Graphene Oxide Coatings as Tools to Prevent Microbial Biofilm Formation on Medical Device

Margherita Cacaci, Cecilia Martini, Cinzia Guarino, Riccardo Torelli, Francesca Bugli, and Maurizio Sanguinetti

Abstract

The clinical challenge on surface engineering of medical devices to prevent microorganisms adhesion and biofilm formation, has become an essential aspect for medical implants. Antibacterial properties of Graphene Oxide (GO) have been demonstrated across a broad spectrum of bacteria, and the different mechanisms of action with which this nanomaterial interacts with the microbial surface have been elucidated in detail. Innovative protective coatings based on graphene film and hydrogel could represent an innovative solution for the prevention of nosocomial pathogens colonization on implantable device. This brief review mainly focuses on the applications of graphene in nanomedicine with a particular deepening on the antibacterial properties of GO and GO-based nanomaterials. In order to evaluate the possible future applications of GO as an anti-biofilm coating material for medical devices, studies on the ability of graphene coated surface to prevent microbial adhesion are also discussed. A concise review on *in vitro* toxicity and *in vivo* safety is also presented.

M. Cacaci, F. Bugli (✉), and M. Sanguinetti
Dipartimento di Scienze di Laboratorio e Infettivologiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy

Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy
e-mail: Francesca.bugli@unicatt.it

C. Martini and C. Guarino
Istituto di Microbiologia, Università Cattolica del Sacro Cuore, Rome, Italy

R. Torelli
Dipartimento di Scienze di Laboratorio e Infettivologiche, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy

1   Introduction

Health care-associated infections (HAI) represent a major public health threat in hospital setting. According to World Health Organisation (WHO), the definition of HAI is: "Health care-associated infections, or "nosocomial" and "hospital" infections, affect patients in a hospital or other health-care facility, and are not present or incubating at the time of admission. They also include infections acquired by patients in the hospital or facility but appearing after discharge and occupational infections among staff" (Boev and Kiss 2016). They are considered the sixth leading cause of death, with an incidence from 4% to 10% (Cloutier et al. 2015). Medical devices, such as

catheters, cardiac pacemakers, joint prosthesis, prosthetic heart valves and dentures, represent one of principal source of nosocomial infection (Percival et al. 2015; Sabir et al. 2017; Boisvert et al. 2016). Microorganisms are able to adhere to biotic and abiotic surfaces, producing biofilm, composed of cells embedded in a self-produced matrix of extra-cellular polymeric substances (EPS). The main components of EPS are proteins, polysaccharides and extracellular DNA (Del Pozo et al. 2018; Ciofu et al. 2017). The biofilm formation proceeds through distinct stages, in the last stage the cells detach from the matrix and disseminate. Biofilm allows microorganisms to survive long period on the surfaces as they are in a starvation state, with low nutrient need (Kumar et al. 2017); therefore, the cells in biofilm are more resistant to the host immune response and to antimicrobial therapies making these infections hard to be treated, as antibiotics usually act against planktonic cells that are actively reproducing.

The main microorganisms isolated from medical devices health-care associated infections are gram-positive and gram-negative bacteria, such as *Staphylococcus aureus*, *Enterococcus faecalis, Escherichia coli*, *Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumanii* and yeasts, particularly*, Candida* species *(*Sohail et al. 2018; Jain et al. 2019; Alcántar-Curiel et al. 2018; Touil et al. 2018; Percival et al. 2015; Sabir et al. 2017).

Research focuses on the development of new coating material that can prevent the adhesion and subsequent biofilm formation of bacteria and yeast on medical devices (Francolini et al. 2017, Cyphert and von Recum 2017, Swartjes et al. 2015). In recent years, the use of nanotechnologies has become an interesting approach to prevent: the use of metal or carbon nanoparticles appears to be simple, safe, not expensive and it is possible to overcome the problem of the antibiotic resistance (Polívková et al. 2017; Karahan 2018). Several antimicrobial nanoparticles have been identified, like iron oxide, zinc oxide, copper oxide but till now only silver nanoparticle (AgNps) is currently under clinical trial for evaluation as antimicrobials

(Bao et al. 2011; Allahverdiyev et al. 2011; Srividya et al. 2017; Karahan 2018). Therefore, side-effects, such as AgNps accumulation in the tissue and subsequent inflammation that can be toxic for human body, have been reported (Karahan 2018).

Among the carbon nanomaterial, graphene is a two-dimensional ultra-thin nanomaterial composed only of hybridized-sp^2 carbon atoms, arranged in a hexagonal structure. Andrej Gejm and Konstantin Novosëlov, from the University of Manchester, synthetized graphene for the first time in 2004 (Novoselov et al. 2004). It is the first two-dimensional crystalline material ever produced. This material presents several physical properties: stretchability, electrical conductivity, huge surface area and high thermal conductivity (Palmieri et al. 2016; Karahan 2018; Compton and Nguyen 2010).

Since the synthesis, several derivatives have been studied, such as Graphene Oxide (GO) and reduced GO (rGO). Particularly, GO is a precursor of large-scale synthesis of graphene and it is prepared by the oxidation of graphite, making a single monomolecular layer of graphite heavily oxygenated, bearing hydroxyl and epoxide functional groups on their basal planes, in addition to carbonyl and carboxyl groups located at the sheet edges. The presence of these functional groups makes GO sheets strongly hydrophilic (Zhu 2010; Dreyer 2010).

Numerous are the potential applications of GO in biomedicine, such as biological and molecular imaging, drug/gene delivery, cancer therapy, tissue scaffold and antibacterial agent (Yousefi et al. 2017; Depana et al. 2011; Yao et al. 2012; Xia et al. 2019; Pulingam et al. 2019).

In this report, we provide a review of the studies conducted on the antimicrobial activity of GO, especially on the ability to inhibit the microorganism adhesion and biofilm formation on solid phase graphene oxide.

2   GO Antimicrobial Properties

The antimicrobial activity of GO has been widely reported, even if most of the studies focus on the activity of GO in solution while only few reported
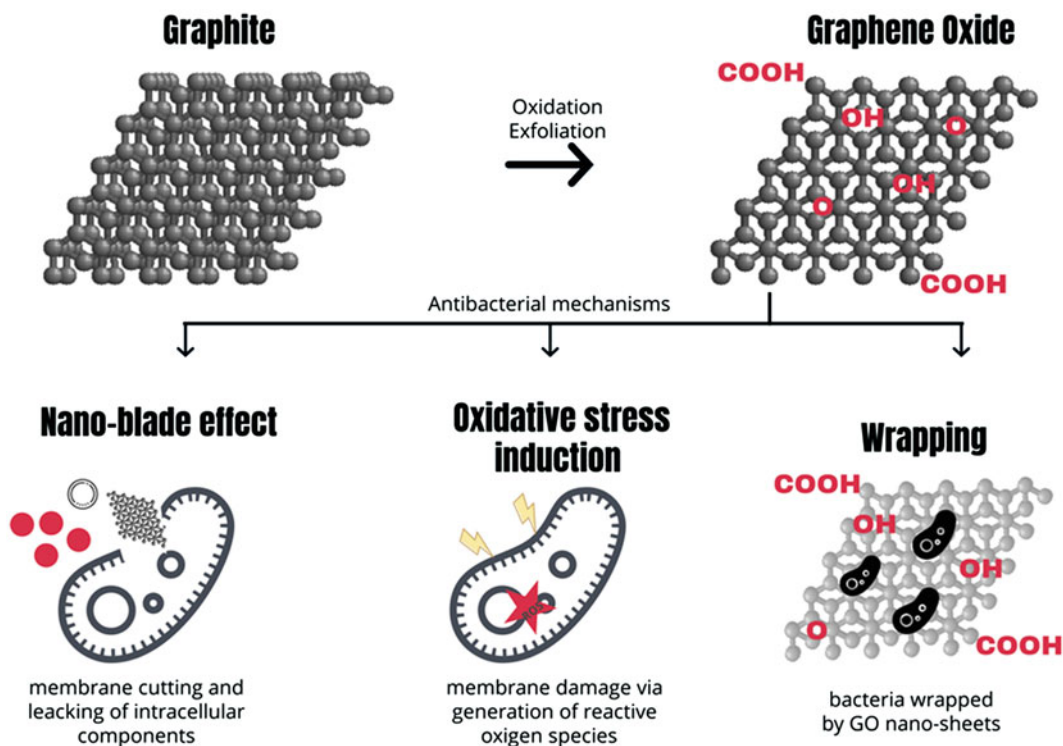
Fig. 1 Schematic representation of the three different GO antibacterial mechanisms of action

the activity of GO as coating material in solid phase (Zou 2016; Yousefi et al. 2017; Krishnamoorthy 2012).

Three main GO antimicrobial activities (Mangadlao 2015), are reported and represented in Fig. 1:

1. GO sheets sharp edges can physically interfere with microorganism by cutting the membrane with subsequent intracellular leakage of cytoplasmic constituent and death of the microorganism; this mechanism is called nano-knife or nano-blade effect (Akhavan and Ghaderi 2010; Pham et al. 2015; Castrillón 2015);
2. GO can induce oxidative stress, this phenomenon is caused by a chemical reduction of graphene sheets by bacteria (Gurunathan 2012; Salas et al. 2010; Liu et al. 2011)
3. GO can wrap and isolate microorganisms from the environment so that they cannot find nutrition, stopping proliferation; this mechanism has

been mainly observed in solution phase rather than in GO coated surfaces (Palmieri et al. 2017; Perreault et al. 2015).

It has been reported how different solvents, the method used to prepare the graphene oxide, the size, the dispersion state and other factors influence the antimicrobial activity of GO.

In a study published in 2017, Palmieri et al. investigated the activity of GO in different solvents, such as sodium chloride (NaCl), magnesium chloride ($MgCl_2$), ultra-pure water and Phosphate-buffered saline (PBS) and in the growth media LB (Luria Bertani broth). GO was used at concentrations ranging from 3 up to 200 μg/mL. After GO incubation with *Escherichia coli* and *Staphylococcus aureus* the antimicrobial effect of GO was assessed by the colony counting method. This work clearly shows how the solution and the different GO concentrations affect the antibacterial properties:

in water, for example, GO is highly stable, presenting a homogenous solution and the main mechanism is nano-blade, cutting the bacteria membranes. In the solution containing electrolytes, the GO quickly aggregates, and the main antibacterial effect is the bacteria wrapping. Interestingly, when GO is dispersed in nutrient media such as LB, it creates aggregates that cannot be resuspended by vortex. The antimicrobial effect was visible in a concentration-dependent manner after 5 h incubation but disappeared after 24 h, when the number of bacteria saturated the GO available in solution (Palmieri et al. 2017). In addition to the antimicrobial properties that GO possess in solution, GO has also been extensively studied for its ability to inhibit microbial adhesion and biofilm formation.

3 Antimicrobial Activity of GO-Coated Surfaces

GO material can be used to coat surfaces, to obtain anti-adhesion and anti-biofilm activity either alone or in combination with other substances.

Different methods have been used to prepared GO-based coating, such as spin coating and electrospinning, vacuum filtration, electroless plating, phase inversion, electrophoretic deposition, hydrogel self-assembly/crosslinking, wet chemical reduction, solvent evaporation and solutions casting (Hu et al. 2010; Duan et al. 2015; Liu et al. 2014; Kumar et al. 2016; Carpio et al. 2012; Nine et al. 2015).

Preliminary studies based on GO-coated surfaces were conducted in 2010. Hu and colleagues created GO and rGO paper by vacuum filtration and tested it against *E. coli*. They either measured the metabolic activities of *E.coli* in the presence of GO nano-sheets *via* a luciferase-based ATP assay and the viability, counting the colony forming unit. Data collected demonstrated a decreased metabolic activity and viability of the bacteria after the interaction with GO paper (almost 85% of viability loss after 2 h incubation using 85 μg/ml GO) while the rGO paper was less effective against bacteria (Hu et al. 2010). The

authors further analysed the biocompatibility of GO. Trasmission Electron Microscopy (TEM) analysis suggests that GO can be internalized within A549 cells *via* endocytosis. The biocompatibility assays showed that GO nanosheets at 20 g/mL exhibited no cytotoxicity to A549 cells after 2 h incubation and a slight decrease in cell viability after 24 h. GO nanosheets at higher concentration (85 g/mL) displayed an increased cytotoxicity (50%) within 24 h.

Another study (Akhavan and Ghaderi 2010), focuses on Go and rGO surfaces formed by electrophoretic deposition on stainless steel substrates. A mechanism of membrane disruption has been reported also in this case, but rGO appeared to be more active than GO. The better antibacterial activity of the rGO was motivated by its more sharpened and charged edges with a consequent major bacterial cell membrane damage caused by direct contact. The authors claim that the Gram-negative *E.coli*, characterized by an outer membrane was more resistant to membrane impairment caused by the rGO than the Gram-positive *S. aureus*. In 2015, Perrault et al. explored the different antimicrobial mechanism of GO nano-sheet both in suspension and on GO-coated surfaces, analysing the impact of nano-sheet size on antimicrobial activity. They demonstrated that, on GO-coated surfaces, the higher antimicrobial activity is exploited on GO-smaller sheets size, through a mechanism of bacterial cells oxidation, especially when sizes of 0.10 and 0.01 μm^2 are tested, obtaining 50% and 30% of cell viability, respectively. The main antibacterial effect is due to the bacterial cell oxidation, confirmed by the author by measuring the percentage of glutathione (GSH) oxidation that increases when the sheets size is decreasing. In suspension, the higher antimicrobial activity was obtained using larger GO sheets: in particular, the number of viable bacteria is 0.5% when 0,65 μm^2 sheets size are used. The GO sheet area inhibited bacterial development by a cell entrapment mechanism, so the largest sheets are more able to wrap the bacteria (Perreault et al. 2015). A paper, published in 2017, highlights the importance of the orientation of the GO sheets for its antimicrobial

activity. The antibacterial activity of GO films with different alignments of the sheets was measured using *E. coli* as a model. Bacteria were deposited for 3 h on the HEMA-GO (Polyhydroxyethylmethacrylate-GO) film. The vertically aligned sharp edges have enhanced antibacterial activity, showing decreased cell viability (56%) compared to random aligned (75.3%) and planar aligned sheets (81.8%) (Lu 2017). Authors showed that either a mechanism of membrane perturbation and oxidative stress contributed to the anti-bacterial activity of the film.

Another way to obtain GO coated surfaces is to mix with hydrogel to obtain a uniform and stable antimicrobial surface. In a paper published in Papi et al. 2016, Papi et al. described a hydrogel made of GO and agar and its activity against *S. aureus*, *E. coli*, and the yeast *Candida albicans*. Particularly, they used the laser induced super cavitation technology to reproduce on the surface of the GO-hydrogel the natural antimicrobial pattern of the *Cancer Pagurus*. This formulation showed anti-microbial activity due to the GO blade effect already described and the anti-adhesive pattern reproduced on the surface of the GO hydrogel (Papi et al. 2016).

3.1 Inhibition of Microbial Adhesion and Biofilm Formation on GO-Coated Surface

As already described, biofilm related infections represent a crucial point in clinical setting (Singhai 2012).

Anti-biofilm activity of graphene oxide has been described in the last few years. Di Giulio et al., investigated the anti-biofilm activity of GO against the principal wounds pathogens: *P. aeruginosa*, *S. aureus* and *C. albicans* (Di Giulio et al. 2018). They analysed the effect of GO in the inhibition biofilm formation and in the ability to interfere with mature biofilm. For the evaluation of the inhibition, microorganisms were put in contact with 50 mg/L of GO in 96 wells-flat bottom plate for 24 h and the biofilm biomass was quantified, reflecting a significant reduction in the wells treated with GO for the

microorganisms under study; the greatest reduction was observed for *S. aureus*. GO appears to be able to act also in mature biofilm, possibly penetrating in the extra-cellular matrix of biofilm; indeed, author revealed a reduction of the biomass after the exposure of the mature biofilm to 50 mg/L of GO and a detachment of the cells from the biofilm. An interesting study was conducted by Zhang et al. 2018. The effect of GO on *P. aeruginosa* biofilm was evaluated after short (4 h) and long-term (7 days) exposure. GO at 3 different concentrations (10,20,40 mg/L) was used to evaluate the inhibition of biofilm formation. The bacteria were put in suspension with the different GO concentrations and then incubated in a 96-wells plate to evaluate the biofilm production. A biofilm biomass reduction, more evident at 40 mg/L concentration, was observed. The authors therefore decided to analyse the effect obtained after 7 days of exposure (20 mg/L GO), no differences compared to the control have been detected in biomass production, suggesting that GO may not have long-term effect. The effect of GO on quorum sensing (QS) of *P. aeruginosa*, implicated in the biofilm formation of this bacteria, have been therefore explored. No impact on the expression of QS-related genes has been detected after either short and long-term exposure but GO affects biofilm formation by absorbing QS signals or related products (such as proteases) in the short term exposure but the altered biofilm formation appears to be restored during the long-term treatment, as the level of proteases and other products increase and any-change detected in the short term exposure are not present after 7 days, indicating the need to analyse the effect on the biofilm in long period with further experiments.

Song et al. tried to determine the anti-biofilm mechanism of graphene oxide, analysing the GO activity against *E. coli* and *Bacillus subtilis* biofilm formation. Bacteria were inoculated in 24-wells plate using different concentrations of GO (0, 10, 20, 40, 80, 160 mg/L) for 48 h and the biofilm biomass quantified by crystal violet staining. They found that at low GO concentration (10 mg/ml) the biofilm formation was enhanced compared to the control, while at 20 and 40 mg/ml of GO no significant differences

have been detected; the biofilm biomass decreased substantially when high GO concentration (160 mg/ml) was tested. The authors speculated that, at low GO concentration, the cell not in contact with GO might utilize the released cytoplasmatic materials as nutrients for growth, promoting the biofilm formation. The amount of oxidised GSH was furthermore quantified. About 25% of GSH was oxidized in the presence of 10 mg/L GO, and the loss of oxidation increased with the increasing GO concentrations, indicating the oxidative stress one of the anti-biofilm mechanism of GO (Song et al. 2018).

Few studies reported the activity of GO coated surface against microorganism adhesion and biofilm formation. In 2017, Thampi and collaborators deposited GO on polycarbonate urethane (PCU) membrane by a simple method of electrospraying, creating a thin layer of GO surrounding the membrane (GOPCU). PCU is a widely used material for medical devices. They measured the adhesion of the Gram-positive *S. aureus* and the Gram-negative *Pseudomonas aeruginosa* to PCU and GOPCU surfaces, detecting, by plate count of viable bacterial colony, a reduction of adhesion of 85.5% and 63.5%, respectively. Authors generically attribute this antibacterial activity to various factors such as size (without actually giving precise information about it), or formation of reactive oxygen species (ROS) by surface functional groups, as already described by Perrault in 2015. GOPCU appears to be biocompatibility, as the hemocompatibility assay conducted showed no adhesion or aggregation of platelets on GOPCU and proliferation assay on fibroblast mammalian cells showed a good percentage of survival (Thampi et al. 2017).

In a paper published in 2017 (Yadav et al. 2017), GO coated surfaces where prepared using two different methods, the traditional Hummer's method and an improved method that improves the efficiency of the oxidation process providing a greater amount of hydrophilic oxidized graphene material. This result was achieved by excluding the $NaNO_3$ and increasing the amount of $KMnO_4$ regularly used for the traditional Hummer's method.

These two methods of graphene deposition, resulting in two different surfaces that differ for their nano-sheets size, morphology and exposition of functional groups. They examined the rate of biofilm formation of the bacteria *E. coli* and *S. aureus* either by crystal violet assay and microscopy imaging, revealing that both the GO formulations inhibit cell adhesion and biofilm formation.

The GO concentration and the different methods of deposition impact on the ability to inhibit biofilm formation in Gram-positive and Gram-negative bacteria, maybe due to the different cell wall, resulting in different interaction between the microorganism and the surface (Yadav et al. 2017). To explore the physical and chemical mechanism underlying the anti-bacterial activity of the GO surfaces tested, the oxidation of GSH was evaluated. The percentage loss of glutathione increased with the increase of GO concentration that led to ROS mediated oxidative stress.

In another study, GO was used to coat PVDF (Polyvinylidene fluoride) membrane by vacuum-filtration. Also in this study, the microorganisms taken as models are the Gram-positive *S. aureus* and the Gram-negative *E. coli.* Bacteria were incubated in contact with the surface for 2, 4 and 6 h. The bactericidal and anti-adhesive effect was evaluated by colony forming unit quantification, live/dead assay and the biofilm formation rate quantified by crystal violet assay (Farid et al. 2018) revealing low rate of survival and cell growth on GO surfaces. The bactericidal effect increases with the increasing time of incubation. Crystal violet quantification revealed inhibition of biofilm formation. The anti-biofilm activity displayed+ has been attributed by the authors to physiochemical properties of the surface: GO membrane has a hydrophilic surface compared to the PVDF membrane leading to a minor adsorption of substances released from the bacteria after exposure with GO that can represent nutrient source for bacteria. Moreover, the exposure of carboxylic functional groups of the surface determines a negatively-charge GO surface that can inhibit the initial adhesion of negative-charged *S.aureus* and *E.coli* due to electrostatic repulsion.

Biofilm was further monitored under continuous flow condition over 48 h. The lower biofilm formation formed on GO coated PVDF membrane compared to pristine PVDF was related mainly to the formation of a "wrinkled surface with sharp exposed nano-sheet edges which serve as nano-blades that strongly impair the cell's outer membranes by piercing or laceration. The thickness of biofilm formation on GO coated membrane was 56.5 μm in contrast to the thickness of the biofilm developed on the virgin PVDF membrane, 110,3 μm. Moreover, the stability of the biofilm was evaluated washing the membrane after measuring the thickness, that revealed to be high on the untreated membrane (106,2) and low on the GO treated membrane (4,8 μm).

The authors investigated whether the mechanical disruption or the chemical oxidation could be the mechanism responsible for GO bactericidal effect. To examine the physical destruction of the membranes, the intra-cytoplasmic material released upon membrane destruction has been quantified and the cell integrity analysed by TEM. Loss of membrane integrity and release of intracellular components confirmed the physical destruction of GO surfaces after interaction with GO. The GO-induced oxidative stress has been confirmed by measuring the increased intracellular level of ROS in *E.coli* and *S.aureus* following contact with GO surfaces. Authors speculate that the intracellular ROS induction is mainly attributed to the presence of oxygen-containing functional groups on the surface of GO (Farid et al. 2018).

3.2 GO Functionalization

GO can be combined with other substances either to enhance its antimicrobial activity or to improve the stability of the coating. Different studies evaluated the antimicrobial properties of GO mixed with nanoparticles, such as silver (Xie et al. 2017), zinc oxide (Jones et al. 2008; Chen et al. 2016), gold (Hussain et al. 2014), titanium dioxin (He et al. 2013) or with other polymers, such as chitosan (Konwar et al. 2016; Chen et al.

2013) or antibacterial substances like lysozyme (Bera et al. 2018) or curcumin (Bugli et al. 2018).

Silver nanoparticle is one of the most studied nanoparticles as it presents good features, such as broad antimicrobial activity, high resistance to oxidation and high-thermal conductivity (Yousefi et al. 2017, De Faria et al. 2014). In Zhao et al. 2018 Zhao et al., synthesize a polyethyleneimine (PEI)-modified and AgNP-decorated GO nanocomposite (GO–PEI–Ag) with a size of around 5 nm that displayed antimicrobial activity against Gram-negative *E.coli*, *Acinetobacter baumanii*, *Shigella sonnei*, the Gram-positive bacteria *S. aureus* and the fungi *Aspergillus fumigatus* and *C. albicans*. This composition can kill the adhered bacteria resulting in a complete biofilm formation inhibition. Cell membrane disruption and intra-cytoplasmic leakage appear to be the main inhibition mechanism. Moreover, this formulation showed long-term stability compared to GO-Ag and biocompatibility as HeLa cells treated for 8hs and 24 h using 10, 20 and 50 μg/mL of GO−PEI − Ag were 86.1, 81.4, and 80.2% of viability, respectively, for the 8 h group and 80.0, 77.9, and 72.0%, respectively, for the 24 h group (Zhao et al. 2018).

GO can be also combined with antibacterial substances that can enhance its efficacy in killing and inhibiting bacteria adhesion, such as lysozyme, one example is the work of Duan et al. They prepared a GO-lysozyme surface resulting in a effective activity against *Escherichia coli* (Duan et al. 2015).

In 2016, an antimicrobial film composed by chitosan-iron oxide coated GO has been realized; specifically, iron oxide coated GO nanomaterial was prepared by a modified co-precipitation method and, in a second step, chitosan hydrogel was incorporated thought hydrogen bonding and electrostatic interaction. The antimicrobial activity was compared to the individually synthesized chitosan GO and chitosan iron oxide hydrogel against methicillin-resistant *S. aureus* (MSRA), *S. aureus*, *E. coli* and *Candida albicans*; authors noticed an improved activity of chitosan-GO nanocomposite respect to the individual former type of films. This mixed GO appeared also to be biocompatible as the haemolysis test conducted on

human erythrocytes displayed low to mild haemolytic activity (0,37%) and, moreover, the cytotoxicity assay on L929 fibroblast cell line showed a much higher viability (80–93%) compared to the negative control. (Konwar et al. 2016).

Curcumin is a yellow–orange polyphenol compound that possesses a wide range of pharmacological activities due to its anti-inflammatory, anticarcinogenic, and anti-infectious properties. The major issue in curcumin-based therapies is the poor solubility of this hydrophobic compound and the cytotoxicity at high doses. Bugli et al. (2018) load the curcumin on the surface area of GO and demonstrated its effectiveness against *Staphylococcus aureus* MRSA. Curcumin (CU) is a hydrophobic compound, it is able to spontaneously absorb on GO surface by π-π stacking interactions, without the need of chemical agents, leading to the creation of a 'green' and safe antimicrobial solution. GO and curcumin showed a synergic effect as MIC of GO alone ranges from 2.35 to 18.75 µg/ml, MIC of curcumin alone against MRSA ranges from 125 to 256 µg/ml while the MIC of the GO-CU combination are between 1.06 and 2.8 µg/ml. Furthermore, this formulation has no cytotoxic effects on eukaryotic cells.

GO-CU nanocomposite was investigated by the same group (Palmieri et al. 2018) for its ability to inhibit biofilm formation of the fungus *C. albicans,* often isolated from medical devices related infections. While GO seems to not have effect in the adhesion of Candida cells on GO-coated plastic discs, GO-CU shows a partial inhibition of adhesion and poor biofilm formation of *C.albicans*. Furthermore, PEG (polyethylene glycol) was used to coat GO discs either alone or with curcumin. The GO-CU-PEG nanocomposite showed anti-adhesion property and the ability to act as drug release surface: CU is released in the media when the coating is in ddH$_2$O showing anti-fungal effect.

Frigols and collaborators recently presented a zinc alginate graphene-oxide film (Frigols et al. 2019) with strong anti-bacterial activity against *S. aureus* and *S. epidermidis* enhanced by the release of Zn^{2+} in the solution. Although the antibacterial properties of this innovative material

can only be attributed to the presence of Zn^{2+}, GO stabilizes and promotes long-term ion release, confirming the potential drug-release technology of the graphene oxide-based coating.

Table 1 shows some studies focusing on the antimicrobial properties of surfaces covered with graphene with different coating methods and types of functionalization. Anti-biofilm activity tested microbial species and biocompatibility are also revised in the table.

4 Go-Based Antibacterial Materials Safety Profile in Mammals

With the perspective to promote the clinical use of GO, it is fundamental to evaluate the short- and long-term cytotoxicity profiles of GO towards eukaryotic cells. Despite the benefits that GO possesses for antibacterial applications, such as high surface area and exceptional solubility in aqueous environments, its biocompatibility is at the moment controversial. In the biological environment, GO-based nanomaterials with their peculiar physico-chemical surface are straight exposed to direct contact with cells and biological macromolecules. In recent years several publications (Zhang et al. 2016; Ou et al. 2016; Wang et al. 2011) have deepened the potential toxic effects of GO in mammalian cells and animals. Zhang B. and co-workers (Zhang et al. 2016) highlighted a widespread toxic effect of GO in mammalian systems due to cell membrane disruption and lysosomal and mitochondria dysfunction with massive ROS generation. GO toxicity was closely linked to three different parameters: GO concentration, lateral size and surface functionalization. These factors are, not surprisingly, the same ones that influence the antibacterial properties of GO (Ou et al. 2016).

4.1 GO Concentration

GO dose-dependent toxicity in cells lines and animals has been demonstrated in recent years

Table 1 Antimicrobial activity of GO

Material	Coated surface	Surface coating method	Microorganism tested	Biofilm inhibition	Biocompatibility	GO concentration tested	References
GO/rGO	Paper	Vacuum filtration	E.coli	none	Adenocarcinomic alveolar basal epithelial cells A549 cells (20 μg/ml)	20, 85 μg/ml	Hu et al. (2010)
GO/rGO	Stainless steel	Electrophoretic deposition	E.coli S.aureus	none	none	Not indicated	Akhavan and Ghaderi (2010)
PVK-GO	Indium tin oxide (ITO).	Electrophoretic deposition	E.coli	yes	none	1 mg/ml	Santos et al. (2011)
PLA/PU/GO	Fibrous paper	Electrospinning	E.coli S.aureus	yes	Osteoblastic cell line MC3T3-E1 (GO: 3, 5%)	GO:3, 5%	An et al. (2013)
GO	Silicon dioxide (SiO$_2$)	Spin-coating	Halomonas spp.CAM2	yes	none	Not indicated	Parra et al. (2015)
GO-Lys	Water membrane	Phase inversion	E.coli	none	none	512, 256, 128, 64, 32 μg/ml	Duan et al. (2015)
GO	Mixed cellulose filter	Vacuum filtration	E.coli S.aureus	none	none	200 μg/ml	Perreault et al. (2015)
Ag-HAP-GO	Titanium	Electro-deposition	E.coli, S.aureus	none	PBMC	Not indicated	Janković et al. (2015)
GO	None –study in suspension	–	E.coli S.aureus	none	None	2 to 200 μg/ml	Palmieri et al. (2016)
Agar-GO	Plastic disc	Hydrogel self-assembly	E.coli, S. aureus, C. albicans	none	none	1 mg/ml	Papi et al. (2016)
GO	Polycarbonate urethane (PCU) membrane	Electrospraying	S. aureus P.aeruginosa	none	L-929 mammalian fibroblast cells	Not indicated	Thampi et al. (2017)
GO	96-well plate	Drop-casting and slow oven drying	E.coli S.aureus	yes	none	50, 200 μg/ml	Yadav et al. (2017)
GO-HEMA	Glass substrates	Magnetic fields-UV irradiation	E.coli	none	none	200 μg/ml	Lu et al. (2017)
GO	PVDF membrane	Vacuum-filtration	E.coli S.aureus	yes	none	100 μg/ml	Farid et al. (2018)

(continued)

Table 1 (continued)

Material	Coated surface	Surface coating method	Microorganism tested	Biofilm inhibition	Biocompatibility	GO concentration tested	References
GO–PEI–ag	None- in suspension	–	E.coli S.aureus A.baumanii Shigella sonnei Candida albicans Aspergillus fumigatus	Yes	Epitelian cervix adenocarcinoma cells HeLa cells (10,20,50 µg/ml)	1 to 128 µg/ml	Zhao et al. (2018)
Chitosan-iron oxide GO hydrogel	Hydrogel	Gel-casting Co-precipitation	E.coli, S.aureus S.aureus MRSA C.albicans	none	Human erythrocytes L929 fibroblastic cells	Not indicated	Konwar et al. (2016)
GO-CU	None- in suspension	–	S.aureus MRSA	none	3 T3 fibroblast cell line (GO: 50 µg/ml CU: 200,300 µg/ml)	GO:1 mg/ml CU: 100,200,300 µg/ml	Bugli et al. (2018)
GO-CU GO-PEG GO-CU-PEG	Plastic disc Central venous catheters	Drop-casting	C.albicans	yes	VERO kidney's epithelial monkey cell line (PEG 5%)	GO:1 mg/ml CU:2 mg/ml PEG:5%,10,20%	Bugli et al. (2018)
GO-Zn^{2+}-SA1 and SA2	Glass petri dish	Chemical route	S.aures S.epidermidis MRSE	no	Human keratinocyte HaCaT cells (1% diluted GO-Zn^{2+}-SA1 and SA2)	1%w/w GO	Frigols et al. (2019)

When available, the safe concentration of GO resulted in the biocompatibility test has been indicated (). Abbreviations: *GO* graphene oxide, *GO–PEI–Ag* polyethyleneimine (PEI)-modified and AgNP-decorated GO nanocomposite, *PVK* polyvinyl-N-carbazole, *PVDF* Polyvinylidene Difluoride, *HAP-GO* silver graphene oxide hydroxyapatite, *PBMC* peripheral blood mononuclear cells, *CU* curcumin, *PEG* polyethylene glyco, *IPLAVPU* Polylactic Acid/Polyurethane, *Lys* Lysozyme, *SA* sodium alginate, *MRSA* methicillin-resistant *Staphylococcus aureus*, *MRSE* methicillin-resistant *Staphylococcus epidermidis*, *Hema* Hydroxyethyl methacrylate

by numerous studies. The main toxic effects result in cell apoptosis, liver and kidney lesion and lung fibrosis. Wang et al. (Wang et al. 2011) analyzed GO cytotoxic effects on human dermal fibroblast cells (HDF). In this study, different concentration of GO, ranging from 10 to 100 µg/ml were exposed to HDF for 24 h. Significant increase in cytotoxicity was observed at 50 µg/ml with decreased cell survival rate and increased apoptosis. GO sheets that enter the cells were found in the cytoplasm and very few within the nucleus. The internalized GO was mainly distributed inside the lysosomes and mitochondria and the size of GO sheets was between 100 and 200 nm. Another study from Lammel and co-workers (Lammel et al. 2013) showed that the exposure of HepG2 cells to both GO and carboxylated graphene nanoplatelets (CXYG) displayed a dose and time dependent increase in ROS production. The predominating size distribution of GO and CXYG determined by means of dynamic light scattering was 385 and 1,110 nm respectively. Both graphene-based nanomaterials penetrate into the cell by impairing the membrane phospholipid bilayer. These data were recently confirmed by a study from Duan and collaborators (Duan et al. 2017) in which they demonstrated that graphene sheets, both pristine and GO with a lateral sizes ranging from 200 nm to 700 nm, caused the formation of pores in the cytoplasmic membranes of A549 and Raw264.7 cells, consequently reducing cell viability. Other studies confirm the fact that the amount of cellular uptake increases with the exposure time and increasing dose. (Wang et al. 2011, 2015; Lee et al. 2017). In *in vivo* studies, dose and surface modification are considered the main parameters that modulate graphene-based nanomaterials bio-distribution and toxicity. One of the most basic pre-clinical *in vivo* studies demonstrated that GO, with an average size of 200 nm, if injected *via* tail-vein in mice at a concentration of 0.25 mg/kg does not affect lifetime, while a high GO dose of 0.4 mg/kg showed chronic toxicity (Wang et al. 2011).

4.2 GO Lateral Size

GO lateral size may influence its cellular uptake and bio-distributions in mammalian organs.

Gurunathan and collaborators (Gurunathan et al. 2019) profiled a significant size and dose dependent toxicity of different sized GO nanosheets with an average size of 100 and 20 nm on two different germ cell lines. Smaller flakes resulted more cytotoxic in a dose depending manner showing higher cellular internalization with loss of cell viability and cell proliferation. A study from Ma et al. (2015) highlighted that large GO flakes showed evident binding with plasma membrane and minor phagocytosis, eliciting robust activation of NF-kB pathway while small GO sheets were highlighted much more internalized into cell cytoplasm. Liao and coworkers investigated the effects of graphene on human erythrocytes and shown that 350 nm-sized graphene could induce strong hemolysis compared to 3 µm-sized graphene sheets (Liao 2015). Drastic membrane disruption by nanoscale sized graphene could be related to the strong electrostatic interactions between the graphene surface and the lipid bilayer of the erythrocyte membrane. On the other hand, low toxicity of microscale sized graphene sheets may be attributed to their lower overall surface areas.

4.3 GO Surface Functionalization

Recent perspective strategies for future biological applications of GO-based nanomaterials include several surface functionalization strategies to improve their safety profile. GO owns a strong hydrophobic surface that allow the formation of a protein corona in biological environment, thus minimizing the physical interactions between GO and cell membranes and reducing cytotoxicity (Hu et al. 2011). This study evaluated the effect of fetal bovine serum (FBS), a common component in cell culture medium, on GO cytotoxicity. At 1% concentrations of FBS, GO showed concentration-dependent cytotoxicity.

Interestingly, the cytotoxicity of GO was much lower at 10% FBS, the concentration usually employed in cell culture medium.

The influences of surface charge have also been highlighted in GO-induced toxicities because the electrostatic repulsion between GO and non-phagocytes plays an important role in particle internalization (Ou et al. 2016). Also, in *in vivo* studies, bio-distribution of graphene nanomaterials is influenced by surface functionalization which in turn modulates their toxicological profiles. (Kanakia 2014). In a study by Yang et al. (Yang et al. 2011), radiolabeled PEG functionalized GO and rGO were administrated intragastrically to female BALB/c mice and bio-distribution. After 4 h of injection, was mainly observed in stomach and intestine, disappearing 1-day post feeding. The same study highlighted the bio-distribution of the same PEG functionalized GO and rGO after intra peritoneal administration. Conversely to oral administration, intraperitoneal injection resulted in high accumulation of graphene nanoparticles on liver and spleen, still evident 30 days after administration.

5   Conclusion

Preventing the bacterial colonization of biomedical devices is the key for limiting the spread of hospital-acquired infections. Antibacterial coatings have become a very active field of research, strongly stimulated by the urgent and increasing need to identify innovative strategies instead of using traditional antibiotics. This review focuses on the antibacterial properties of GO, analyzing the different mechanisms of action exerted to impair bacteria integrity. The antibacterial mechanisms of graphene oxide have still to be completely understood, but most of the current findings and advances support its antibacterial efficacy. Most of the studies agree on the importance of precise physicochemical parameters that most influence the GO bacteriolytic and bacteriostatic properties, like the GO size, the GO concentration and the quantity of hydroxyl groups on the surface. These

characteristics impact on the different interaction of GO with the microbial surface: cutting bacteria membranes by the sharp edges, inducing oxidative stress and wrapping bacteria between large GO sheets. Several studies reported in this article focus on the functionalization of the GO with different compounds, some with known antimicrobial activities but not usable individually for intrinsic toxicity (Xie et al. 2017), others highly hydrophobic that can be delivered by GO nano sheets (Bugli et al. 2018). It is now largely accepted that bacteria can attach to solid substrates, in sessile structured communities called biofilms, where they can persist for long periods, acting as a pathogens reservoir. In this regard, the prospect of exploiting the antimicrobial properties of GO to coat medical devices and prevent microbial adhesion represents a challenge of great impact on human health. Key topic of this brief review focused on GO-coated surface ability to interfere with microbial adhesion. Data on this subject are still rather limited and deriving from different methods of GO deposition on as many different surfaces. Overall, the results are promising and demonstrate an effective capacity of GO-coated surfaces to interfere with microbial adhesion and biofilm formation. Plastics are the most widespread material used for medical devices construction, for weight, cost, and performance purposes. To translate results obtained from such a considerable number of studies, it is mandatory to standardize the best method of covering specific materials used for medical devices (e.g. polyethylene, polypropylene, polyvinyl chloride, polycarbonate), accurately assessing the stability and biocompatibility. Further, the present review offers a concise overview of research on biosafety of GO towards mammals, both *in vitro* and *in vivo*. Toxicity of GO and graphene family nanomaterials is deeply reviewed in current literature, but the research is still in its infancy and it is hard to conclude the potential risks to human health associated with the use of such innovative nanomaterials. As an extraordinary material with remarkable and unique properties, we are convinced that graphene will have a significant impact on many aspects of our life, ranging from general biotech up to biomedical

applications, like "anti-biofilm devices". The enormous increase in research and development of graphene will replace most of the materials on the market so far.

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