Biocompatibility and biodegradability of 2D materials: graphene and beyond

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The potential risks associated with two-dimensional (2D) nanomaterials may cause serious concerns about their real applications and impact in biological systems. In addition, the demonstration of biodegradability of these flat nanomaterials is essential in living organisms. Here, we summarise the state-of-the-art in the field of biocompatibility and biodegradability of graphene-related materials (such as 2D materials like MoS_2, BN or WS_2). The impact of chemical functionalisation on the potential control of the biodegradability profile of these structures is also discussed.

The development of two-dimensional (2D) materials, especially after the isolation of graphene in 2004, has stimulated enormous interest due to their unique properties.[1] In fact, although graphene is probably the tip of the iceberg (since it is the most widely studied), there are other 2D materials (Fig. 1), which contribute to the mass of the same iceberg and are still unexploited and unexplored.[2]

The numerous applications of these layered nanomaterials including their functionalised derivatives are undeniable, due to their extraordinary physicochemical properties.[3,4] Many reviews have previously summarised 2D materials spanning from their technological[5–7] to their biomedical uses.[8,9] Therefore, being aware of the environmental impact and possible risks on health of 2D nanomaterials it is imperative to address these concerns in a proactive manner,[9,10] particularly in the case of bio-applications. In this context, this highlight focuses on the biocompatibility and biodegradability of 2D nanostructures reported over the last few years, including the role played by surface functionalisation.

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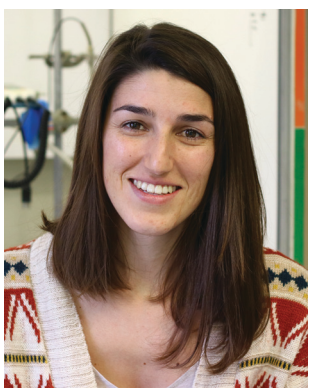

Cristina Martín

Cristina Martín finished her Chemistry studies in 2012 in the University of Castilla-La Mancha (Spain) and obtained her PhD in Chemistry in 2016 from the Universities of Trieste (Italy) and Castilla-La Mancha, working under the co-supervision of Prof. Maurizio Prato and Prof. Ester Vázquez. During her PhD, she spent 3 months at the University of Brighton (UK) under the supervision of Prof. Matteo Santin. As a postdoctoral researcher she worked in 2016–2017 at IRICA (Spain). In October 2017 she joined the group of Dr Alberto Bianco as postdoc. Her research focus is mainly on carbon nanostructures and their biodegradation and biomedical applications.

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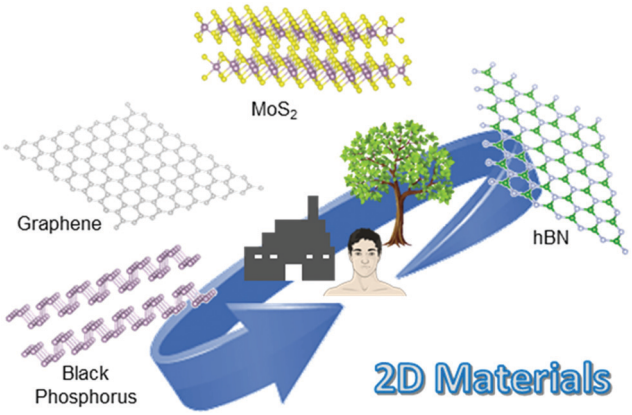

Fig. 1 Molecular schematics of some of the most developed 2D materials beyond graphene.

2. Biocompatibility

The ability of materials to interact with cells, tissues or a living body without causing harmful effects is known as biocompatibility. Comparing the biocompatibility by cytotoxicity studies of 2D nanomaterials, which differ in composition, size, number of layers, or functionalisation degree, helps to better understand the mechanisms responsible for any toxic effect, really assessing the hazard potential of these materials.

Regarding graphene-based materials (GBMs), Pinto *et al.* reviewed in 2013 their biocompatibility based on existing work.[11] The authors concluded that some studies reported the decrease or the slight decrease of bacterial and mammalian cell viability, after exposure to GBMs. However, systematic studies of the effect of the particle size on cell viability were still lacking. Moreover, knowledge of the long-term cytotoxicity of GBMs or the effect of these nanomaterials on cell signalling, among other biological processes, was just beginning to be unraveled. Soon after, it was found that pristine and functionalised graphene cause negligible ($>0.2\%$) hemolysis in red blood cells (up to 75 μg mL^{-1}).[12] In another interesting study, graphene synthesized through the chemical vapor deposition (CVD) method on copper foils was explored as a substrate to promote the cardiomyogenic differentiation process of mesenchymal stem cells.[13] The nanomaterial did not exhibit any sign of cytotoxicity for the stem cell cultures, and the cell signalling molecules involved in the cardiomyogenic differentiation were upregulated. More recently, some of us showed that CVD graphene[14] and graphene oxide papers[15] could act as neuronal and other mammalian cell substrates favouring growth with no cytotoxic effects.

Gurunathan *et al.*[16] described the synthesis of graphene and GBMs, and highlighted their biocompatibility in the context of biological applications. The authors concluded that more studies on toxicity *versus* biocompatibility were needed, especially using *in vivo* models. Very recently we reported an extensive survey on safety assessment of GBMs by analysing the most up-to-date data on their biocompatibility. We classified a range of examples in different 3D graphs according to each particular GBM and each specific impact (*i.e.* on macrophages, on lungs, on the gastrointestinal tract).[17] The general conclusion of this comprehensive work is that GBMs can be categorised according to their physicochemical characteristics and cannot be considered as a single type of materials. Indeed, GBMs differ according to three key parameters: the number of layers, dimension, and carbon-to-oxygen atomic ratio, and these parameters modulate the toxicity of each specific GBM, as postulated earlier by some of us.[18,19] We observed a predictable pattern of effects for the selected examples, but there are still missing gaps to be filled with alternative GBMs for more systematic characterisation.

Regarding the influence of surface functionalisation on nanomaterial biocompatibility, many factors need to be taken into consideration. The GBM synthesis method in most cases tunes the functionalisation degree of the sheets, influencing for instance the cellular internalisation and other biological processes. It has been demonstrated that reduced graphene oxide (rGO)

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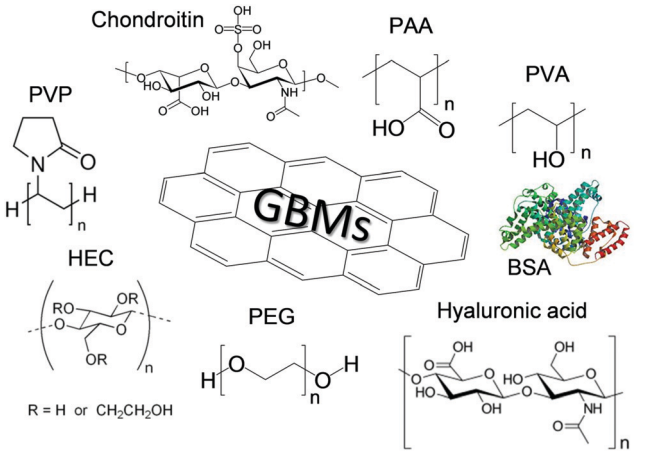

Fig. 2 Functionalisation strategies to enhance the biocompatibility of GBMs.

functionalised with a biocompatible biopolymer, which makes it highly stable in water, showed good cytocompatibility towards endothelial cells even at very high concentrations (*i.e.* 100 μg mL^{-1}).[20] In another work, graphene nanoplatelets were modified with poly(vinyl alcohol) (PVA), hydroxyethyl cellulose (HEC), poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone), chondroitin, glucosamine, and hyaluronic acid (Fig. 2).[21] All materials resulted in low haemolysis up to 500 μg mL^{-1}, which further decreased after polymer adsorption, reaching the best results with PVA and HEC. However, differences regarding biocompatibility were observed, being improved using PVA. The authors explained the differences in terms of the encapsulation and agglomeration of the graphene platelets by PVA, decreasing the cell interaction/internalisation. In addition, the behaviour of few-layer graphene (FLG) was assessed during three months *in vivo*,[22] finding that the PEGylation of FLG significantly reduced the histological abnormalities in comparison to the unmodified materials.

Jasim *et al.* had previously shown that a very large proportion of intravenously injected thin GO sheets functionalised and radiolabelled with In-111 were able to be excreted rapidly in the urine without causing kidney (or other tissue) damage.[23] In addition, the critical role played by the thickness of functionalised graphene oxide (GO) sheets in tissue accumulation and urinary excretion has been recently evaluated.[24] This pilot study provides an initial correlation between GBM structures and pharmacological profiles to understand how 2D structures behave *in vivo*. Yang *et al.* previously reported a quantitative evaluation of the biodistribution of GO after administration using ^{125}I-labeled nanosized GO further functionalised with PEG.[25] The biodistribution revealed a higher accumulation in the spleen compared to that in the liver at all time points. In a slight contrast with these results, a clear improvement of biocompatibility both *in vitro* and *in vivo* has been demonstrated for GO after surface modification with poly(acrylic acid) or PEG.[26] All these representative examples covering different types of GBMs illustrate how the characteristics of the starting material and its chemical modification influence their biological impact, eventually allowing the development of safer GBMs by-design.

Similar to GBMs, the atomic composition, the exfoliation process and the lateral dimensions of transition metal dichalcogenides (TMDCs), hexagonal boron nitride (hBN, also termed "white graphene") or black phosphorus (BP) are key factors in determining their biocompatibility.[27–30] The group of Pumera has studied the role played by the chalcogen atoms in the cytotoxicity of TDMCs.[31–33] The differences in the chemical reactivity of each TMDC are related to the release of the chalcogens, resulting in higher toxicity. In general terms, selenium and vanadium play an important role in the toxicity, and ditellurides show higher cytotoxicity than disulphide containing materials.[27] The exfoliation process is also important; however, its correlation with the levels of cytotoxicity is not very clear yet, since there are several works resulting in contrasting data. As it has been previously discussed for GBMs, the functionalisation of these alternative 2D nanomaterials can lead to control of their biocompatibility.[34] Among the series of related papers, it has been reported that the functionalisation of MoS_2 sheets with lipoic acid-modified PEG increased their physiological stability and biocompatibility.[35]

The concentration of the 2D nanomaterial or the type of cell lines used in the experiments also plays an important role. In fact, a few studies have shown dose-dependent toxicity using different cell lines.[36] Dose-dependent toxicity has also been observed for BP sheets using human lung carcinoma epithelial cells,[37] while BP quantum dots did not induce inflammatory responses.[38]

Concerning the *in vivo* impact of this type of materials, the most important studies today have focused on functionalised 2D materials. For example, iron oxide decorated MoS_2 PEGylated nanosheets have been used for chelator-free radiolabelling and multimodal imaging guided photothermal therapy.[39] This multifunctional conjugate was used for *in vivo* experiments, since the PEGylation endows the nanocomposite with enhanced biocompatibility and a more favourable pharmacokinetic profile. BP nanosheets have also been proven to be robust delivery platforms for cancer theranostics.[40] The drug-loaded PEGylated BP nanosheets showed excellent long circulation confirmed by pharmacokinetic experiments, good biocompatibility, and enhanced antitumor effects both *in vitro* and *in vivo*.

In conclusion, the mechanisms and material parameters responsible for tissue damage induced by 2D materials still need to be thoroughly examined. More investigations need to be performed to reveal the possible health risks of both 2D nanomaterials and related composites and hydrids.[41–43]

3. Biodegradability

Biodegradability refers to the ability of microorganisms to modify and alter the structure of a material by their metabolic or enzymatic action (Fig. 3). The complete clearance from the body and the biodegradation of 2D nanostructures need to be demonstrated in order for these materials to be approved for clinical use and to validate their safe use. Therefore, understanding the mechanisms leading to biodegradation has been associated to biocompatibility. Several techniques such as

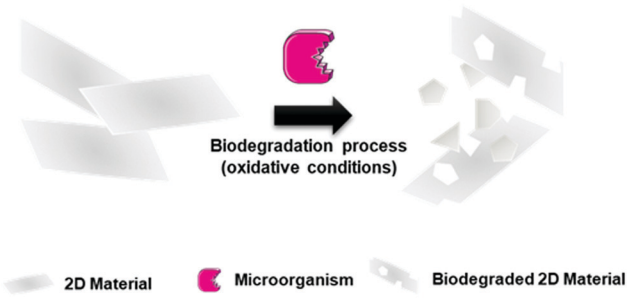

Fig. 3 Schematic representation of 2D material biodegradation.

Raman, mass spectrometry and transmission electron microscopy can be used to assess biodegradation, particularly following the morphological and structural changes of the nanomaterials during this process.[44]

GBMs, and carbon nanomaterials in general, were assumed to be structurally persistent. However, subsequent work evidenced that oxidative enzymes (*i.e.* peroxidases) are able to catalyse the degradation of graphene oxide or carbon nanotubes in test tubes, *in vitro* and *in vivo*.[45–48] In addition, our very recent review carefully evaluated the role of the material properties including the number of layers, the lateral dimension and the C/O ratio in the degradation ability of each specific GBM (Fig. 4).[17] In a comparative study it has been demonstrated that the degradation of GO sheets by hypochlorite was faster than that of 1D oxidised carbon nanotubes or nanohorns.[49] The research performed in the last few years allowed us to confirm also the importance of the nanomaterial dispersibility,[50] the synthetic strategy and the role played by surface functionalisation in the biodegradation process.

Among GBMs, GO is the most studied 2D material in the biological context due to its versatile surface modification and

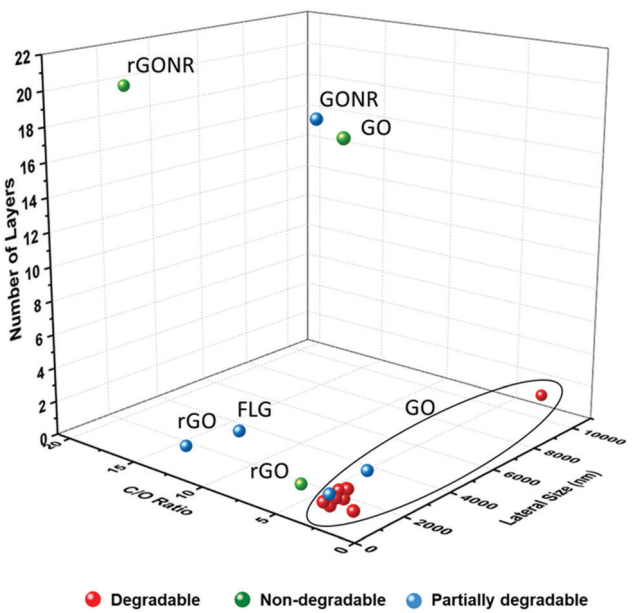

Fig. 4 Categorisation of the GBMs tested in studies on degradation reported in the literature (GONR, graphene oxide nanoribbons). Reprinted from ref. 17. Copyright 2018 American Chemical Society.

the aqueous dispersibility. The investigation of the effect of surface coatings on the biodegradation of GO and its derivatives revealed that both PEG and BSA (bovine serum albumin) protect the material from degradation by horseradish peroxidase (HRP).[51]

In view of those results, the authors of the study designed an intermediate cleavable disulfide bond strategy that rendered the 2D nanomaterial biodegradable with negligible toxicity. Our group devised a new system to "attract" enzymes toward GO by functionalising the nanomaterial with coumarin and catechol, which are natural ligands of HRP.[52] Not only GO but also single-layer graphene and few-layer graphene have been recently investigated. We have studied the biodegradability of these materials by human myeloperoxidase (MPO) and in the presence of degranulating human neutrophils, cells that are able to secrete high concentrations of MPO when activated.[53] The degradation of both single- and few-layer graphene was proved, concluding that these water-dispersed pristine carbon nanostructures are not biopersistent. Finally, we also demonstrated that alternative artificial enzymes, like DNAzymes consisting of a PS2.M-hemin complex that mimic HRP, can degrade GO.[54] However, additional studies to test not only *in vitro* but also *in vivo* degradation and elimination of GBMs are necessary, in order to exclude their possible long-term accumulation and persistence.

Interestingly, very little is still known on the biodegradation possibilities of other non-carbonaceous 2D materials like hBN, MoS_2, graphitic C_3N_4, 2D clay materials or BP monolayers.[55–59] The possibility of HRP, MPO and photo-Fenton reaction to degrade hBN was assessed by our group in 2016.[60] We saw that HRP does not degrade hBN up to 60 days, while partial oxidation was observed by using MPO after 35 h and nearly complete oxidation/degradation of hBN occurred by photo-Fenton reaction within 100 h. We also examined in a different work the biodegradability of water dispersible pristine and functionalised MoS_2 nanosheets.[61] Interestingly, both nanostructures showed a much quicker degradation in the presence of low concentrations of H_2O_2 without any enzymes compared to HRP or MPO treatments. In fact, it has also been demonstrated that MoS_2 nanosheets are thermodynamically and kinetically unstable in the presence of O_2, being degraded under ambient conditions in various oxidising aqueous environments.[62] The *in vivo* long-term biodistribution, excretion and toxicity of PEGylated TMDC nanosheets have also been reported.[63] This study demonstrated that PEGylated TMDCs made of MoS_2, WS_2 and TiS_2 can accumulate in the reticuloendothelial system after intravenous injection. However, only MoS_2-PEGylated TMDC was degraded and excreted within one month due to its different chemical properties compared to modified WS_2 and TiS_2. The degradation ability of these alternative 2D nanostructures renders feasible their further development in the creation of new hybrid materials able to combine biodegradability and biocompatibility with biomedical applications such as, for example, photothermal cancer therapy.[64]

4. Conclusion and future prospects

Although a lot of research has been carried out in the field of biocompatibility and biodegradability of 2D nanomaterials, the

progressive rise of such materials is indisputable,[65] and the behaviour of the new 2D heterostructures that continue to appear needs to be assessed. Their safety evaluation should not only comprise the most recent GBMs that arise from novel strategies or that are characterised by modified surfaces, but also 2D materials like Si_2BN,[66] borophene,[67] ZnO,[68] among others that are of novel chemical consistency. In addition, the majority of the studies performed until now have been carried out *in vitro*, while critical validation tests should be extended to whole model organisms. Finally, the fundamental aspects and the mechanisms of their biological effects and actions are still poorly understood. The design of new artificial enzymes mimicking natural systems could help to fulfil this purpose. Therefore, more research has to be performed to cover the demand of rapid and high-content screening by generating large data sets that help to measure the safety of 2D materials.[69] That is the only way to ensure the relevance of these materials in mass market technological and biomedical applications.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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