameters used in preparing GO and the size of particles produced. From the data collected, it is noteworthy that although the size of the final nanoparticle decreases as the sonication's strength increases, several outliers exist in the trend. This may be due to the variations in methods employed for producing and characterizing GO such as variations in the type of graphite, the presence or absence of carboxylation, reaction conditions, and size measurement. In addition, ball milling or pre-oxidation steps conducted at the beginning of the synthesis may also affect the efficiency of size reduction by probe sonication.

As far as biomedical applications are concerned, using chemicals to exfoliate or crack GO (outside of the chemical exfoliation that occurs from graphite oxidation) is rarely observed compared to using sonication. However, several methods have been proposed for using chemical reagents to flake graphene and its derivatives. As an example, a study by Hernandez *et al.* (2008) demonstrated that certain solvents such as *N*-methylpyrrolidone (NMP), in addition to bath ultrasonication, can disperse graphene at a maximum concentration of 0.01 mg/mL due to the matching enthalpy of specific solvents to the energy required to flake graphene sheets (Hernandez *et al.* 2008).

It is important to note that besides methods dedicated specifically to flaking graphene and its derivatives, chemical flaking can also be achieved by modifications in the GO synthesis method. A decrease in the C/O ratio (an increase in functionalization with oxygen-based functional groups) is commonly observed to produce GO of smaller particle size (single-layered sheet or otherwise). This happens due to the flaking of GO mid-reaction due to the formation of epoxy groups on the surface of individual sheets, subsequently causing repulsive forces between sheets to induce flaking. In addition, it is stated that cracking may also occur due to the breaking of the carbon-oxygen bonds on the sheet in a process commonly called "molecular scissors." The basis of these concepts forms the general rule of thumb, whereby the C/O ratio is inversely related to the particle size (Li *et al.* 2006), which should be considered in the preparation of GO as a drug carrier.

Table 2. Mechanical exfoliation of GO by probe sonication.

Probe ultrasonication strength and total duration	Type of graphite source	Approximate size/ hydrodynamic diameter of GO particles		Size measurement method	Purpose of experiment	Ref
		Before sonication	After sonication			
20W, 1 h	Graphite flakes[a] (0.2 mm or less)	900 nm	Less than 500 nm	DLS	Functionalized GO-PLA-PEG drug carriers	Angelopoulou *et al.* (2015)
30W, 1 h	Natural graphite (30 µm)	2,400 nm	350 nm	DLS	Safety studies involving GO	Chen *et al.* (2012)
40W, 20 min	Graphite powder[b] (325 mesh)	n/d	100 nm[c]	TEM	GO aptamer complex for biosensing	Wang *et al.* (2010)
200W, 2 h	Natural graphite	n/d	250–400 nm	AFM	Gene delivery	Imani *et al.* (2015b)
200W, 2 h	Natural graphite	n/d	250–400 nm	SEM, TEM, AFM	Carboxylation assay	Imani *et al.* (2015a)
400W, 1 h	Native graphite[d] (0.6–0.7 µm)	Less than 40 nm	30 nm	TEM, AFM	Oral delivery of quercetin	Rahmanian *et al.* (2014)
500W, 1 h	n/d	n/d	500 nm	SEM, TEM	Oral delivery of pingyangmycin	Liu *et al.* (2019)

Table 2. Cont.

500W, 1 h 40 min	Native graphite[c]	n/d	Less than 150 nm[c]	TEM, AFM	Delivery of camptothecin and doxorubicin	Zhang *et al.* (2010)
570W, 2.5 h	n/d	n/d	140 nm	TEM, AFM	Functionalized GO-PEG drug carriers	Pei *et al.* (2020)
750W, 20kHz, 1 h	n/d	n/d	120 nm (AFM) 140 nm (DLS)	AFM, DLS	Delivery of oridonin and methotrexate	Chai *et al.* (2019)
750W, 40% intensity, 2 h	n/d	450 nm	45 nm	DLS	Functionalized GO-Pluronic drug carriers	Jang *et al.* (2015)
750W, 30% intensity, 2 h	n/d	420 nm	38 nm	DLS	Functionalized GO-Pluronic drug carriers	Sahu *et al.* (2013)

[n/d] Not disclosed
[a]Staudenmaier method
[b]Pre-oxidation
[c]Nanoparticles obtained from supernatant following 12,000–13,000 rpm centrifugation (size fractionation)
[d]Ball milling or grinding with NaCl for initial graphite size reduction

CONCLUSIONS AND FUTURE PERSPECTIVES

The production and synthesis methodology of graphene-based delivery systems has been improving over the years, expanding their application into the biomedical field. Their versatility in terms of structure and functionalization should be exploited to produce a stable and efficient drug delivery carrier. Ensuring the carrier's safety is very important for biological applications, and studies have shown the possibility of improving their biocompatibility using certain approaches such as biopolymer coating with chitosan.

Based on the available data, several key points must be considered in developing graphene-related materials for biomedical applications. Graphene prepared through chemical vapor deposition leads to an increased production of reactive oxygen species, which may cause a significant increase in the apoptosis rate of neuronal cells. This production method is the least suitable for graphene production for biomedical applications. In addition, the amount of chemical reagents used in the synthesis process is also a critical factor, which influences the content of metal ions in the product that will be immediately available to the cells upon *in vivo* administration. This must be controlled during production to prevent serious implications during treatment.

GO and rGO have been shown to have good biocompatibility with red blood cells and blood components. Hence, they have a longer circulation time in the blood than other nanomaterials. Surface modifications also play a crucial role in enhancing the biocompatibility of graphene while mitigating its liver accumulation without inflicting significant harm to the body. PEGylation has been demonstrated as a promising approach to alter the *in vivo* properties of GO, rendering it more biocompatible and facilitating its gradual elimination from the body. These approaches hold tremendous potential for promoting graphene-based materials' safe and effective use in various biomedical applications. This could be the starting point for future exploration with more innovative approaches such as polymer coating and dispersion to be incorporated into the development process.

Along the way, more data would be needed to modify and enhance their biomedical and pharmaceutical applications. It is believed that the full potential of graphene as a delivery system is yet to be discovered. This versatile material should be further explored and as more data becomes available, its potential in biomedical applications will become clearer.

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