Graphene-based Engineered Living Materials

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Published in [Small Methods]

The full version of this paper is available online on:

https://onlinelibrary.wiley.com/doi/full/10.1002/smtd.202300930

DOI:

https://doi.org/10.1002/smtd.202300930

Keywords: engineered living materials, graphene-based nanostructures, living graphene, smart living materials

With the rise of engineered living materials (ELMs) as new sustainable and smart systems for different engineering and biological applications, the global interest in the development of more advanced living materials is on the rise. In this race, graphene-based nanostructures can be an effective tool to fabricate ELMs beyond limitations. By using graphene-based materials as building units and microorganisms as the designers of final materials, next-generation ELMs with structural properties of graphene-based materials and the smartness of microorganisms can be developed. However, some challenges need to be addressed to fully take advantage of graphene-based nanostructures for the design of next-generation ELMs. This review covers the most recent advances in the fabrication and application of graphene-based ELMs. We first categorized fabrication strategies for the preparation of graphene-based ELMs and then systematically investigated the advantages and disadvantages of each fabrication category. Next, the reported potential applications of graphene-based ELMs are covered. Moreover, challenges for the fabrication of next-generation graphene-based ELMs are identified and discussed. Based on our systematic overview of publications, nanotoxicity due to synthetic and structural parameters is the main challenge that limits the concentration of graphene-based nanostructures in ELMs. Finally, we conclude with some possible design principles that potentially can be used to solve these challenges.

1. Introduction

Engineered living materials (ELMs), as a nascent field of interest on the edge of biomaterials engineering, biological engineering, and materials science, hold great potential for the development of next-generation smart and high-resolution designed materials for a variety of applications in different advanced technologies, from biomedical to engineering.[1] The final goal in this field is to design biological materials or systems, consisting of natural or engineered microorganisms, that can not only add features such as smart response to condition changes (pH, temperature, etc.) and self-healing to the system but also the option of natural design and assembly to biologically mimic the final material (in situ growth of biomaterials).[2]

Although ELMs have opened a new chapter in the design of biomaterials, the environmental concerns regarding the release of microorganisms into the environment have raised some questions about the wide application of these materials in the industry.[3] Encapsulation of microorganisms can be an effective solution to this challenge.[4] Microorganisms can be encapsulated/trapped in the structure of porous materials, such as carbonous and polymeric hydrogels, to reduce the chance of their release into the environment.[5] The microorganism's leakage in such a system depends on the pore texture of the hydrogel and the adhesion mechanism of the microorganism to the surface of the hydrogel.[6] Consequently, the chemical nature (the type of the material) and the texture (pore size, pore volume, etc.) of the encapsulating hydrogel are influential factors in the successful encapsulation of microorganisms for the development of hydrogel-based ELMs.

Graphene-based nanostructures are one of the most interesting classes of nanostructures for application in a variety of different engineering and biological fields.[7] Different types of graphene-based nanostructures have been used for biological applications.[8] Moreover, these nanostructures hold great potential for interactions (either constructive or destructive) with living microorganisms, making them interesting choices for the development of advanced ELMs.[8] However, challenges that can arise in the development of graphene-based ELMs are of great importance and worth close consideration.

Despite recent progress in the field of ELMs,[9] the effective convergence of distinctive structural and functional attributes of biotic and abiotic elements of an ELM is another challenge yet to be addressed. These inherent structural differences get much more important when building units in the structure of ELMs are inherently incompatible components like graphene-based nanostructures and living microorganisms.[10] Although several reports are available in the literature on the biocompatibility of graphene and its derivative 2D and 3D structures,[11] the nanotoxicity and genotoxicity of these materials are still in question.[12] In particular, there are several reports on the nanotoxicity of graphene oxide (GO), graphene quantum dots (GQDs), and graphene nanosheets against extracellular biopolymer-producing microorganisms, such as bacteria and algae, that can limit the application of these materials in the fabrication of graphene-based ELMs.[13]

In this work, recent progress and main unsolved challenges in the field of graphene-based ELMs as one of the most recent types of ELMs are overviewed. First, recent reports in the literature on graphene-based ELMs are covered. Based on the available reports, the main strategies for the fabrication of graphene-based ELMs are categorized into four categories in this work, and each category is introduced. Then, the structural properties and potential applications of graphene-based ELMs are overviewed. Finally, possible paths that can be followed to solve the remaining challenges in the design of next-generation graphene-based ELMs are suggested and the prospects for the impact of graphene-based ELMs on some important application fields are illuminated.

2. Graphene-based ELMs

We categorize graphene-based structures in this review into nanosheets, quantum dots, three-dimensional, and nanocomposite geometrical categories (**Figure 1 a**). This classification is based on the impact of each geometry type on the viability of microorganisms through the fabrication process and within the structure of final living hydrogels. Graphene is a two-dimensional nanosheet with a thickness of one carbon atom. The sp^2-bonded carbon atoms in the structure of graphene are densely packed in a honeycomb lattice. However, such a single-layer nanosheet not only is hard to synthesize on a large scale but also difficult to process into applicable structures. Moreover, the formation of different types of molecular defects in the structure of graphene nanosheets is inevitable.

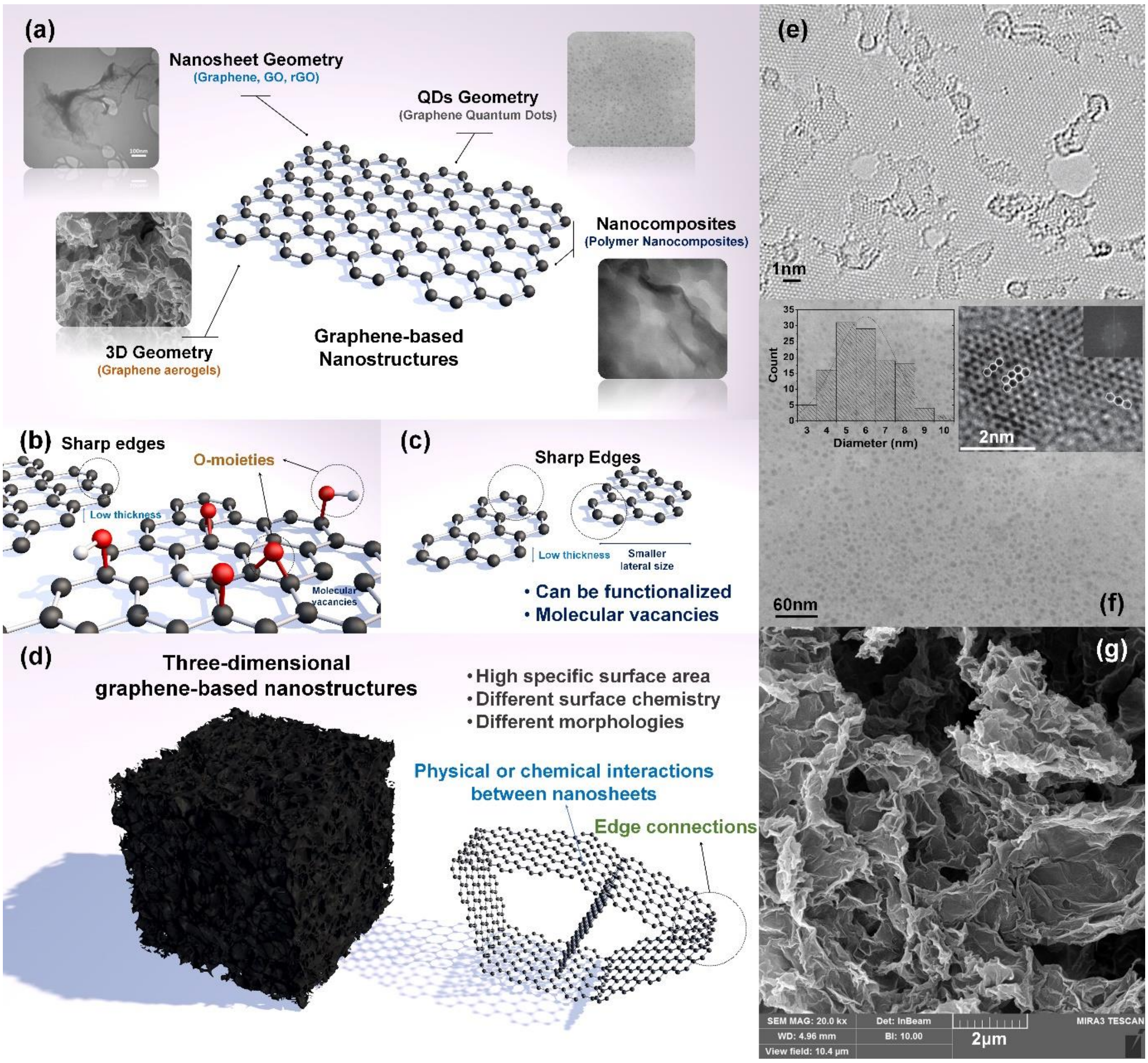

Figure 1. Classification of graphene-based nanostructures: General classification based on the geometry (a) including nanosheets (transmission electron microscopy, TEM, image of graphene oxide, GO, reproduced with permission.[14] 2020, Elsevier.), quantum dots (TEM image of nitrogen-doped graphene quantum dots, N-doped GQDs, reproduced with permission.[15] 2021, Elsevier.), three dimensional (scanning electron microscopy, SEM,

image of reduced GO, rGO, aerogel, reproduced with permission.[16] 2023, Elsevier.), and nanocomposite (TEM image of polysulfide/GO nanocomposite reproduced with permission.[17] 2020, Elsevier.); Main atomic characteristics of GO (b) and GQDs (c) that can affect the toxicity of these graphene-based nanosheets against microorganisms; the arrangement of rGO nanosheets in the three-dimensional structure of graphene-based porous materials is different from other types of graphene-based nanostructures and in these porous materials, the interconnection between nanosheets leads to less sharp edges in the structure (d); Atomic resolution TEM image of a single layer rGO indicating the presence of molecular vacancies (e); (Reproduced with permission.[18] 2010, American Chemical Society.) TEM of N-doped GQDs indicating the size range of these quantum dots (Reproduced with permission.[15] 2021, Elsevier.) and atomic resolution TEM image of a single GQD indicating the presence of sharp edges and molecular vacancies (Reproduced with permission.[19] 2013, Wiley.) (f); and SEM image of rGO aerogels indicating the 3D porous structure of these graphene-based materials (g) (Reproduced with permission.[16] 2023, Elsevier.)

GO has a thickness of 1-1.5 nm and is decorated with different O-moieties on both the basal plane and edges (Figure 1 b). Compared to graphene, GO has slightly more potential for the design of graphene-based ELMs, as O-moieties on the edges of GO reduce slightly the chance of harm to the membrane of the living microorganism.[20] However, the formation of reactive oxygen species (ROS) and the related toxicity are more possible in the presence of O-moieties on the edges and basal plane of GO nanosheets.[20]

The reduction of GO (thermal or chemical) leads to the formation of rGO.[21] Compared to GO, rGO has fewer O-moieties on the basal plane and edges, and hence, the surface chemistry and molecular properties of rGO nanosheets are different from GO nanosheets.[21] The number of vacancies in the structure of rGO is more than GO, as the reduction process increases the number of such defects (Figure 1 b and e).[22]

Graphene quantum dots (GQDs) are similar in geometry to graphene-based nanosheets but with a lateral size of less than 20 nm (Figure 1 c).[7] In the graphene-based nanostructures family, GQDs are the only type with the potential for cell uptake by the microorganism.[23] Due to their low lateral dimensions (Figure 1 f), GQDs can pass through the cell membrane of microorganisms and affect the DNA of the microorganism.[24] Consequently, the cell viability in the presence of GQDs and their derivatives not only depends on the common factors discussed earlier but also the cell penetration and impacts on the DNA.[24]

One of the most compatible graphene-based geometries for the development of ELMs is the three-dimensional one.[25] In this geometry, rGO nanosheets are connected through physical or chemical interactions (Figure 1 d).[26] Interconnections between nanosheets are mostly edge-connections and hence, the number of nanosheet edges in the structure of graphene-based three-dimensional structures is much lower than the nanosheet geometry (Figure 1 g). Graphene-based nanosheets and GQDs can be incorporated into the structure of polymers, ceramics, and metals to fabricate graphene-based nanocomposites.[27] In the nanocomposite form, graphene-based nanosheets are mostly fixated in the structure of a matrix, and as a result, the cytotoxicity of the final nanocomposite toward microorganisms depends on not only the graphene-based nanosheets but also the matrix.[28] When introduced into the structure of a nanocomposite, the sharp-edges-related cytotoxicity of graphene-based nanosheets is less likely to limit the applicability of graphene-based nanosheets for the design of ELMs. However, the surface area of graphene-based nanosheets in this form is mostly involved in the interfacial interactions with the matrix and is not fully available for microorganism adhesion.

2.1. Strategies for the fabrication of graphene-based ELMs

Graphene-based ELMs can be classified into four main categories: (1) graphene-based ELMs designed and manufactured by a living microorganism, (2) graphene-based nanostructures loaded with a living microorganism after fabrication, (3) graphene-based ELMs manufactured by a living component and loaded with another living microorganism, and (4) graphene-based ELMs produced with a living microorganism introduced into the structure of a graphene-based nanostructure through the fabrication process and remained alive after the fabrication process.

2.1.1. Strategy I: Bacteria as the designer of the ELM

In the first strategy (Strategy I), a microorganism manufactures a hydrogel in the presence of graphene-based nanostructures and then, remains alive in the structure of the hydrogel. In this strategy, graphene-based nanostructures are mostly introduced into the culture media of a microorganism, and through a biosynthesis process, the microorganism forms the graphene-based hydrogel (**Figure 2**).

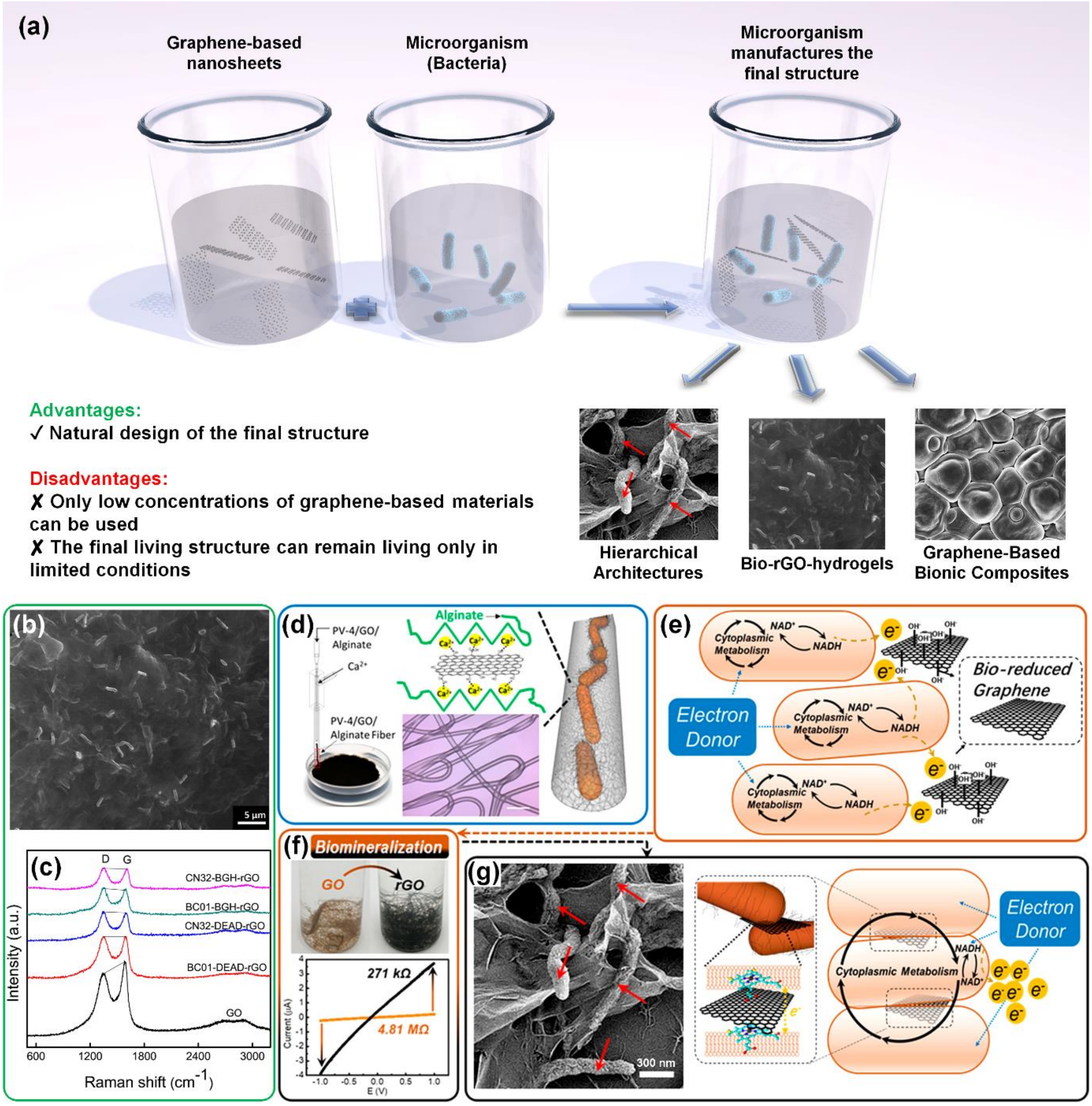

Figure 2. Strategy I for the fabrication of graphene-based ELMs: a schematic presenting the first strategy for the fabrication of ELMs including main advantages and disadvantages and SEM images of hierarchical architectures (Reproduced with permission.[29] 2020, American Chemical Society.), bio-rGO hydrogels (Reproduced with permission.[30] 2018, Elsevier.), and graphene-based bionic composites (Reproduced with permission.[31] 2016, American Chemical Society.) prepared using strategy I (a); SEM image (Reproduced with permission.[30] 2018, Elsevier.) of *Shewanella putrefaciens* loaded self-assembled bio-rGO-hydrogel (CN32-BGH-rGO) prepared using the first strategy (b); Raman spectra (Reproduced with permission.[30] 2018, Elsevier.) of CN32-BGH-rGO, *Shewanella xiamenensis* loaded bio-rGO-hydrogel (BC01-BGH-rGO), rGO cultivated with dead *Shewanella putrefaciens* (CN32-DEAD-rGO) and *Shewanella xiamenensis* (BC01-DEAD-rGO), and GO (c); The fabrication strategy of living filters prepared by co-axial microfluidic device assembly from *Shewanella*

loihica (PV-4), GO, and alginate (d) (Reproduced with permission.[29] 2020, American Chemical Society.); The bio-reduction mechanism of GO by alginate encapsulated *Shewanella loihica* (e) (Reproduced with permission.[29] 2020, American Chemical Society.); Impact of bio-reduction of GO on color and electrical resistance of fabricated living filters (f) (Reproduced with permission.[29] 2020, American Chemical Society.); and a SEM image and a schematic presentation of the bacteria integration by rGO nanosheets that led to the assembly of living filters (Reproduced with permission.[29] 2020, American Chemical Society.).

Different graphene-based nanostructures can be used in the design of hydrogels using this biosynthesis strategy. However, since graphene-based nanostructures should remain dispersed in the culture media through the biosynthesis process to reach a homogenous final hydrogel, most of the available reports in this strategy category are limited to graphene-based nanosheets (Figure 2 a).

The fermentation of microorganisms such as fungi in the presence of graphene-based nanostructures that lead to the formation of graphene-based bionic composites can be classified in this strategy category.[31] Such a process leads to the formation of graphene-based living materials that contain living fungi (see Figure 2 a for SEM of final bionic composites). However, available reports on such protocols are limited to low concentrations of graphene-based nanostructures such as graphene nanoplatelets (stacked nanosheets with a thickness of 8−15 nm).[31] Therefore, the final living composite mostly represents the structural properties of the yeast film rather than a graphene-based living material.

GO-based porous ELMs can be prepared by introducing the *Shewanellaceae* bacteria family on the basal plane of GO nanosheets (Figure 2 b). Adhered bacteria can then form a self-assembled hydrogel structure by forming crosslinks between GO nanosheets.[30, 32] Moreover, culturing *Shewanella* with GO nanosheets in the culturing media results in the formation of electroactive and self-assembled 3D reduced graphene oxide (rGO)/bacteria hybrid biofilms.[33] In such a culture media, GO nanosheets trap bacteria on their basal plane through a “fishing” process.[33] As evidenced through Raman analyses (higher I_D/I_G ratios of bio-reduced samples in Figure 2 c, compared to GO), bacteria reduce GO nanosheets and through the self-assembly process, a 3D microporous biofilm can form. With bacteria embedded in a 3D rGO-based structure, such a biofilm is a living graphene-based hydrogel that can present enhanced bidirectional electron transfer and a net current density change of 820 $\mu A/cm^2$ (from -60 to -880 $\mu A/cm^2$).[33]

In general, three stages of interactions are expected in a media containing GO nanosheets and bacteria: (1) colloidal instability between bacteria and GO, (2) reversible aggregation, and (3) irreversible aggregation.[34] When the separation distance between GO and bacteria is greater than 54 nm, the mobility of the bacteria leads to the surficial contact of the bacteria and GO nanosheets. However, the attraction energy between GO and bacteria is not yet high enough which leads to the surface attachment of bacteria on the GO basal plane.[34]

When the separation distance is between 54 nm and 5-6 nm, the bacteria attach to the surface of GO in a reversible form. This step is called: "reversible aggregation". At this stage, bacteria/GO interactions are still reversible. Moreover, the irreversible bacteria/GO aggregation takes place when the separation distance is less than 5-6 nm.[34] At this stage, the attachment of the bacteria to the GO surface is irreversible and the bacteria adhesion to the basal plane of GO takes place.

Based on the aforementioned theory,[34] bacteria attachment to the surface of GO can be crucial for the development of graphene-based ELMs. This theory provided insight into the adhesion behavior of bacteria on the basal plane of GO. However, the interaction mechanism of bacteria and GO edges has not been included in the provided discussions. Moreover, this interesting theory can be completed with more work on the impact of the bacteria's outer layer membrane on the aggregation process. Moreover, since GO nanosheets are expected to be reduced by the bacteria after adhesion (the irreversible aggregation), the impact of such reduction and elimination of O-moieties on the interaction mechanism of GO and microorganisms should also be studied.

Using the bottom-up approach, functionally coherent and electrochemically active biohybrids can be developed for the design of living bio-carriers (Figure 2 d).[29] Living catalysts such as *Shewanella loihica* (*S. loihica*) can be designed to act as conductive bacterial electron transfer pathways in the structure of living composites.[29] In this strategy, each bacteria's electrically conjugated metabolic pathways play a role in forming an extracellular electron transfer network with a large active surface area throughout the structure of the living composite. This is done by the bioreduction of GO into conductive rGO through the metabolism of *S. loihica* (Figure 2 e).[29] When these bacteria are bio-printed in an ink consisting of the bacteria, GO and alginate, a 3D hierarchical living framework with a large number of extracellular electron transfer pathways can be reached (Figure 2 f).[29] Culturing the printed scaffold leads to the bioreduction of GO into rGO. The formed rGO nanosheets then act as conductive joints between the redox center of cytochromes in electroactive conformations (Figure 2 g).

The bioreduction process can also be used to design and fabricate high-resolution sensors using Strategy I. When a low concentration of *Shewanella oneidensis* (1 ml solution with the OD600 of 8) is mixed with a low concentration of GO (1 ml solution with a concentration of 2 mg/ml) and the mixture solution is injected into a capillary tube, the bioreduction of GO by the microorganism results in the formation of biohydrogel sensors that can present high molecular-level sensing properties.[35] The fabricated microfiber-like whole-cell electrochemical biosensor can present a linear calibration curve ranging from 1 nM to 10 mM with a sensing limit of 0.60 nM.[35] The increase of the local cell density with embedding the microorganism on the basal plane of rGO nanosheets leads to an improvement in the electron exchange efficiency and as a result, the high sensitivity.[35]

Reduced graphene oxide (rGO) nanosheets can be incorporated into the bacterial cellulose (BC) hydrogels using this biosynthesis protocol to fabricate BC/rGO nanocomposites.[36] Culturing *Komagataeibacter xylinus* (*K. xylinus*) in a Hestrin-Schramm (HS) medium containing low concentrations of rGO nanosheets (< 1 wt.%) can lead to the fabrication of electrically conductive hydrogels.[36a] GO nanosheets can also be used to fabricate BC/GO composite pellets using the same fabrication strategy and through culturing *K. xylinus* in a GO-containing culture media. The concentration of GO also is reported to be very low, probably to prevent possible cytotoxicity for *K. xylinus* through the composite biosynthesis process.[37]

It should be noted here that the removal of the bacteria from the final nanocomposite hydrogel is a standard step in such biosynthesis protocols and as a result, these hydrogels cannot be classified as living hydrogels after the fabrication process is over.[36b] Moreover, Similar to other fabrication strategies, graphene-based nanostructures used in this manufacturing strategy should not have any cytotoxicity, nanotoxicity, or genotoxicity to prevent any unwanted impacts on the manufacturing process as well as on the living of the microorganism in the structure of the final hydrogel.[38] Therefore, the content of rGO and GO that can be introduced into the culture media is limited, considering the cytotoxicity of both rGO and GO nanosheets at high loading content for microorganisms.[39]

The most important advantage of this fabrication strategy is the formation of naturally designed and generated graphene-based ELMs. Since the microorganism is the designer of the system in this strategy, the formation of naturally randomized structures that can mimic the natural environments for the final application of the ELM is very likely in this strategy. However, the applicability of the final ELM prepared using this strategy is limited to the conditions in which the designer microorganism will remain alive and this limits the application fields of graphene-based ELMs produced via this strategy.

2.1.2. Strategy II: Loading graphene-based nanostructures with bacteria

In the second strategy (Strategy II), graphene-based nanostructures are first fabricated from precursors and then a microorganism is introduced into the structure of the fabricated hydrogel to convert it into a living hydrogel for different applications (**Figure 3 a**). As a result, graphene-based nanostructures used in this strategy should not have toxicity for the microorganism that will be inserted in their structure and should be structurally able to provide the microorganism with the conditions of metabolism.

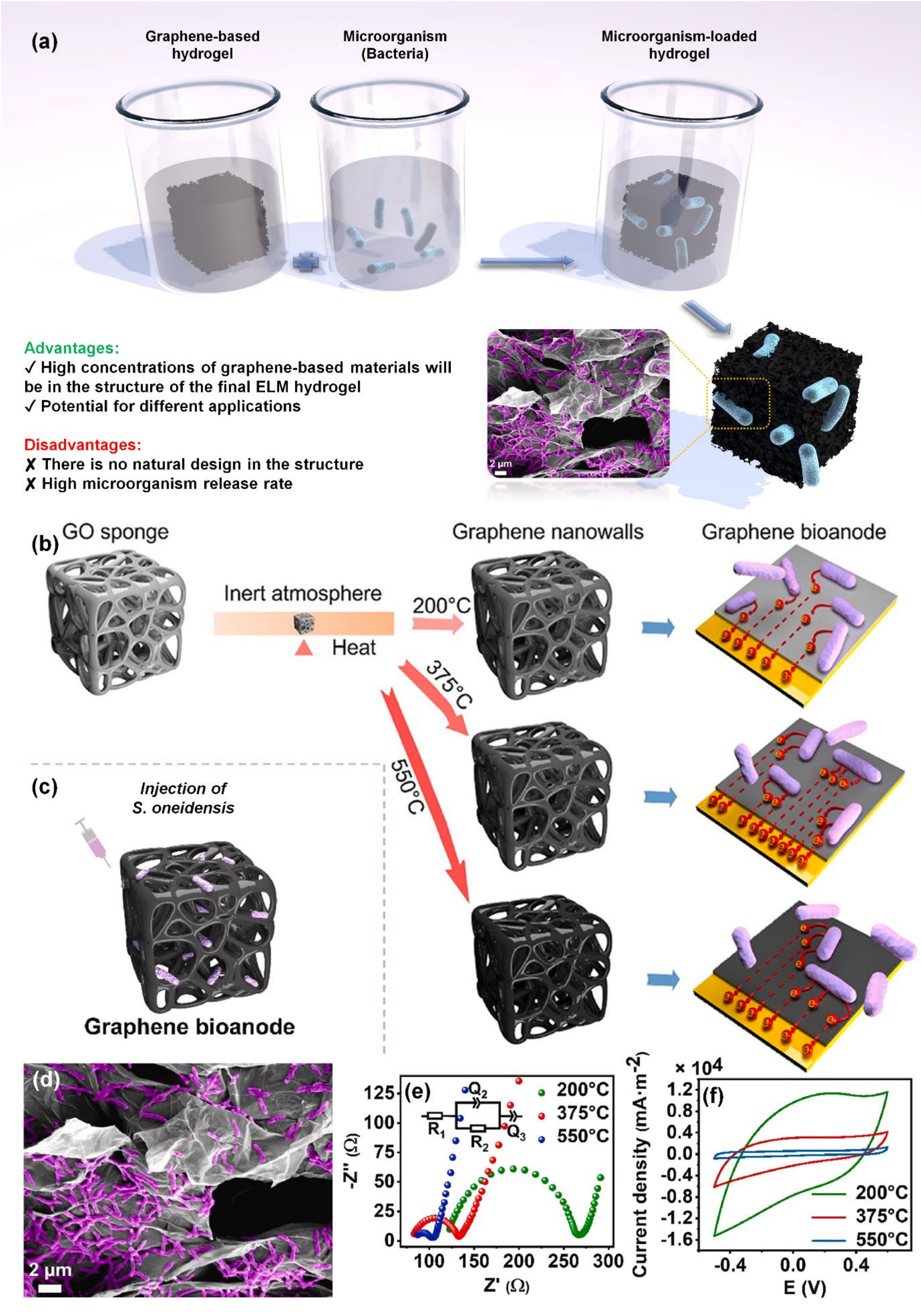

Figure 3. Strategy II for the fabrication of graphene-based ELMs: a schematic of the second strategy for the fabrication of graphene-based ELMs including the introduction of a living microorganism into the structure of a graphene-based nanostructure (a); The fabrication

strategy of graphene-based three-dimensional scaffolds (b) and *S. oneidensis*-loaded bioanodes (c); (Reproduced with permission.[25] 2023, Elsevier.) SEM image of *S. oneidensis*-loaded bioanodes (d); (Reproduced with permission.[25] 2023, Elsevier.) Nyquist graphs (e) and cyclic voltammetry (f) results of the *S. oneidensis*-loaded graphene-based bioanodes prepared using scaffolds reduced at different temperatures. (Reproduced with permission.[25] 2023, Elsevier.)

Microorganisms that can be introduced into the structure of graphene-based nanostructures in this strategy can be either different types of bacteria or even programmed bacteria capable of providing different biomedical-related properties. For instance, *Escherichia coli* (*E. coli*) can be engineered to secrete bacteriocin lysostaphin, an enzyme capable of killing *Staphylococcus aureus* (*S. aureus*).[40] The engineered *E. coli* can be trapped in the structure of a hydrogel to fabricate a new class of microorganism-encapsulated ELM with the potential to kill antibiotic-resistant bacteria such as *S. aureus*. Moreover, microorganisms with engineering properties like self-healing or energy generation/harvesting properties can also be trapped in the structure of hydrogels to fabricate new biocompatible devices.[41]

Leng et al.[25] used the lyophilization process to fabricate GO scaffolds and then reduced GO nanosheets in the structure of the fabricated GO scaffold through the thermal reduction process at different reduction temperatures (Figure 3 b). Then, they injected *Shewanella oneidensis* (*S. oneidensis*) as a living microorganism into the structure of reduced scaffolds to fabricate a living three-dimensional nanostructure for electrochemical energy generation applications (Figure 3 c). The reduced graphene-based porous scaffolds acted as a proper lightweight host scaffold for microorganisms (Figure 3 d). Electrical conductivity in the final bioanode is directly related to the temperature of the thermal reduction process (Figure 3 b) and the charge transfer resistivity in the structure of bioanodes reduced from 146.2 Ω to 30.4 Ω with increasing the reduction temperature from 200 °C to 550 °C (Figure 3 e). Moreover, the elimination of O-moieties through the thermal reduction process led to an increase in the contact angle of the rGO sponge scaffold.[25]

Cyclic voltammetry (CV) curves of the *S. oneidensis*-loaded bioanodes (Figure 3 f) prepared using scaffolds reduced at different thermal reduction temperatures (the voltage scan range of −0.5 V–0.6 V at a scan rate of 5 mV/s) confirmed the electron transfer from bacteria to the scaffolds through the presence of redox peaks from the bacteria membrane proteins and the mediated flavins at 0 V and −0.3 to −0.4 V (vs. Ag/AgCl electrode).[25] Considering the impacts of hydrophilicity and capacitive current, the authors reached a Janus effect with a reduction temperature of 200 °C resulting in a hydrophilic interface with stimulated metabolic processes slow electron transfer and a reduction temperature of 550 °C leading to a conductive interface with a fast electron transfer but such less biocompatibility.[25] Consequently, the largest bio-current can be achieved by employing an intermediate reduction temperature of 375 °C for a balanced combination of both surface hydrophilicity and electron transfer.[25]

The main advantage of this strategy is the potential of the final living material for different applications, as the type of microorganism introduced into the living hydrogel is selectable. However, the release rate of the microorganism from the final graphene-based ELMs prepared via this strategy can be high, as the scaffold loaded with the microorganism may not be able to fully trap the microorganism. Consequently, the precise tune of the surface chemistry of the graphene-based scaffold that hosts the microorganism is essential in this strategy.

2.1.3. Strategy III: Sequential and co-cultivation

In the third strategy (Strategy III), graphene-based ELMs manufactured by a microorganism are loaded by another microorganism to fabricate a living hydrogel (**Figure 4 a**). This can be achieved by either a sequential order or through the formation of a microorganism community (Figure 4 b). In the sequential process, the primary and designer microorganism should be first removed from the fabricated graphene-based ELM and the new microorganism should then be loaded into the structure to fabricate graphene-based ELMs.

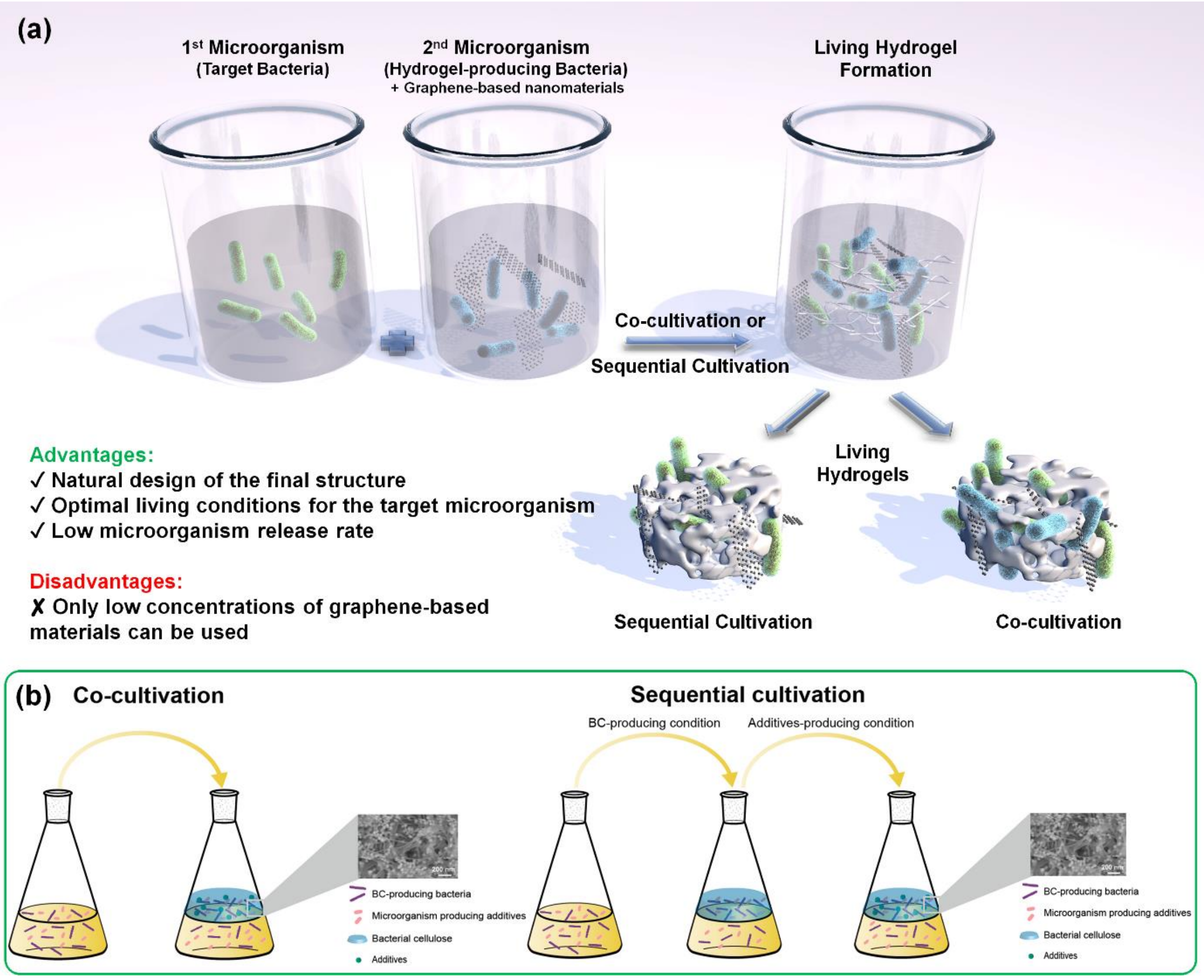

Figure 4. Strategy III for the fabrication of graphene-based ELMs: a schematic demonstrating two possible methods for the fabrication of graphene-based ELMs in the third strategy, sequential cultivation and co-cultivation (a); An example of co-culturing a bacterial cellulose-producing bacteria and an additive-producing bacteria using sequential cultivation and co-cultivation (b). (Reproduced with permission.[42] 2022, Elsevier.)

The main challenge in this process would be the complete removal of the designer microorganism from the fabricated scaffold before the introduction of the final microorganism. Moreover, since the structure will be designed by a microorganism, likely, the scaffold can likely host the new microorganism and provide it with the nutrition conditions required for it to be vial. However, a challenge would be to retrain the biocompatibility and structural properties of the scaffold through the removal of the first microorganism.

In the second type of protocol, a community of microorganisms can be cultured (or even co-cultured) in the presence of graphene-based nanostructures. Intra-microorganism interactions and the survival of the loaded microorganism are the main challenges in this strategy. Based on a related publication by Gilbert and coworkers[43] for the co-culture of bacterial cellulose-producing *Komagataeibacter rhaeticus* and *Saccharomyces cerevisiae* yeast, functional bacterial cellulose-based living materials can be fabricated by co-culturing different microorganisms. Inspired by living materials such as plant leaves, the production and modification of the final ELMs can be done by different microorganisms.[43] This strategy can be extended to graphene-based ELMs by introducing graphene-based nanostructures into the co-culture media of bacterial cellulose-producing bacteria and yeast.

2.1.4. Strategy IV: 3D bioprinting

In the fourth strategy (Strategy IV), a graphene-based structure is manufactured using a living microorganism in the precursor material of a 3D scaffold. 3D printing of bioinks containing a living microorganism is an example (**Figure 5**). The microorganism should remain alive through the fabrication process and after manufacturing the final hydrogel. Consequently, neither the bioink nor the final hydrogel should have any toxicity to the microorganism and should also provide the microorganism with the necessary conditions for living. Moreover, this strategy can be used to fabricate 4D-printed structures, in which a graphene-based bioink containing a microorganism is first 3D printed, and the structure is then further developed by a microorganism after 3D printing and with time.

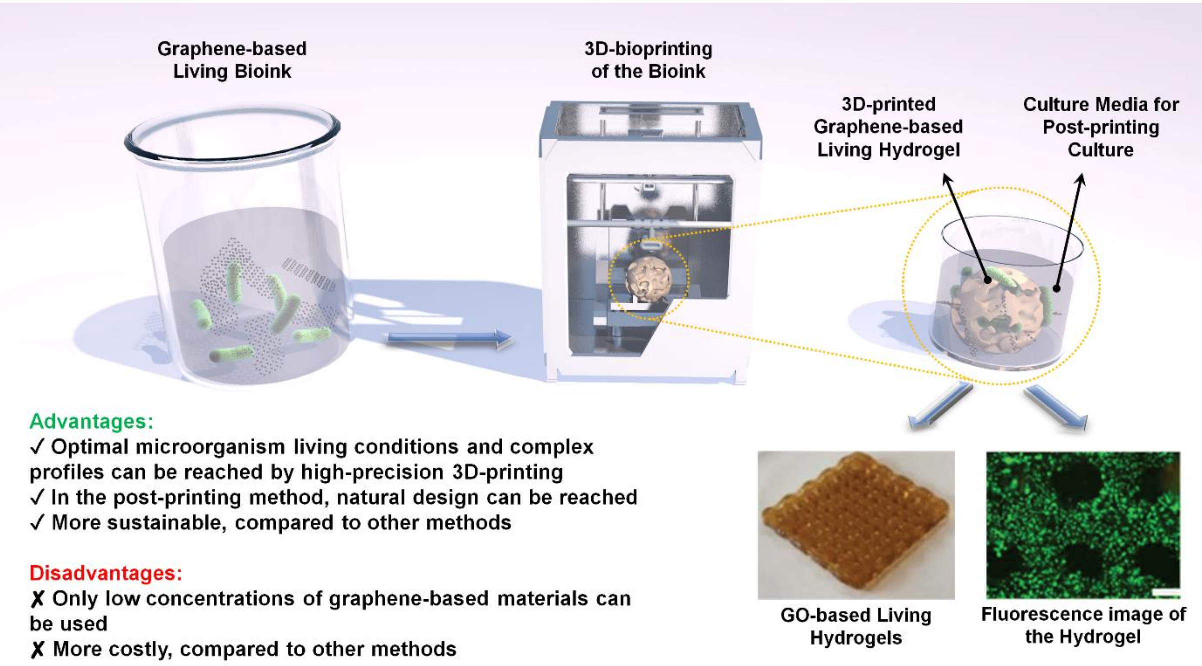

Figure 5. Strategy IV for the fabrication of graphene-based ELMs: a schematic of the 3D-bioprinting of graphene-based ELMs from graphene-based microorganism-containing bioinks resulting in the formation of living hydrogels (digital and fluorescence images are for GO-

based living hydrogels 3D printed using mesenchymal stem cells-containing bioinks, Reproduced with permission.[44] 2019, Royal Society of Chemistry.).

Alginate/GO solutions containing human mesenchymal stem cells can be used as bioinks for the 3D printing of stem cell-laden 3D scaffolds (**Figure 6 a**). Although the content of GO nanosheets used in studies available in this field is relatively low,[44] graphene-based materials such as GO can affect the conditions of the 3D printing process, even at low concentrations (Figure 6 b-c). Moreover, GO act as an antioxidant component and a protein adsorption agent in the structure of final 3D-printed ELMs and as a result, protect cells from oxidative stress environments (Figure 6 d-e).[44] Furthermore, the incorporation of GO nanosheets facilitates osteogenic differentiation. However, the main component in such bioinks is still a polymer such as alginates, and hence, the final structure is more of a 3D printed graphene/polymer engineered living nanocomposite rather than a graphene-based ELM.

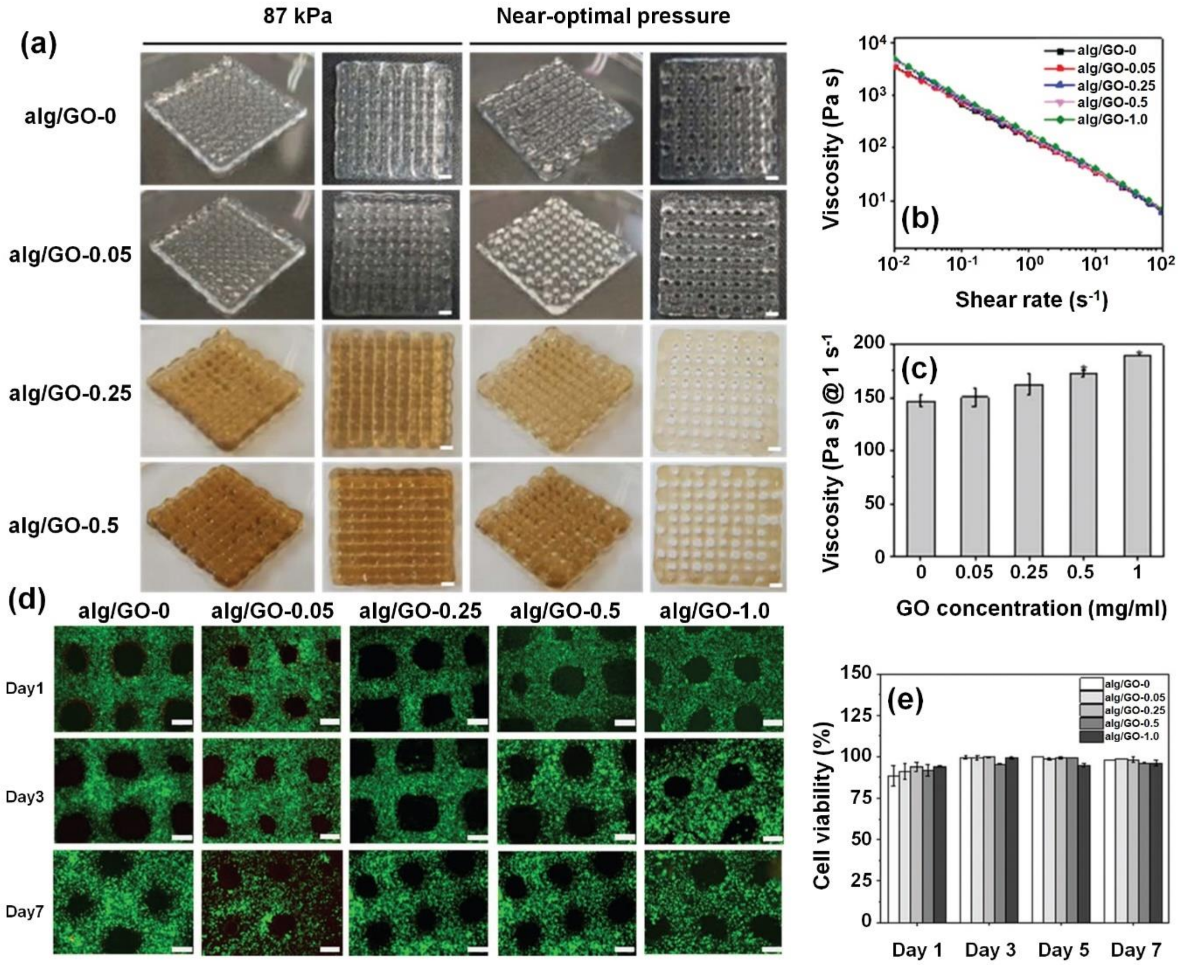

Figure 6. Digital images of 3D-printed scaffolds (a) prepared using different concentrations of GO in alginate-based bioinks (scale bars = 2 mm); Flow curves (b) and point viscosities (c) at a shear rate of 1 s^{-1} of alginate/GO bioinks; Live/dead fluorescence images of 3D-printed

scaffolds with mesenchymal stem cells in bioinks (d) after different time intervals of incubation (scale bars = 300 μm); Cell viability of 3D printed scaffolds after during one week of incubation (e). (Reproduced with permission.[44] 2019, Royal Society of Chemistry.)

Similar strategies were also reported in the literature for the fabrication of 3D-printed microorganism-containing structures. For instance, graphene nanoribbons can be introduced into a 3D-printing electronic ink and cyanobacterial colonies into a separate bioink.[45] The simultaneous 3D printing of these inks can lead to the formation of living structures.[45] However, graphene-based nanostructures in such strategies are not the main elements of the living structure, but more of a conductive path for electron transfer in the living system.[45]

2.2. The importance of nanotoxicity and biocompatibility

Since all four discussed strategies for the fabrication of graphene-based ELMs are biological processes, special attention should be placed on using graphene-based nanostructures. In general, three aspects should be carefully considered when graphene-based nanostructures are in use for biological applications: (1) interactions with biological components of the system including cells, proteins, and tissues; (2) structural and physicochemical characteristics of graphene-based nanostructures; and (3) the outcomes of biological activities in the system containing graphene-based nanostructures.[46]

The main microorganism-based interaction that may affect the fabrication of graphene-based ELMs is the interaction between the microorganism's interface and graphene-based nanostructures. In general, two sets of mechanisms have been proposed for the interactions on the interface of bacteria and graphene-based nanostructures. In the first set of mechanisms, the antibacterial properties of graphene-based nanostructures are of interest.[10] Considering the nanosheet-based geometry of single-layer and multilayer types of graphene, GO, rGO, and GQDs, the interaction mechanism known as the "nanoknife effect" has been proposed widely as the most important antibacterial mechanism of graphene-based nanosheets.[47] The nanoknife effect (**Figure 7**) leads to the destruction of the microorganism cell's wall resulting in the extraction of phospholipids and the release of thiols (R-SH) which in turn can lead to the formation of hydrogen peroxide.[8] Except for graphene-based nanomaterials in the form of nanocomposites and/or aggregated structures, this mechanism is the most possible interaction mechanism for the interface of living microorganisms and graphene-based nanostructures.[12a] The nanoknife effect not only leads to the physical damage of the microorganism's cell wall but also is reported to result in destructive mechanisms such as lipid extraction and DNA destructive interactions (Figure 7 a). Such damage to the microorganism's structure leads to the death of the microorganism, even in the presence of low concentrations of graphene-based nanosheets (Figure 7 b).

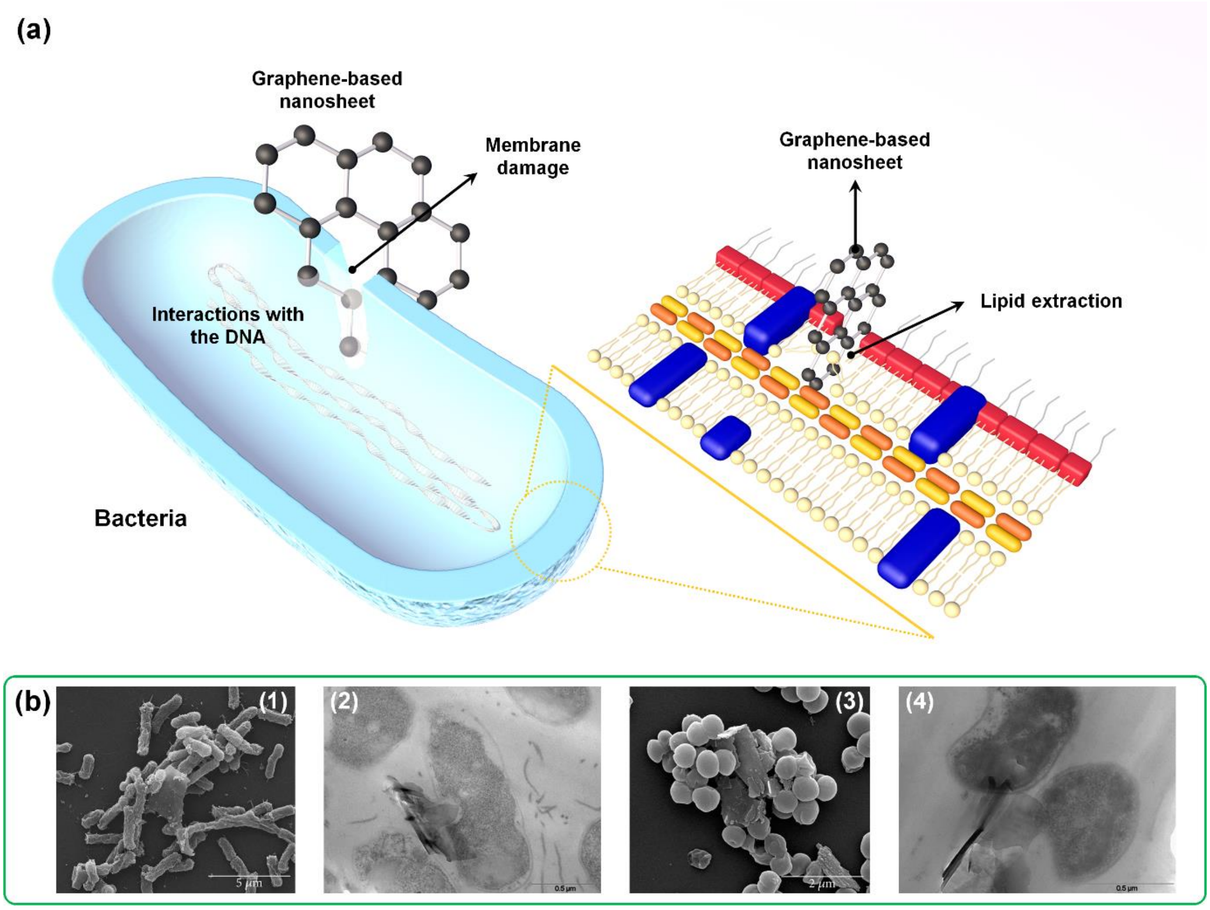

Figure 7. A schematic of the membrane damage for the bacteria in the presence of graphene-based nanosheets due to the sharp edges of nanosheets (a); Microscopy evidence of bacteria cell damage in the presence of GO nanosheets (b) including SEM (1,3) and TEM (2,4) images of *E. coli* (1,2) and *S. aureus* (3,4) after 24 h of incubation in the presence of 80 μg/mL of GO. (Reproduced with permission.[48] 2019, Hindawi.)

Microorganism wrapping in graphene-based nanosheets is another antibacterial mechanism of these nanomaterials with 2D nanosheet geometry that can isolate the microorganism from the culture medium. With a lack of access to nutrients necessary to live and grow, the wrapped microorganisms die.[49] GO,[49b] rGO,[49a] and graphene[50] nanosheets prepared from precursors with a high aspect ratio (graphite flakes and graphite powders with a large mech size) are more expected to present this antibacterial behavior. Graphene-based nanosheets prepared from precursors with a lower aspect ratio (graphite powders with a low mesh size) as well as GQDs are expected to have the nanoknife effect as their antibacterial mechanism.[51]

Even though a large number of reports are available on the antibacterial properties of graphene-based nanosheets,[12b, 52] there are a series of reports on the microorganism growth-enhancement properties of these nanosheets.[53] Moreover, available reports on the successful growth of bacterial cellulose-producing bacteria in the presence of graphene-based nanomaterials suggest that the interaction mechanism of graphene-based nanomaterials and microorganisms directly relates to the structural properties of the graphene-based nanomaterials.[54]

Biological activities in systems used for the fabrication of graphene-based ELMs cover a wide range of main and side activities that can alter through the metabolism of the microorganism in the presence of graphene-based nanostructures.[46] This includes reactions that can affect the structure of graphene-based nanostructures[55] and the ones that can affect the microorganism. Moreover, such reactions can result in components that can change (limit/improve) the media surrounding the final graphene-based ELMs in favor of or against the metabolism of the living microorganism. This becomes more important when a graphene-based living hydrogel ELM containing a microorganism is placed in a biological environment such as the human body.

GO nanosheets have been reported to reduce in a culture media of the model bacteria like *E. coli* and *Shewanella*.[56] This behavior relates to the glycolysis process of the bacteria in an anaerobic condition containing glucose.[57] In such a process, extracellular electron transfer is known to be mediated by membrane cytochromes as well as self-secreted electron mediators.[55] In general, GO can act as both an electron acceptor and donor in contact with microorganisms.[53a] Compared to other types of graphene-based nanosheets, GO has a larger amount of O-moieties on the edges and the basal plane. The presence of these functionalities is reported to limit the microorganism's cell wall rupture by sharp edges.[55]

As one of the youngest members of the graphene-based nanostructures family, GQDs have quite interesting interaction properties with microorganisms. Compared to other graphene-based nanostructures with the same nanosheet/nano-disk geometry, GQDs have shown lower toxicity toward healthy cells through in vivo studies.[58] Moreover, the antibacterial properties of GQDs are believed to be closely related to the source and size of these nanomaterials. [59] GQDs with high selective antibacterial properties can be achieved via C60 as the source.[51] The C60-derived GQDs are active against *S. aureus* and inactive against *E. coli*, *Bacillus subtilis* (*B. subtilis*) and *P. aeruginosa*.[51] Similarly, carbon nanotubes-derived GQDs have higher toxicity towards *S. aureus* than *P. aeruginosa*,[60] suggesting that GQDs fabricated from different sources can present different behaviors against bacterial activities.[58] The main impact of the source is on the functionality and shape of GQDs, which can impact the formation of reactive oxygen species (ROS) and the cellular uptake of GQDs.[61]

The internalization of graphene-based materials, and graphene-based nanosheets in particular, affects different cell types differently.[62] Consequently, the reported impacts of graphene-based nanosheets on internalization, genes, intracellular organelles, etc. should be considered for biological applications of graphene-based ELMs when graphene-based nanosheets are the main building units of the final hydrogel.[62] Moreover, the time of exposure is a very important factor in the biological applications of graphene-based ELMs.[12a]

In addition to nanotoxicity based on structural characteristics and surface/edge chemistry, soluble acidic impurities that form through the synthesis/assembly and remain in the structure of graphene-based nanostructures can also lead to antibacterial properties. Barbolina et al.[63] studied the antibacterial properties of highly purified and insufficiently purified GO solutions against *E. coli* and *S. aureus*. When well purified, GO solutions with a concentration of 1 mg/ml reached a pH of 5.5, which was less acidic than an insufficiently washed GO solution with a pH of 3.5 (8 and 2 cycles of washing-centrifugation for highly and insufficiently purified GO solutions, respectively). No significant inhibition or stimulation effects were observed with the exposure of both bacteria strains to highly purified GO for 2 h and 4 h.[63] Consequently, the purity of graphene-based materials should also be considered as an influential factor in the applicability of these materials for the fabrication of graphene-based ELMs.

2.3. Potential applications of graphene-based ELMs

Although the number of publications in the field of graphene-based ELMs is limited, these studies suggest a wide range of potential applications for these materials. A summary of possible applications for graphene-based ELMs is presented in **Figure 8 a**. Renewable energy harvesting is one of the most interesting fields of applications for these living materials. With the introduction of living microorganisms like *S. Oneidensis*, graphene-based bioanodes for renewable energy generation can be fabricated (Figure 8 b). A maximum current density and an average current density of 135.35 mA/m^2 and 88.01 ± 28.02 mA/m^2 can be reached using such bioanodes within 24 h.[25] Moreover, the final graphene-based living bio-anode can produce a stable bio-current just a few hours after the assembly of the system.[25] Reaching a balance between electrical conductivity (fewer O-moieties) and biocompatibility (more O-moieties) in living bio-anodes prepared using graphene-based ELMs loaded with bacteria is essential for the design of bio-based energy generation systems.[25]

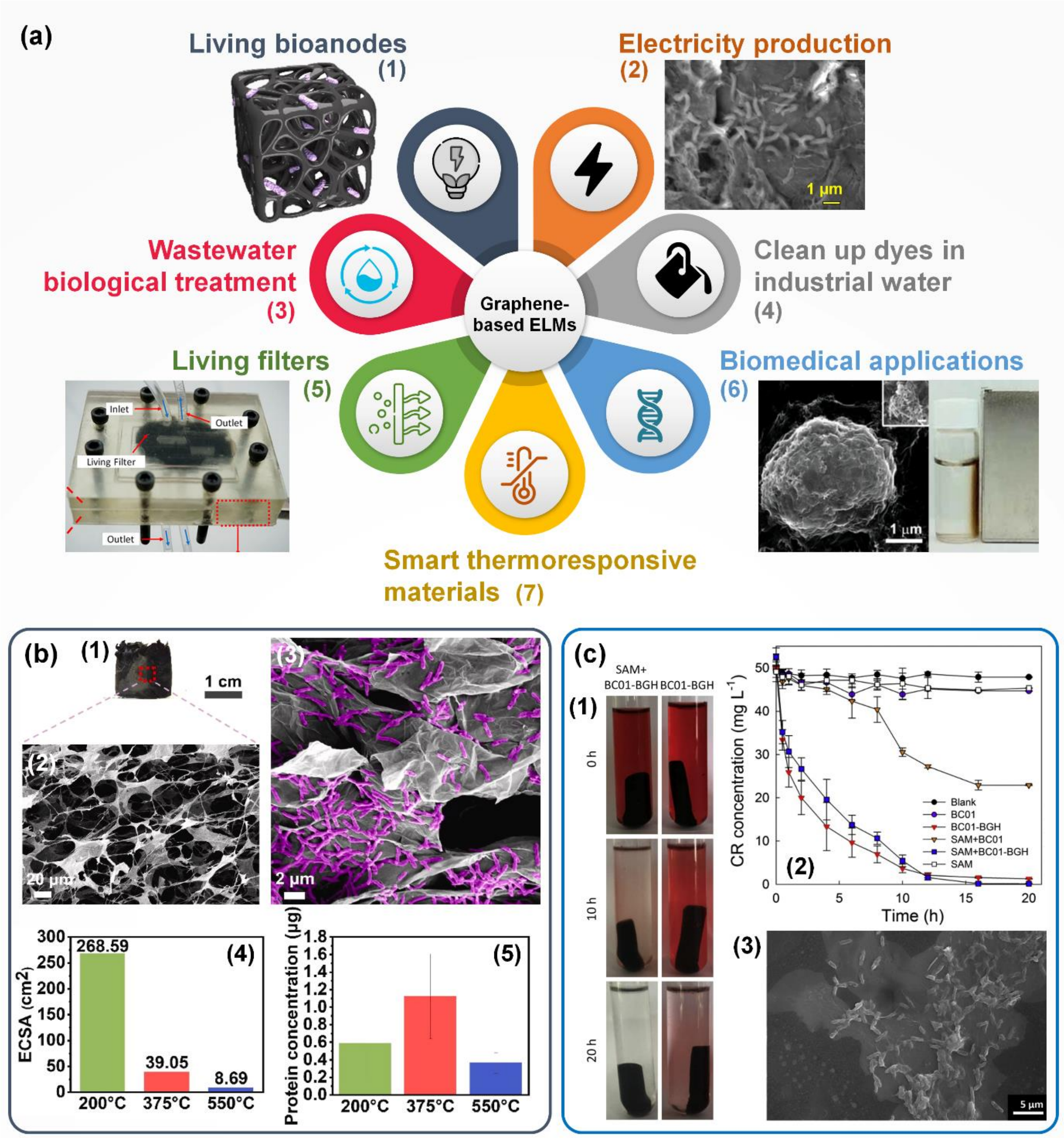

Figure 8. An overview of potential applications suggested in the literature for different types of graphene-based ELMs including (a): (1) living bioanodes for renewable energy generation (schematic reproduced with permission.[25] 2023, Elsevier.), (2) electricity-producing hydrogels prepared from rGO and sludge (SEM image reproduced with permission.[64] 2016, MDPI.), (3) bacteria-induced GO hydrogels for wastewater biological treatment reported in ref.[30], (4) bacteria-encapsulated rGO hydrogels for the removal of various dyes in wastewaters reported in ref.[32a], (5) bacteria-containing living hybrid materials as electrochemically active living filters (digital image reproduced with permission.[29] 2020, American Chemical Society.), (6) GO nanosheaths containing living yeast cells applicable in the design of biosensors and biomedical devices (SEM and digital images reproduced with

permission.[65] 2012, Wiley.) and (7) functionalized graphene/bacteria systems for the design of thermosensitive smart materials reported in ref. [66]; Living graphene-based hydrogels for energy generation (b): (1) digital and (2) SEM images of graphene-based porous nanostructures before injection of the electricity-producing bacteria, as well as (3) SEM image of bacteria-loaded living materials,(4) electrochemical surface area (ECSA) results of graphene-based scaffolds prepared at different reduction temperatures, and (5) protein concentration of energy producing living materials after cyclic voltammetry analyses; (Reproduced with permission.[25] 2023, Elsevier.) Wastewater biological treatment using graphene-based living materials (c): (1) digital images demonstrating the performance of BC01-BGH-rGO hydrogels in the removal of the toxic dye Congo Red (CR) by time, (2) CR concentration reduction for different living biohydrogels, and (3) SEM image of the BC01-BGH-rGO living hydrogel. (Reproduced with permission.[30] 2018, Elsevier.)

Yoshida et al.[64, 67] reduced GO by incubating GO nanosheets in the presence of seawater and coastal sand. Then, by introducing *Geobacter* microorganism species into the structure of fabricated hydrogels, a group of electricity-producing hydrogels was fabricated ((2) in Figure 8 a). Their results suggest that rGO structures with an electrical conductivity of 23 mS/cm can be fabricated using such a bioreduction process and a stable production of electricity can be achieved (179-310 $\mu A/cm^3$).[64, 67]
Shen et al.[30] used the bio-reduction process of GO nanosheets using *Shewanella* to fabricate bio-rGO hydrogels with the potential for the biological treatment of wastewater containing metallic contaminations (Figure 8 c). Such a system can reduce Cr(VI) into Cr(III) within 20 h under anaerobic conditions.[30] Also, these types of hydrogels can present high reduction efficiencies of 99.8 and 97.3% for the reduction of organic red and blue colors in 55 h of treatment, which suggests the high potential of these materials for the decolorization of industrial wastewater.[32a]
Deng et al.[29] developed an electrochemically active biohybrid material through the incubation of *S. loihica* in the presence of GO nanosheets followed by the microfluidic assembly of living filters and the design of a bioreactor using these living filters ((5) in Figure 8 a). Using such a design for the bioreactor and living filters they reached a rapid Cr(VI) conversion within the first 4 h of wastewater treatment.[29] The microfluidic assembly of living filters, which can be considered a post-fabrication design for graphene-based ELMs had a significant impact on the diffusion limit of filters for the reduction of Cr(VI).[29]

Yeast cells can also be encapsulated in GO-based nanosheaths, as suggested by Yang and coworkers,[65] to fabricate living hybrid materials with the potential for the design of biosensors and biomedical devices ((6) in Figure 8 a). However, since encapsulation can lead to the isolation of the microorganism from the culture media, providing channels for the nutrition of the living microorganism is a crucial design consideration in such systems, and more work is needed to confirm the successful applicability of such systems.

Tan et al.[66] designed a thermoresponsive smart hybrid material by controlling the interface of graphene and bacteria using the surface functionalization of graphene by poly(ethylene glycol)-block-(poly-N-isopropylacrylamide) copolymer. Functionalized graphene can have temperature-dependent antibacterial properties and as a result, can act as a smart system for temperature control. Moreover, electroactive bacteria-induced reduction of GO nanosheets can also lead to the formation of microfiber-like biohydrogels applicable in miniature whole-cell electrochemical sensing.[35] High-resolution sensing that can be provided with such a system can make the idea of fast point-of-care sensing.[35]

Graphene-based hydrogels can also be used as conductive scaffolds for the development of living hydrogels with cells encapsulated in the porous structure of the hydrogel.[68] When graphene-based nanostructures are introduced into gelatin methacryloyl (GelMA) hydrogels, conductive bioscaffolds with high biocompatibility can be designed.[68] Such a system can present improved proliferation behavior under an electric field. The design of electro-responsive cell growth and proliferation of living hydrogels can lead to the development of a new class of cell-laden bioscaffolds for neural tissue engineering.[68]

The most important advantage of graphene-based ELMs in any aforementioned applications, compared to non-living materials, is the presence of a living component in the system that can lead to the smartness of the final hydrogel.[66] The structural and responsive properties of graphene-based ELMs can be defined by controlling parameters such as pH, temperature, etc.[66] More importantly, graphene-based ELMs can be considered sustainable materials, as they can form through the natural metabolism of a microorganism.[69] However, since the concentration of graphene-based materials in these ELMs was relatively low in almost all summarized studies, the mechanical properties of graphene-based ELMs reported up to now were much lower than common engineering materials.[29-30] Consequently, controlling the fabrication conditions and microstructural properties of graphene-based ELMs can be the key to next-generation ELMs.

3. Design principles of next-generation graphene-based ELMs

In this section, some suggestions for the fabrication of next-generation graphene-based ELMs with (1) high contents of graphene-based nanomaterials and (2) biocompatibility for the living and proliferation of microorganisms are presented. Suggestions in this section are mostly around the structural improvements that can be applied to graphene-based nanostructures, as engineering, modification, and programming of microorganisms for the fabrication of ELMs is not the purpose of this paper. This is because although microorganism programming can lead to the easier culturing of microorganisms in the presence of graphene-based nanostructures, the toxicity of graphene-based nanostructures that is the main obstacle for the fabrication of graphene-based ELMs will remain the main challenge, even after programming the microorganism. This challenge will then limit the applicability of ELMs, specifically when the foreseen applications are in biological fields.

3.1. The roles of lateral size and thickness

The lateral size of graphene-based nanosheets can significantly affect the microorganism viability through the fabrication process of graphene-based ELMs. When the lateral size of graphene-based nanosheets is large (~0.01 μm^2), physical wrapping of the microorganism with graphene-based nanosheets results in the isolation of the microorganism from the culture media and as a result, strong toxicity against the active microorganism.[70] Consequently, graphene-based nanosheets with lower lateral size ranges can be better choices for the fabrication of graphene-based ELMs. However, it should be noted that most of the available works are concerned with culture systems containing low concentrations of graphene-based nanosheets[12a] and the impact of high concentrations of graphene-based nanosheets on the viability of microorganisms applicable in the design of graphene ELMs should be studied in future.

The rigidity of nanosheets also affects the behavior of graphene-based nanosheets against microorganisms.[52a] The rigidity of nanosheets depends on two factors: (1) the thickness of nanosheets and (2) the content of O-moieties in the structure of nanosheets. The rigidity of graphene-based nanosheets (GO, rGO, and graphene nanosheets) increases with an increase in the number of layers. An increase in the rigidity with an increase in the thickness of nanosheets leads to a change in the type of interaction between nanosheets and the microorganism from wrapping behavior to edge-wise contact.[52a]

Although no report is available in the literature on the impact of the thickness of nanosheets on the contact behavior of microorganisms applicable in the design of graphene-based ELMs, it is expected that an increase in the thickness of graphene-based nanosheets results in less sharp edges and as a result, a decrease in the amount of the nanoknife effect contact behavior.[52a] However, an increase in the number of nanosheets can lead to a decrease in the available surface area for the development of graphene-based ELMs. Consequently, finding an optimum number of nanosheets (from single layer to multi-layered and expanded morphologies) for the fabrication of ELMs seems crucial, and further studies in this field are necessary.

3.2. Aggregation of nanosheets

The aggregation of graphene-based nanosheets (graphene, GO, and rGO) can lead to less content of sharp edges and as a result, can reduce the nanoknife effect in the culture media of the microorganism. Reduction of GO nanosheets into rGO in a static reduction condition can lead to the aggregation of nanosheets, due to the high tendency of reduced forms of graphene-based nanosheets to aggregate. Static chemical reduction processes of GO mostly lead to the self-assembly of GO into rGO three-dimensional structures.[15] Such porous structures can be good candidates to host microorganisms for the fabrication of ELMs. Moreover, the thermal reduction of GO nanosheets mostly leads to aggregated rGO nanosheets. The thermal stress through the thermal reduction process mostly prevents the formation of an assembled structure, and this method can be more effective for the fabrication of rGO nanosheets.

3.3. The impact of surface functionalities

For different applications, different electrical and thermal conductivity ranges are required and consequently, the surface functionality of graphene-based nanostructures should be controlled to reach the desired properties. For instance, high electrical conductivity and hydrophilicity in energy harvesting applications of graphene-based ELMs can be reached through the design of the synthesis process.[25] Electron transfer at the interface of the microorganism and the graphene-based nanostructure on the one hand, and the impact of the surface functionalities on the biocompatibility of graphene-based nanostructures, on the other hand, should be controlled to reach high-performance graphene-based energy harvesting ELMs.[25]

In general, rGO has a strong antibacterial activity against different microbial species and may not be the best option for designing graphene-based ELMs.[71] In the presence of O-moieties on the basal plane and edges of graphene-based nanosheets (GO nanosheets), nanosheets are more flexible, and as a result, the main challenge in such a condition is the wrapping of the microorganism with nanosheets.[49b] When O-moieties are eliminated from the structure of graphene-based nanosheets (rGO nanosheets), the rigidity of nanosheets increases and as a result, the chance of microorganism wrapping by nanosheets reduces. However, the nanoknife effect becomes more effective for the interaction of nanosheets with the microorganism in such a reduced form of nanosheets.[49b]

As a general strategy, the fabrication of graphene-based nanostructures with fewer sharp edges and high O-moieties on the surface can be an effective strategy for the fabrication of graphene-based ELMs. This can be reached by the formation of high-concentration graphene-based nanosheets containing nanocomposites. Moreover, if aggregated structures and three-dimensional structures without any sharp edges and a biocompatible surface are introduced into the culture media of extracellular biopolymer-producing microorganisms, naturally designed graphene-based ELMs can be fabricated. The important point is that the aggregation process can also eliminate the chance of microorganism wrapping effect by graphene-based nanostructures and can provide the microorganism with a suitable substrate for the attachment and generation of ELMs.

4. Conclusion

Graphene-based ELMs are an important member of the rising ELMs family that can play a leading role in the future of these sustainable and smart materials. Most recent publications suggest that graphene-based ELMs hold great potential for different applications, from water treatment and renewable energy harvesting to biomaterials. Based on the recent advances in the literature, graphene-based ELMs can be fabricated through four main fabrication strategies. Sustainable hydrogels can be reached in strategies where bacteria is the designer of the system, and the final hydrogel is produced through culturing or co-culturing of a biopolymer-producing bacteria. However, in all these four fabrication strategies, the main challenge is the direct relationship between the concentration of graphene-based nanostructures and the nanotoxicity of these materials for living microorganisms. Designing and defining the microstructure of graphene-based materials by controlling the concentration of edges possibly is the most efficient method that can be used to simultaneously increase the concentration of graphene-based nanostructures and improve the living conditions of the microorganisms in the structure of graphene-based ELMs. Moreover, proper purification protocols for the removal of acidic synthesis residues in the structure of graphene-based nanostructures can significantly affect the impact of these materials on the viability of living microorganisms through the fabrication process of graphene-based ELMs. Using structural modification strategies, next-generation graphene-based ELMs can be designed and fabricated, leading the way for a future with smarter and more sustainable materials in different engineering applications.

Acknowledgements

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 101034324. Moreover, the authors would like to acknowledge the financial support provided by the ULB programme: actions blanches. NG acknowledges funding from UKRI/European Commission for (SUMO), grant agreement: 101071203.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

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This article provides a comprehensive overview of the latest scientific advancements in the design and production of graphene-based engineered living materials. Based on available reports, four main approaches for producing these living hydrogels are recognized and discussed in detail. Moreover, potential engineering applications and design principles for next-generation graphene-based living materials are overviewed.

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Graphene-based Engineered Living Materials