Biocompatibility Considerations in the Design of Graphene Biomedical Materials

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Graphene-based materials (GBM) have outstanding properties that have proven highly beneficial in several proof-of-concept investigations on their biomedical potential. They can be used as suspensions of nanosheets for nanomedicine purposes as well as components of macroscale products in medical devices or tissue engineering/regenerative medicine products. However, the clinical translation of these preclinical concepts is hampered by the incomplete understanding of the biocompatibility of GBM in general, and the limited or lack of safety considerations in most preclinical proof-of-concept studies. In this review, the main safety aspects to be considered for the design of biocompatible materials based on GBM that interface with the human body or its fluids are outlined. Guidance to overcome some of the unique challenges presented by graphene biomedical materials are provided, stressing the need to consider safety challenges as early as possible in the design phase of the candidate biomedical product to raise its chance for clinical translation.

1. Introduction

Graphene, the poster child of 2D materials, is a nanomaterial consisting of atom-thick planar sheets of sp² bonded carbon atoms arranged in a honeycomb shape. It was first isolated from graphite by Novoselov and Geim in 2004 at the University of Manchester,[1] work that was later recognized with the Nobel Prize in physics in 2010.

As a material, it combines many unique and astounding properties, including but not limited to the highest charge-carrier motility of any known material \((2.5 \times 10^6 \text{ cm}^2 \text{ V}^{-1} \text{ S}^{-1})\) giving it outstanding properties as an electrical conductor.[2] It is also known to have extremely high thermal conductivity, excellent optical transparency (in thin films), the highest known mechanical strength in its perfect crystalline form, and one of the largest surface areas of any nanomaterial as all constituting atoms are at the surface.[3] It can also be readily chemically functionalized, enabling this material to be used both in dry or liquid states. As a result, graphene has been nicknamed the wonder material because the list of properties that are combined in this atom-thin layer seems limitless.[4]

Not surprisingly, this unique combination of properties have attracted the interest of researchers working in practically every field of technology development,[5] and medicine is no exception to this; graphene can improve the performance of many existing biomedical technologies as well as opening the possibility of creating brand new applications.[5,6] However, despite a large number of publications in all these fields and the rising number of commercial goods containing graphene, to date no graphene-based biomedical materials have been successfully translated into real-patient use.

There are a number of reasons for this fact and in many cases these can be put down to the relative infancy of the field. Main reasons include the need to improve techniques for synthesizing and handling graphene materials in a reproducible and standardized fashion for a consistent production of medical prototypes, and ultimately the production scale-up of the designed medical product. However, there are also instances where the development approach taken by researchers has not considered enough – or at all – the potential safety implications of their intended biomedical materials containing graphene-based materials (GBM),[5,7] increasing the risk of later stage failures for lack of biocompatibility or for not complying with regulatory frameworks used for the safety testing of biomedical materials prior to clinical translation. Any failure of a new graphene-based biomedical material for these reasons, even at a very early stage of development, has the potential to delay the clinical translation of graphene-based technologies as a whole by adversely affecting scientific, clinical, industrial, and public opinion.
In the following mini-review, the main safety considerations toward the development of biocompatible GBM biomedical materials are therefore highlighted. After a rapid overview of the different types of GBMs and their potential as biomedical solutions, the main safety aspects to be considered when designing biomedical materials based on GBMs that will interface with cells, organs, biofluids, or matrices are outlined. Clear distinctions between macroscale (i.e., tissue engineering scaffolds and medical devices) and nanoscale (i.e., nanomedicines) applications of GBMs and specific safety considerations in this respect are made. In view of the lack of clinical history with these new biomaterials and the safety concerns around their nanoforms, it is proposed that considering safety aspects right from the initial stages of developing graphene-based biomedical materials would greatly accelerate their translation to the clinic.

2. Graphene-Based Materials and Potential Applications in Medicine

Graphene and its derivatives exist in many forms with varying lateral dimensions, thickness, and functionalization (Figures 1 and 2).\(^8\) Despite being generally “nano” in one dimension (i.e., thickness), GBMs can be produced either as materials with lateral dimensions below the micrometer (i.e., nanosheets) or as macroscale products (up to meter-sized films), owing to advances in graphene synthesis methods.\(^9\) This size versatility is highly valuable for the wide range of dimensions that apply in material-based biomedical solutions,\(^9\) from implantable medical devices or tissue engineering scaffolds (later referred to as macroscale application) to injectable colloidal suspensions of nanomedicines (nanoscale application).\(^10,11\) They can also differ in thickness, with GBMs being defined as having one layer (single layer), few layers (2–5 layers) or multilayers (2–10 layers); above 10 being considered as the threshold between what should be called graphene and graphite.\(^12\) Finally GBMs can be readily functionalized by different methods,\(^13\) which open up a wider range of possible medical applications for these planar platforms.

In the section below, the main forms of graphene derivatives and how they are being considered for some exemplar biomedical applications will be reviewed, paying attention to the two main scales (macro and nano) of GBM biomedical materials.

2.1. Pristine Graphene

Novoselov et al. first used the “scotch-tape technique” to form atom-thin graphene sheets through the microexfoliation of graphite.\(^14\) Given its ability to form very high quality single layer graphene, this simple technique is still used in research but is not practical for large-scale production, hence commercial applications. A variety of other production techniques have been since developed,\(^9\) including ball-milling graphite to form graphene nanoplatelets (a mechanized version of the scotch-tape technique leading to few layer graphene).\(^15,16\) However it is the chemical vapor deposition (CVD) method that has emerged as the main production method for large-scale, high quality, single layer graphene.\(^17,18\) In this technique, methane is thermally degraded at high temperature (around 1000 °C), resulting on the deposition of carbon atoms to form graphene onto a metallic surface (usually copper or nickel). This last technique has therefore great potential for producing large film of conductive graphene.\(^19\)

For medical applications, single to few layer graphene films are primarily being considered as surfaces for the development of recording\(^20,21\) and stimulating\(^22\) bioelectronic devices (Figure 3)\(^23\) or biosensors,\(^24\) in which the carbon lattice of few micrometers in lateral dimensions will interface directly with either cells or biofluids, respectively.\(^25,26\) CVD can also be used to produce 3D graphene “foams” by deposition of carbon atoms onto nickel sponge,\(^27\) which can then be etched to leave a pristine graphene-based 3D structure.\(^10\) These foams have been explored for use as cell scaffolds for tissue engineering,\(^28–30\) and CVD was also proposed for the production of graphene film transferred onto the surface of devices such as dental implants, as a mean to transfer graphene properties to the coated substrate, such as shielding against corrosion.\(^31\)

One of the issues with graphene as biomedical material is that a graphene lattice is by nature hydrophobic, being completely impermeable to everything except protons.\(^9\) Given this, graphene nanosheets in aqueous phase are limited somewhat by their tendency to aggregate and drop out of solution. To circumvent this hurdle, various methods of liquid phase exfoliation of graphene from graphite based on the properties of chemicals...
to intercalate between graphene sheets have been developed.\textsuperscript{[32]} These methods have been recently adapted to the biomedical arena, using biocompatible exfoliating agents.\textsuperscript{[33]} At single cell level, these suspensions of graphene nanosheets are primarily being considered as nanovectors for the delivery of agents used in therapy or diagnosis. At larger scale, these nanosheet suspensions can also be used for the production of thin films via spray-coating, spin-coating, or inkjet printing methods, and for similar biomedical applications as the ones proposed for CVD graphene films, or for coating the surface of biomedical devices with complex 3D structure, such as cardiovascular stents.\textsuperscript{[34]}

2.2. Graphene Oxide

Because graphene nanosheets cannot form well-dispersed liquid suspensions without the addition of chemicals, many researchers have turned to graphene oxide (GO) sheets as reference nanomaterials for use in medical applications.\textsuperscript{[35]} Indeed, the oxide groups on the materials confer a hydrophilic nature and enable them to disperse in water without any additive.\textsuperscript{[36]} Moreover, GO can be produced in relatively large batches from graphite using chemical reactions such as the Hummers' method.\textsuperscript{[37–39]} The GO nanosheets resulting from these reactions are however not homogenous and typically exist as distributions of varying lateral dimensions, degree of functionalization and proportions of the different chemical functionalities present.\textsuperscript{[40,41]} In addition, impurities such as graphite or manganese may be present in the final product, if no purification steps are performed.\textsuperscript{[41,42]} These properties can also vary according to production batch, source of graphite, and the manufacturer\textsuperscript{[43]} and have direct impact on the response of biological systems to the GO used.\textsuperscript{[44–46]}

Despite these limitations, GO nanosheets still have the large surface area of 2D materials and owing to their distinctive surface oxygen groups can be readily chemically functionalized in
a somewhat easier way than pristine graphene. For these reasons, GO nanomaterial suspensions have attracted considerable research interest for imaging and diagnostic applications as well as vectors for drug and gene delivery. Along these injectable formulations, GO can also be deposited into films and membranes by a number of techniques. However, due to its aqueous dispersibility, films consisting purely of GO would be liable to instability in biological solutions, with the risk to release large amounts of individualized nanosheets into the biological environment. To this end, researchers developing “bulk” material medical applications using GO (as opposed to injectable nanomaterial suspensions) have tended to focus either on a reduced version of graphene oxide or composite materials.

### 2.3. Reduced Graphene Oxide

Compared to pristine graphene, the electronic conductance of GO is severely reduced, but the conductivity can be recovered by the reduction of the material. The resulting material is generally known as reduced graphene oxide (rGO) and the conductivity of rGO is proportional to its oxidation degree (i.e., the lower the degree, the higher the conductivity). Reduction of GO can be performed using a variety of chemical agents including hydrazine, l-ascorbic acid, and hydrohalic acids. It has also been performed thermally using lasers, steam, and dry heat. Moreover, the degree of reduction can be tuned by altering the chemical concentrations of reagents, pressure, temperature, or time. Therefore, just like GO, there is no one type of rGO but many possible variants of it. Noticeably, the resulting product of GO reduction will not only depend on the starting GO materials (varying with graphite origin, method of liquid phase oxidation, and degree of oxidation) but also the applied reduction process, as reduction can alter the amount and type of chemical functionalities present at the surface of rGO, the size of the resulting nanosheets in comparison to the starting ones and introduce chemical contaminants.

For medical purpose, while electrical conductivity of rGO does not quite match that of pristine graphene, it still compares favorably to many other materials. Moreover, the ability to use solution-based processes to create GO films, foams, and other structures followed by the reduction of the material in situ can confer advantages in using rGO compared to pristine graphene for a variety of bioelectronics applications, wherein depositing a single crystal (one atom thin film) without introducing defects is not an easy task. Furthermore, the enhanced stability of rGO-based films in aqueous solutions has also led to higher research interest in using rGO rather than GO materials for creating cell scaffolds and other implanted devices.

### 2.4. Graphene Composites

Although the number of biomedical opportunities offered by the three families of GBMs mentioned above is already high, researchers have looked to combine these materials with others to generate composites, expanding the number of combination to almost infinity.

Creating graphene-based composites can indeed confer a number of functional advantages to either the graphene materials or the partnering materials/matrix. In many cases these advantages are to do with providing a structure to host the graphene nanosheets. For instance, polymers have been used to distribute graphene nanosheets into functional highly ordered 3D structures for tissue engineering. Adjusting the relative composition of composite can also be used to control the mechanical properties or the pore size of partnering materials. In other studies, proteins and peptide sequences
have been used in graphene composites to improve the attachment of cells to the surface of tissue scaffold in regenerative medicine applications.[73,81]

When used as nanosheets, a number of metallic materials have been combined with graphene to enhance graphene properties; for instance, combining graphene with silver nanoparticles or iron oxide nanoparticles has been shown to have greater antibacterial effects.[82,83] Gold nanoparticles can also be used to modify the photothermal properties of graphene material for uses in biomedical imaging and cancer therapy.[84,85]

Overall, the versatility of GBMs especially when combined with other materials is what makes those materials one of the most exciting platforms in many areas of materials for healthcare (Figure 4).[11,86] However, irrespective of how great biomedical applications exploiting the properties of GBMs can be, it is their biocompatibility profile that will ultimately determine whether or not GBMs will translate into the clinic. It is therefore important to understand how GBMs perform in this respect.

3. Contribution of Graphene-Based Materials to Biocompatibility

3.1. Biocompatibility of Materials in Medicine

The first widely accepted definition of biocompatibility was proposed by Williams in 1987 and could be summarized by three following tenets,[87] namely that a material implanted into the body must perform its function successfully, that the biological response of the body should be appropriate for and justified by the intended application, and that this response and its appropriateness may vary according to the specifics of one application to another. This definition clearly links biological responses (and hence possible adverse effects) to application; making a clear distinction between what biocompatibility entails for biomaterials and what most people would name toxicity. As a result, one material can be considered biocompatible for a specific application but may cause toxic reaction if not used according to its designed application.

The decades following the Williams’s definition saw a great deal of progress in the development of biomaterials technology, guided by the Hippocratic maxim “do no harm.” In general, the approach taken by researchers during this period was to minimize the interaction between the tissue and the biomaterial. In other words, they aimed to generate inert materials that did not elicit local or systemic toxicity and generated only a minor local inflammatory response. For example, chemically reactive carbon steels in orthopedic implants were replaced by stainless steels and subsequently by passivating cobalt, titanium, and platinum alloys. This approach had a great impact on the success of these devices by reducing the inflammatory response of the body to the implanted materials, and there are now over 160,000 hip and knee replacements carried out each year in England and Wales alone.[88]

Orthopedic prostheses such as these have had a profound impact on the quality of life of millions of patients worldwide. Yet they are also a prime example of the complexity of assessing the response of biological tissues to materials and inadequacy of even long-established approaches to designing biomaterials. Indeed intact cobalt–chrome implants had been
thought to be benign, eliciting a relatively minor local inflammation and no immediately obvious toxicity. However, it is now apparent that the nanometer-scale wear debris from articulating cobalt–chrome surfaces in metal-on-metal hip prostheses can be profoundly toxic, causing localized tissue necrosis and have even been linked to systemic complications such as motor and psychosocial disorders. The complications arising from these effects in patients treated with metal-on-metal hip prostheses had attracted a lot of media interest and led eventually to medical device alerts from regulatory agencies and the discontinuation of several designs of orthopedic prostheses.

The contrast between the apparent biocompatibility of metal-on-metal hip prostheses in preclinical development and the adverse reactions in patients stands as a cautionary tale for all scientists and engineers currently developing novel biomaterial applications. It is a perfect example of the need to carefully consider a very large range of properties of a material during the determination of its biocompatibility. Both intrinsic (physicochemical) properties coming from the design and selection of materials, and characteristics appearing following the interaction with the biological system environment should be considered (see Table 1). And these biocompatibility considerations are even more important for emerging biomaterials with no clinical history and limited toxicological profile understanding, such as graphene-based materials.

3.2. How Graphene-Based Materials can Enhance Biocompatibility

In recent years, there has been a shift in scientific consensus around the definition of biocompatibility. Inert materials capable of sustained survival within the body with only minor inflammatory reaction have been requalified as "biotolerable" materials, while true biocompatible materials now require a functional integration into the host tissue with scar-free healing. However, functional integration of this type presents a much greater technical challenge for material development than that required for "simple" implant safety. And to date, a major barrier to the creation of fully integrated biomaterials has been the inability to create functional devices that are not only safe but also soft and flexible enough to match the mechanical compliance of the tissue. Indeed, mismatch in the elastic moduli between a biomaterial and the host tissue is known to trigger a number of different undesirable biological responses.

Owing to their flexibility as planar atom-thin materials, graphene and associated materials are therefore expected to make a significant difference in this area of tissue integration of biomaterials. This is in part due to their ability to create soft, flexible materials that maintain their mechanical properties but acquire enhanced abilities (such as greater stability, less brittleness/dissolution profile, or conductivity) through the addition of GBMs in the resulting composite.

A further advantage of graphene materials, unlike some other biomaterials such as metals, is the ability of cells to adhere directly to a variety of graphene surfaces and to spread and proliferate on these surfaces without apparent adverse effects (Figure 5). This ability has been demonstrated in numerous publications focusing on the development of tissue engineering/regenerative medicine scaffolds. Several material properties underpin the ability of cells to adhere at their surface, and surface chemistry is the main characteristic that controls extracellular protein adhesion, hence cell anchorage. While a systematic comparison of the three main types of possible GBM surface (graphene, vs graphene oxide, vs reduced graphene oxide) is not existing yet; there are matching reports evidencing that GBM-based surfaces facilitate cell adhesion, most likely due to a facilitated adhesion of proteins, which then favor cell anchorage. Moreover, it is possible to engineer the response of cells in contact with a material surface through a number of techniques, such as nanopatterning, which can help direct cell orientation, differentiation, and tissue formation. Because they can be easily sprayed, printed, coated, or transferred on any substrate or surface, in a customized fashion, GBMs are therefore of great interest to researchers in this area.

While GBMs seem to offer some advantages to make other materials more compatible with the body, their own biocompatibility/toxicity profile is however a matter that should not be neglected in the design of new GBM-based biomaterials.

4. General Considerations for the Biocompatibility of Graphene-Based Biomedical Materials

Due to the relative young age of GBMs as candidate biomaterials, the response of the body to injected or implanted GBMs (in preclinical models) is not yet fully understood or conclusive.
Indeed, there have been conflicting reports in the literature with some suggesting that implanted GBMs are benign,[36,105–107] and others indicating adverse responses including cytotoxicity,[108,109] inflammatory cell recruitment,[45,109,110] and tissue fibrosis.[46,110] Overall, the field of GBM biocompatibility appears immature and in need of more systematic assessment of GBM potential side effects before any overarching conclusion(s) can be made.[111] There are, however, few fundamental rules that can be followed to help avoid adverse biological reactions to GBMs when designing new biomedical materials based on these planar materials.

4.1. Consideration 1: Material Properties Matter

Discrepancies in the literature on GBM biocompatibility can be primarily ascribed to differences in the physicochemical characteristics of the materials used.[112,113] As discussed above, two materials that might be both named “graphene” may in fact be entirely different materials, because their lateral dimensions, thickness, oxidation degree, surface chemistry, formulation, and thus the way they interact with biological systems differ.[12,40,41] It is therefore not correct to make general statements on GBM safety or toxicity as these statements are likely true only in very particular circumstances depending on the properties of the GBMs used and conditions of GBM–tissue interface that depend on the intended medical applications. Instead, the direct relationship between material properties (a.k.a. structure) and their impact on cells or tissues (a.k.a. activity) should be defined. This so-called structure–activity relationship will help in guiding the design of safer GBM-based biomaterials using material properties that have been proven to not induce adverse effects.[44,114]

In addition, when performing biocompatibility assessment, all material properties should be considered: not only the intrinsic physicochemical properties of GBMs at the end of production, but also the biological properties of the materials that are acquired once GBMs start to interact with biological systems (e.g., adsorption of biomolecules, potential to biotransform or biodegrade, or ability to create extracellular or intracellular free radicals) (as mentioned in Table 1).[115]

Among all material properties that can be ascribed for GBM adverse effects, as previously reviewed in details,[46,111,112,116,117] lateral dimensions,[118] surface chemistry and reactivity,[46] thickness,[119] agglomeration,[120] and the presence or absence of biomolecule corona seem to be the most essential toxicity determinants for GBM nanoforms (Figure 6).

By extension, surface properties such as surface chemistry and reactivity, coatings (with biomolecules or chemical contaminants), or the nanotopography are believed to be the key factors of GBM biocompatibility at the macroscale, whereby the surface of GBMs is generally the intended component to be used (e.g., in tissue engineering scaffolds or medical devices).[73]

In both nanoscale and macroscale applications of GBMs, it therefore emerges that a thorough physicochemical and biological characterization of the used materials should be performed. And it follows that the characteristics providing the safest profile for the intended application should be selected in a safe-by-design approach to maximize biocompatibility and prevent unexpected failures, as seen for metal-on-metal implants.

4.2. Consideration 2: Chemical Contamination Should Be Avoided

A number of chemical compounds, which can be highly toxic, are routinely used in the production or processing of GBMs; therefore even trace residues of these compounds can have important impacts on the biological response to GBMs. Indeed, GBMs being planar materials, these chemical compounds will be directly present at their surface; i.e., they will be bioavailable, hence able to compromise the interface between GBMs and biological systems (extracellular matrix, cells, or tissues). In addition, taking into consideration the high surface area and bounding properties of GBMs, there is a high chance that a toxic amount of these chemical contaminants remains attached to the surface of GBMs if no preventive measures are taken. To limit these negative responses, two approaches could be implemented: i) identify potential harmful chemicals in the production or processing of GBMs, and substitute them for more benign alternatives,[118] or ii) ensure a thorough cleaning/purification of the
final materials to ensure the elimination of toxic residues, if substitution is not possible.\[41\]

One typical example of such inherent contamination from GBM processing would be the use of polymethylmethacrylate (PMMA) in the wet transfer of CVD graphene films or laminates. In bulk form, PMMA is relatively inert in respect to biological systems (hence its clinical use as a bone cement). But in nano- or micro-particle trace form (such as what can be expected to be found as a result of graphene film transfer) it can be proinflammatory and cytotoxic.\[121\] To limit this issue, the use of a dry transfer protocol for CVD graphene films\[122\] utilizing the comparably benign poly vinyl alcohol (PVA) rather than PMMA could be favored.

Another example is the choice of reduction method when preparing rGO samples from GO. In the literature, many researchers choose to use hydrazine as a reducing agent, but this is known to be highly cytotoxic\[123\] and carcinogenic,\[124\] consequently any hydrazine residues could have severe consequences for the biological response to the material surface. As an alternative, l-ascorbic acid (which occurs naturally within the body)\[58\] or high temperature\[61,64\] can be used to reduce GO without the possibility of introducing toxic contaminants.

A third example would be the choice of chemical compound used for mechanical or liquid phase exfoliation of graphene sheets from graphite. While most chemists would use an organic solvent to exfoliate graphene or other 2D materials,\[32\] it is advisable to use biocompatible compounds when GBMs or 2D materials are intended for biomedical use.\[16,33,125\]

4.3. Consideration 3: Sterilization and Depyrogenation are Not Negligible Issues

Sterility in the context of biomaterials can be defined as an absence of living microorganisms on a device, which may or may not be pathogenic. However, sterile medical implants can still be pyrogenic due to the remaining presence of microorganism fragments, known as endotoxins, such as lipopolysaccharides (LPS) from bacterial walls, which can lead to innate or adaptive immune reactions.\[126\] This in turn can lead to a range of undesirable outcomes, including rejection for implants or infusion reaction for injectable nanoformulations.\[127\]

Any materials that are intended for implantation or injection must therefore be sterilized and depyrogenated. And this rule applies not only for clinical setting but also for preclinical evaluation, including the biocompatibility/hazard assessment of candidate materials. Indeed sterility and nonpyrogenicity of candidate materials are essential to prevent any false or biased results, even more so for nanomaterials including GBMs due to their high surface area and binding properties.\[115,128,129\] Assessing the level of endotoxins (or worse microorganisms) in GBMs is therefore a paramount parameter of preclinical studies that should not be neglected,\[130\] as those biological contaminants may skew the results in particular when evaluating materials inflammogenicity.\[131\]

For medical devices made of conventional materials such as stainless steel, sterilization and depyrogenation can be achieved by several methods that have been validated by medical device regulators including dry heat (typically >220 °C), steam (i.e., autoclave), ionizing radiation (e.g., gamma radiation from Cobalt 60), or gas (typically ethylene oxide).\[132\] However, some properties of GBMs can be altered by the use of these regulatory-approved processes. A typical example is the use of steam or dry heat that will lead to the reduction of GO or further reduction of rGO. To circumvent those issues, nontemperature dependent processes such as gas sterilization should be preferred, in particular in cases where GBM films are present at the surface of the devices to be implanted. Alternatively, the sterilization and reduction of rGO materials can be designed as a single processing step where the means for sterilization doubles as a mean for reduction, as long as sterility is maintained beyond this point.
However, in some cases such as aqueous GBM suspensions or gel-like GBM composites, none of these conventional methods will be applicable; and sterilization by filtration would be inadequate because a large amount of materials will be lost in the filter. In this instance, aseptic processing of the materials from the very first stages of material production may therefore be more appropriate. For example, the Hummers’ method reaction typically used to produce GO involves high temperatures and low pH, enough to effectively sterilize and depyrogenate the materials. By ensuring that all subsequent processing steps are performed under aseptic conditions using depyrogenated glassware and containers, the sterility and nonpyrogenicity of the final product should be assured. Similarly when producing pristine graphene nanosheet suspensions, dry heating the starting material (i.e., graphite) before performing liquid phase exfoliation under similar aseptic conditions as the ones described above may be a sensible approach.

Importantly, the use of alternative sterilization methods other than the aforementioned processes recognized by regulators due to the unique requirements of GBMs should not be perceived as a barrier to clinical translation. However, where an unconventional sterilization method is utilized, it is likely that regulatory agencies will ask for additional evidence demonstrating the sterility and nonpyrogenicity of the final products. This is another reason for microbial contamination and endotoxin assessment to be part of the routine biocompatibility profiling of candidate GBM biomaterials.

5. Specific Considerations for Macroscale Applications of GBMs in Medicine

The three rules outlined above will be applicable to both nanoscale (i.e., injectable nanomedicines) and macroscale (i.e., implantable devices and scaffolds) forms of biomedical materials based on GBMs and should aid in the development of at least biotolerable materials based on GBMs. However, they are in no way a guarantee of developing materials that will be successfully translated into the clinic. A rigorous program of dedicated characterization and safety testing would be required to satisfy the concerns of healthcare regulatory agencies worldwide, and before commercialization of any candidate GBM-based medical product happens. While the key criteria for a safe design of GBMs in nanoscale application such as nanomedicines have been summarized in the above section and reviewed elsewhere, there are a few additional considerations for the macroscale applications of GBMs in candidate implantable medical devices or scaffolds that should be highlighted. These considerations relate not only to GBM characteristics, but also to the regulatory framework of implantable product safety assessment, with a particular focus on devices, which are likely the type of GBM biomedical applications that will achieve clinical translation in the near future.

5.1. Full Physicochemical Characterization in Both “Bulk” and “Nano” Forms

As mentioned previously, a prerequisite for the biocompatibility testing of any GBM is a deep understanding of the physical and chemical properties of that material. Indeed, the underlying mechanisms for any biological response to GBMs invariably lie within the properties of the specific graphene material that interface with the considered biological tissue. For GBMs that will feature in medical devices or tissue engineering scaffolds, this rule still applies. And more importantly, full characterization should apply not only to the bulk form but also the starting nanoscale GBM form used during the fabrication process of these bulk materials (i.e., CVD layer; nanosheet suspensions used to form laminates, membranes, thin films, or composites). This is in part due to the fact that in many cases the performance of the overall device or scaffold will be governed by the properties of these starting nanomaterials. But it is also as a necessity for achieving regulatory compliance. Indeed, for regulatory approval of medical devices in the EU, US, and many other worldwide markets, it is necessary to demonstrate compliance with the international standard ISO 13485, which covers quality control across the full production process of new medical devices (e.g., the ability to repeatedly produce the same final product with the same functionality and properties, using the same starting products). In order to meet this standard, amongst many other criteria, it is necessary to demonstrate that the properties and performance of the device are not changing over time. Complete physicochemical characterization of both the starting nanomaterials (as primary particles nanoform) and the final “bulk” version, for every production batch, can hence be used to evaluate changes in physicochemical properties between batches, or over time. Which physicochemical properties of the starting materials are of greatest interest will vary considerably depending on the specifics of the intended application. However, Table 2 sets out some of the properties of graphene materials that are of interest for many biomedical applications and the techniques commonly used to assess those properties.

5.2. Overtime Stability of Implanted Materials

The main concern raised by numerous researchers over the safety of GBMs has primarily focused on their nanofoms, as nanosheet liquid suspensions or aerosols. It therefore follows that the key concern with the safety of implanted materials containing GBMs will be with the release into the body of GBM nanofragments coming from the implanted bulk material, or the delamination of full GBM film from coated surfaces (Figure 7). Consequently, and as part of their integrated preclinical characterization/biocompatibility assessment, it is becoming imperative to carefully assess the wear and degradation profiles of GBM implanted materials under conditions as close to what they will experience when in clinical use as possible. Particular emphasis should be placed on determining the ability of bulk material/composites to degrade and form GBM by-products, as well as the physicochemical properties and biodistribution of those degradation products. Identifying mechanisms of degradation product release and the possible adverse effects of these by-products should be the next steps toward a thorough understanding of the biocompatibility profile of GBM implants.

A growing number of medical implants are designed to be temporary and are gradually but fully broken down by the body over time (e.g., resorbable bone screws or sustained release...
As of now, drug delivery devices). However, given the concerns around the safety of GBM nanosheets described above, mostly coming from a still incomplete understanding of their safety profile, the first GBM implants to be clinically translated are unlikely to be of this kind. Instead, GBM implants will likely be intended for transient use, following the path of preclinical proof-of-concept demonstrations such as intraoperative neural recording electrodes, where small piece of graphene attached to a substrate will interface with brain surface for few hours only.[21] Even in this case, preclinical researchers will still need to ensure that GBM delamination/degradation is not happening fast when these transient implants are implanted in or removed from their intended location. Alternatively, and to avoid degradation concerns, permanent GBM implants will need to be specifically designed and engineered to remain inert, stable, and to not release any GBM wear or degradation by-products. Both proposals clearly highlight the need for dedicated research on this aspect of the stability of GBM components in respect to the bulk material to help design composite biomedical materials from which GBMs are not being released.

Of note when considering this issue of GBM fragment release from bulk matrices or delamination from coated surfaces, there have been a series of reports demonstrating that GBM nanoflakes might not be as biopersistent as first thought. Different research groups have indeed demonstrated that various types of GBMs have the ability to biodegrade or biotransform into less reactive forms,[135–138] or clear naturally from the body.[139] Therefore, even if GBM by-products were to be released, these fragments might not be in a high enough amount to cause adverse effects and might disappear quickly from the body, as a result of these physiological degradation or elimination processes. In more details, one study reported that phagocytic cells were able to degrade GO and that the resultant products were nontoxic.[140] Another study showed that intravenously injected pristine graphene nanoplatelets were accumulating in organs of the mononuclear phagocyte system (e.g., liver, spleen), in which they were undergoing degradation in macrophages.[137] In both cases, degradation was attributed to an oxidative enzyme-based mechanism (Figure 8).[135] Finally, it was demonstrated that it is possible for thin GO sheets, regardless of their lateral dimensions, to be cleared from the blood stream by urinary excretion,[139] and that this process does not impact kidney function.[141] However, the stacking of GO sheets into thicker nano-objects appears to impair this urinary clearance, with greater accumulation in organs of the mononuclear phagocytic system or the lung vasculature.[119] Interestingly, some research group are now integrating these degradation properties in the design of new GBM biomedical systems,[142] with the aim to create materials with greater/faster degradation ability, which will comply well with the trend of having resorbable implanted biomaterials as mentioned above.

A more systematic understanding of what specific conditions and material properties underpin the differences between the apparently conflicting reports of adverse biological reactions to GBMs and the safe clearance of GBM nanoforms from the body could hence be used to engineer graphene-based biomedical materials with enhanced biocompatibility, even if GBM fragments were to be released over time. The long term fate of these fragments and potential to induce adverse effects should also be of interest in future biocompatibility testing of implanted GBM based products.

5.3. The Likely Regulatory Classification of Biomedical Materials Based on GBMs

Throughout this review, the term “biomedical materials” has been used as a generalization when referring to graphene-based biomedical products. It is important for the reader to note, however, that biomedical products are typically categorized by regulatory agencies as either medical devices, medicines (including drugs and biologics), or advanced therapeutic medicinal products (ATMPs). ATMPs consist of therapeutics

"Figure 7. Overtime stability issues related to graphene-based biomedical materials that could lead to the release of GBM fragments in the body."
based on the delivery of tissues, cells, or genes to a patient to treat disease. These differ to transplants in that the tissues, cells, or genes must have been treated or modified in a laboratory setting in some way prior to implantation. The regulatory requirements placed on these different categories of biomedical products differ greatly and it is important for researchers developing new graphene-based biomedical materials to consider how their innovations will be classified.

For the purpose of this section on macroscale biomedical use of GBMs, we have assumed that the majority of graphene-based biomedical products in this category will be classified as medical devices. To that end, researchers may want to refer to the regulatory requirements of major markets such as the EU (Regulation (EU) 2017/745 of the European Parliament)\[143\] and the USA (FDA 2018 Medical Device Safety Action Plan) for further details on safety testing.\[144\] Information on the regulatory systems in place for medical devices in any global territory can also be found in the WHO Global Atlas of Medical Devices 2017.\[145\] However, as discussed previously, these regulations may not be applicable to all new graphene-based biomedical products, and will depend on their specific characteristics. For instance, graphene-based nanovehicles for targeted drug delivery would likely be classified as a medicine rather than a device. On the other hand, graphene-based nanovesicles for the delivery of genetic material could likely be classified as ATMP. Similarly, an acellular GBM-based scaffold for supporting the repair of native tissues (for example in peripheral nerve injury) could be classified as a medical device; however, if non-native cells, such as stem cells, were included in that scaffold prior to implantation, it is likely to be classified as an ATMP.

5.4. Regulatory Assessment of Medical Device Biocompatibility: A Further Push Toward Early Stage Safety Testing of Graphene-Based Biomedical Products

Medical device regulations are complex and contain requirements (such as on the packaging of medical devices) that may only become relevant as a device is heading toward first-in-man use. ISO standards provide guidance on specific aspects of medical device assessment that may be more beneficial to researchers at the preclinical stages of development. These standards are adopted by the majority of countries around the world and compliance with them can help to reconcile differences in the regulation of biomedical products in different global territories. Indeed, in many instances medical device regulations are based on these standards and compliance with them is a regulatory requirement.

For instance, irrespective of the chemical composition of the material(s) used, the international standard ISO 10993 provides guidance on the biocompatibility tests appropriate for new medical devices in development.\[146\] To achieve the regulatory compliance necessary for clinical translation, this standard has to be followed in combination with ISO 14971 risk management framework.\[147\] ISO 10993 categorizes medical devices according to the nature of their contact with the human body and the duration of that contact (Table 3). The duration of contact is defined as being limited (≤24 h), prolonged (>24 h to 30 d) or permanent (>30 d).

By definition, the potential risk to the health of patients from the medical device will both change in its nature and increase in its severity with increased duration of interface and increased intimacy of interface with the internal tissues. The overall risk to patients posed by a new medical device is therefore a product of both the nature of the interface with the body and the duration of that interface. As a consequence, the tests performed during the biological evaluation of a new medical device and the burden of evidences expected by medical regulatory agencies for authorizing this new medical device will increase proportionally to the expected risk. For instance, a new design of daily contact lens will require a less rigorous biological evaluation than a permanently implanted deep brain-stimulating electrode.

In addition, ISO 10993 provides a framework for possible biological investigations that depend on the nature of the medical device.\[146\] But it is not prescriptive, expert opinion is still required to review and judge the investigations required for each new medical device depending on the specific characteristics of that device. Possible biological outcomes that may require consideration include (but are not limited to): sterilization, cytotoxicity, irritation, acute systemic toxicity, subchronic and chronic systemic toxicity, carcinogenesis, genotoxicity, reproductive/developmental toxicity, hemocompatibility, and other organ-specific toxicities. The intended use will hence guide the selection of assays that are relevant.

With these regulatory considerations in mind, the lack of clinical history with GBMs and the possible variation in their physicochemical properties (i.e., each GBM is different) and macroscale applications (medical devices or tissue engineering/regenerative medicine products) mean that the biological evaluation and regulatory validation of new medical devices and products based on GBMs could likely be a long and rigorous process.\[148\] It is expected that thorough in vitro and in vivo investigations will be required to demonstrate the safety/biocompatibility of these devices and allow regulatory agencies to authorize commercialization. To facilitate this process, proactive measures such as the integration of biocompatibility testing directly in the development phase of proof-of-concept
biomedical materials based on GBMs have been implemented in some large research consortium.[6,21,23] The aim of such initiative is to speed up the translation of academic proof of concept to the clinical setting by demonstrating that improved functionality is not achieved while neglecting biocompatibility. Moreover, integrating safety considerations early on in the development of biomedical materials can help when modifying the design of GBMs (e.g., changes in GBM surface functionalization) to make the final product safer without hindering its GBM-enhanced functionality.

6. Conclusion and Perspectives

Graphene and its related materials undoubtedly have the potential to make an enormous impact in medicine, both by improving the performance of existing medical technologies and by enabling the development of technologies that were previously not possible. However, for the present, there remains a great deal that is unknown with regards to the interactions between GBMs and biological systems and the consequences of these interactions, fostering the need for further research in this area, if graphene technology is to achieve its great potential in medicine.

Historically, researchers have used the clinical history of materials such as polymers and metals to help guide the safety assessment of new related materials, and improve the design of the next generation of biomaterials. However, the properties of GBM are unique and the development of graphene-based biomedical materials cannot be approached in the same way. In view of the lack of clinical history with these planar materials, preclinical researchers are advised to implement safety/biocompatibility considerations as early as possible in the design and development of their candidate graphene-based biomedical materials if they want to achieve a fast clinical translation.

In this mini-review, we have tried to address some of the unique biocompatibility challenges presented by the clinical translation of graphene-based biomedical materials and provide some basic guidance as to how to go about overcoming them. We have proposed three key solutions to help promote a tolerable biological response from an early stage in the development of graphene-based biomedical materials, namely: i) perform a thorough materials characterization and design the GBMs in line with a safe-by-design approach and with respect to the structure–activity relationship, ii) avoid chemical contamination and think about alternative production methods specific to the biomedical application, and iii) consider a sterilization strategy in line with the intended application and GBM function, as early as possible. In addition, unlike with many “conventional” biomedical materials, researchers will have to consider the physicochemical and biological characteristics of both the whole biomedical material (e.g., device, scaffold, or nanovector system) and its constituent nanomaterials. The evolution of these characteristics over time with respect to the specific application of the material (e.g., protein adsorption or the release of wear products) would also have to be considered. Last but not least, the intended application and the material design will impact the regulatory classification of the candidate biomedical materials, which in turn will impact how biocompatibility should be tested. It is therefore advisable to integrate these criteria early on in the development to ensure that the appropriate

Table 3. Regulatory classification of medical devices.

| I | II | III |
| --- | --- | --- |
| Surface contacts | External communicating devices | Implanted devices |
| Skin (e.g., compression bandages) | Indirect blood path (e.g., blood administration sets) | Tissue/bone (e.g., joint replacement) |
| Mucosal membranes (e.g., contact lenses) | Tissue/dentin (e.g., dental cements) | Blood (e.g., heart valves) |
| Breached or compromised surface (e.g., wound dressings) | Circulating blood (e.g., dialysis tubing) | |

Figure 9. The challenges of graphene-based materials biocompatibility.
biocompatibility assessment allowing clinical translation has been applied during the preclinical development.

Reconciling the material properties required for a benign interface with the body and those required for the effective function of the biomedical materials is likely to be a great technological challenge (Figure 9), but one that is very much worth addressing when considering the vast potential of GBMs for biomedical applications. There is also a great deal that remains to be understood in the interactions between the body and GBMs, and as with any medical technology, there is risk inherent in the unknown. It is therefore likely that the first graphene-based devices to be clinically translated will be those intended for topical use or short-term transient implantation.

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Conflict of Interest

C.J.B. is declaring being the co-founder of Honeycomb Biotechnology Ltd. registered in England and Wales with company number 11608210.

Keywords

biocompatibility, graphene, medical products, safety testing

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