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**EXPLORING ANTIBACTERIAL GRAPHENE-BASED
BIOMATERIALS FOR HEMODIALYSIS CATHETERS**

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Science is not finished until it is communicated.

Sir Mark Walport
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Happiness is only real when shared, and so is a PhD.

Abstract

End-stage renal disease (ESRD) is characterized by the complete failure of both kidneys, and is a limiting condition that affects 0.1% of the world population. Hemodialysis (HD) catheters, despite highly associated with bacterial infections and elevated treatment costs, continue to be the most commonly used device for vascular access in HD patients. Catheter-related bloodstream infections are a life-threatening problem, posing significant risks to patient morbidity and mortality. Inefficacy of current treatment options – lock solutions and systemic antibiotic administration – strengthens the need for surface modification of HD catheters with bactericidal and/or anti-adhesive agents.

Considering the antibacterial profile of graphene-based materials (GBMs), the main goal of this thesis was the development of a biomaterial containing GBMs for application in HD catheters. To achieve this, GBMs with different intrinsic features (oxidation degree, platelet size, and thickness) – reduced graphene oxide (rGO), graphene oxide (GO), few-layer graphene (FLG), few-layer graphene oxide (FLGO) – were used to produce surfaces using different techniques – vacuum filtration, dip coating, and perfusion. Particular attention was given to the impact of these parameters on the surface features and on the biological response, focusing primarily on bacteria.

A fundamental study on the interaction of stand-alone graphene-based surfaces produced by vacuum filtration revealed that, unlike thickness, oxidation degree of GBMs strongly impacted surface morphology, topography, and chemistry of the films. GO and FLGO films were more oxidized, smoother, and hydrophilic. rGO and FLG were, as expected, less oxidized, rougher, and more hydrophobic. All films promoted glutathione oxidation to some extent, indicating their potential to induce oxidative stress in biological systems. Human plasma proteins, which mediate most of the biological interactions, adsorbed less to oxidized films than to rGO and FLG. Bacterial interaction with the surfaces (adhesion and viability) was found to be bacteria-dependent, with clinically relevant bacteria adhering also less to oxidized films, despite *S. epidermidis* and *E. coli* adhering even less than *S. aureus* and *P. aeruginosa*. All materials allowed fibroblasts adhesion, proliferation, and viability, as well as blood cells attachment, without causing hemolysis (<5%). A lower adhesion of blood cells was however observed in more oxidized surfaces. As such, surfaces containing high levels of oxygen were demonstrated to be good candidates for the development of antibacterial biomaterials for application in HD catheters.

As light-responsive materials, GBMs films antibacterial performance was explored upon stimulation with low power near-infrared (NIR) radiation. Films' exposure to NIR drastically improved their ability to kill planktonic (up to ~99%) and adherent (up to ~85%) methicillin-resistant *S. aureus*, and *S. epidermidis*. Upon irradiation, a mild photothermal effect was observed in the supernatant, with the temperature reaching 39 °C – 42 °C, while the surface temperature of non-oxidized FLG films increased to 51.3 °C versus 56 °C for oxidized films. Further exploitation on the mechanism of action of the

surfaces revealed that both films prompted total glutathione oxidation when irradiated, despite FLG films inducing higher reactive oxygen species (ROS) generation than FLGO. This suggests that antioxidants depletion occurred preferentially by ROS-dependent pathway (photodynamic effect) for FLG versus ROS-independent pathway for FLGO films.

Envisaging the application of GBMs onto HD polyurethane (PU) catheters, FLG and FLGO materials, which are cheaper and easier to produce than GO – also commercially denominated graphene nanoplatelets (GNP) and oxidized GNP (GNPox) – were incorporated in PU through two different strategies: melt-blending, producing a bulk composite, and dip coating, where a composite layer is deposited on top of a PU film. GNP exposure at the surface strongly impacted the antibacterial profile: GNP absence at the surface of composites yielded no significant effects on bacteria, while by varying GNP:PU ratio and GNP concentration, coatings showed improved GNP exposure, depicting an antibacterial profile. Oxidized GNP-containing coatings induced higher antibacterial effect than non-oxidized forms, particularly GNPox with smaller lateral size, where a homogeneous layer of “fused” platelets was formed on PU, leading to a 70% reduction in bacterial adhesion and 70% bacterial death. Considering that smaller and oxidized GBMs are therefore the most promising to yield antimicrobial functionality to PU catheters, GO and FLGO were used for the first time to coat the inner lumen of commercially available HD catheters using perfusion. A coating that covers homogeneously the surface was produced, and exploratory experiments unraveled the potential of these coatings to prevent bacteria adhesion and migration through the coated surface.

Overall, this thesis strengthens the importance of carefully considering the GBMs intrinsic features and the surface production techniques in order to efficiently design an antibacterial coating. Smaller particles with higher oxidation degree can reduce bacterial adhesion while exerting a bactericidal effect, with possibility of extending the antibacterial action to planktonic bacteria when activated using a low energy light, thus showing great potential to prevent biofilm formation and the consequent development of catheter-related infections.

Resumo

A falência renal (insuficiência renal crônica, fase 5) traduz-se na perda de função de ambos os rins, e é uma condição limitante que afeta 0.1% da população mundial. Os cateteres de hemodiálise (HD), apesar de associados a infecções bacterianas e a elevados custos de tratamento, continuam a ser o dispositivo mais utilizado para acesso vascular em pacientes a realizar HD. Infecções da corrente sanguínea com origem no cateter de HD são um problema grave, representando riscos significativos e aumentando a morbidade e mortalidade dos pacientes. A ineficácia das opções de tratamento atuais – soluções de manutenção do cateter e administração sistêmica de antibióticos – reforça a necessidade de modificação da superfície dos cateteres de HD com agentes bactericidas e/ou anti-adesivos.

Considerando o perfil antibacteriano dos materiais à base de grafeno (GBMs), o objetivo principal desta tese consiste no desenvolvimento de um biomaterial contendo GBMs para aplicação em cateteres de HD. Para isso, GBMs com diferentes características intrínsecas (grau de oxidação, tamanho e espessura das folhas de grafeno) – óxido de grafeno reduzido (rGO), óxido de grafeno (GO), grafeno com multi-camadas (FLG), óxido de grafeno com multi-camadas (FLGO) – foram usados para produzir superfícies através de diferentes técnicas – filtração por vácuo, revestimento por imersão, e perfusão. Foi realçado o impacto desses parâmetros nas características da superfície e na resposta biológica, com foco na ação contra bactérias.

Um estudo fundamental sobre a interação dos filmes constituídos apenas por GBMs, produzidos por filtração a vácuo, revelou que, ao contrário da espessura, o grau de oxidação dos GBMs tem um grande impacto na morfologia, na topografia e na química da superfície dos filmes. Os filmes de GO e FLGO têm superfícies mais oxidadas, mais lisas, e hidrofílicas, enquanto as superfícies dos filmes de rGO e FLG são, como esperado, menos oxidadas, mais rugosas, e mais hidrofóbicas. Todos os filmes promoveram a oxidação de glutatona, evidenciando o seu potencial para induzir stress oxidativo nos sistemas biológicos. As proteínas do plasma humano, que medeiam a maioria das interações biológicas, adsorvem menos a filmes oxidados do que a filmes de rGO e FLG. A interação das bactérias com estes filmes (adesão e viabilidade) foi considerada dependente do tipo de bactérias, com bactérias clinicamente relevantes aderindo também menos aos filmes oxidados, apesar da *S. epidermidis* e *E. coli* aderirem ainda menos que *S. aureus* e *P. aeruginosa*. Todos os filmes permitiram a adesão, proliferação e viabilidade de fibroblastos, bem como de componentes do sangue, sem, no entanto, causar hemólise (<5%). Menor adesão destes componentes foi, no entanto, observada em filmes mais oxidados. Assim, superfícies contendo elevados níveis de oxigênio demonstraram ser candidatos promissores para o desenvolvimento de biomateriais antibacterianos para aplicação em cateteres de HD.

Considerando a responsividade à luz dos GBMs, o desempenho antibacteriano dos filmes de GBMs foi também explorado após estimulação com radiação infravermelha próxima (NIR) de baixa potência. A irradiação dos filmes aumentou drasticamente a sua capacidade de matar *S. aureus* resistente à

metecilina e *S. epidermidis*, tanto em suspensão (até ~ 99%) como aderidas à superfície (até ~ 85%). Foi observado um efeito fototérmico moderado após irradiação, com a temperatura do sobrenadante a atingir 39 °C – 42 °C, enquanto a temperatura da superfície dos filmes FLG aumentou para 51.3 °C e para 56 °C no caso dos filmes oxidados (FLGO). A exploração do mecanismo de ação das superfícies revelou que ambos os filmes provocaram oxidação total da glutathione quando irradiados, apesar dos filmes de FLG induzirem maior produção de espécies reativas de oxigênio (ROS) do que os filmes de FLGO. Estes resultados sugerem que a depleção de antioxidantes ocorre preferencialmente pela via dependente de ROS (efeito fotodinâmico) para os filmes de FLG, contrariamente a uma via independente da produção de ROS para os filmes de FLGO.

Tendo em vista a aplicação dos GBMs em cateteres de HD de poliuretano (PU), FLG e FLGO, mais baratos e fáceis de produzir do que GO – e comercialmente denominados nanoplaquetas de grafeno (GNP) e de grafeno oxidado (GNPox) – foram incorporados em PU através de duas estratégias diferentes: fusão-mistura, produzindo um compósito, e revestimento por imersão, onde um compósito é depositado na superfície de um filme de PU. A exposição dos GNP à superfície influenciou significativamente o perfil antimicrobiano: a ausência de GNP na superfície dos compósitos não promove ação antibacteriana, enquanto que a alta exposição de GNP obtida nos revestimentos (ao variar a relação GNP:PU e a concentração do GNP) revela um perfil antimicrobiano mais eficiente. Revestimentos com GNP oxidado têm maior efeito antibacteriano do que com formas não oxidadas, particularmente com GNPox com menor tamanho lateral, verificando-se uma camada uniforme de folhas de grafeno “fundidas” formada no PU, levando a uma redução de 70% na adesão e 70% da morte bacteriana.

Considerando que materiais com menor tamanho lateral e maior grau de oxidação são os mais promissores para tornar os cateteres de PU antibacterianos, GO e FLGO foram usados pela primeira vez para revestir o lúmen interno de cateteres de HD disponíveis comercialmente, usando um sistema de perfusão. Formou-se um revestimento superficial uniforme, que em resultados exploratórios revelou potencial para prevenir a adesão e migração de bactérias através da sua superfície.

No geral, esta tese reforça a importância de considerar cuidadosamente as características intrínsecas dos GBMs e a técnica de produção da superfície, de modo a desenvolver com eficiência um revestimento antibacteriano. Superfícies produzidas com plaquetas de grafeno mais pequenas e com maior grau de oxidação reduzem a adesão bacteriana enquanto exercem um efeito bactericida, verificando-se ainda a possibilidade de extensão da ação contra bactérias em suspensão quando estas superfícies são ativadas por uma luz NIR de baixa energia. Estes dados revelam assim o grande potencial destas superfícies para prevenir a formação de biofilme e, consequentemente, o desenvolvimento de infeções relacionadas com a utilização do cateter de HD.

Abbreviations List

A

AFM – Atomic Force Microscopy
 AVF – Arteriovenous fistula
 AVG – Arteriovenous graft

B

BSA – Bovine serum albumin

C

CFU – Colony forming unit
 CKD – Chronic kidney disease
 CRBSI – Catheter-related bloodstream infection
 CRT – Catheter-related thrombosis
 CVC – Central venous catheters
 CVD – Chemical vapor deposition

E

eGFR – Estimated glomerular filtration rate
 EPD – Electrophoretic deposition
 EPS – Extracellular polymeric substances
 ePTFE – expanded-Polytetrafluoroethylene
 ESRD – End-stage renal disease

F

FBS – Fetal bovine serum
 FDA – Food & Drug Administration
 FLG – Few-layer graphene
 FLGO – Few-layer graphene oxide
 FTIR – Fourier-transform infrared spectroscopy

G

G – Graphene
 GBMs – Graphene-based materials
 GBMox – Oxidized graphene-based materials
 GNP – Graphene nanoplatelets

GNPox – Oxidized graphene nanoplatelets
 GO – Graphene oxide
 GSH – Glutathione
 Gt – Graphite
 GtO – Graphite oxide

H

HD – Hemodialysis

L

LB – Langmuir-Blodgett

M

MHM – Modified Hummers' method
 MLG – Multi-layer graphene
 MLGox – Oxidized Multi-layer Graphene
 MRSA – Methicillin resistant *S. aureus*

N

NIR – Near-infrared

O

OPT – O-phthalaldehyde

P

PBS – Phosphate buffered saline
 PD – Peritoneal dialysis
 PDT – Photodynamic therapy
 PI – Propidium iodide
 PTT – Photothermal therapy
 PU – Polyurethane

R

RBCs – Red Blood Cells
 rGO – Reduced graphene oxide
 ROS – Reactive oxygen species
 RRT – Renal replacement therapies

ABBREVIATIONS LIST

RT – Room temperature

S

SEM – Scanning Electron Microscopy

T

TCPET – Tissue culture polyethylene
terephthalate

TEM – Transmission Electron Microscopy

TPUs – Thermoplastic polyurethanes

TSA – Tryptic Soy Agar

TSB – Trypticase Soy Broth

X

XPS – X-Ray Photoelectron Spectroscopy

XRD – X-Ray Diffraction

Author's contributions

The work performed directly under the scope of this thesis resulted in the following contributions:

PUBLICATIONS:

Papers in international peer-reviewed journals

1. P.C. Henriques, A.T. Pereira, D. Bogas, J.R. Fernandes, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Graphene films irradiated with safe low-power NIR-emitting diodes kill multidrug resistant bacteria. *Carbon* 2020 (IF 8.821).
2. P.C. Henriques, A.T. Pereira, A.L. Pires, A.M. Pereira, F.D. Magalhães, I.C. Gonçalves. Graphene Surfaces Interaction with Proteins, Bacteria, Mammalian Cells and Blood Constituents: The Impact of Graphene Platelet Oxidation and Thickness. *ACS Applied Materials & Interfaces*, 2020, 12, 18, 21020–21035 (IF 8.456).
3. I. Borges*, P.C. Henriques*, R.N. Gomes, M. Pestana, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Exposure of Smaller and Oxidized Graphene on Polyurethane Surface Improves its Antimicrobial Performance. *Nanomaterials*, 2020, 10(2), 349 * **equal contribution** (IF 4.034).
4. P.C. Henriques, I. Borges, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Fabrication and antimicrobial performance of surfaces integrating graphene-based materials. *Carbon*, 2018, 132:709-732 (IF 7.466).

Patents

PPP 20191000031489. (2019.12.20). ANTIMICROBIAL GRAPHEN COATING. Ana Patrícia Henriques, Inês Borges, Natacha Rosa, Manuel Pestana, Artur Pinto, Fernão Magalhães, Inês Gonçalves.

PRESENTATIONS IN SCIENTIFIC MEETINGS:

Oral communications

1. P.C. Henriques, A.T. Pereira, A.L. Pires, A.M. Pereira, F.D. Magalhães, I.C. Gonçalves. Graphene Surfaces Interaction with Proteins, Bacteria, Mammalian Cells and Blood Constituents: the Impact of Graphene Platelet Oxidation and Thickness. NanoPortugal, Online Conference (2020).
2. P.C. Henriques, A.T. Pereira, A.F. Maia, F.D. Magalhães, I.C. Gonçalves. Graphene-based surfaces love affair with bacteria, mammalian cells and blood constituents. Is there a perfect match? 30th Annual Conference of the European Society for Biomaterials (ESB), Dresden, Germany (2019).

3. P.C. Henriques, F.D. Magalhães, I.C. Gonçalves. Graphene-based materials (GBMs) containing surfaces to prevent bacterial infections and thrombus formation. Summer School for Biomaterials for Medical Devices and Regenerative Medicine, Bordeaux (2018).
4. P.C. Henriques, I. Borges, N. Rosa, C.N. Mota, A.M. Pinto, F. D. Magalhães, I.C. Gonçalves. Graphene/Polyurethane coatings for antimicrobial surfaces: exploring different coating strategies. International Symposium on Bioinspired Macromolecular Systems, Aveiro, Portugal (2017).

Poster communications

1. P.C. Henriques, I. Borges, N. Rosa, M. Pestana, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. From 2D to 3D: using graphene coatings to develop antimicrobial hemodialysis catheters. 11th World Biomaterial Congress (WBC), Online Conference (2020).
2. P.C. Henriques, A.T. Pereira, A.F. Maia, F.D. Magalhães, I.C. Gonçalves. The love affair between graphene-based surfaces and bacteria, mammalian cells and blood constituents. Is there a perfect match? Graphene Week, Helsinki, Finland (2019).
3. P.C. Henriques, I. Borges, N. Rosa, C.N. Mota, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Graphene/Polyurethane coatings for antimicrobial surfaces: exploring different coating strategies. International Symposium on Bioinspired Macromolecular Systems, Aveiro, Portugal (2017).
4. P.C. Henriques, I. Borges, C.N. Mota, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Coating polyurethane with graphene: exploring different strategies. 6th China-Europe Symposium on Biomaterials in Regenerative Medicine, Porto, Portugal (2017).
5. P.C. Henriques, I.S. Borges, C.N. Mota, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Different approaches for graphene incorporation into polyurethane. II Jornadas Nacionais de Caracterização de Materiais, Aveiro, Portugal (2017).

AWARDS:

1. Travel Grant awarded to present the work "P.C. Henriques, A.T. Pereira, A.F. Maia, F.D. Magalhães, I.C. Gonçalves. The love affair between graphene-based surfaces and bacteria, mammalian cells and blood constituents. Is there a perfect match?" at Graphene Week 2019, Helsinki, Finland (2019).
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3. Best graphical abstract and pitch presentation of the work "P.C. Henriques, F.D. Magalhães, I.C. Gonçalves. Graphene-based materials (GBMs) containing surfaces to prevent bacterial infections

and thrombus formation” at Summer School: Biomaterials for Medical Devices and Regenerative Medicine, Bordeaux, France (2018).

4. Honorable Mention 2nd Prize for the pitch presentation of the work “P.C. Henriques, I.S. Borges, C.N. Mota, A.M. Pinto, F.D. Magalhães, I.C. Gonçalves. Different approaches for graphene incorporation into polyurethane” at II Jornadas Nacionais de Caracterização de Materiais, Aveiro, Portugal (2017).

ORGANIZATION OF SCIENTIFIC CONFERENCES:

1. Organizing committee of the 2nd Doctoral Congress in Engineering (DCE), Symposium on Biomedical Engineering (June 2017).

Other contributions in related topics during the thesis period:

Papers in international peer-reviewed journals

1. A.T. Pereira, P.C. Henriques, K.H. Schneider, A.L. Pires, A.M. Pereira, M.C.L. Martins, F.D. Magalhães, H. Bergmeister, I.C. Gonçalves. Graphene-based materials: the key to a successful application of pHEMA as a blood contacting device. *Biomaterials Science*, 2021, under revision.
2. A.T. Pereira, P.C. Henriques, P. Costa, M.C.L. Martins, F.D. Magalhães, I.C. Gonçalves. Graphene oxide-reinforced poly(2-hydroxyethyl methacrylate) hydrogels with extreme stiffness and high-strength. *Composites Science and Technology*, 2019, 184, 107819 (IF 6.309).
3. P.C. Henriques, L.M. Costa, C.L. Seabra, B. Antunes, R. Silva-Carvalho, S. Junqueira-Neto, A.F. Maia, P. Sampaio, P. Oliveira, A. Magalhães, C.A. Reis, F. Gartner, E. Touati, J. Gomes, P. Costa, M.C.L. Martins, I.C. Gonçalves. Orally administrated chitosan microspheres bind *Helicobacter pylori* and decrease gastric infection in mice. *Acta Biomaterialia*, 2020, 114, 206-220 (IF 7.242).

Oral communications

1. A.T. Pereira, P.C. Henriques, K. Schneider, C. Grasl, P. Costa, M.C.L. Martins, F.D. Magalhães, H. Bergmeister, I.C. Gonçalves. Tuning Poly(2-hydroxyethyl methacrylate) Hydrogels Mechanical Properties Through Graphene Oxide Incorporation, *Advanced Materials*, Online Conference (2020).
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CHAPTER I

Motivation, Objectives, and Thesis Structure

1. Motivation

Chronic kidney disease had a global prevalence of 9.1% in 2017, corresponding roughly to 700 million cases worldwide [1]. The last stage of the disease, where both kidneys fail, affected approximately 0.1% of the world population, meaning 7.6 million people [2], with the number of diagnosed patients increasing at a rate of 5 – 7% per year [3]. Reaching this point requires patients to perform either a kidney transplant (occurring in 30% of the cases) or life-sustaining dialysis.

Hemodialysis (HD) represents the most widely adopted modality of kidney replacement therapy, with long-term vascular access being performed through a surgically created arteriovenous communication – either an autologous fistula (AVF) or a synthetic graft (AVG) – or through a central venous catheter (CVC). Although both AVF and AVG are preferred for vascular access, CVCs remain a common form of vascular access in patients initiating HD therapy (70% to 80% of patients in US [4]) and in chronic patients (30 to 40% [5]) [6], with the prevalence of permanent catheters as HD access increasing [7, 8]. Amongst other complications, widespread usage of CVCs – varying greatly among countries [4] – exposes patients to an enhanced risk for catheter-related infections [5]. Immediately upon catheter insertion inside the body, plasma proteins adsorb on the catheter surface, setting the tone for interaction with blood components and bacteria, which can be originally from the outside environment (exit site) or from contaminated fluids. Once adherent, bacteria initiate the development of a biofilm that promotes their protection and proliferation. This event leads to the establishment of infection, and consequent need for catheter replacement, which might increase the hospitalization time, the cost of treatment (about 1 billion USD per year in the USA [9]) and mortality rates.

Current treatment options – antimicrobial lock solution and systemic antibiotic administration [10] – fail to terminate and prevent infection, revealing the necessity for surface modification of HD catheters with bactericidal and/or anti-adhesive agents. Strategies relying on antibiotic/antiseptic impregnated catheters have reduced catheter-related infections when comparing with non-impregnated ones, however few randomized clinical trials have been directed to HD patients [9, 11]. Furthermore, the agents commonly used – silver compounds and nanoparticles, anticoagulants (e.g. heparin), antibiotics (e.g. gentamicin and chlorhexidine) – raise concerns regarding toxicity, secondary effects, and/or antimicrobial resistance – considered one of the biggest threats to public health nowadays, both globally and in the EU region [12, 13]. In addition, if bacteria manage to attach and develop a biofilm, prophylactic and local antibiotics may have difficulty in penetrating the protective matrix, losing efficiency and allowing the establishment of the infection.

Therefore, the high number of catheter-related infections and the associated concerns with the use of antibiotic-based options, demand for safer and non-antibiotic based antimicrobial catheters, efficient at reducing the risk of infections.

Graphene, first isolated in 2004, and graphene-based materials (GBMs) have been explored for antimicrobial purposes since 2010 [14]. Given their biocompatibility and light-responsive ability, they

have been explored for different biomedical and healthcare applications [15-18]. GBMs' use as a surface rather than a suspension has been far less explored, and their effect has been neglected or underestimated by the presence of other materials and antimicrobial agents. When compared with antibiotics or metal nanoparticles, GBMs offer a safer antibacterial performance, with no known mechanisms of bacterial resistance. Furthermore, a competing advantage for industrial partners lies with graphene production costs that nowadays with the new synthesis techniques can be 100 times cheaper than before [19].

This envisages the use of graphene for coating of medical instruments, implants, catheters and surgical equipment as a competitive and promising approach to deliver low cost and efficient antimicrobial materials.

2. Objectives

The ultimate goal of this thesis concerns the development of an antibacterial graphene-based biomaterial for application in hemodialysis (HD) catheters. Envisaging this application, the adopted strategy focused on exploring the antibacterial potential of different graphene-based materials (GBMs) when integrated in a surface. The main goal was to identify the best production strategy and surface parameters in order to obtain an efficient antibacterial coating. The approach followed in this work involved three main specific objectives:

1. Assessment of the reported strategies to produce graphene-based surfaces and their antibacterial performance

Considering the discrepant reported data on graphene's antibacterial performance and their increasing variability, a literature review focused on the production methods and antimicrobial performance of different graphene-based surfaces was performed.

2. Identification of the interactions between GBMs films and biological systems – proteins, bacteria, mammalian cells, and blood components

The literature review highlighted the impact of surface features on the final interaction with bacteria, highly dependent also on the GBMs intrinsic features. A fundamental study was therefore performed to determine how GBMs with different oxidation degree and platelet thickness affected the surface features of stand-alone GBMs films, and consequently the interaction with the major biological systems in our body – proteins, bacteria, mammalian cells, and blood components.

Considering the ability of GBMs to absorb near-infrared (NIR) radiation and its potential usage for different antimicrobial applications, the NIR-triggered killing effect of previously produced GBMs films was also explored.

The mechanisms of action of both non-irradiated and irradiated films towards bacteria were investigated in order to understand how to identify and optimize the best antibacterial surface.

3. Incorporation of GBMs in polyurethane (PU) catheters to make them antimicrobial

GBMs incorporation in PU – the most commonly used material to produce HD catheters – was firstly attempted on flat PU substrates by dip coating. This could promote GBMs exposure contrarily to composites – produced by melt-blending –, enhancing the interaction with microorganisms. The impact of GBMs with different oxidation degree and platelet size on the surface features was explored as well as on the antibacterial performance of the surfaces. Envisaging the application on HD catheters, a graphene-based coating was applied directly on commercially available HD catheters using perfusion. This could bring added benefits in terms of regulatory process and translation to the clinics. The antibacterial action of the coatings was explored in static and flow conditions.

3. Thesis Structure

This thesis is divided into eight chapters, with two of them comprising a literature overview on the thesis subject, four containing the main experimental work, and the final chapter comprising the concluding remarks, as represented in the flowchart.

Chapter I sets the tone for the thesis, containing the motivation under the development of this work, the main objectives established, and how this thesis is structured.

Chapter II provides a general introduction on chronic kidney disease and on the complications associated with hemodialysis catheters. It briefly gives background on why addressing catheter-related bacterial infections has such economic and human impact, and on what has been done to solve this problem.

Chapter III is the cornerstone for the development of this thesis, highlighting the antibacterial potential of graphene-based surfaces, but also reinforcing the need for more studies on the impact of surface features and production techniques of graphene-integrating surfaces on their antibacterial performance. This chapter is based on the following published review article: “P.C. Henriques, *et al.*, Fabrication and antimicrobial performance of surfaces integrating graphene-based materials. *Carbon*, 2018; 132:709-732”.

Chapter IV initiates the experimental work and aims to answer the question: “How do GBMs films interact with our major biological systems?”. It reports a fundamental study on how GBMs films with different intrinsic characteristics, namely oxidation degree and thickness, impact surface features. Using four different GBMs (rGO, GO, FLG and FLGO) to produce surfaces by vacuum filtration, oxidation degree was found to be the most crucial parameter affecting the surface features. It then explores how these features affect protein adsorption and interaction with the three major biological systems: bacteria (four clinically relevant bacteria), human mammalian cells (fibroblasts), and blood components (blood platelets and white and red blood cells). Oxidized surfaces demonstrated to better prevent adhesion and bacteria viability, while allowing mammalian cells to attach, with low adhesion of blood platelets and white and red blood cells. This chapter is based on the following published original article: “P.C. Henriques, *et al.*, Graphene Surfaces Interaction with Proteins, Bacteria, Mammalian Cells and Blood Constituents: The Impact of Graphene Platelet Oxidation and Thickness. *ACS Applied Materials & Interfaces*, 2020, 12, 18, 21020–21035”.

Chapter V explores the light-responsiveness of FLG and FLGO films focusing on answering the question “Can GBMs films act as stand-alone photoactive antibacterial surfaces?”. FLGO is cheaper and easier to produce than GO while presenting similar antibacterial properties, since the thickness did

not show to be relevant as a surface. This chapter explores the potential of near-infrared (NIR) radiation to boost GBMs surfaces' antibacterial performance, revealing their augmented action upon exposure to NIR light, particularly towards planktonic bacteria and with oxidized surfaces. Continuous irradiation caused local mild heating, glutathione depletion, and reactive oxygen species exacerbated production, which led to an intensified oxidative stress response, resulting in bacterial death. This chapter is based on the following original article that has been recently submitted for peer-review and publication in *Advanced Materials Journal*: "P.C. Henriques, *et al.*, Graphene films irradiated with safe low-power NIR-emitting diodes kill multidrug resistant bacteria".

Chapter VI reports the incorporation of GBMs into polyurethane (PU), the most commonly used polymer to produce catheters, and addresses the question "Can GBMs be incorporated in polyurethane to make it antibacterial?". Composites were initially produced by Inês Borges (a researcher from our group) using melt-blending, however, absence of antibacterial properties – possibly due to lack of GBMs exposure – prompted the exploitation of dip coating approach with PU as a binding agent, in a collaborative work. FLG and FLGO materials were used for the same aforementioned reasons, however, named in this chapter GNP (graphene nanoplatelets) and GNPox (oxidized graphene nanoplatelets), their commercial name. In this experimental work, GNP powders with different lateral size and oxidation degree (GNP-M5, GNP-M15, GNP-M5ox, GNP-M15ox) were used to produce the surfaces, and the impact of these parameters on GNP exposure and antimicrobial potential was investigated. This work confirmed that also as coatings, surfaces produced with smaller and oxidized GNP concordantly presented better antibacterial performance. This chapter is based on the following published original article: "I. Borges*, P.C. Henriques*, *et al.*, Exposure of Smaller and Oxidized Graphene on Polyurethane Surface Improves its Antimicrobial Performance. *Nanomaterials*, 2020, 10(2), 349, * equal contribution".

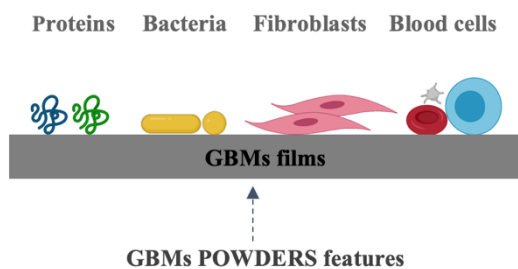
Chapter VII initiates the translation of the 2D graphene-based coating into a 3D catheter. As an exploratory work, it aims to answer the question: "Can oxidized graphene materials be applied as a coating on HD catheters to make them antibacterial?". It explores the application of smaller and oxidized graphene materials (GO and GNP-M5ox) as a coating on the inner lumen of PU hemodialysis catheters using perfusion. This technique was explored for the first time as a coating strategy for GBMs, revealing that a homogeneous and well distributed coating can be achieved in the inner lumen of the catheter. Antibacterial performance was evaluated at i3S and at Nottingham University, as part of a collaboration with Professor Roger Bayston. Preliminary results revealed the coating ability to prevent bacterial adhesion and migration throughout the coated surface, even though under flow conditions and after forcing bacterial adhesion, adhesion can occur.

Chapter VIII finishes this thesis with a general discussion, correlating results from Chapters IV, V, VI and VII, and with the concluding remarks, providing also suggestions for future research.

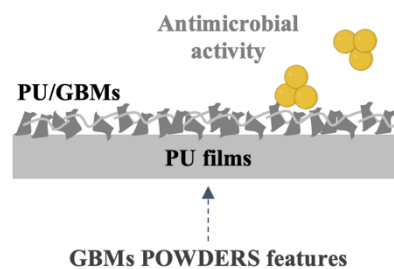
Exploring antibacterial graphene-based biomaterials for hemodialysis catheters

Chapter II and III: Literature Review

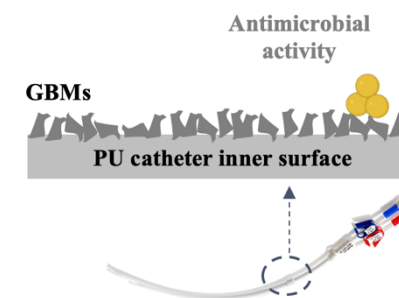
Chapter IV – How do GBMs films interact with our major biological systems?



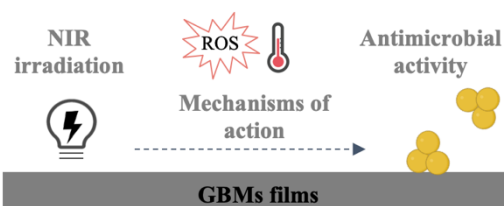
Chapter VI – Can GBMs be incorporated in polyurethane to make it antibacterial?



Chapter VII – Can oxidized graphene materials be applied as a coating on HD catheters to make them antibacterial?



Chapter V – Can GBMs films act as stand-alone photoactive antibacterial surfaces?



Chapter VIII General Discussion, Concluding Remarks, Future Perspectives



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CHAPTER II

Why are hemodialysis catheters a problem?

1. Chronic kidney disease: when kidneys fail

Kidneys play a critical role in the filtration of metabolic waste from the blood, removal of excess fluid from the body, and maintenance of the osmotic and water balance, having also endocrine functions. Nephrons are the functional unit of the kidney, existing approximately one million in each kidney [1].

Chronic kidney disease (CKD), a condition characterized by a gradual loss of kidney function, had a global prevalence of 9.1% in 2017, affecting roughly 700 million people [2]. This progressive decline of kidneys ability to properly remove harmful toxins and excess fluid from the body – leading to their accumulation – is quantitatively reported by the estimated glomerular filtration rate (eGFR), which differentiates the five stages of CKD (Table 1). When in stage 4, patients are recommended to start dialysis due to severe loss of kidney function and prognosis of progressive loss. Once reached stage 5, which corresponds to the total kidney failure – a condition also named end-stage renal disease (ESRD) and where GFR is below 15% – both kidneys can no longer function on their own and renal replacement therapies (RRT) need to be considered.

Table 1. Chronic kidney disease (CKD) stages, symptoms and associated estimated glomerular filtration rate (eGFR). From <https://www.freseniuskidneycare.com/about-chronic-kidney-disease/stages>.

CKD stage	Description	eGFR
Stage 1	Kidney damage with normal kidney function	> 90%
Stage 2	Mild loss of kidney function	60–89%
Stage 3	3a: Mild to moderate loss of kidney function;	3a: 45–59%
	3b: Moderate to severe loss of kidney function	3b: 30–44%
Stage 4	Severe loss of kidney function	15–29%
Stage 5 (ESRD)	Kidney failure	< 15%

ESRD is a limiting condition that affects approximately 0.1% people worldwide (7.6 million) [3], with the number of diagnosed patients increasing at a rate of 5 – 7% per year [4]. Between 2000 and 2016, and in the USA alone, there was an increase of 86% in the prevalent cases of ESRD [4]. The elderly represents the most rapidly growing segment of the ESRD population [5], with 67.7% of patients in RRT being over 65 years. Portugal has one of the highest incidence and prevalence rates of ESRD

among European countries [6], and demographic projections state that by 2050 about 32% of the Portuguese population should be aged 65 and over, and thus are at a greater risk [6].

Both CKD and ESRD represent an enormous economic burden to the healthcare systems worldwide. The global ESRD market size was valued at USD 68 billion in 2019, and is anticipated to reach USD 172 billion by 2027, due to increasing number of patients suffering from ESRD, rising prevalence of hypertension and diabetes, and introduction of technologically advanced products, such as low maintenance dialysis equipment and artificial kidneys [7]. Patients with ESRD require renal replacement therapies that although life-sustaining represent high cost treatments for patients and for the medical system. The number of people receiving such therapies exceeds 2.5 million and is projected to double to 5.4 million by 2030 [8].

1.1 Renal replacement therapies (RRT)

Renal replacement therapies (RRT) aim to substitute the kidneys by performing their function of removing toxins and metabolic waste products from the blood, while maintaining the volumetric and osmotic balance [1]. Currently available modes of RRT include kidney transplantation and dialysis. Between 1990 and 2017, all-age incidence of kidney transplantation and dialysis increased by 34.4% and 43.1%, respectively, reflecting the increased availability of these therapies [2]. At the end of 2018, approximately 4.1 million patients underwent dialysis treatment or received a donor organ, with around 3.4 million people worldwide depending on dialysis treatment [9]. Other alternatives have however been explored: Fresenius Medical Care's subsidiary Unicyte AG reached a pre-clinical milestone with nanoscale extracellular vesicles that can restore the kidney function of patients with CKD [9].

Kidney transplantation is the preferred option, and combined with medication and a healthy diet can restore normal kidney function, providing better quality of life and survival. Transplant success rates are high, with transplants from live donors having a 90 to 95% success rate. However, there are a number of barriers to kidney transplantation, especially the patient's socio-economic status [3], the constant shortage of donors with an average waiting time up to 3.6 years, the chances of host rejection (complications associated with immunosuppression and immunosuppressant use) and loss of functionality of the graft over time (posttransplant lymphoproliferative disorder) [1, 10].

Dialysis corresponds to the filtration and cleaning of blood, being achieved through hemodialysis (HD) or peritoneal dialysis (PD). In HD, the blood is removed from the body and filtered outside by a machine that uses a filter to remove waste and excess fluid. It can be performed at a dialysis center or at home. In PD, the blood is cleaned while still inside the body rather than in a machine: a catheter is placed into the patient's abdomen and fluid is added to collect and remove waste from the body. PD is administered either continuously or intermittently, and it is typically performed at home. Both processes are very time-consuming (weekly sessions), representing significant constraints for the patient. Artificial kidneys are portable dialysis devices that have also been explored to improve patients' quality

of life, with the first human clinical trials of blood purification technologies for designing Wearable Artificial Kidney 2.0 approved by the FDA in 2014 [7]. Their effectiveness is however limited by the negative interactions between blood and the artificial materials used, resulting in clotting, inflammation, and increased patient morbidity and mortality. These drawbacks have been significantly improved by new biomaterials that provide similar levels of mass transfer but a lesser degree of coagulation and inflammation [1]. An example is the new hemodialysis machine developed by Fresenius Medical Care that aims to prevent blood clotting without the use of any medication.

Although selection of the dialysis modality is a multifactorial decision [11], HD is the preferred option in most cases. In the USA, in 2016, among the prevalent cases of ESRD, 63.1% followed HD as their renal replacement therapy, 7.0% used PD, and 29.6% had a functioning kidney transplant [4]. According to an annual report in 2018, 72% of total 3.362.000 end-stage renal patients are undergoing HD worldwide [9].

1.1.1. Hemodialysis (HD)

Effective HD requires repeated and reliable access to the bloodstream. This can be achieved through a surgically created arteriovenous communication, which can be either an autologous fistula (AVF) or a graft (AVG) – autologous (saphenous and femoral veins) or synthetic (generally ePTFE) –, or through a central venous catheter (CVC) [12] (Figure 1). Bioartificial grafts have been explored as an alternative to vascular access [10], however without reaching the clinical setting. Vascular access complications remain the main reason for HD patient hospitalization [1, 12].

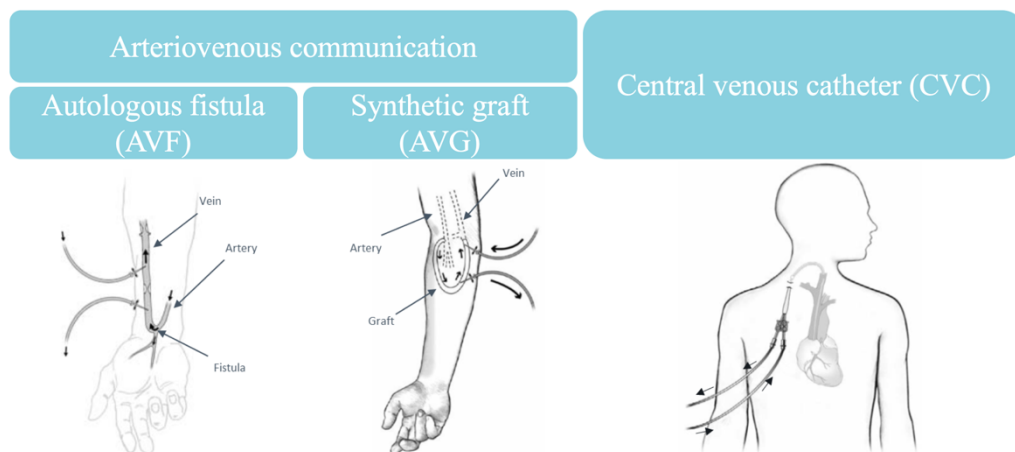


Figure 1. Vascular access for hemodialysis. Access to the bloodstream can be achieved through a surgically created arteriovenous fistula (AVF) or graft (AVG), or through a central venous catheter (CVC).

AVFs are considered the best vascular access for HD treatment, associated with lower risk of mortality than AVGs and CVCs [12, 13]. Nevertheless, 80% of patients with incident ESRD and 20%

of patients with prevalent ESRD in the United States still use a CVC for vascular access [14]. Increase in CVC usage is related with increase comorbidities associated with AVF and AVG [15, 16] and with their ability to be inserted quickly and easily, allowing immediate access for HD. They provide urgent and temporary access during acute kidney injury, overcoming the limitations of the long maturation periods of AVF, and the depletion of usable vessels suitable to be used as a fistula [13, 16, 17]. CVCs can be permanent (tunneled) or temporary (non-tunneled) and cuffed or non-cuffed HD catheters [18]. Chronic hemodialysis catheters are almost always tunneled, providing effective vascular access for months or years. They are inserted subcutaneously from the site of the insertion, with a bonded cuff (ePTFE) that allows anchoring – tissue ingrowth that immobilizes the catheter below the skin surface – and prevention of bacterial migration, thus reducing the risk of infection [19, 20]. This has made the use of tunneled cuffed catheters an acceptable form of HD vascular access, despite presenting significantly higher complication rates than the other options of vascular access [13, 17]. Recently, regenerative strategies for vascular access have also been explored: Fresenius Humacyte, Inc developed a human acellular blood vessel, Humacyl [9].

Historically, long-term access devices were made from medical-grade silicone rubber. Advances in material technology have resulted in the transition from silicone-based dialysis catheters to the use of polyurethane (PU) [20]. PU is a polymer often synthesized from the reaction between an isocyanate and a polyol molecule in the presence of either catalyst or ultraviolet light activation (Figure 2). They are composed of long-chain linear polymers without crosslinks, which are weakly bonded at room temperature, but become free to slide past one another under sufficient thermal energy – such as provided by the body [20]. This allows them to be rigid outside the body, facilitating the catheter insertion, while softening and becoming more flexible once inside the body, reducing mechanical trauma and irritation, and making it more compliant within a vein. Thermoplastic polyurethanes (TPUs) are widely used in the manufacturing of catheters due to their availability, excellent mechanical and physical properties as well as good biocompatibility and hemocompatibility [21, 22]. Compared with silicone catheters of the same French size, PU high tensile strength allows the production of catheters with thinner walls, and consequently with larger inner lumens, multiple lumens and smaller external diameters, thereby maximizing blood flow within the vessel [20]. However, due to their hydrophobic character, they perform only moderately well in resistance to bacterial adhesion, being susceptible to bacterial colonization and having a higher risk of infection [22, 23].

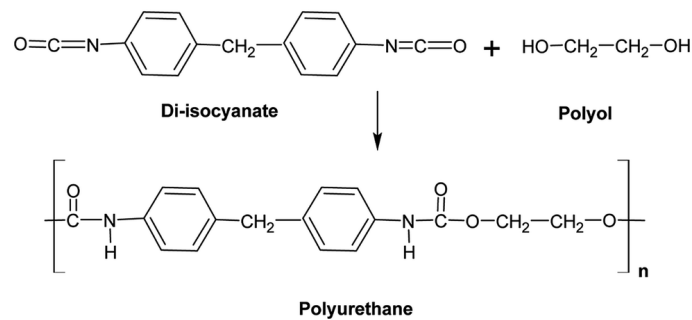


Figure 2. Polyurethane chemical structure. From [21].

Although catheter materials are selected to be biocompatible and hemocompatible, catheter-associated complications are still inevitable.

2. Catheter-associated complications

Apart from catheter kinking, cracking or breaking during insertion, major catheter-associated complications include thrombosis and infection [16, 24] (Figure 3).

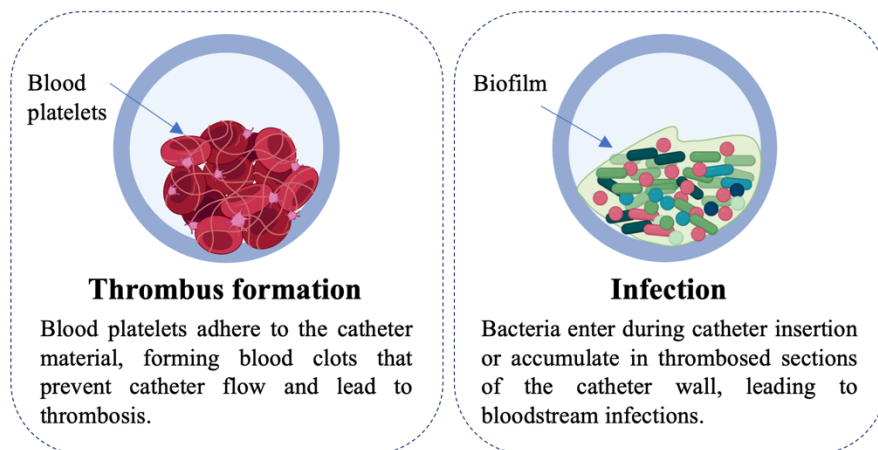


Figure 3. Most frequent catheter-associated complications that lead to catheter replacement.

Similar to any other biomaterial, upon insertion of the catheter into the bloodstream, a conditioning film composed of plasma proteins is rapidly formed on its surface, with catheters' surface hydrophobicity increasing susceptibility to non-specific protein adsorption. Protein adsorption is the onset for the formation of a fibrin sheath, initially composed of fibrinogen, albumin, lipoproteins, and

coagulation factors, and formed within 24 h of insertion of the catheter [25]. It causes activation of the blood coagulation cascade, platelet adhesion, activation, and aggregation, being one of the most common causes of thrombotic occlusion. Planktonic microorganisms can also bind to the adsorbed proteins such as fibronectin, fibrinogen, collagen, vitronectin and laminin through several specific adhesins expressed on their surface [25], starting to colonize the medical device. A biofilm can be formed as early as 24 h after catheter placement [25, 26]. Once organisms spread down from the catheter hub along the inner surface of the catheter forming an adherent biofilm, thrombosis at the end of the catheter can occur, and the organisms may extend onto the outer side of the catheter within the fibrin sheath.

Proteins within the thrombus, such as fibrinogen and fibronectin, or isolated platelets can also attract and promote bacterial attachment to the catheter surface, thereby increasing the risk of catheter infection [1, 25, 27]. On the other hand, adherent bacteria can provide a niche for thrombus formation, and interact with platelets, resulting in platelet activation/aggregation that subsequently can lead to blood coagulation and thrombosis [1, 25, 27]. This association has led to an emphasis on preventing catheter-related thrombus as an additional mechanism for reducing catheter-related infections [25, 28].

All of these complications cause catheter malfunction and possible occlusion, compromising the blood flow debit and failing on attaining and maintaining an extracorporeal blood flow sufficient to perform HD without significantly lengthening the treatment [13]. As a result, catheters need to be removed or replaced frequently, causing unfavorable effects on morbidity and mortality, and dramatically increasing costs for the healthcare system. When compared with a fistula, central venous catheters carry a relative risk of death 1.6 times greater and a fivefold increase of infection.

2.1. Thrombus formation

It is estimated that catheter-related thrombosis occurs in up to 66% of adults with a long-term CVC. This event can lead to increased morbidity, due to pulmonary embolism, post-thrombotic syndrome, and persistent vascular compromise [13, 25]. The need for catheter exchange can also increase the risk of infection, leading to increased costs of care. Catheter-associated thrombosis can be classified as extrinsic when the thrombus forms outside the catheter and intrinsic when the thrombus is either intraluminal or attached to the surface of the device (Table 2) [29].

Table 2. Classification of catheter thrombosis [29].

Extrinsic
Mural thrombus
Atrial thrombus
Central vein thrombosis
Intrinsic
Intraluminal
Catheter tip thrombus
Fibrin sheath

Flow problems are generally associated with intrinsic thrombi, where the flow is reduced or stagnated – contrarily to the constant blood flow on the exterior of the catheter that reduces extrinsic thrombosis. Intrinsic thrombi may be intraluminal, or attached to the catheter tip, or they may surround the catheter in a sheath-like configuration [29]. Intraluminal clots, which account for 5–25% of all catheter occlusions, may cause complete catheter obstruction [25]. More common are the formation of thrombus associated with fibrin sheath formation, where a layer of loosely attached fibrin and other components surrounds the catheter from the point where it enters the vein, with the possibility of continuing through the lining of the catheter tunnel [29]. As the sheath extends downward, it eventually closes over the tip of the catheter. In this position, it can be disrupted by outward pressure to create a flap-valve that will allow entry but prevents withdrawal of blood and fluid [29].

Blood–catheter interface triggers protein adsorption, adhesion and activation of platelets and leukocytes, complement activation and coagulation [1]. Therefore, the physicochemical properties of the catheter material such as surface wettability and roughness may influence the propensity to thrombosis [25]. In general, a hydrophobic surface is more receptive to platelet adhesion, and rough material surfaces have microscopic crevices that may adsorb fibrinogen and permit platelet adhesion and activation of the intrinsic coagulation pathway [25, 30].

Thrombus formation and catheter occlusion can be fought through the use of anti-coagulant therapy, where anticoagulants inhibit both surface-induced coagulation as well as tissue-based coagulation [1]. Anticoagulant locking solutions instilled into the catheter at the conclusion of HD have been used [19], with unfractionated heparin being the anticoagulant of choice [1, 19]. Several others have been attempted, such as direct platelet inhibitors and other systemic inhibitors of the coagulation cascade [1]. Sodium citrate can also be used given its calcium chelating properties (which anticoagulates blood prior to the dialyzer blood inlet; calcium is then added back to the blood prior to it returning to the patient), however this method is cumbersome, complicated, and thus costly to employ [1].

Heparin-coated catheters have also been reported to decrease infection rate without the risks of systemic antibiotic exposure or bacterial resistance [13]. As it exhibits anticoagulant activity via interaction with the plasma protein antithrombin as well as some electrostatic repulsion of charged platelets, it can also reduce bacterial trapping within fibrin clots and sheaths. Hydrophobic and

electrostatic interactions also decrease direct bacterial adhesion onto catheter polymer [20]. However, as HD catheters may remain inserted for several months, heparin has the potential to cause adverse effects, such as bleeding, allergic reactions, and increase the risk of osteoporosis with long-term use [31]. Locking solutions with both an anticoagulant and antimicrobial agent (such as sodium citrate and antiseptic agents) have been explored to decrease the risk of catheter infections and thrombosis compared to heparin locking solution [19].

2.2. Catheter-related infections

Infection is the second leading cause of death among patients with ESRD [32], and more than two-thirds of access-related bloodstream infections are due to catheter infections [28]. Catheter-related bloodstream infections (CRBSI) are the most serious complication in patients with prolonged CVC dependence [14, 16], and are clinically defined as a systemic blood infection (bacteremia) where the catheter has been identified as the source of the infection [25].

CRBSI are a major cause of patient morbidity and mortality [33]. The risk of sepsis with a CVC is 2-fold to 5-fold higher than with an AVG and AVF [34]. Tunneled cuffed catheters present 53% higher risk of mortality, 2-fold higher risk of fatal infection and 68% higher risk of hospitalization than AVF [28]. CRBSI are the most common indication for premature catheter replacement (30%–60%), accounting for an economic burden of more than \$23 billion annually in the USA and between €35.9 and €163.9 million in four European countries (France, Germany, Italy, and the UK) [24, 33].

The majority (around $\frac{3}{4}$) of catheter-related infections are caused by Gram positive organisms, and the remaining less than $\frac{1}{4}$ are Gram negative (Table 3) [14, 35]. *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), is the most common causative organism (3–74%), followed by *Staphylococcus epidermidis* (7–42%) and other coagulase-negative *Staphylococci* [28]. Reported rates of MRSA range from 2.3% to 27.3%, and up to 35% of colonized patients subsequently develop MRSA infections within 1 year [14, 32]. Fungal infections are also a concern, with *Candida albicans* being the predominant fungal species isolated from medical devices [36]. The severity of presentation and clinical consequences of CRBSI are related to the infecting organism: *S. epidermidis* CRBSI tends to present with atypical symptoms, while *S. aureus* CRBSI is more likely to require hospitalization or intensive care admission [37]. Polymicrobial infections accounted for 14% of CRBSIs and were associated with longer duration of prior catheter use [37].

Table 3. Distribution of microorganisms commonly associated with CRBSIs [28, 35, 37].

Gram positive	
<i>S. aureus</i>	$\frac{3}{4}$ of infections
<i>S. epidermidis</i>	
Other coagulase negative <i>Staphylococci</i>	
Gram negative	
<i>E. coli</i> , <i>Enterobacter</i> species and <i>Klebsiella</i> species	$\frac{1}{4}$ of infections

Bacterial colonization of the catheter can occur due to 1) catheter insertion and manipulation of the vascular access site, 2) bacteria migration from the catheter-skin interface via an extraluminal or intraluminal route, or 3) contaminated intravenous fluids [13, 25, 38]. Biofilm distribution is related with the duration of catheter placement. Colonization of the extraluminal surface, likely originating from the skin, predominates soon after insertion and in short-term catheters that have been in place less than 10 days. Colonization of the intraluminal surface occurs generally due to hub contamination and increases progressively with time, becoming predominant after 30 days of placement of long-term devices [25, 39, 40]. In patients with CVCs, the major source of CRBSI is the bacterial biofilm formed in the inner surface of the CVC [13].

Similar to other medical devices, the general mechanism of infection in HD catheters starts with the adsorption of proteins at the surface (Figure 4). Planktonic bacteria migrate to the surface via Brownian motion, and due to different forces, including van der Waals and surface electrostatic charges and hydrophobic interactions, adhere reversibly to the conditioning films established. As the adherent bacteria multiply, form colonies and secrete extracellular polymeric substances (EPS), the process becomes irreversible and bacteria permanently anchor to catheter surface. These aggregates of bacteria protected from external disruptors, as drugs and the host immunity, are called biofilm. Bacteria living within a biofilm have different properties from the free-floating bacteria of the same species, as the dense and protective environment of the biofilm allows them to cooperate and communicate in different ways [41]. Biofilm bacteria also excrete toxins that can reversibly block important host defensive processes. After maturation and growth of the biofilm, single cells or aggregates of cells may detach from the biofilm due to fluid shear, or as a result of internal biofilm processes, leading to infection [28]. Biofilms also cause the deterioration of surface properties and functions, limiting the service life of the catheter [42].

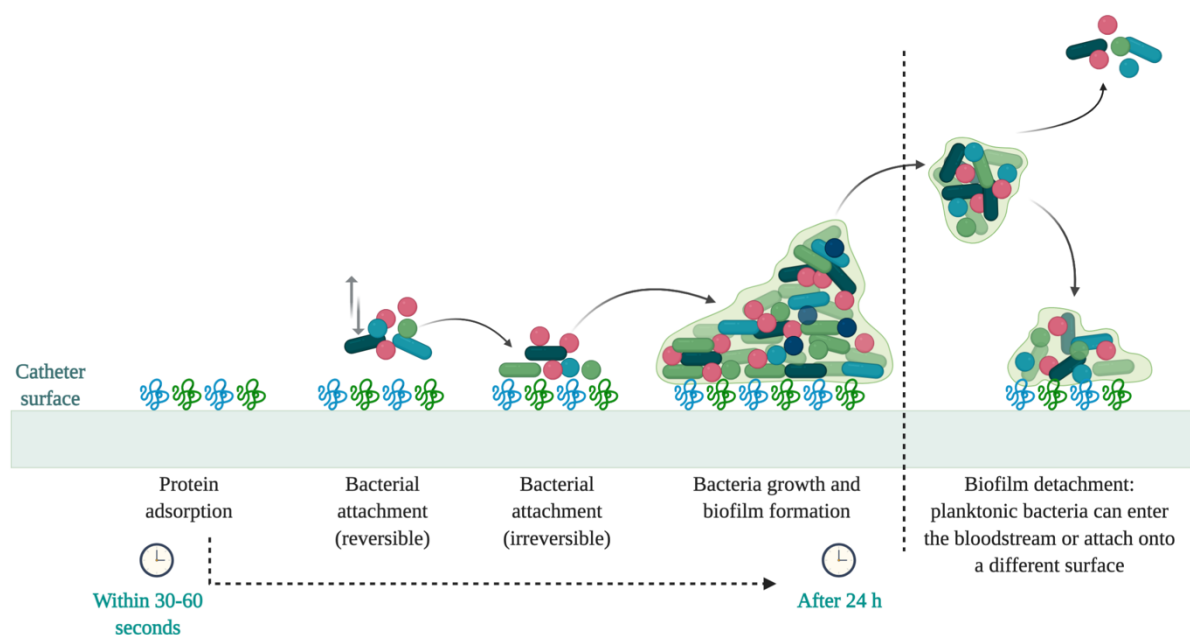


Figure 4. Bacterial colonization and biofilm formation in hemodialysis catheters.

Biofilms at different stages of maturity showed different susceptibility to antibiotics, which may give rise to antibiotic resistance [32]. Patients with CKD, particularly those with ESRD who require dialysis and/or kidney transplantation, have some of the highest rates of colonization and infection with antibiotic-resistant bacteria worldwide [28, 32]. These include MRSA, as aforementioned, vancomycin-resistant *Enterococcus* spp. and several multidrug-resistant Gram negative organisms. Antimicrobial resistance limits treatment options and increases the risk of infection-related morbidity and mortality. In addition, the use of traditional antibiotics exerts secondary effects on the healthy human microbiota, further contributing to antibiotic selection pressure and the risk of emergence and propagation of antimicrobial resistance [32].

3. Strategies to fight infections: surface modification as a weapon

Bacterial infection in HD catheters or in any other medical device starts by the attachment of a single cell to the surface. Endowing surfaces with antibacterial performance has therefore become of utmost importance to tackle healthcare-associated infections. Strategies can focus on preventing bacteria from adhering to the surface (anti-adhesive), killing the bacteria that manage to attach on the surface (contact-killing), or eradicating early developed biofilm [36, 42, 43]. Commonly used polymeric materials in catheters production, namely silicone, polyurethane or latex, readily adhere bacteria, and so research has been dedicated to their surface modification and the development of antibacterial coatings [25, 42-48].

Surfaces are generally divided into different categories according to their mechanism of action (Figure 5). An antifouling strategy can be followed, with surfaces incorporating anti-adhesive

molecules that prevent bacteria from adhering. On the other hand, a killing strategy can be explored: surfaces can promote killing of bacteria through a biocide release (leaching) system, aiming to kill or limit bacteria growth both on the surface and in the vicinity [25], or through direct contact upon adhesion to the surface. Some surfaces combine competences and promote killing after contact followed by release of death bacteria, generally upon external stimulation (light, pH, temperature) [47].

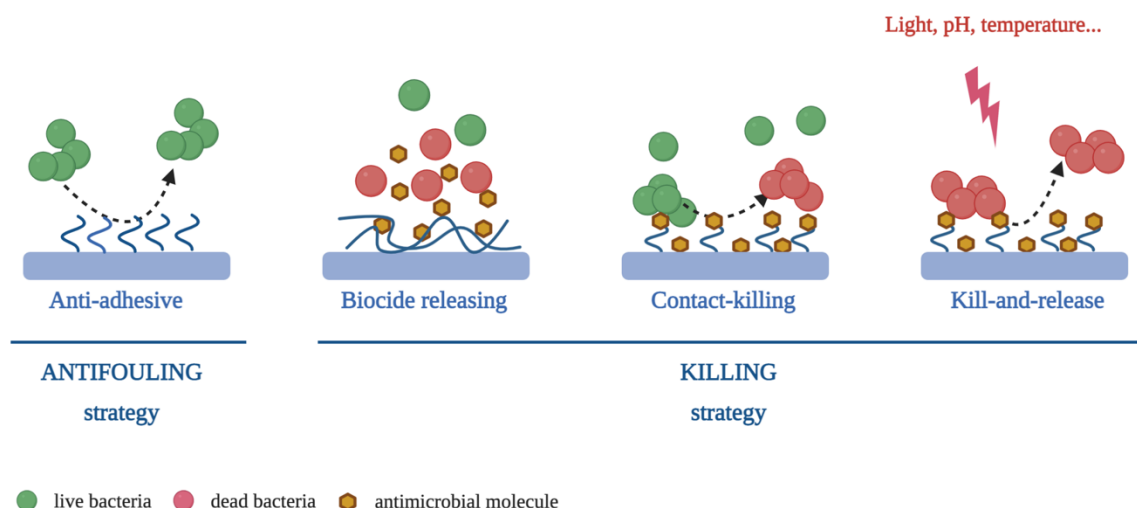


Figure 5. Mechanisms of action towards bacteria of different surface modification strategies.

Bacterial adhesion is a complex process affected by the physicochemical properties of the surface and bacteria (e.g. hydrophobicity and surface charge) as well as by the environment where the adhesion takes place (e.g. pH, the presence of proteins or electrolytes) [25].

An antiadhesive surface can be created by modification of surface topography or chemistry. Tuning of surface features, as roughness, wettability, or surface energy, can be explored, with smoother and hydrophilic surfaces generally reducing protein adsorption and bacterial adhesion [43, 49, 50] – even though the presence of the conditioning film may change the surface characteristics of the substrate [25]. Modification of the surface chemistry can be attained by grafting polyhydrophilic and/or electrically neutral (zwitterionic polymers, PEG, pHEMA) polymers, which are known to resist protein adsorption and bacterial adhesion [25]. Introduction of hydroxyl bonds, for instance, can increase surface energy, leading to increase in the hydrophilic nature of the surface, and thus a bacterial reduction by more than 90% [51]. The action of these coatings is related with the formation of a hydration layer near the surface that acts as a physical and energetic barrier preventing protein adsorption [25]. However, if not completely efficient, anti-adhesive surfaces can still allow bacterial adhesion, and due to the lack of a killing effect, permit biofilm formation [52].

Surfaces focused on a bactericidal action can be attained by using different antimicrobial molecules. Antibiotics have been the major weapon to fight bacterial infections, even though their overuse has made them far less effective and contributed to the development of bacterial resistance, which has

become one of the biggest threats to public health today, with costs to the healthcare systems reaching around €1.1 billion [53]. Other molecules have therefore been investigated, including antiseptics (chlorhexidine and triclosan), silver (ions and nanoparticles), nitric oxide (NO), and natural antibacterial molecules (antimicrobial enzymes, antimicrobial peptides, bacteriophages (and their lysins), immunomodulators, quorum sensing inhibitors) [44, 54]. Cationic polymers with inherent antibacterial properties and covalent immobilization of quaternary ammonium compounds have also been widely investigated for use as contact-killing coatings [25, 33]. Carbon nanotubes (CNTs) and graphene-based materials (GBMs) – tailorable and relatively simple and affordable to produce – are other class of materials increasingly explored due to their action in a multimodal manner and therefore might better escape or delay the development of microbial resistance [54]. Silver nanoparticles, on the other hand, are also prone to induce bacterial resistance development since metal ions primarily act via the disruption of internal biochemical targets/pathways similarly to most antibiotics, apart from tissue accumulation and local or systemic side effects (specially in a leaching system, due to release of Ag⁺ ions) [44, 54]. Positively charged nature of such surfaces can likely increase protein adsorption and the antibacterial efficacy will be greatly diminished. Covalently attached, non-leaching biocidal-moieties are expected to provide longer term effects than the use of a releasing agent [55, 56]. Combination of agents can therefore be an interesting solution to increase the bactericidal effect and reduce the possibility of bacterial resistance, such as integration of both anti-adhesive and bactericidal moieties, comprising for instance a zwitterionic polymer and a bactericidal polymer [25]. Tuning of the surface features can also be explored in a contact-killing approach: attracting bacteria may be of interest to potentiate the contact and the killing effect. Since bacteria are negatively charged in neutral media, positively charged surfaces are expected to increase bacterial adhesion over negatively charged surfaces, as well as hydrophobic, rougher surfaces [49, 52].

In bactericidal coatings, thickness must be carefully considered: thin coatings may have limited antibacterial action (with low incorporation yield), while thick coatings may last longer but are more susceptible to cracking, peeling, and flaking off [57]. In some situations, the release of the antimicrobial agent can lead to concentrations below the effective concentration, which can cause bacterial resistance and enhance bacterial attachment and biofilm formation by some bacterial strains [25]. Another issue that must be considered when exploring the killing approach, particularly the contact-killing, is the accumulation of dead bacteria on the surface, masking the functional groups, potentially triggering the activation of the immune system, and serving as a nutrient source for other bacteria that might afterwards attach [25, 58-60]. Kill-and-release surfaces have been developed to prevent this scenario, with the release mechanism being a response to pH alteration, photoactivation or temperature [58-60]. These smart antibacterial surfaces with switchable functions between bacteria killing and bacteria releasing have been increasingly explored as being more suitable to respond to the complex, real world applications [42, 43].

In the case of surface irradiation with light of different wavelengths, this external stimulus is used as a way either to confer or to enhance the surface antibacterial properties [33, 42]. This can be achieved through a local increase in the temperature or in the reactive-oxygen species (ROS) production, when in the presence of photothermal agents and photosensitizers. A broad-spectrum activity can therefore be achieved without occurrence of drug resistance [42, 61-63].

3.1. Strategies explored to fight HD catheters-related infections

Currently, measures to prevent or control infection in HD catheters rely on skin disinfection and on the use of prophylactic topical antimicrobial ointments and dressings at the catheter exit site, associated with the use of anticoagulant and antimicrobial catheter locking solutions [34]. Failure to efficiently achieve that, results in catheter replacement, being possibly the only option to effectively eliminate the risk of further infection-associated problems.

Surface modification of the HD catheters has been attempted, primarily focusing on the release of biocides that can be impregnated into the material, physically adsorbed or covalently attached [53]. The most commonly used biocides include classical antimicrobial agents, such as silver compounds or ions, silver sulfadiazine, or chlorhexidine, and antibiotics, such as rifampin, or minocycline. Impregnation requires a homogeneous dispersion of the biocide throughout the polymeric matrix, and can be achieved either during the production phase (during the melt processing of the catheter) or *a posteriori*, by swelling the material in a suitable solvent containing the desired agent [53, 66]. Physical adsorption, on the other hand, can be achieved by simply submerging the material into a biocide-containing solution and allowing the biocidal compounds to bind through noncovalent interactions [53]. Biocides and other antimicrobial agents, such as antifouling or bactericidal moieties, can also be incorporated in the polymer via spray coating or dip coating, where covalent attachment may take place [25, 53, 67].

The most common implemented strategy relies on antibiotic-impregnated CVCs, namely with rifampicin-miconazole [68] and chlorhexidine-silver sulfadiazine [69], which have been shown to reduce the risk of infection and catheter colonization when comparing to standard catheters [68, 69]. Other impregnated catheters show 2% lower rates of CRBSIs compared to those with standard non-impregnated catheters, and there was a 9% lower probability of bacterial colonization on the impregnated catheters [69]. However, this reduction in bacterial colonization was dependent on the type of patients and facilities [69]. Other examples include Vantex from Edwards Lifesciences, a PU CVC made with Oligon agent (silver, platinum, and carbon black) that showed a statistically significant reduction of > 99.9%, and Bactiguard®, an antimicrobial coating based on a thin layer of noble metal alloy (silver, gold, palladium) that presented a lower hemolysis rate than the chlorhexidine–silver sulfadiazine coating, a lower potential for blood coagulation activation and 90% reduction in microbial adhesion compared to the unmodified surface [70]. In both situations and in most studies and randomized clinical trials assessing antibacterial efficiency of modified CVCs, application towards HD

patients was not specifically addressed, and so conclusions cannot be easily drawn [71]. Silver-containing antimicrobial products are also available in the healthcare market, as is the case of VentriGuard ventricular catheter (by Neuromedex) that utilizes small amounts of ionized silver incorporated in a non-metallic porous molecular structure [56], however a long debate still persists over the possible inactivation of silver-mediated antibacterial activity in physiological fluids and over the low biocompatibility index of silver determined by the low threshold concentration for cytotoxic effects, especially when in form of nanoparticles [67].

Few have addressed specifically these patients, as is the case of minocycline-rifampin-coated HD catheters that decreased the risk of catheter-related infection in patients with acute renal failure [72]. Arrowgard® Blue Acute HD catheter, from Teleflex Incorporated, is another CVC that permit access for acute HD, with chlorhexidine and chloracetate incorporated in the internal surface, and chlorhexidine and silver sulfadiazine on the external surface [73]. A recent review from Balikci, *et al.* reports surface modification strategies for hemodialysis catheters, where a table summarizing the recent studies on antimicrobial coatings of HD catheters can be found [18].

Apart from modification of the catheter surface, other strategies have been followed, such as modification of the catheter cap. CurostTM, commercialized by 3M, is a cap containing isopropanol that allows disinfection of the outside part of catheter hubs. ClearGuardTM HD, commercialized by ICU Medical, is on the other hand an antimicrobial cap that disinfects the inner lumen of the catheter hub. The cap contains a rod coated with chlorhexidine that extends into the catheter hub and that, upon contact with the lock solution, releases chlorhexidine, killing bacteria [74].

Overall, and even though great progresses have been made, complete elimination of bacteria is difficult to achieve [75, 76]. There are many requirements that infection-resistant materials must fulfill in order to be effective, including 1) antimicrobial activity against a broad spectrum which can be achieved by any combination of reduction or prevention of adherence of bacterial and fungal microorganisms; reduction or prevention of biofilm formation; bacteriostatic and/or bactericidal properties; 2) adequate, long-term duration of antimicrobial activity; 3) antimicrobial activity must be present on the outer and inner surfaces of the device; 4) the polymer modification must not affect the physical properties of the biomaterial, i.e. stability, durability, elasticity and strength, thrombogenicity, cytotoxicity [77-79]. Apart from the demanding conditions, randomized clinical trials to assess the efficacy of these surface treatments on non-tunneled and tunneled catheters in HD patients are still few, and implementation in the clinics may be hindered by the high costs associated with coated catheters [73].

4. Graphene-based materials: a solution?

Graphene-based materials (GBMs) offer an enormous opportunity for the development of novel antimicrobial surfaces, particularly considering their ability to escape or delay antimicrobial resistance,

due to their ability to act towards bacteria through different mechanisms, thus making it harder for bacteria to adapt and develop resistance. Intrinsic features of the different GBMs, such as oxidation degree, platelet size, thickness, and conductivity, make them of particular interest to specific applications, with a unique level of chemical and physical versatility and robustness [36]. Furthermore, their remarkable broad-band optical absorption ability allows them to generate heat when the light-excited electrons relax to the ground states, making them photothermal agents [42, 63]. Their intrinsic oxidative potential and ability to induce oxidative stress can also be further potentiated upon light irradiation, making them interesting photodynamic agents [61].

These materials can be applied in different modalities, falling under four major categories: (i) antimicrobial colloids; (ii) contact-killing surfaces, (iii) release-killing surfaces, and (iv) light-activated disinfectants (i.e., photothermal/photodynamic inactivation) [54]. Even though GBMs can be used alone, being the only active component, GBMs are often utilized to reinforce the mechanical and antibacterial properties or to improve the drug stability and distribution in the material, acting also as killing-release platforms when loaded with silver nanoparticles, for instance [45].

It is established that GBMs interaction with biological systems differs according to their physical arrangement [77], and so different antimicrobial performance can be observed for GBMs suspensions or surfaces. By depositing GBMs-based colloids on a surface, different GBMs-integrating surfaces can be produced: GBMs free-standing films, coatings or composites, with different associated surface features [78]. Discrepant data have however been reported regarding the bactericidal action of GBMs-integrating surfaces, demanding a deeper understanding of the impact of surface features on the interaction with bacteria.

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CHAPTER III

**Can graphene-based materials improve the
antibacterial performance of surfaces?**



Review article

Fabrication and antimicrobial performance of surfaces integrating graphene-based materials



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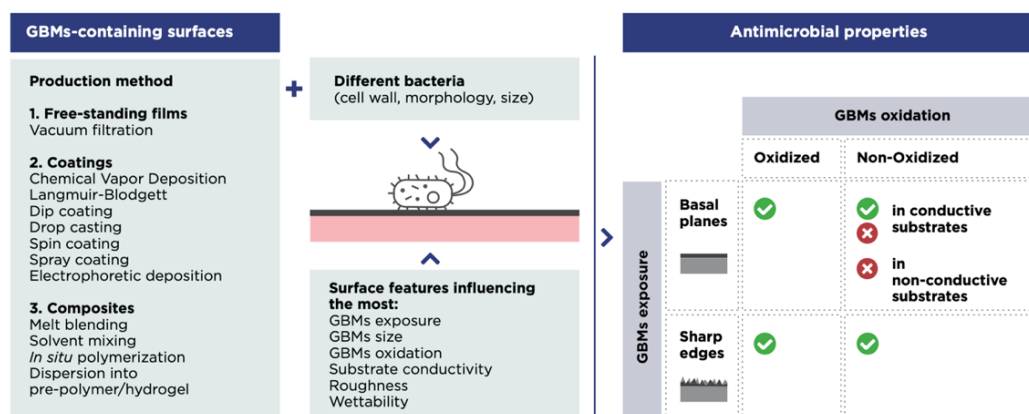
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Abstract

Although graphene-based materials (GBMs) have been thoroughly explored, their use in antimicrobial surfaces is still a developing field. This review overviews the different methods for fabricating GBMs-containing surfaces (free-standing films, coatings or bulk composites) and their antibacterial properties. The difficulty in controlling the broad number of factors affecting interactions between GBMs and bacteria hampers the establishment of clear cause-effect relations. Nevertheless, it is clear that GBMs size, exposure, oxidation, as well as surface conductivity and roughness are the main surface features influencing the antimicrobial properties. Depending on the production method, GBMs basal planes and/or sharp edges are exposed, having a major impact on bacteria through electron transference, piercing of the membrane or pore formation, amongst others. Each of these effects leads to production of oxidative stress and/or bacterial membrane disruption and, consequently, to bacterial death. While oxidized graphene-containing surfaces are antimicrobial when either basal planes or sharp edges are exposed, graphene-containing surfaces are mainly effective when sharp edges are protruding, except for few studies showing effect due to graphene basal planes when coated over conductive materials. As such, this review enlightens and clarifies the surface features most strongly affecting bacteria, providing researchers the necessary tools to produce antibacterial GBMs-containing surfaces with tuned mechanisms of action.

Graphical Abstract



1. Introduction

Since its first isolation from graphite (Gt) in 2004, graphene (G) has attracted worldwide interest. Its particular structure with hybridized sp^2 bonding confers G a hydrophobic and conductive nature as well as exceptional mechanical, thermal and optical properties. These properties are also related with its physical structure composed of both sharp edges and basal planes. Its interesting features have allowed G to be used either alone [1, 2] or in combination with other materials, generally improving their native properties [3-5].

Several graphene-based materials (GBMs) have been developed, differing in terms of physical structure, namely morphology, number of layers, lateral dimensions and defects (Figure 1). Even though the term “graphene” (G) is often used indistinctly, it should be used only to refer to a single sheet of carbon atoms packed together in a crystal lattice. The misuse of this term motivated a *Carbon* Editorial containing some recommendations for the nomenclature of these materials [6]. As such, bilayer or trilayer graphene refers to 2 or 3 graphene sheets packed together, few-layer graphene (FLG) refers to between 2 to about 5 graphene sheets, and multi-layer graphene (MLG) - also called graphene nanoplatelets (GNP) - refers to stacks of between 2 to about 10 graphene sheets packed together. Graphite (Gt) is formed by more than 10 sheets. All these structures can be oxidized, with suffix “O” or “ox” being added to designate the oxidized form (*e.g.* GO, MLGox, GtO).

Different methods have been explored and modified in order to obtain high quality GBMs, involving top-down (starting from graphite) and bottom-up (starting from alternative carbon sources) approaches. These have been thoroughly reviewed [7-15] and are summarized in Figure 1. The number of methods has been increasing, as more researchers become interested in developing green, simple, efficient and low-cost methods.

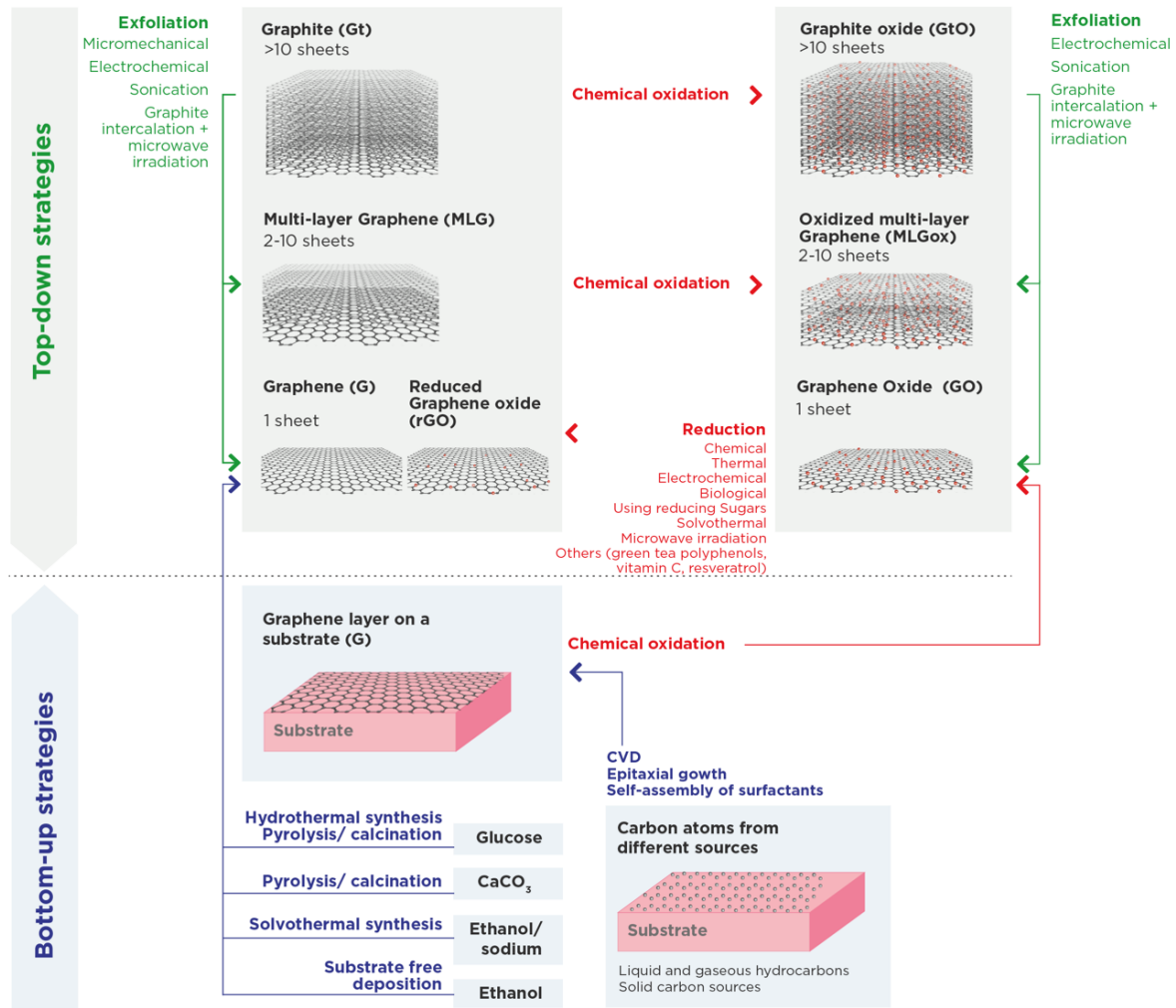


Figure 1. Graphene-based materials (GBMs) and their production methods. Top-down strategies that yield G and derivatives in the form of liquid dispersions or powders [13, 16-20], including oxidation of Gt into GtO and GO [21-24], with GO reduction to rGO occurring through several routes [25-30]. Bottom-up strategies that produce G either as a dispersion or powder [31-39] or as a layer on top of a substrate (metallic or non-metallic) [40-43].

Depending on the synthesis procedures, GBMs can end up with different chemical (functional groups, degrees of oxidation) and physical (size, thickness, edge length, defect density) features [7, 44-49]. GO, for instance, can contain an uncertain number of water molecules intercalated between the oxidized carbon layers as well as a variable type and coverage of oxygen-containing functional groups [50, 51]. In addition, proper washing of the GBMs has been shown relevant to eliminate low weight contaminants and increase the pH to a more neutral level [46]. Post-production separation processes or treatments, such as centrifugation and sonication, can also change the physical-chemical features of the sheets, with oxidation degree and lateral size being reduced as centrifugation speed increases [52], and sheets with a smaller area being obtained with longer and more energetic sonications [46, 53].

GBMs have been mainly explored as powders or in suspension, with numerous review articles addressing their features and advancements concerning production methods, properties and applications [8, 9, 12, 54, 55]. Antibacterial properties have only started to be explored more recently, in 2010, boosting the exploration of these materials for such applications. From then on, GBMs effect on bacteria [56-58] and mammalian cells [59, 60] has been intensively studied, unravelling the complexity and controversy of the subject [61, 62].

Incorporation of GBMs into surfaces has been receiving more attention due to the enormous range of promising applications, including electronics, optics, and bioengineering. Application as antibacterial surfaces is a topic of particular interest, with the goal of preventing bacterial colonization in medical/health environments, without representing harm to humans, while being cost-effective and with long lasting effects [63]. A few authors have analyzed the interaction between bacteria and GBMs-containing surfaces [18, 50, 53, 57, 64-66], but given the complex and diverse surface/bacteria interface, research is still on-going regarding the effects and mechanisms of action of these surfaces.

GBMs-containing surfaces can be divided in three main types, namely films (free-standing films made only of GBMs), coatings (GBMs or GBMs + polymer applied on a substrate), and bulk composite materials (GBMs dispersed within polymer or ceramic matrices, for instance). The production methods of these GBMs-containing surfaces in addition with the characteristics of the GBMs and experimental conditions (as materials' concentration and exposure time) [67-71] will have an impact on the surface properties, and thus on its antimicrobial/antibacterial action and biocompatibility (Figure 2) [51].

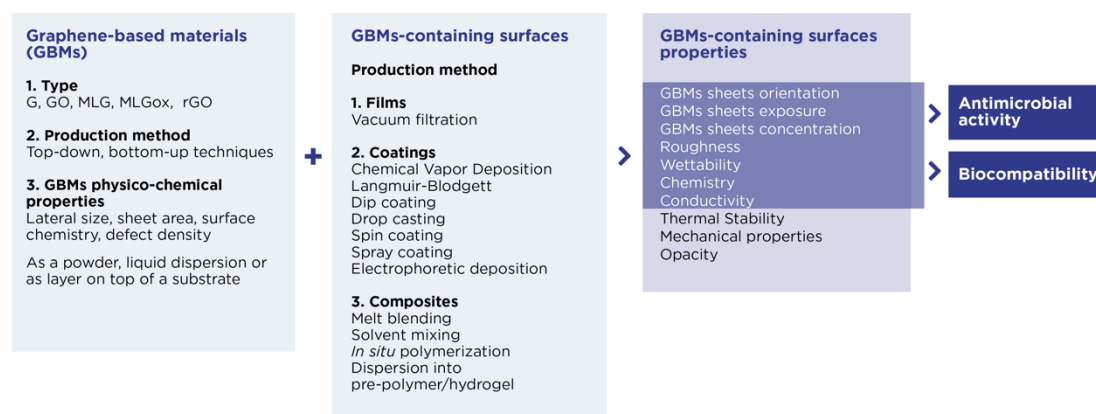


Figure 2. Interaction between the different factors affecting the antimicrobial properties of GBMs-containing surfaces. G = graphene, GO = graphene oxide, rGO = reduced graphene oxide, MLG = multi-layer graphene, MLGox = oxidized multi-layer graphene.

The type of bacteria or microorganisms analyzed [40, 72], namely the morphology (coccus, bacillus) and the nature of the cell wall (Gram positive and Gram negative), will also be crucial to determine the surface's antimicrobial performance. The cell envelope composition may be the primary barrier and

distinctive factor to the antimicrobial action of the GBMs: Gram positive bacteria possess a less resistant cell wall, although thicker, constituted only by a peptidoglycan layer (30-100 nm), while Gram negative possess a thinner peptidoglycan layer (5–10 nm) but has an outer membrane of lipopolysaccharides (7.5-10 nm) (Figure 3). The outer membrane is crucial to protect organisms from the environment by excluding toxic molecules while providing an additional stabilizing layer around the cell [73].

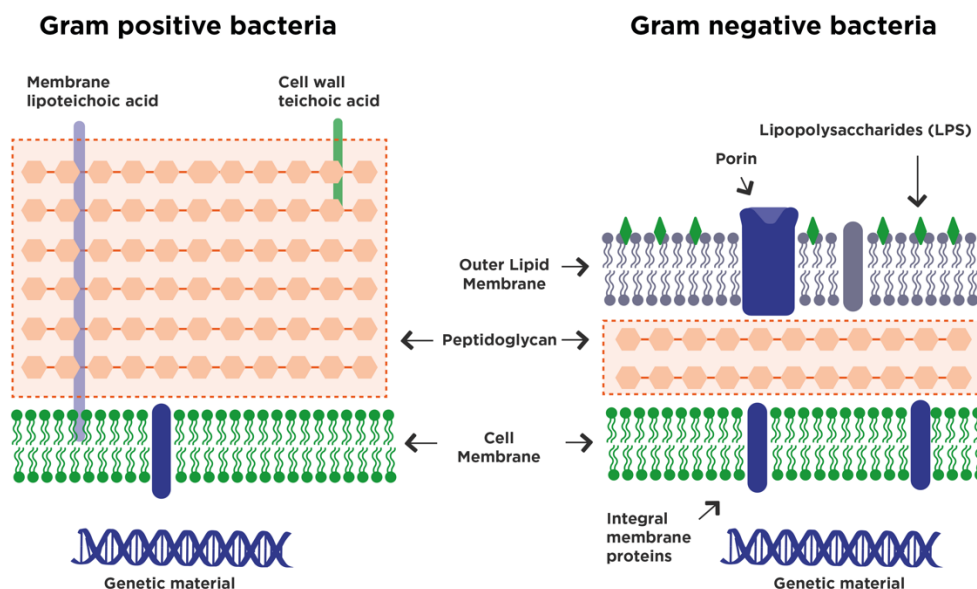


Figure 3. Cell envelope structure of Gram positive and Gram negative bacteria.

The aim of this review is therefore to contribute to clarify which are the surface features that most strongly affect bacteria, and to provide the necessary tools to produce antibacterial GBMs containing surfaces with tuned mechanisms of action. The production and antimicrobial properties of the different types of surfaces integrating GBMs are presented and discussed, followed by an overview of the different proposed mechanisms of action for these surfaces. Finally, the most relevant points are highlighted as conclusions.

2. GBMs-containing surfaces

As aforementioned, graphene-containing surfaces can be divided into 1) free-standing GBMs films, 2) coatings and 3) bulk composite materials. The following sections aim to establish a connection between the production method and features of each type of these surfaces and their action towards bacteria. To note that despite not considered in this review, functionalization of the GBMs with other antimicrobial molecules can also be a strategy to improve surface antibacterial performance [74, 75].

2.1. Free-standing GBMs films

Free-standing film-like or paper-like materials obtained from GBMs have attracted great attention due to their potential use for chemical filters, molecular storage and supercapacitors [76-78].

2.1.1. Production of free-standing GBMs films

These materials are mainly produced by vacuum filtration, a simple and inexpensive technique, that allows films to be peeled off from the filtration membrane and used as a free-standing paper [2, 79], transferred onto a different substrate [78], or tested while still on the membrane [53] (Figure 4.A).

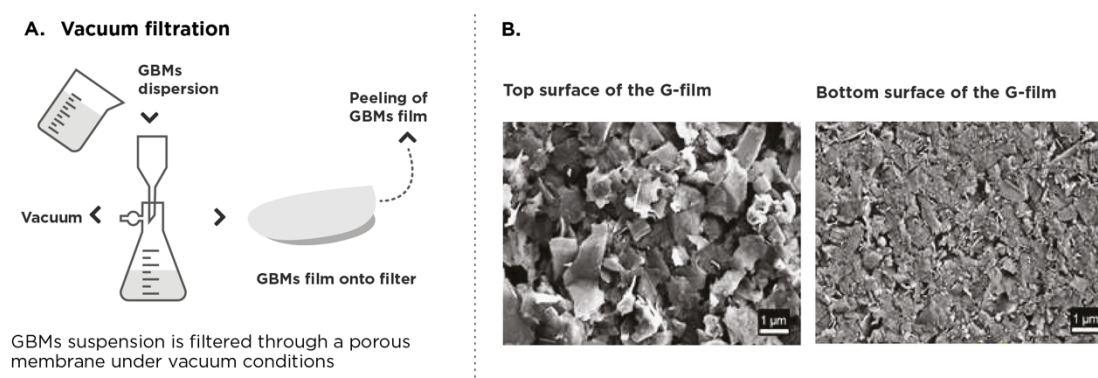


Figure 4. Vacuum filtration technique: mode of operation (A). SEM images of vacuum filtered G films: different topographies were found for the top surface and for the bottom surface in contact with the filter membrane (B). Adapted with permission from [18]. Copyright (2017) American Chemical Society.

Film thickness can be controlled by adjusting the volume and concentration of the colloidal GBMs suspension [2], allowing the production of films ranging from a single layer to up to 10 layers [12, 78, 80]. Wrinkling of the sheets has been reported to inherently occur in large-scale films [12]; however, a wrinkled surface geometry can be intentionally introduced through a pre-strained filter. Wavy wrinkles and nanoscale grooves can be found with sharp edges popping up from the wrinkles at different length scales [81].

Macroscopically, all these films are robust, flexible and stable, with homogeneous surfaces [2, 18, 53, 77, 82]. Microscopically, they normally present a random deposition of the GBMs (either G, GO, rGO) with their sharp edges and basal planes exposed and available to interact with contacting microorganisms (Figure 4.B) [18, 53]. Aryal *et al.* [82] reported a rough surface, with rGO deposited in layers and with a higher specific surface area comparing to carbon paper (consists of graphitic carbon - commercially available), also with outstanding conductive properties [82]. Musico *et al.* [83] produced GNP and GO films and reported the same random deposition of the GNP and GO sheets, with GNP presenting sharper edges than GO sheets. The presence of the GO sheets increased surface

hydrophilicity, due to the presence of oxidized functional groups that form hydrogen bonds with water [76]. Perreault *et al.* [53] demonstrated that more sheets were exposed when GO sheets with smaller sizes ($0.01\ \mu\text{m}^2$) were used [53]. A higher edge density was also found by Pham *et al.* (Figure 4.B) [18] on the bottom surface of G vacuum filtration produced films. Although G sheets were protruding from both sides of the film, the bottom surface possessed sheets with smaller edge lengths, though with a higher edge density across surface, while the top surface presented sheets with an increased lateral/average size and edge length. The angle of orientation of the G sheets was also different, with the sheets from the top presenting an angle closer to the one related with the maximum activity on bacteria (90°) [84]. Chen *et al.* [77] also reported a looser structure on the top part of the film, with the bottom side being more well-packed and ordered (associating this with the different stages of the vacuum filtration process).

All these surface features, as sharp edges and basal planes exposure, sheets size and orientation, edge density or wettability, will influence the interaction with microorganisms, as discussed in the next section.

2.1.2. Antimicrobial properties of free-standing GBMs films

Few studies have been conducted in order to evaluate free-standing films antibacterial properties (Table 1). Most studies include Au or Ag NPs in order to make free-standing films more antibacterial and thus will not be considered.

Regarding the effect of GBMs type and oxidation degree, Hu *et al.* [2] showed that both GO and rGO surfaces could effectively inhibit bacterial growth: GO completely suppressed *E. coli* growth, and a limited number of colonies was found on the rGO surface [2]. The films were not microscopically analyzed, and thus no correlation can be established with GBMs orientation at the surface. A similar effect was observed by Musico *et al.* [83], with GO presenting a higher inhibitory effect on both *E. coli* and *B. subtilis* bacteria than GNP, as well as a higher percentage of glutathione (GSH) oxidation, associated with an increased production of reactive oxygen species (ROS). This effect was attributed to the presence of $-\text{COOH}$ and OH^- functional groups on GO surface.

Table 1. Effects of free-standing GBMs films produced by vacuum filtration on bacteria.

GBMs production method	GBMs/surface properties	Bacteria	Antibacterial effect	Ref.
GO: MHM	Macroscopic, free-standing, robust, and flexible paper	<i>E. coli</i>	GO: completely suppressed <i>E. coli</i> growth	[2]
rGO: GO reduced by hydrazine			rGO: limited number of colonies on the surface	
GNP: commercial	Random deposition of GNP and GO	<i>E. coli</i>	GO: higher inhibitory effect on both <i>E. coli</i> and	[83]
GO: MHM from GNP		<i>B. subtilis</i>	<i>B. subtilis</i> bacteria than GNP	
GO: MHM	Variable GO sheet area 0.65, 0.29, 0.10 and 0.01 μm^2 smaller sheets $\rightarrow$ more defects (reactive sites + possible sites for O_2 adsorption)	<i>E. coli</i>	GO: 0.65 μm^2 = 27% 0.29 μm^2 = 39% 0.10 μm^2 = 50% 0.01 μm^2 = 70% GSH oxidation increased from 49% to 71% as the sheet area decreased from 0.65 μm^2 to 0.01 μm^2	[53]
GO: MHM		<i>E. coli</i> <i>S. aureus</i> <i>M. smegmatis</i>	<i>E. coli</i> and <i>S. aureus</i> viability decreased to ~20% of control values <i>M. smegmatis</i> viability decreased to ~30% of control values	[81]
Gt: commercial	Variable roughness top surface $\rightarrow$ edge length = 137.3 ± 93.9 nm, orientation = 62.1° , edge density = $7.7 \mu\text{m}/\mu\text{m}^2$ bottom surface $\rightarrow$ edge length = 79.7 ± 56.7 nm, orientation = 37.2° , edge density = $10.8 \mu\text{m}/\mu\text{m}^2$	<i>P. aeruginosa</i>	Gt: <i>P. aeruginosa</i> = <i>S. aureus</i> : increased number of bacteria adhered (with increased viability)	[18]
G: liquid phase exfoliation		<i>S. aureus</i>	G: Top surface of the film: <i>P. aeruginosa</i> = 87.6%; <i>S. aureus</i> = 43.1% Bottom surface: <i>P. aeruginosa</i> = <i>S. aureus</i> = ~75% G smaller size (0.09 μm^2) = ~23% G larger size (0.55 μm^2) = ~10% GO small size (0.09 μm^2) = ~41%	
G: laser induced graphene	Variable G sheet area 0.09 μm^2 and 0.55 μm^2	<i>P. aeruginosa</i>	G smaller size (0.09 μm^2) = ~23% G larger size (0.55 μm^2) = ~10% GO small size (0.09 μm^2) = ~41%	[85]
GO: MHM		<i>B. cereus</i>	Bacterial attachment and subsequent growth	[86]
rGO: GO reduced by hydrazine	rGO: conductive surface	<i>E. coli</i>	Increase in bacteria growth of 2 and 3 times	[87]
GO: MHM			Preferential attachment and growth in areas containing higher amounts of GO	
GO: MHM	Rough surface, with rGO deposited in layers and with a higher specific surface area	<i>S. ovata</i>	Bacterial adhesion and biofilm development	[82]
rGO: GO reduced by hydrazine				

MHM = modified Hummers method.

Singh *et al.* [85] and Perreault *et al.* [53] went further and took a closer look at the surface of the produced films, aiming to understand the effect of particle size on the antibacterial performance of the surface. Singh *et al.* [85] observed an increased killing effect on *P. aeruginosa* when in contact with G smaller size particles (0.09 μm^2 , ~23 %) in comparison with larger ones (0.55 μm^2 , ~10 %); however, an even increased effect was observed when small size GO was used (~41 % killing). Perreault *et al.* [53] reported the same relation between size and the antibacterial effect for GO films: a stronger antimicrobial effect on *E. coli* (73, 61, 50 and 30 % cell viability) was respectively observed when smaller GO sheets (average sheet area of 0.65, 0.29, 0.10 and 0.01 μm^2) were used to produce the films, contrarily to what occurred when GO is in suspension (where the opposite relation is observed [86]). Also, GSH oxidation increased from 49 % to 71 % as the sheet area decreased from 0.65 μm^2 to 0.01 μm^2 [53]. These effects were related with the increased surface area and small edge features of smaller sheets. They also have more defects, and thus more reactive sites and possible sites for O_2 adsorption and consequently ROS production and GSH oxidation [53]. In addition, oxidized particles have a higher oxygen content, which can also enhance the effect through oxidative stress production.

Roughness of the surface also influences its antibacterial ability, as demonstrated by Zou *et al.* [81] with GO films. *E. coli* (Gram negative, rod-shaped, $d \cong 0.76 \mu\text{m}$) and *S. aureus* (Gram positive, spherically-shaped, $d \cong 0.90 \mu\text{m}$) were strongly affected by GO films with ~0.5 μm roughness (viability decreased to ~20 % of control values) while *M. smegmatis* (Gram positive, rod-shaped, $d \cong 0.62 \mu\text{m}$) were affected majorly by the GO film with ~0.845 μm roughness (decrease in viability to ~30 % of control values). This effect was related to the tight contact of the GO substrate with the cell wall or

cytoplasmic membrane, which may cause significant membrane stress and disruption of the cell membranes [81].

The relevance of GBMs type, sharp edges exposure and surface roughness was strengthened by Pham *et al.* [18], who reported major differences in the antibacterial properties of Gt or G films against *P. aeruginosa* and *S. aureus*. In fact, more bacteria (and with increased viability) were attached to Gt surface (smoother surface) when comparing to G films, where G sheets were protruding, creating a rougher surface. Moreover, the presence of GBMs with a small edge length, near vertical orientation and higher edge density improved the antibacterial activity of the surface, with small and round bacteria surviving more when these features were missing [18]. In fact, the top surface of the film, with an increased edge length (137.3 ± 93.9 nm) and a favorable orientation (62.1° , closer to the maximum killing efficiency occurring at 90° [84]) in the surface was highly lethal for Gram negative *P. aeruginosa* (87.6 % cell death), but less effective against Gram positive *S. aureus* (43.1 % cell death). On the other hand, the surface contacting the filter, with a smaller edge length (79.7 ± 56.7 nm) and poor orientation (37.2°), but with a higher edge density ($10.8 \mu\text{m}/\mu\text{m}^2$ in comparison to $7.7 \mu\text{m}/\mu\text{m}^2$), was found to be efficient in killing both bacteria (~75 % death) (Figure 5). As such, a higher edge density (with more sharp edges exposed) seems to be more relevant and favorable for killing bacteria, especially spherical-shaped bacteria (larger in diameter). More basal planes exposed, on the other hand, seem to allow bacteria to survive more, since smoother surfaces are available for bacteria to adhere, as in the case of *S. aureus*. This increased survival occurs despite the fact that the bacteria is Gram positive and thus reportedly more susceptible to the effect of the GBMs [40, 71, 72, 89].

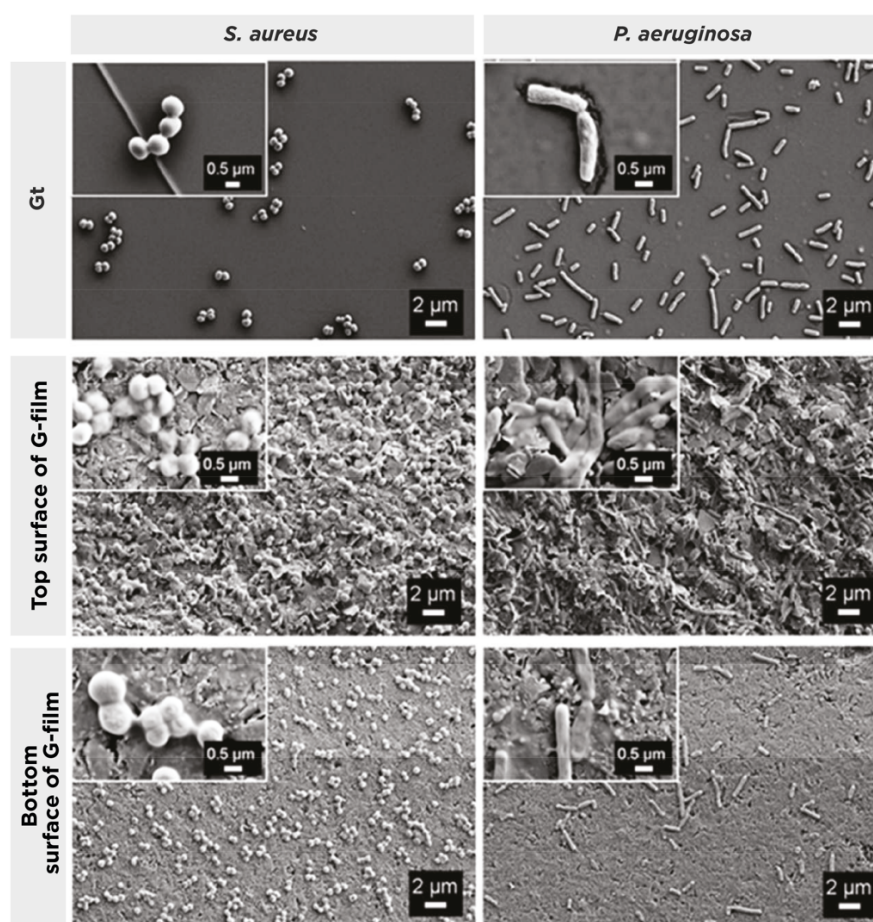


Figure 5. SEM images of *S. aureus* and *P. aeruginosa* when in contact with Gt (control), the top surface of the G film and the surface contacting the filter (bottom surface). Adapted with permission from ([18]). Copyright (2017) American Chemical Society.

Although the presence of exposed sharp edges that pierce the bacterial membrane has been commonly suggested as a mechanism of antibacterial action [53], a few works argue the low probability of this happening [18, 90]. Pham *et al.* [18] associate the effect towards bacteria with the insertion of the G sheet into the membrane leading to pore formation, causing alteration in the osmotic pressure, thus leading the bacteria to swell and die [18]. Although not assessed, ROS production could also be occurring due to the increased reactivity and possible O₂ adsorption on defect sites, that consequently could be causing oxidative stress. A recent force spectroscopy study proposed that the interactions between the bacteria membrane (negatively charged) and the GO sheets (hydrophilic and negatively charged) are mainly repulsive, and so the edge blade effect is not likely to occur [90]. However, O₂ can still adsorb on defect sites, and thus the oxidative effect may be the fundamental effect in these cases. In this context, some authors report no antimicrobial effects for GBMs films [86], even suggesting that bacteria attachment and growth is promoted [87]. In a study from Ruiz *et al.* [87], an increase in bacteria growth of 2 and 3 times occurred on GO retained on filtration membranes compared to membranes

without GO. There was even a preferential attachment and growth in the areas of the membrane containing higher amounts of GO. Films composed of rGO fabricated by Park *et al.* [86] also allowed bacterial (*B. cereus*) attachment, and subsequent growth [86], as well as rGO free-standing films produced by Aryal *et al.* [82] that also exhibited good biocompatibility, allowing *Sporomusa ovata* to adhere and develop a biofilm. An improved electron transference between the microorganism and the rGO film was also observed, with *S. ovata* being a catalyst for the acetate production from CO₂. This study raises doubts regarding the mechanisms of action towards bacteria, since here electron transference occurs with no lack of viability being detected.

The reason for these seemingly contradictory results has not yet been identified. The absence of information does not allow to identify possible differences between surface morphology in these and the previous works. However, considering previous studies, large GBMs sheets where bacteria could adhere without directly contacting with the sharp edges could be an explanation.

2.2. GBMs-containing coatings

Coatings are a layer of material adhered onto the surface of a bulk material (substrate) with the purpose of achieving a surface with intended properties. GBMs-based coatings have been widely explored in different areas, focusing on the protection of metals or biomedical devices from corrosion [41, 91], oxidation [92, 93] or bacterial adhesion and colonization [40].

2.2.1. Production of GBMs-containing coatings

Numerous techniques can be implemented for the production of GBMs integrating coatings, resulting in GBMs deposition in a flat or random orientation (Figure 6).

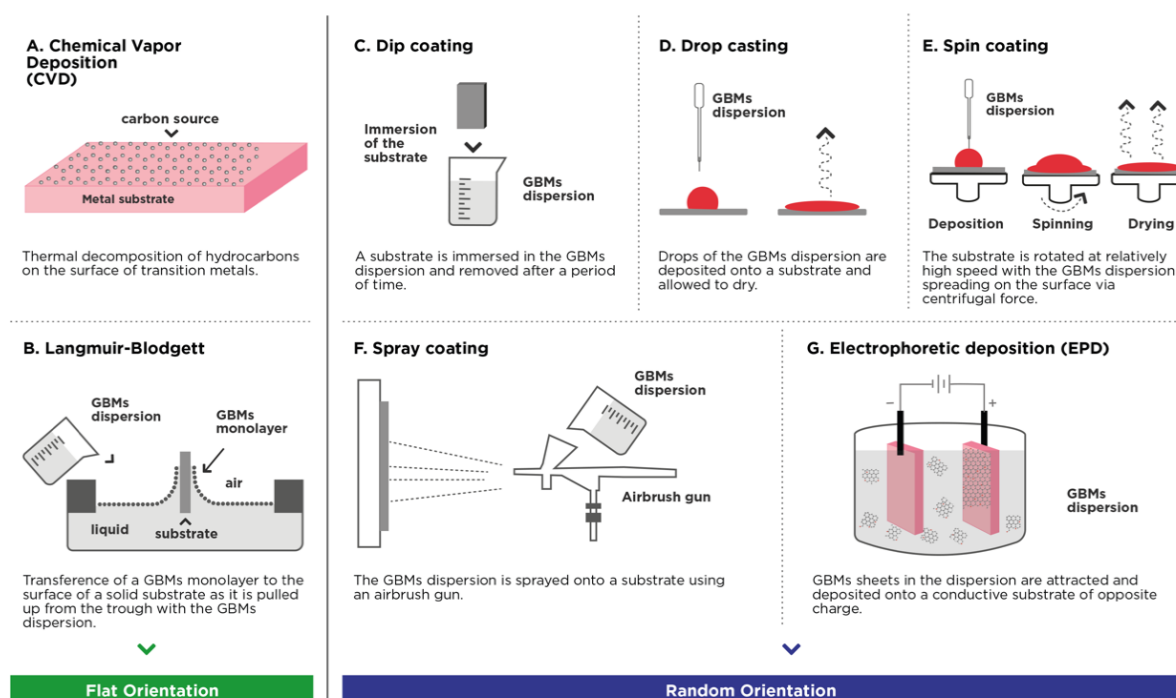


Figure 6. Approaches for production of GBMs coatings: methods that confer a flat orientation of the GBMs sheets onto the surface (chemical vapor deposition (CVD) [40, 88, 94] and Langmuir-Blodgett (LB) [89]), and methods that confer a random orientation of GBMs (dip coating [95], drop casting [96], spin coating [97, 98], spray coating [99] and electrophoretic deposition (EPD) [72, 100]).

The orientation of the sheets at the surface is strongly related with the roughness of the surface. When a flat orientation is obtained, only the basal planes of the GBMs are exposed, therefore producing a smoother surface. On the other hand, when GBMs are randomly exposed at the surface, not only the basal planes but also the sharp edges are exposed, producing a surface with variable roughness. Apart from the production technique, features as the GBMs sheets size and edge length contribute to the topography and morphology of the surface. Wettability is another variable surface feature mostly dependent on the selected GBMs (and synthesis method) and on the exposed functional groups at the surface as well as on their availability. In fact, very recently Yadav *et al.* [51] demonstrated the importance of the synthesis processes on the final characteristics of the GBMs and consequently on the chemical and physical features of the surface. GO-coated surfaces were produced using GO obtained by MHM (GO_H) and dominated by carboxylic-rich groups and a larger sheet size (1.2 μm), and GO produced by an improved method based on Marciano's (GO_I), possessing epoxide- and hydroxyl-rich surface functionalities and a smaller size. GO_H surface presented increased roughness, with a wrinkled/folded surface, hydrophilic (contact angle of 33°) and with a non-uniform thickness. On the other hand, GO_I presented a smoother surface, less porous, slightly less hydrophilic (contact angle of 46°), forming a thinner film. In addition, the deposition methodology of the GO on the substrate was also shown to influence the features of the surfaces [51].

The need for a binder can also greatly influence not only the roughness of the surface (diminishing it) and GBMs exposure (reducing it) but also its wettability. Since CVD and LB generally do not use binders, they may allow a better GBMs sheets exposure, although only of their basal planes; on the other hand, in techniques as drop casting or dip coating that usually require a binder, GBMs can be more easily covered or embedded on the coating. Polymers can be used as binders, ensuring good adhesion and mechanical stability of the coating [101]. GBMs content can also influence the way sheets organize thus influencing its overall surface features [51].

Several approaches have however been explored to avoid the use of polymer binders, such as grafting of GBMs onto the surface of the substrates. Perreault *et al.* [102] and Hegab *et al.* [103] grafted GO onto the surface of a thin film composite (TFC) polyamide membrane through either an EDC/NHS chemistry [102] or poly L-Lysine (PLL) intermediary using either layer by layer (LbL) or hybrid (H) grafting strategies [103], to improve surface-based interactions, and leading to an increase in hydrophilicity. GO/PLL-H presented a smoother surface than the GO/PLL-LbL (36 nm and 47 nm (RMS) respectively) and a greater GO grafting density in the GO/PLL-H membrane surface.

CVD, LB, spin coating and EPD are techniques that allow an accurate control of the thickness and of the surface coverage, while dip coating, drop casting and spray coating frequently result in a non-uniform deposition of the GBMs dispersion, with poor film thickness control [104].

Table 2 summarizes the requirements, influencing factors and advantages/disadvantages of the main methodologies to produce GBMs coatings.

GO is often used as the starting material in several coating strategies. Reduction to G, when desired, is performed before or after application onto the substrate, mainly through chemical reduction or thermal annealing. Both the pH value of the reduction solution as well as the reaction temperature have been shown to greatly influence the structure and dispersibility of the composites [105].

Table 2. Overview of the GBMs-containing coatings production methods: features, influencing factors, advantages and disadvantages.

Production method	GBMs orientation*	GBMs requirement	Substrate requirement	Influencing factors	Advantages	Disadvantages	Ref.
Chemical vapor deposition (CVD)	Flat	—	- Preferably metals (influences the quality and the features of the G film)	- Carbon source - Temperature - Pressure - Substrate roughness and conductivity	- High quality and uniform G film - Controllable thickness - G layer may be transferred to another substrate	- Transference to a new substrate may cause deformities and contamination - Difficulty in scaling up - High T required (650-1000 °C) - Ultra-high vacuum conditions - Produces only G films - Residual toxicity if catalysts are used	[64] [40] [91] [66,106–110]
Langmuir-Blodgett (LB)	Flat	- G in NMP, DCE - GO in a water/methanol mixture or acetone, chloroform, THF and DMF	- Preferably hydrophilic (PET, mica, glass, quartz)	- Particle density - Expansion/compression velocity - Substrate wettability - Coating cycles	- Transparent, large-area, single-layer films - Good uniformity - Controllable thickness - Deposition at RT	- Requires specific expensive equipment - Requires deposition of few layers to fully cover the substrate – more time consuming - Wrinkles - Difficulty in controlling thickness (dependent on several factors); solution accumulates - Requires a long drying step - Films with non-uniform thickness - Limited quality of films - Wrinkles (reduce surface coverage)	[89] [111–115]
Dip coating	Random	- GBMs in dispersion - rGO, GO, G	- Preferably hydrophilic	- Velocity at which the substrate is pulled up - Dispersion features (viscosity, concentration) - Temperature - Coating cycles - Adsorption times - Substrate shape	- Low-cost - Simple - Large area coverage - Deposition at RT - Deposition in both sides simultaneously	- Difficulty in controlling thickness (dependent on several factors); solution accumulates - Requires a long drying step - Films with non-uniform thickness - Limited quality of films - Wrinkles (reduce surface coverage)	[95] [116]
Drop casting	Random	- GBMs in dispersion	- Flat substrate	- Solvent - Solvent evaporation rate - Dispersion features (concentration, volume) - Substrate surface	- Simple - Low-cost - Deposition at RT - No waste of material	- Difficulty in controlling thickness - Poor uniformity	[24] [117–119] [51]
Spin coating	Random	- GBMs in dispersion	- Flat substrate	- Solvent - Dispersion features (viscosity, concentration, volume) - Parameters spin process (spinning time, rotational speed, acceleration)	- Uniformity of the coating - Controllable thickness - Deposition at RT - Very thin films can be quickly obtained - Fast drying times (may be a disadvantage)	- Limited substrate dimensions - Coated one side at a time - Requires specific equipment (spin coater) - Waste of material (the majority is flung off the side)	[12] [13] [98] [120,121]
Spray coating	Random	- GBMs dispersion in polar solvents	—	- Dispersion features (concentration, viscosity) - Solvent (polarity, pressure and evaporation rate) - Flow rate - Spraying duration - Spraying pattern - Distance substrate/nozzle - Substrate temperature	- Low-cost - Large area coverage - Deposition at RT - Substrates may have different sizes and shapes	- Difficulty in controlling thickness (dependent on several factors) - Requires specific equipment (airbrush gun) - Difficulty in controlling homogeneity	[79] [99] [122,123]
Electrophoretic deposition (EPD)	Random	- GBMs dispersions in solvents (ethanol, isopropanol, acetone, acetonitrile, DMF) - Water should not be used - G sheets need to be modified with positive or negative charge (–50 mV to +50 mV)	- Must be conductive	- Dispersion features (viscosity, concentration and particle size and charge) - Solvent - Deposition time - Equipment (applied voltage) - Substrate conductivity	- Easy scalable - Cost effective - Good uniformity - Dense packing - Controllable thickness - Deposition at RT - Substrates may have different sizes and shapes - High and controlled deposition rate - Short formation time	- Limited thickness of insulating deposits (e.g. GO) - Possibility of side electrochemical reactions - Requires specific equipment	[72] [124–128]

NMP = *N*-Methyl-2-pyrrolidone, DCE = 1,2-Dichloroethene, THF = tetrahydrofuran, DMF = dimethylformamide, RT = room temperature; * flat = only basal planes exposed; random = both basal planes and sharp edges exposed.

2.2.2. Antimicrobial properties of GBMs-containing coatings

Table 3 congregates works reported in literature regarding the effects of GBMs integrating coatings on bacteria, being representative of the current understanding.

Table 4. Effects of GBMs-containing coatings on bacteria.

GBMs-containing coating	GBMs production method	Production method	GBMs/GBMs coatings properties	Bacteria	Antibacterial effect	Membrane damage	Ref.
G-coated Cu G-coated Ge G-coated SiO ₂	<u>CVD</u>	<u>CVD</u> : G-coated Cu: G on Cu G-coated Ge: G on Ge G-coated SiO ₂ : G on Ge + transference by PMMA to SiO ₂ substrate	Monolayered films with high crystalline quality	<i>E. coli</i> <i>S. aureus</i>	After 24 h G-coated Cu = 100% G-coated Ge = some bacterial growth G-coated SiO ₂ = 0%, continuous bacterial growth Similar for <i>S. aureus</i> cells, however more susceptible	G-Cu and G-Ge = severe membrane disruption and cytoplasm leakage G-SiO ₂ = no evident membrane destruction	[40]
G-coated SiO ₂	<u>CVD</u>	<u>CVD</u> : G-coated SiO ₂ : G on Cu (commercial) + transference by PMMA to SiO ₂ substrate	single layer with wrinkles	<i>Halomonas</i> spp. CAM2	After 72 h SiO ₂ = 0%, highest concentration of live bacteria G-coated SiO ₂ = 0%, greatly reduced bacterial adhesion	No damage found, intact membranes	[129]
G-coated Cu G-coated Au	<u>CVD</u>	<u>CVD</u> : G-coated Cu: G on Cu G-coated Au: G on Cu + transference by PMMA to Au substrate	G-coated Cu = monolayer with some bi- and tri-layer islands; entirely covers the Cu surface G-coated Cu (partially) = single hexagonal domains; monolayer, bi- or tri-layer flakes; does not fully cover the Cu G-coated Au = continuous films of G	<i>E. coli</i> <i>S. aureus</i>	After 24 h bare Cu <i>E. coli</i> = 94%; <i>S. aureus</i> = 100% G-coated Cu and G-coated Au <i>E. coli</i> = <i>S. aureus</i> = 9% G-coated Cu (partially) <i>E. coli</i> = 46%; <i>S. aureus</i> = 34%	No	[110]
G-coated Cu	<u>CVD</u>	<u>CVD</u> : G-coated Cu: G on Cu (commercial)	single layer graphene (SLG)	<i>E. coli</i>	After 24h Fresh Cu = 100% G-coated Cu = 0% G coating substantially suppress interaction between bacteria and Cu substrate	No	[91]
G-coated Au	<u>CVD</u>	<u>CVD</u> : G-coated Au: G on Cu + transference by PMMA to Au substrate	n/s	<i>E. coli</i>	After 24h G-coated Au: 0%, allowing proliferation	No	[57]
GO-coated PET	<u>MHM</u>	<u>LB</u> : GO-coated PET	GO-LB films: fully exfoliated sheets t = 1 nm	<i>E. coli</i>	After 2 h Bare PET = 13% 1 layer GO = ~40% 2 layers GO = ~75% 3 layers GO = 89%	—	[89]
G/AgNWs/EVA/PET	<u>CVD</u>	<u>Spin coating</u> : AgNWs on EVA/PET substrate <u>CVD</u> : G on AgNWs/EVA/PET substrate	n/s	<i>C. albicans</i> <i>E. coli</i> <i>S. aureus</i>	<i>C. albicans</i> EVA/PET substrate = ~15% G/EVA/PET substrate = ~15% AgNWs/EVA/PET substrate = ~50% G/AgNWs/EVA/PET substrate = ~50% <i>E. coli</i> EVA/PET substrate = ~25% G/AgNWs/EVA/PET substrate = ~50% <i>S. aureus</i> EVA/PET substrate = ~25% G/AgNWs/EVA/PET substrate = ~45%	Yes	[66]
rGO/AuNPs/AgNWs coating	<u>MHM</u>	<u>Spin coating</u> : GO/AuNPs/AgNWs on glass slides and Si wafers rGO: All GO containing-films exposed to hydrazine	AgNWs L = 20–40 µm D = 100–130 nm	<i>E. coli</i>	After 24h presence of top rGO coverage promotes adhesion however, clean glass slide = 34 colonies/slide hybrid film-covered glass slides = 100% GO-MoS ₂ > MoS ₂ > GO effect	Yes	[130]
GO-MoS ₂ coated SiO ₂	<u>MHM</u>	<u>Spin coating</u> : GO-coated SiO ₂ (5 times) MoS ₂ -coated SiO ₂ (5 times) GO-MoS ₂ coated SiO ₂ : spin coating of GO dispersion + dry in a vacuum desiccator for 24 h + spin coating MoS ₂ dispersion	Roughness: GO = 0.9 nm MoS ₂ = 0.5 nm GO-MoS ₂ = 1.2 nm Thickness: GO = 1.2 nm MoS ₂ = 2.2 nm	<i>E. coli</i>	After 2 h GO-MoS ₂ = 61% After 4 h GO-MoS ₂ = 80% After 6 h GO-MoS ₂ = 96.4%	GO-MoS ₂ : Cell volume loss = 67.2% Dry mass loss = 78.8%	[120]
GO-coated cotton fabrics	<u>MHM</u>	<u>Dip coating</u> : GO-coated cotton fabrics	n/s	<i>S. iniae</i> <i>E. coli</i>	After 6 h <i>S. iniae</i> = 68%; <i>E. coli</i> = 46% After 12 h <i>S. iniae</i> = 86%; <i>E. coli</i> = 62% After 24 h <i>S. iniae</i> = 100%; <i>E. coli</i> = 74%	—	[71]
GO-coated PS	<u>GO₁</u> : <u>MHM</u> <u>GO₂</u> : improved method (Marcano et al. [23] with slight modifications)	<u>Drop casting</u> : GO ₁ /GO ₂ -coated PS 96-well microtiter plate + slow oven drying (50 °C, 4 h)	GO ₁ : mostly COOH groups size = 1.2 µm t = ~3.9 nm higher surface roughness GO ₂ : epoxy, C-OH groups t = ~1 nm size = 200 nm	<i>E. coli</i> <i>S. aureus</i>	After 24 h <i>E. coli</i> GO ₁ = promoted proliferation GO ₂ = 36% at 60 µg, 90% at 150 µg, 100% at 180/200 µg <i>S. aureus</i> GO ₂ = 65% at 60 µg, 88% at 150 µg, 81% at 200 µg	—	[51]
GO-coated nitinol	<u>MHM</u>	<u>Drop casting</u> : GO-coated nitinol and GOGel-coated nitinol (GO mixture with gelatin)	t = 1 nm (single-layer)	<i>E. coli</i>	After 24h GO@NiTi > GOGel@NiTi > NiTi antibacterial effect	Yes	[119]
GO nanowalls (GONW)	<u>MHM</u>	<u>EPD</u> : GO-coated stainless steel	t = single- and/or multilayer sheets	<i>E. coli</i> <i>S. aureus</i>	After 1 h <i>E. coli</i> = 59%; <i>S. aureus</i> = 74%	Efflux of RNA: <i>E. coli</i> = 30 ng ml ⁻¹ <i>S. aureus</i> = 38 ng ml ⁻¹	[72]
reduced GONW (RGONW)	<u>MHM</u>	<u>EPD</u> : rGO-coated stainless steel + followed Hr reduction			After 1 h <i>E. coli</i> = 84%; <i>S. aureus</i> = 95%	Efflux of RNA: <i>E. coli</i> = 43 ng ml ⁻¹ <i>S. aureus</i> = 56 ng ml ⁻¹	

CVD = Chemical Vapor Deposition, LB = Langmuir-Blodgett, EPD = Electrophoretic Deposition, PET = Polyethylene terephthalate, EVA = ethylene-vinyl acetate, HAP = hydroxyapatite, PMMA = poly(methyl methacrylate), PS = polystyrene, MHM = modified Hummers method, t = thickness; l = length, n/s = not studied, Hr = hydrazine reduction, RT-A = room temperature in air.

For surfaces to mitigate attachment and proliferation of bacteria, several parameters must be taken into account, as the surface free energy, wettability, surface topography in terms of pore size and their density and surface roughness [51].

CVD is one of the techniques that allows exposure of only GBMs basal planes, being for that reason a good method to study the influence of these structures alone on bacteria viability. Li *et al.* [40] followed this strategy and concluded that: 1) basal planes do have an action against bacteria (both *E. coli* and *S. aureus*), and 2) the substrate material underneath the G film affects the antibacterial activity of the film. In fact, G films presented an effect against bacteria only when on top of conducting (Cu, copper) and semi-conducting (Ge, germanium) substrates, in opposition to G on top of insulating materials (SiO₂, silicon dioxide). Authors relate this effect with the ability of the G/substrate system to act as an electron pump, driving away the electrons from the bacterial membrane, producing ROS-independent oxidative stress. For this to happen, some conductivity is required in order to form the underlying circuit for electron transfer [40]. Despite using different bacteria (*Halomonas* bacteria), Parra and co-workers [129] also reported that insulating G-coated SiO₂ surfaces did not present bactericidal effects. However, bacterial adhesion was greatly inhibited, despite the expected favored bacterial adhesion and interaction with the hydrophobic tail of the outer membrane of the Gram negative bacteria tested, given G's hydrophobicity. This was correlated with modifications of the material surface energy (caused by the G coating) and electrostatic interaction between material and bacteria [129].

Not all works corroborate this theory, however. Dellieu *et al.* [110] and Parra *et al.* [91] refute the importance of the substrate conductivity by stating that the viability and proliferation of bacteria (*E. coli* and *S. aureus*) were not affected when in contact with a G film entirely covering both Cu [91, 110] and Au substrates [110]. Additionally, both authors reported that G per se and with only basal planes exposed has no antibacterial effect, since only when partially covered Cu surfaces were in contact with bacteria (Dellieu *et al.* [110]), an antibacterial effect was observed. This effect was associated with the release of Cu²⁺ ions from the substrate, in an inverse proportion to the surface coverage. In fully covered surfaces, G impermeability prevents the escape of ions due to a smaller pore size of the G layer comparing to Cu²⁺ ions [41]. In conclusion, they contradict the electron transference theory proposed by Li *et al.*, stating that both chemically and biologically, the theory presents some significant flaws. Szunerits *et al.* [57] followed the same conclusions, by reporting CVD-deposited G films on Au as promoters of bacterial (*E. coli*) growth, with no morphological changes being detected [57].

LB is another technique that produces flat surfaces. For antimicrobial analysis studies, mainly GO films have been produced with this technique. Considering the controversy on the effect of the basal planes, Mangadlao *et al.* [89] produced GO films on PET substrates (non-conductive, with reduced antibacterial activity - 13%) through LB and confirmed that contact with sharp edges is not fundamental for the antibacterial activity of the material and also that conductive substrates are not a requirement for the GO-coated surface to be antibacterial [89]. However, in this case, the antimicrobial effect might be arising from the oxygen-containing groups present on the basal planes of GO. This is corroborated by the fact that the antibacterial activity was dependent on the number of layers deposited on the PET substrate: increasing the number of layers, a broader area of the substrate was covered, more oxygen-

containing groups were exposed and therefore more bacteria could be inactivated [89]. The surface oxides may then be reduced by electron transfer from antioxidant enzymes causing the formation of ROS. In addition, as the GO is deposited onto the surface, more defects may be produced causing either a direct oxidation (since these defects are highly reactive) or an increase in the O₂ adsorption and consequent on oxidation ability. This may occur independently of the lack of sharp edges exposed and of the low favorable interaction between negatively charged bacteria and GO. For all this, and to avoid bias of results, inert substrates should be used when the aim is to study the antimicrobial properties of GBMs alone.

Although with GO in suspension, Hui *et al.* stressed the importance of the basal planes, showing that bactericidal effect could only be observed in samples where the GO basal planes were exposed in opposition to a protein-covered GO surface [94]. This study also raises the question about the adsorption of proteins and molecules in an *in vivo* situation or when in contact with body fluids. Would the antimicrobial activity of the GBMs-containing surface be masked? Papi *et al.* [131] reported that indeed a protein corona forms around GO sheets in suspension [131], a process that can also occur when GBMs are part of a surface. In fact, this may cause GBMs to be reported as non-antibacterial if the proper medium is not used and this is not taken into consideration.

Sharp edges or a mixture of both sharp edges and basal planes can be achieved through techniques as spin coating, dip coating or EPD. These surfaces present a rather rough surface, with a more complex interface with bacteria.

Akhavan and Ghaderi [72] evaluated the effect of GO and rGO surfaces produced by EPD onto stainless steel towards *E. coli* and *S. aureus*. A random orientation of the GO and rGO sheets was found at the surface, with sharp edges greatly exposed. In this case, the impact of the substrate conductivity and the effect of the oxygen-containing groups may be hindered by the presence of the sharp edges, with authors proposing 1) sharp edges cause membrane damage, and since rGO has sharper edges, it has a higher antibacterial activity than GO surfaces and 2) the edges of the GO and rGO nanowalls are good electron acceptors, leading to cell membrane damage. In addition, rGO is conductive while GO is electrically insulating, which somehow may interfere with the charge transfer processes in bacteria/rGO and bacteria/GO systems. Moreover, the type of bacteria tested was also a relevant factor, with Gram positive bacteria being more susceptible to the action of the surface than Gram negative [72]. Electron microscopy images showing the interaction between the bacteria and the surfaces would have been interesting in order to realize exactly with which structures bacteria interacts with. Krishnamoorthy *et al.* [71] reported a time dependent antibacterial activity of a GO coating on cotton fabrics (non-conductive), where GO sharp edges were well exposed. The antimicrobial effect seemed to arise from the sharp edges of the GO, with authors attributing the effect to the direct contact between the material and the bacteria or to the oxidative stress caused, however no further studies were performed to understand whether the membrane of the bacteria was punctured by the sharp edges or the oxidative stress led to the membrane damage. The same study with G randomly exposed would allow to compare

the antibacterial activity of both (GO and G), clearly understanding the effect of oxidation. Musico *et al.* [83] produced PVK-GO and PVK-GNP surfaces on top of non-conductive materials (cellulose) and also reported antibacterial effect, attributed to the presence of –COOH and OH- groups that induced the production of ROS. Although with less sharper edges, PVK-GO increased antibacterial effect, either due to the better dispersion in the matrix and therefore more exposure of the GO sheets or due to the presence of a higher number of oxygen-containing groups [83]. Zhao *et al.* [119] reported bactericidal effect of GO-coated nitinol surfaces produced by drop casting, with a significant inhibition of *E. coli* growth comparing to NiTi control surface. Although conductive, nitinol does not have inherent antimicrobial activity (besides, the coating covered the surface entirely). Therefore, the damages on the bacteria membrane and cytoplasm leakage may be attributed only to the GO coating. The mechanism of action proposed by Zhao *et al.* comprised the interaction with the basal planes of the GO (that caused membrane stress) rather than with the sharp edges of the structures [119].

Grafting of GBMs onto surfaces has also been attempted and was shown efficient in reducing the amount of GBMs used and in maximizing the interaction with bacteria. In fact, Perreault *et al.* [102] demonstrated that by grafting GO onto polyamide membranes, the number of viable *E. coli* cells was reduced by 64.5 % after contact for just 1 h. This and similar studies also reinforce the theory that conductive substrates are not necessary to render GBMs-containing surfaces antibacterial.

Recently, Yadav *et al.* [51] explored the influence of several factors on the antimicrobial properties of GO-coated polystyrene substrates. Although also using drop casting, different surface features (roughness/porosity and chemistry) were reported simply by using GO produced differently (GO_H and GO_I) and thus having different functional groups and sizes. GO_H surface possessed a modest rough surface and non-uniform thickness, which is favorable to bacterial attachment and biofilm formation by facilitating the anchoring mechanism. On the other hand, the GO_I-coated surface was smooth, which may have prevented the adhesion of bacteria (although other studies have reported the opposite [18]). Here, once again, surfaces had a different action towards *E. coli* and *S. aureus*, with GO_I acting stronger towards *E. coli* despite being a Gram negative bacteria. Also, by being more hydrophobic, the GO_I-coated surfaces should favor bacterial attachment. Therefore, results suggest that other factors may be influencing the interaction between the bacteria and the surface, namely the bioadhesive material released from bacteria that is composed both of hydrophilic and hydrophobic proteins, a ratio that may vary according to bacterial species. This adds even more complexity to the subject.

The effect on bacteria is generally reported based on the analysis of the cytoplasmic content efflux or CFU's counting, lacking the deeper understanding of the interaction between GBMs and bacteria. Considering that the most effective structures/factors against bacteria would be known, the production process of the surfaces could then be optimized.

Kholmanov and co-workers [130] fabricated a rGO/AuNPs/AgNWs hybrid conductive coating on glass substrates, ascribing the antimicrobial effect to the presence of the rGO sheets in the top layer of the coating, which caused oxidative stress and disruption of the membrane due to direct contact with

the sharp edges of the rGO sheets. Although no further studies were performed in order to confirm the mechanisms of this action, pure rGO films without AgNWs presented similar results [130]. On the other hand, Zhao *et al.* [66] studied the antimicrobial activity of G/AgNWs coating on a EVA/PET substrate, attributing the bactericidal action to the sustainable release of Ag^+ ions from the AgNWs, potentially enhanced by their electrochemical corrosion (and defect boundaries found on the G monolayer). In agreement with Yadav *et al.* [51], G was considered to only play a role in reducing the attachment of the microorganisms tested, being responsible for smoothing the surface of the composite, encapsulating the AgNWs, and making it more hydrophobic [66]. Apart from the residual presence of oxygen-containing groups on Kholmanov's work, the different outcomes may indeed be related with the production method: while in the first case the GO layer was deposited by spin coating followed by hydrazine treatment, which gives rise to randomly exposed sharp edges [130], in the latter scenario, the G layer was produced by CVD, thus presenting a flat morphology [66]. The mechanisms of action suggested by the authors rely on membrane damage, in the former case due to membrane oxidative stress (caused by direct contact of the bacteria with sharp edges of the rGO platelets, which disrupts the outer membrane and causes oxidative stress) and in the latter caused by the Ag^+ ions, although no further studies were conducted to prove these theories. Kim *et al.* [120] have recently reported the synergistic effect of GO and MoS_2 , both deposited by spin coating onto SiO_2 . In this work, GO was found mostly horizontally deposited with its basal planes exposed rather than the sharp edges. The mechanism of action was stated to be the ROS-independent oxidative stress (GO- MoS_2 showed enhanced oxidative ability when compared to GO and MoS_2 alone) that through charge transference may cause disruption of the bacterial membrane. ROS-dependent pathway, on the other hand, was found to have a minor role [120]. Although using a SiO_2 substrate, reported by some authors as responsible for G's lack of antibacterial properties, GO surfaces produced presented some antibacterial properties (although inferior when compared with the MoS_2 alone and GO- MoS_2 films), suggesting that oxidation may be important to support GBMs antibacterial properties.

2.3. GBMs-containing bulk composite materials

GBMs incorporation into a matrix has been thoroughly explored, specifically as a way to improve mechanical, thermal or electrical properties of a material, even when added in extremely small amounts [12-15, 132]. The antimicrobial properties of these materials however have rarely been addressed. The recent use of GBMs in water desalination, gas separation, food processing and wound dressing has increased the need for investigation regarding their antibacterial effect [133].

2.3.1. Production of GBMs-containing bulk composite materials

GBMs composites can be produced by four different methods. By melt blending, the final form of the material is obtained through extrusion, injection molding [134] or hot pressing [135]. By solvent

mixing, the GBMs/polymer dispersion is casted into a mold [52, 135], spread as a film by doctor blading [136, 137], used for electrospinning [138-140], or for 3D printing [141-147]. Finally, either by *in situ* polymerization [148, 149] or by dispersion into pre-polymer/hydrogel [150], dispersions are casted into a mold or spread as a film (Figure 7) [14, 151, 152].

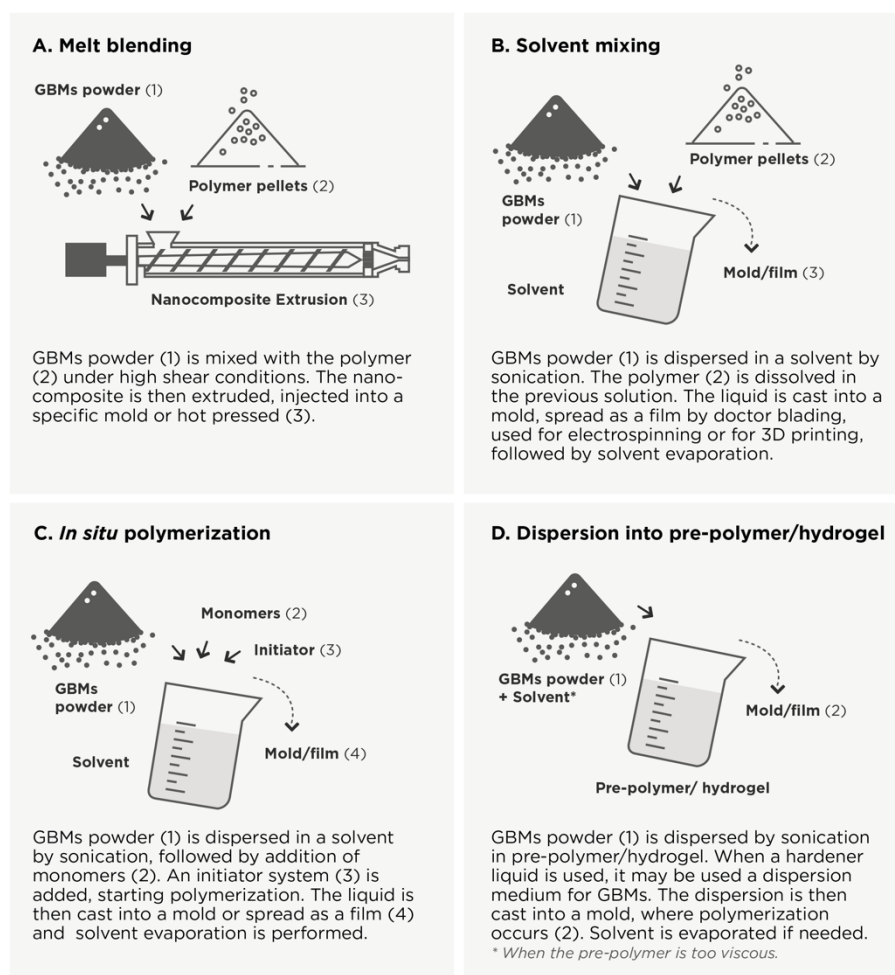


Figure 7. Methods for production of GBMs composites.

A combination of techniques can also be implemented, as for instance *in situ* polymerization and melt blending, aiming to enhance the dispersion of the GBMs in the polymer matrix while still using a low cost and more feasible method for fabricating composites on a large scale [153].

The different production techniques have been associated with different levels of GBMs dispersion, with properties of the produced composites being strongly dependent on the dispersion of the filler on the matrix [14, 151, 152]. Melt blending may lead to some degree of aggregation, with Kim *et al.* [135] reporting that good exfoliation cannot be obtained during the melt blending procedure. Also, sheets appeared more oriented and with some stacking. On the other hand, when solvent mixing or *in situ* polymerization were used, sheets were curved and randomly oriented, with better dispersions being

achieved [135]. This good dispersibility is also facilitated due to the pre-dispersion of GBMs in a solvent or liquid polymer. Table 4 gathers some information about the different production techniques and some associated features.

The interaction between GBMs and polymers is strongly dependent on the characteristics of the GBMs platelets (as the level of exfoliation achieved prior to, or during mixing) and on the polarity of the polymer matrix. For water soluble polymers (hydrophilic) [154, 155], as polyacrylamide (PAM), PVA and poly(allylamine), GO can be directly incorporated without the use of potentially toxic organic solvents [156]. These organic solvents may however be an option to improve dispersibility and avoid agglomeration of the GBMs within the matrix, especially when using G or rGO [99, 155]. G aggregation in aqueous solutions and in a range of solvents has been avoided by the use of stabilizers, as polyvinylpyrrolidone (PVP) [138] and gelatin [119].

Apart from these, several polymers have been mixed with GBMs forming composites including natural polymers, such as chitosan (CS) [157], cellulose [158], agar [150] and poly (lactic acid) (PLA) [137], and synthetic polymers, including polystyrene (PS) [159], poly(vinyl alcohol) (PVA) [160], poly(ethylene) (PE) [161], poly(N-isopropylacrylamide) (PNIPAM) [162], polypropylene [92], poly(propylene carbonate) (PPC) [163] and polyurethane (PU) [164-168] among many others [13, 14]. Pinto *et al.* [136] showed that GO or GNP incorporation into PLA matrices changed surface topography and wettability. An increase in roughness was observed due to the incorporation of both GO and GNP, as also reported in other studies [169]. Furthermore, water contact angles decreased when GO was incorporated, and slightly increased when GNP was used. However, when using hexadecane, in both situations, the contact angle dropped to 0°. Although chemical composition did not change with incorporation of either GO or GNP, surface wettability did, suggesting that even at low concentrations (0.4 wt.%) GO and GNP seem to be exposed at the surface, even though they might be slightly covered with polymer.

Although an increase in roughness is sometimes reported [136, 170, 171], a top layer is generally covering the GBMs, showing gradually-sloped structures without obvious exposure of the GBMs edges or basal planes. To overcome this and improve GBMs exposure at the surface, laser based approaches [150] or oxidative etching [172] have been explored. Papi *et al.* [150] produced agar-GO hydrogels and shaped an antibacterial pattern on their surface in order to also increase the exposure of the GO sheets. In Lu *et al.* [172] work, oxidative etching was used to expose GO sheets after studying the different orientation of the GO sheets within the pHEMA matrix. The etched composites showed different topographies depending on the orientation of the GO sheets: when a vertical orientation was achieved, visible sharp ridges were detected while when GO sheets were randomly distributed, sloped ridges and deep valleys could be noticed. When horizontally aligned, a much smoother surface was designed, attributed to the exposure of the GO basal planes [172].

Table 5. Overview of the producing techniques of GBMs-containing composites and some of their features, advantages and disadvantages.

Production method	Features	Advantages	Disadvantages	Refs
Melt blending	- Obtains flat composite slabs if using hot pressing - Obtains other shape composites if using injection molding - Few or no GBMs exposed at the surface - Rather smooth surface	- Economically attractive - Environmentally friendly - Highly scalable - Avoids using organic solvents - Can be used for both polar and non-polar elastomers	- Expensive, specific equipment - Feeding the melt-mixer might be a troublesome task (due to low density of GBMs) - Lower degree of dispersion (due to high viscosity of the polymer along with use of higher filler fractions) - Poor GBMs exfoliation and dispersion - High temperatures and high shear forces can compromise thermal stability and damage GBMs - Difficulty to remove air bubbles	[135,173–175]
Solvent mixing	Casting - Obtains flat composite slabs - Ultrasonication improves dispersibility and reduces aggregation size - Incorporation of GO into the matrix introduces roughness on the surface Doctor blading - Obtains flat composite slabs - Thinner films - Better dispersion of GO than GNP in polymer matrix - GBMs affect the surface morphology and wettability - Agglomerated platelets at higher loadings (2 wt %) Electrospinning - Allows the production of fibers (much smaller in diameter than those produced by conventional techniques) - With G incorporation, an increase in flow rate and voltage favors the production of aligned fibers with a smaller diameter - G increases viscosity resulting in fibers with larger diameters (although still below 1 μm) - Depending on the amount of G incorporated, on its size and fiber diameter, G may or may not be exposed at the surface 3D printing - GO shows tunable rheological properties, which vary greatly with its concentration in the aqueous dispersion: from liquid-like to gel-like behavior - Gel-like behavior is required for 3D printing - A good dispersion of GO in the polymer matrix has been observed with flake-like structures with sharp edges increasingly appearing as the loading of GO increases	- Simple procedure - Does not require expensive equipment - Allows for a straightforward scale-up - Good dispersions of GBMs in polymer matrix even without a chemical modification of the filler - The dispersion process can be better controlled - GBMs might be easily exposed at the surface, through control of the fiber diameter - May avoid the use of organic solvents - Allows a controlled deposition of materials - GBMs might be easily exposed at the surface	- May require organic solvents - Slow solvent evaporation may allow re-aggregation of the GBMs - High cost for disposal of the organic solvents - Total solvent evaporation required to guarantee elimination of residual solvents, avoiding toxicity or undesired plasticization of the material - Sensitive procedure since parameters as quantity and quality of solvents, mixing time and speed, sonication etc. can affect dramatically the outcome of the process	[136–138,144,154,176–178]
<i>In situ</i> polymerization	- Allows the production of flat surfaces or specimens with different shapes	- Good dispersion of GBMs in the polymer matrix - Does not require expensive equipment - Allows the use of insoluble and thermally unstable polymer composites	- Demands a low elastomer viscosity - The polymer macromolecular chains may become attached to G, hampering the production of an interconnecting network	[15,174,179–182]
Dispersion into pre-polymer/hydrogel	- Using amines, a simultaneous solidification of the composite occurs with the reduction of the GO		- If there is a strong electrostatic interaction between GO and the polymer, the self-assembly takes place immediately upon contact, leading to a sharp increase in viscosity and possible difficulty in dispersion	[183] [150] [184]

2.3.2. Antimicrobial properties of GBMs-containing composites

The effectiveness of the antibacterial action depends on the type and amount of incorporated GBMs as well as on the material production technique. A summary of the effects of GBMs-containing composites on bacteria can be consulted in Table 5.

Table 6. Effects of GBMs-containing composites on bacteria.

Material	GBMs production method	Composite production method	GBMs and composites properties	Bacteria	Antibacterial effect	Membrane damage	Ref
PE/GO-MPC composite	GO: Modified Brodie method + sonication GO-MPC: GO and MPC in water + sonication	Melting blending: pre-mixed PE and GO-MPC blended + sheeted through a two-roll mill at 130 °C	GO: Size = single layer (0.5 µm × 1.5 µm) t = 1–1.2 nm GO-MPC: single layer t = 1 nm Homogeneous distribution of GO/MPC into PE	<i>E. coli</i> <i>S. aureus</i>	PE/GO-MPC (0.2 wt %) = no activation of adhered bacteria	–	[185]
GO/CS composite	GO: MHM + ultrasonication	Solvent mixing: CS + acetic acid + GO + stirring + ultrasonication + stirring + Casting method (50 °C, overnight)	GO: l = 1 µm t < 1 nm GO-CS composite: t = 200–500 nm small area GO: area < 50 µm ² lateral size = 5 µm large area GO: area = 7000 µm ² lateral size = 100 µm	<i>S. aureus</i>	After 3 h CS = –77% CS/GO (1.5 wt %) = –78% CS/GO (3 wt %) = –82% CS/GO (6 wt %) = –87%	–	[170]
CS/small area rGO composite CS/large area rGO composite	Small area GO: HM from Gt powder Large area GO: simplified HM from Gt flakes rGO: GO reduced by NaOH	Solvent mixing: CS + GO or rGO + acetic acid + Casting method + peeled off	GO: lateral size = 5 µm large area GO: area = 7000 µm ² lateral size = 100 µm	<i>P. aeruginosa</i>	After 12 h CS = GO = rGO = no bacterial effect CS/GO (small and large, 0.3 wt %) = many colonies found, poor effect CS/GO (small and large, 0.3 wt %) = 100% (independent on the concentration and size of rGO)	–	[4]
CS/PVP/GO composite	GO: MHM + filtration + sonication + centrifugation	Solvent mixing: CS + acetic acid + stirring + PVP solution + GO solution + sonication + Casting method (slow evaporation)	GO: t = 0.9 nm lateral dimensions = few micrometers CS/PVP: relatively smooth surface without defects CS/PVP/GO: increase in surface roughness; GO appears to protrude from the polymer matrix	<i>E. coli</i> <i>S. aureus</i>	After 12 h <i>E. coli</i> and <i>S. aureus</i> CS = –85% CS/PVP = slightly lower bactericidal potential than CS CS/PVP/GO (1, 2, 3 wt %) = improved bactericidal capacity in the presence of GO	–	[171]
PLA/PU/GO composite	GO: MHM + ultrasonication	Solvent mixing: PLA and PU dissolved into the GO suspension + ultrasonication + stirring + Casting method (60 °C, 24 h) + peeled off	n/s	<i>E. coli</i> <i>S. aureus</i>	After 4 h <i>E. coli</i> PLA/PU/GO (3 wt %) = 54% PLA/PU/GO (5 wt %) = 89% <i>S. aureus</i> PLA/PU/GO (3 wt %) = 54% PLA/PU/GO (5 wt %) = 91% After 24 h <i>E. coli</i> PLA/PU = 94% PLA/PU/GO (3 wt %) = 100% PLA/PU/GO (5 wt %) = 100% <i>S. aureus</i> PLA/PU = 98% PLA/PU/GO (3 wt %) = 99% PLA/PU/GO (5 wt %) = 100%	Yes	[139]
G/PVA-biocide composite	G: Expandable graphene (EG) + Microwave irradiation + Blending + Ultrasonic bath at 65 °C for 6 h	Solvent mixing: PVA and biocide (qPVB/Cl–) in DMF at 80 °C + G + Casting method	G: t = 0.1 nm	<i>E. coli</i> <i>S. aureus</i>	<i>E. coli</i> G/PVA = increased bacterial growth G/PVA (1% biocide) = 92% G/PVA (5% biocide) = 95.8% G/PVA (10% biocide) = 97.1% <i>S. aureus</i> G/PVA = increased bacterial growth G/PVA (1% biocide) = 92.3% G/PVA (5% biocide) = 99.6% G/PVA (10% biocide) = 99.7%	–	[186]
GO/pHEMA composite	GO: commercial	Solvent mixing: HEMA + GO + EGDMA + ACVA + Casting method (between 10 glass slides) + magnetic field (6 T, 30 min, 20 °C) + UV light + etching (UV/O ₃)	GO: t = –0.8 nm	<i>E. coli</i>	Vertical-GO film = 44% Random-GO film = 24.7% Planar-GO film = 18.2%	–	[11]
Agar/GO hydrogel	GO: commercial	Solvent mixing: Luria Bertani-Agar solution + GO + Casting method + laser printing	n/s	<i>E. coli</i> <i>S. aureus</i> <i>C. albicans</i>	Agar/GO = 40% <i>S. aureus</i> Agar/GO = 53% <i>C. albicans</i> Agar/GO = 30% Increased reduction when patterning was added	–	[150]
CS-PVA/G nanofiber membrane	G: Micromechanical cleavage: Scotch tape	Solvent mixing: PVA + CS + stirred + G + DMF + ultrasonication + Electrospinning	G: few layers Nanofibers diameter = 120 nm	<i>E. coli</i> <i>Agrobacterium</i> Yeast	After 12 h CS-PVA/G (4 wt %) = 100% <i>E. coli</i> and <i>Agrobacterium</i> = limited number of cells near the membrane Yeast = no difference	–	[187]
GO/PVDF nanofiber membranes	GO: HM	Solvent mixing: PVDF in DMAc in acetone + Electrospinning	GO/PVDF: Pore size = 0.28–0.35 µm t = 36–62 µm	<i>E. coli</i> <i>Bacillus</i>	After 2 h: <i>E. coli</i> PVDF/GO powder = 90.4%; PVDF/GO (0.1 wt%) = 93.3%; PVDF/GO (0.2 wt%) = 99.6% <i>Bacillus</i> PVDF/GO powder = 99.5%; PVDF/GO (0.1 wt%) = 98.9%; PVDF/GO (0.2 wt%) = 95% MIC (sulphur NPs rGO) < MIC (rGO) and MIC (sulphur NPs) HPU/sulphur NPs rGO (2 wt % sulphur NPs rGO) = growth and fouling inhibition	–	[188]
HPU/sulphur NPs decorated rGO composite	GO: MHM + ultrasonication Sulphur NPs rGO: thiosulfate solution + GO solution + 30 min sonication + lemon juice and 60 min stirring + 10 min sonication	In situ polymerization: PCL + BD + DMAc + sulphur NPs rGO + TDI + monoglyceride of castor oil addition (110 °C, 2.5 h)	n/s	<i>E. coli</i> <i>S. aureus</i> <i>C. albicans</i>	AgO/G1 and AgO.5G1 = poor antibacterial activity (AgO.5G1 better) Ag1G1 = 100% Ag5G1 = 100% <i>S. aureus</i> AgO/G1 and AgO.5G1 = poor antibacterial activity Ag1G1 = almost 100% (2 colonies found) Ag5G1 = 100% Inhibition zone: Ag1G1 and Ag5G1 > AgO/G1 and AgO.5G1	Yes	[189]
Ag/G polymer hydrogel	GO: MHM + 1 h sonication + glucose addition as reducing agent + ammonia in AgNO ₃ solution	In situ polymerization: crosslinking of Ag/G composites (in different weight ratios: 0, 0.5, 1, 5 denoted as AgO/G1, AgO.5G1, Ag1G1, Ag5G1) with AA + BIS + APS + Casting method (65 °C, 4 h) + peeled off	AgNPs size = 11.5–39.0 nm	<i>E. coli</i> <i>S. aureus</i>	AgO/G1 and AgO.5G1 = poor antibacterial activity (AgO.5G1 better) Ag1G1 = 100% Ag5G1 = 100% <i>S. aureus</i> AgO/G1 and AgO.5G1 = poor antibacterial activity Ag1G1 = almost 100% (2 colonies found) Ag5G1 = 100% Inhibition zone: Ag1G1 and Ag5G1 > AgO/G1 and AgO.5G1	–	[148]
PU/SRGO-PEI composite	GO: MHM + exfoliation SRGO-PEI: GO in DMF + PEI in DMF + HATU + heating (63 °C) + stirring 8 h (mid conditions) SRGO: slightly reduced GO	In situ polymerization: SRGO-PEI in DMF + sonication + polyisocyanate + stirring (2 h, 80 °C) + PCL + catalyst + defoamer + Casting method (RT)	GO: size = 9.81 (±3.89) µm SRGO-PEI: size = 0.87 (±2.66) µm	<i>E. coli</i>	After 16 h to 24 h PU film = numerous bacteria; biofilm PU/GO (1 wt%) = less cells; scattered PU/SRGO-PEI (1 wt%) = almost no cells	–	[149]

PE = polyethylene, MPC = 2-Methacryloyloxyethyl phosphorylcholine, CS = chitosan, PLA = poly(lactic acid), PU = polyurethane, PVA = Poly(vinyl alcohol), PVDF = polyvinylidene fluoride, t = thickness; l = length, n/s = not studied, HM = Hummers method, MHM = modified Hummers method, NaOH = sodium hydroxide, DMAc = N,N-dimethylacetamide, DMF = N,N-dimethylformamide, Hr = hydrazine reduction, RT = room temperature in air, HEMA = 2-hydroxyethyl methacrylate, EGDMA = ethylene glycol dimethacrylate, ACVA = 4,4'-Azobis(4-cyanovaleic acid).

From the different polymers that have been conjugated with GBMs, most of them already have inherent antibacterial characteristics, such as polyvinyl-N-carbazole (PVK) [190] or chitosan (CS) [4], and so the conjugation aims to improve their native properties. Others seek the conjugation with GBMs

to provide them the antimicrobial nature, such as PE [185] or PLA [139]. The incorporation of different molecules as boosters of the antimicrobial effects is also very common, although not the main objective of this review.

Melt blending has been poorly explored for production of antimicrobial surfaces, most likely due to limited GBMs exposure at the surface. In fact, our group found low antibacterial activity towards *S. epidermidis* of GNP/polyurethane composites, which was related to the reduced number of GNP particles exposed [191]. Nonetheless, Jin *et al.* [185], produced polyethylene (PE)-modified GO composites with 2-(methacryloyloxy)ethyl phosphorylcholine (antibiofouling) (PE-GO-MPC) that showed bacteriostatic activity against *E. coli* and *S. aureus*. The antibacterial activity was associated with the presence of GO and with the large number of -COO^- groups that adsorbed the cytoplasm of bacteria and engendered flocculation, thus killing bacteria [185]. However, analysis of the surface features was not performed, and therefore no relation between the latter and antibacterial activity could be firmly established.

Materials produced by solvent mixing have shown better antibacterial performance when compared to melt blending composites. Mazaheri and co-workers [170] found significant antibacterial activity on CS/GO composites produced by casting against *S. aureus*, despite the inherent antibacterial effect of CS alone. The antibacterial performance and roughness of the surface increased with GO content [170], with CS presenting a smooth surface. GO concentrations varied from 1.5 to 6 wt %, higher amounts than the ones used by Lim *et al.* [4], who also produced CS/GO composites (0.3 wt %) by the same technique and evaluated their activity towards *P. aeruginosa*. However, Lim *et al.* [4] found no effect for these composites. This can be attributed not only to the reduced amounts of GO used, which may not be enough to reach the surface of the composite (being lost in the polymer matrix) but also to the different bacteria used – *S. aureus* is a Gram positive, round-shaped bacterium while *P. aeruginosa* is a Gram negative, rod-shaped bacterium – with Gram positive bacteria being generally reported as more susceptible. Interestingly, rGO/CS composites also produced [4] presented an increased antibacterial effect over GO/CS composites, completely inhibiting *P. aeruginosa* growth independently on the concentration and size of rGO (100% viability loss even at the lowest concentration of 0.3 wt %) [4]. Here, the more sharpened edges of rGO [72] may somehow be more exposed or the conductivity of rGO in opposition to the electrically insulating GO may be affecting. Nonetheless no detailed analysis of the surface of the composite was performed in order to be sure. Since CS, rGO and GO alone did not show growth inhibition, a synergistic effect between CS and rGO was proposed. A change in the surface morphology was also found for CS/PVP/GO films produced by Mahmoudi *et al.* [171]: with addition of 1 wt % GO, the surface became rougher, with nanosheets appearing to protrude from the polymer matrix; by increasing the GO concentration (3 wt %), more wrinkles and a more organized structure was found. The CS/PVP/GO composites antibacterial activity against *S. aureus* and *E. coli* was also found dependent on the GO content. Even though, the effect of GO was only slightly noticed and when no CS was present. This may be related to the use of lower amounts of GO (1, 2, 3 wt %) and once

again with the inability of GO to achieve and protrude from the surface. Therefore, it would have been interesting to further evaluate the reason behind the antibacterial effect on bacteria. The major player however seems to be CS.

Also using solvent mixing, An *et al.* [139] produced GO/PLA/PU composite films able to withstand their effect over time: a concentration dependent effect on bacteria at least after the first 3 h [139], however after 24 h the antibacterial effect was rather similar and effective (100%) against both bacteria independently on the GO concentration. However, PLA/PU films also showed considerable bacterial growth inhibition, which suggests the strong impact of the PLA/PU mixture on the antibacterial effect of the composite. In fact, in the FLG/PVA-biocide nanocomposites produced by Cao and co-workers [186] no antibacterial activity was found when only in the presence of FLG/PVA [186]. The biocide was found responsible for the antimicrobial activity, in a concentration dependent way. Using a different production method, electrospinning, Lu *et al.* [187] reported G/CS/PVA nanofibers with antimicrobial effect towards *E. coli* and *Agrobacterium* (prokaryotic cells), however yeast cells (eukaryotic cells) were still able to grow when in their presence. The effect of these fibers on bacteria may be related with the presence of the CS polymer, with the production method – although also solvent mixing, through electrospinning GBMs may be easily exposed when compared to casting – and with the type of GBM incorporated: G may be more easily interacting with bacteria than FLG (increased thickness) [186]. Regarding the different effect towards eukaryotic and prokaryotic cells, the effect was associated with the electron transference from the G to the cells, with eukaryotic cells being less susceptible to GBMs effect than prokaryotic, given the difference in the nuclear envelope. When comparing GtO and GO deposition in polyvinylidene fluoride (PVDF) nanofiber membranes, the effect was similar, suggesting the importance of the oxygen-containing groups rather than the GBMs type or thickness [188].

Although the main focus of the work was GO surface functionalization with AgNPs, Liu *et al.* [169] observed that PLA-1%GO nanocomposite presented little antibacterial activity. A change in the topography was observed, however a thin layer of PLA may be covering the GO sharp edges, leading to a loss of the cutting effect due to their binding with the PLA matrix [169].

Overall and as expected, when incorporated in a polymer matrix, GBMs have to be used in higher concentrations in order to have the same or similar effect. Thakur *et al.* [189] showed exactly that with rGO combined with sulphur nanoparticles for incorporation on HPU (hyperbranched polyurethane) through *in situ* polymerization followed casting [189]. While sulphur-rGO alone showed the lowest MIC against bacteria (*S. aureus* and *E. coli*) and fungi (*C. albicans*), revealing a synergistic effect of both (each one also with low MIC), incorporation in the HPU caused a decrease in the effect, requiring a higher dose to show the same inhibitory effect (from 13.7 µg/mL for sulphur-rGO to 33.7 µg/mL for HPU/sulphur-rGO against *S. aureus*, for instance).

Apart from sulphur, other molecules with inherent antibacterial activity, as PEI [149], have been incorporated. However, once again, the reduced antibacterial effect of GO may be associated with the

deficit in exposure of the SRGO platelets, thus hampering the contact with bacteria. Authors also reinforce the idea that roughness/smoothness and wettability may contribute to the antiadhesive effect, in this case with smooth and hydrophilic surface being beneficial for the prevention of bacterial attachment [149].

An improvement regarding antibacterial properties can be obtained by increasing the GBMs exposure at the surface of the composite. Papi *et al.* [150] reported that when exposing GO sheets at the surface of agar-GO hydrogels, a reduction of 10% in bacterial growth was achieved when comparing to agar alone. The combination of the GO exposure and the shaping of a specific antibacterial pattern caused an improved reduction of the bacterial growth of 53% for *S. aureus* and 40% for *E. coli* [150]. Lu *et al.* [172] reported very recently similar results when exposing GO at the surface of GO/pHEMA composites, with the presence of GO significantly reducing bacterial viability, however only when in a vertical configuration, in opposition to a nonaligned (random) or horizontally aligned (planar) samples. The similar topography between composites without GO and with horizontally aligned GO sheets was translated into a similar antibacterial effect. Here, not only the GO exposure was crucial to the antibacterial effect of the surfaces but also the alignment of the GO sheets. The vertical orientation of the GO sheets was associated with an increased edge density and with a preferential orientation for membrane disruption. To note that data are influenced not only by the cytotoxicity caused by the GO but also by the ability of bacteria to adhere to the surface, with pHEMA surface being smooth and hydrophilic, therefore inherently reducing bacterial adhesion. In this case, the difference in topography does not seem to influence bacterial viability, since both in randomly and horizontally exposed, bacteria are viable, retaining their morphological integrity. Authors tried to explore the mechanism of action using a model system, although in suspension, reporting that GO unequivocally induces both physical disruption of lipid bilayers and chemical oxidation where GO mostly works as an electron shuttle between the glutathione and dissolved oxygen (required for the oxidation) with poor ROS production [172]. In both cases, direct contact is required to occur.

Although several works have explored the use of 3D printing to produce composite materials with GBMs [144-146], to our knowledge, the antimicrobial activity of these composites has not yet been explored. Since this technology allows a more accurate control of the material's deposition, it would be relevant to explore new ways to better expose the GBMs. Also, perhaps a combination of strategies could be explored.

Overall, comparisons between studies are difficult to conduct, since not always a full morphological characterization of the material is performed, particularly regarding the structure of the surface. Also, the mechanisms of action underlying the antimicrobial effect of composites have been poorly explored.

3. Antimicrobial mechanisms of action of surfaces integrating GBMs

Overall, direct contact with GBMs at the surface (either edges or basal planes) is a requirement to have antibacterial action, with no loss of bacteria viability being found when no contact is established [53]. Upon contact with the antimicrobial agents, materials can either cause an inhibition of bacteria attachment (anti-fouling) [192], prevent bacteria growth, keeping them in a stationary stage (bacteriostatic) [193, 194], or cause effective death of bacteria (bactericidal) [63, 195]. Bacteria can thus acquire a flattened or deformed shape or suffer a direct leakage of intracellular cytoplasm, both leading to loss of integrity and consequently cell death.

According to the surface features of the GBMs-containing surfaces, different mechanisms of action can explain the effect of GBMs-containing surfaces towards bacteria (Figure 8). When bacteria are exposed only to basal planes, two possible actions have been reported to occur: i) electron transference between GBMs and bacteria membrane, with GBMs being good electron acceptors [40, 187] and ii) reduction of the O₂ adsorbed on defect sites and edges of the GBMs [53] by antioxidant enzymes, as glutathione (involving once again electron transference). On the other hand, when sharp edges are exposed, both the aforementioned events can occur, as well as iii) physical insertion of the GBMs sharp edges in the bacteria membrane (nano-knife effect) [72, 81], causing a direct extraction of phospholipid molecules, iv) protein-protein bonding disruption, due to the lipophilic nature of G sheets that favorably enter the hydrophobic interface between contacting proteins, leading to their destabilization [196] and v) pore formation, due to the interaction between lipophilic GBMs and hydrocarbons tails of the lipids in the bacterial membrane [18].

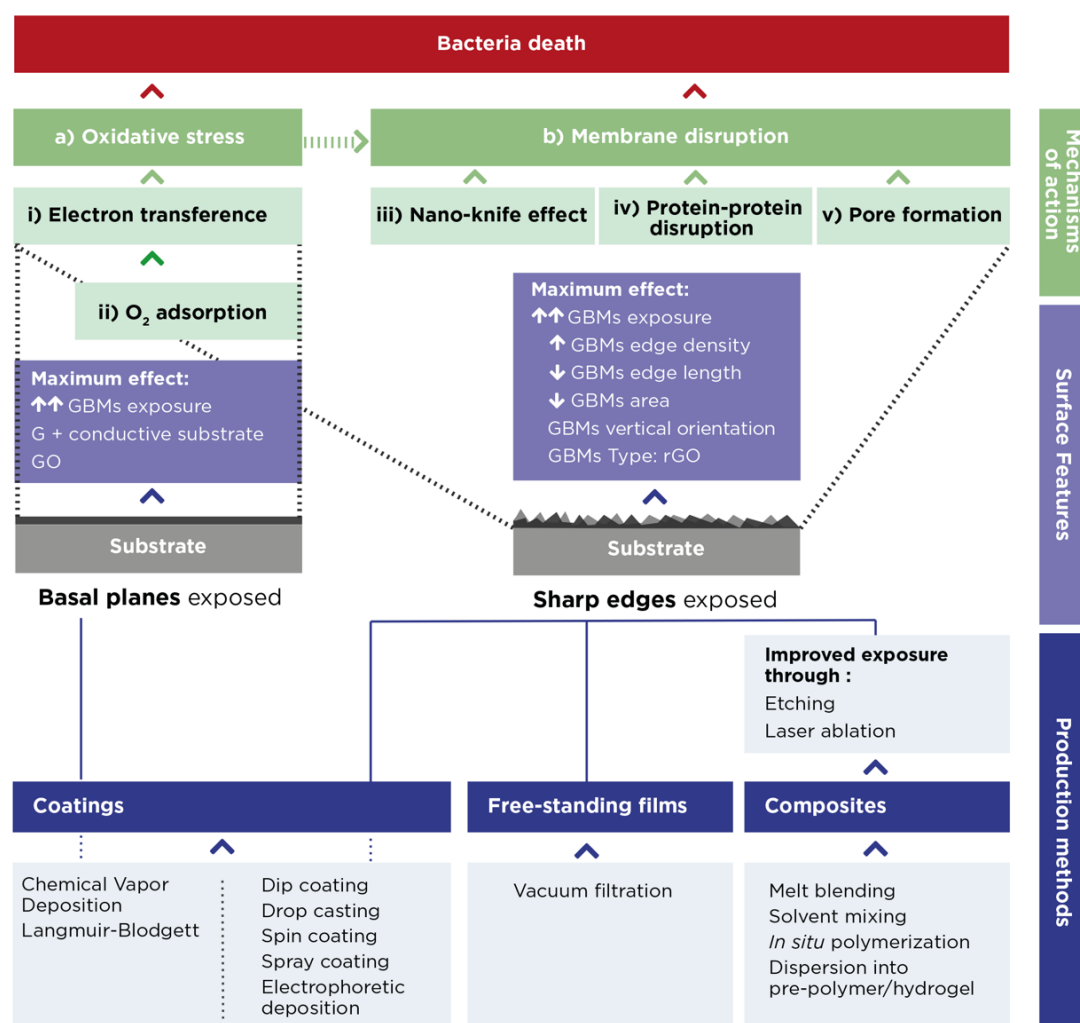


Figure 8. Relation between GBMs-containing surfaces production methods, surfaces features and mechanisms of action.

These events can have as consequences a) the induction of oxidative stress [197, 198] and/or b) the disruption of the bacterial membrane [72, 81]. Oxidative stress can be induced either by a reactive oxygen species (ROS)-dependent pathway, due to an unbalance in intracellular ROS levels (accumulated after O₂ adsorption on defect sites and edges), or by a ROS-independent pathway, in which GBMs disturb or oxidize a vital cellular structure or component causing a disruption of a microbial process (occurring through electron transfer for instance) [70]. Disruption of bacteria membrane is the most commonly reported outcome. The sharp edges can penetrate the membrane causing a physical damage or can interact with the components of the membrane, causing a destabilization and further loss of membrane integrity, without directly destroying it [192].

Different surface features can be optimized in order to enhance the antibacterial performance of the surface, both when only basal planes or sharp edges are exposed (Figure 8). Some features might directly influence the mechanism of action tuning the interaction with the bacteria. GBMs lipophilicity

is an example, with lipophilic materials (as G and rGO) interacting more favorably with the outer membrane of Gram negative bacteria [18, 72]. For hydrophilic materials, as GO, where the interaction may be reduced, the O₂ adsorption on defect sites and edges and further reduction to ROS may be more likely to occur. The less sharp edges of the GO in comparison with the rGO sheets can also support this hypothesis. Defect sites and edges, present in a higher extent in rGO for instance, are highly reactive sites and thus also able to directly cause oxidation without the need of O₂ adsorption [53]. The shape (coccus or bacillus) of the bacteria and size also play a role, with small and round bacteria having more chances of survival in rough surfaces.

4. Conclusions

GBMs must be exposed at the surface in order to directly contact with microorganisms. From the GBMs-containing surfaces, both free-standing films and coatings present a better GBMs exposure when comparing to GBMs-containing composites. Notwithstanding, antibacterial activity is still observed for GBMs-containing composites. In this case, roughness or wettability can be more influential parameters, preventing most of all bacterial adhesion: a hydrophilic surface can more easily prevent adhesion of hydrophobic components, such as the hydrophobic components of extracellular polymeric substances or dissolved organic matter from the environment. Roughness is a controversial parameter and its influence will depend on the topography of the surface and whether it is micro or nano, with generally rougher surfaces having more surface area and allowing more bacterial adhesion. However, the bioadhesive material released from bacteria, a mixture of both hydrophilic and hydrophobic proteins (a ratio that depends on the bacterial species), can alter the interaction with the surface. Strategies to expose GBMs in composites, as oxidative etching and laser ablation, improve significantly their antibacterial performance.

G basal planes have been reported to cause damage to bacteria due to electron transference, particularly when coating a conductive substrate. On the other hand, exposure of GO basal planes in non-conductive surfaces exerts killing effect on bacteria. In these cases, O₂ adsorption on the surface of the GBMs can be occurring, being related with ROS production and oxidative stress, causing further damage to bacteria. When G sharp edges are exposed (either alone or together with basal planes), the antibacterial activity increases, and here not only the electron transference can occur but also the physical insertion on the membrane, protein-protein bonding disruption and pore formation.

Apart from GBMs exposure, GBMs edge density and length, GBMs orientation, size, wettability will undoubtedly influence the antimicrobial performance of the surface. Considering all the works presented, in order to produce a surface with an antimicrobial profile one should 1) increase the GBMs exposure (preferentially sharp edges, with an increased edge density and vertical orientation), 2) consider the GBMs type (small, GBMs with oxidized groups might act more due to oxygen-containing groups, while rGO and G might act due to their sharper edges), 3) focus on the wettability of the GBMs

(hydrophilic materials may prevent more bacterial adhesion while hydrophobic ones might cause them to adhere, however may interact more with the membrane) and 4) consider the final roughness/smoothness of the surface (smoother surfaces may have less adhesive points for bacteria).

Knowledge about the target bacteria (nature of the cell wall, morphology and size) is also crucial to understand their weaknesses in order to more effectively define the characteristics of the surface. Gram positive bacteria are generally more affected by the GBMs-containing surfaces; however, the outer membrane of Gram negative bacteria can be a favorable target for lipophilic materials (as G and rGO) that will most likely interact physically with the membrane. The shape (coccus or bacillus) of the bacteria and size also play a role, with small and round bacteria having more chances of survival in surfaces with plane spaces for their adhesion. Eukaryotic cells, on the other hand, are less susceptible to the action of GBMs.

The bacteria-surface interface is therefore a complex and intricate environment, with different and variable factors interfering at the same time, and so each factor must be considered as part of a complex environment. Also, preventing bacterial adhesion, where wettability and roughness might be crucial, is different from causing bacterial death, where type of GBMs, sharp edges exposure, presence of defect sites, edge density, sheets orientation might be the factors playing a major role.

Further studies are still necessary to clarify the influence of some variables on the antibacterial performance of GBMs-containing surfaces in order to develop the most effective surface.

5. Future perspectives

Graphene is an interesting material with a broad spectrum of applications, however there are still many gaps to fill in, particularly regarding its antimicrobial properties.

GBMs will continue to be explored and so guidelines should be provided regarding the main points to be considered about GBMs and about the tests that should be performed to evaluate the antimicrobial activity. The elaboration of an ISO standard is recommended.

More fundamental studies need to be conducted after a thorough characterization of the materials, in order to clearly understand how the physical and chemical features of the GBMs and their incorporation onto a surface can impact the interaction with the bacteria and other microorganisms. Only with this knowledge and a complete understanding of the interfacial process, materials with proper features can be designed. Particularly, only then the relevance of the GBMs exposure will be clarified.

Coating of materials in the biomedical field is a promising area for GBMs with still lot to explore. The growing number of antibiotics-resistant bacteria and the possibility of biofilm formation are still two major problems, causing antimicrobial materials and anti-fouling coatings or composites crucial to increase the efficiency of the current materials. The development of safe materials, toxic only against bacteria and biocompatible towards human cells, is the ultimate goal for biomedical applications.

Assurance needs to be given about the long-term biocompatibility and stability of G-based coatings and composites.

Translation from the bench to bedside is still a challenge. *In vivo* dynamics need to be considered carefully, especially since proteins and other molecules can significantly inhibit GBMs antibacterial activity through prevention of graphene interaction with bacteria.

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CHAPTER IV

**How do GBMs films interact with our
major biological systems?**

Graphene Surfaces Interaction with Proteins, Bacteria, Mammalian Cells, and Blood Constituents: The Impact of Graphene Platelet Oxidation and Thickness

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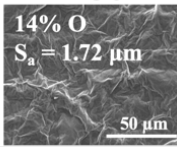
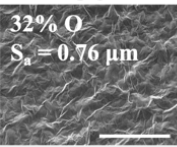
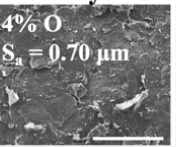
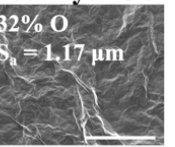
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Abstract

Graphene-based materials (GBMs) have been increasingly explored for biomedical applications. However, interaction between GBMs-integrating surfaces and bacteria, mammalian cells, and blood components, that is, the major biological systems in our body, is still poorly understood. In this study, we systematically explore the features of GBMs that most strongly impact the interactions of GBMs films with plasma proteins and biological systems. Films produced by vacuum filtration of GBMs with different oxidation degree and thickness depict different surface features: graphene oxide (GO) and few-layer GO (FLGO) films are more oxidized, smoother, and hydrophilic, while reduced GO (rGO) and few-layer graphene (FLG) are less or nonoxidized, rougher, and more hydrophobic. All films promote glutathione oxidation, although in a lower extent by rGO, indicating their potential to induce oxidative stress in biological systems. Human plasma proteins, which mediate most of the biological interactions, adsorb less to oxidized films than to rGO and FLG. Similarly, clinically relevant bacteria, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, adhere less to GO and FLGO films, while rGO and FLG favor bacterial adhesion and viability. Surface features caused by the oxidation degree and thickness of the GBMs powders within the films have less influence toward human foreskin fibroblasts; all materials allow cell adhesion, proliferation and viability up to 14 days, despite less on rGO surfaces. Blood cells adhere to all films, with higher numbers

in less or nonoxidized surfaces, despite none having caused hemolysis (<5%). Unlike thickness, oxidation degree of GBMs platelets strongly impact surface morphology/ topography/chemistry of the films, consequently affecting protein adsorption and thus bacteria, fibroblasts and blood cells response. Overall, this study provides useful guidelines regarding the choice of the GBMs to use in the development of surfaces for an envisioned application. Oxidized materials appear as the most promising for biomedical applications that require low bacterial adhesion without being cytotoxic to mammalian cells

Graphical abstract

Graphene-based materials films		rGO	GO	Few-layer G	Few-layer GO
		14% O $S_a = 1.72 \mu\text{m}$ 	32% O $S_a = 0.76 \mu\text{m}$ 	4% O $S_a = 0.70 \mu\text{m}$ 	32% O $S_a = 1.17 \mu\text{m}$ 
Plasma proteins 	Adsorption	+	+/-	++	+/-
	Adhesion	++	+	++	+
Bacteria 	Death %	Dependent on bacteria			
	Adhesion	+	++	++	++
Fibroblasts 	Adhesion	++	+	++	+
	Hemolysis	-	-	-	-

S_a = roughness

Keywords: graphene oxide; films; protein adsorption; bacterial adhesion; antimicrobial; biocompatibility; hemocompatibility.

1. Introduction

Graphene is the thinnest, strongest but at the same time one of the lightest materials in the world, being one atom thick, 200 times stronger than steel, and weighing $\sim 0.77 \text{ mg/m}^2$. These features combined with graphene and graphene-based materials' (GBMs) high thermal and electric conductivity, cytocompatibility, and antimicrobial properties have widened their use as platforms for healthcare and biomedical purposes [1-5]. Recent applications include biosensors for biomarkers, cellular, and pathogen detection, surface coating of implants and membranes, stem cell engineering, cardiac and neuronal tissues regeneration, and microbial fuel cells [6, 7]. The requirements that these surfaces need to fulfill in order to meet the desired biological responses can however be significantly different.

GBMs-integrating surfaces and biological systems coexist in a dynamic and complex interface. It is established that GBMs interaction with biological systems differs according to their physical

arrangement [8], despite biological studies on GBMs suspensions [9, 10] being more extensive than on surfaces containing GBMs [11]. In addition, although antimicrobial properties, safety and biological effects of GBMs-integrating surfaces have been explored and summarized in some reviews, consistency has been more often achieved regarding the effect towards mammalian cells than microorganisms. These surfaces are generally not harmful to different mammalian cell types [12-14], but can have a double effect towards microorganisms: some studies support a bactericidal or anti-adhesive profile [15-17], while others strongly reject this idea [18, 19]. These diverging opinions may arise from the vast number of parameters influencing the behavior of surfaces containing GBMs. It has been recognized that surface features such as roughness, chemistry, wettability, and surface charge have a major contribution toward surface activity [20, 21]. Conclusions however can be hard to draw due to increase in GBMs' variability and availability [1], either associated with GBMs intrinsic features (thickness, oxidation degree and lateral size) [17] or production method [8]. Production technique of the surface, which affects GBMs arrangement [1], and the presence of other components, which may hinder GBMs' real effect [1], can also contribute to the different observed effects. Finally, lack of thorough surface characterization can hamper the establishment of a cause-effect relation between surface features and biological responses. Moreover, the specificities of the different biological systems (e.g., type of cell, cell wall structure, morphology), and experimental conditions (e.g., presence of plasma proteins, incubation time) have also profound impact.

These facts emphasize the need for a deeper understanding of the predominant surface features affecting biological responses, so that GBMs-integrating surfaces can be more efficiently designed considering the final purpose. In addition, the fact that most studies generally focus on one or two of the three biological systems (bacteria [19, 20, 22-26], cells [12, 13] or blood [23]) also limits the withdrawal of coherent conclusions. Therefore, and although GBMs are thriving in healthcare applications, there is still a clear need to provide quantitative experimental results that directly establish a clear link between GBMs physicochemical properties and cell function by connecting GBMs–protein and protein–cell interfaces, as recently highlighted by Kumar *et al.* [27].

To address these burning questions, we designed a systematic study to investigate how GBMs with different intrinsic characteristics, namely oxidation degree and thickness, impact surface features, and then how these parameters affect protein adsorption and interaction with the three major biological systems: bacteria, human mammalian cells, and blood components. We narrowed down the testing variables by (i) focusing on one production technique, namely vacuum filtration, (ii) eliminating the use of other materials, by producing surfaces that are composed of only GBMs (GBMs films), and (iii) exploring the impact of GBMs intrinsic features, as oxidation degree and thickness, on the surface properties of such films. The surface of these GBMs films was characterized regarding chemical functionality, crystallite arrangement, topography, wettability, oxidative potential, and protein adsorption profile. Finally, the interaction with biological systems was investigated through assessment of (1) antibacterial performance toward clinically relevant Gram+/Gram– bacteria with different

morphology (coccus/ bacillus); (2) *in vitro* biocompatibility toward human mammalian cells; and (3) *in vitro* blood cells adhesion and hemolytic potential.

This knowledge can be directly translated into the design and development of membranes, films, and coatings or any other surface comprising GBMs, considering the required features for a specific application.

2. Materials and Methods

Four different GBMs films made of reduced graphene oxide (rGO), graphene oxide (GO), few-layer graphene (FLG), and few-layer graphene oxide (FLGO) are studied. As described in Section 2.1, GO, FLG, and FLGO aqueous suspensions were first synthesized and then used to produce micrometer thick films. rGO film was afterward obtained from GO film, as described in Section 2.2.

2.1. Synthesis of graphene-based materials (GBMs) powders

Carbon graphite (Gt) micro powder (99% purity, 7-11 μm diameter) and FLG (xGnP® grade M, ~ 5 μm diameter according to the supplier) were purchased from American Elements (California, USA) and XG Sciences (Lansing, USA), respectively. GO and FLGO were obtained by oxidation of Gt and FLG, respectively, using modified Hummers method [28]. Briefly, either Gt or FLG (3 g) were added to H_2SO_4 (Sigma)/ H_3PO_4 (Thermo Fischer Scientific) mixture (4:1, 150 mL) followed by cooling and the addition of KMnO_4 (18 g, Sigma). The reaction was kept at 35 °C and stirred for 2 h; the mixture was cooled to 0 °C and distilled water (450 mL) added slowly. Excess KMnO_4 was eliminated by adding H_2O_2 until oxygen release stopped. After overnight resting, materials were repeatedly centrifuged at 4000 rpm during 20 min until pH of supernatant reached the pH of distilled water (assuring the removal of acidic materials or contaminants [29]), rendering either GO, after sonication for 6 h in an ultrasonic bath, or FLGO materials.

2.2. Production of GBMs films

GO, FLG, and FLGO films were produced by vacuum filtration technique (Figure S1): 45 mg of GBMs in aqueous dispersions were filtered through a nylon membrane (0.20 μm pore size, NY2004700, Merck) and dried at 37 °C overnight. rGO films were then produced from GO films through a thermal reduction process (400 °C, 1 h) under argon atmosphere in a tubular furnace. rGO and FLG films were slightly compressed after production, for 5 s under ~ 16 kg/mm^2 , using a hydraulic press (Graseby Specac). This pressing is necessary in the case of rGO films to reduce voids left during the reduction process, associated with water evaporation and gas release as CO_2 and CO. In the case of FLG films, pressing helps preventing particle detachment. All films were cut into 9 mm diameter discs using a

stainless-steel puncher and sterilized with ethylene oxide (EO Gas series 3+). GBMs film thickness was determined via a 0–10 mm dial thickness gauge, Mitutoyo NO 2046F (N° 7301), graduation of 0.01 mm. At least five measurements of different samples were performed.

2.3. Characterization of GBMs powders and films

GBMs powders (GO, FLG, and FLGO) were characterized regarding morphology by transmission electron microscopy (TEM) and chemistry by X-ray photoelectron spectroscopy (XPS). GBMs films (rGO, GO, FLG and FLGO in a dry state) were characterized regarding atomic composition and functional groups by XPS, crystallinity and structural properties by X-ray diffraction (XRD), surface topography by scanning electron microscopy (SEM) and atomic force microscopy (AFM), and wettability by contact angle measurements.

2.3.1. Transmission Electron Microscopy (TEM)

Morphology of GBMs powders was determined by TEM. Visualization was performed after dispersion of dry powders in water and dropping the suspension on 200 mesh copper TEM grids. Images were acquired using a JEOL JEM 1400 TEM (Tokyo, Japan) coupled to a digital camera CCD Orious 1100 W (Tokyo, Japan).

2.3.2. X-Ray Photoelectron Spectroscopy (XPS)

Chemical composition and oxidation degree of both GBMs powders and films were investigated by XPS analysis. Measurements were performed at Centro de Materiais da Universidade do Porto (CEMUP, Porto, Portugal) using a Kratos Axis Ultra HSA (Kratos Analytical, U.K.) equipment with a monochromatic Al X-Ray source operating at 15 kV (90 W). Survey spectra were acquired at 80 eV and C 1s and O 1s high-resolution spectra at 40 eV. Electric charge effect was corrected by setting the reference of the C 1s peak to 284.6 eV. Deconvolution of high-resolution spectra was performed with CasaXPS processing software version 2.3.16, using background type Shirley. For C 1s deconvolution, Gaussian–Lorentzian (70:30) function was used to fit all peaks with exception of sp^2 carbon peak that due to its asymmetric nature was fitted using an asymmetric Lorentzian function (LF) with asymmetry parameter of 0.14.[30] Peak positions established based on literature data were sp^2 C=C (284.2–284.5 eV), sp^3 C–C (284.8–284.9 eV), C–OH (285.3–286.0 eV), C–O–C (286.1–286.6 eV), C=O (287.5–287.9), O=C=O (288.8–288.9 eV) and π – π (290–292 eV) [30]. Deconvolution of O 1s high-resolution spectra was performed using a Gaussian–Lorentzian (70:30) function where peaks were established based on Ganguly, *et al.* [31].

2.3.3. X-Ray Diffraction (XRD)

Crystallites interlayer d -spacing, number of layers, and size (thickness) of GBMs within the films were obtained by XRD analysis, carried out on a Rigaku SmartLab diffractometer operating with 45 kV and 200 mA under Cu K α radiation of wavelength $\lambda \sim 1.540593 \text{ \AA}$ in a Bragg–Brentano geometry. Samples were measured in the 2θ range of $5\text{--}50^\circ$ with a step of 0.01° . To increase the crystallite size statistics, samples were measured in a rotative system ($30^\circ \text{ min}^{-1}$). Crystallite interlayer d -spacing was extracted from the Rigaku PDXL XRD analysis software based on the Bragg's law equation and thickness was calculated by Scherrer's equation considering a Scherrer constant of 0.89. The number of layers was determined by dividing the thickness by the d -spacing adding one layer to achieve the correct number of layers.

2.3.4. Scanning Electron Microscopy (SEM)

Morphology of GBMs films was observed by SEM. Surfaces were adhered onto carbon tape and coated with a thin layer of Au/Pd by sputtering to improve their conductivity. Visualization was performed using a FEI Quanta 400FEG ESEM/EDAX Genesis X4M SEM with an acceleration voltage of 10 kV.

2.3.5. Atomic Force Microscopy (AFM)

Surface topography imaging of GBMs films was studied by AFM. A PicoPlus scanning probe microscope interfaced with a Picoscan 5500 controller (Keysight Technologies, USA) was used with Picoview 1.20 software (Keysight Technologies, USA). Each sample was imaged with a $100 \times 100 \text{ }\mu\text{m}^2$ piezoelectric scanner from scanning areas of $50 \times 50 \text{ }\mu\text{m}^2$ and $5 \times 5 \text{ }\mu\text{m}^2$. A silicon cantilever tip (AppNano, U.S.A.) in intermittent tapping mode was used with a spring constant (k) of 26 N/m with a frequency of 296 kHz and with a speed of 1.0 Hz. The 2D and 3D images and surface profiles were collected using WSxM 5.0 software while surface roughness was determined using SPIP software. Roughness was characterized by a set of amplitude and spacing parameters, namely surface roughness (S_a), root-mean-square surface roughness (S_q), skewness (S_{sk}), kurtosis (S_{ku}), summit density (S_{ds}), largest peak height value (S_p), largest valley depth value (S_v), peak to peak height (S_z) and ten-point average roughness (S_{10z}), explained in detail in Table S1. Two samples of each type were analyzed, and the reported roughness values are mean values from three randomly chosen location images.

2.3.6. Contact angle measurements

Wettability of GBMs films was evaluated by static water contact angle measurements using the sessile drop method (OCA 15, DataPhysics, Instruments GmbH). Drops of $4 \text{ }\mu\text{L}$ of $d\text{H}_2\text{O}$ were placed on samples surface and images were acquired. Water contact angle was calculated using SCA20

software (DataPhysics Instruments GmbH) after droplet fitting using the Young–Laplace mathematical function. Measurements were repeated at least three times, and results are reported as mean value.

2.3.7. Oxidative potential of GBMs films

Oxidation of glutathione (GSH) mediated by GBMs films under acellular conditions was investigated using Ellman's reagent 5,5'-dithio-bis-(2- nitrobenzoic acid) (Thermo Scientific), which quantifies the thiol groups of GSH. O-phthalaldehyde (OPT) fluorometric assay was also used to quantify GSH oxidation at pH 8; further details on the materials and methods are provided in Supporting Information.

GBMs films were incubated with 300 μ L of GSH (0.8 mM, Sigma) in reaction buffer (0.1 M sodium bicarbonate (pH 8), containing 1 mM EDTA) for 2 and 24 h at 37 °C in the dark. After incubation, 50 μ L of this solution was mixed with 500 μ L of the reaction buffer and 10 μ L of Ellman's reagent (10 mM in reaction buffer) for 15 min in the dark, allowing its reaction with the thiol groups of GSH to yield fluorescent TNB. GSH in reaction buffer without GBMs films was used as the negative control, whereas GSH in H₂O₂ (1 mM) was used as the positive control of oxidation. GSH was quantified spectrophotometrically at 412 nm and the loss of GSH was calculated by the equation:

$$Loss\ of\ GSH\ \% = \frac{\text{absorbance of negative control} - \text{absorbance of sample}}{\text{absorbance of negative control}} \times 100\%$$

2.4. Protein adsorption to GBMs films

Protein adsorption to GBMs films was conducted using a pool of four donors of human plasma, to reduce the impact of plasma donor variability. A drop of 20 μ L of human plasma was added on top of GBMs films and incubated for 3 h at 37 °C under static conditions. After that, surfaces were gently rinsed thrice with *d*H₂O to remove nonbounded proteins. Adsorbed proteins were solubilized with 100 mM Tris pH 8.5, 1% sodium deoxycholate, 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM chloroacetamide, and protease inhibitors for 10 min at 95 °C at 1000 rpm (Thermomixer, Eppendorf). Each sample was processed for proteomics analysis following the solid-phase-enhanced sample-preparation (SP3) protocol as described in Hughes *et al.* [32]. Enzymatic digestion was performed with Trypsin/LysC (2 μ g) overnight at 37 °C at 1000 rpm. Peptide concentration was determined using the quantitative fluorometric peptide assay (Thermo Scientific). Five hundred nanograms of peptides from each condition were injected in the nanoLC-MS/MS for protein identification and quantification. This equipment is composed of an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) as described in detail in Supporting Information [33]. Briefly, a trap column of 50 cm by 75 μ m inner diameter EASY-Spray (ES803, PepMap RSLC, C18, 2 μ m, Thermo Scientific, Bremen, Germany) was used,

and separation was generated by mixing (A) 0.1% FA, and (B) 80% ACN with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B), and 10 min (hold 99% B). Raw data was processed using Proteome Discoverer 2.4.0.305 software (Thermo Scientific) and searched against the UniProt database for the *Homo sapiens* Proteome 2019_09. At least two unique peptides were considered for protein analysis. Two independent experiments were conducted.

2.5. Antibacterial performance of GBMs films

Four different clinically relevant bacterial strains were used to assess the influence of cell envelope structure (Gram⁺ and Gram[−]) and morphology (coccus and bacillus) (Table S2 and Figure S2 for more information on the different bacteria and cell envelope differences) on their antimicrobial susceptibility to GBMs surfaces.

2.5.1. Bacterial strains and culture conditions

Surfaces were challenged with *Staphylococcus epidermidis* (ATCC[®] 35984[™]), *Staphylococcus aureus* (ATCC[®] MRSA 33591[™]), *Pseudomonas aeruginosa* (ATCC[®] 27853[™]) and *Escherichia coli* (ATCC[®] 25922[™]), obtained from American Type Culture Collection (ATCC) (Table S2). Bacteria were grown in Trypticase Soy Agar (TSA, Merck) plates overnight at 37 °C. Two colonies were collected, inoculated into 5–10 mL of Trypticase Soy Broth (TSB, Merck) and cultured overnight at 37 °C under stirring at 150 rpm.

2.5.2. Antibacterial assay

Bacterial cultures were adjusted to 10^5 – 10^6 CFUs/mL in fresh TSB medium supplemented with 1% (v/v) human plasma, aiming to mimic the presence of proteins that naturally occurs in physiological conditions. GBMs films transferred to 24-well suspension plates were incubated with 300 μ L of bacterial suspension at 37 °C under static conditions. The remaining empty wells were filled with *d*H₂O to avoid evaporation. After 2 h or 24 h incubation, samples were rinsed twice with 0.085% sodium chloride (NaCl) and prepared either for fluorescence staining or SEM observation to evaluate the number, viability, and morphology of bacteria adherent to GBMs films.

Fluorescence staining using the LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (L13152, Invitrogen) determined viability of adherent bacteria. A mixture of SYTO9 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 480/500$ nm) green fluorescence and propidium iodide (PI) ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 490/635$ nm) red fluorescence nucleic acid stains was incubated with GBMs films for 15 min in the dark at room temperature (RT). Samples were rinsed with NaCl and transferred to uncoated 24-well μ -plates (82406, IBIDI, Germany) with the

surface facing the bottom of the plate. Fluorescence images were acquired using a high-content screening microscope IN Cell Analyzer 2000 (GE Healthcare, Nikon 40×/0.95 NA Plan Apo objective) using a CCD Camera (CoolSNAP K4). Three replicates of each GBMs film were evaluated, acquiring nine fields per sample (each field across a z-section of ~25 µm, with a z-step of 1 µm) with a total spanning area of 1.44 mm². Z-sections were projected on one plane using the IN Cell Investigator Developer Toolbox v.1.9.2 (GE Healthcare). Bacteria identification and segmentation was obtained through machine-learning customized algorithms generated for each wavelength using Ilastik [34]. The generated individual signal probability images were fed into CellProfiler (3.1.5) software [35], which returned the number of viable and dead bacteria.

SEM observation was conducted to reveal changes on bacteria morphology. Samples were fixed with a freshly prepared solution of 1.5% glutaraldehyde (VWR) in 0.14 M sodium cacodylate (Merck) buffer for 30 min at RT, rinsed twice with dH₂O and dehydrated in a graded ethanol series (50, 60, 70, 80, 90, and 99% for 10 min each). Hexamethyldisilazane (Sigma) was added to each sample and left to dry overnight. Samples were mounted, coated and visualized in the SEM as previously described in Section 2.3.4.

2.6. *In vitro* biocompatibility of GBMs films

In vitro biocompatibility of GBMs films was evaluated by direct contact assay towards human fibroblasts, according to the international standard ISO 10993-5;12 [36, 37].

2.6.1. Cell line and culture conditions

Human foreskin fibroblasts (HFF-1) cell line (ATCC[®] SCRC1041TM) was grown in Dulbecco's modified Eagle's medium (DMEM+, Gibco, Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) and 1% (v/v) penicillin/streptomycin (Pen/Strep, Biowest) at 37 °C, in a humidified atmosphere of 5% CO₂. Media was refreshed every 3 days.

2.6.2. Cell adhesion, proliferation and viability assay

GBMs films were transferred to 48-well TCPS plates; cells were seeded on top (10⁴ cells/well) and maintained for 1, 7, and 14 days under the above-mentioned conditions. Cells seeded directly on TCPS plates were used as positive control of viability, and cells seeded on TCPS and fixed with 70% (v/v) methanol (10 min) were used as negative control of viability.

After the above-mentioned time points, seeded GBMs films rinsed with PBS were incubated for 20 min at 37 °C with a mixture of Live/Dead Viability/Cytotoxicity kit (L-3224, Thermo Fisher Scientific), which is composed of Calcein AM (1 µM) and Ethidium-1 (4 µM), and Hoechst 33342 stain (5 µL/10

000 μ L PBS, Thermo Fisher Scientific). Samples were rinsed with PBS and transferred to uncoated 24-well μ -plate (82406, IBIDI) with the surface facing the bottom. Fluorescence images were obtained by IN Cell Analyzer 2000: Calcein AM ($\lambda_{\text{ex}}/\lambda_{\text{em}} = \sim 494/\sim 517$ nm) enters live cells and emits green fluorescence, Ethidium-1 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = \sim 528/\sim 617$ nm) enters cells with damaged membranes producing a bright red fluorescence and Hoechst 33342 ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 350/461$ nm) enters all cells and emits blue fluorescence when bound to dsDNA. Cell counting and viability was determined using IN Cell Investigator Developer Toolbox v.1.9.2 (GE Healthcare). Three replicates with 16 fields each were analyzed.

2.7. Hemocompatibility of GBMs films

GBMs films capacity to adhere blood cells (red blood cells, white blood cells, and platelets) was investigated as well as surfaces' hemolytic potential. Human whole blood and buffy coats were obtained from São João Hospital (Porto, Portugal) according to ethical approval established with INEB/i3S (Protocol 90/19).

2.7.1. Whole blood assay

GBMs films were transferred to a bovine serum albumin (BSA) blocked plate (1% (w/v) BSA in PBS, 1 h, 37 °C) to avoid platelet activation by the plate polystyrene. Human whole blood from a healthy volunteer was added and incubated at 37 °C for 1 h in a horizontal shaker at 70 rpm. Samples were rinsed with PBS to remove weakly attached cells and prepared for observation of cell adhesion and activation either through SEM or fluorescence microscopy using the glutaraldehyde-induced fluorescence technique (GIFT).

For SEM observation, samples were prepared as aforementioned in Section 2.5.2.

For GIFT, adherent cells were fixed with a freshly prepared solution of 1.5% glutaraldehyde (Merck) in PBS buffer for 30 min at RT. After fixation, samples were rinsed three times with PBS and placed on microscope slides; one drop of fluorescent mounting medium (Vectashield-H-1000; Vector Laboratories) was added and a coverslip placed on top of each sample. Blood components were visualized with an inverted fluorescence microscope (Axiovert 200M, Zeiss, Germany) using FITC filter ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 490/525$ nm).

2.7.2. Hemolysis assay

Red blood cells were isolated from human buffy coats by differential centrifugation at 400g during 30 min in the presence of Histopaque-1077 (Sigma). Supernatant (containing plasma components and mononuclear cells) was discarded, and the lower layer (containing the red blood cells) was rinsed three times with PBS. Purified red blood cells were diluted to a concentration of 2×10^8 cells/mL and 300

μL added to GBMs films and incubated during 3 h at 37 °C in a 48-well plate. Plates were centrifuged at 4000 rpm for 15 min and 100 μL of supernatant collected from each well to black 96-well plates. The absorbance was read at 380, 415, and 450 nm using a microplate reader spectrophotometer. The amount of released hemoglobin (Hb) was calculated using the following equation:

$$Hb \text{ (mg.dL}^{-1}\text{)} = \frac{(2 \times A_{415} - (A_{380} + A_{450})) \times 1000}{E}$$

where A_{415} , A_{380} , and A_{450} represent the absorbance values of samples at 415 nm, 380 nm, and 450 nm, respectively, and E the molar absorptivity of oxyhemoglobin at 415 nm.

The hemolytic potential of the different films was determined according to the following equation:

$$\% \text{ Hemolysis} = \frac{Hb \text{ sample}}{total \text{ Hb}}$$

where total Hb is the correspondent value for 100% hemolysis with Triton 1%. The assay was performed with five replicates.

3. Results

3.1. Characterization of GBMs powders

Gt and FLG were oxidized into GO and FLGO, respectively, by modified Hummers method. Successful oxidation was confirmed by the increase in the content of oxygen-containing groups from 8.3% (Gt [38]) and 3.5% (FLG) to 30–32% (GO and FLGO), demonstrated by XPS elemental composition analysis and C 1s high-resolution spectra (Figure S3, C 1s top panel). This was corroborated by the deconvolution of O 1s high-resolution spectra (Figure S3, O 1s top panel), which revealed the presence of high amounts of aliphatic C–O bonds in oxidized materials as oppose to FLG, where aromatic C–O bonds appear, associated with the C=C bonds of the graphene characteristic rings. TEM images show exfoliated GO flakes with lateral size around 4 μm and high transparency, suggesting a high exfoliation [39], and confirm FLG and FLGO lateral size of around 5 μm (according to manufacturer information), with visible higher number of layers (Figure S3, bottom panel).

3.2. Characterization of GBMs films

Regarding chemical functionalities, GBMs films present atomic percentages (Figure 1, top panel) similar to the starting powder materials: GO and FLGO surfaces showed a similar degree of oxidation (~32%), while FLG presented a low but existing percentage of oxygen (3.5%). rGO film spectrum confirms GO reduction, showing a weaker oxygen peak intensity and lowering of the oxygen-containing groups to 13.5%. On the surface of all films, epoxy (C–O–C) was the most prevalent group,

associated with basal planes of the sheets, in detriment of carboxylic ($\text{O}-\text{C}=\text{O}$) and carbonyl ($\text{C}=\text{O}$) groups, linked with the edges of the sheets [17]. Thermal reduction structurally repaired rGO due to increased interaction between sp^2 clusters that were once isolated by oxygen-functionalized and defective areas (sp^3 carbon clusters). This is demonstrated by an increase on the percentage of the $\text{C}=\text{C}$ bonds in the C 1s high-resolution spectra of rGO film [40], which consequently led to the appearance of aromatic C–O bonds on the O 1s spectrum (Figure S4), indicative of the re-establishment of these bonds (also present in high amounts in FLG films). Total removal of oxygen-containing groups would however be unlikely through thermal reduction due to high and diverse binding energies of the groups: hydroxyl ($\text{C}-\text{OH}$) groups attached to basal planes of sheets are easily eliminated when comparing to these groups at the edges, as well as $\text{O}-\text{C}=\text{O}$ groups; $\text{C}=\text{O}$ groups present high stability, with no particular binding energy being found for $\text{C}-\text{O}-\text{C}$ groups [41]. rGO presents indeed higher amounts of $\text{C}-\text{O}-\text{C}$ and $\text{C}=\text{O}$ groups as opposing to $\text{O}-\text{C}=\text{O}$ and $\text{C}-\text{OH}$.

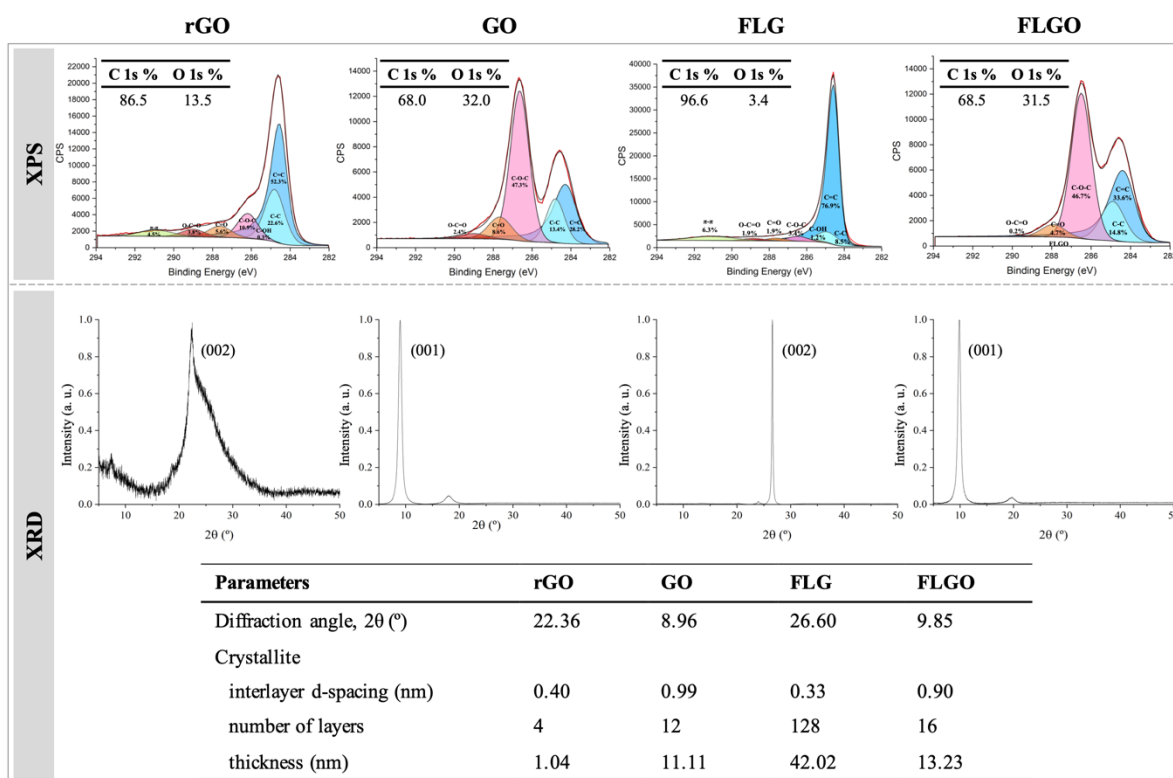


Figure 1. Chemical characterization of GBMs films: atomic percentage of carbon and oxygen, and exposed functional groups extracted from XPS spectra (top panel); XRD patterns of GBMs films (001 and 002 corresponding to characteristic lattice reflections) and table with extracted values of diffraction angle (2θ), and crystallite interlayer d -spacing, number of layers, and thickness (bottom panel). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

GBMs films' crystalline arrangement was verified by XRD patterns (Figure 1, bottom panel). rGO film presents a broad diffraction peak at $2\theta = 22.36^\circ$, corresponding to the reflection plane of 002 and a d -spacing of 0.40 nm. Its broadness is indicative of a rGO film poorly ordered along the c-axis stacking direction [42] and means that, as quantitatively depicted in Figure 1 (bottom panel), the sheets within the film possess a small number of layers and reduced crystallite thickness. This can be the result of the reduction process since it causes not only oxygen groups decomposition but also exfoliation of the sheets [40]; some rearrangement of the carbon atoms in the basal plane can also occur due to availability of thermal energy [43, 44]. GO film possesses a diffraction peak at $2\theta = 8.96^\circ$ as the result of a drastic increase of lattice d -spacing to 0.99 nm. The sharpness of the peak suggests a long-ranged highly ordered structure along c-axis [39]; this may be derivative of aligned intact graphene domains. The increase in interlayer d -spacing indicates the presence of oxygen functionalities and water molecules attached to GO sheets, thus corroborating the oxidation of the surface. The FLG surface's intense and sharp peak at $2\theta = 26.60^\circ$ indicates a more compact and layered structure but with a smaller d -spacing, demonstrative of a less oxidized structure and more similar to the one observed in graphite (e.g., $2\theta = 26.50^\circ$, the reflection 002 corresponding to a d -spacing of 0.34 nm [45]). A shift of 2θ to the left from 26.60° to 9.85° translates an increase in the d -spacing from 0.33 to 0.90 nm but still with an ordered structure along the c-axis, confirming the oxidized character of the FLGO film. The presence of still some reflection at 20° in the GO and FLGO is indicative of some remnant graphitic structures in the sample.

Surface morphology and topography of the films were characterized by SEM and AFM (Figure 2). SEM images show that FLG film has platelets randomly deposited on the surface with more abundant and sharper edges exposed. Oxidized surfaces, GO and FLGO, exhibit a compact and continuous structure, where oxygen-containing groups seem to interact with each other through strong hydrogen bonds. This causes adjacent sheets to “fuse” together, forming randomly distributed wrinkles and ridged protrusions (asperities). The reduction process of GO films into rGO caused the formation of macroscopic voids, associated with the expansion of CO and CO₂ gases between sheets [40]. The presence of these voids was translated into a higher thickness ($\sim 81.6 \mu\text{m}$) of the rGO film comparing to the remaining surfaces (35–45 μm) measured using a thickness gauge.

Both SEM and AFM ($50 \times 50 \mu\text{m}$) images reveal that films have different surface microroughness, being higher for rGO films ($S_a \cong 1.72 \mu\text{m}$), followed by FLGO ($S_a \cong 1.17 \mu\text{m}$), GO ($S_a \cong 0.76 \mu\text{m}$), and FLG ($S_a \cong 0.70 \mu\text{m}$) films (Figure 2 and Figure S5).

Smaller scanning areas of the films can better provide insights on surface interaction with biological systems, especially bacteria that are smaller than mammalian cells; therefore, $5 \times 5 \mu\text{m}$ AFM images were acquired to infer on surface nanotopography (Figure 2, bottom panel). Although the morphology of the wrinkles is similar between rGO, GO, and FLGO films, the surface of rGO film is different from the oxidized ones. It presents low peak density (S_{ds}) with a large height variation, which is evidenced

by the higher root-mean-square roughness ($S_q = 0.43 \pm 0.13 \mu\text{m}$) and the large values of the maximum height values (S_z and S_{10z}); the surface is also composed of valleys with plateau areas ($S_{sk} < 0$) and slightly wider peaks (reduced S_{ku} but still $S_{ku} > 3$). GO film surface has a high peak density (S_{ds}), mostly with sharp and narrow peaks ($S_{ku} > 3$) rather than with holes ($S_{sk} \gg 0$), but with a smaller distance between peaks and valleys (smaller S_z and S_{10z}). FLG and FLGO films present a more homogeneous surface regarding height distribution (closer to a Gaussian distribution ($S_{sk} \sim 0$)), with FLG presenting a high peak density (S_{ds}) and a high height variation (S_z and S_{10z}). The two explored scanning areas (Figure 2 and Figure S5) reveal the presence of different roughness levels within the same sample, although the same trend is observed in both profile graphs: microroughness for rGO and FLG surfaces versus nanoroughness for GO and FLGO. This reasons with GO and FLGO sheets being per se atomically rough because of their nonplanar arrangement due to defects and functionalization [40].

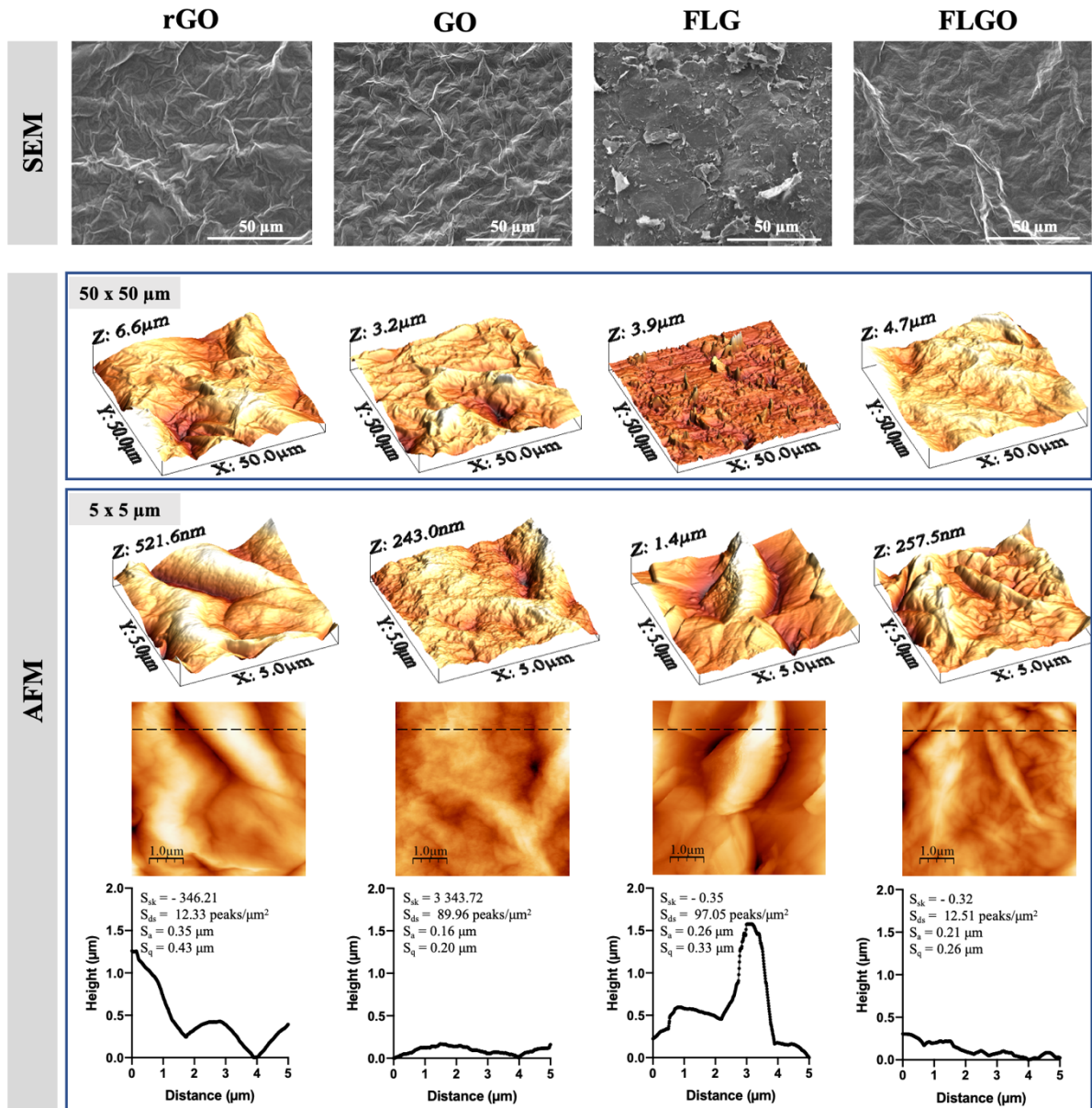


Figure 2. Morphological and topographical characterization of GBMs films: morphology obtained by SEM (scale bar = 50 μm) and 3D and 2D images (scale bar = 10 μm), roughness parameters, and height profiles from two scanning areas (50 × 50 μm and 5 × 5 μm) provided by AFM. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Concerning surface wettability, rGO films present a more hydrophobic nature (~83°) than FLG films (~56°), while GO and FLGO surfaces are the most hydrophilic with water contact angles of 33° and 42°, respectively (Figure 3).

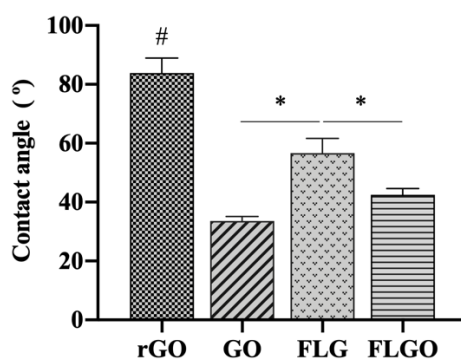


Figure 3. Contact angle measurements of GBMs films. #, *: statistically significantly different from all other samples (#) and from each other (*) (one-way ANOVA, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

The concentration of oxidized groups, distribution of pristine and oxidized regions in oxidized materials, and morphology of sheets and their arrangement on the surface, can affect not only surface chemistry and topography but also wettability of the surface [46]. In GO and FLGO films, the high concentration of oxygen-containing groups increase the interaction between the water droplet and the surface, conferring it a more hydrophilic character (within the range for GO reported in literature, 20–60° [26, 46]). In rGO films, the rougher, more heterogeneous surface with larger height variation ($S_{10z} \cong 8.14 \mu\text{m}$; $50 \times 50 \mu\text{m}$ AFM images) may reduce the interaction of the droplet with the surface, increasing the contact angle value, despite the presence of some oxygen-containing groups (13.5%). Both FLG and rGO films' less hydrophilic character reinforce the idea suggested by Wei *et al.* [46] through molecular dynamics simulations that surface topography might be more relevant to wettability than surface chemistry.

Figure 4 shows the oxidative potential of GBMs films after 2 and 24 h incubation with GSH determined by Ellman's assay. After 2 h, GO films showed the highest ability to oxidize GSH (86.9%), followed by FLGO (50.5%) and FLG (44.0%), while rGO has reduced capacity. The oxidative potential increased over time with almost all GSH being oxidized after 24 h; rGO films are the exception, reaching only 26.3% loss of GSH. The increased content of oxygen-containing groups on oxidized films emerge as a potential source for glutathione oxidation [8], associated with the presence of more reactive defect sites that adsorb more O_2 and water molecules, which increase surface oxides and hydroxyl radicals formation. On FLG films, GSH oxidation might be related with their conductive nature, acting as an electron acceptor [9].

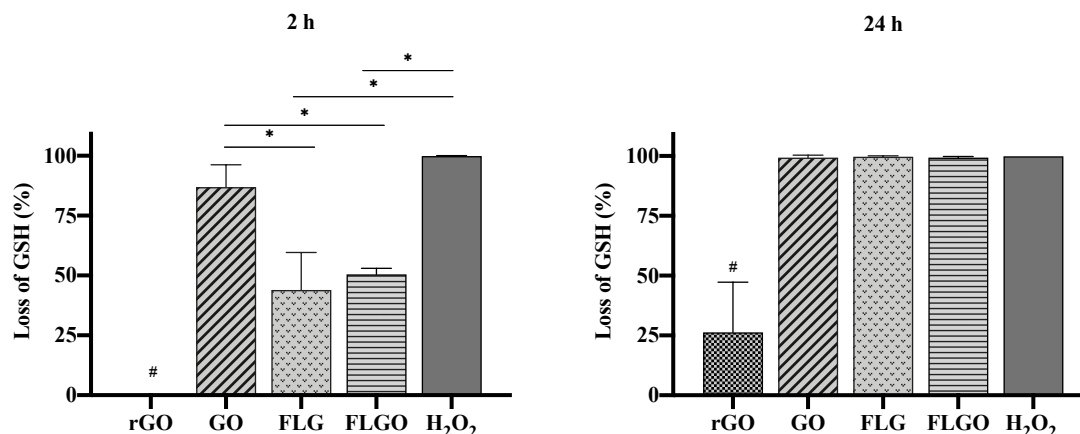


Figure 4. Oxidation of glutathione (GSH) by GBMs films: loss of GSH (%) after 2 and 24 h incubation with the surfaces determined by Ellman's assay. GSH in H₂O₂ (1 mM) was used as the positive control of oxidation. #, *: statistically significantly different from all other samples (#) and from each other (*) (one-way ANOVA, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

O-phthalaldehyde (OPT) fluorometric assay (Figure S6) corroborated these results. Both assays confirm that GO, FLG, and FLGO films have the ability to oxidize glutathione (GSH), an endogenous antioxidant that plays a crucial role in reducing reactive oxygen species (ROS). It states therefore their potential to induce oxidative stress toward biological systems either by an oxygen-mediated pathway (direct formation of ROS) or by depletion of cellular antioxidants.

3.3. Protein adsorption to GBMs films

After digestion of the proteins present in plasma and adsorbed to the GBMs films, the amount of peptide per microliter was quantified. A total of 2259.6 ng/ μ L was found in plasma, 506.5 ng/ μ L in rGO films, 235.8 ng/ μ L in GO films, 975.3 ng/ μ L in FLG films, and 204.5 ng/ μ L in FLGO films. In the same line, Figure 5 shows that higher amounts of adsorbed proteins can be found in reduced or nonoxidized films (26.5% on rGO and 49.3% on FLG), contrary to oxidized surfaces that adsorb less proteins (8.3% on GO and 7.6% on FLGO), highlighting the impact of GBMs oxidation degree on protein adsorption. The most abundant proteins in human plasma and also in GBMs films are albumin (~75%), immunoglobulins (~8%), serotransferrin (~4%), and fibrinogen (~1–2%, depending on the surfaces), despite these percentages being proportional to the total amount of adsorbed proteins. When considering proteins with an abundance higher than 1%, differences can be found between the GBMs films. Proteins such as complement C3 and α -2-macroglobulin adsorb more to oxidized surfaces, while proteins as α -1-antitrypsin and haptoglobin are found preferentially on reduced and nonoxidized films.

Apolipoprotein A-I (the major component of high-density lipoprotein (HDL)) is identified only in oxidized surfaces without being within the 1% most abundant proteins in the plasma.

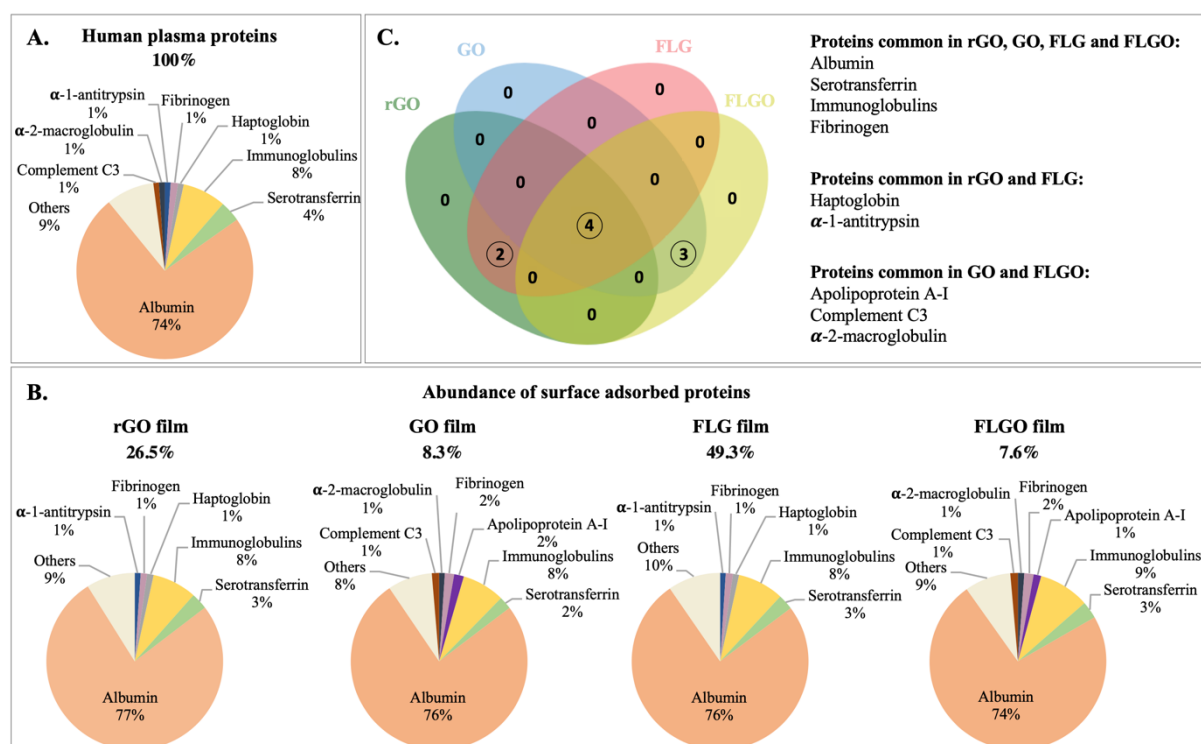


Figure 5. Human plasma protein adsorption to GBMs films. (A) Proteins present in human plasma and (B) adsorbed on GBMs films with respective percentages of total protein adsorption and most abundant proteins. (C) Venn diagram of adsorbed proteins with an abundance higher than 1% in GBMs films. Proteins were identified by nanoLC-MS/MS. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Table S3 presents a list of all proteins identified on GBMs films and human plasma, together with their abundances. The corresponding heat map (Figure S7) shows that for every single protein, the amount of adsorbed proteins is always lower in oxidized films. It also emphasizes (as seen in Figure 5) that even though some proteins are within the 1% most abundant on a surface, when considering the total amount of adsorbed proteins on each film, their abundance may be in fact smaller than on other surfaces where it does not get to the 1% more abundant. This is the case of α -2-macroglobulin and complement C3, for example, which belong to the 1% more abundant proteins in oxidized films but are present in higher amounts on rGO and FLG than in these surfaces. The heat map also shows protein distribution and a clear overall predominance of adsorbed proteins on FLG and rGO (darker colors). Indeed, these surfaces have more proteins with high abundances ($>1 \times 10^8$) than oxidized surfaces.

The composition of this protein layer represents the final biological identity of the GBMs films that will eventually interact with the different biological systems. Proteins adsorbed in higher amounts are

mainly related with complement activation (complement factor H, complement C3), coagulation (fibrinogen, antithrombin-III), lipid metabolism (apolipoproteins: apo A-I, apo B-100), and protease inhibitors (α -1-antitrypsin and α -2-macroglobulin).

3.4. Antibacterial performance of GBMs films

Since several bacterial features have been linked with different susceptibility to GBMs, GBMs films' antibacterial performance was assessed toward *S. epidermidis* (coccus/Gram+), *S. aureus* (coccus/Gram+), *P. aeruginosa* (bacillus/Gram–), and *E. coli* (bacillus/Gram–) (Figure 6), a panel of clinically relevant implant infection-associated bacteria (Table S2).

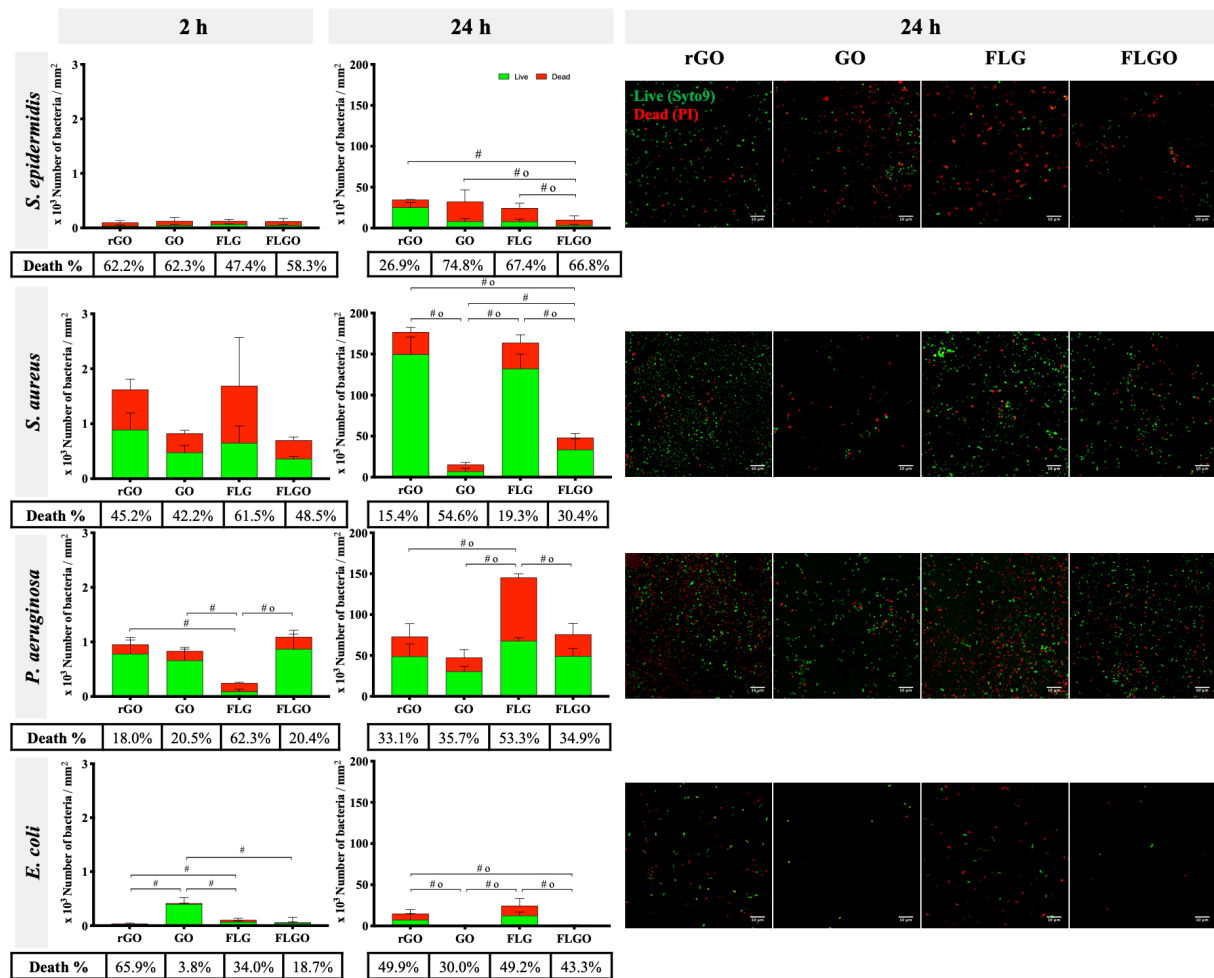


Figure 6. Antibacterial performance of GBMs films toward *S. epidermidis*, *S. aureus*, *P. aeruginosa*, and *E. coli* determined by live/dead staining. Graphs represent the number of live/dead bacteria adherent on the surfaces after 2 and 24 h. Adherent bacteria after 24 h are shown in fluorescence images obtained by IN Cell Analyzer 2000 (scale bar = 10 μ m). Zoomed graphs and images of 2 h are shown in Figure S8. #, o: statistically significant differences between samples regarding total number (#) and dead (o) adherent bacteria (Kruskal–Wallis, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

In general, adherent bacteria increase over time in all surfaces (from 2 to 24 h, Figure 6 and Figure S8) with a higher number of bacterial adhesion associated with *S. aureus* and *P. aeruginosa* (up to 150×10^3 bacteria/mm²). In the first 2 h, coccus-shaped Gram+ bacteria seem to be more susceptible to the action of surfaces, depicting a higher percentage of bacterial death, as demonstrated by loss of membrane integrity in the live/dead assay (e.g. for GO surfaces, ~62% and ~42% death for *S. epidermidis* and *S. aureus* versus ~27% and ~4% for *P. aeruginosa* and *E. coli*). After 24 h, *S. epidermidis* still present the highest death percentage across all surfaces, reaching 75% in GO films, and with FLGO films significantly adhering less bacteria than any other surface, while still presenting a death percentage of 66.8%. The thickness of the platelets within the films does not significantly influence the bactericidal effect; the only exception occurred with *S. epidermidis*, where there are significantly different death percentages between oxidized surfaces produced with different thickness (GO and FLGO). The impact of the degree of oxidation and roughness is more evident, as depicted in Figure 6: FLGO surfaces adhere significantly less bacteria than FLG surfaces, similar to GO surfaces that adhere significantly less *E. coli* and *S. aureus* than rGO films. This general antiadhesive action of oxidized surfaces (GO and FLGO) is possibly due to the presence of higher percentages of oxygen-containing groups, lower amounts of adsorbed proteins, and/or surface nanotopography. Contrarily, less or nonoxidized rougher surfaces, as rGO and FLG films, adsorb higher amounts of proteins, which is also translated into high adhesion levels and reduced number of dead bacteria. The exception is *P. aeruginosa* that adhere and die similarly in rGO, GO, and FLGO films; the sharp edges of FLG seem therefore to be acting preferably toward rod-shaped bigger bacteria, although FLG ability to induce oxidative stress, as demonstrated through GSH oxidation, can be contributing.

The surface effect is also dependent on the type of bacteria (Figure 6) with different outcomes being observed for bacteria with similar membrane features (*S. epidermidis* and *S. aureus*, and *E. coli* and *P. aeruginosa*, Table S2 and Figure S2): *S. epidermidis* (coccus/Gram+) are more susceptible to all surfaces with low adhesion levels already after 2 h and an accentuated bactericidal effect after 24 h with the exception of rGO surfaces; *S. aureus* (coccus/Gram+) on the other hand show higher adhesion levels, particularly in less or nonoxidized surfaces, but are more susceptible to the killing effect after 2 h opposing to 24 h; *P. aeruginosa* (bacillus/Gram-) also present high adhesion levels, despite lower than *S. aureus*, but are more affected by the surfaces, showing higher killing susceptibility in rGO and FLG after 24 h; *E. coli* (bacillus/Gram-) present low adhesion levels in all surfaces both after 2 and 24 h and increased death percentages after 24 h, especially for oxidized surfaces.

Morphology of adherent bacteria obtained by SEM after 24 h reveals both signs of biofilm formation and bactericidal effects of some of the surfaces (Figure S9). Biofilm formation cues are more evident in surfaces with high number of adhered and viable bacteria such as rGO surfaces toward *S. aureus* and FLG surfaces toward *E. coli*. Comparison with other studies [47] suggests the presence of extracellular extrusions and a viscous polysaccharide layer (slime), associated with cell–cell network formation and cell–surface attachment, and capsular exopolysaccharide colanic acid particles that are present on

bacteria and on the surface. Bactericidal effects are also noticed and corroborate the live/dead assay: holes, membrane corrugation, and deformation (cell surface roughness suggests it has been damaged) with some bacteria showing a collapsed structure. Regarding distribution, bacteria do not seem to attach preferentially; they adhere on basal areas and ridges as well as on sharp edges in the case of FLG surfaces.

Overall, although adherent bacteria increase over time (Figure 6 and Figure S8), oxidized surfaces (GO and FLGO) present low adhesion levels in opposition to rougher and less or nonoxidized surfaces (rGO and FLG). Higher oxidation degree, lower protein adsorption, and smoother (with nanotopography) surfaces seem therefore advantageous to prevent bacterial adhesion.

3.5. Biocompatibility of GBMs films

GBMs films effect toward human mammalian cells was explored regarding morphology, proliferation, and viability of adherent fibroblasts (Figure 7).

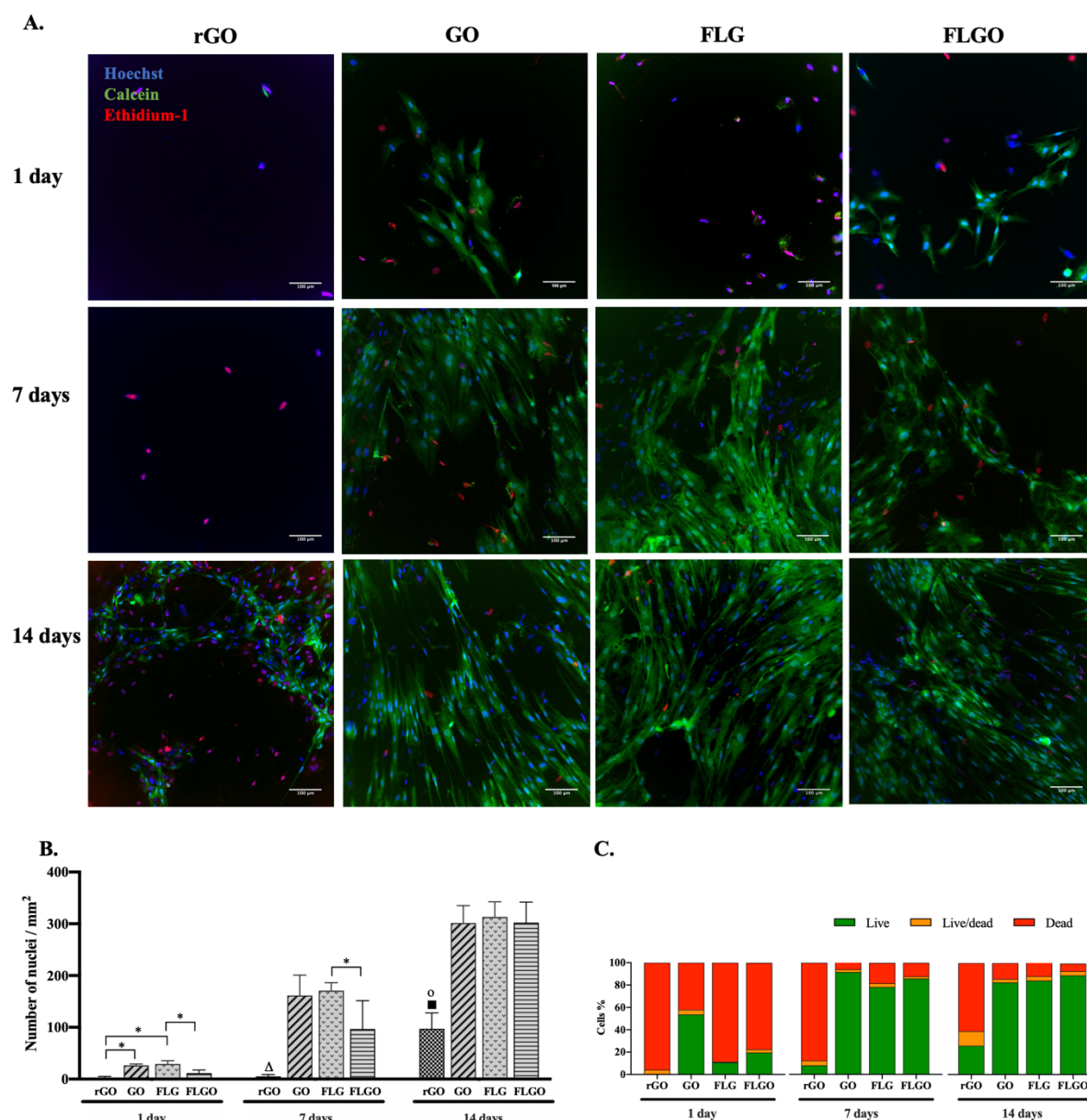


Figure 7. Morphology, proliferation, and viability of HFF-1 fibroblast cell line on GBMs films after 1, 7, and 14 days. (A) Fluorescence images of cells stained with Hoechst (blue, total), Calcein (green, viable), and Ethidium-1 (red, dead), obtained by IN Cell Analyzer 2000 (objective 20 $\times$, scale bar = 100 μ m). (B) Cell proliferation as the total number of nuclei per mm² and (C) cell viability, both parameters calculated based on the stained images. Statistically significant differences (*) between each other, (Δ) from all other materials at day 7, ($\blacksquare$) from rGO at day 1 and 7, ($\circ$) from all other materials at day 14; FLG, GO, and FLGO present statistically differences over time (one-way ANOVA, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

After 1 day of culture, and similar to TCPS culture controls (Figure S10), cells exhibit a well-defined cytoskeleton, spindle-like morphology, and elongated shape on GO and FLGO films, adhering significantly more than to rGO films, where fewer adherent cells with a round morphology are observed

(Figure 7). Over 14 days, fibroblasts proliferated, with the number of cells increasing significantly and reaching up to 300 cells/mm² demonstrating films cytocompatibility and capability to enhance cell proliferation; rGO films are the exception, taking longer to recover and only reaching ~100 cells/mm². rGO surfaces, despite having some oxygen-containing groups, present a rougher surface (S_q of 0.43 μm) with pronounced negative S_{sk} (meaning higher height variations) with high plateaus and sharp deep valleys. Because of this topography and hydrophobic character, cells might have more difficulty during initial attachment.

3.6. Hemocompatibility of GBMs films

GBMs films hemolytic potential and effect on blood components (red blood cells, white blood cells, and platelets) adhesion were investigated (Figure 8).

All surfaces present a hemolysis percentage smaller than 5%, which according to ISO 10993-4, classifies them as hemocompatible. Incubation with human whole blood led to adhesion of blood components onto all surfaces; specifically, red and white blood cells and platelets adhered in higher numbers to rGO and FLG surfaces (more hydrophobic) as opposed to oxidized surfaces (GO and FLGO), implying the advantage of a higher oxidation degree on inhibiting/reducing blood components adhesion by increasing surface hydrophilicity and reducing protein adsorption. Platelets on rGO and FLG films are also in more activated states, spread or fully spread with membrane disintegration and granules distribution across the cytoplasm (Figure 8 and Figure S11). Some small clots are also observed: agglomerates of platelets in different activation states (from nonactivated to spread and dendritic) with extended filamentous actin rich filopodia and lamellipodia [48] within fibrin networks (Figure 8 and Figure S11). Red blood cells on FLG present different morphologies; however, given the low hemolysis potential of these surfaces, this effect may be associated with an hypertonic state or alterations in membrane protein distribution that may be associated with oxidative stress caused by the surface or with response to white blood cells activation [49].

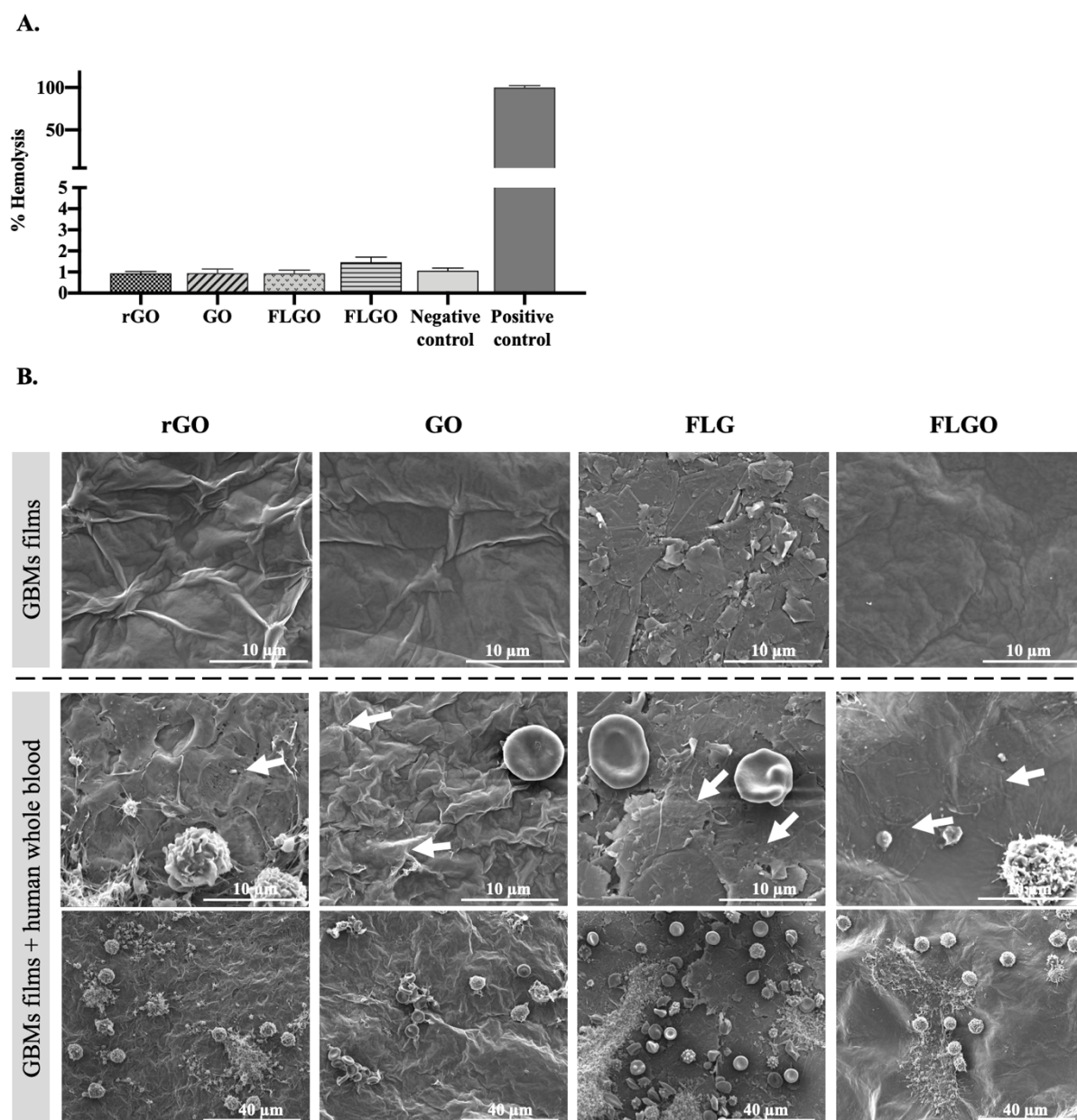


Figure 8. Hemocompatibility profile of GBMs films. (A) Hemolysis assay after incubation with red blood cells for 3 h and (B) blood components adhesion to GBMs films after whole blood incubation for 1 h under dynamic conditions. Images obtained by SEM (scale bar = 10 and 40 μm). White arrows = adhered and activated platelets. Standard surfaces used as controls on the whole blood assay are shown in Figure S12. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

4. Discussion

In this study, we demonstrate that GBMs inherent characteristics, specifically oxidation degree and thickness, do confer distinctive features to the surfaces, in terms of chemistry, roughness, and wettability. These surface features affect protein adsorption, having more impact on bacteria and blood

components than on human cells such as fibroblasts. How this happens is unraveled by the connection between surface physicochemical characterization and *in vitro* biological tests.

Oxidation degree is the major factor causing differences on the surface by crucially impacting chemistry and dictating the way sheets organize at the surface. This arrangement consequently impacts roughness and wettability, which largely affect protein adsorption. Oxidized films, produced with graphene oxide (GO) and few-layer graphene oxide (FLGO), are smoother and hydrophilic, leading to the adsorption of lower amounts of proteins. On the other hand, less or nonoxidized films, produced with reduced GO (rGO) and few-layer G (FLG), are rougher and hydrophobic, leading to increased protein adsorption. This tendency regarding the impact of surface roughness and wettability has been also reported for other surfaces [27, 50]. Surface chemistry can be masked by protein adsorption, contrarily to topography that has been shown not to change, with surfaces presenting the same topographical cues [27].

Bacteria, mainly due to their smaller size in comparison to mammalian cells and blood components, seem to be particularly affected by surface features in terms of adhesion and viability, although differences in their membrane composition and organization can also impact this susceptibility (Figure S2).

Similar to protein adsorption, in films produced with oxidized materials (GO and FLGO), the increased number of oxygen-containing groups combined with reduced roughness conferred the surface a hydrophilic character, causing a reduction in bacterial adhesion. In particular, carboxylic functional groups can be contributing to the reduction in adhesion of negatively charged bacteria (due to electrostatic repulsions) [51]. Hydroxyl and epoxy groups, present in higher amounts, could be contributing to the bactericidal effect of these surfaces through an increase in the surface oxides and hydroxyl radicals formation. This occurs mainly due to the presence of more reactive defect sites that adsorb more O₂ and water molecules, as suggested by the higher oxidative potential of these surfaces after incubation with glutathione (GSH) [17]. Since these surfaces are in principle insulating and lack exposure of sharp edges, oxidative stress seems to be the major pathway leading to bacterial death, either by depletion of cellular antioxidants from the environment or by an oxygen-mediated process that induces ROS formation, which will consequently cause GSH oxidation [52]. Even though the influence of surface features on bacterial adhesion has been established, the impact of roughness has not yet reached a consensus [21, 53, 54]. In oxidized surfaces, lower surface roughness reduces the contact points with bacteria, particularly in GO surfaces, where the peak density is high and lateral peak-to-peak distances are small. In combination with a hydrophilic character and low protein adsorption, these surfaces show lower bacterial adhesion. Although evidence from Perreault *et al.* [52] suggests an increase in antibacterial activity when GO sheet area decreases, no significant differences were observed between GO and FLGO surfaces (GO has a smaller lateral size); differences were only found in the adhesion of some bacteria.

On the other hand, in rGO and FLG films the increased roughness with more valleys and wider peaks allows a match with bacteria size, as has been reported to occur [20, 54, 55]. The combination between more available surface for direct contact and a more hydrophobic character increases protein adsorption, and similarly bacterial adhesion. The impact on bacteria viability will however depend on the material and on their oxidative potential [55]. rGO poor antibacterial activity may be related with its lower ability to induce oxidation of GSH, and consequently induce oxidative stress. In the case of FLG films, the increased peak density and exposed sharp edges may underlie the higher death percentage observed in bacteria with the exception of *S. aureus*. The latter is smaller and can fit within the rugosity of the surface, while bigger bacteria will directly contact with sharp edges, as suggested by Pham *et al.* [54]. Even though a perpendicular alignment relative to the surface has been reported as necessary for facilitating penetration of the bacterial membrane [14], random deposition of the sharp edges can still be effective [54], as highlighted in our study in FLG surfaces. A reduction on the FLG lateral size could have been advantageous, according to Pham *et al.*, who reports a higher bactericidal effect when using graphene with shorter exposed edge lengths [54]. Bacterial death was demonstrated by a disruption of the cell integrity, illustrated by the live/dead assay and bacteria morphology on SEM images. In addition to the effect of the sharp edges, the higher oxidative potential of FLG films in the presence of GSH reveals a possible combination of mechanisms of action toward bacteria. Our study also supports the theory that the existence of exposed sharp edges is not mandatory for antimicrobial activity [25, 26], expressed by the dead bacteria on oxidized and rGO surfaces, which lack exposure of sharp edges. As aforementioned, these surfaces may be causing bacteria death through an oxidative stress pathway rather than by physical piercing of the membrane. On rGO surfaces, the reduction of epoxy groups can lower the oxidative potential of the surfaces, which associated with increased roughness translate into higher bacterial adhesion and lower death.

The high number of adherent bacteria in rGO and FLG surfaces and the reduced bactericidal effect, particularly on rGO films, can be associated with the accumulation of dead bacteria that can mask the GBMs effects and provide a source of nutrient for live bacteria [14, 26, 56]. As aforementioned, the high amount of adsorbed plasma proteins can also be covering the surface of the films hampering their direct effect on bacteria. These events are reduced in GO and FLGO surfaces given their higher hydrophilicity and less adsorption of proteins and hydrophobic components, such as some bioadhesive molecules produced by bacteria [57]. The use of high initial inoculum, simulating extreme conditions, can also influence the results. In a real scenario, however, the initial bacterial inoculum should be lower than on these experiments, thus possibly allowing the remaining live bacteria on the surfaces to be eradicated by host immunity.

Equally affecting the antimicrobial outcomes are the features of the contacting microorganisms [26]. These include bacteria physiological state [58], membrane hydrophobicity and charge [55], bioadhesive hydrophilic and hydrophobic proteins released from bacteria [8], and bacteria wall composition. Gram+ bacteria have thicker peptidoglycan layers (20–80 nm) than Gram– species (7–8 nm) (Figure S2),

generating more rigid, stress-bearing outer surfaces [59], even though peptidoglycan thickness within species can vary. The lower tendency of *S. epidermidis* to adhere GBMs films could be associated with the lower affinity of this bacteria to plasma proteins present in the incubation media when comparing with *S. aureus* and *P. aeruginosa* [60, 61]. These last bacteria are coagulase positive, possessing the capacity to produce fibrin fibers from fibrinogen, and thus establish a stronger interaction with plasma proteins (even when using 1% plasma in culture medium [62]) and consequently with the interface, which promotes their protection and viability. The nonpolar nature of FLG surface and hydrophobic regions of the lipid external membrane of the Gram- bacteria may justify the increased bacterial effect toward *P. aeruginosa* and *E. coli*; the same may be happening with the nonpolar areas of rGO.

Biocompatibility studies suggest that GBMs oxidation degree has a minor effect on cells adhesion and viability, since fibroblasts adhere and survive both in FLG and FLGO surfaces. On the other hand, surface roughness, ranging from 0.16–0.35 μm (with S_z going from 1.00 to 2.24 μm), seems to contribute majorly and in a scale-dependent way. Although bacteria can fit within some of these features due to their smaller size (from 1 to 5 μm), fibroblasts (with around 20 μm) may be too large to directly sense these effects, except when roughness increases (S_a 1.72 μm) and the surface becomes hydrophobic, as in the case of rGO films, where cells adhere and proliferate less. In some studies, surfaces with larger roughness (GO-wrinkled substrates, 2–24 μm) significantly induce cell alignment and cause changes in cell morphology (compactness, rigidity) [13]; in other works, cells preferentially adhere to areas with nanowrinkles over flat areas, even though the former are reduced (rGO) and the latter oxidized (GO)[15], stressing out once again the impact of roughness over chemistry toward cells. rGO films are also thicker than the remaining surfaces, which according to supporting literature can change surface roughness and mechanical properties [63], providing also mechanical cues to cells [64]. Surface wettability also affects cell proliferation, considering that the initial phase of attachment involves physicochemical linkages between cells and surfaces through ionic forces or indirectly through an alteration in the adsorption of conditioning molecules (such as growth factors and proteins) [65]. Generally, cell attachment is usually more efficient on hydrophilic than on hydrophobic surfaces [66] with negatively charged surfaces inducing cell adhesion through electrostatic interactions. High hydrophobicity and lower protein adsorption can be behind the effect observed in rGO films, while in the case of FLG surfaces the higher amount of proteins on the surface might be contributing to making it more hydrophilic, thus improving its biological performance. In fact, after 14 days all surfaces supported fibroblasts growth independently of the oxidation degree and thickness. Some studies report negligible influence of chemistry [17, 67] and wettability [66] on cell adhesion, highlighting the effect of roughness [68], especially if the differences between the surfaces are not significant [67], which can be the case between GO and FLGO surfaces. Cells' viability has been reported even when sharp edges are exposed if their height ranges from 60-100 nm [14], either due to different membrane composition (Figure S2), size difference between cells and bacteria, or production of extracellular matrix, validating

our results for cellular viability in FLG films. Considering cell viability, oxidative stress induced by GBMs films has a lower impact towards cells than bacteria.

Blood components are also a major biological system in our body. Although platelets and red and white blood cells adhered to all films, rougher and hydrophobic surfaces, as rGO and FLG, follow the pattern of promoting adhesion. This correlates with higher protein adsorption on these surfaces, particularly of proteins associated with complement activation (complement factor H, complement C3) or coagulation (fibrinogen, antithrombin-III). Even though oxidized surfaces also adsorb some of these proteins, their significant lower amount reduces their impact on the overall cellular adhesion. Thampi *et al.* [16] also observed that hydrophilic surfaces with similar roughness to ours reduced platelet adhesion, expressing a combinatory effect between roughness and hydrophilicity. Although some studies with GO in suspension report red blood cell morphology alterations and hemolysis (up to 96%) in a concentration-dependent manner [69], our group previously confirmed lack of harmful effects with FLG and particularly FLGO [28]. We herein demonstrated that surfaces produced using the same FLG and FLGO show no signs of hemolysis (below 5% in all cases). FLG films show adhered red blood cells with altered morphology with the spherical shape of spherocytocytes with pointed spicules, despite being considered as normally found in the bloodstream of healthy people.

Contrarily to oxidation degree, GBMs platelet thickness has a smaller impact on surface features. When comparing GO and FLGO films, which have similar surface chemistry and wettability, small differences are observed in roughness; this translates into small differences regarding protein adsorption, bacteria, and mammalian cells response. Since the surfaces that have more distinct roughness also have different chemical composition, the impact of surface roughness cannot be completely isolated.

Apart from the GBMs intrinsic features, surface properties depend on the production method of GBMs-containing surfaces, as recently reviewed by our group [1]. However, surfaces produced by dip [70] and spray coating [16], for instance, have been reported to have similar surface morphologies to the ones we obtained by vacuum filtration when using GO and FLGO, with randomly exposed deposited platelets forming a compact, wrinkled film. This suggests that similar conclusions can be drawn regarding the biological behavior of surfaces produced by other techniques but with comparable features, thus increasing the significance of these conclusions.

5. Conclusions

The intrinsic features of GBMs, namely thickness and oxidation degree have different effects on the surface properties of films produced by vacuum filtration. GBMs thickness loses impact when these are integrated as a surface, even though films produced with GO and FLGO, which have similar chemical composition, presented different roughness. On the other hand, oxidation degree of GBMs platelets altered significantly the surface chemistry, roughness, and wettability of the formed films. The higher

content of oxygen groups, hydrophilic character and nanoroughness of GO and FLGO films, reduce the adsorption of plasma proteins. This, associated with their high ability to induce oxidative stress, leads to reduced bacterial adhesion and viability and low adhesion of blood platelets and white and red blood cells. rGO and FLG lead to the formation of rougher and more hydrophobic films with less oxygen-containing groups. Such surfaces promote high protein adsorption and increased bacterial and blood components adhesion. Mammalian cells, larger in size, are less sensitive to the chemistry of the surface, behaving similarly in oxidized and nonoxidized surfaces, except when roughness increases and the surface becomes hydrophobic, as in the case of rGO films, where cells adhere and proliferate less.

This fundamental and systematic study provided insights on the importance of the features of the GBMs (mainly oxidation) that are used in the development of GBMs-integrating surfaces, and on the surface parameters that consequently influence the interaction with proteins and the three major biological systems. This knowledge can be used to guide the development of GBMs-integrated surfaces when a specific medical application is envisaged.

6. Acknowledgements

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Supplementary Information

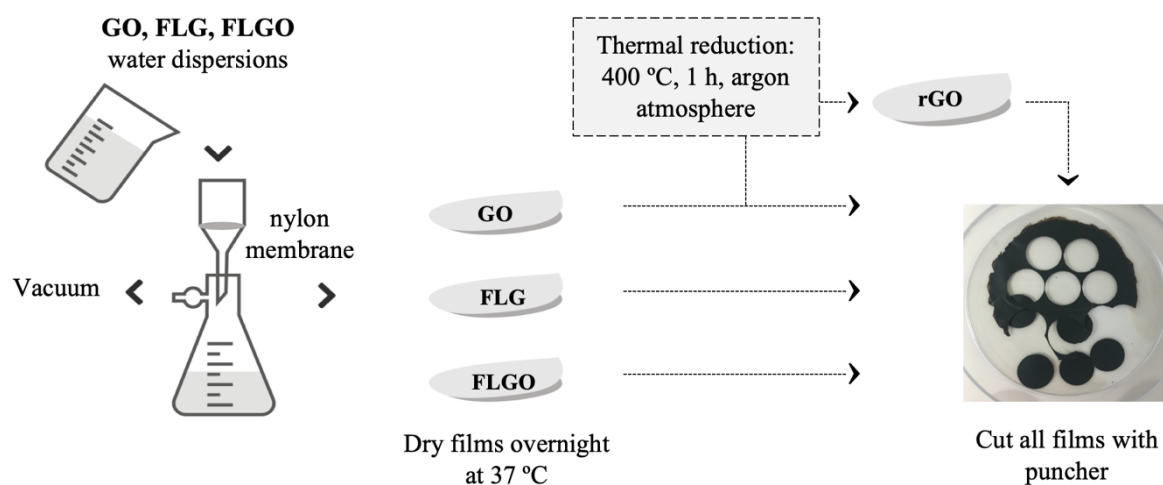
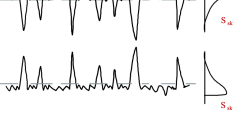
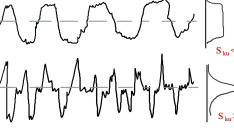
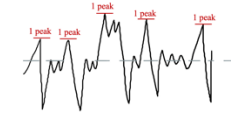
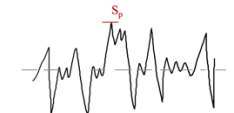
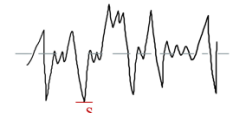
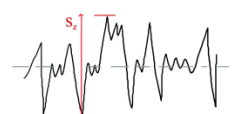
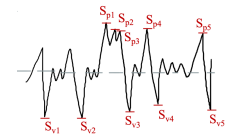


Figure S1. Experimental setup for the production of GBMs films through vacuum filtration. GO, FLG and FLGO aqueous suspensions were used to produce films, with rGO being obtained afterwards from thermal reduction of GO film. GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide, rGO = reduced graphene oxide.

Table S1. Description of the amplitude – height distribution (S_a , S_q , S_{sk} , S_{ku} , S_p , S_v , S_z , S_{10z}) – and spacing (S_{ds}) roughness parameters obtained by atomic force microscopy (AFM) and extracted using SPIP software.

Surface parameters	Meaning		Formula	Observations
Roughness average (S_a , μm)	Average absolute deviation of the roughness irregularities from the mean line.		$\frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} z(x_k, y_l) $	It makes no distinction between peaks and valleys; depends only on the average profile of heights, and so S_a can be the same for surfaces with different roughness profiles.
Root-mean-square (RMS) (S_q , μm)	RMS height of the surface roughness. Standard deviation of the height distribution.		$\sqrt{\frac{1}{MN} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} [z(x_k, y_l)]^2}$	More sensitive to large deviations from the mean plane (peaks and valleys) than S_a . Statistical measurement. Most commonly reported measurement of surface roughness.
Surface Skewness (S_{sk})	Describes the asymmetry of the height distribution histogram.		$\frac{1}{MNS_q^3} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} [z(x_k, y_l)]^3$	$S_{sk} < 0 \rightarrow$ surface with holes; plateaus with sharp deep valleys; $S_{sk} = 0 \rightarrow$ symmetry regarding peaks and valleys (with as many peaks as valleys), Gaussian height distribution; $S_{sk} > 0 \rightarrow$ flat surface with peaks; wide valleys with narrow sharp peaks. S_{sk} can be used to distinguish between two profiles having the same S_a or S_q values but with different shapes.
Surface Kurtosis (S_{ku})	Describes the “peakedness/sharpness” of the surface topography.		$\frac{1}{MNS_q^4} \sum_{k=0}^{M-1} \sum_{l=0}^{N-1} [z(x_k, y_l)]^4$	$S_{ku} < 3 \rightarrow$ broader height distributions, few peaks and low valleys; $S_{ku} = 3 \rightarrow$ surface with Gaussian height distribution; $S_{ku} > 3 \rightarrow$ narrow height distribution, pronounced peakedness of profile, has relatively many high peaks.
Density of Summits (S_{ds} , $1/\mu\text{m}^2$)	Number of local maximums (summits) per area.		$\frac{\text{number of local maximums}}{(M-1)(N-1)\delta x \delta y}$	Calculation of S_{ds} can vary depending on the peaks/summits definition. Commonly, a peak is defined as any point that is higher than its nearest neighbors, i.e. the two points on either side in a profile, and the surrounding four or eight points on a surface. Several approaches for peak definition can be found in Crawford, R. J. <i>et al.</i> [2]. Sensitive to noise peaks, and so should be interpreted carefully.
Maximum peak height (S_p , μm)	Largest peak height value.			Provide an idea about the maximum and minimum height values (extremes) on the surface.
Maximum valley depth (S_v , μm)	Largest valley depth value.			
Peak-peak height (S_z , μm)	Height difference between the highest and lowest pixel in the image.		$S_z = z_{\max} - z_{\min}$	where $z_{\max}$ and $z_{\min}$ are the height of the highest local maximum and lowest local minimum, respectively.
Ten-point height (S_{10z} , μm)	Average height of the five highest local maximums plus the average height of the five lowest local minimums.		$\frac{\sum_{i=1}^5 z_{pi} + \sum_{i=1}^5 z_{vi} }{5}$	where z_{pi} and z_{vi} are the height of the i^{th} highest local maximums and the i^{th} lowest local minimums, respectively. More sensitive to occasional high peaks or deep valleys than S_a .

Note: information based on the user's guide of SPIP software, Gadelmawla, E. S. *et al.* [1] and Crawford, R. J. *et al.* [2].
 MN = size image (area of the scanned image).

Table S2. Bacterial strain features.

Bacterial strain	Wall	Morphology	Size (μm)	Coagulase	Other features
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<i>S. epidermidis</i> (ATCC 35984)	Gram +	coccus	diameter 1-2	-	• Nonmotile • Forms grape-like clusters • Biofilm formation • Less adhesion to plasma proteins
<i>S. aureus</i> (ATCC MRSA 33591)	Gram +	coccus	diameter ~ 0.8	+	• Nonmotile • Adheres to extracellular matrix and plasma proteins
<i>P. aeruginosa</i> (ATCC 27853)	Gram -	bacillus	width: 0.5-1.0 length: 1-5	+	• Motile (polar flagella) • Water-soluble pigments production • Biofilm formation
<i>E. coli</i> (ATCC 25922)	Gram -	bacillus	width: ~ 0.5 length: ~ 2	-	• Motile (flagellated) • Biofilm formation in rich medium

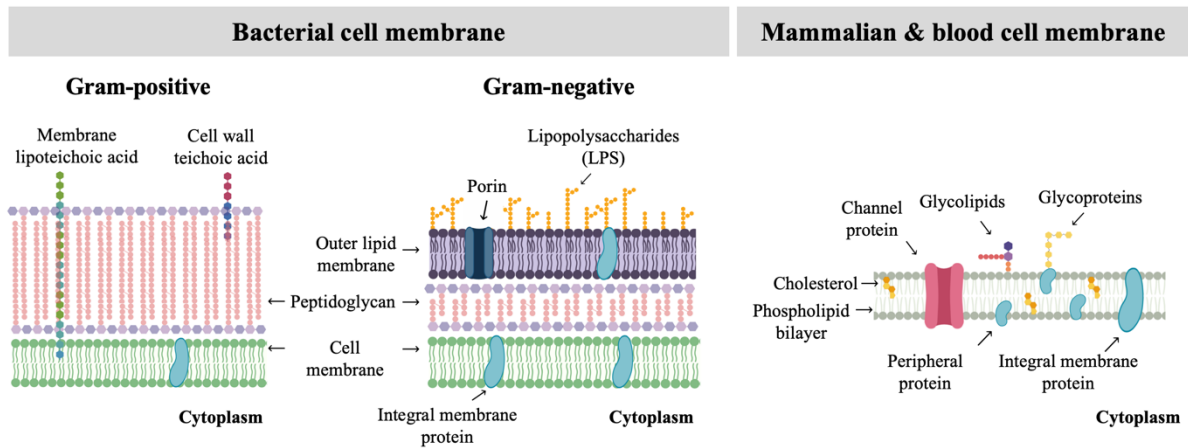


Figure S2. Bacterial (Gram+ and Gram-) and mammalian & blood cell membrane composition. *Note: mammalian and blood cell (platelets, leukocytes and erythrocytes) membranes will vary mostly depending on the phospholipids, glycoproteins and receptors exposed.* Created with BioRender.com.

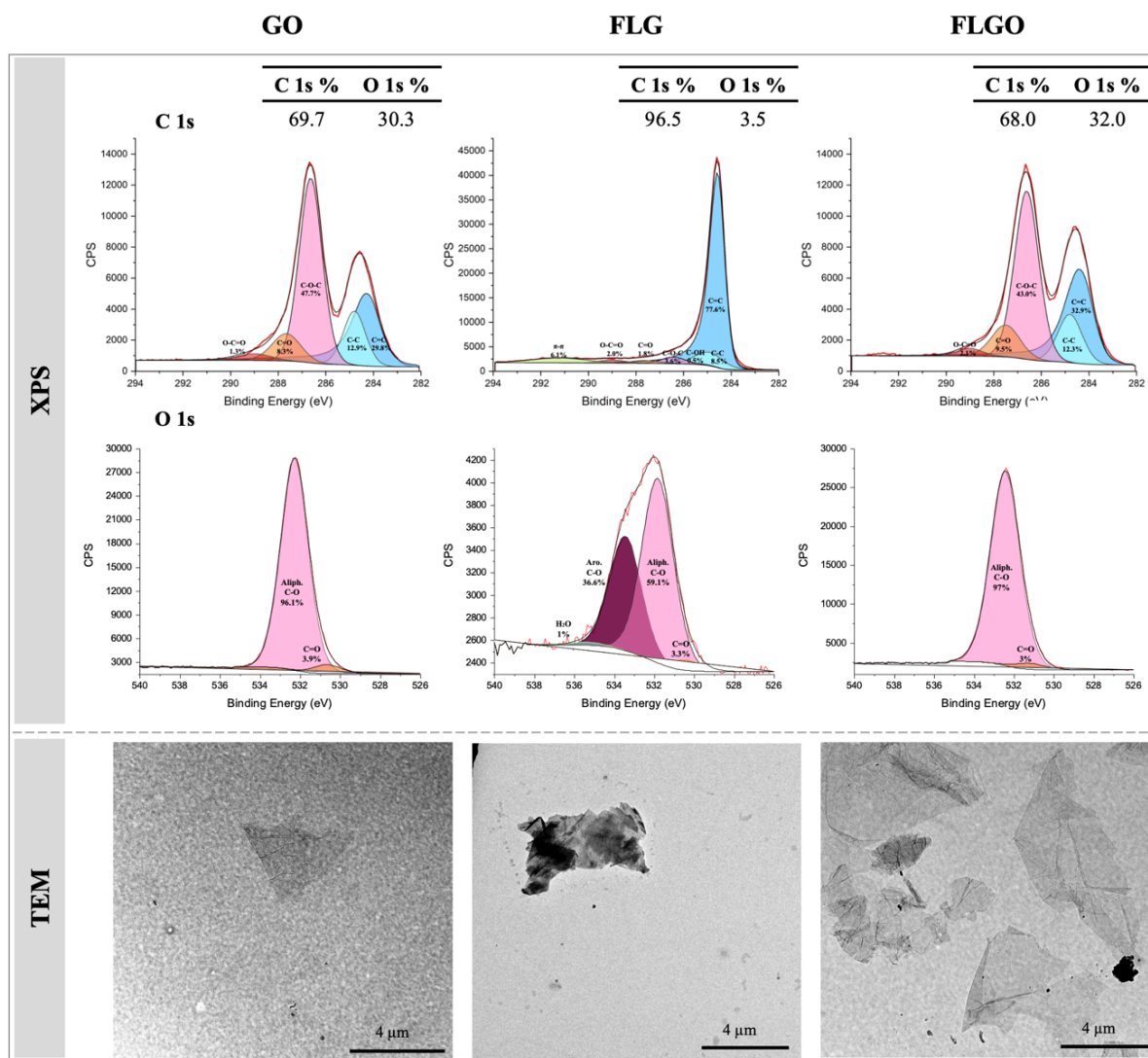


Figure S3. Chemical characterization and morphology of graphene-based materials (GBMs) powders: XPS atomic composition, and C 1s and O 1s high-resolution spectra (top panel), and TEM images (bottom panel, scale bar = 40 μm). GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

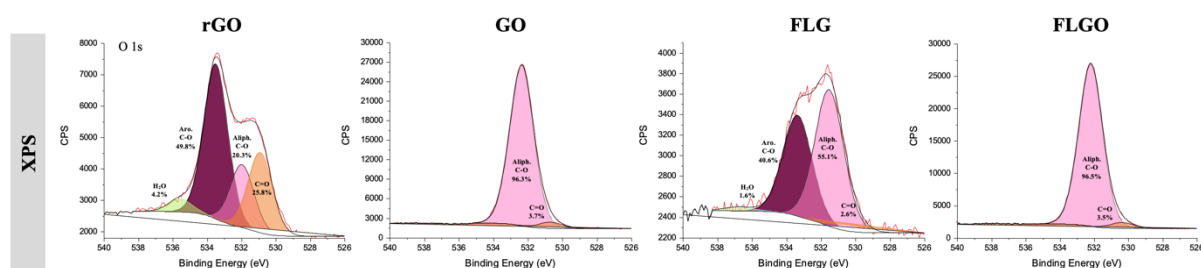


Figure S4. XPS O 1s high-resolution spectra of graphene-based materials (GBMs) films. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

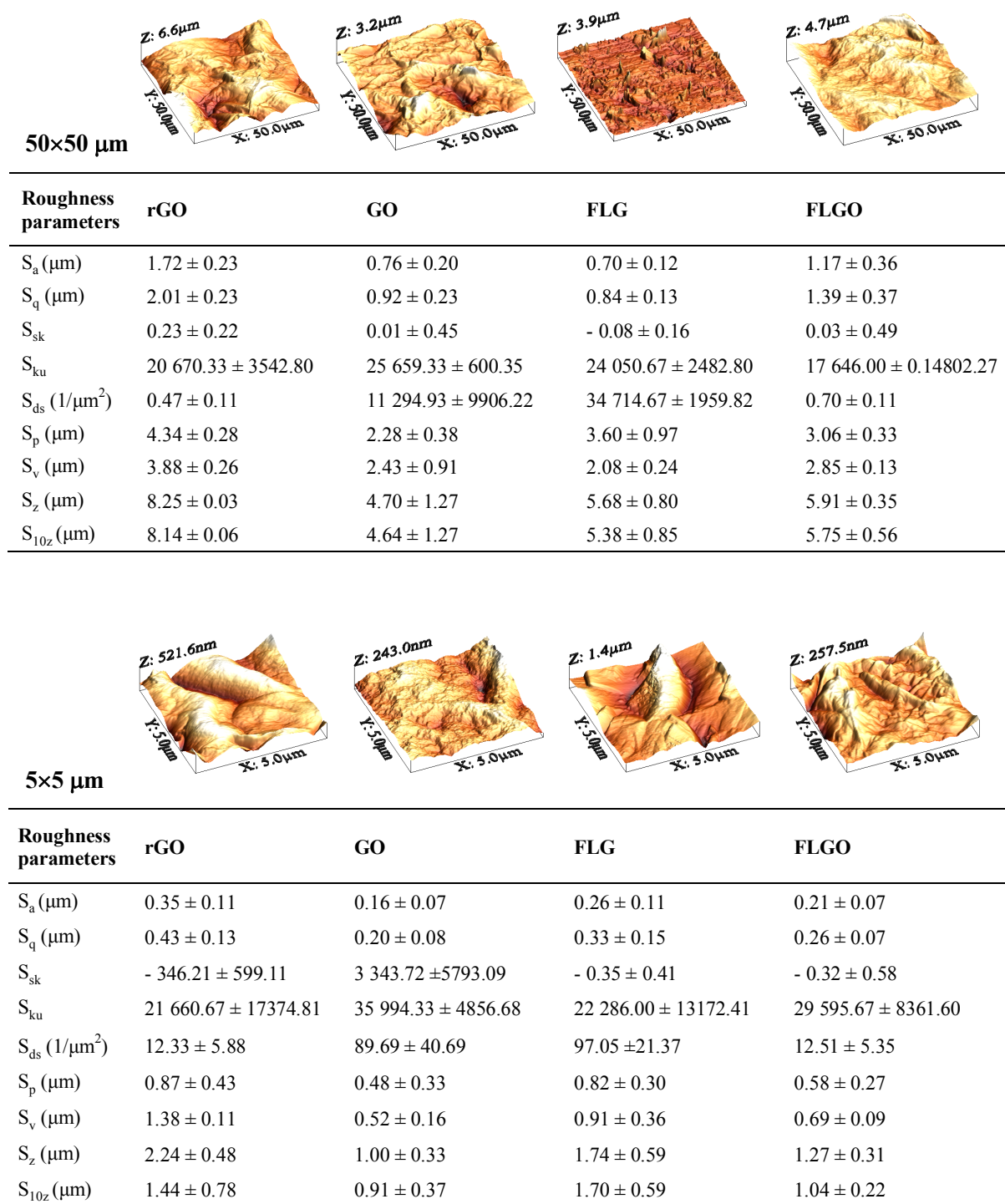


Figure S5. Topographical analysis of GBMs films over scanning areas of 50 x 50 μm and 5 x 5 μm : 3D images of the surfaces and AFM roughness parameters. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Oxidative potential of GBMs films: O-phthalaldehyde (OPT) fluorometric assay

Oxidative potential of GBMs films was also determined by O-phthalaldehyde (OPT) fluorometric assay [3]. GBMs films were incubated with 300 μL of GSH (0.8 mM, Sigma) in reaction buffer (0.1 M sodium bicarbonate (pH 8), containing 1 mM EDTA) for 2 h and 24 h at 37 $^{\circ}\text{C}$ in the dark. After incubation, 50 μL of this solution was mixed with 950 μL of the reaction buffer and 50 μL of OPT (0.1% (w/v) in methanol) for 15 min in the dark. GSH in reaction buffer without GBMs films was used as the negative control, whereas GSH in H_2O_2 (1 mM) was used as the positive control of oxidation. Fluorescence was measured using an excitation wavelength of 350 nm and an emission wavelength of 420 nm. The loss of GSH was calculated by the equation:

$$\text{Loss of GSH \%} = \frac{\text{absorbance of negative control} - \text{absorbance of sample}}{\text{absorbance of negative control}} \times 100\%$$

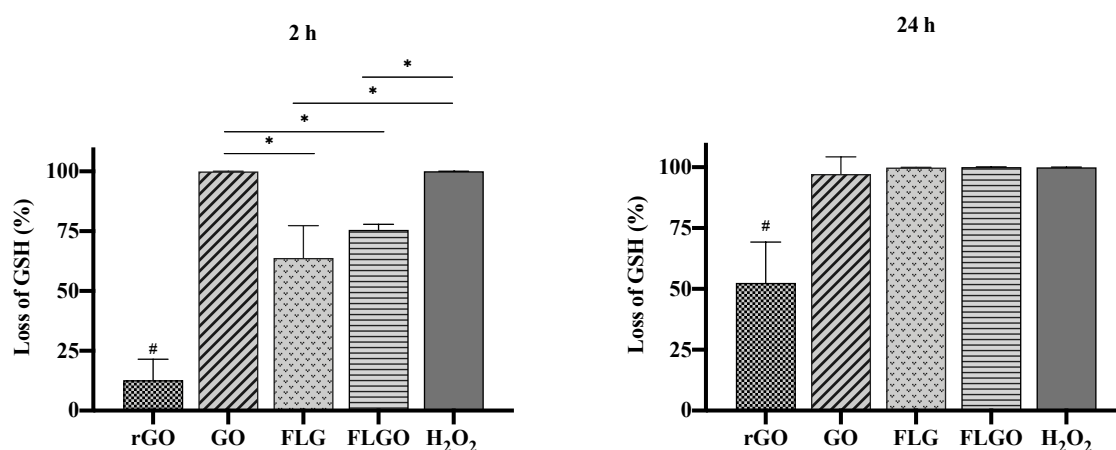


Figure S6. Oxidation of glutathione (GSH) by GBMs films: loss of GSH (%) after 2 h and 24 h incubation with the surfaces determined by o-phthalaldehyde (OPT) fluorometric assay. GSH in H_2O_2 (1 mM) was used as the positive control of oxidation. #, * - statistically significantly different from all other samples (#) and from each other (*) (one-way ANOVA, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Protein adsorption to GBMs films: detailed materials and methods

Proteins were solubilized with 100 mM Tris pH 8.5, 1% sodium deoxycholate, 10 mM tris(2-carboxyethyl)phosphine (TCEP), 40 mM chloroacetamide and protease inhibitors for 10 minutes at 95 $^{\circ}\text{C}$ at 1000 rpm (Thermomixer, Eppendorf). Each sample was processed for proteomics analysis following the solid-phase-enhanced sample-preparation (SP3) protocol as described in Hughes *et al.* [4]. Enzymatic digestion was performed with Trypsin/LysC (2 micrograms) overnight at 37 $^{\circ}\text{C}$ at 1000 rpm. Protein identification and quantitation was performed by nanoLC-MS/MS. This equipment is composed by an Ultimate 3000 liquid chromatography system coupled to a Q-Exactive Hybrid Quadrupole-

Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). Samples were loaded onto a trapping cartridge (Acclaim PepMap C18 100Å, 5 mm x 300 µm i.d., 160454, Thermo Scientific) in a mobile phase of 2% ACN, 0.1% FA at 10 µL/min. After 3 min loading, the trap column was switched in-line to a 50 cm by 75µm inner diameter EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Bremen, Germany) at 300 nL/min. Separation was generated by mixing A: 0.1% FA, and B: 80% ACN, with the following gradient: 5 min (2.5% B to 10% B), 120 min (10% B to 30% B), 20 min (30% B to 50% B), 5 min (50% B to 99% B) and 10 min (hold 99% B). Subsequently, the column was equilibrated with 2.5% B for 17 min. Data acquisition was controlled by Xcalibur 4.0 and Tune 2.9 software (Thermo Scientific, Bremen, Germany).

The mass spectrometer was operated in data-dependent (dd) positive acquisition mode alternating between a full scan (m/z 380-1580) and subsequent HCD MS/MS of the 10 most intense peaks from full scan (normalized collision energy of 27%). ESI spray voltage was 1.9 kV. Global settings: use lock masses best (m/z 445.12003), lock mass injection Full MS, chrom. peak width (FWHM) 15s. Full scan settings: 70k resolution (m/z 200), AGC target 3e6, maximum injection time 120 ms. dd settings: minimum AGC target 8e3, intensity threshold 7.3e4, charge exclusion: unassigned, 1, 8, >8, peptide match preferred, exclude isotopes on, dynamic exclusion 45s. MS2 settings: microscans 1, resolution 35k (m/z 200), AGC target 2e5, maximum injection time 110 ms, isolation window 2.0 m/z , isolation offset 0.0 m/z , spectrum data type profile.

The raw data was processed using Proteome Discoverer 2.4.0.305 software (Thermo Scientific) and searched against the UniProt database for the *Homo sapiens* Proteome 2019_09. The Sequest HT search engine was used to identify tryptic peptides. The ion mass tolerance was 10 ppm for precursor ions and 0.02 Da for fragment ions. Maximum allowed missing cleavage sites was set 2. Cysteine carbamidomethylation was defined as constant modification. Methionine oxidation and protein N-terminus acetylation were defined as variable modifications. Peptide confidence was set to high. The processing node Percolator was enabled with the following settings: maximum delta Cn 0.05; decoy database search target FDR 1%, validation based on q-value. Protein label free quantitation was performed with the Minora feature detector node at the processing step. Precursor ions quantification was performing at the processing step with the following parameters: unique plus razor peptides were used for quantification, precursor abundance was based on intensity, normalization was based on total peptide amount.

Table S3. List of proteins identified on the surface of GBMs films and in human plasma, identified by MS: protein ID, protein name, molecular weight (MW, kDa) and abundances of adsorbed proteins on GBMs films (corresponding to the average of abundances on the surfaces on both experiments) and present in plasma. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Protein ID	Protein name	MW (kDa)	Abundance of adsorbed proteins on GBMs films				Plasma
			rGO	GO	FLG	FLGO	
P60709	Actin, cytoplasmic I	41.7	4.34E+06	3.54E+06	9.74E+06	4.91E+06	2.20E+07
P43652	Afamin	69	1.94E+07	5.64E+06	4.37E+07	4.23E+06	8.78E+07
P02763	Alpha-1-acid glycoprotein 1	23.5	1.27E+07	2.77E+06	3.34E+07	1.61E+06	5.93E+07
P19652	Alpha-1-acid glycoprotein 2	23.6	4.79E+06	4.76E+05	9.56E+06	4.03E+05	3.54E+07
P01011	Alpha-1-antichymotrypsin	47.6	3.42E+07	1.27E+07	8.51E+07	1.50E+07	1.56E+08
P01009	Alpha-1-antitrypsin	46.7	6.07E+08	1.70E+08	1.06E+09	1.33E+08	2.25E+09
P04217	Alpha-1B-glycoprotein	54.2	1.74E+07	4.11E+06	4.05E+07	4.72E+06	1.57E+08
P08697	Alpha-2-antiplasmin	54.5	1.83E+07	6.05E+06	4.76E+07	4.85E+06	1.12E+08
P02765	Alpha-2-HS-glycoprotein	39.3	5.97E+07	1.51E+07	1.67E+08	1.16E+07	4.87E+08
P01023	Alpha-2-macroglobulin	163.2	4.75E+08	1.82E+08	9.72E+08	1.87E+08	2.28E+09
P01019	Angiotensinogen	53.1	6.73E+06	1.58E+06	1.39E+07	1.39E+06	3.56E+07
P01008	Antithrombin-III	52.6	5.10E+07	9.31E+06	8.38E+07	1.13E+07	2.99E+08
P02647	Apolipoprotein A-I	30.8	4.55E+08	3.12E+08	9.64E+08	2.22E+08	1.26E+09
V9GYM3	Apolipoprotein A-II	14.9	3.16E+07	4.61E+07	9.85E+07	2.16E+07	1.01E+08
P06727	Apolipoprotein A-IV	45.4	2.75E+07	1.34E+07	4.12E+07	1.02E+07	6.14E+07
P04114	Apolipoprotein B-100	515.3	1.06E+08	3.92E+07	2.02E+08	4.00E+07	4.88E+08
P02654	Apolipoprotein C-I	9.3	6.51E+06	4.63E+06	1.30E+07	3.17E+06	5.45E+07
P02655	Apolipoprotein C-II	11.3	5.68E+05	3.21E+05	9.38E+05	1.25E+05	1.14E+06
P02656	Apolipoprotein C-III	10.8	6.26E+06	3.10E+06	1.59E+07	2.60E+06	2.98E+07
C9JF17	Apolipoprotein D (Fragment)	24.1	2.34E+07	5.77E+06	7.46E+07	4.15E+06	1.56E+08
P02649	Apolipoprotein E	36.1	1.01E+07	5.47E+06	1.92E+07	7.04E+06	5.77E+07
O14791	Apolipoprotein L1	43.9	1.26E+07	4.69E+06	2.20E+07	4.64E+06	8.06E+07
O95445	Apolipoprotein M	21.2	6.53E+05	2.46E+05	1.28E+06	8.48E+04	1.54E+05
O75882	Attractin	158.4	1.04E+06	3.35E+05	1.88E+06	3.67E+05	9.06E+06
P02749	Beta-2-glycoprotein 1	38.3	6.21E+07	1.19E+07	9.45E+07	1.11E+07	2.33E+08
P04003	C4b-binding protein alpha chain	67	1.74E+07	2.71E+06	2.36E+07	3.39E+06	4.84E+07
Q961Y4	Carboxypeptidase B2	48.4	2.31E+06	3.25E+05	4.78E+06	3.95E+05	6.95E+06
P22792	Carboxypeptidase N subunit 2	60.5	2.11E+06	3.84E+05	5.34E+06	6.16E+05	1.78E+07
P49747	Cartilage oligomeric matrix protein	82.8	1.39E+05	4.99E+04	8.53E+04	3.13E+04	4.46E+05
O43866	CD5 antigen-like	38.1	6.43E+06	1.54E+06	9.19E+06	2.88E+06	5.83E+07
B4E1Z4	cDNA FLJ55673, highly similar to Complement factor B	140.9	4.82E+07	1.80E+07	8.36E+07	1.35E+07	2.17E+08
P00450	Ceruloplasmin	122.1	8.36E+07	2.30E+07	1.42E+08	2.54E+07	3.81E+08
P10909	Clusterin	52.5	2.64E+07	1.36E+07	9.70E+07	1.13E+07	2.03E+08
P00740	Coagulation factor IX	51.7	5.99E+05	3.09E+05	1.92E+06	3.40E+05	6.30E+06
P00742	Coagulation factor X	54.7	3.08E+05	1.90E+05	6.81E+05	3.37E+05	2.56E+06
P03951	Coagulation factor XI	70.1	1.05E+05	1.45E+05	6.00E+04	4.42E+05	1.06E+06
P00748	Coagulation factor XII	67.7	1.76E+06	3.37E+05	2.44E+06	6.89E+05	1.58E+07
P00488	Coagulation factor XIII A chain	83.2	8.23E+05	1.02E+05	1.23E+06	1.47E+05	2.20E+06
P05160	Coagulation factor XIII B chain	75.5	5.41E+05	2.35E+05	7.56E+05	2.59E+05	3.09E+06
P02745	Complement C1q subcomponent subunit A	26	7.82E+05	9.04E+04	1.30E+06	1.07E+05	2.87E+06
P02746	Complement C1q subcomponent subunit B	26.7	5.51E+05	1.12E+06	1.59E+06	1.21E+06	6.53E+06
P02747	Complement C1q subcomponent subunit C	25.8	3.00E+06	1.41E+06	6.63E+06	1.60E+06	2.07E+07
B4DPQ0	Complement C1r subcomponent	81.8	4.11E+06	1.10E+06	4.71E+06	1.29E+06	1.36E+07
P09871	Complement C1s subcomponent	76.6	6.99E+06	2.89E+06	1.69E+07	1.72E+06	2.24E+07
P01024	Complement C3	187	4.23E+08	2.33E+08	1.04E+09	2.22E+08	2.18E+09
A0A0G2JPR0	Complement C4-A	192.8	9.52E+07	3.15E+07	1.69E+08	3.65E+07	6.21E+08
A0A0G2JL54	Complement C4-B	187.6	3.57E+06	8.23E+05	9.16E+06	9.95E+05	2.68E+07
P01031	Complement C5	188.2	6.25E+06	3.89E+06	1.39E+07	3.10E+06	3.54E+07
P13671	Complement component C6	104.7	5.40E+06	1.69E+06	8.54E+06	1.82E+06	2.76E+07
P10643	Complement component C7	93.5	1.64E+06	9.31E+05	3.92E+06	1.13E+06	2.46E+07
P07357	Complement component C8 alpha chain	65.1	2.78E+06	1.02E+06	5.43E+06	1.18E+06	1.79E+07
P02748	Complement component C9	63.1	2.31E+06	1.38E+06	9.39E+06	1.59E+06	1.79E+07
P08603	Complement factor H	139	7.25E+07	1.75E+07	1.65E+08	2.03E+07	3.06E+08

B1AKG0	Complement factor H-related protein 1	30.8	3.34E+06	1.29E+06	6.50E+06	1.32E+06	1.66E+07
P36980	Complement factor H-related protein 2	30.6	4.34E+05	1.83E+05	7.08E+05	1.43E+05	2.54E+06
P05156	Complement factor I	65.7	1.09E+07	2.49E+06	2.25E+07	2.94E+06	5.32E+07
P08185	Corticosteroid-binding globulin	45.1	2.26E+06	2.72E+05	3.16E+06	5.09E+05	1.84E+07
Q16610	Extracellular matrix protein 1	60.6	1.32E+06	6.16E+05	3.22E+06	4.32E+05	5.31E+06
E9PG08	Fetuin-B	34.5	6.86E+05	2.21E+05	1.38E+06	1.76E+05	2.36E+06
P02671	Fibrinogen alpha chain	94.9	6.30E+08	3.08E+08	1.43E+09	3.16E+08	3.49E+09
P02675	Fibrinogen beta chain	55.9	4.24E+08	1.23E+08	8.29E+08	1.12E+08	1.82E+09
P02679	Fibrinogen gamma chain	51.5	6.95E+08	2.88E+08	1.33E+09	2.34E+08	2.27E+09
P02751	Fibronectin	272.2	9.59E+07	4.72E+07	1.52E+08	3.66E+07	6.66E+08
B1AHL2	Fibulin-1	78.3	9.69E+05	7.60E+05	3.77E+06	6.95E+05	6.09E+06
P21333	Filamin-A	280.6	4.94E+05	3.47E+05	1.06E+06	5.15E+05	2.17E+06
A0A0A0MT01	Gelsolin	84.7	2.72E+07	1.17E+07	5.76E+07	9.34E+06	8.89E+07
H0YBE4	Glutathione peroxidase 3 (Fragment)	8.8	1.10E+06	1.70E+05	8.52E+05	1.11E+05	3.63E+06
P00738	Haptoglobin	45.2	6.72E+08	1.44E+08	1.14E+09	1.37E+08	2.23E+09
P00739	Haptoglobin-related protein	39	6.11E+06	1.18E+06	9.35E+06	9.96E+05	1.56E+07
P69905	Hemoglobin subunit alpha	15.2	9.44E+05	3.68E+05	4.63E+05	5.20E+05	9.96E+06
P68871	Hemoglobin subunit beta	16	1.65E+06	7.45E+05	3.28E+06	6.59E+05	1.13E+07
P02790	Hemopexin	51.6	1.78E+08	3.92E+07	2.66E+08	4.09E+07	7.42E+08
P05546	Heparin cofactor 2	57	8.60E+06	4.68E+06	1.77E+07	3.89E+06	3.45E+07
P04196	Histidine-rich glycoprotein	59.5	2.02E+07	9.13E+06	3.52E+07	1.23E+07	1.28E+08
Q14520	Hyaluronan-binding protein 2	62.6	3.55E+06	6.44E+05	8.89E+06	1.23E+06	1.35E+07
P01876	Immunoglobulin heavy constant alpha 1	37.6	2.88E+08	4.86E+07	5.52E+08	6.69E+07	1.96E+09
A0A0G2JMB2	Immunoglobulin heavy constant alpha 2 (Fragment)	36.5	4.78E+06	7.37E+05	1.48E+07	1.15E+06	5.04E+07
P01880	Immunoglobulin heavy constant delta	42.3	7.62E+05	4.17E+05	2.53E+06	5.51E+05	8.59E+06
P01857	Immunoglobulin heavy constant gamma 1	36.1	5.18E+08	1.17E+08	8.91E+08	1.37E+08	1.60E+09
A0A286YFY4	Immunoglobulin heavy constant gamma 2 (Fragment)	43.8	1.32E+09	3.26E+08	2.74E+09	3.66E+08	5.00E+09
A0A4W9A917	Immunoglobulin heavy constant gamma 3 (Fragment)	41.3	6.91E+08	1.97E+08	1.33E+09	1.97E+08	2.50E+09
A0A286YFJ8	Immunoglobulin heavy constant gamma 4 (Fragment)	43.8	1.22E+07	2.01E+06	2.91E+07	2.43E+06	4.76E+07
P01871	Immunoglobulin heavy constant mu	49.4	2.07E+08	9.22E+07	3.94E+08	8.15E+07	1.14E+09
A0A0C4DH31	Immunoglobulin heavy variable 1-18	12.8	1.16E+07	8.73E+06	2.15E+07	5.56E+06	3.84E+07
A0A0B4J1V0	Immunoglobulin heavy variable 3-15	12.9	1.43E+07	4.86E+06	2.86E+07	6.93E+06	6.16E+07
P01772	Immunoglobulin heavy variable 3-33	13.1	3.73E+06	1.69E+06	3.62E+06	1.52E+06	1.58E+07
P01767	Immunoglobulin heavy variable 3-53	12.8	7.13E+05	4.85E+05	1.04E+06	6.01E+05	5.48E+06
P01780	Immunoglobulin heavy variable 3-7	12.9	3.16E+07	1.10E+07	3.83E+07	1.20E+07	8.71E+07
A0A0B4J1V6	Immunoglobulin heavy variable 3-73	12.8	1.17E+06	6.80E+05	2.18E+06	4.79E+05	4.66E+06
P01782	Immunoglobulin heavy variable 3-9	12.9	4.26E+06	2.57E+06	9.19E+06	2.17E+06	2.54E+07
A0A0C4DH34	Immunoglobulin heavy variable 4-28	13.1	2.43E+05	7.27E+05	6.84E+05	1.07E+06	3.59E+06
P01825	Immunoglobulin heavy variable 4-59	12.9	7.60E+06	5.93E+06	9.68E+06	4.38E+06	1.12E+07
A0A0B4J1U7	Immunoglobulin heavy variable 6-1	13.5	2.71E+06	1.09E+06	3.33E+06	9.88E+05	8.89E+06
D6RD17	Immunoglobulin J chain (Fragment)	18	1.90E+06	7.54E+05	4.58E+06	9.02E+05	2.51E+07
P01834	Immunoglobulin kappa constant	11.8	1.44E+09	4.62E+08	2.89E+09	4.67E+08	4.11E+09
A0A075B6S5	Immunoglobulin kappa variable 1-27	12.7	4.94E+06	2.99E+05	4.04E+06	5.87E+05	1.36E+07
A0A075B6S9	Immunoglobulin kappa variable 1-37 (non-functional) (Fragment)	12.7	3.46E+05	1.55E+05	5.14E+05	1.51E+05	7.70E+06
P01602	Immunoglobulin kappa variable 1-5	12.8	3.96E+06	3.29E+06	6.24E+06	5.09E+06	6.41E+07
P01593	Immunoglobulin kappa variable 1D-33	12.8	1.10E+05	1.56E+05	2.70E+05	1.99E+05	5.73E+06
A0A075B6P5	Immunoglobulin kappa variable 2-28	12.9	4.60E+07	7.51E+06	7.36E+07	8.19E+06	1.56E+08
A2NJV5	Immunoglobulin kappa variable 2-29	13.1	8.43E+06	2.38E+06	1.50E+07	3.88E+06	2.76E+07
P06310	Immunoglobulin kappa variable 2-30	13.2	6.35E+06	1.86E+06	1.36E+07	2.26E+06	2.14E+07
P04433	Immunoglobulin kappa variable 3-11	12.6	9.85E+06	4.33E+06	1.96E+07	4.79E+06	5.11E+07
P01619	Immunoglobulin kappa variable 3-20	12.5	1.07E+07	6.19E+06	2.03E+07	6.61E+06	1.09E+08
A0A087WSY6	Immunoglobulin kappa variable 3D-15	12.5	3.15E+06	2.07E+06	5.35E+06	1.93E+06	2.25E+07
P06312	Immunoglobulin kappa variable 4-1	13.4	3.21E+06	1.66E+06	5.98E+06	2.33E+06	2.86E+07
P0DOY2	Immunoglobulin lambda constant 2	11.3	1.13E+09	3.82E+08	1.83E+09	4.64E+08	5.34E+09
A0A0B4J1U3	Immunoglobulin lambda variable 1-36	12.5	3.60E+05	8.62E+04	8.99E+05	1.00E+05	6.37E+05
P01703	Immunoglobulin lambda variable 1-40	12.3	3.79E+06	1.12E+06	6.57E+06	8.47E+05	2.55E+07
P01699	Immunoglobulin lambda variable 1-44	12.2	4.00E+07	1.03E+06	5.57E+07	9.30E+05	1.86E+07
P01701	Immunoglobulin lambda variable 1-51	12.2	1.47E+07	1.52E+06	2.49E+07	1.24E+06	1.65E+07
P01706	Immunoglobulin lambda variable 2-11	12.6	1.52E+06	2.79E+05	3.63E+06	4.24E+05	8.86E+06

A0A075B6K4	Immunoglobulin lambda variable 3-10	12.4	1.00E+07	3.79E+06	2.00E+07	2.87E+06	4.55E+07
P01714	Immunoglobulin lambda variable 3-19	12	8.77E+05	6.34E+05	1.78E+06	5.84E+05	6.28E+06
P80748	Immunoglobulin lambda variable 3-21	12.4	3.78E+06	8.55E+05	6.46E+06	1.28E+06	2.74E+07
P01718	Immunoglobulin lambda variable 3-27	12.2	5.03E+06	1.80E+06	6.51E+06	2.67E+06	2.80E+07
A0A075B6K5	Immunoglobulin lambda variable 3-9	12.3	1.84E+05	3.52E+04	2.03E+05	3.68E+04	1.11E+06
A0A075B6H9	Immunoglobulin lambda variable 4-69	12.8	5.99E+05	3.61E+05	1.92E+06	5.06E+05	3.06E+06
P01721	Immunoglobulin lambda variable 6-57	12.6	7.16E+05	1.24E+05	1.03E+06	2.10E+05	2.25E+06
A0A075B6I0	Immunoglobulin lambda variable 8-61	12.8	6.11E+05	4.99E+04	1.55E+06	7.50E+04	1.15E+06
P15814	Immunoglobulin lambda-like polypeptide 1	22.9	6.51E+06	2.83E+06	1.60E+07	2.25E+06	2.63E+07
A0A0B4J231	Immunoglobulin lambda-like polypeptide 5	23.1	3.70E+07	9.81E+06	6.21E+07	7.94E+06	8.16E+07
P35858	Insulin-like growth factor-binding protein complex acid labile subunit	66	1.37E+06	2.30E+05	2.50E+06	3.68E+05	6.25E+06
P05106	Integrin beta-3	87	1.54E+05	1.51E+05	4.90E+05	2.79E+05	5.47E+05
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	101.3	1.26E+07	9.43E+06	2.78E+07	9.56E+06	1.27E+08
P19823	Inter-alpha-trypsin inhibitor heavy chain H2	106.4	4.75E+07	1.20E+07	1.13E+08	1.39E+07	3.15E+08
A0A087WW43	Inter-alpha-trypsin inhibitor heavy chain H3	75	9.21E+05	5.00E+05	2.40E+06	3.62E+05	5.96E+06
B7ZKJ8	ITIH4 protein	103.8	4.96E+07	1.15E+07	8.87E+07	1.40E+07	1.68E+08
P01042	Kininogen-1 OS=Homo sapiens	71.9	3.79E+07	1.35E+07	7.42E+07	1.43E+07	1.70E+08
P51884	Lumican	38.4	1.21E+06	4.79E+05	3.41E+06	2.60E+05	2.80E+06
P35579	Myosin-9	226.4	8.04E+04	1.63E+05	2.99E+05	2.72E+05	2.22E+05
Q96PD5	N-acetylmuramoyl-L-alanine amidase	62.2	4.22E+06	9.44E+05	6.57E+06	8.87E+05	1.94E+07
P04180	Phosphatidylcholine-sterol acyltransferase	49.5	1.02E+05	2.22E+04	1.68E+05	6.80E+03	1.10E+06
P80108	Phosphatidylinositol-glycan-specific phospholipase D	92.3	3.28E+05	1.20E+05	7.41E+05	5.61E+04	1.12E+06
P36955	Pigment epithelium-derived factor	46.3	2.68E+06	6.37E+05	3.26E+06	6.96E+05	1.31E+07
P03952	Plasma kallikrein	71.3	6.72E+06	3.98E+06	1.35E+07	5.25E+06	3.82E+07
P05155	Plasma protease C1 inhibitor	55.1	3.51E+07	6.48E+06	6.35E+07	7.46E+06	1.19E+08
P00747	Plasminogen	90.5	6.29E+07	1.99E+07	1.35E+08	2.05E+07	3.21E+08
E9PAQ1	Properdin	45.1	2.83E+05	4.55E+04	6.25E+05	6.42E+04	2.04E+06
P02760	Protein AMBP	39	1.87E+07	6.86E+06	4.53E+07	6.09E+06	1.02E+08
P00734	Prothrombin	70	2.39E+07	7.31E+06	5.10E+07	7.35E+06	1.94E+08
Q5VY30	Retinol-binding protein	23	7.34E+06	1.88E+06	1.39E+07	3.69E+06	8.72E+07
A0A096LPE2	SAA2-SAA4 readthrough	23.3	1.35E+06	7.78E+05	3.15E+06	1.39E+06	2.60E+07
P02787	Serotransferrin	77	1.71E+09	4.07E+08	2.92E+09	5.05E+08	8.10E+09
P02768	Serum albumin	69.3	4.31E+10	1.33E+10	7.86E+10	1.18E+10	1.57E+11
P02743	Serum amyloid P-component	25.4	1.32E+07	5.48E+06	2.87E+07	4.54E+06	9.41E+07
P27169	Serum paraoxonase/arylesterase 1	39.7	9.10E+06	3.74E+06	2.02E+07	2.63E+06	4.89E+07
Q9Y490	Talin-1	269.6	3.72E+05	4.92E+05	1.89E+05	2.52E+05	9.43E+05
E9PHK0	Tetranectin	17.8	1.84E+06	5.10E+05	3.01E+06	4.67E+05	6.10E+06
P05543	Thyroxine-binding globulin	46.3	2.03E+06	1.46E+06	5.48E+06	1.10E+06	5.72E+06
P02766	Transthyretin	15.9	8.73E+07	2.02E+07	1.37E+08	1.26E+07	4.44E+08
P02774	Vitamin D-binding protein	52.9	1.77E+08	5.46E+07	3.62E+08	4.18E+07	6.41E+08
P07225	Vitamin K-dependent protein S	75.1	4.52E+06	1.18E+06	7.87E+06	1.50E+06	1.81E+07
P04004	Vitronectin	54.3	1.93E+07	2.97E+06	4.46E+07	4.91E+06	1.26E+08
P04275	von Willebrand factor	309.1	6.27E+05	3.11E+05	9.74E+05	2.58E+05	2.28E+06
P25311	Zinc-alpha-2-glycoprotein	34.2	1.60E+07	4.43E+06	3.80E+07	4.42E+06	6.48E+07

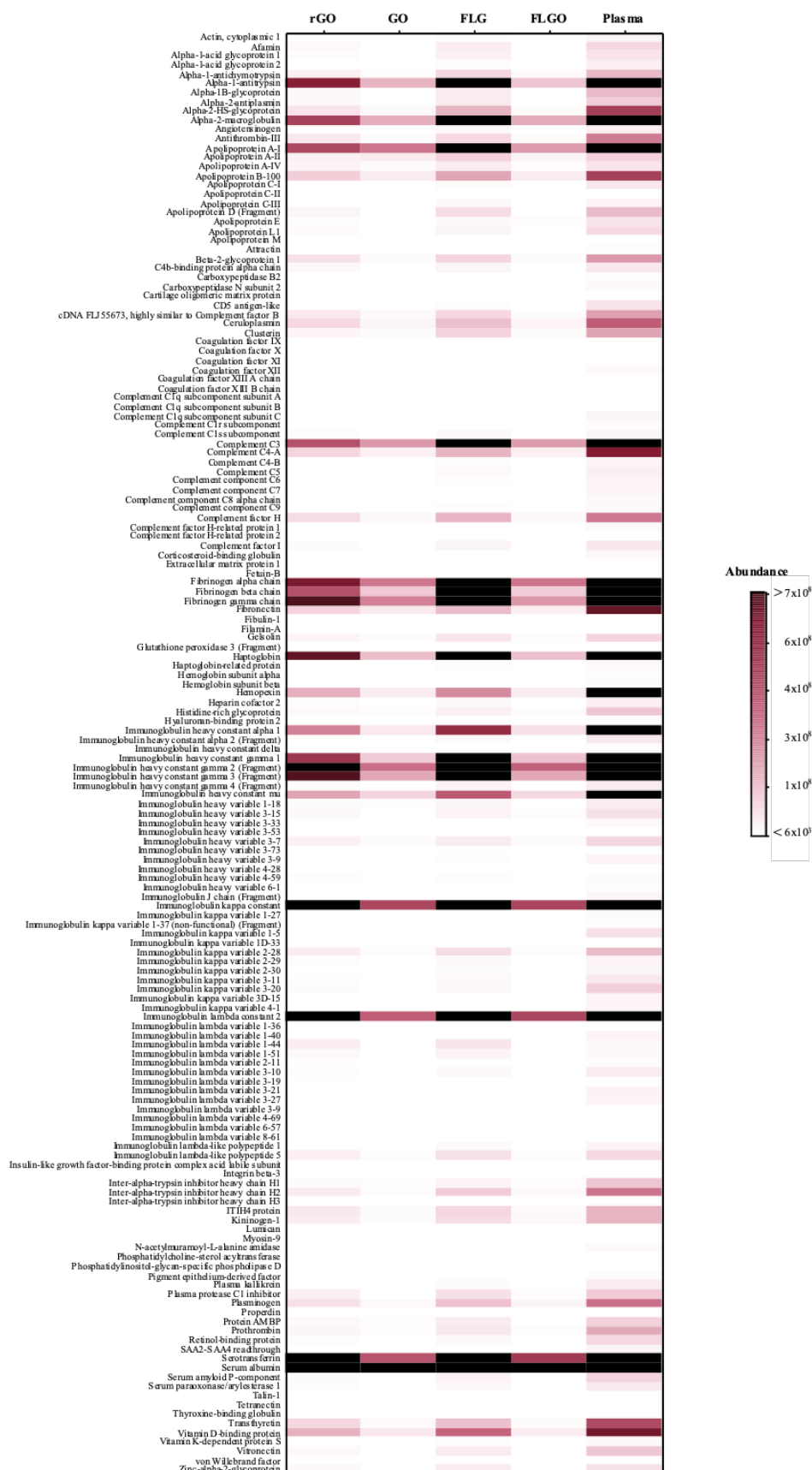


Figure S7. Heat map of abundances of the total proteins present on GBMs films and on human plasma. rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

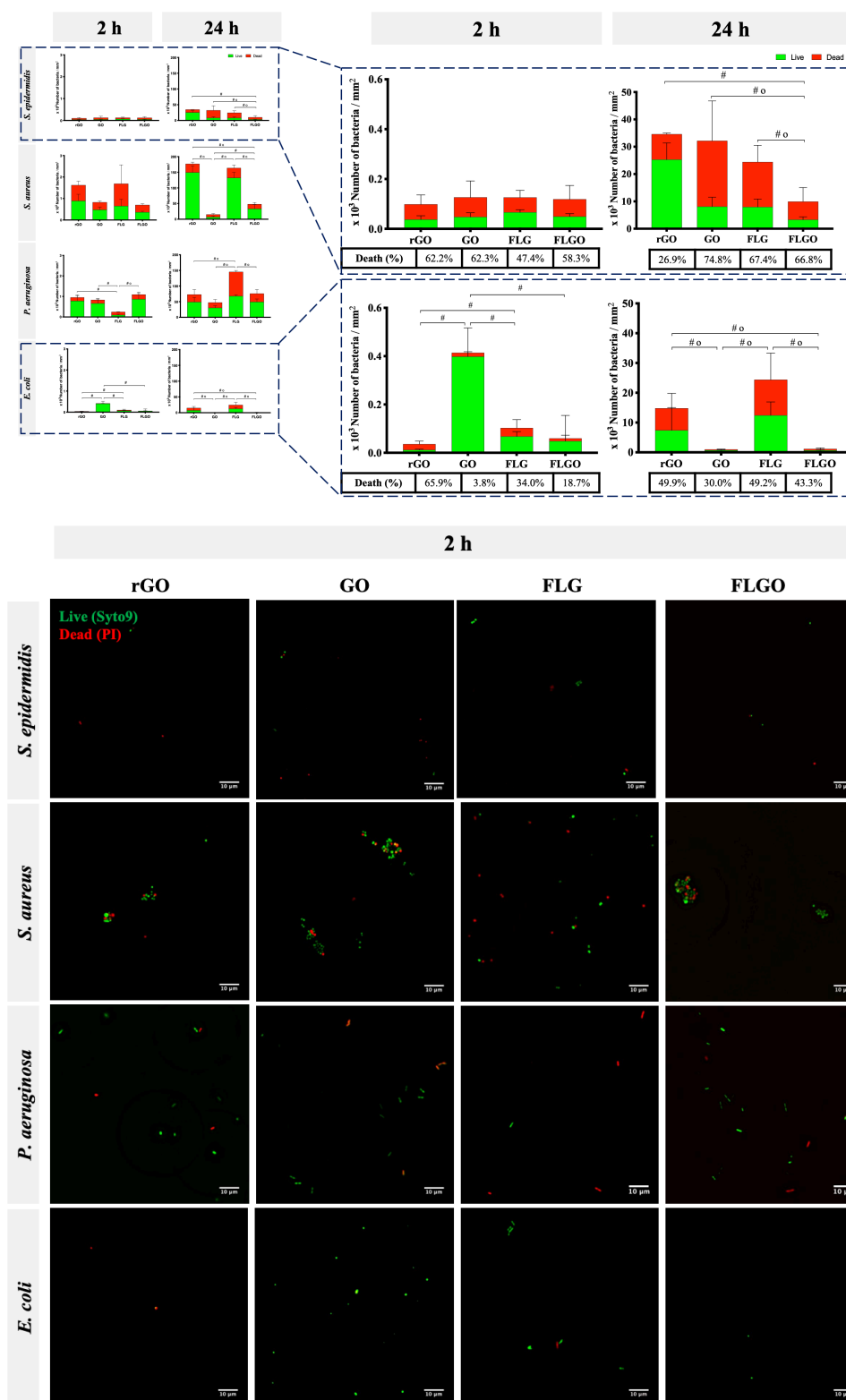


Figure S8. Antibacterial profile of GBMs films. Expanded graphs of antibacterial activity towards *S. epidermidis* and *E. coli* (top panel) and fluorescence images of live/dead staining after 2 h, obtained by IN Cell Analyzer 2000 (scale bar = 10 μm). #, o – statistically significant differences between samples within total number (#) and dead (o) adherent bacteria (Kruskal-Wallis, $p < 0.05$). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

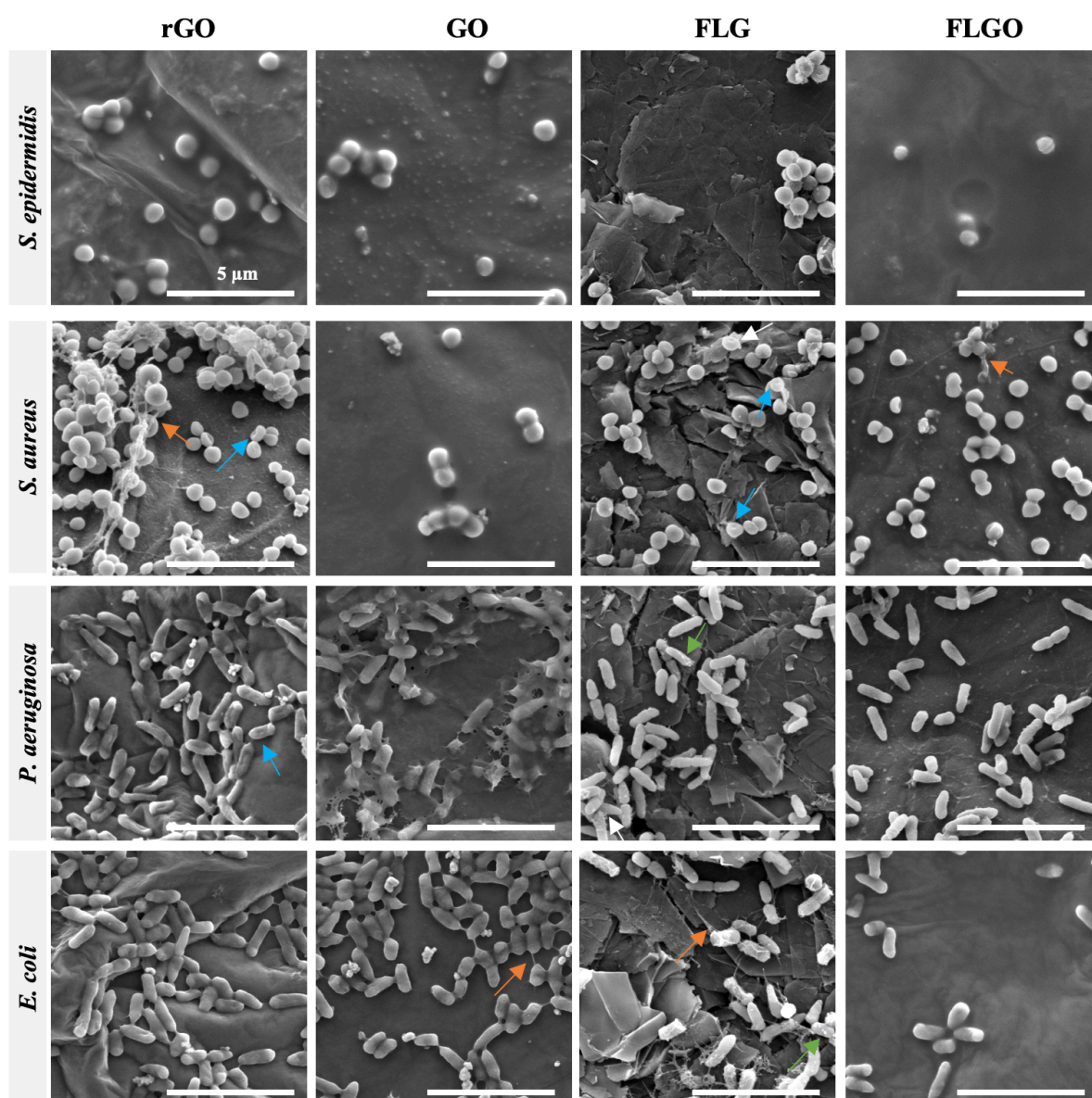
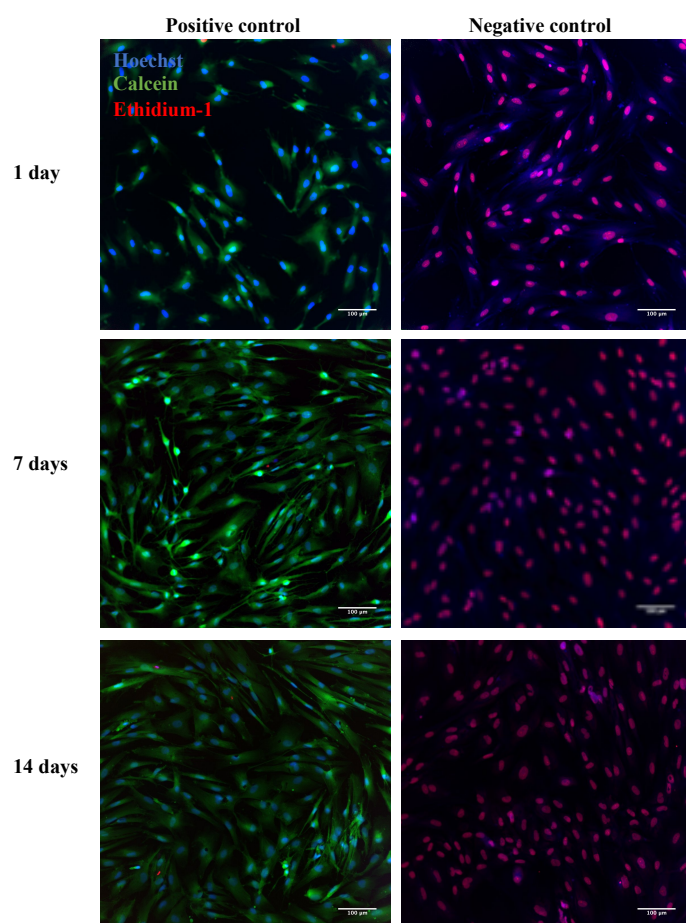
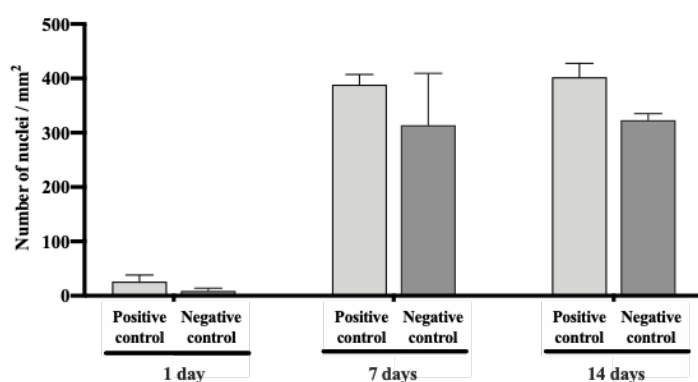


Figure S9. Morphology of bacteria after adhesion to GBMs films over 24 h. Observation of signs of biofilm formation – extracellular extrusions and a viscous polysaccharide layer (slime) (orange arrows), capsular exopolysaccharide colanic acid particles [5, 6] (green arrows) attached around and on top the bacterial cells – and signs of bactericidal effect: holes, membrane corrugation and deformation (blue arrows). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

A.



B.



C.

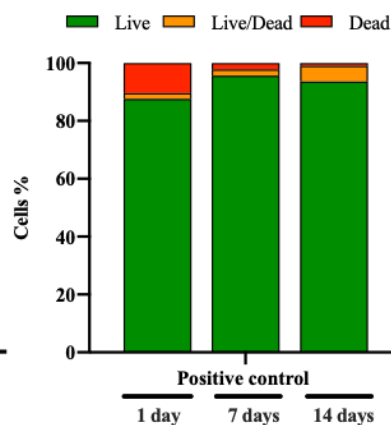


Figure S10. Morphology, proliferation and viability of HFF-1 fibroblast cells after seeding and culture on control samples over 14 days. (A) Fluorescence images of cells stained with Hoechst (blue, total), Calcein (green, viable), and Ethidium-1 (red, dead), obtained by IN Cell Analyzer 2000 (objective 20x, scale bar = 100 µm). (B) Cell proliferation as number of nuclei/mm² and (C) cell viability (percentage). Positive control of viability: cells seeded directly on TCPS plates; negative control of viability: cells seeded on TCPS and fixed with 70% (v/v) methanol (10 min).

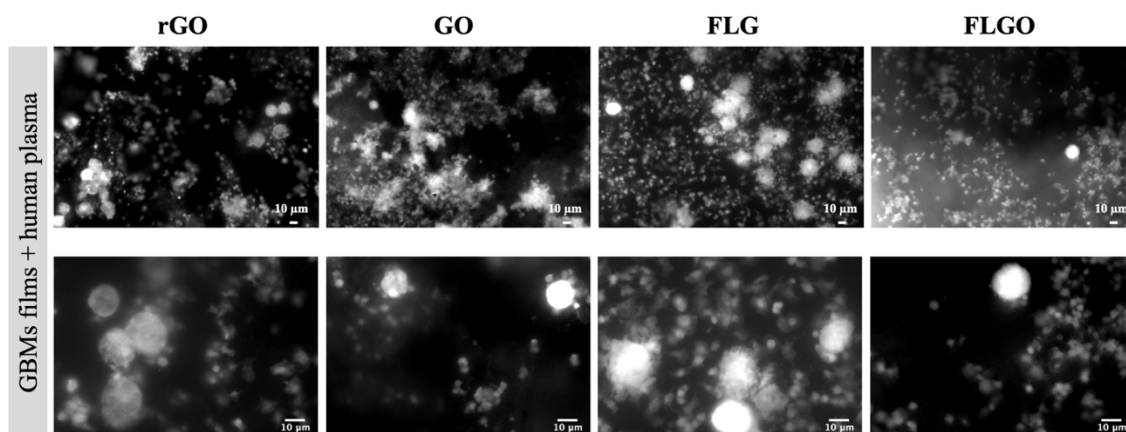


Figure S11. Blood components adhesion to GBMs films after human plasma incubation for 1 h. Images obtained by GIFT (scale bar = 10 μm). rGO = reduced graphene oxide, GO = graphene oxide, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

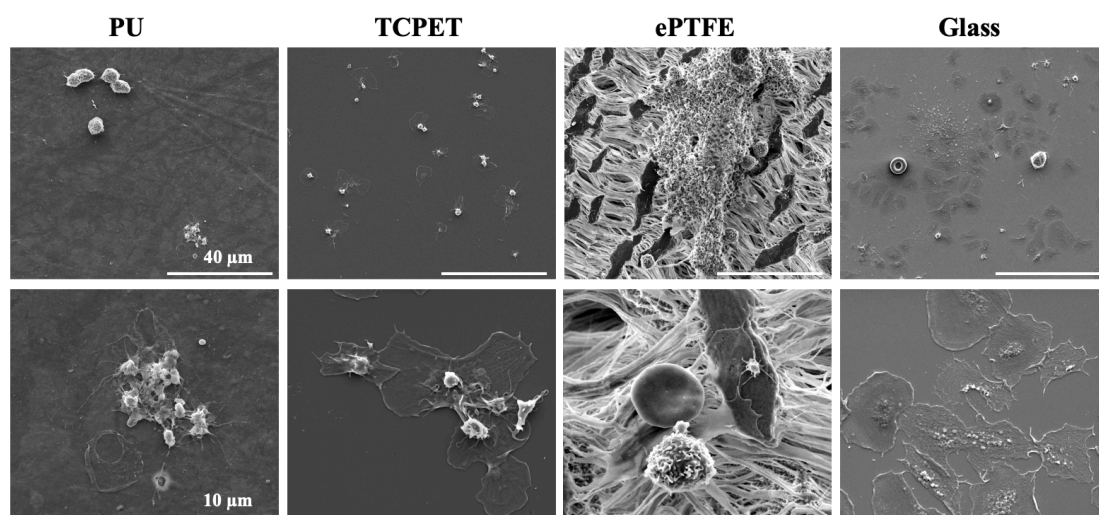


Figure S12. Standard surfaces used as controls on the whole blood assay: PU = polyurethane, TCPET = tissue culture polyethylene terephthalate, ePTFE = expanded polytetrafluoroethylene, glass. Images obtained by SEM (top row: scale bar = 40 μm, and bottom row: scale bar = 10 μm).

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CHAPTER V

**Can GBMs films act as stand-alone
photoactive antibacterial surfaces?**

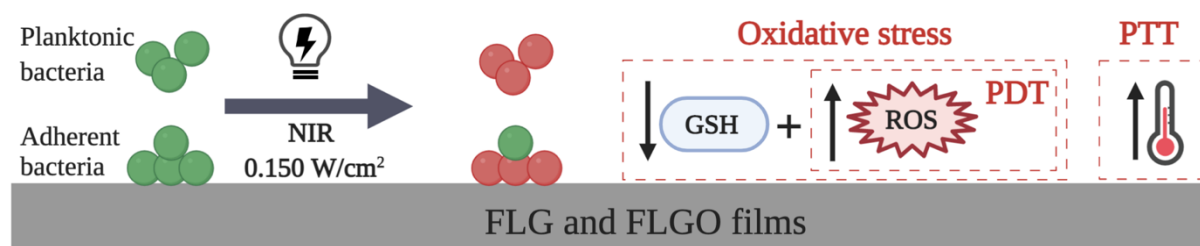
Graphene films irradiated with safe low-power NIR-emitting diodes kill multidrug resistant bacteria

Patrícia C. Henriques, Andreia T. Pereira, Diana Bogas, José R. Fernandes, Artur M. Pinto, Fernão M. Magalhães, Inês C. Gonçalves

Abstract

Bacterial adhesion to surfaces is the onset of biofilm formation and a hard problem to tackle, aggravated by the rise in drug-resistant bacteria, responsible for more than 500000 deaths globally/year. This work reports few-layer graphene (FLG) and few-layer graphene oxide (FLGO) as stand-alone light-responsive platforms to develop smart antibacterial surfaces. Films exposure to low intensity NIR drastically improves their ability to kill planktonic (up to ~99%) and adherent (up to ~85%) methicillin resistant *S. aureus* and *S. epidermidis*. Upon irradiation, a mild photothermal effect is observed in supernatant, with temperature rising from 37 °C to 39 °C–42 °C, while surface temperature of non-oxidized FLG films increases to 51.3 °C versus 56 °C for oxidized films. Both films prompt total glutathione oxidation when irradiated, despite FLG films induce higher ROS generation than FLGO, suggesting antioxidants depletion occurs preferentially by ROS-dependent pathway (photodynamic effect) for FLG versus ROS-independent pathway for FLGO films. This proof-of-principle study demonstrates that safe, low-intensity NIR irradiation is a valuable and effective tool to boost graphene surfaces' antibacterial performance through a synergistic photothermal and photodynamic effect. These stand-alone NIR-activated graphene-based platforms arise as simple and economical disinfection surfaces/systems, with widespread use in medical and non-medical applications.

Graphical Abstract



Antibacterial performance of stand-alone graphene films is strongly augmented upon exposure to low-intensity near-infrared (NIR) radiation. Planktonic bacteria are eliminated (99%) and adherent bacteria reach 85% death with few-layer graphene oxide (FLGO) films. Irradiation causes surface heating (up to 56°C), glutathione (GSH) depletion, and reactive oxygen species (ROS) overproduction, intensifying oxidative stress and bacterial death. Graphene films appear as effective stand-alone bactericidal light-activated platforms.

Keywords: graphene-based materials; near-infrared (NIR); antibacterial coatings; photodynamic; photothermal; graphene oxide; sterilization; free-standing films.

1. Introduction

Prevention of bacterial adhesion and consequent biofilm formation is a demanding and costly task. It is, however, of utmost importance in different contexts directly related to human health and life, from medicine and dentistry, to food processing and water treatment [1]. Different problems may arise when these issues are not efficiently treated, including microbial-induced corrosion, contamination of drinking water systems, oral diseases, failure of biomedical implants and several other industrial and environmental problems, such as marine fouling [1-3].

Endowing surfaces with antibacterial properties has been a widely explored strategy [4-8]. Conventional solutions have focused on the use of biocidal agents, as antimicrobial nanoparticles or antibiotics, with the latter being still the major weapon to fight bacterial infections. However, antibiotics overuse has contributed to the development of bacterial resistance, one of the biggest threats to public health today [9, 10]. Therefore, despite remarkable advances in the development of bactericidal surfaces, no completely efficient solution has been found.

Photoactive bactericidal surfaces have been attracting particular attention, since killing of bacteria is achieved using light combined with an appropriate photoactive agent, instead of biocides [11]. Antimicrobial photothermal therapy (PTT) and photodynamic therapy (PDT) are two promising light-based methods. PTT, a physical antibacterial method due to the absence of biocide release, is based on the irradiation of a photothermal agent that converts light into heat, leading to a local temperature

increase. As a consequence, membrane permeability increases, expression of heat shock proteins is upregulated, and cellular components denaturation occurs, eventually leading to bacterial death [11, 12]. In PDT, irradiation and excitation of a nontoxic photosensitizer starts the process of energy or electrons transference to tissue oxygen molecules, inducing the production of singlet oxygen ($^1\text{O}_2$) and/or other reactive oxygen species (ROS), respectively [12, 13]. This leads to oxidation of different biological molecules, ultimately leading to bacterial death [11, 14].

Contrarily to antibiotics, the development of resistance by bacterial cells is highly unlikely when using PTT or PDT [15, 16]. This is mostly due to: 1) non-selective and multi-targeted action, which affects or destroys numerous molecular microbial targets (e.g. membrane, proteins, and nucleic acids); 2) fast and severe cell damage caused by these approaches, which disables cross-generation adaptivity; 3) ROS short lifetime and short diffusion length, which prevents the expression of protective factors; 4) ROS low dark toxicity, which does not elicit the adaptive survival mechanism in bacteria [17].

The individual use of these therapies can be, however, insufficient to fully eliminate bacteria. In PTT, for instance, temperature may need to rise up to 70 °C (depending on the microorganism and irradiation time) [18], which may be prejudicial to surrounding tissues [19]. In addition, PTT is generally implemented using lasers or high-power LEDs [20], with irradiation intensities higher than the irradiance limit of skin tolerance set by American National Standards Institute – ANSI ($\sim 0.33 \text{ W/cm}^2$ at 808 nm) [11, 21, 22]. Regarding PDT, even though with lesser adverse effects than PTT, its efficiency is dependent on local oxygen levels, which at the site of infection are generally low [11]. This gives rise to reduced concentrations of different ROS and $^1\text{O}_2$, with reduced lifetime, causing them to be rapidly balanced out by internal mechanisms [12, 16].

To overcome these limitations, combination of PTT and PDT has been explored [23, 24]. Reports show that a small amount of ROS can enhance the thermal susceptibility of bacteria, decreasing the required temperature for elimination, while hyperthermia can disrupt the bacterial membrane to facilitate the permeation of ROS into bacteria [23, 25]. This has been accomplished using different molecules [13, 26] (gold nanomaterials [22], metal nanoparticles, inorganic nanomaterials, two-dimensional materials, such as graphene-based materials (GBMs)) or different wavelengths to achieve complementary performances [11, 22, 27, 28].

GBMs show great potential to be used in these light-based therapies, due to their remarkable broadband optical absorption ability that arises from the closely spaced energy levels of the loosely held π electrons. This allows for heat generation when the light-excited electrons relax to the ground states. Such high photothermal conversion efficiency, boosts their use as photothermal agents [11, 20, 29, 30]. Even though also absorbing visible (blue and red) and UV light, the absorption of NIR irradiation by GBMs is of particular interest. Due to its long wavelength and low interaction with biological components (in the biological therapeutic window of 700–950 nm, where light tissue absorption and scattering is reduced), NIR can achieve a high penetration depth and minimal off-target heating [31, 32]. GBMs have also been reported as having some ability to induce oxidative stress in biological systems

[33], via a ROS-independent (glutathione oxidation [34]) and ROS-dependent pathways (inducing the formation of ROS and further inducing ROS-mediated programmed bacterial death [30]) [14, 24, 35]. Their native properties can therefore be potentiated by NIR stimulation. GBMs have however been mostly used in combination with other materials or as immobilization platforms for other molecules, namely metallic nanoparticles [36, 37], antibiotics [38], or photosensitizers (as chlorin e6) [17], and in the larger part of the reported works, GBMs were studied as suspensions in water rather than deposited on surfaces [39-44].

In this study, we investigated the use of stand-alone few-layer graphene (FLG) and few-layer graphene oxide (FLGO) films as NIR-activated bactericidal platforms. In addition, we explored the mechanism of action underlying the NIR-triggered killing effect, based on surface morphology and chemistry, changes in temperature, glutathione oxidation (oxidative potential), and ROS generation. The bactericidal effect towards Staphylococci, in particular *S. aureus* and *S. epidermidis*, is demonstrated, which has profound impact on the prevention of medical infections [45-48]. This should stimulate the use of such graphene-based platforms in medical settings, such as coatings for medical devices, instruments, or medical implants [49]. It is particularly noteworthy the usage of a low power LED-based NIR source, with irradiance below the recommended threshold for skin burn. The more widespread use of these approaches for non-medical sterilization purposes, as light-activated disinfectants [50], may also be of great relevance.

2. Materials and Methods

2.1. Production of FLG and FLGO films

Few-layer graphene (FLG) (~5 μm diameter, xGnP® grade M) was commercially acquired from XG Sciences (Lansing, USA) and oxidized into few-layer graphene oxide (FLGO) using the modified Hummers method as previously described by us [34, 51]. FLG and FLGO films were produced by vacuum filtering FLG and FLGO aqueous dispersions (45 mg) through a nylon membrane (0.20 μm pore size, NY2004700, Merck) [34]. Films were dried at 37 °C overnight and cut into 9 mm diameter discs using a stainless-steel hole puncher. FLG films were slightly compressed after production, for 5 sec under ~16 kg/mm², using a Graseby Specac press, to prevent particle detachment. All films were sterilized with ethylene oxide (EO Gas series 3+) at Centro Hospitalar Universitário de São João.

2.2. NIR irradiation of FLG and FLGO films

In the following experiments, FLG and FLGO films were either kept in the dark (NIR off) or exposed to NIR light (NIR on). A low-power LED-based source with peak emission of 812 nm and irradiance of 0.150 W/cm² was used to irradiate the films over 60 min. In order to expose bacteria for at least 30 min to the maximum temperature, 45 min was selected as the irradiation time for the antibacterial assays.

The impact of films physicochemical, photothermal and photodynamic properties on the antibacterial activity was explored.

2.2.1. Impact of NIR irradiation in antibacterial properties

Surfaces were challenged with methicillin resistant *Staphylococcus aureus* (ATCC® MRSA 33591™) and *Staphylococcus epidermidis* (ATCC® 35984™). Bacteria were grown in Trypticase Soy Agar (TSA, Merck) plates overnight at 37 °C. Two colonies were collected, inoculated into 5 mL of Trypticase Soy Broth (TSB, Merck) and cultured overnight at 37 °C under stirring at 150 rpm.

Bacterial cultures were adjusted to 10^5 – 10^6 CFUs/mL in fresh TSB medium supplemented with 1% (v/v) human plasma, aiming to mimic the presence of proteins that naturally occurs in physiological conditions. GBMs films were transferred to 48-well suspension plates and incubated with 300 µL of this bacterial suspension at 37 °C under static conditions. The remaining empty wells were filled with deionized water (dH_2O) to avoid evaporation. After 2 h incubation, samples were either kept in the dark (NIR off) or NIR irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²), at room temperature (RT). Silicone films were used as control surfaces. The supernatant of each sample was then collected, and surfaces rinsed two times with new TSB to remove non-adherent bacteria (washing media also recovered and added to supernatant). Both supernatant and surface of the films were analyzed to assess planktonic and adherent bacteria, respectively (Figure S1), as described below.

2.2.1.1. Planktonic bacteria

Viability of planktonic bacteria was evaluated by colony forming units using the agar plate culture method. For that, serial dilutions (10^{-1} , 10^{-2} , 10^{-3}) of the supernatants were prepared and three drops of 10 µL of each dilution plated in TSA. Plates were incubated overnight at 37 °C and CFUs counted. Bacteria viability was also confirmed by alamarBlue™ assay. For that, resazurin was added to supernatants (10% v/v) and incubated for 2 h at 37 °C. RFUs were measured ($\lambda_{ex}/\lambda_{em}$: 530 nm/590 nm) in a microplate fluorometer (Spectra Max GeminiXS, Molecular Devices, California, USA) and correlated with the metabolic activity of bacteria present in the medium. Eight replicates of each film were evaluated.

2.2.1.2. Adherent bacteria

Viability of adherent bacteria was determined by fluorescence staining using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152, Invitrogen). A mixture of SYTO9 ($\lambda_{ex}/\lambda_{em}$ = 480/500 nm, green fluorescence) and propidium iodide (PI) ($\lambda_{ex}/\lambda_{em}$ = 490/635 nm, red fluorescence) nucleic acid stains was incubated with samples for 15 min in the dark at RT. Samples were transferred to uncoated 24-well µ-plates (#82406, IBIDI, Germany) with surface facing the glass bottom of the plate. Fluorescence images were acquired using a high-content screening microscope IN Cell Analyzer 2000

(GE Healthcare, Nikon 40×/0.95 NA Plan Apo objective) using a CCD Camera (CoolSNAP K4). Three replicates of each film were evaluated, acquiring 9 fields per sample (each field across a z-section of ~25 μm, with a z-step of 1 μm), with a total spanning area of 1.44 mm². Z-sections were projected on one plane using the IN Cell Investigator Developer Toolbox v.1.9.2 (GE Healthcare). Bacteria quantification was performed as previously described by us [34].

2.2.2. Impact of NIR irradiation in surface physicochemical features

NIR radiation impact on surface morphology and chemistry (elemental composition and oxidation degree) of films was evaluated by Scanning Electron Microscopy (SEM) and X-Ray Photoelectron Spectroscopy (XPS), respectively. Films were continuously irradiated for 45 min (812 nm, 0.150 W/cm²).

2.2.2.1. Scanning Electron Microscopy (SEM)

SEM analysis was performed using a Phenom XL SEM in a Secondary Electron Detector (SED) mode and with an acceleration voltage of 10 kV. FLG and FLGO films were adhered onto carbon tape and coated with a thin layer of Au by sputtering to improve their conductivity.

2.2.2.2. X-Ray Photoelectron Spectroscopy (XPS)

XPS analysis was conducted using a Kratos Axis Ultra HSA (Kratos Analytical, U.K.) equipment with a monochromatic Al X-Ray source operating at 15 kV (90 W). Survey spectra were acquired at 80 eV and C 1s and O 1s high-resolution spectra at 40 eV, setting the reference of the C 1s peak to 284.6 eV. Deconvolution of high-resolution spectra was performed with CasaXPS processing software version 2.3.16, using background type Shirley. C 1s deconvolution was performed as previously described by us [34].

2.2.3. Impact of NIR irradiation in photothermal properties

The light-to-heat conversion capacity of films upon NIR irradiation was also investigated. Films were transferred to a 48-well suspension plate, immersed in 300 μL of TSB, and incubated for 2 h at 37 °C. After that, samples were exposed to NIR light (812 nm, 0.150 W/cm²), and the temperature of both the surrounding medium and the surface of the films was recorded each 15 min, during 60 min, using a type K thermocouple microprobe. Three replicates were used per condition and two independent experiments were conducted. Silicone films were used as a control surface.

2.2.4. Impact of NIR irradiation in photodynamic properties

Glutathione (GSH) levels, a key endogenous antioxidant, and reactive oxygen species (ROS) generation, are two measures of oxidative stress in biological systems. Both can contribute to the photodynamic effect and therefore were quantified.

2.2.4.1. GSH oxidation assay

To determine GSH levels, GBMs films were incubated with 300 μ L of GSH (1.5 mM, Sigma) in reaction buffer (0.1 M sodium bicarbonate (pH 8), containing 1 mM EDTA) for 2 h at 37 °C. After incubation, samples were either kept in the dark (NIR off) or NIR irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). After that, 50 μ L of the supernatant was mixed with 500 μ L of the reaction buffer and 10 μ L of Ellman's reagent (10 mM in reaction buffer) for 15 min in the dark, allowing its reaction with the thiol groups of GSH to yield fluorescent TNB. GSH was quantified spectrophotometrically at 412 nm and the loss of GSH was calculated. GSH in reaction buffer without films was used as negative control, whereas GSH in H₂O₂ (1 mM) was used as positive control of oxidation.

2.2.4.2. DCFH-DA assay

To assess the generation of ROS, GBMs films were incubated alone or with bacteria for 2 h at 37 °C, followed by addition of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, ThermoFisher) at 10 μ M. Samples were either kept in the dark (NIR off) or NIR irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Fluorescence intensities were measured at 527 nm with excitation at 495 nm in a microplate fluorometer (Spectra Max GeminiXS, Molecular Devices, California, USA). Bacteria exposed to radiation were considered as basal activity and subtracted to the fluorescence intensities of films + bacteria.

3. Results

3.1. Antibacterial properties of NIR-irradiated FLG and FLGO films

Antibacterial performance of FLG and FLGO films was evaluated towards planktonic and adherent methicillin-resistant *S. aureus* and *S. epidermidis* after irradiation with a safe, low power (0.150 W/cm²) near-infrared (NIR) (812 nm) diode.

Comparing with non-irradiated samples (NIR off), viability of planktonic bacteria was drastically reduced after 45 min irradiation (NIR on). Almost 90% of bacteria were eliminated when in the presence of FLG films and close to 99% with FLGO films (Figure 1), independently of the bacterial strain. Results were consistent in both techniques used to evaluate bacteria viability, namely CFU counting and metabolic activity. Furthermore, in silicone, used as control irradiated material, no bacterial death was observed, demonstrating that the antibacterial action is induced by the NIR irradiated GBMs.

Furthermore, control bacterial culture showed no reduction of viability upon irradiation, indicating that NIR irradiation alone was harmless to both bacterial strains.

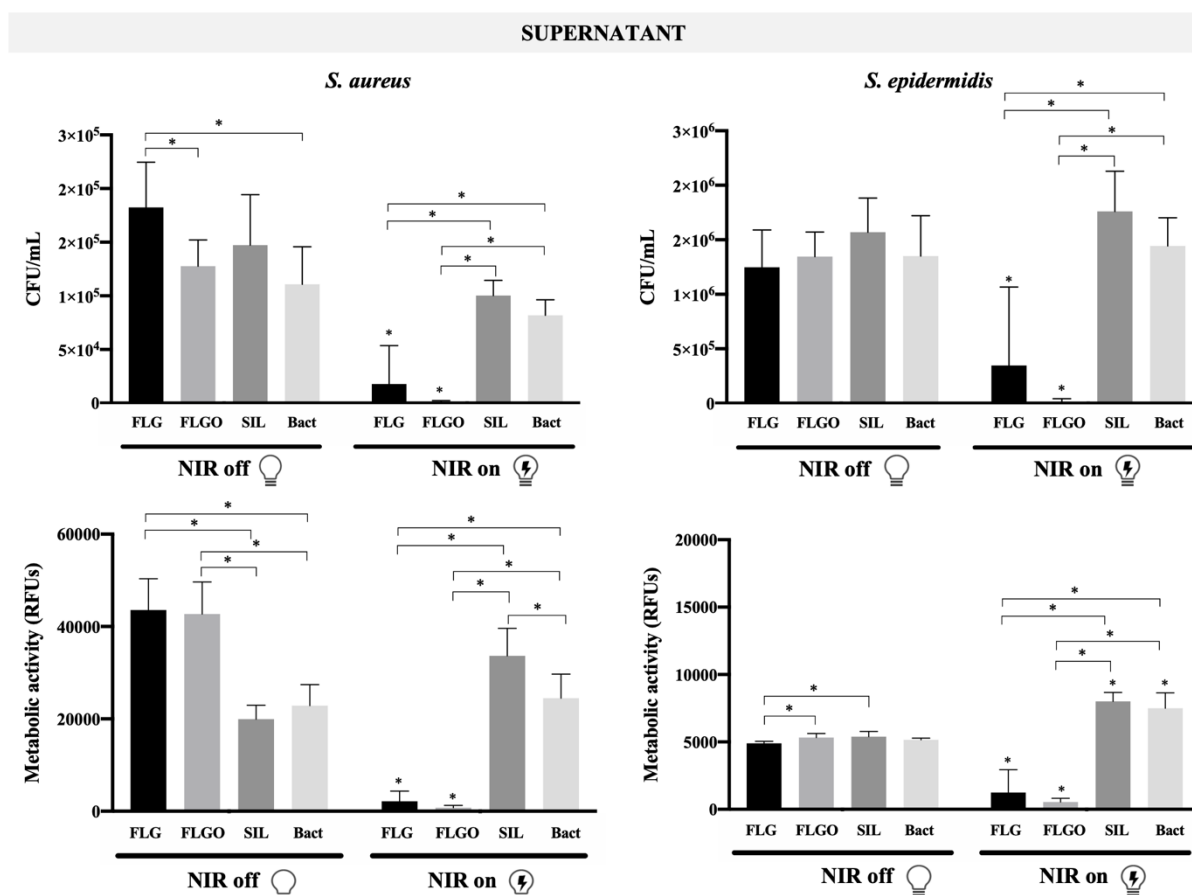


Figure 1. Antibacterial performance of FLG and FLGO films towards planktonic bacteria. After 2 h incubation with *S. aureus* and *S. epidermidis*, FLG and FLGO films were either kept in the dark (NIR off) or irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Bacteria from the supernatant were evaluated by CFU counting and bacterial metabolic activity measurement. Silicone films were used as a control material. Bacteria cultured in the absence of any material was used as the control of the irradiation effect on the bacteria viability. *statistically significant differences between samples, $p < 0.05$: one-way ANOVA test to compare films within the same condition (either NIR off or NIR on), and unpaired t-test with Welch's correction to compare the same film between NIR off or NIR on conditions; *alone indicates significant differences from its counterpart in NIR off condition. FLG = few-layer graphene, FLGO = few-layer graphene oxide, SIL = silicone. 8 replicates of each material in each independent assay ($n = 3$).

Adherent bacteria also showed an increased death percentage (reaching around 80% of death) and a significant reduction in adhesion as a result of NIR irradiation (NIR on), particularly for methicillin-resistant *S. aureus* and when in the presence of FLGO films, (Figure 2, and Figure S2 and S3 for fluorescent microscopy images). In the absence of irradiation (NIR off), *S. aureus* was already more susceptible to the action of surfaces, with *S. epidermidis* showing a more pronounced death increase upon irradiation (NIR on). This is denoted by the increased uptake of propidium iodide (PI) dye that

only enters bacteria with compromised membrane. No differences were seen in silicone surfaces before and after irradiation, validating that the observed bactericidal effect of NIR irradiation results from the presence of GBMs films.

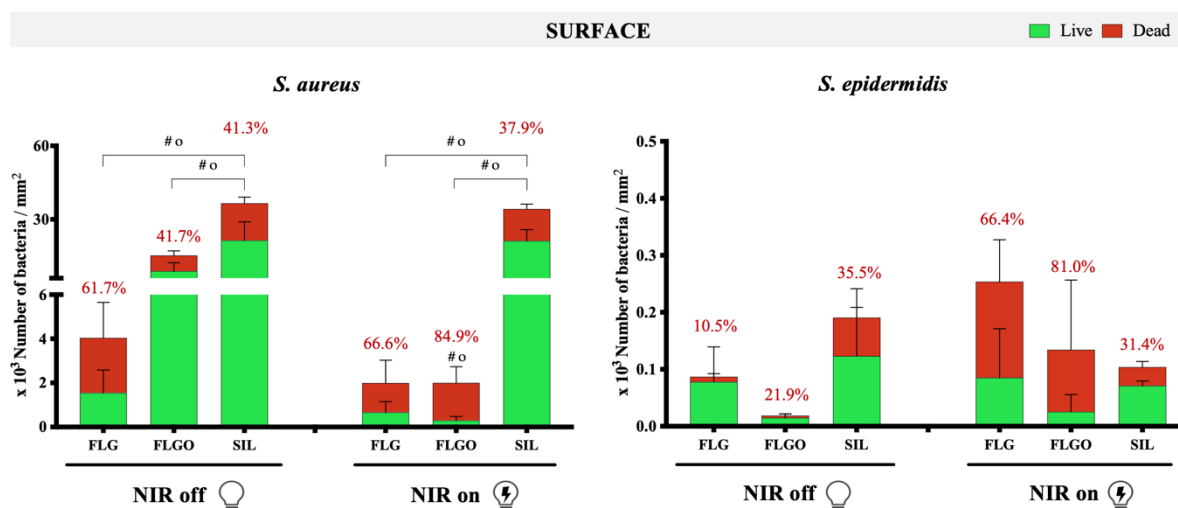


Figure 2. Antibacterial performance of FLG and FLGO films towards adherent bacteria. After 2 h incubation with *S. aureus* and *S. epidermidis*, FLG and FLGO films were either kept in the dark (NIR off) or irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Adherent bacteria were stained with Syto9 (live) or PI (dead), visualized by fluorescent microscopy (Figure S2 and Figure S3), and counted. Red numbers represent the percentage of dead bacteria. Silicone films were used as a control material. #, o – statistically significant differences between samples regarding total number (#) and dead (o) adherent bacteria ($p < 0.05$): one-way ANOVA to compare films within the same condition (either NIR off or NIR on) and unpaired t-test with Welch's correction to compare the same film between NIR off or NIR on conditions. FLG = few-layer graphene, FLGO = few-layer graphene oxide, SIL = silicone. 3 replicates of each material in each independent assay ($n = 3$).

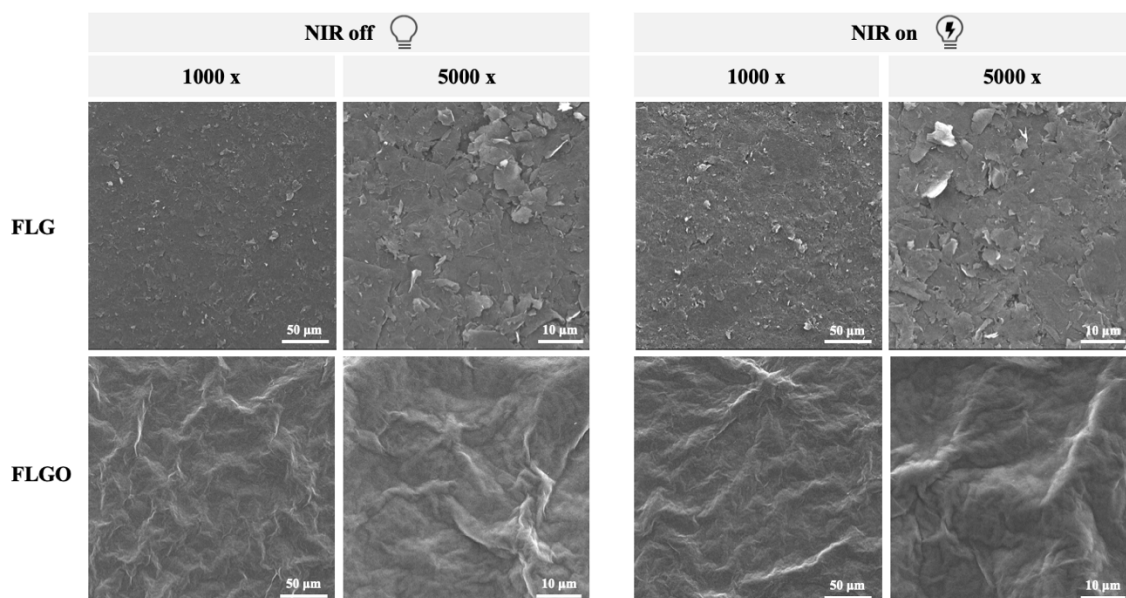
3.2. Mechanism of action

3.2.1. Surface physicochemical features

Exposure to NIR irradiation for 45 min caused no alterations in neither surface morphology (Figure 3.A) nor chemistry (Figure 3.B) of the FLG and FLGO films. Surface features were similar in both conditions (NIR off and NIR on), with sharp edges protruding in FLG films and wrinkles with similar morphology and size being observed in FLGO films. Oxidized materials presented high amounts of oxygen (~30%) and maintenance of the overall elemental composition upon irradiation, with a carbon/oxygen (C/O) ratio of 28.4 (NIR off) vs 26.75 (NIR on) for FLG films, and 2.17 (NIR off) vs 2.23 (NIR on) for FLGO films. The percentages of chemical groups, and in particular oxygen-containing groups (hydroxyl groups (C–OH), epoxy groups (C–O–C), carbonyl groups (C=O), carboxyl groups (O–C=O)), were also similar (Figure S4), confirming that neither oxidation nor reduction was induced by NIR radiation.

In the absence of irradiation, surface morphology and chemistry seem to contribute to the inherent bactericidal or anti-adhesive activity of FLG and FLGO films. However, upon exposure to NIR radiation, something else than just these features *per se* is driving the overall mechanism of action.

A. Surface morphology: SEM



B. Surface chemistry: XPS

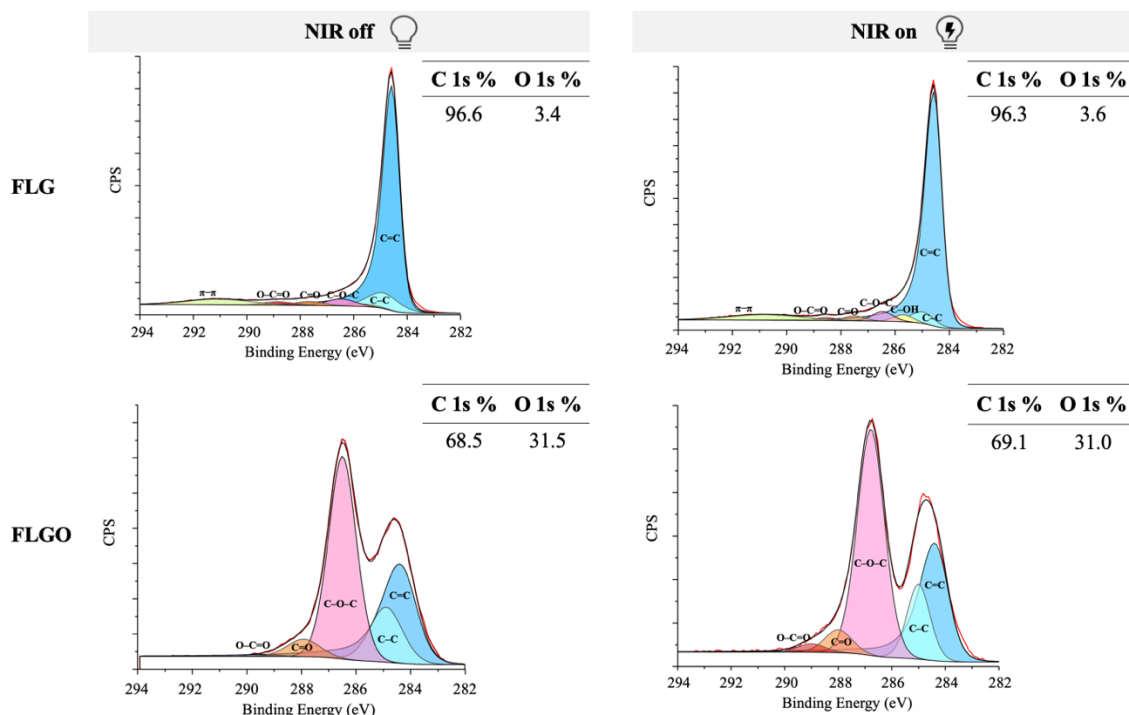


Figure 3. Physicochemical features of FLG and FLGO films. (A) surface morphology and (B) chemistry of films when kept in the dark (NIR off) and when irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Morphology and topography obtained by SEM (1000 $\times$ and 5000 $\times$ magnifications, scale bar = 50 μm and 10 μm , respectively). Atomic percentages of carbon (C 1s) and oxygen (O 1s) extracted from XPS survey spectra. Chemical group percentages are shown in Figure S4. FLG = few-layer graphene, FLGO = few-layer graphene oxide.

3.2.2. Photothermal effect

The photothermal effect relies on a local increase in the temperature to kill bacteria. Photothermal properties of FLG and FLGO films were assessed in conditions similar to the antibacterial assays (after 2h incubation in culture media at 37 °C) through evaluation of the temperature of the supernatant and surface of samples over 60 min (Figure 4). In the presence of both FLG and FLGO films, temperature of the supernatant increased during the first 30 min, remaining similar afterwards and within the suitable range for bacterial growth and viability (between 33 °C to 41 °C [52]). Surface temperature, however, showed a steep increase during the first 15 min, attaining 50 °C in FLG and 55 °C in FLGO films. After 45 min irradiation, temperatures reached 51.3 °C and 56 °C in FLG and FLGO films, respectively, allowing adherent bacteria to be subjected to high temperatures for at least 30 min, thus potentiating the photothermal-induced bactericidal effect.

In the absence of GBMs films, temperature of the culture media (TSB) stayed below 37 °C, similarly to when silicone films were irradiated. This shows that NIR alone has no capacity to induce an increase in temperature, proving the excellent photothermal efficiency derived from GBMs films, even with low intensity radiation.

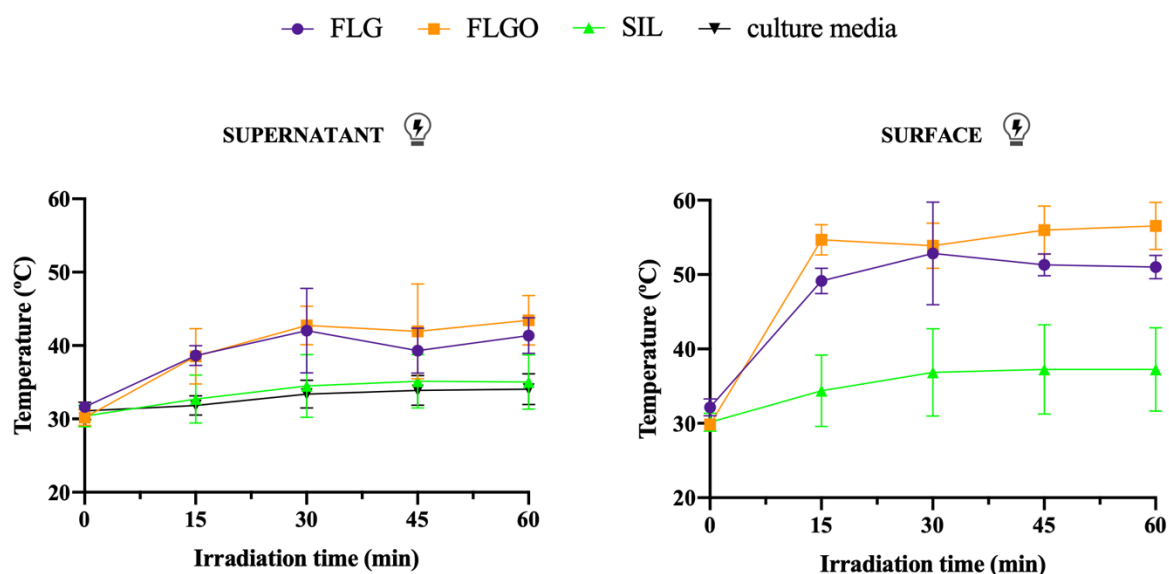


Figure 4. Photothermal performance of FLG and FLGO films after 2 h incubation with culture media (TSB) followed by irradiation (NIR on) for 60 min (812 nm, 0.150 W/cm²). NIR-induced temperature change was assessed on films surrounding supernatant and surface using a type K thermocouple microprobe. FLG = few-layer graphene, FLGO = few-layer graphene oxide, SIL = silicone.

3.2.3. Photodynamic effect

The photodynamic effect relies on ROS production to kill bacteria. This boost in ROS production disrupts the balance between ROS generation and elimination, leading to oxidative stress. GSH, an

endogenous antioxidant, is crucial to maintain this balance; its elimination potentiates the photodynamic effect by failing to eliminate ROS.

The intrinsic oxidative potential of FLG and FLGO films caused a GSH oxidation of around 75% (NIR off). Exposure to NIR irradiation (NIR on) prompted a significant increase in GSH oxidation to 95% (Figure 5.A), as denoted by a gradual paling of its typical yellow color, due to reaction between GSH and Ellman's reagent, quantitatively translated into a decrease in the absorbance at 412 nm. Silicone surfaces showed negligible oxidative potential, independently of the irradiation by NIR light.

Oxygen-containing groups on FLGO films can potentiate GSH oxidation [53], considering that more reactive defect sites are available to adsorb O₂ and water molecules, which increases surface oxides and hydroxyl radicals formation. Apart from having reactive points, FLG films conductive nature may also be stimulating GSH oxidation, with FLG acting as an electron acceptor [54].

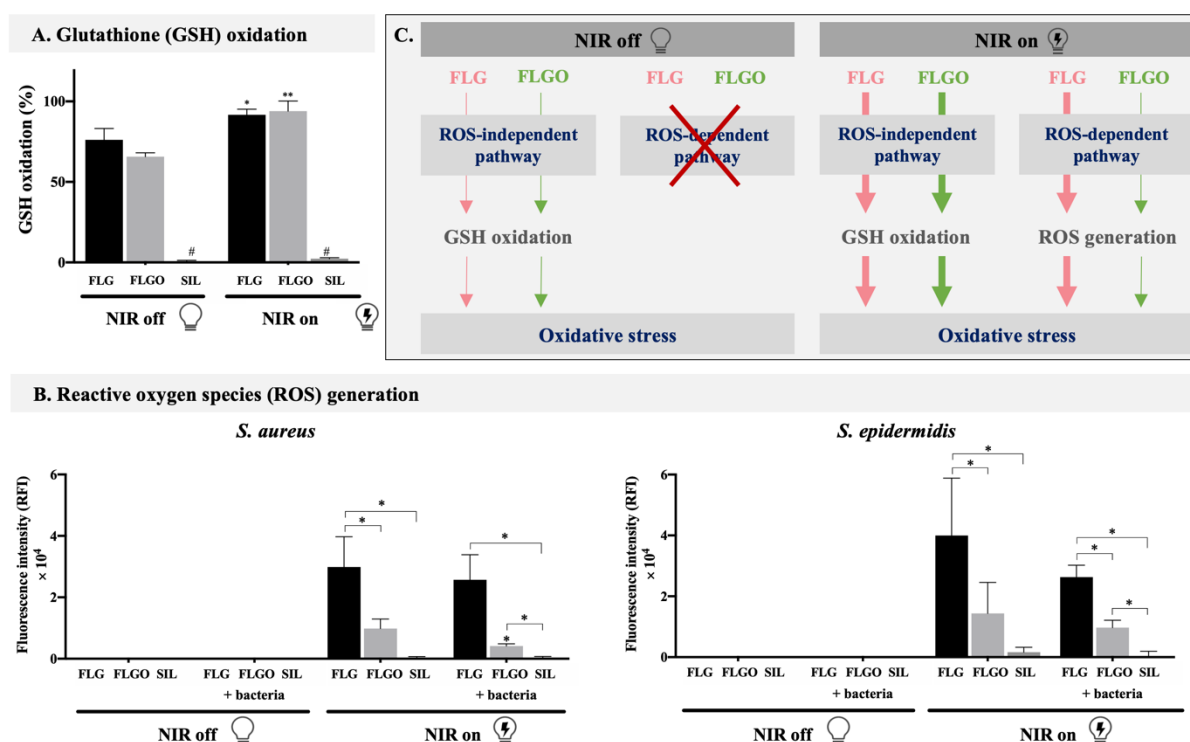


Figure 5. Photodynamic properties of FLG and FLGO films. (A) determination of glutathione (GSH) depletion by Ellman's reagent and (B) reactive oxygen species (ROS) generation by films and by films in contact with bacteria (*S. aureus* and *S. epidermidis*) using DCFH-DA probe, when samples were kept in the dark (NIR off) or irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). (C) Scheme summarizing the putative pathways for occurrence of oxidative stress. * Statistical significance, $p < 0.05$: one-way ANOVA to compare films within the same condition (either NIR off or NIR on) and unpaired t-test with Welch's correction to compare the same film between NIR off or NIR on conditions. * alone indicates significant differences from its counterpart in NIR off condition. FLG = few-layer graphene, FLGO = few-layer graphene oxide, SIL = silicone.

Agents with photodynamic action potentiate ROS production. In the absence of NIR stimulation (NIR off), all films elicited negligible amounts of DCF fluorescence, suggesting the lack of ROS in the presence of both films and films with bacteria (Figure 5.B). Exposure of films to NIR irradiation (NIR on) caused a significant increase in DCF fluorescence, indicating a NIR-triggered ROS generation as a consequence of the association between the GBMs films and NIR radiation. Silicone films showed residual levels of ROS production, independently of the condition. When the films are in the presence of bacteria, ROS levels are maintained independently of the strain used. This suggests that either bacteria are managing/balancing out ROS overproduction, which is unlikely considering their high death percentage, or ROS molecules are interacting with the bacterial membrane, leading to ROS elimination and bacterial damage or death [55, 56].

Based on both assays, an explanation for what is happening can be hypothesized (Figure 5.C). In the absence of NIR (NIR off), FLG and FLGO films actively contribute to GSH oxidation, however they did not mediate the generation of ROS. On the other hand, exposure to radiation (NIR on) augmented FLG and FLGO capacity to oxidize GSH (represented as thicker arrows) while also inducing ROS production. Despite with similar GSH oxidation, FLG showed significantly higher capacity to induce ROS production than FLGO, thus suggesting ROS-mediated killing as the dominant factor in the antibacterial activity of FLG films. The lower ability to induce ROS production indicates that FLGO films may act primarily through a ROS-independent pathway. Combination of both GSH oxidation and ROS production potentiates oxidative stress, due to inability of ROS neutralization and consequent accumulation. This increases the efficacy of the photodynamic effect, leading to collapse of cellular functions through the damage of DNA and vital enzymes and bacterial death [50].

4. Discussion

In this study, we investigated the use of stand-alone FLG and FLGO films as NIR-activated bactericidal platforms, and provided insights on the mechanism of action underlying the NIR-triggered killing effect.

Previous studies have reported an increase in the bactericidal action of different materials upon light activation (gold materials [57, 58], red [59] and black [60] phosphorous, ruthenium nanoparticles [61]), with intensity and duration strongly influencing the overall outcome [11, 23, 62]. Graphene-materials have been used as photothermal or/and photodynamic agents, potentiating the overall antibacterial action of a complex system [20, 29, 30, 63-65] (in combination with other materials) rather than acting alone (the system itself). Moreover, most of them use much more powerful light sources ($1\text{--}48\text{ W/cm}^2$), which despite allowing shorter irradiation times (seconds to few minutes), exceeds the skin tolerance threshold values set by ANSI (maximum permissible exposure of skin at 808 nm is 0.33 W/cm^2 , with an exposure time of 10–1000 s). This is therefore not suitable for clinical usage.

Our implementation of NIR irradiation demonstrates that FLG and FLGO films are efficient NIR-activated bactericidal platforms on their own, showing an outstanding bactericidal action against planktonic bacteria upon NIR exposure (almost 100% efficiency), with death of adherent bacteria reaching 65% for FLG films and 85% for FLGO films. This was achieved by low-power irradiation, which has also much lower cost per mW, is safer, lighter, and presents a higher area of irradiation/treatment than lasers [66].

Bacteria response to environmental stimuli is different when they are in suspension or embedded in a matrix and attached to a surface [11], and the process of inhibiting the development of bacteria on the surface can be different than in suspension [53]. Action towards adherent bacteria can potentially be further improved by increasing the irradiation time or augmenting the thickness of the films (increasing concentration of graphene present), as the efficacy of the thermal treatment has been shown to depend on the magnitude of the temperature increase, which depends on these parameters and on the photothermal ability of the agents [20, 63].

The antibacterial activity of FLG and FLGO films towards adherent bacteria had been previously evaluated in the absence of external light stimulation [34]. The bactericidal effect of the films is however highly potentiated upon NIR radiation, demonstrating its key role, independently on the bacterial strain and bacterial adhesion. While the higher adhesion of *S. aureus* may be related with high amounts of adhesins present on *S. aureus* surface and their higher affinity to adsorbed plasma proteins on the surface [67], the lower adhesion of *S. epidermidis* may be particularly relevant considering their role in medical-related infections.

In general, the antibacterial mechanism of action of graphene-based surfaces has been associated with 1) physical/mechanical disruption of the membrane, which leads to the release of the intracellular content, 2) exacerbated generation of ROS that causes oxidative stress through a ROS-dependent pathway, and 3) disruption of a specific signaling process or structure that causes oxidative stress through a ROS-independent pathway [68]. Not only exacerbated ROS production promotes oxidative stress, but also elimination of natural antioxidants, an event that potentiates the unbalance. Furthermore, elevated temperatures contribute to the antibacterial action, with most bacteria viability decreasing when subjected to a temperature greater than 45–50 °C, mostly due to enzyme denaturation and protein and lipids damage on the cell membrane [26, 42].

Different mechanisms of action are therefore proposed depending on the physicochemical nature of the films (surface morphology and chemistry, thus FLG and FLGO films) and on the external stimulation by NIR radiation (NIR off and NIR on) (Figure 6).

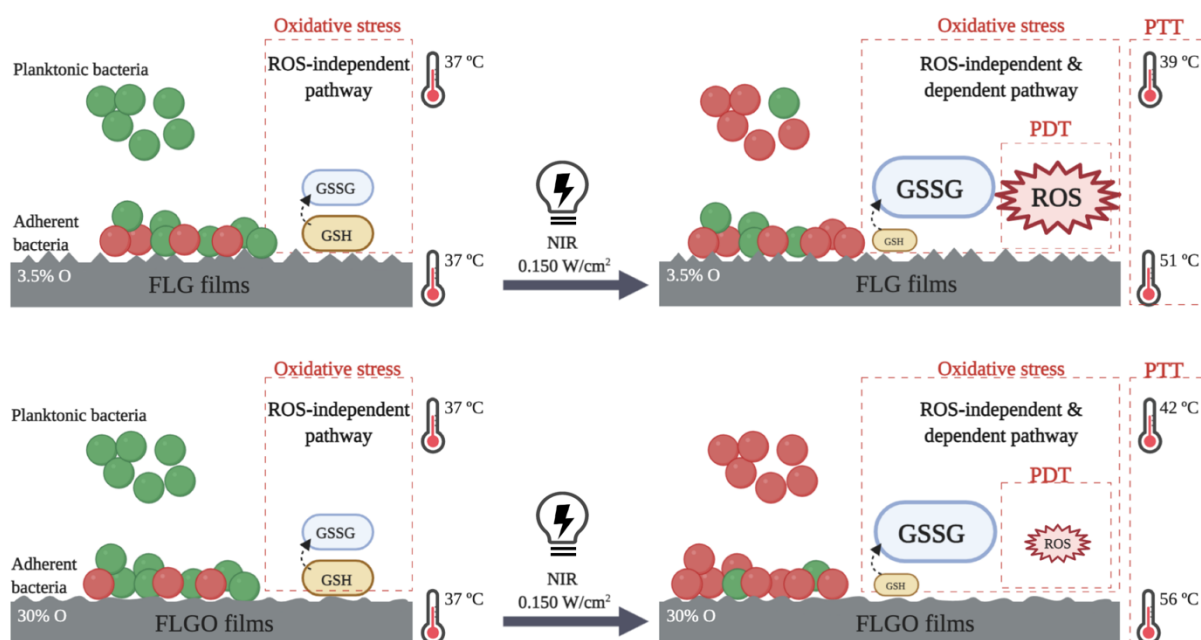


Figure 6. Mechanism of action of FLG and FLGO films towards bacteria when kept in the dark (NIR off, left) and upon irradiation (NIR on, right). GSH = glutathione, GSSG = glutathione disulfide (oxidized glutathione), ROS = reactive oxygen species, PDT = photodynamic therapy, PTT = photothermal therapy, NIR = near-infrared, FLG = few-layer graphene, FLGO = few-layer graphene oxide.

Physical damage of the bacterial membrane has been mostly associated with FLG sharp edges, which in our films are protruding from the surface. However, action of the edges of the oxidized materials, as GO [63] and rGO [69], has also been reported towards bacteria, with some authors referring to this physical effect has having a higher contribution to the materials' antibacterial action than oxidative stress [63, 69]. In the present work, incubation of films with bacteria for 2 h may promote membrane disturbance and/or irreversible damage due to interaction with the sharp edges from FLG films or with the wrinkles from FLGO films. In addition, electron transference and charge interaction can occur due to the conductive nature and presence of oxygen-containing groups in FLG and FLGO films, respectively, further contributing to the overall destabilization of the membrane. Considering the undetectable ROS production in the absence of light stimulation, physical damage associated with intrinsic ability to cause GSH oxidation seems to be powering the observed antibacterial action, supporting a ROS-independent pathway of oxidative stress.

Upon NIR exposure, even though surface chemistry and morphology may weaken the bacterial membrane, the major contribution to the overall antibacterial activity comes from the photothermal and photodynamic action of the graphene materials. The photothermal properties of graphene, *i.e.*, its ability to convert light into heat, causes surface temperature to rise to 51.3 °C in the presence of FLG and to 56 °C with FLGO films. The small temperature difference between both films, however, suggests that the improvement in the antibacterial performance would be mainly attributed to the photodynamic action. This is associated with the ability to induce ROS production, which combined to GSH depletion, can

greatly contribute to oxidative stress – as the degree of oxidative stress relies on competing actions of ROS generation and ROS scavenging reactions. Based on the results of DCFH-DA assay, FLG films potentiate ROS generation in a higher extent than FLGO films. Even though oxidized materials are generally associated with higher ROS production due to higher density of oxygen-containing functional groups [56], the conductive nature of FLG films may allow them to act as an electron pump (causing disruption or oxidation of cellular structures without ROS production and through charge transfer from extracellular membrane to graphene). Adsorption of environmental oxygen can still occur, but in a smaller extent.

The higher depletion of GSH by the films due to augmented oxidative potential upon irradiation, greatly contributes to enhance PDT efficiency, since GSH presence acts as ROS scavenger [70]. This can be particularly relevant in the case of FLGO films where GSH depletion can potentiate the effect of the lower levels of ROS, while implying that a predominant ROS-independent pathway for oxidative stress is taking place. Temperature may also be playing a more active role in the mechanism of action of FLGO irradiated films, considering the lower ROS production and the greater bactericidal action. During irradiation, exposure to high temperatures for at least 30 min may be weakening bacteria membrane structure, which associated with ROS overproduction and GSH depletion may allow excessive amounts of ROS to enter bacteria more easily, contributing to the disruption of the intracellular redox balance. The presence of dissolved O₂ in the media can also be contributing to ROS levels, as demonstrated by Sang *et al.*, [71] where its deficiency resulted in declined ROS levels. Singlet oxygen (¹O₂) produced can also enhance the permeability and thermal sensitivity of the bacterial cell membrane [59]. On the other hand, the presence of ROS can also contribute to increase the permeability of damaged bacterial membranes, making them more sensitive to heat, and thus accelerating the leakage of intracellular content from bacteria [27].

Finally, considering the maintenance of the surface chemistry and morphology, stand-alone films and coatings produced by other techniques using the same GBMs should provide a similar PTT/PDT action upon NIR irradiation. These materials can therefore be applied in various healthcare applications where bacterial adhesion and infection is a concern (such as medical devices and implants), and in other non-medical applications also requiring sterilization and disinfection (such as food packaging and water treatment). Even though our approach was designed to operate with NIR light in the transparency window of tissues, making it compatible with an *in vivo* setting, in non-medical applications irradiation intensity can be increased, thus potentially increasing efficiency and reducing the exposure time to obtain the same performance. Therefore, we strongly believe that these platforms can be used for effective and efficient sterilization for a diverse range of applications.

5. Conclusions

Stand-alone FLG and FLGO films can be broadly used as light-activated platforms for the effective killing of bacteria (up to ~99% for planktonic and ~85% for adherent bacteria) under low power near-infrared light exposure (0.150 W/cm²). The system acts through a synergistic and interdependent combination of photothermal (PTT) and photodynamic (PDT) actions, with PDT efficacy being enhanced by the films' ability to reduce GSH levels (high oxidative potential). Upon exposure to NIR, FLG films performance towards bacteria occurs through a combined action of high surface temperature (51.3 °C), ROS overproduction and elevated GSH depletion. This suggests a predominant ROS-dependent oxidative stress pathway, with the action of sharp edges contributing positively to the overall antibacterial effect. Showing higher bactericidal action than FLG but lower production of ROS, FLGO films are hypothesized to act primarily through a ROS-independent pathway, based on GSH depletion and increased membrane destabilization due higher temperatures generated by NIR absorption (56 °C).

Altogether, our results report GBMs as stand-alone efficient antibacterial PTT/PDT platforms, when irradiated with low power LEDs. This opens the path for new treatments for resistant bacterial infections and also for innovative disinfection surfaces/systems, with widespread use in a range of medical and non-medical applications.

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Supplementary Information

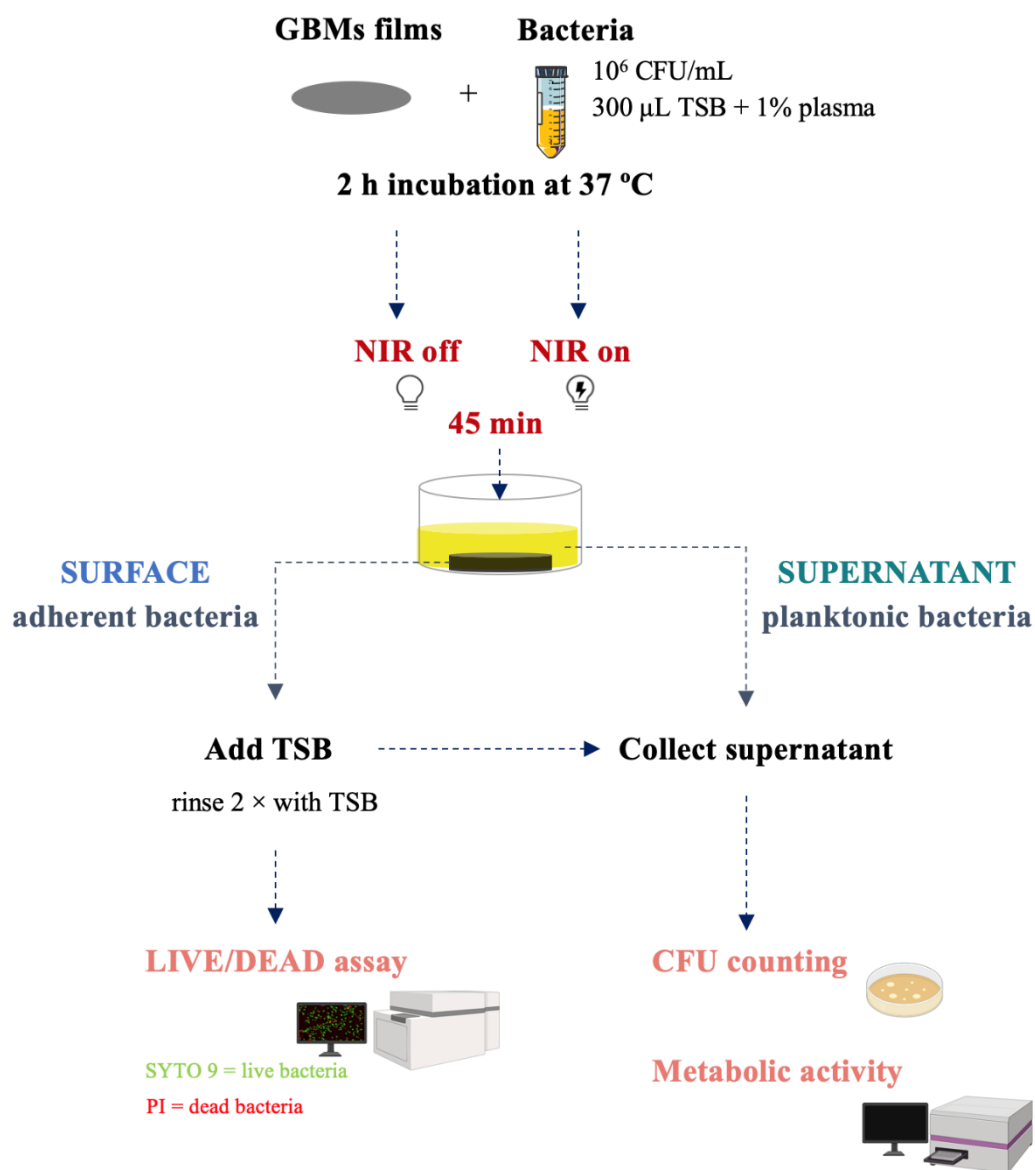


Figure S1. Antimicrobial assay procedure. After 2 h incubation of FLG and FLGO films with *S. aureus* or *S. epidermidis* to allow bacterial adhesion samples were either kept in the dark (NIR off) or irradiated (NIR on, 812 nm, 0.150 W/cm²) for 45 min. Viability of adherent and planktonic bacteria was analyzed by live/dead fluorescent assay, and colony forming unit (CFU) and metabolic activity assays, respectively.

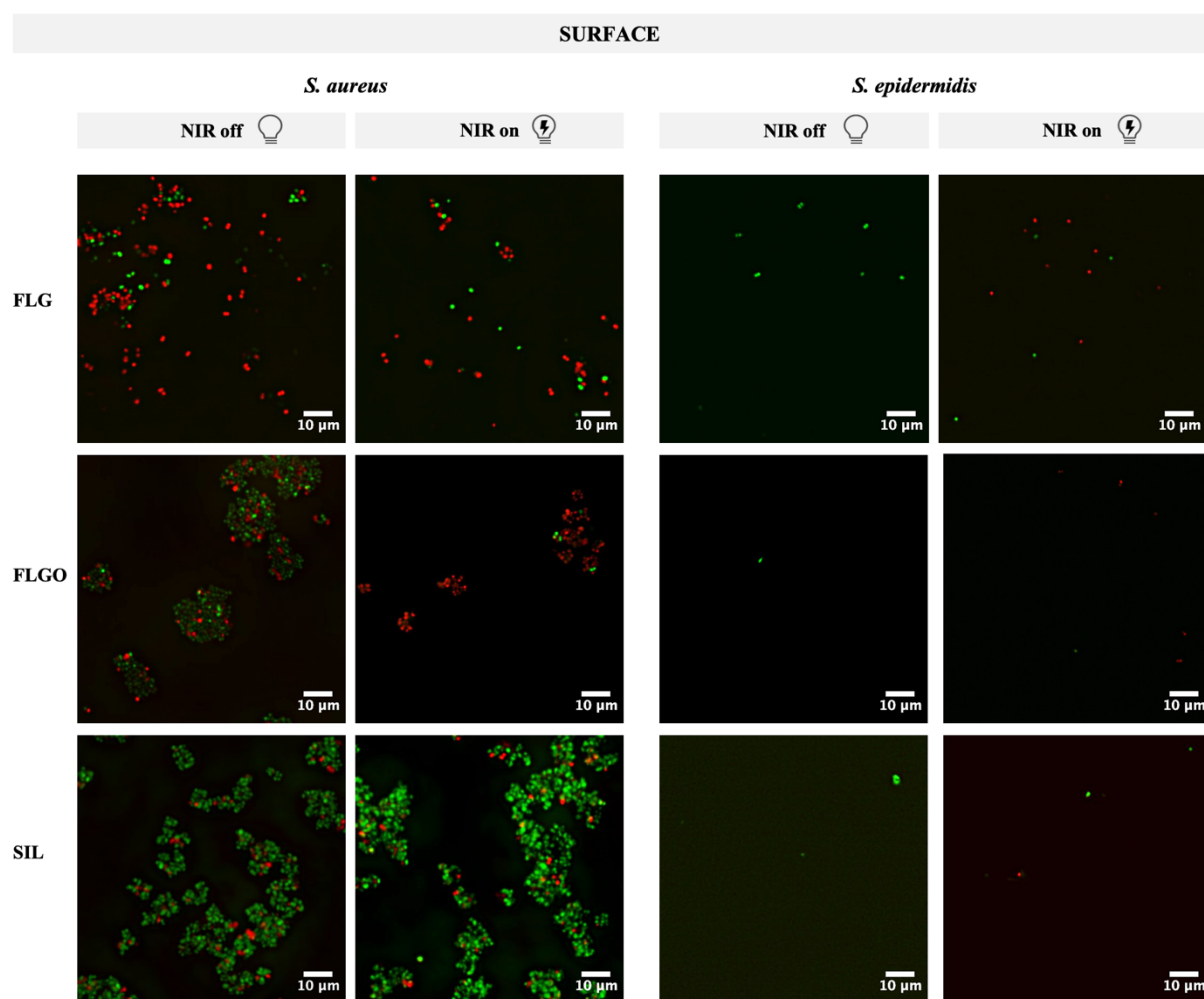


Figure S2. Adherent bacteria on FLG, FLGO and SIL films. After 2 h incubation with *S. aureus* and *S. epidermidis*, FLG and FLGO films were either kept in the dark (NIR off) or irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Adherent bacteria were stained with Syto9 (live) or PI (dead) and images were obtained by IN Cell Analyzer 2000 (scale bar = 10 μ m).

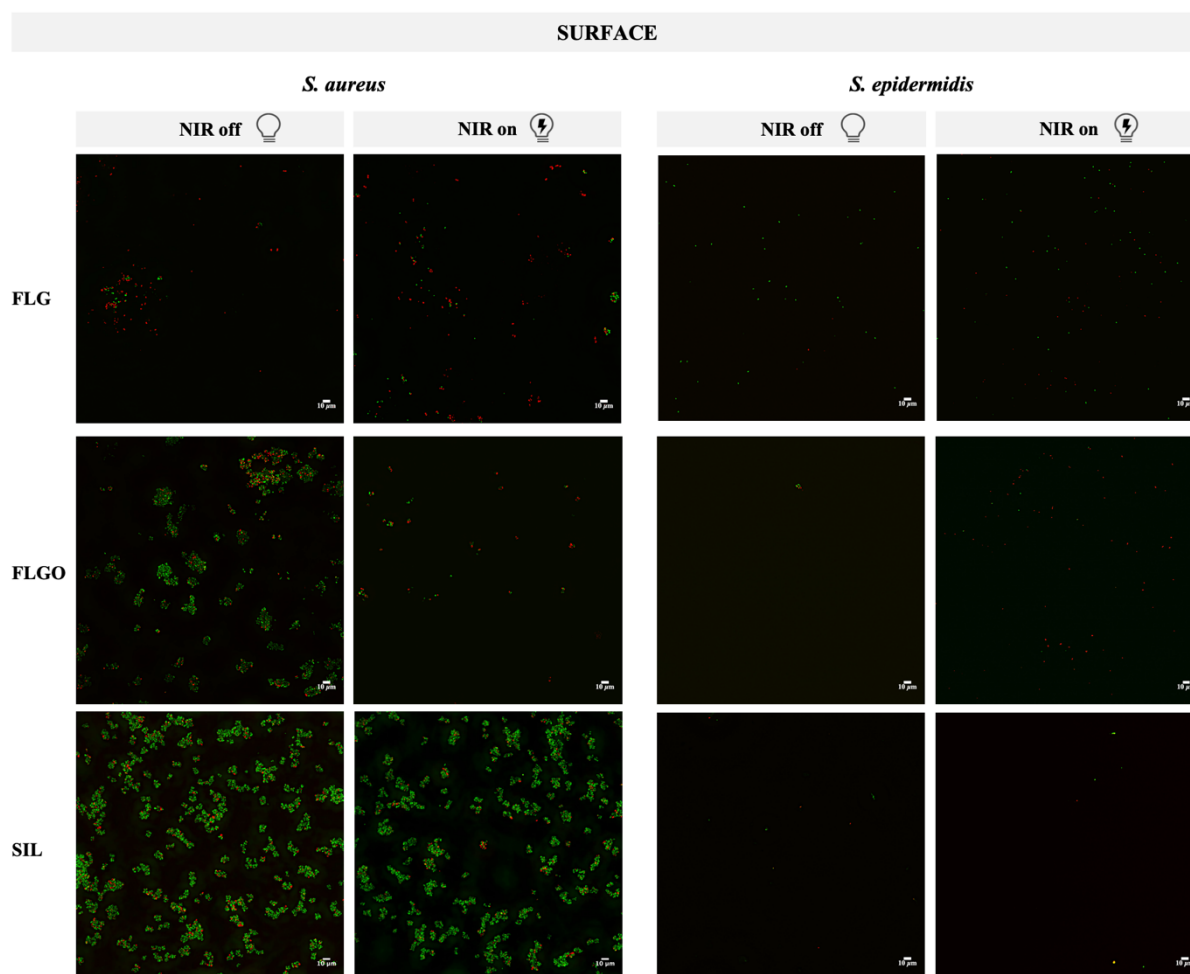


Figure S3. Zoom out of adherent bacteria on FLG, FLGO and SIL films. After 2 h incubation with *S. aureus* and *S. epidermidis*, FLG and FLGO films were either kept in the dark (NIR off) or irradiated (NIR on) for 45 min (812 nm, 0.150 W/cm²). Adherent bacteria were stained with Syto9 (live) or PI (dead) and images were obtained by IN Cell Analyzer 2000 (scale bar = 10 μ m).

	NIR off 		NIR on 	
	FLG films	FLGO films	FLG films	FLGO films
C=C	76.9%	33.6%	80.2%	23.0%
C-C	8.5%	14.8%	4.5%	21.5%
C-OH	1.2%	---	2.2%	---
C-O-C	3.4%	46.7%	3.4%	48.3%
C=O	1.9%	4.7%	1.7%	5.7%
O-C=O	1.9%	0.2%	1.1%	1.4%
π-π	6.3%	---	6.9%	---

Figure S4. Chemical composition of the surfaces when kept in the dark (NIR off) or irradiated (NIR on) for 45 min. Exposed functional groups percentage extracted from XPS survey spectra: double carbon bonds (C=C), simple carbon bonds (C-C), hydroxyl groups (C-OH), epoxy groups (C-O-C), carbonyl groups (C=O), carboxyl groups (O-C=O), and pi-pi bonds (π - π).

CHAPTER VI

**Can GBMs be incorporated in polyurethane
to make it antibacterial?**



Article

Exposure of Smaller and Oxidized Graphene on Polyurethane Surface Improves its Antimicrobial Performance

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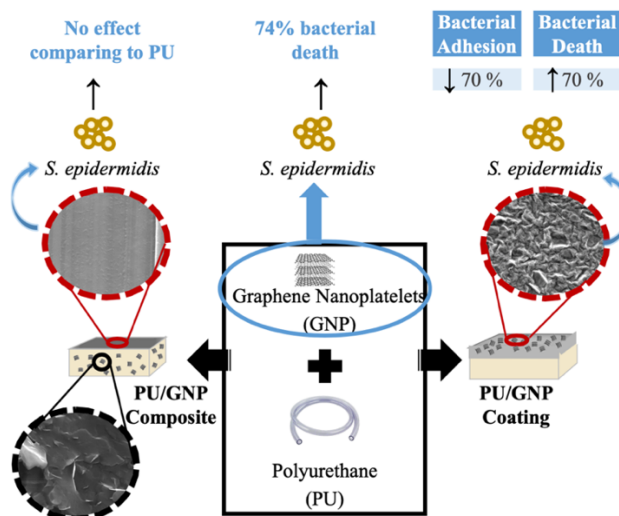
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Abstract

Catheter-related infections are a common worldwide health problem, highlighting the need for antimicrobial catheters. Here, antibacterial potential of graphene nanoplatelets (GNP) incorporated in the commonly used polymer for catheter manufacture—polyurethane (PU)—is investigated. Two strategies are explored: melt-blending, producing a composite, and dip coating, where a composite layer is deposited on top of PU. GNP with different lateral sizes and oxidation degrees—GNP-M5, GNP-M15, GNP-M5ox, GNP-M15ox—are applied in both strategies, and the antimicrobial potential towards *Staphylococcus epidermidis* of GNP dispersions and GNP-containing PU evaluated. As dispersions, oxidized and smaller GNP powders (GNP-M5ox) inhibit 74% bacteria growth at 128 µg/mL. As surfaces, GNP exposure strongly impacts their antimicrobial profile: GNP absence at the surface of composites yields no significant effects on bacteria, while by varying GNP: PU ratio and GNP concentration, coatings enhance GNP exposure, depicting an antimicrobial profile. Oxidized GNP-containing coatings induce higher antibacterial effect than non-oxidized forms, particularly with smaller GNPOx, where a homogeneous layer of fused platelets is formed on PU, leading to 70% reduction in bacterial adhesion and 70% bacterial death. This pioneering work unravels how to turn a polymer clinically used to produce catheters into an antimicrobial surface, crucial to reducing risk of infection associated with catheterization.

Graphical Abstract



Keywords: biomaterials; catheter-related infections; bacterial adhesion; composites; coatings.

1. Introduction

Catheters are medical devices routinely used in patients in different healthcare centers [1]; catheter-related infections (CRI) are however a recurrent and threatening problem, especially if progressing to bloodstream infection, the third leading cause of hospital-acquired infections. These can cause morbidity, prolonged hospitalizations and ultimately death [2], being associated with elevated medical costs and therefore representing a serious worldwide health problem [3, 4]. CRI are mainly caused by colonization of the catheter hub or tubing by microorganisms from the patient's skin or external sources—*Staphylococcus epidermidis* being the major responsible one [5]—but also by contamination of the catheter lumen by circulating bacteria [3, 6, 7]. Treatment involves catheter replacement or removal, and antibiotic therapy—inefficient in most cases of biofilm formation [3] and associated with bacterial resistance [7]. To overcome and prevent CRI, apart from better clinical practices during catheter insertion/manipulation, new technologies and products have been continuously applied in the clinics, namely impregnation of catheters and dressings with antiseptics or antibiotics [3, 4, 8]. Other strategies are being investigated, focusing on conferring anti-adhesive and/or bactericidal properties through surface modification of the medical-grade polyurethane (PU)—a thermoplastic elastomer from which most catheters are made of [9, 10]. Strategies include physical and chemical modifications such as incorporation or coatings with antibiotics or antiseptics [11-13], topography modification with micropatterns [14], functionalization or coating with antimicrobial peptides (AMPs) [15] and antimicrobial nanomaterials, such as silver nanoparticles (Ag NPs) [16], titanium oxide (TiO₂) NPs [17], carbon nanotubes (CNT) [18], bioactive compounds [19], and graphene oxide (GO) [20]. Approaches

that depend on eluting bactericidal agents have many drawbacks, namely the limitation of the amount loaded, difficulty in controlling the release rate and the concentration of the released agents during the application period [21, 22]. In particular, antiseptics and antibiotics can induce bacteria resistance as a consequence of their prolonged use [23] and loose efficiency along time due to leaching [8]. Therefore, permanent modification of the catheter surface with antimicrobial properties is the ideal solution, aiming preventive strategies that act at the frontline by avoiding bacteria adhesion, colonization and consequent biofilm formation.

Graphene-based materials (GBM), a new family of nanomaterials, have attracted a lot of attention in the scientific community, with increasing interest and potential in biomedical applications [24-28]. The antibacterial effect of these materials was first explored in suspension in 2010 by Hu *et al.* [29] and since then increasing number of studies have described GBM as having antibacterial activity, making them strong candidates for antimicrobial applications [30-32]. Antimicrobial properties of these materials have been addressed in a large number of reviews [33-39], but mostly in suspension; their antimicrobial performance is however different when incorporated in surfaces [40, 41]. The mechanisms of action have been thoroughly explored, with recently published reviews covering the most important conclusions so far. As suspensions, GBM act either through wrapping, isolating bacteria from the environment, or through insertion or nano knife, physically piercing the membrane, although oxidative stress can also be stimulated [31, 42]. As surfaces, they act after direct contact with bacteria, with predominant mechanisms depending on the exposure of either basal planes or sharp edges: electron transference and oxidative stress induction occur mostly when only basal planes are exposed, while besides these, physical damage of the membrane (due to insertion or protein-protein destabilization) can also occur when both structures contact bacteria [40, 43, 44]. Very few studies report the use of GBM to confer antibacterial properties to PU [45-47]: An *et al.* produced polylactic acid (PLA)/PU composite films reinforced with GO with antibacterial activity against both *Staphylococcus aureus* and *Escherichia coli* [45]; in another work, hyperbranched polyurethane/reduced GO decorated with sulphur nanoparticles (HPU/SRGO) composite showed microbial inhibitory effects against *S. aureus*, *E. coli* and *Candida albicans*, inhibiting the growth rate and causing membrane disruption [46]; in the study of Tang *et al.*, PU composites incorporated with GO and with slightly reduced GO modified with polyethylenimine (SRGO-PEI) showed an antiadhesive effect towards *E. coli* [47]. However, most of the times, either PU or GBM are combined with other polymers or materials, preventing the understanding of their effective role on the antibacterial performance; GBM type and oxidation state as well as the influence of the method of incorporation and degree of GBM exposure at the surface are seldom evaluated.

This work aims to incorporate graphene nanoplatelets—GNP, composed of 2 to 10 graphene sheets—into PU through two different methods compatible with catheter fabrication process—melt-blending and dip coating—and investigate the antimicrobial potential of the obtained surfaces for application on PU catheters aiming to prevent catheter-related infections. The antibacterial potential

towards *S. epidermidis* of GNP powders with different lateral size and oxidation degree—GNP-M5 (5 μm), GNP-M15 (15 μm) and oxidized forms, GNP-M5ox, GNP-M15ox—was initially assessed and the impact of these parameters on GNP exposure and antimicrobial potential of the obtained surfaces was investigated. Since in GNP-related literature, evaluation fails in the complete assessment of their antibacterial properties and adaptability to different mechanisms of antimicrobial surface design (antimicrobial-releasing, contact-killing and non-adhesiveness) [48], in our study bacteria viability and quantification were conducted, both in the supernatant and adherent to the surface, by adaptation of ISO 22196.

2. Materials and Methods

2.1. Oxidation of Graphene Nanoplatelets (GNP)

Graphene nanoplatelets of grade M (xGnP[®] Graphene Nanoplatelets Grade M) with 5 μm (GNP-M5) and 15 μm of diameter (GNP-M15) were purchased from XG Sciences (Lansing, USA). Oxidized forms of GNP-M5 and GNP-M15, designated GNP-M5ox and GNP-M15ox, were produced by a modified Hummers' method (MHM) using a GNP-M:KMnO₄ mass ratio of 1:6. 4 g of GNP were added to a mixture of 160 mL of 95%–97% H₂SO₄ (VWR, Darmstadt, Germany) and 40 mL of H₃PO₄ (VWR, Germany) [49-51]. Oxidized GNP (GNPox) was repeatedly washed by resuspending in distilled water (*d*H₂O) and centrifuging until the pH of the decanted washing water was close to the one of *d*H₂O. To obtain dried GNPox for composite production, freeze-drying was performed at a temperature of $-80\text{ }^{\circ}\text{C}$ and at a pressure of 0.008 mBar for three days.

2.2. Production of GNP-Containing Polyurethane (PU)

GNP-containing polyurethane surfaces were produced using two approaches: melt-blending, originating PU/GNP composites that were composed of PU with GNP as nanofiller, and dip coating, yielding PU/GNP coatings, where a thin composite layer of GNP dispersed in PU was deposited on top of PU films.

2.2.1. Production of PU/GNP Composites

Pellets of Tecothane TT-1095A medical grade polyurethane (PU) were purchased from Lubrizol LifeSciences (Ohio, USA). PU composites with non-oxidized (GNP-M5 and GNP-M15) or oxidized (GNP-M5ox and GNP-M15ox) GNP powders were produced by melt-blending in a Micro-Compounder Xplore (DSM) MC5 (5 mL capacity, vertical, co-rotating twin-screws) at $200\text{ }^{\circ}\text{C}$ and mixing for 3 min with a screw speed of 50 rpm, followed by injection molding performed using the Xplore micro injection moulder IM5.5. (Xplore Instruments BV, Sittard, The Netherlands). Different GNP contents were tested (0.5, 1, 5 and 10 wt%) and dog bone shaped composites obtained (Figure S1).

2.2.2. Production of PU/GNP Coatings

2.2.2.1. PU Films

PU films were prepared by solvent casting [52]. Briefly, PU pellets were dissolved at 12.5% w/v in tetrahydrofuran (THF, JMGS, Odivelas, Portugal), and 80 mL of this solution were casted into clean glass petri dishes of 14 cm of diameter. Petri dishes were covered with aluminum foil and left at room temperature (RT) for 48 h for slow evaporation of THF; films were removed and cut into squares of $2 \times 2 \text{ cm}^2$.

2.2.2.2. PU/GNP coatings on PU films

Coating formulations containing PU and GNP were prepared by solvent mixing. Briefly, GNP dispersions were prepared in dimethylformamide (DMF, Fisher Scientific, UK) at different concentrations (0.25, 0.5 and 1 mg/mL) with mechanical stirring and three periods of 1.5 min ultrasonication using a Hielsher UIP 1000 probe (1000 W, 20 kHz). Then, PU solution in DMF (50 mg/mL) was added to the GNP dispersions in different GNP to PU weight proportions (1:1, 1:0.5 and 1:0.25—diminishing the quantity of PU in the coating formulation), under continuous stirring followed by three periods of 1.5 min ultrasonication, to obtain homogenous PU/GNP dispersions in DMF. PU films were immersed vertically in the coating formulations for 30 s and dried vertically at 60 °C for 12 h in a vacuum oven.

2.3. Characterization of GNP Powders and GNP-Containing Polyurethane

2.3.1. Transmission Electron Microscopy (TEM)

Size of GNP powders was confirmed based on images obtained from TEM using a JEOL JEM 1400 TEM (Tokyo, Japan) coupled to a digital camera CCD Orious 1100 W (Tokyo, Japan) at the Histology and Electron Microscopy Service (HEMS, i3S, Porto, Portugal). For that, a small amount of dry GBMs were dispersed in water and dropped on 200 mesh copper TEM grids.

2.3.2. Scanning Electron Microscopy (SEM)

Morphology of GNP powders and GNP-containing polyurethane—PU/GNP composites and PU/GNP coatings—was observed using a high-resolution SEM FEI Quanta 400FEG, with acceleration voltage of 10 or 15 kV. Powder samples and PU/GNP coatings were placed directly on conductive carbon tape. Composites were fractured transversely after freezing with liquid nitrogen, placed on carbon tape and both the surface and the transversal fracture surface were analyzed. To improve surface conductivity, all samples were sputtered with Au/Pd using a SPI Module Sputter Coater equipment for 50 s using a 15 mA current.

2.3.3. X-Ray Photoelectron spectroscopy (XPS)

Chemical composition and oxidation degree of GNP powders and selected PU/GNP coatings were determined by XPS analysis. Measurements were performed using a Kratos Axis Ultra HSA equipment with a monochromatic Al X-Ray source operating at 15 kV (90 W). The analyzer pass energy was 80 eV for survey spectra and 40 eV for C 1s high-resolution spectra. Analyzed scanning area was close to 5 mm² and photoelectron take-off angle was 90° for powders and 70° for coating analysis. The effect of the electric charge was corrected by setting the reference of the C 1s peak to 284.6 eV. Spectra deconvolution was performed with CasaXPS processing software (version 2.3.16), using background type Shirley and Gaussian–Lorentzian (70:30) function to fit all peaks with exception of sp² carbon peak, fitted using an asymmetric Lorentzian function with asymmetry parameter of 0.14 [53]; peak positions were established based on literature data [54].

2.3.4. X-Ray Diffraction (XRD)

Oxidation degree, thickness, interlayer spacing between sheets, and crystallinity of GNP powders were determined by XRD analysis. Measurements were performed in a SmartLab Rigaku® diffractometer (Rigaku, Tokyo, Japan) that operates with 45 kV and 200 mA and has a Cu-K α radiation source (wavelength of 1.540593 Å) in a Bragg-Brentano configuration. Samples were measured in a rotative system (30 deg min⁻¹), in the range of 5–50 theta (2 θ), with a step of 0.01°. Spectra were baseline corrected.

2.3.5. Raman Spectroscopy

Presence of GNP on the surface of selected PU/GNP coatings was evaluated by Raman spectroscopy. Spectra were acquired using a confocal Raman microscope, LabRAM HR800 UV, Horiba Jobin-Yvon, with a laser excitation of 633.3 nm and acquisition time of 20 sec in a depth of 50 μ m. A 100 $\times$ objective (Olympus) lens was used. Between two and four spectra ($\sim$ 19 μ m² each) were acquired for each sample, followed by baseline correction, and results are presented as mean of the spectra.

2.3.6. Water Contact Angle

Wettability of GNP-containing polyurethane was evaluated by water contact angle measurements using the dynamic sessile drop method and performed with an OCA 15 goniometer equipped with a video CCD camera and SCA 20 software (DataPhysics, Filderstadt, Germany). Samples were placed in a closed thermostatic chamber (25 °C) saturated with *d*H₂O to prevent droplet evaporation. Drops of 4 μ L of *d*H₂O were placed on samples surface and images were taken 30 times/min during 2 min. Droplet profiles were fitted using Young-Laplace mathematical function. Water contact angles were calculated by extrapolating the time-dependent curve to zero. Results are the average of six measurements on six independent samples.

2.3.7. Rubbing Test

GNP adhesion of the PU/GNP coatings to the PU films was determined by rubbing a white eraser on samples surface with constant pressure. The resultant shade of grey on the rubber surface was used as a qualitative indication of GNP detachment.

2.4. Antibacterial Activity Assays

2.4.1. Bacterial Strains and Growth Conditions

Staphylococcus epidermidis (*S. epidermidis*, ATCC 35984), clinically isolated from catheter sepsis, was obtained from the American Type Culture Collection. Bacteria were grown in Trypticase Soy Agar (TSA, Merck, Darmstadt, Germany) plates overnight at 37 °C and used immediately or stored at 4 °C up to one week. Two colonies were collected, inoculated into 5 mL of Trypticase Soy Broth (TSB, Merck) and cultured overnight at 37 °C under stirring at 150 rpm.

2.4.2. Antibacterial Activity of GNP Dispersions

Overnight culture of *S. epidermidis* was adjusted to 2×10^5 Colony-Forming Units (CFUs)/mL in fresh TSB medium. GNP-M5 and GNP-M15 powders and respective oxidized forms (GNP-M5ox and GNP-M15ox) were dispersed in *d*H₂O using ultrasound bath; 2-fold serial dilutions were prepared with concentrations ranging from 2 to 1024 µg/mL. 100 µL of each of the different dilutions were added to 100 µL of the initial bacterial inoculum, obtaining a final bacteria concentration of 1×10^5 CFU/mL and GNP concentration ranging from 1 to 512 µg/mL. Incubation was performed for 18–24 h at 37 °C under static conditions in a 96-well plate. Controls of TSB, inoculated TSB and materials suspensions in TSB (without bacteria) were performed. To avoid water evaporation, empty wells of the 96-well plate were filled with *d*H₂O and the plate was placed in a container with moistened paper. The determination of the minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) is commonly performed by visual analysis of bacterial growth inhibition on the bottom of the wells and by plating the content of the first three wells where visible growth is not observed, respectively [55]. However, since the presence of GNP turns the dispersions black, visual assessment of bacterial growth was not possible in this case. As such, for MIC determination, the metabolic activity of bacteria was evaluated by Alamar blue assay. For that, 20 µL of resazurin were added to each well and incubated for 3 h at 37 °C. Then, 100 µL were transferred to a 96-well black plate and the relative fluorescence units (RFUs) (λ_{ex} : 530 nm and λ_{em} : 590 nm) were measured in a microplate fluorometer (Spectra Max GeminiXS, Molecular Devices). Wells where reduced metabolic activity was detected, and therefore considered MICs, were selected for MBC evaluation. This was performed by CFUs counting, where viable bacteria are counted by the agar plate culture method. For that 10-fold serial dilutions were made and three drops of 10 µL per dilution were plated in TSA and incubated overnight at 37 °C. Finally, the CFUs were counted for cell viability evaluation.

2.4.3. Antibacterial Activity of GNP-Containing Polyurethane

PU/GNP composites were cut into squares of $1 \times 1 \text{ cm}^2$ and PU/GNP-coated films were cut into disks of 14 mm diameter. All samples were rinsed 3 times with $d\text{H}_2\text{O}$ to remove possible impurities resultant from the cutting process, dried with argon (Ar) and sterilized using ethylene oxide.

The antibacterial activity of the surfaces was assessed following an adaptation of the standard “ISO 22196—Measurement of antibacterial activity on plastics and other nonporous surfaces”. Overnight culture of *S. epidermidis* was adjusted to a target concentration of 6×10^5 CFUs/mL in fresh TSB medium. A drop of 15 μL bacterial suspension was placed on top of surfaces and a 9 mm diameter polypropylene (PP) sterile film was placed on top to promote contact between bacteria and the sample surface. Empty wells were filled with $d\text{H}_2\text{O}$ and plates placed inside a container with moistened paper to avoid water evaporation. Samples were incubated for 24 h under static conditions at 37 °C. PU controls were used to measure viable cells immediately after inoculation to determine the recovery rate of bacteria from the test specimens. Experiments were conducted with five replicates of each sample and replicates of each sample were incubated with only TSB medium as contamination controls. After incubation, samples were rinsed with 1.5 mL of fresh TSB to detach the PP film and loosely adherent bacteria. The supernatant was collected to assess planktonic bacteria and the samples surfaces were analyzed regarding adherent bacteria.

2.4.3.1. Adherent Bacteria

Bacteria adherent to the surfaces were assessed by staining total and dead bacteria through DNA staining. Samples were rinsed twice with sterile phosphate-buffered saline (PBS), and for total bacteria staining the composites were incubated with 5 $\mu\text{g/mL}$ Hoechst 33342 (Molecular Probes, Oregon, USA) at 37 °C and coatings with 5 μM Draq5 (BioStatus, Leicestershire, UK) at RT for 15 min and protected from light, due to autofluorescence issues of PU. Samples were then incubated with 1.25 $\mu\text{g/mL}$ propidium iodide (PI) (Molecular Probes, Oregon, USA) at RT and protected from light, which only penetrates bacteria with damaged membranes (dead), staining them red. Afterwards, samples were rinsed with PBS and transferred to an uncoated black 24-well μ -plate (IBIDI, Munich, Germany) with the surface facing the bottom. Images of nine fields per sample were acquired using the high-content screening microscope IN Cell Analyzer 2000 (GE Healthcare Life Sciences, Illinois, USA) in the DAPI (455 nm), Cy5 (705 nm) and Cy3 (605 nm) channels with a Nikon 40x/0.95 NA Plan Fluor objective. Quantification of bacteria on the images was performed using the ImageJ software.

2.4.3.2. Planktonic Bacteria

Metabolic activity of planktonic bacteria was evaluated by Alamar blue assay. Resazurin was added to supernatants (10% v/v) and incubated for 1 h at 37 °C. RFUs were measured (λ_{ex} : 530 nm; λ_{em} : 590 nm) in a microplate fluorometer (Spectra Max GeminiXS, Molecular Devices, California, USA) and correlated to the metabolic activity of bacteria present in the medium. Viable cell count was also

performed using the agar plate culture method. For that, serial dilutions (10^{-2} , 10^{-4} , 10^{-6}) of the supernatants were prepared and three drops of 10 μL of each dilution plated in TSA. Plates were incubated overnight at 37 °C and CFUs counted.

3. Results and Discussion

This work explores the antimicrobial potential of graphene-based materials (GBM), in particular graphene nanoplatelets (GNP), for application on polyurethane (PU) catheters in order to prevent catheter-related infections. To our knowledge, modification of PU alone using GNP for antimicrobial applications has never been reported before, even though the two have been combined for other applications such as the improvement of mechanical, thermal and electrical properties [56-63]. Therefore, it was important to test different types of GNP by varying the platelet size and oxidation and, moreover, test different incorporation strategies such as the production of composites and the application as a coating. For this, four distinct types of GNP were explored, namely commercial non-oxidized GNP-M5 and GNP-M15, with two different lateral sizes (5 and 15 μm), and their respective oxidized forms, GNP-M5ox and GNP-M15ox, produced by chemical oxidation through the modified Hummers method.

3.1. GNP Powders

GNP powders were morphologically and chemically characterized before assessing their antibacterial potential. Difference between platelets lateral size was confirmed by TEM; both TEM and SEM demonstrated differences in their structure and morphology, with platelets morphology being closely related to their oxidation state (Figure 1a,b). Non-oxidized GNP-M5 and GNP-M15 powders presented a planar conformation, flat and smooth surfaces with irregular and sharp edges, as well as a more compact structure—denoted by the darker areas in the TEM images. Platelets were closely associated with each other due to inter-platelets hydrogen bonds and hydrophobic interactions that lead to a higher aggregation and agglomeration [64]. Oxidized GNP powders lost the planar conformation, with flakes appearing highly wrinkled, with folded sharp edges. This can occur due to formation of intra-platelet hydrogen bonds through the oxygen-containing functional groups present at the platelets' edges and basal planes [49]. XPS analysis confirmed GNP oxidation and chemical composition (Figure 1c): non-oxidized GNP presented low oxygen content, with atomic percentage of O 1s being lower than 3%—this small amount of oxygen is associated with GNP preparation process that consists in graphite intercalation and thermal exfoliation, leading to addition of small amounts of oxygen groups mostly at the platelets edges [49]. GNP oxidation increased the oxygen content up to 30%, associated with intercalation of oxygen-containing groups and water molecules. The oxidation process led to a decrease in the sp^2 carbon bonds, with reduction of the C=C groups from $\sim 70\%$ to $\sim 30\%$, leading to a decrease of the sp^2/sp^3 ratio from 7.1 in GNP-M5 to 2.3 in GNP-M5ox and from 8.5 in GNP-M15 to 1.9 in GNP-M15ox. Predominant oxygen-containing groups in GNP-M5ox are epoxy groups (C-O-C), followed by

carbonyl (C=O) and carboxyl (O-C=O) groups, while GNP-M15ox also shows the presence of hydroxyl (C-OH) groups. Oxidation is corroborated by the XRD patterns of GNP powders (Figure 1d), evidenced by the shift to the left of the 2θ diffraction angle. Non-oxidized GNP powders presented sharp and strong diffraction peaks (002 lattice) at $2\theta = 26.5^\circ$, indicative of their ordered, stacked structure and reduced interlayer spacing between sheets ($d = 3.4$ nm). Oxidized powders exhibited a lower diffraction angle (001) at $2\theta \approx 11.0^\circ$, corresponding to an increase in the interlayer spacing between sheets ($d = \sim 8.0$ nm), associated with the presence of oxygen-containing groups.

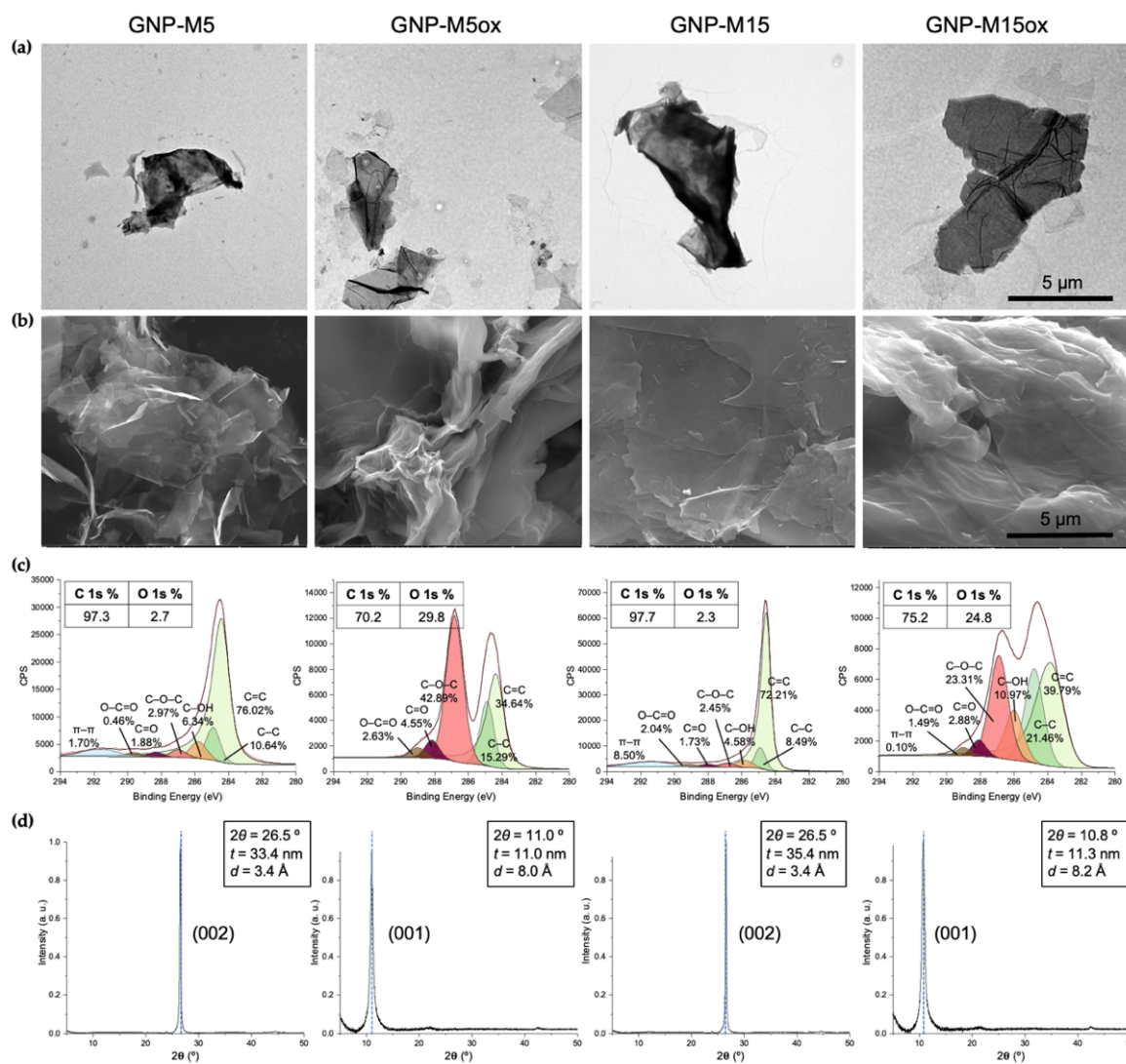
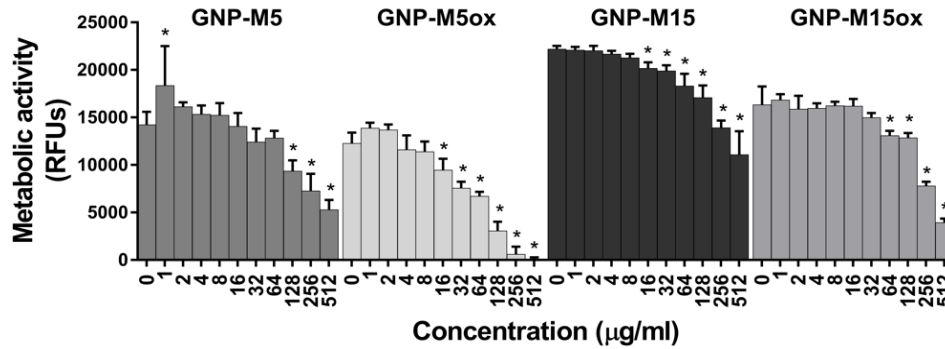


Figure 1. Characterization of graphene nanoplatelet (GNP) powders (GNP-M5, GNP-M5ox, GNP-M15 and GNP-M15ox): (a) TEM images (first row) and (b) SEM (second row) images (scale bar = 5 μm), (c) XPS atomic composition and carbon high-resolution spectra with respective functional groups, and (d) XRD patterns with diffraction angle (2θ), thickness (t) and interlayer spacing between sheets (d).

Antibacterial assessment revealed a decrease in metabolic activity with increasing GNP concentration, reaching reductions of 63%, 100%, 50% and 76% for the 512 $\mu\text{g/mL}$ dispersions of GNP-M5, GNP-M5ox, GNP-M15 and GNP-M15ox, respectively (Figure 2a). Bacteria viability decreased

particularly when in contact with oxidized materials, namely with 74% and 57% killing when in contact with 128 $\mu\text{g/mL}$ of GNP-M5ox and 256 $\mu\text{g/mL}$ of GNP-M15ox, respectively; the highest decrease of bacteria viability of 82% and 69% occurred with 512 $\mu\text{g/mL}$ dispersions of GNP-M5ox and GNP-M15ox, respectively (Figure 2b). In the GNP-M5 dispersions, bacteria were metabolically less active (Figure 2a) but not dead since growth was observed in the viable cell counting method (Figure 2b).

(a)



(b)

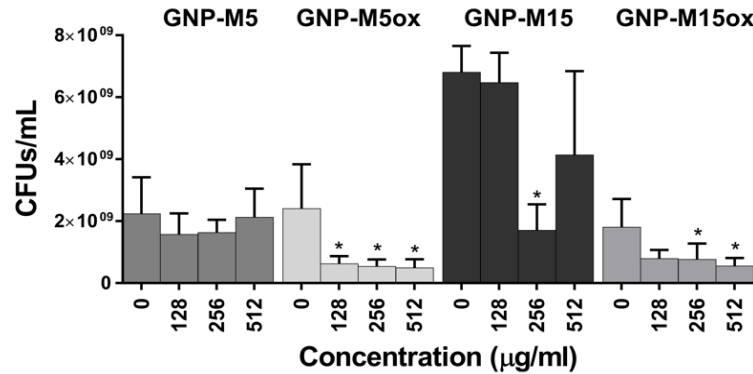


Figure 2. Antibacterial activity of GNP dispersions: (a) metabolic activity determined by resazurin assay and (b) bacteria viability of *S. epidermidis* determined by viable cell count after 24 h incubation with GNP-M5, GNP-M5ox, GNP-M15 and GNP-M15ox aqueous dispersions. * Statistically significantly different from control (one-way ANOVA (A) and Kruskal–Wallis (B); $p \leq 0.05$).

When comparing GNP with different sizes, GNP-M5 led to a higher decrease on the metabolic activity than GNP-M15 (Figure 2a); the same tendency occurred for the GNP-M5ox and GNP-M15ox. This suggests that smaller particles (5 μm) have higher bacterial inhibitory effect. When comparing particles with different oxidation degree, higher oxidation led to a higher reduction in bacteria viability, leading to greater CFUs/mL reduction (Figure 2b). Bacteria were metabolically inactive but still viable when cultured with non-oxidized GNP and, on the other hand, metabolically inactive and dead when cultured with oxidized GNP. As powders in suspension, GNP-M5ox showed to possess stronger antibacterial activity than the other materials, expressed by a significant decrease of both metabolic activity (100%) and cultivable bacteria counting (82%) when 512 $\mu\text{g/mL}$ were used. The impact of GNP

lateral size was also explored on Rago *et al.* work [65], with lower thickness and smaller size GNP exhibiting stronger antibacterial activity towards *Streptococcus mutans* (500 µg/mL) than larger and thicker ones, with reductions of cell viability of 80% and 65%, respectively. However, the effect of GNP oxidation, namely comparison between different GNP (FLG or MLG) and GNPox alone on the antibacterial properties has, to our knowledge, never been reported. Most of the studies explore the relation between GO and reduced graphene oxide (rGO) in dispersion [29, 66-70]—*E. coli* expressed a viability loss up to 98.5% when treated with 85 µg/mL GO for 2 h, higher than with rGO (≈90%) [29]; when increasing the concentration of GO and rGO to 150 µg/mL, the same tendency was maintained with *E. coli* presenting a viability loss of 87% and 81%, respectively [69]; increasing even more the concentration (250 µg/mL) and extending the period of incubation (18 h) with GO and rGO led to complete inhibition of the growth of *Listeria monocytogenes* and *Salmonella* [70]. Liu and co-workers [66] corroborated the higher antibacterial activity of smaller and more oxidized materials, demonstrating an increased antimicrobial potency for GO (0.31 µm) > rGO (2.75 µm) > graphite (6.87 µm) > graphite oxide (6.28 µm) dispersions (40 µg/mL) expressed by 70%, 46%, 26% and 15% of *E. coli* viability loss after 2 h. GNP and GNPox behaved similarly in our study, with smaller and oxidized particles having higher antibacterial potential.

3.2. PU/GNP Composites

GNP incorporation in the PU matrix was visually confirmed through an obvious change in color—from transparent PU into black opaque composites—even for the lower amount of GNP (0.5 wt%), due to the high specific surface area of the nanoplatelets (Figure S1). The effective incorporation of GNP in the PU matrix was corroborated by SEM of the fracture surfaces (Figure S2): in comparison with neat PU that has a smooth cross-section and surface, PU/GNP composites presented a rougher surface with apparent irregular stripes, as also reported by Tang and co-workers upon GO incorporation [47]. A homogenous dispersion of fillers was achieved, with an increase of GNP concentration leading to more platelets with sharp edges in the PU matrix and to a rougher fracture. Composites containing oxidized GNP (GNP-M5ox and GNP-M15ox) presented platelets with wrinkled appearance and folded edges, as observed in the oxidized GNP powders. A good adhesion at the interface between the GNP and the PU was observed, even without using any compatibilizers, as observed by others [56, 58]; some aggregates could be found in composites containing the highest GNP amount (10 wt%), particularly when larger and oxidized GNP were used. However, surface morphology of the composites was rather similar to the neat PU surface, with very few platelets being exposed—may be partially or completely covered by a layer of polymer—and usually found in a planar orientation, independently of the type and loading of the GNP incorporated (Figure 3a).

Regarding surface wettability, all composite samples presented similar water contact angles, associated with the poor GNP exposure at the surface, and higher than 90°, independently on the oxidation degree. Only when incorporating GNP with larger lateral size significant differences from the

neat PU were observed: incorporation of GNP-M15 (0.5, 1 and 5 wt%) contributed to a significant increase in PU hydrophobicity (angle increase around 4–5°) whereas GNP-M15ox (10 wt%) incorporation led to a significant decrease in PU hydrophobicity (angle decrease of 7°) (Figure 3b).

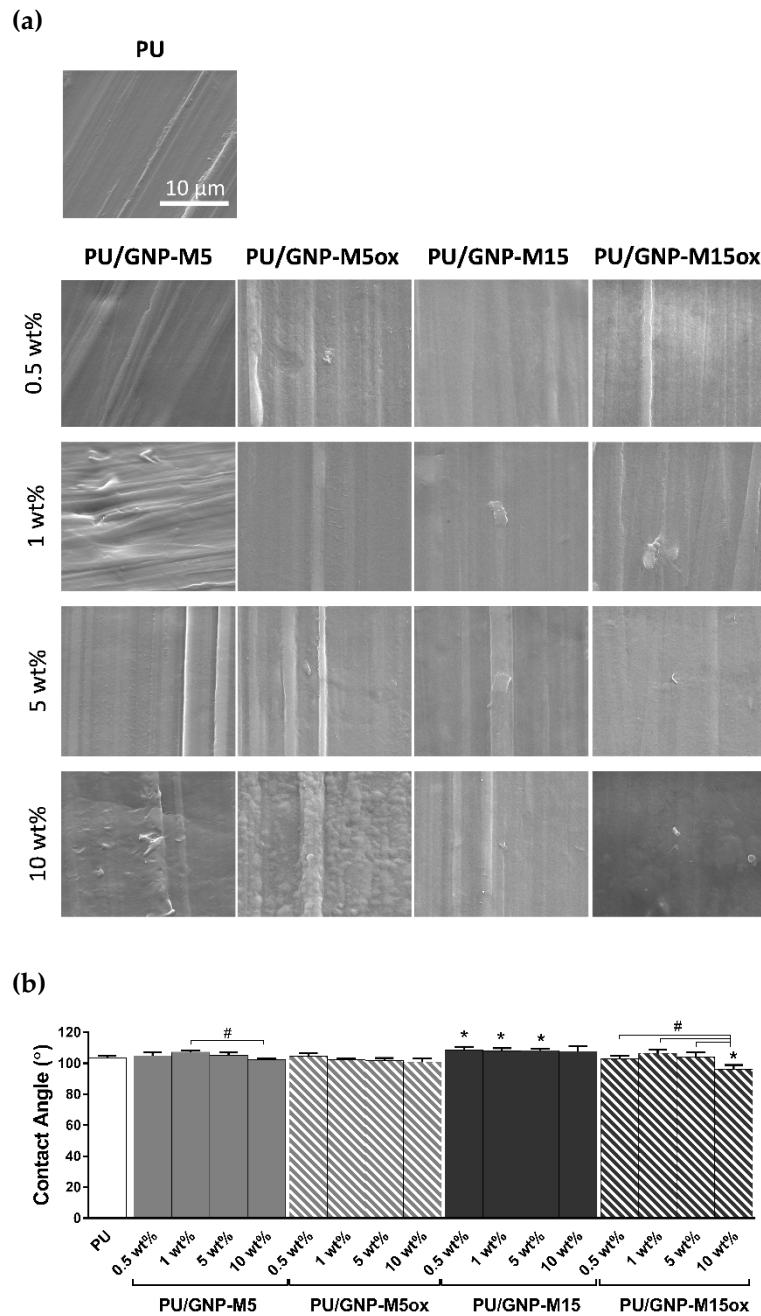


Figure 3. Characterization of polyurethane (PU)/GNP composites: (a) surface topography and (b) wettability of PU and PU/GNP composites with different GNP content produced by melt-blending. Images were obtained by SEM (scale bar = 10 μ m) and wettability determined by water optical contact angle measurements. * and # statistically significantly different from control (PU) and differences between samples, respectively (one-way ANOVA; $p \leq 0.05$).

The antibacterial potential of these composites upon contact with *S. epidermidis* was low, both towards adherent and planktonic bacteria: no statistically significant differences were observed in the

number of total or dead adherent bacteria compared to neat PU for any of the composites (Figure 4 and Figure S3) nor in the bacteria viability and metabolic activity of planktonic bacteria (Figure 4b and Figure S4). Common LIVE/DEAD kits could not be used due to the presence of green fluorescent stains: initial tests demonstrated PU autofluorescence in this channel. Incorporation of the different GNP onto PU neither decreased the bacterial adhesion nor increased the bactericidal activity of PU.

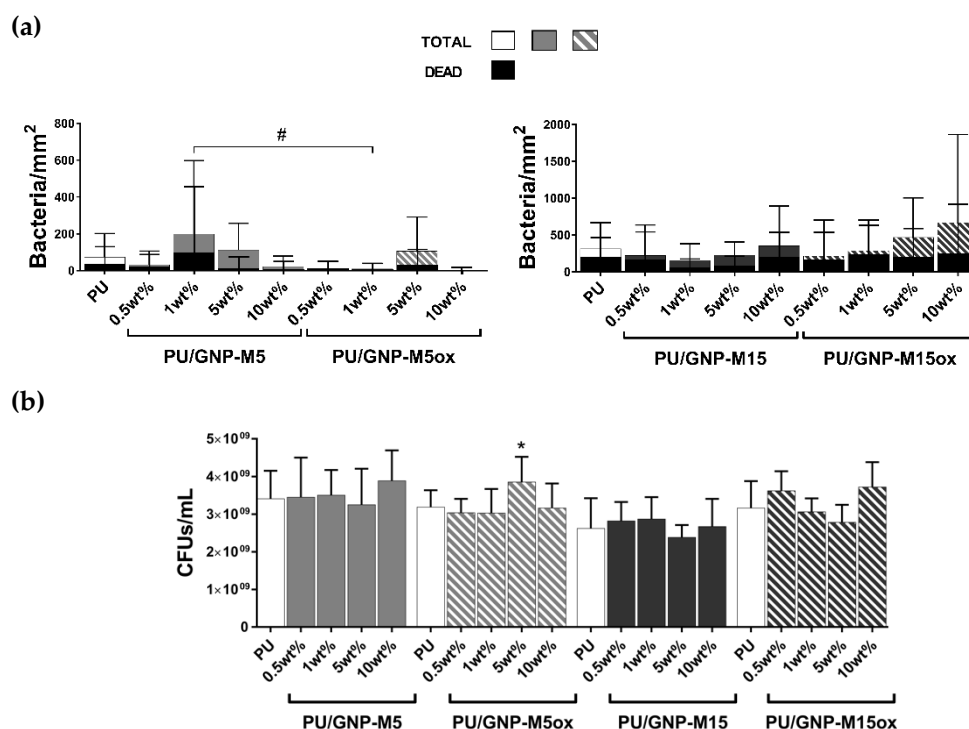


Figure 4. Antibacterial activity of PU/GNP composites after 24 h incubation with *S. epidermidis*: **(a)** total and dead adherent bacteria per mm^2 determined after total/dead staining and **(b)** planktonic viable bacteria collected in supernatant determined through viable cell counting. * and # statistically significantly different from PU (control) and differences between samples, respectively (Kruskal–Wallis (A) and one-way ANOVA (B); $p \leq 0.05$).

Melt-blending represents an economically attractive and industrially scalable method for efficiently dispersing nanofillers in polymers, being compatible with the manufacturing processes for catheter production [9, 71]. Besides, this method does not involve the use of solvents, representing an environmentally friendly method that avoids concerns with human health during processing and with toxicity of remaining solvent residues [72]. However, although melt-blending allowed GNP to be effectively dispersed within the polymer matrix, surface characterization showed that this was not a good method to expose GNP at the surface and, consequently, favor GNP contact with bacteria; this can justify the lack of antibacterial properties of the composites.

3.3. PU/GNP Coatings

Composite dispersions containing PU and different GNP—GNP-M5, GNP-M5ox, GNP-M15 and GNP-M15ox—were applied as a coating onto PU substrates by dip coating. GNP concentration was varied (from 0.25 to 1 mg/mL) to understand if there was a direct relation between GNP concentration and exposure at the surface whereas the ratio of GNP to PU was increased (from 1:1 to 1:0.25) to evaluate if the PU content could be lowered and still ensure GNP adherence to the substrate. A change in color was visible for all samples, ranging from transparent PU to black or brownish color when GNP or oxidized GNP were incorporated, respectively; increasingly darker appearance occurred as GNP concentration increased (Figure S5). All coatings produced from different batches were homogenous. Comparing to composites obtained by melt-blending (Figure 3a), these PU/GNP coatings presented a mesh-like appearance (attributable to solvent evaporation and consequent PU precipitation) and much higher amounts of GNP platelets exposed at the surface (Figure 5). Particularly in coatings with non-oxidized GNP (GNP-M5 and GNP-M15) many platelets with sharp edges were bursting from the coating surface and at different orientations, although some of these platelets were also partially or completely covered with polymer; especially in coatings with higher GNP-M15 loading (1 mg/mL) some platelet aggregation took place. When decreasing the PU content (GNP:PU ratio increasing from 1:1 to 1:0.25), the surface acquired a more mesh-like and irregular topography with platelets less covered by polymer as particularly observed with GNP-M5 incorporation: the lower amount of PU present in the solution, although not enough for complete uniform surface coverage, allowed platelets to stick to the PU film substrate. As expected, the number of platelets and aggregates observed at the surface increased with GNP concentration. Individualized platelet-like structures were scarcely seen in the coatings with oxidized GNP (Figure 5): coatings with GNP-M5ox presented homogeneous creased surfaces composed of apparently fused wrinkled GNP-M5ox sheets with some protruding edges; coatings with GNP-M15ox also had areas with a similar wrinkled topography, but the presence of spherical wrinkled agglomerates was also visible. The wrinkled topography and formation of spherical structures indicates strong polymer–GNPox interactions [61], behavior related to the morphology and surface chemistry of the oxidized GNP where oxygen-containing groups present at the surface may have formed hydrogen bonds with the N-H groups present in the PU molecule, leading to strong interfacial interactions [45, 73]. In the liquid-phase mixing method used, when PU was mixed with oxidized GNP sheets, they preferentially stuck together, causing agglomerated oxidized GNP/PU structures to be formed upon solvent evaporation [45]. Differences in lateral size can also contribute to the different morphology observed, both with non-oxidized and oxidized platelets, where the greater the sheet size the more irregular the surface, due to the aggregation of the GNP sheets and interaction with the polymer. Similar surface topographies were obtained by our group by dip or spray coating silicone films, obtaining exposed platelets and aggregates or creased structures when using either GNP-M5 or GNP-M5ox [50]. Flat coatings without sharp edges, identical to the coatings containing GNP-M5ox obtained here were also reported by others using GO [20]. Thampi *et al.* also observed wrinkled topography when spraying GO

onto electrospun polycarbonate urethane (PCU) membranes [74]. Overall, coatings with GNP:PU ratio of 1:0.5, and specially with GNP concentration of 0.5 mg/mL, seemed to present more uniform and thus more promising surfaces.

GNP coatings' adherence to the PU substrate was confirmed by the rubbing test with no GNP detachment being observed for all coatings, except for the coatings containing higher GNP content (1 mg/mL), with the resistance of the coating decreasing as the GNP:PU ratio increased. This result is not surprising as PU in solution works as binder between GNP and the PU film surface.

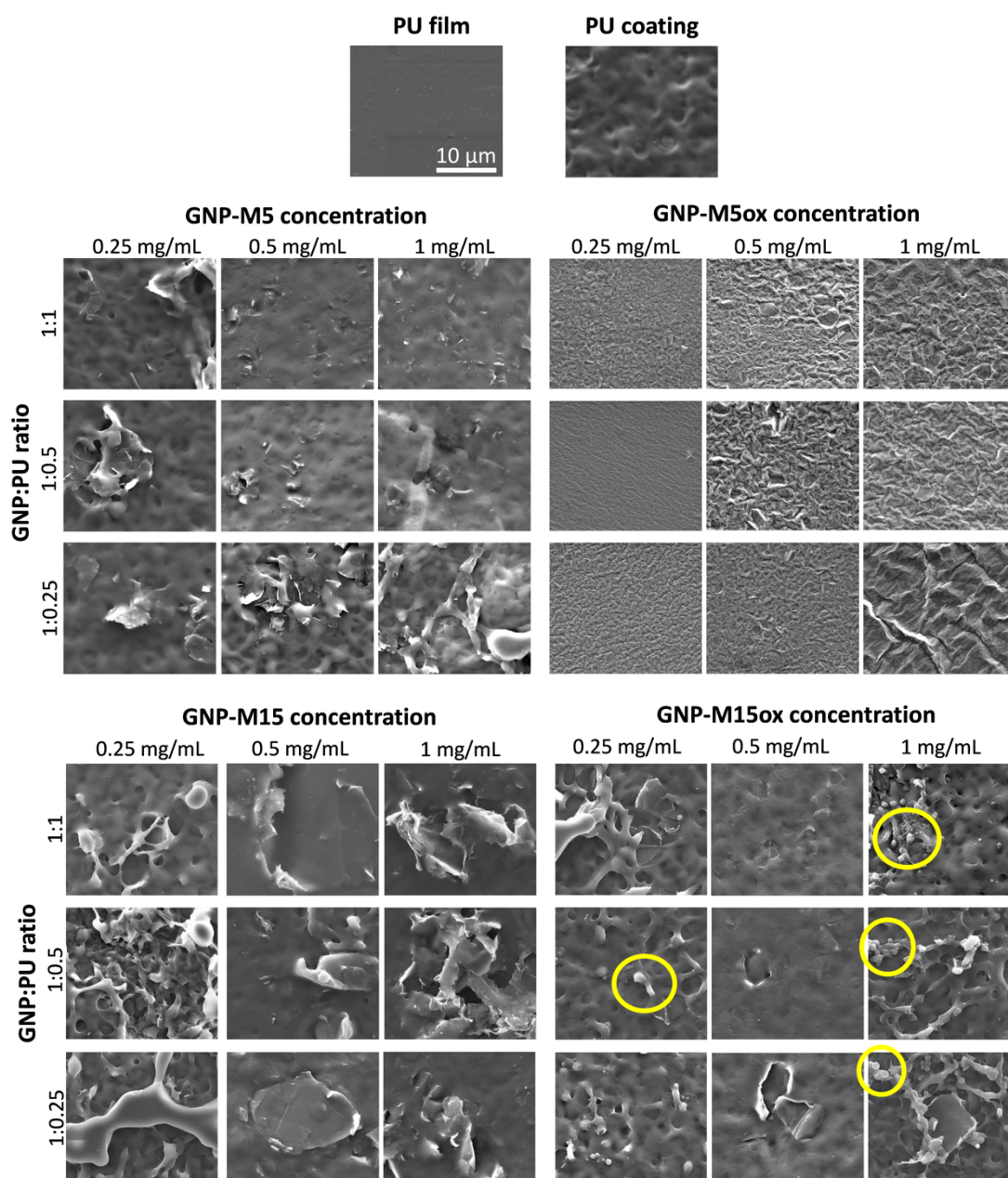


Figure 5. Characterization of PU/GNP coatings: surface topography of PU film (substrate), PU coating (PU coating on PU substrate) and PU/GNP coatings with different GNP concentration and GNP:PU ratio produced by dip coating with different GNP concentration and GNP to PU weight ratio. Images were obtained by SEM (scale bar = 10 μm); yellow circles indicate spherical agglomerates observed in the surfaces.

Regarding surface wettability, both PU film (substrate) and PU coating on PU film exhibited a water contact angle of 85° (Figure 6), less hydrophobic than PU produced by melting and injection (103°) (Figure 3b). Some variability regarding PU Tecothane surface wettability has been found throughout the literature—surfaces produced by solvent casting presented angles ranging from 90° to 73° [75, 76], with the difference between studies relying on the solvent used to dissolve PU: DMF and THF, respectively; surfaces produced by solvent polymerization and curing presented a contact angle of 103° [77]—suggesting that not only the production method impacts surface wettability but also the materials interacting with the polymer. When producing the coating with PU/GNP-M5 composite, wettability change was only detected for the formulation with 0.25 mg/mL GNP and 1:1 GNP:PU ratio and with 1 mg/mL and 1:0.25 GNP:PU ratio, with the water contact angle of PU decreasing to 77° and increasing to about 100° , respectively. As observed in composites, when producing coating with larger GNP-M15 platelets, the hydrophobicity increased significantly in many samples and more evidently for 0.25 mg/mL GNP and GNP:PU ratio of 1:0.25, with an increase of the angle to 104° . Increasing the GNP-M15 concentration (1mg/mL) led to a significant increase in the hydrophobicity (from 85° (neat PU) to 93°), which is justified by the presence of more platelets and aggregates exposed at the surface, forming hydrophobic sites. Similar results have been reported for poly(vinyl acetate) (PVAc) with incorporation of 0.1 wt% GNP [78]. The impact of oxidation degree was less notorious independently of the size of the platelets (PU/GNP-M5ox and PU/GNP-M15ox), being roughly the same for all samples with no obvious tendency observed, which correlates with the similar topography observed for all these surfaces. There was however a decrease of the angle (to 78°), and thus an increase of the hydrophilicity, for 0.5 mg/mL GNP-M15ox and GNP:PU ratio of 1:1 and 1:0.5, suggesting that surface topography might play a role in wettability, with these surfaces being apparently smoother than the remaining conditions. When increasing the concentration (from 0.25 mg/mL to 1 mg/mL) for the same GNP:PU ratio (1:1), surface hydrophobicity decreased (from 99° to 95°), even though not significantly. At the highest concentration of GNP-M15ox, when reducing the amount of PU, an increase in hydrophobicity was observed (to 109°), even though more platelets are exposed; once again, surface topography may overrule chemistry on the effect of GNP exposure. It would be expected that incorporation of oxidized GNP increased the hydrophilicity due to the hydrogen bond interactions between the oxygen-containing groups present in GNP and water. Our group has previously reported a water contact angle decrease of $\sim 9^\circ$ on PLA/GO films compared to pristine PLA films [79]; others have also shown that incorporation of GO into PU decreased the water contact angle [80]. However, wettability is affected not only by chemical composition but also by surface roughness. Interestingly, for the coatings there seems to be a relation between the extent of irregularities at the surface, more randomized mesh and bigger defects, and the higher changes of water contact angle. In the composites, an increase of the hydrophilicity was also only observed with the highest GNP-M15ox loading (10 wt%), with surface roughness being similar between conditions.

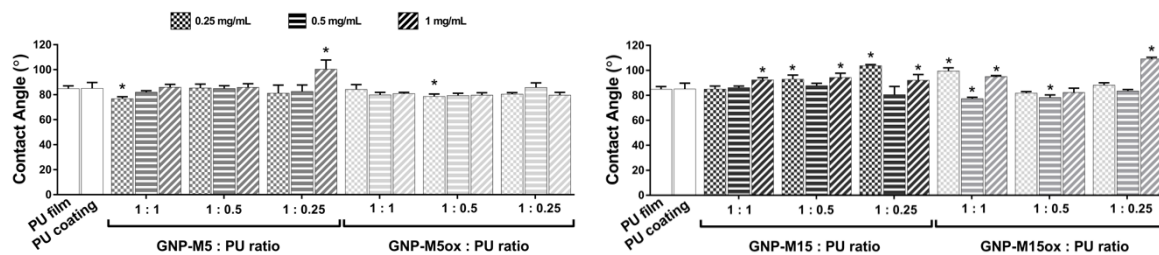


Figure 6. Characterization of PU/GNP coatings: surface wettability of PU (control) and PU/GNP coatings with different GNP concentration and GNP:PU ratio, obtained by water optical contact angle measurements. * statistically significantly different from control (PU) (one-way ANOVA; $p \leq 0.05$).

Surface chemistry of the PU/GNP coatings was further explored using XPS and Raman spectroscopy, being PU/GNP coatings with intermediate GNP concentration (0.5 mg/mL) and intermediate GNP:PU ratio (1:0.5) selected for this analysis (Figure 7). XPS spectra showed that the PU/PU coating is mainly constituted by carbon (87.8%), oxygen (9.4%) and nitrogen (2.8%), as expected. Coating the surface with PU/GNP-M5ox and PU/GNP-M15ox increased the amount of oxygen to ~16%, as noticed by the increase of the oxygen peak in the survey, highlighting the presence of the oxygen-containing groups from the oxidized GNP in the upper 10 nm of the coatings. Raman spectra showed the characteristic bands of PU in all the analyzed surfaces, demonstrating the PU presence in all coatings. The presence of GNP-M5 and GNP-M15 in the coatings could not be confirmed since the strong, sharp G band (~1585 cm^{-1} [81]) typical from these materials overlapped with the C=C bond from PU at 1617 cm^{-1} . For PU/GNPox, the presence of strong and broad D (~1310 cm^{-1}) and G (~1615 cm^{-1}) bands, characteristic of oxidized forms of graphene can be easily identified. G peak corresponds to the bond stretching of sp^2 carbon atoms, while the D band is associated with the structural defects present in the GNPox rings introduced due to their oxidation process [82]. Therefore, XPS and Raman spectra confirmed the presence of both PU and GNP or GNPox at the surface of the composite coating, presenting different surface chemistry.

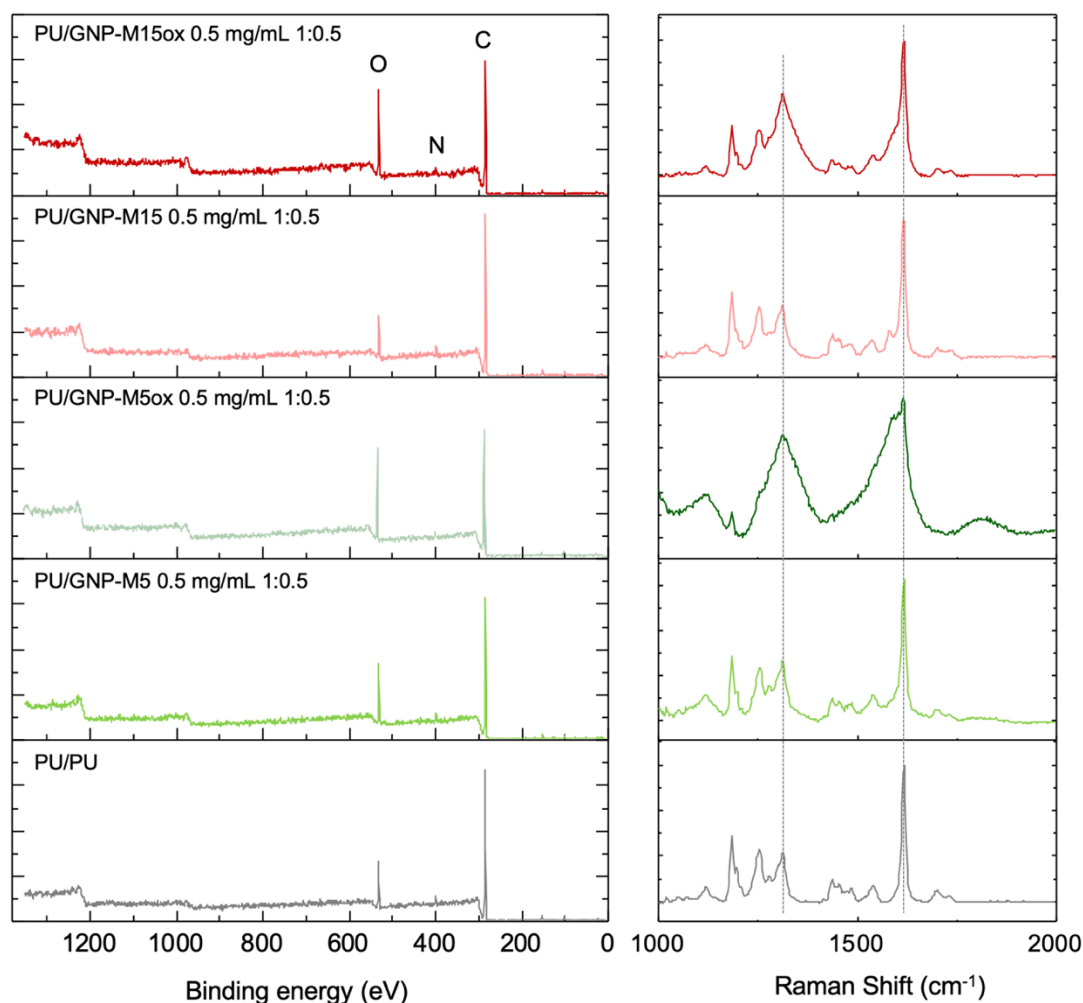


Figure 7. Characterization of surface chemistry of PU/PU and PU/GNP coatings with intermediate GNP concentration (0.5 mg/mL) and intermediate GNP:PU ratio (1:0.5): XPS survey spectra (left panel) and Raman spectra (right panel).

In order to study the impact of either GNP concentration and PU:GNP ratio on the antibacterial potential towards *S. epidermidis*, one of the variables was maintained while varying the other: GNP concentration of 0.5 mg/mL was fixed while varying PU:GNP ratio (1:1, 1:0.5 and 1:0.25) and the other way around, fixing the PU:GNP ratio of 1:0.5 and varying GNP concentration (0.25, 0.5 and 1 mg/mL). Regarding total adherent bacteria (Figure 8), there was a significant decrease of 37% when using GNP-M5 at 0.5 mg/mL and GNP:PU ratio of 1:0.5 when comparing to bare PU film. Increasing the concentration to 1 mg/mL or the GNP:PU ratio to 1:0.25 caused a significant increase of dead bacteria to 78% and 85%, respectively. These conditions contained higher amounts of sharp graphene nanoplatelets, suggesting the role of these structures on the effective killing of bacteria after contact with the surface. In oxidized samples with the same lateral size (GNP-M5ox) there was a reduction in bacterial adhesion up to 70% (ranging from 44% to 70% in different conditions) while increasing bacterial death (ranging from 53% for the lower concentration and ~70% in all other conditions); the exception was the condition with the highest amount of GNP-M5ox (1 mg/mL), showing high bacterial

adhesion, although at the same time high killing ability (around 70%). The wrinkled morphology of the surfaces with GNP-M5ox associated with the oxidation of the surface may be behind this effect, preventing bacteria to adhere in high number while prompting some oxidative stress mechanism. Increasing platelets lateral size (GNP-M15) led to an increase in bacterial adhesion and death (reaching 62% and 71% bacterial death) when comparing to PU, specifically in conditions with lower amount of PU on the composite coating (0.5 mg/mL, ratio of 1:0.25) and with higher amount of GNP (1 mg/mL), suggesting that the exposure of the sharp edges of non-oxidized GNP and associated increase of surface area/roughness promotes bacterial adhesion despite killing them. Contrarily to GNP-M5ox, GNP-M15 oxidation (GNP-M15ox) significantly boosted bacterial adhesion in most of the conditions. The lower amounts of oxygen-containing groups in platelets with higher lateral size seems to prevent the formation of a continuous layer as observed in PU/GNP-M5ox surfaces, resulting in a surface with high roughness exposing both GNPox and PU, which can promote bacterial adhesion. The percentage of dead bacteria was on the other hand significantly higher than in PU and their non-oxidized counterparts, reaching 67% in conditions with lower amount of PU on the composite coating (0.5 mg/mL, ratio of 1:0.25).

Overall, when using non-oxidized GNP-M5 and GNP-M15, the highest bacterial death was observed when higher amounts of GNP are exposed (at the highest GNP:PU ratio of 1:0.25 and 0.5 mg/mL GNP and at the highest GNP concentration of 1 mg/mL). With oxidized GNP, more death was also observed in the coatings with 0.5 mg/mL and 1 mg/mL of GNP-M5ox. Increasing GNP:PU ratio (1:0.25), thus decreasing the amount of PU in the coating formulation, improved the exposure of platelets at the surface, which also seemed less covered by the polymer; this allowed more contact with bacteria. Increasing GNP concentration increased the chances of exposing platelets at the surface and thus the contact with bacteria. This correlates with the literature that states the importance of GBM sheets orientation, exposure and concentration on the antibacterial performance of surfaces integrating GBM [40, 83]. Therefore, increasing platelets exposure increased contact with bacteria, which consequently seemed to potentiate the effects of the sharp edges and basal planes of the GNP platelets, either due to disruption of the cell membrane [36, 84] or oxidative stress induction [41, 85].

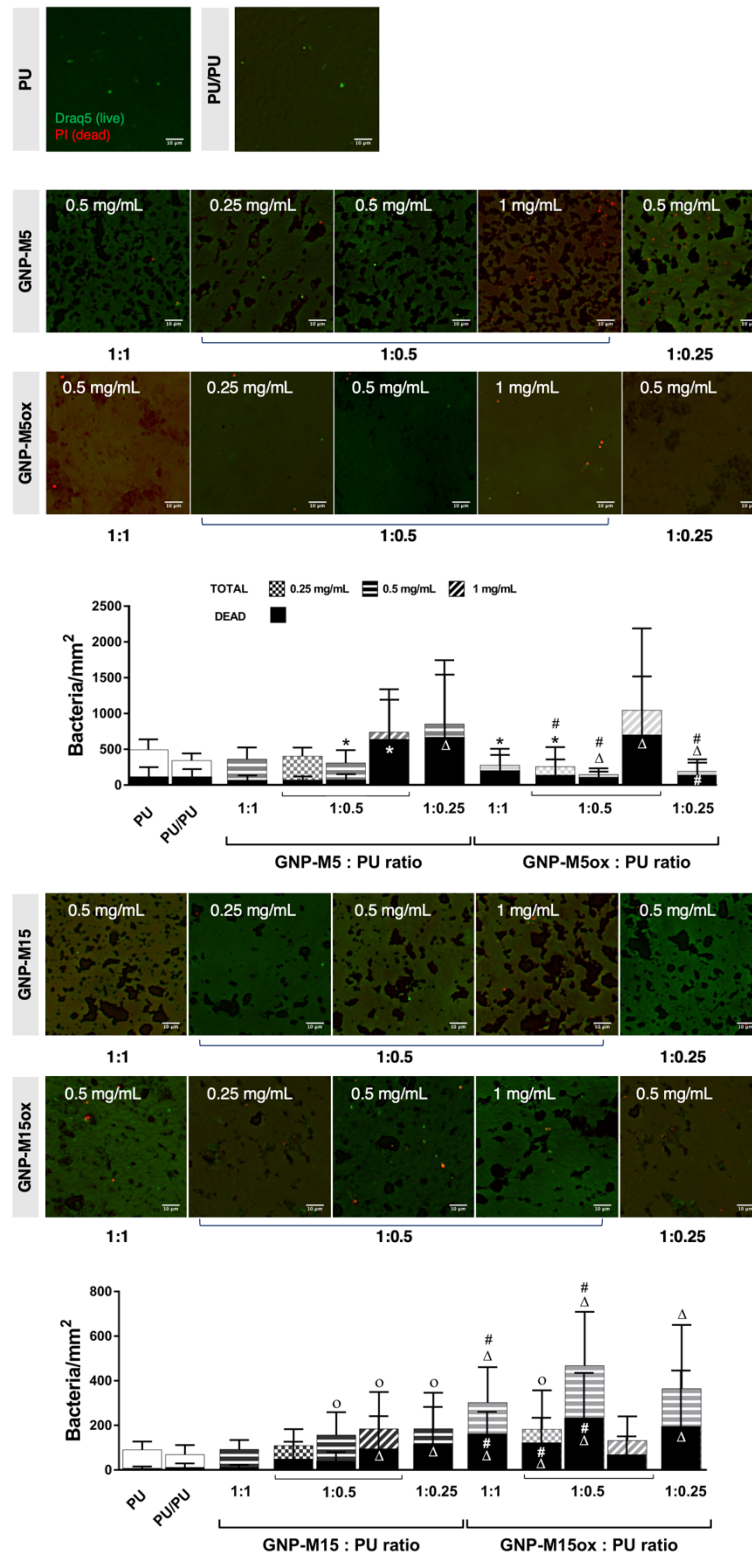


Figure 8. Antibacterial activity of PU/GNP coatings with different GNP concentration and GNP:PU ratio towards adherent *S. epidermidis* after 24 h incubation. Images of live/dead bacteria staining (Draq5/PI) (scale bar = 10 μ m) and total and dead adherent bacteria per mm² determined from total/dead staining. *, o, Δ and # statistically significantly different from control PU film (PU), from PU coating on PU (PU/PU), from both and differences between the correspondent non-oxidized samples, respectively (Kruskal–Wallis; $p \leq 0.05$).

Concerning planktonic bacteria, in most of the surfaces, neither bacteria viability (Figure 9) nor metabolic activity (Figure S6) decreased. This result was also expected since these surfaces do not act as leaching systems and therefore the effects of GNP will only be exerted against the bacteria in contact with the surface. However, specifically in PU/GNP-M5ox surfaces where bacterial adhesion was reduced, viable bacteria on the supernatant significantly increased when compared to PU film, suggesting that bacteria were impaired to adhere but survived and grew in the supernatant.

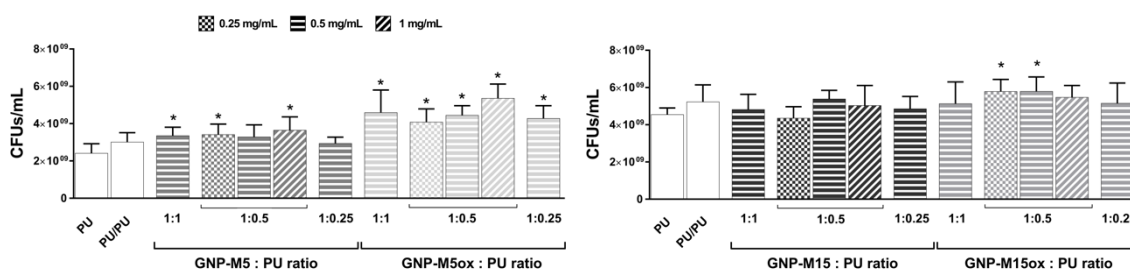


Figure 9. Antibacterial activity of PU/GNP coatings with different GNP concentration and GNP:PU ratio towards planktonic *S. epidermidis* after 24 h incubation determined by viable cell count. *, o, Δ and # statistically significantly different from control PU film (PU), from PU coating on PU (PU/PU), from both and differences between the correspondent non-oxidized samples, respectively (one-way ANOVA (B); $p \leq 0.05$).

As aforementioned, PU/GNP composites produced by melt-blending lacked GNP exposure at their surface, therefore failing on improving the antibacterial properties of PU. This was overcome through the application of PU/GNP composite as a coating on PU by dip coating, where higher GNP exposure at the surface was achieved, which, in some of the conditions, was translated into a reduction/increase in bacterial adhesion and/or increase in bacterial death. GNP exposure is therefore crucial for the antibacterial activity of the GBM-integrating surfaces, being potentiated when increasing the amount of platelets present and/or reducing the presence of the polymer. This affects the way GBM are deposited and oriented on the surface, thus impacting bacteria-GBM interaction and ultimately the antibacterial activity of the surfaces [40]. This relation of GBM exposure with the antibacterial activity of GBM-containing surfaces has been suggested in other studies, as recently by Cheng *et al.* [83]. Even though antibacterial activity has been observed both when basal planes [84-86] or sharp edges are exposed [87], a direct contact between bacteria and the surface must occur, as reinforced by the absence of antibacterial effects both on the bacteria incubated with the composites and on bacteria collected in the supernatant with both surfaces.

Apart from GNP exposure, we have also established the influence of oxidation degree by reporting higher antibacterial activity on PU/GNP coatings containing GNPOx than on coatings with non-oxidized GNP, as also supported by a study comparing other GBM (namely GO with rGO [29]) and by our previous work with GNP/silicone coatings [50]. Lateral size also played a role, with smaller platelets being more promising regarding inhibition of bacterial adhesion, particularly, when smaller oxidized particles are used, as GNP-M5ox. This was also supported by several works, as by Perreault *et al.* that

demonstrated that smaller GO sheets presented higher antimicrobial effect, attributed to oxidative mechanisms associated with the higher defect density of smaller sheets, which allow more oxygen to be adsorbed on them [41]. Bigger platelets (GNP-M15) led to the presence of topography irregularities, which seem to favor bacteria adherence. At the same time, this induces higher percentage of death. Morphology and topography of the surfaces play therefore a crucial role on the antibacterial potential of the surfaces, while on surfaces containing GNP the action towards bacteria can be attributable to mechanical damages provoked by the sharp edges of the exposed nanoplatelets, on surfaces containing GNPox, particularly GNP-M5ox, the wrinkled topography and the absence of sharp edges indicates oxidative stress induction.

Coatings containing GNP-M5ox revealed to be the most promising by having both antifouling and bactericidal effects towards *S. epidermidis*. Therefore, these coatings are the most suitable for the development of an antimicrobial material.

4. Conclusions

This work demonstrates the relevance of GNP oxidation and lateral size on the antimicrobial profile of GNP both as dispersions and surfaces. Oxidized and smaller particles (GNP-M5ox) in suspension induced stronger activity against *S. epidermidis*, decreasing bacteria viability in 74%. The impact of GNP exposure on the surface was stated by comparing surfaces produced by melt blending—bulk PU/GNP composite—and dip coating—PU/GNP composite layer on top of a substrate. Absence of GNP exposure at the surface of PU/GNP composites resulted in the lack of antibacterial effect towards *S. epidermidis*. On the other hand, PU/GNP coatings yielded surfaces with well-adhered and exposed nanoplatelets, revealing an improvement on antibacterial and anti-adhesive properties. Coatings containing smaller platelets (GNP-M5 and GNP-M5ox) caused higher antibacterial activity than coatings with larger platelets (GNP-M15 and GNP-M15ox). Coatings with GNP-M5 showed the highest increase of bacterial death (up to 85%). The most promising surfaces were however the PU/GNP coatings containing GNP-M5ox at a concentration of 0.5 mg/mL and GNP:PU ratio of 1:0.5, which presented both antifouling (70% reduction of bacterial adhesion) and bactericidal activity (70% bacterial death) towards *S. epidermidis* when comparing to bare PU surface.

As such, dip coating technique can be explored as a promising approach to easily transform clinically used catheters into efficient antimicrobial catheters, prolonging their lifetime by reducing the commonly associated risk of infections, thus impacting the life of millions of people. These data should also be considered in the process of designing new antimicrobial polyurethane surfaces in a wide range of applications.

5. Acknowledgments

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Supplementary Information



Figure S1. Images of the PU and PU/GNP composites produced by melt-blending after injection molding.

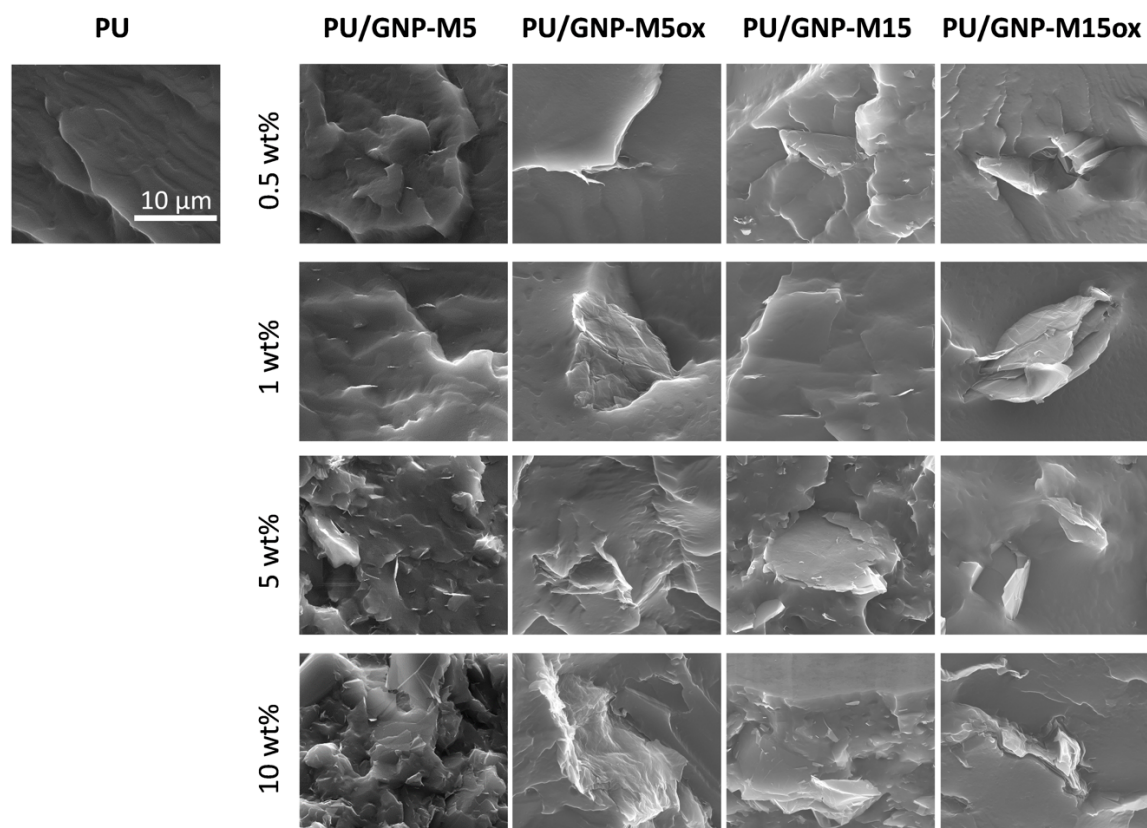


Figure S2. SEM images of PU and PU/GNP composites with different GNP content. Images were taken to the transversal fracture to analyse the composite matrix (scale bar = 10 μm and valid for all images).

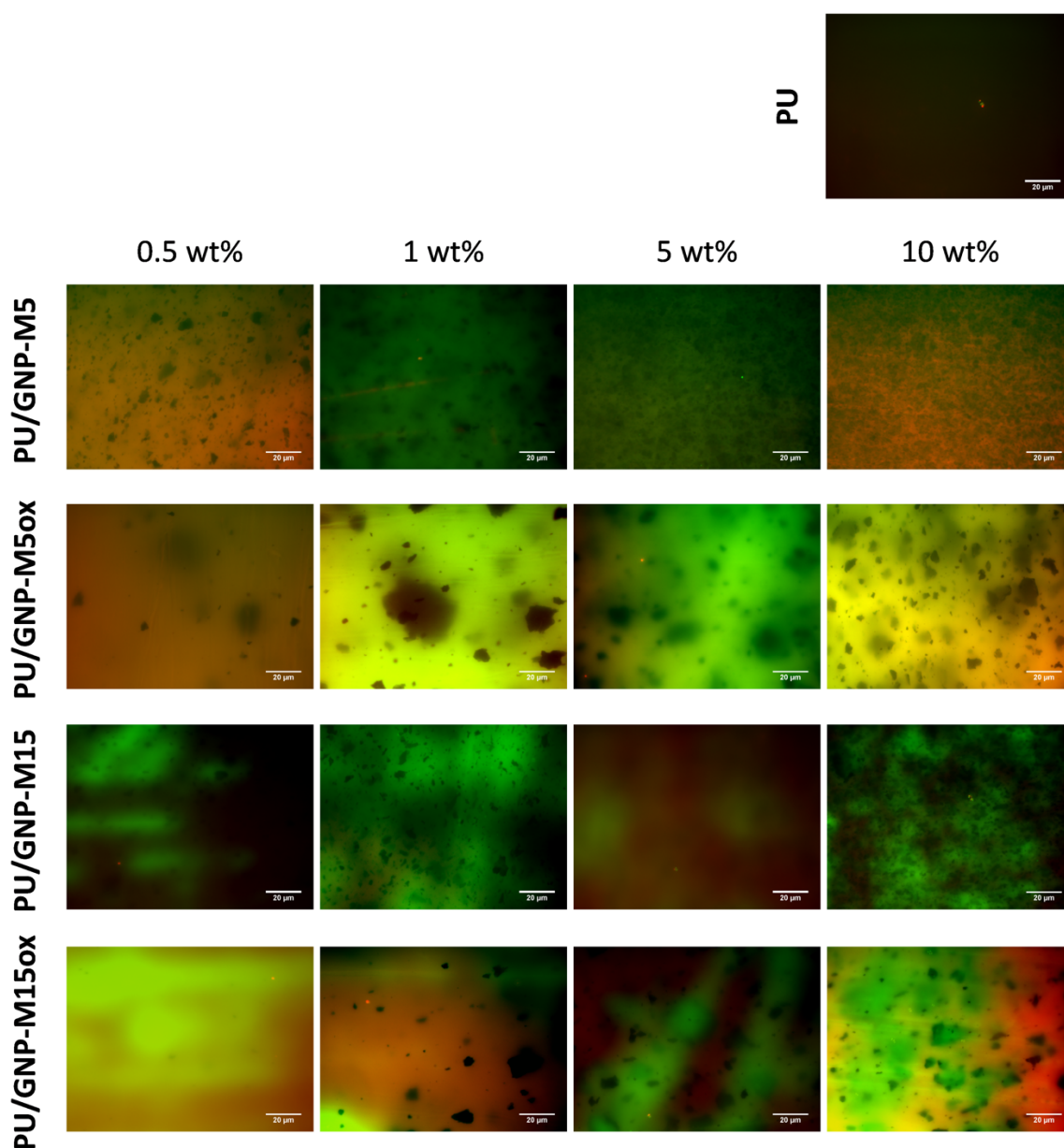


Figure S3. Representative images of the total/dead bacteria staining (Hoechst/PI) of adherent *S. epidermidis* after 24 h incubation on PU/GNP composites (scale bar = 20 µm). Due to the lower number of bacteria per mm² found at the surfaces and the small area of each field, no or very few bacteria are found in the fields, being these images representative of the total number of bacteria presented in Figure 4A.

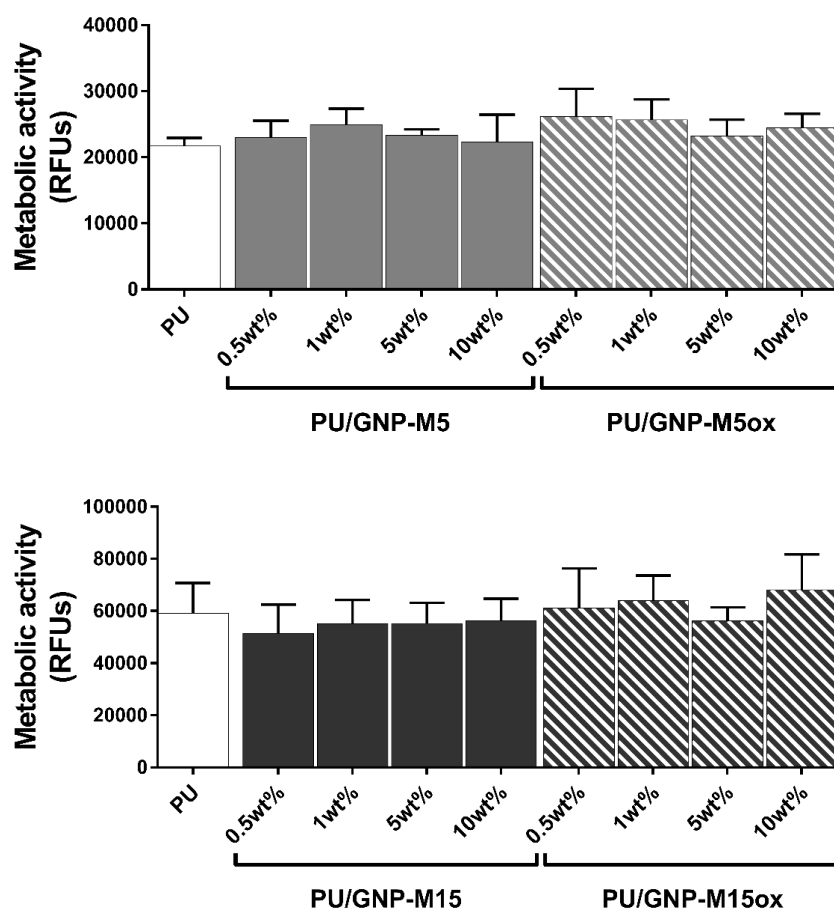


Figure S4. Metabolic activity of *S. epidermidis* planktonic bacteria after 24 h incubation on PU/GNP composites.

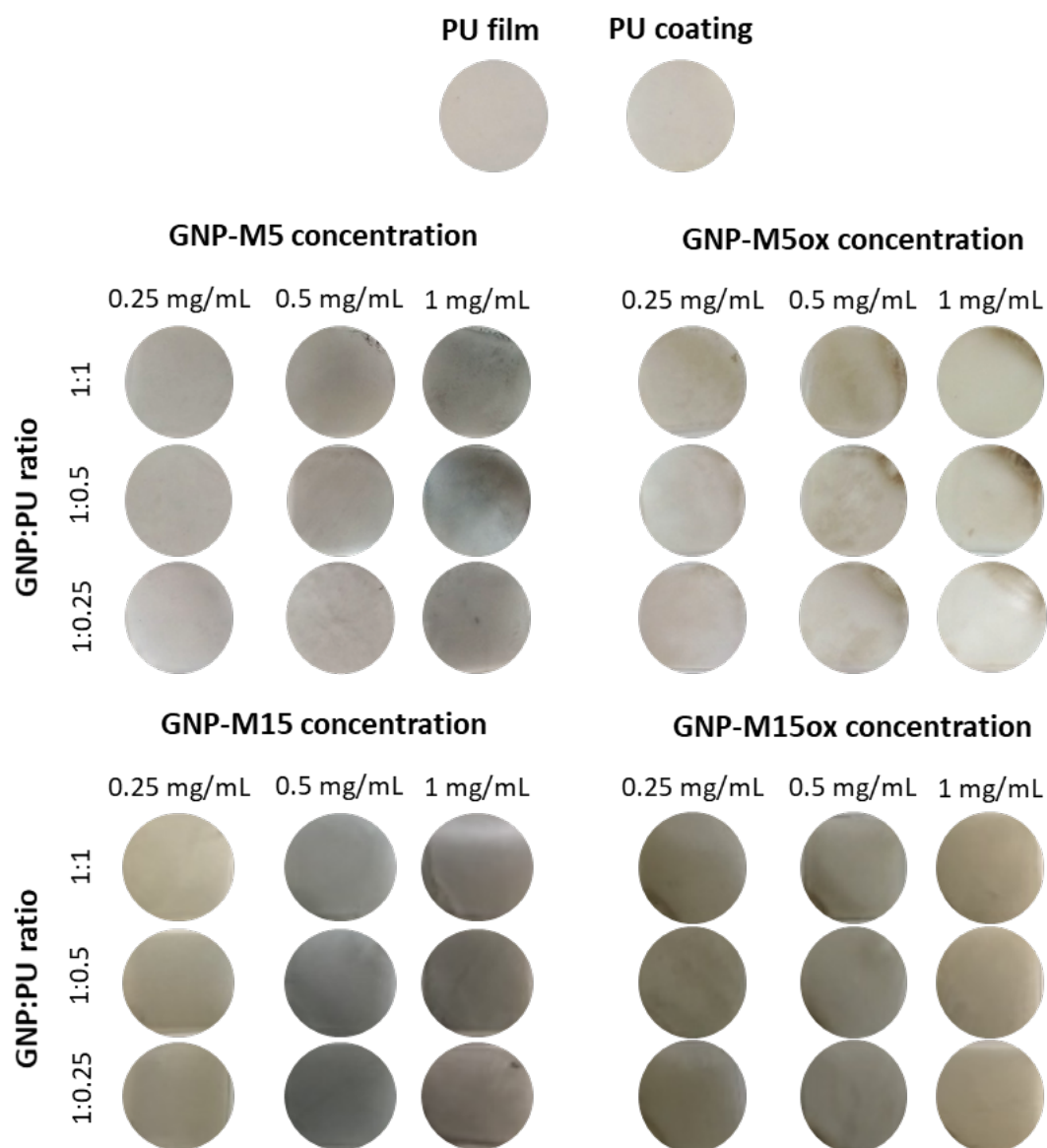


Figure S5. Images of the PU film produced by casting, PU coating on PU film and PU/GNP coatings on PU film.

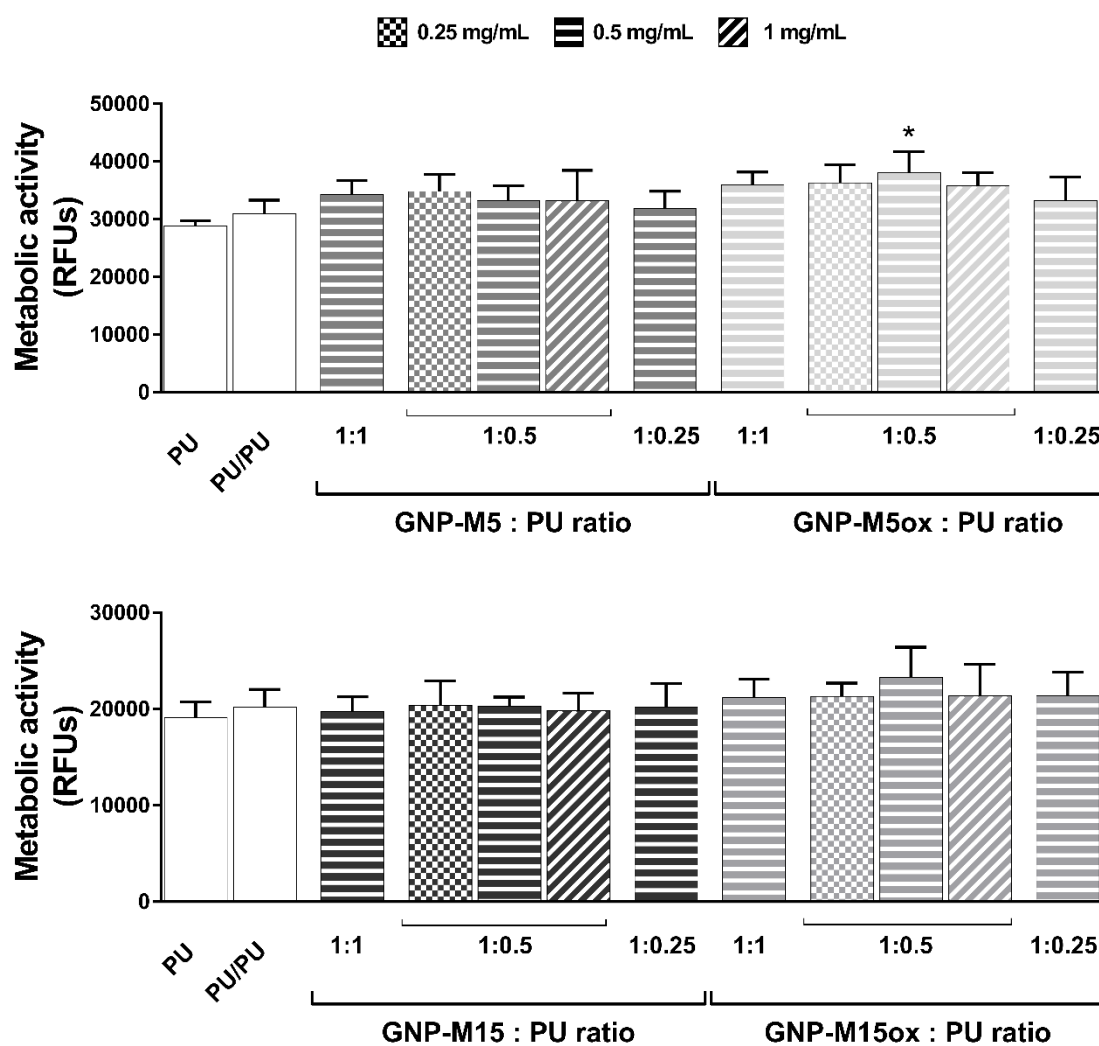


Figure S6. Metabolic activity of *S. epidermidis* planktonic bacteria after 24 h incubation on PU/GNP coatings.

CHAPTER VII

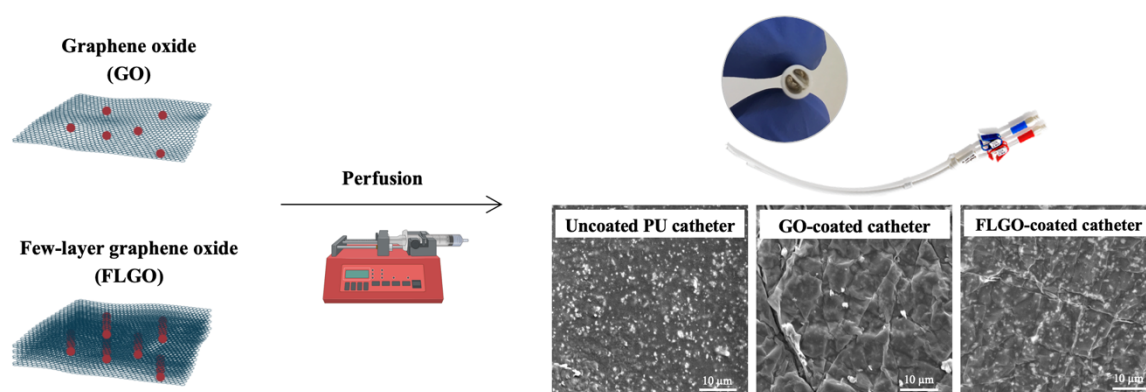
**Can oxidized graphene materials be applied
as a coating on HD catheters to make them
antibacterial?**

Oxidized graphene coatings as antibacterial agents on hemodialysis catheters

Abstract

Insertion of a catheter into the body is one of the most common procedures in hemodialysis (HD) and the potential onset for bacterial infection. Catheter complications are a major cause of morbidity and mortality for HD patients and increase the burden on the healthcare system. Aseptic practices, systemic antibiotic administration, and antimicrobial lock solutions have been the cornerstone of infection control measures. Antibiotic-impregnated and silver coated catheters have been the foundation for research improvements, however, short efficacy period, rise in bacterial resistance, and toxicity demand the development of new strategies. Graphene-based materials stand out as promising antimicrobial agents due to their associated cytocompatibility and lack of known bacterial resistance mechanisms. Our previous studies revealed that oxidized graphene materials reduce bacterial adhesion while exerting a bactericidal effect. In this study, graphene oxide (GO) and few-layer graphene oxide (FLGO) were applied as a coating of commercially available polyurethane (PU) hemodialysis catheters by perfusion through the internal lumen, and the antibacterial performance investigated. Morphological characterization by scanning electron microscopy demonstrated the efficient and homogeneous application of both materials on the internal lumen of the 3D catheter as a thin layer, with wrinkles and grooves. The ability of coatings to resist bacterial adhesion, colonization, and migration was explored towards Gram positive *Staphylococcus epidermidis*. Preliminary results showed that coatings prevent bacterial adhesion and migration through the coated surface under static conditions, despite allowing growth under low flow conditions. Further studies on the single-cell interaction with the surface are required in order to validate the results and understand how surface features are impacting the antibacterial performance of the coatings.

Graphical abstract



Keywords: hemodialysis catheters; graphene oxide; few-layer graphene oxide; perfusion; coating; polyurethane.

1. Introduction

Bacterial colonization of medical surfaces, including catheters during hemodialysis (HD), involves the transition from a free-floating lifestyle into a sessile, protective, surface-attached style, which culminates in the development of a biofilm and consequent establishment of infection [1]. This transition is a multifactorial process, determined by the biological, chemical, and physical properties of the environment, the surface features and the bacterial cells [1]. According to the National Institutes of Health (NIH), bacterial biofilms are responsible for 65% of microbial diseases, for more than 80% of chronic infections [2], and for around 70% of all bloodstream's infections [3]. These biofilms formed on the catheters can be the source of persistent infections that are recalcitrant to systemic antibiotic therapy – currently the only available treatment, combined with antimicrobial locking solutions and implementation of antiseptic practices [1, 4, 5] – and are the major cause of patient morbidity, with mortality rate reaching 12% – 25% [4, 6]. This often leads to the inevitable and premature replacement of the catheter, being thus associated with a high economic burden [6].

Approximately 75% of the microorganisms causing infection are Gram positive bacteria, most commonly *Staphylococcus aureus* (*S. aureus*), *S. epidermidis* and coagulase-negative staphylococcus (CNS), while 25% corresponds to Gram negative bacteria. Catheter-related infections can result from the accumulation of microorganisms on the external surface (extraluminal) and on the lumen of the catheter (intraluminal) [7-9], with biofilm-embedded bacteria being found on catheters as soon as 24 hours after insertion [10]. To prevent and fight the establishment of biofilms on the surface of catheters, a number of strategies has been explored in conceptual 2D polymer substrates or directly on 3D catheters.

Focus has been given to impregnation – homogeneous dispersion of the biocide throughout the polymeric matrix [11] – of the polymer of interest with classical antimicrobial agents, most commonly antibiotics and antiseptics, such as rifampin, minocycline, chlorhexidine, or silver sulfadiazine [8, 12, 13]. Silver nanoparticles, deposited on the surface through techniques as photochemical deposition, photo-reduction, or ion beam assisted deposition [8], have also been explored due to its antimicrobial profile, but toxicity and bacterial resistance have been major concerns [8]. Coating of only the surface of the catheter can also be achieved, through physical or covalent attachment and using techniques as dip coating or spray coating [11]. Several reviews reporting surface modification strategies for catheters (HD and other types) have been published, denoting the vast amount of research that has been conducted in this field [4, 8, 11, 14, 15].

Complete elimination of bacteria is however difficult to achieve [16], with reduction in both bacterial colonization and incidence of catheter-related bacteremia in critical care patients reaching only 30–50% [12]. In addition, only few modified catheters have been tested in randomized clinical trials specifically for HD use, which prevents evidence-based recommendations. Examples of catheters tested for HD patients include minocycline-rifampin-coated HD catheters that decreased the risk of catheter-related infection in patients with acute renal failure [17], and Arrowgard® Blue Acute HD catheter (Teleflex Incorporated), with chlorhexidine and chloracetate incorporated in the internal surface, and chlorhexidine and silver sulfadiazine on the external surface, that reduces bacterial colonization by 60% and catheter-related bacteremia by 80% [12]. The continuous use of antibiotics in these strategies also raises concerns about secondary effects on the healthy human microbiota and bacterial resistance [20], emphasizing the need for safe and non-antibiotic based antimicrobial catheters efficient at reducing the risk of infections.

Polyurethane (PU) is the polymer commonly used to produce HD catheters, and so modification of its surface has been sought, mostly on a laboratorial scale. The type of PU, the presence of proteins, and surface features seems however to strongly affect the outcomes. For instance, phosphonated PU showed the lowest adhesion of *S. aureus* (either in the presence of pre-adsorbed 1% albumin or fibrinogen) contrarily to quaternized aminated and sulfonated [19]. Phosphonated Pellethane, however, did not show any potential as infection-resistant material in Flemming's work [20], while quaternized amine PU showed promising ability to reduce bacterial colonization both in the presence or absence of 0.5% albumin [20]. PU has been modified with micropatterning [21], silver nanoparticles [22, 23], S-nitrosated poly (sulfhydrylated polyester)/poly(methyl methacrylate) (shown to release nitric oxide) [24], anti-quorum sensing enzymes, quaternary ammonium compounds, antimicrobial peptides, metallic compounds or bactericidal polymers [8].

Graphene-based materials (GBMs) are very interesting materials, with antimicrobial, cytocompatible and hemocompatible character, and with the advantage of lacking known mechanisms of bacterial resistance. Their use to confer PU antimicrobial properties has however been poorly explored [25, 26]. Previous work in our group showed that oxidized graphene-based materials (GBMox) can be used to

confer anti-adhesive (70% less adhesion) and bactericidal (70% more death) performance to PU (Tecothane) [29]. Smaller and oxidized particles were found to perform better, and only when exposed at the surface of the polymer, specifically as a coating [27, 28].

This study proposes the translation of the 2D conceptual graphene-based coating in PU films into commercially available HD catheters, in order to make them antimicrobial. Acknowledging the good performance of oxidized graphene nanoplatelets in previous works [27-29], graphene oxide (GO) and few-layer graphene oxide (FLGO) were used to coat the inner lumen of the catheter. Particles were immobilized at the PU surface using perfusion – corresponding to the passage of the GBMs dispersion through the interior of the catheter by means of pressure. This technique has potential of translation for industrial catheter manufacturing and that to the best of our knowledge is being used for the first-time as a coating technique of GBMs. The antibacterial effectiveness of the coating was assessed regarding its ability to prevent adhesion, colonization and migration under static and flow conditions.

2. Materials and Methods

2.1. Synthesis of oxidized materials

Carbon graphite micropowder (99% purity, 7–11 μm diameter) and few-layer graphene (xGnP grade M, $\sim 5\ \mu\text{m}$ lateral size) were commercially acquired from American Elements (California, U.S.A.) and XG Sciences (Lansing, U.S.A.), respectively. Materials were oxidized by modified Hummers method into graphene oxide (GO) and few-layer graphene oxide (FLGO), as previously described by us [29, 30], rendering GO and FLGO in aqueous dispersions. GO was further sonicated for 6 h in an ultrasonic bath to obtain exfoliated graphene sheets. Chemical characterization and morphology of GO and FLGO powders using XPS and TEM can be found in the Supplementary Information of Chapter IV (Figure S3), showing that materials have 30% degree of oxidation, different lateral size, and different number of layers [29].

2.2. Production and characterization of oxidized graphene-coated catheters

Long term duration, dual lumen, Hemoflow (HFS40E) polyurethane (PU) catheters were commercially acquired from Medcomp[®].

GO and FLGO dispersions in tetrahydrofuran (THF) were kept under stirring after preparation and were sonicated for 8 min immediately before use. Perfusion of GO/THF and FLGO/THF dispersions through the inside of the PU catheter was performed using a syringe pump (NE - 1010) held vertically to avoid deposition of GO or FLGO particles on one side of the catheter (Figure S1). This process was repeated for each of the two lumens of the catheter. Coating parameters, as GBMs concentration in THF, flow rate, and perfused volume, were optimized in PU tubes (data not shown) to determine the best formulation. Scanning Electron Microscopy (SEM) was used as a screening technique to assess

morphology, distribution, and stability of the coatings. The following set of conditions was selected: GBMs concentration of 6 mg/mL in THF, flow rate of 5 mL/h, and perfused volume of 5 mL. Coated catheters were left to dry in the fume hood for a couple hours and then overnight at 37 °C. Catheters were cut transversally into 1 cm pieces, and longitudinally opened, removing the central part, in order to obtain two samples with the internal surface exposed. These samples were used in subsequent assays, after sterilization with ethylene oxide (EO Gas series 3+) at Centro Hospitalar Universitário de São João.

Surface morphology was characterized by SEM analysis using a Phenom XL SEM in a Secondary Electron Detector (SED) mode and with an acceleration voltage of 10 kV or 15 kV. Uncoated and graphene coated-catheters were adhered onto carbon tape and coated with a thin layer of Au by sputtering to improve their conductivity.

2.3. Antibacterial performance of oxidized graphene-coated catheters

2.3.1. Bacterial strains and growth conditions

The internal surface of catheters was challenged with Gram positive *Staphylococcus epidermidis* (*S. epidermidis*).

In the studies performed at i3S (bacterial adhesion assay), a commercially obtained strain from the American Type Culture Collection (ATCC 35984) was used. After growing overnight in Trypticase Soy Agar (TSA, Merck) plates at 37 °C, two colonies were collected, inoculated into 5 mL of Trypticase Soy Broth (TSB, Merck) and cultured overnight at 37 °C under stirring at 150 rpm. Bacterial culture was adjusted to 10^5 – 10^6 CFUs/mL in fresh TSB medium supplemented with 1% (v/v) human plasma, to resemble the naturally occurring presence of proteins in physiological conditions.

In the studies performed at Queen's Medical Center, Nottingham University (exit site challenge and *in vitro* challenge assays), a clinical isolate from a ventriculoperitoneal (VP) shunt (silicone) was used. After growing overnight in TSA plates at 37 °C, a few colonies were collected and grown in 20 mL of TSB for 4 h at 37 °C under stirring at 200 rpm. Bacterial suspension was then spectrophotometrically adjusted to $\sim 10^8$ CFU/mL in fresh TSB medium.

2.3.2. Bacterial adhesion assay

Catheters ability to prevent bacterial adhesion was investigated by incubating uncoated, GO-coated and FLGO-coated catheters (sectioned catheters with 1 cm long) with *S. epidermidis* (Figure S2). A 10 μ L drop of bacterial suspension (10^5 – 10^6 CFUs/mL in TSB supplemented with 1% plasma) was added on top of the exposed internal lumen, and attachment was allowed to occur for 2 h at 37 °C. Afterwards, the drop was aspirated and the surface was carefully rinsed with TSB to remove non-adherent bacteria. In order to assess the effect of the surface towards adhered bacteria, samples were afterwards maintained in a low nutrient medium (1% TSB) over 72 h, allowing bacteria survival, but preventing them from proliferating. Bacterial attachment was assessed by SEM images after the 2 h initial adhesion, and after

24 h or 72 h re-incubation. Samples were fixed with a freshly prepared solution of 1.5% glutaraldehyde (VWR) in 0.14 M sodium cacodylate (Merck) buffer for 30 min at RT, rinsed twice with deionized water (dH_2O) and dehydrated in a graded ethanol series (50, 60, 70, 80, 90, and 99% for 10 min each). Samples were finally dried by critical point drying (variation of temperature 4 °C to 33–38 °C maximum pressure of 1000–1400 Psi, 5 cycles of 25 min). Catheters were mounted, coated and visualized in a SEM as previously described in section 2.2.

2.3.3. Exit site challenge assay

Catheters ability to prevent bacteria migration from the “exit site” (outside of the catheter) through the catheter track was evaluated by the exit site challenge [31]. A mixture of 0.4% bromocresol purple solution and 50% Muller Hinton agar in water was prepared, poured into a sterile conical bottom polystyrene container (Greiner Bio-One 201152) and allowed to solidify. One end of the inner surface of the catheter (1 cm long) was inoculated with a drop of bacteria ($\sim 10^8$ CFU/mL) for 1 h at 37 °C. After carefully washing non-adherent bacteria, catheters were inserted into the agar through the non-inoculated end and incubated at 37 °C. Growth and/or migration of bacteria through the catheter track was assessed visually for up to 72 h.

2.4.4. *In vitro* challenge assay

Catheters ability to resist bacterial colonization under flow conditions (0.33 mL/min) was evaluated by the *in vitro* flow challenge model – the assay was conducted using the same apparatus and procedure as described by Bayston *et al.* [31]. Briefly, catheters were aseptically introduced through glass cylinders and connected to a peristaltic pump. Lumens of the catheters were then perfused with neat TSB from a reservoir at approximately 500 mL per day, with the liquid being discarded into a waste collection vessel. To challenge catheters, perfusion was stopped and 1 mL of the bacterial suspension ($\sim 10^8$ CFU/mL) was inoculated down the catheters: clamps were applied for 1 h to stop flow and allow attachment. Clamps were then removed and perfusion with TSB was re-started. Samples of effluent were aseptically collected periodically (24, 72 and 96 h after the adhesion) from the outlet of the catheters (before reaching the waste collection vessel) and CFU counting was performed to determine viable bacteria.

3. Results

3.1. Graphene-based coatings on PU catheters

Morphological assessment of GO and FLGO-coated catheters revealed a homogeneous and complete coverage of the original surface of the inner lumen of the PU catheter (Figure 1). A good distribution of the coating throughout the entire extension of the catheter was confirmed by assessing different parts of the catheter by SEM. In both coated surfaces, and in particular in the FLGO-coated PU surface, the

spherical white structures pre-existing in the PU catheters were clearly observed in higher magnification images, suggesting the thin thickness of the coating. Both coatings presented randomly distributed wrinkles with ridged protrusions (asperities) that seem to “fuse” together. This topography resembles the vacuum filtered films and coatings produced by dip coating on PU substrates previously developed by us, using the same GBMs [27, 29]. Perfusion was therefore considered a good method to deliver a homogenous internal coating of PU catheters.

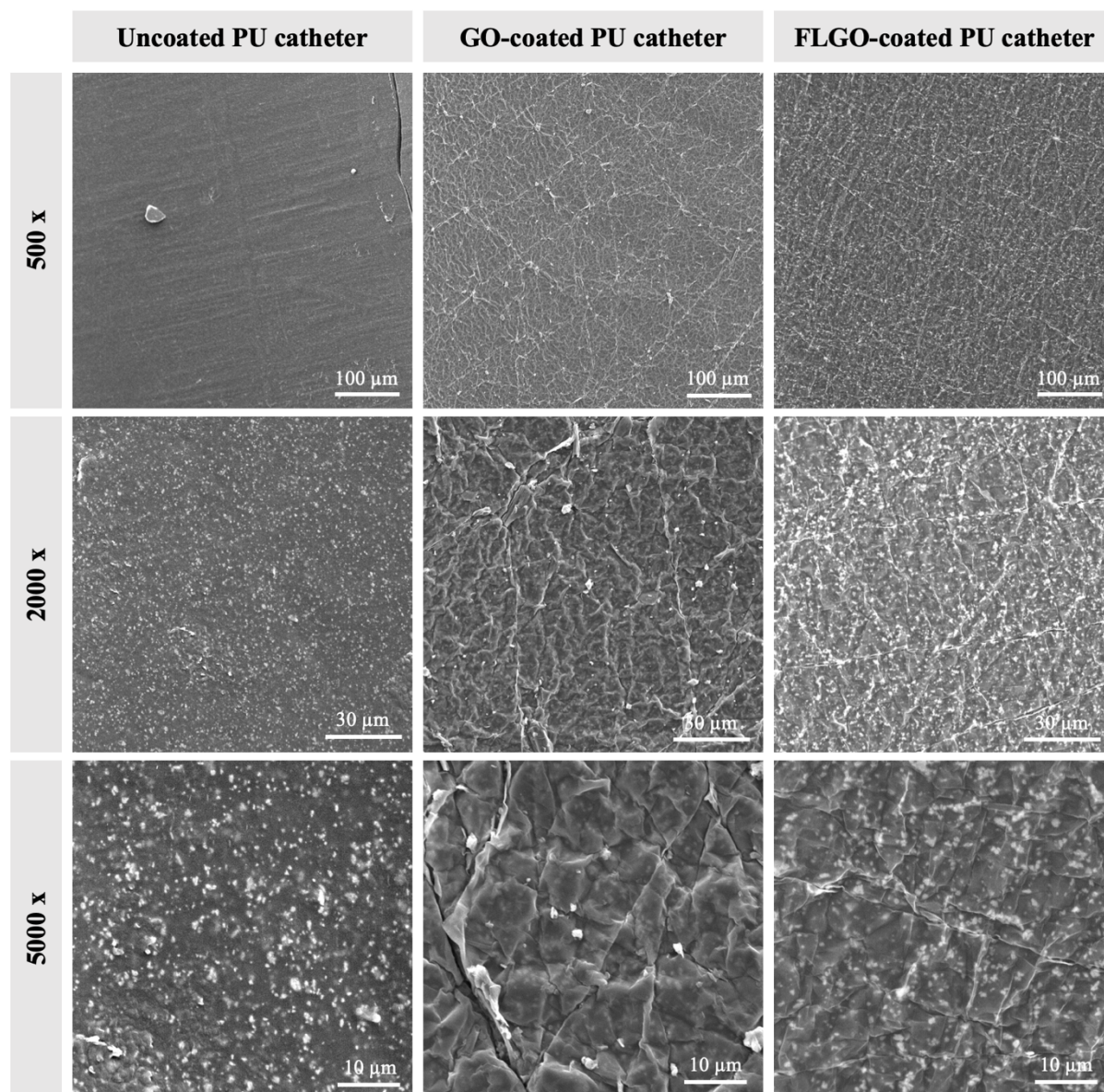


Figure 1. Surface morphology of the inner lumen of uncoated, GO-coated and FLGO-coated PU catheters. Images obtained by SEM (500×, 2000× and 5000× magnifications, scale bar = 100, 30 and 10 μm, respectively). PU = polyurethane, GO = graphene oxide, FLGO = few-layer graphene oxide.

3.2. Antibacterial performance of graphene-based coatings on PU catheters

The onset for catheters related infections is the adhesion and accumulation of bacteria on the surface of the catheter [8]. As such, bacterial adhesion to the coated-catheters was assessed as well as their ability to prevent bacterial migration and colonization (exit site challenge). Flow dynamics also impacts bacterial adhesion and colonization of the surface [1], and so the ability of bacteria to colonize the surface under flow conditions was explored.

S. epidermidis was only clearly observed on uncoated PU catheters at the 72 h time point (Figure 2, bottom row, highlighted with the white arrows). In the remaining time points, only the spherical white structures pre-existing in the PU catheters could be noticed. This suggests that bacteria either did not adhere during the 2 h period (at least not in sufficient amounts to be clearly observed) or detached from the surface during incubation. Regarding the effect of the coatings on bacterial adhesion, *S. epidermidis* was not found at the surface of FLGO-coated catheters in any of the time points, and even though the spherical structures observed in the GO-coated surfaces may resemble bacteria (particularly in the 72 h time point), similar structures are observed in the original coating (Figure 2, first row), and so a deeper analysis must be performed in order to clarify this.

The low adhesion of *S. epidermidis* to oxidized graphene-based surfaces had been observed before [27], validating the small amounts found in these coatings.

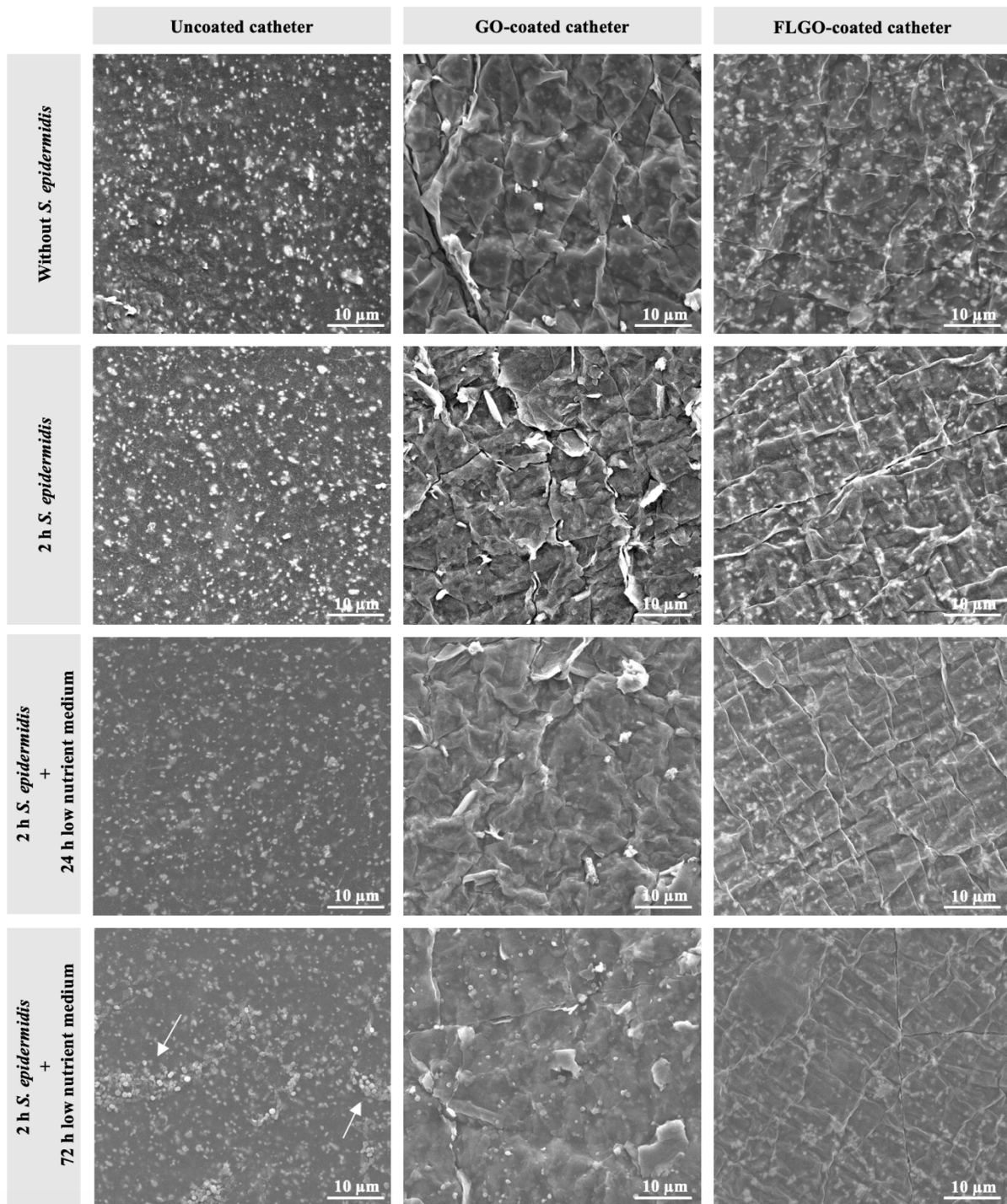


Figure 2. Adhesion assay of *S. epidermidis* in uncoated and GO and FLGO-coated PU catheters after 2 h adhesion and after re-incubation in low nutrient medium (1% TSB) for 24 h and 72 h. White arrows indicate the presence of bacteria. Images obtained by SEM (5000× magnification, scale bar = 10 μm). GO = graphene oxide, FLGO = few-layer graphene oxide.

Bacteria ability to colonize and migrate through the catheter surface was assessed visually by the exit site challenge (Figure 3), as a representation of a real situation of an infection starting at the catheter insertion point. As a proof of concept, only GO-coated catheters were studied regarding their ability to hamper bacterial migration.

After 24 h, white biofilm-like structures were observed in the inoculated end (top part in Figure 3) of both uncoated and GO-coated catheters, indicating bacteria ability to grow and proliferate upon the initial adhesion. After 72 h (bottom part of Figure 3), bacteria growth was clearly demonstrated not only by the biofilm-like structures, but also by the change in the media color from purple to yellow (as a result of the production of lactic acid, indicating that bacteria are growing).

In the uncoated PU catheters, signs of biofilm can be identified both in their internal and external surfaces, and in all the extension of the catheter, suggesting bacteria ability to migrate through. However, in the GO-coated catheter, the presence of bacteria seemed confined to its external surface. This suggests that bacteria are able to grow, proliferate and form biofilm on the PU surface in the catheter external surface, but graphene oxide inner coating seems to prevent this from happening.

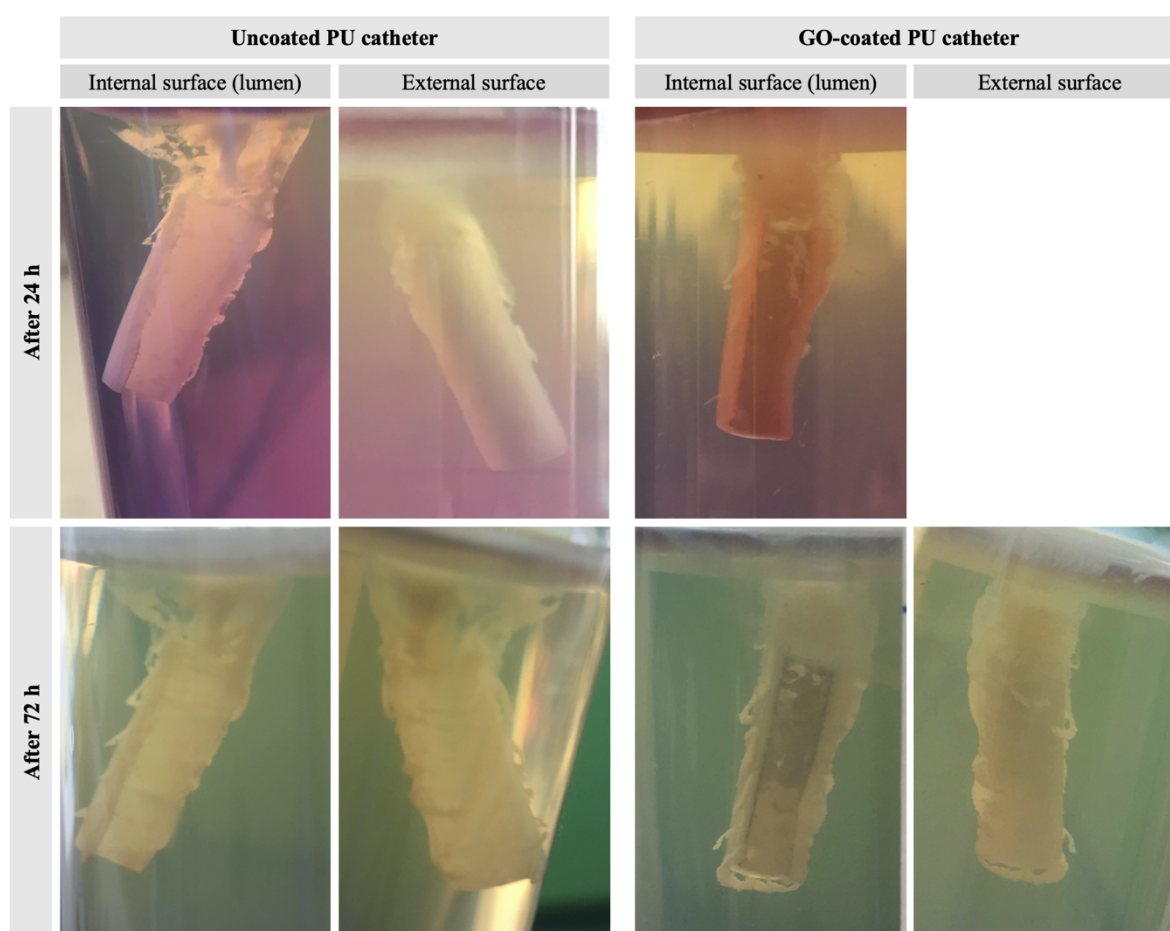


Figure 3. Exit site challenge towards *S. epidermidis* after 1 h adhesion on one end of the catheter. Bacterial colonization can be seen extending down the external and internal surface of the uncoated PU catheter track, while only at the external surface (uncoated part) of GO-coated PU catheter.

After 1 h adhesion (in static conditions) on the entire internal surface of the catheters, flow was applied on the catheters to assess bacteria ability to grow (Figure 4). Over 96 h, collected fluid from uncoated PU catheters demonstrated the presence of bacteria in increasing amounts, suggesting that the surface was not being able to kill adherent bacteria and that live bacteria were detaching from the

surface, thus supporting the need for better antimicrobial surfaces. However, coating of the PU catheters with GO does not seem to improve its performance under flow conditions.

This can be considered the worst-case scenario, where bacteria (in this case also a clinical isolate) are allowed to attach for 1 h followed by a low flow rate that may favor bacterial growth (0.33 mL/min vs 350 mL/min, which is the expected flow rate in a hemodialysis catheter). If in low flow bacteria are able to approach the surface and attach, in high velocity bacteria are transported with the flow having more difficulty to reach the surface [1].

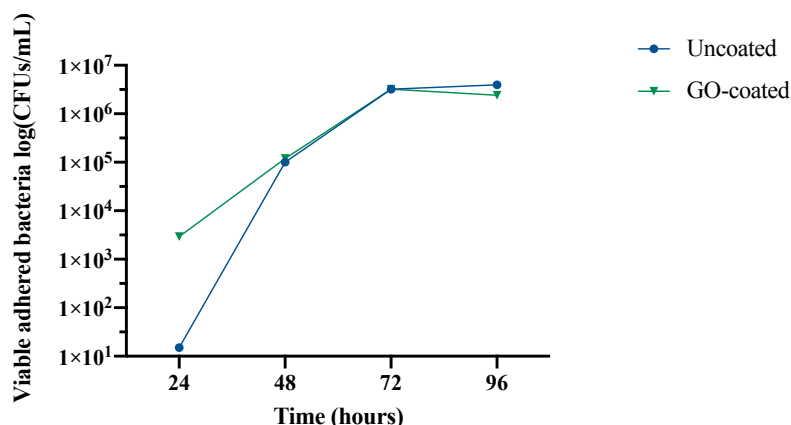


Figure 4. *In vitro* challenge towards clinical isolate *S. epidermidis* for uncoated and GO-coated PU catheters over 96 h.

4. Discussion

This exploratory work proposes the development of an antibacterial graphene-based coating directly applied on commercially available hemodialysis (HD) catheters. Oxidized graphene materials demonstrated efficiency as antibacterial agents when applied in a flat polyurethane (PU) surface through dip coating, even though coating homogeneity and stability revealed the need for further improvements [27], eventually requiring more than one dip to achieve homogeneous coatings. Translation of this approach to HD catheters and assessment of the antibacterial performance was therefore the step to follow. Direct coating with graphene on the surface of catheters, particularly on the inner lumen, is however a challenge. Perfusion – passage of a dispersion through a structure by means of pressure – could be the ideal technique to obtain a uniform and stable graphene-based coating inside the catheter.

Our results demonstrate that perfusion of a dispersion of GO or FLGO in THF is an efficient method to deliver a homogeneous and well distributed coating on the entire internal lumen of the catheter. Surface morphology, known to play a major role on the antibacterial performance of surfaces, resembled the coating obtained in PU flat surfaces when using the same GBMs [27]. GO and FLGO formed a homogeneous film on the catheter internal surface, covering the entire substrate, with wrinkles and grooves, and mostly basal planes exposed. However, differing parameters between the production

technique (dip coating *versus* perfusion), such as flow *versus* static conditions, can influence the surface morphology on a different level, affecting the bacterial response.

Complete exposure of oxidized GBMs with a similar wrinkled-like morphology has demonstrated antibacterial action towards different bacteria in 2D, mostly through an anti-adhesive action [27, 29, 32]. GBMs surfaces are reported to act upon direct contact, even though the oxidative potential of the materials can activate a ROS-independent pathway to oxidative stress. Our results indicate however that the experimental conditions strongly impact the antibacterial outcome. The adhesion assay revealed that *S. epidermidis* was not observed at the surface of GO and FLGO-coated catheters. These results are in line with previous experiments which shown that *S. epidermidis* adhere in low amounts to GBMs surfaces (and less than other bacteria). This can be associated either with the reduced number of bacteria (in 10 μ L around 10000 bacteria are present) or with the reported hydrophilic character of the surfaces. This can also explain why *S. epidermidis* was not able to migrate through the catheter track from the exit site and colonize the coated surfaces of the catheter, contrarily to the uncoated PU ones. Longer catheters with higher distance between the inoculated site and the end of the catheter should be used in order to further validate this result.

Bacteria inability to adhere and migrate was observed when the adhesion was circumscribed to a small region of the catheter, contrarily to the *in vitro* flow challenge assay. Here, the whole internal surface of the catheter was exposed to high amounts of bacteria (10^8 CFU/mL) for 1 h. This could cause bacteria to accumulate at the surface, either live or dead bacteria (if the surface was exerting a bactericidal action), preventing the action towards the remaining bacteria, or even acting as a nutrient source for their growth. In addition, this assay simulated the worst-case scenario where bacteria would be allowed to adhere, under static conditions, being then subjected to low flow in high nutrient media, where flow dynamics potentiates bacterial contact with the surface, since low shear stresses are applied. This sets the perfect conditions for bacterial growth and proliferation. In a real situation, flow rate and shear stress affect the moving bacteria and influence their ability to reach the surface: in low flow rates bacteria are able to approach the surface and attach, while in high flow rates bacteria are transported with the flow without reaching the surface [1]. The low flow rate implemented afterwards can also potentiate bacterial adhesion and grow (20 mL/h), as opposed to the flow rate verified in HD usages (350 mL/min). Still, between HD sessions the flow is steady, and so materials need to be able to prevent adhesion and colonization. Comparing to other catheters with impregnated antibiotics under similar condition [31], our results indicate that oxidized surfaces do not possess a killing action over time, acting preferentially through an anti-adhesive mechanism.

Bacterial adhesion to surfaces is a complex issue with diverse mechanisms occurring simultaneously and being dependent on a plethora of factors, such as physical and chemical properties of the surface, bacterial features, and environmental conditions [1, 8]. Studies need therefore to be carefully considered in order to gather the desired information to draw the right conclusions (for instance, adhesion under static *versus* flow conditions is impacted by different parameters). Surface characterization of the

coatings needs to be fully conducted, specifically regarding roughness, wettability and surface energy, in order to better correlate them with the antibacterial results and the design of the surface. Verification of the stability of the coating must be performed in order to ensure that the coating withstands the elevated flow rates found in HD sessions. Combination of a stable coating and an antiadhesive or bactericidal action that avoids the release of toxic substances (as antibiotics) are great features to make this coating safe. In addition, considering that the implementation in the clinics may be hindered by the high costs associated with coated catheters [12], our simple and inexpensive technology might be the ideal solution. The presence of proteins can also provide topographical, physical and biochemical cues for bacteria to interact and respond [33], with the surface composition of bacteria being also a significant parameter conditioning the adhesion to the surface. The presence of specific adhesins can promote adhesion to the surfaces, as is the case of SdrF and SdrG adhesins in *S. epidermidis* that bind to collagen and fibrinogen, respectively [34], or the production of autolysin E (AtlE) and accumulation-associated protein (AAP) that promote attachment for abiotic surfaces [35]. *In vivo*, this plays a significant role as for instance platelets have integrins that serve as a recognizer for fibrinogen, von Willebrand factor, fibronectin, collagen, and vitronectin, therefore promoting *S. aureus* adhesion. In turn, *S. aureus* promotes aggregation of platelets [35].

Apart from ensuring antibacterial performance, the coated HD catheters have to be highly hemocompatible considering their direct contact with blood. Previous studies in the group have shown that GBMs films with similar features to these coatings cause low adsorption of proteins and do not cause damage of blood components, as erythrocytes, allowing reduced adhesion of blood components [29].

5. Conclusions

Hemodialysis (HD) catheter-related infections are a global and serious problem, driving the exploitation of different strategies to make HD catheters antimicrobial. To the best of our knowledge, this is the first time that graphene, and in particular oxidized graphene-based (GO and FLGO) materials, is proposed as a coating for HD polyurethane catheters applied through perfusion in the internal lumen. Perfusion was an efficient coating technique, delivering a stable and homogeneous coating that covered the entire surface of the catheter. These coatings seem promising to prevent bacteria adhesion, colonization and migration over 72 h, however when the surface was saturated with bacteria and under low flow conditions, no improvements of the antibacterial ability of the coating were verified comparing to uncoated PU catheters.

Further experiments should be performed in order to complete this study using oxidized graphene-coated catheters and validate the biological performance observed in coatings of 2D PU substrates, both towards bacteria and blood components.

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Supplementary Information

Perfusion of GO and FLGO dispersion

6 mg/mL at flow rate of 5 mL/h

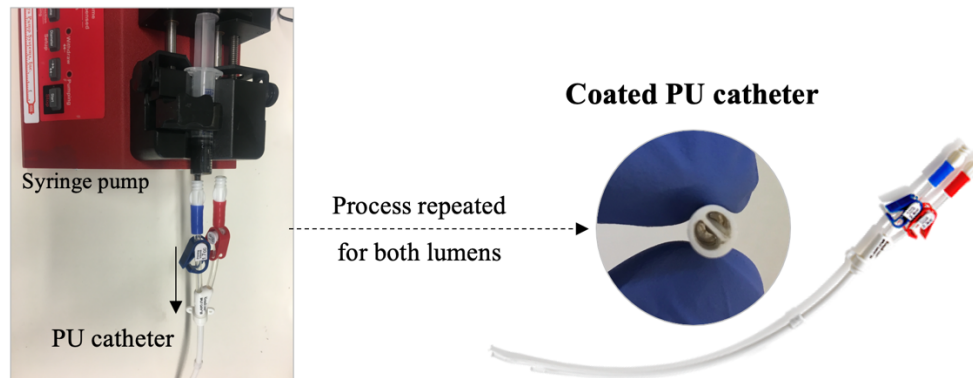


Figure S1. Perfusion setup for GO and FLGO coating of PU catheters. GO/THF and FLGO/THF dispersions (6 mg/mL) were perfused through the inside of the PU catheter at a flow rate of 5 mL/h, and perfused volume of 5 mL. The syringe pump was held vertically to avoid deposition of GO or FLGO particles on one side of the catheter. This process was repeated for each of the two lumens of the catheter. Coated catheters were left to dry in the fume hood for a couple hours and then overnight at 37 °C.

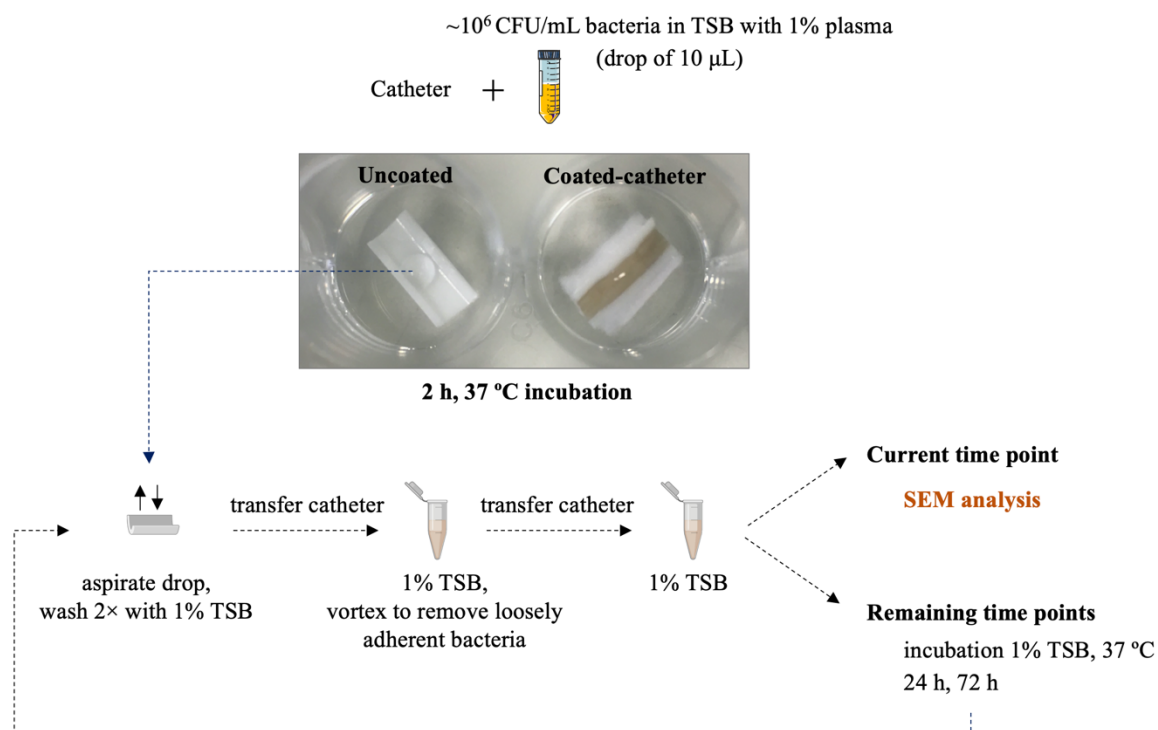


Figure S2. Protocol implemented in the adhesion assay. Catheters were incubated with *S. epidermidis* for 2 h in TSB supplemented with 1% plasma to allow adhesion. After that, non-adherent bacteria were removed, and catheters were prepared for SEM analysis either immediately or after re-incubation in low nutrient medium (1% TSB) for 24 h and 72 h.

CHAPTER VIII

General Discussion, Concluding Remarks, and Future Perspectives

1. General discussion

Hemodialysis (HD) is the most common type of dialysis in patients with end-stage renal disease, and catheters are the most frequent type of vascular access, despite also being the one associated with higher risk of infections [1]. Current treatments rely mostly on the use of antibiotics, which more than a solution can be the reiteration of a rising problem: antimicrobial resistance. Alternative solutions to make catheters antimicrobial are therefore urgent. Graphene-based materials (GBMs) stand out as promising candidates to control bacterial attachment and biofilm formation, and consequently the establishment of a bacterial infection. Designing an improved version of a HD catheter has however two competing demands: the improvement of the antibacterial properties of the surface, and the maintenance, if not the improvement, of its anti-thrombogenic performance. This thesis focused primarily on the antibacterial performance of the surfaces. However, assessment of other biological functions (biocompatibility and hemocompatibility) must be performed before reaching the final design of the catheter surface.

The fundamental study depicted in Chapter IV enabled us to understand the physico-chemical features of GBMs and how they influence surfaces features, and consequently the interaction with the biological systems. Oxidized surfaces, produced with either graphene oxide (GO) or few-layer graphene oxide (FLGO), are more anti-adhesive, adhering less bacteria, than surfaces produced with reduced graphene oxide (rGO) and few-layer graphene (FLG). In particular, clinically relevant *S. epidermidis* and *E. coli* adhere even less than *S. aureus* and *P. aeruginosa*. The use of oxidized surfaces promotes therefore an anti-adhesive approach, more effective considering the final application in HD catheters, as it is preferable to avoid bacterial adhesion than having them adhered and killed on the surface. Low adhesion of *S. epidermidis* to the different GBMs surfaces strengthens the potential of these materials to be used as coatings in medical devices, where this bacterium is responsible for colonization and biofilm formation. This also supported the selection of oxidized surfaces to be explored as catheters coatings in Chapter VII. This bacteria-dependent effect highlighted the importance of not only bacteria membrane structure (Gram positive and Gram negative), size or shape, but also of the different adhesins existing in the bacteria membrane that can change their affinity and interaction with the surface (itself or with adsorbed proteins on the surfaces) [2, 3]. We also revealed that both the randomly exposed sharp edges of FLG surface and the flatter wrinkled-like surface of FLGO were able to kill bacteria. The type of GBMs (G, rGO, GO, FLGO), their alignment at the surface (vertical *versus* horizontal), and the surface production method strongly influence the surface features, and consequently the effect against the different bacteria (with different degrees of susceptibility) [4-7]. Maintenance of these parameters is therefore crucial and can promote preservation of the antibacterial performance. We show that using the same oxidized materials (GO or FLGO) but varying production techniques – vacuum filtration, dip coating, spray coating (data not shown), perfusion –, can lead to the production of surfaces with similar wrinkled-like morphology, causing the bacteria anti-adhesive profile to be preserved.

Apart from surface topography, fluid flow also affects quorum-sensing signaling and thus bacterial response. Biofilm formation depends on the quorum-sensing states of bacteria: *S. aureus* for instance forms biofilms at low cell density, with increase in cell density repressing it [8]. In general, in high fluid flows and independently on the bacterial strain, more bacteria are necessary to initiate quorum sensing, as the flow fluid removes autoinducers [8]. Thus, once again, preventing bacterial adhesion, and consequently biofilm formation, would be a more efficient strategy. Bacterial adhesion is also strongly impacted by the proteins adsorbed onto the surface rather than by the catheter surface itself, a process that occurs immediately after catheter insertion [9]. Chapter IV specifically demonstrated that indeed proteins adsorbed differently to the GBMs surfaces, with adsorbed proteins as fibrinogen promoting bacterial adhesion. The higher amounts of proteins on the non-oxidized surfaces justify higher amounts of bacteria.

The fundamental study in Chapter IV also aimed to address the question regarding the different effect observed towards mammalian cells and bacteria. Similarly to us, other authors reported efficacy of GO and rGO-based surfaces to kill bacteria while promoting mammalian cells proliferation, even when using a different production technique (vacuum filtration *versus* drop casting), and presenting different morphology and wettability [6]. A putative answer lies with the different structure, lipid composition, and stiffness of the mammalian cells *versus* bacteria cell membranes and with their different size [9-11]. Mammalian cells are larger (~20 μm in size, an order of magnitude larger than the bacterial cells, 1–2 μm) and their membrane is more flexible, and therefore they can more easily adapt and interact with the different features of the surface (either wrinkles and grooves or sharp edges). Bacteria, on the other hand, possess a stiffer membrane that is unable to adapt and change according to the surface features, and so if stretched enough can rupture and/or prevent their adhesion. The protective matrix secreted by mammalian cells can also be making them more resistant to the effects of graphene [11]. Tuning nanotopography has been associated with the ability of killing bacteria while being beneficial for mammalian cells [9]. Pandit *et al.* reported that by keeping the height of the vertical graphene coating between 60 and 100 nm, the coating is able to effectively kill bacteria, while being completely harmless to mammalian cells [11]. Apart from the effect of topography and the inherent structural features of the biological systems, their susceptibility to reactive oxygen species (ROS) can also contribute. Our surfaces do not seem to induce the overproduction of ROS (Chapter V), however their ability to oxidize GSH can still disrupt the balance between ROS production and elimination by failing to eliminate ROS (Chapter IV and V). When exposed to NIR irradiation (Chapter V), ROS overproduction was established as the mechanism of action predominant for FLG films. This can rise concerns regarding the potential action towards mammalian cells. Even though not fully elucidated, this can be addressed considering the different sensitivity of bacteria and mammalian cells towards exogenous ROS overproduction, particularly their threshold of exposure levels to ROS [12-14]. In healthy cells, endogenous ROS production is maintained at a low level, and both the endogenous and exogenous ROS amounts are carefully balanced by various antioxidant systems. Bacteria also have the

ability to respond to excess of ROS, increasing expression of ROS detoxification enzymes along with additional protective systems [15]. Bacteria may have however fewer resources, making them more susceptible towards ROS-based therapies.

Combination of GBMs with NIR action can be thought as a strategy to rapidly increase ROS production. Interestingly, it has been suggested that upon exposure to a stressful event or agent, ROS accumulation and cell death continued to occur in bacteria, meaning that once ROS threshold is exceeded, the death process becomes self-driven (i.e., self-sustainable) [16] and in some cases it can also lead to self-destruction [16, 17]. This may be worth exploring since the chances that microbes can develop tolerance or resistance to antimicrobial photodynamic inactivation is considered highly unlikely, since their mechanisms of action acts towards different targets [15].

Despite in low amounts, adhesion of blood components at the surface of GBMs films (Chapter IV) may raise questions about the potential activation of the coagulation cascade when implemented in HD catheters. Adhesion of platelets however may actually potentiate the antibacterial performance of the material, since activated platelets can deliver antimicrobial effector molecules, such as kinocidins and platelet microbicidal proteins [18, 19]. Further studies need however to be performed in order to establish the validation of this hypothesis. Adhesion and proliferation of fibroblasts could also be a strategy worth pursuing for tunneled catheters, since it could be beneficial to ensure stability of the cuff. Unpublished data by our group (manuscript in preparation with P.C. Henriques as co-author) has actually shown that spray coated polyurethane (PU) with oxidized GBMs promotes fibroblast adhesion, unlike uncoated PU, suggesting that such coated catheters could be used without need of a cuff.

This interaction between GBMs and PU, the most common polymer to produce HD catheters, is therefore of great relevance when envisaging the application to HD catheters. Chapter VI was dedicated to understand the best way to incorporate GBMs in PU (composite *versus* coating) in order to make it antimicrobial. Despite preference for a composite approach – simple in manufacturing and regulation –, as highlighted in Chapter III, exposure of GBMs at the surface of the polymer is required for the antibacterial action. From the available techniques depicted in Chapter III, the random exposure of both sharp edges and basal planes provided by dip coating seemed to potentiate the antibacterial action, being also a more easily transferable technique to catheters production (comparing to techniques as spin coating or drop casting). While a higher oxidation degree seemed to potentiate the antibacterial properties of the surface, thickness of GBMs did not significantly influence it (Chapter IV). FLGO was therefore selected to move on considering its cheaper and easier production process than GO, which is important when envisaging an industrial approach. Acknowledging that exposure of smaller and oxidized graphene improved antimicrobial performance of PU flat substrates through dip coating, perfusion of the same materials was explored in commercially available HD catheters. Even though PU was used as a binding agent to ensure coating stability in Chapter VI, a collaborative and parallel work performed in our group using spray coating demonstrated that a good adhesion and stability of the coating could be achieved without the presence of PU. Based on these insights, coating on the inside of

the catheters was performed using a dispersion of FLGO and GO in organic solvent, which *per se* ensured the interaction between the graphene sheets and the polymer. A homogenous oxidized graphene coating on HD PU catheters delivered by perfusion was reported here for the first time, with promising results (Chapter VII). Seeking a safer and more environmentally friendly approach, a water dispersion could be explored with an additional step with polydopamine to promote GBMs coating adherence to PU.

Chapter V demonstrated the great potential of light-responsive GBMs films as smart bactericidal platforms, with NIR irradiation of FLGO films significantly boosting their killing efficiency of the bacteria from the supernatant. Local irradiation of the coated-catheter could be an interesting strategy to improve its antibacterial performance. Tissue penetration depth of NIR light in the first biological window (650 – 950 nm) is around 1 cm [20], with the depth of penetration depending on the wavelength and varying with tissue type. Penetration values were not found specifically for PU, and so experimental evidence should be obtained. The photothermal and photodynamic effects could be isolated and further investigated [21] in order to be specifically tailored according to the envisioned final application.

Bacterial adhesion is a dynamic and diverse process. *In vivo*, free-floating bacteria will have the possibility to contact with the surface, and depending on the affinity may interact or not. In these experiments the high number of bacterial inoculum and the reinforcement of bacterial adhesion needs to be considered as well as their impact in the obtained antibacterial performance. Even though this thesis provided insights on how bacterial adhesion can be controlled on GBMs-integrated surfaces, the *in vivo* scenario will always bring new players and unexpected outcomes.

2. Concluding remarks

This thesis introduced the potential of graphene-based materials (GBMs) as antibacterial agents in coatings of hemodialysis catheters.

GBMs intrinsic features, mostly GBMs oxidation degree and platelet size, were determinant to shape the features of the surface and the interaction with the biological systems, with platelet thickness losing its relevance when integrated in a surface. In spite of the surface production technique – vacuum filtration, dip coating, or perfusion –, smaller particles with higher oxidation degree were consistently shown to improve the antibacterial action of the surface, even though wrinkles size and distribution within the sample varied (which may impact biological behavior). Having hydrophilic character, nano-roughness, and exposed oxygen-containing groups significantly reduced protein adsorption, and consequently bacterial adhesion (with some extent of bactericidal activity), while still allowing mammalian cells adhesion and without causing damage of blood cells. Exposure of the GBMs was fundamental for surfaces to act upon bacteria, with clinically relevant bacteria adhering less to oxidized films, with *S. epidermidis* and *E. coli* adhering even less than *S. aureus* and *P. aeruginosa*. As light-responsive materials, GBMs antibacterial performance was strengthened upon exposure to low power near-infrared (NIR) radiation. Further insights on the mechanism of action of surfaces reinforced the role of oxidative stress over physical puncturing of the membranes, strongly supported by the ability of surfaces to deplete natural antioxidants.

This led to the development of a graphene oxide-based coating that was for the first time applied in the internal lumen of commercially available hemodialysis catheters. Combination of an industrial-like production technique, such as perfusion, with the use of commercially available catheters envisages a faster bench-to-bedside transition. This straightforward and efficient application of graphene-based materials on the surface of HD catheters paves the way for coatings of other medical instruments, implants, and surgical equipment, being a competitive and promising approach to deliver low cost and efficient antimicrobial materials in order to reduce bacterial-associated infections.

3. Future perspectives

Although the work developed in this thesis addressed important knowledge gaps concerning the antimicrobial performance of graphene-based materials integrated surfaces, it also raised questions and new ideas for future studies, aiming not only a better implementation of these materials in hemodialysis catheters, but also for other applications.

Following up on the interesting performance of irradiated GBMs films, the question that naturally arises is: can NIR irradiation improve the antibacterial performance of coated-catheters? To answer this query, a strategy for preventive action could be explored, focusing on the ability of NIR in combination with GBMs to prevent bacterial adhesion and consequent biofilm formation on the surface of catheters. On the other hand, a killing/bactericidal strategy could also be pursued, exploring the ability of this combination to destroy the biofilm once its formed. Implementation of these strategies could be envisaged through a direct application of NIR radiation on the catheter surface or through the development of a catheter cap with a NIR LED incorporated that would maintain the local subjected to contamination under continuous or frequent irradiation, primarily between hemodialysis sessions.

The exquisite interaction between bacteria and the surface of the coated catheter would also be very interesting to explore, since it is the foundation of the antibacterial action observed. Atomic force microscopy combined with single-cell force spectroscopy could tell more about the strength of the interaction between each bacterium and the surface, and the type of surface adhesins driving the interaction (also dependent on the type of adsorbed proteins), so that the coating could be better modified. In further experiments, the use of ATCC strains – which have the advantage of being very well-characterized and thus are ideal for initial and fundamental studies –, can be replaced by clinical isolates, directly collected from hemodialysis catheters. These bacteria may present a higher variety of pathogenicity mechanisms and may have developed resistance or survival mechanisms in the specific clinical context of hemodialysis, thus enriching the outcome.

As mentioned, the improvement of the antibacterial performance of the catheters should occur without compromising their design, material bulk properties, and interaction with the blood. Even though GBMs films were harmless to blood components (not causing hemolysis), this biological behavior must be assessed in the coated catheter and under flow conditions, more closely resembling the blood flow. Stability (under flow conditions) and durability of the coating should be addressed, to ensure safety and a continuous bactericidal action, which can be assessed by incubating the coated catheter for a period of time (and in the presence of bacteria if the aim was to prove antiadhesive action). The mechanical properties of the catheter and the maintenance of its internal diameter after the coating process should be confirmed, in order to ensure suitable handling and insertion, and to avoid disturbance of the blood flow, allowing efficient blood debit. Considering that bacterial adhesion can also occur in the external part of the catheter, modification of the external surface of the catheter could also be

performed for optimal effect against bacteria [22-24]. As mentioned in the Discussion, spray coating could be a promising technique for this purpose.

Considering the intricate relation between bacterial adhesion and thrombus formation, exploitation of the interaction between proteins, bacteria and platelets could provide more insights on how the coating would behave *in vivo* and whether the verified adhesion of platelets can act beneficially towards bacteria. Determination of the type of proteins that are produced by cells (or bacteria) in the presence of GBMs surface could also provide valuable information to better understand this interaction.

Because the *in vitro* experimental conditions rarely fully mimic what is happening *in vivo* [25], the cherry on top of this work would be to test these coated catheters in an *in vivo* infection model. For that, an approach like the one followed by Keum *et al.* could be used. These authors tested the inhibition of *S. aureus* biofilm formation by a polymer coating *in vivo*, by implanting the uncoated or coated urinary silicon catheters subcutaneously at the right flank of a mouse, with the harvested catheters being evaluated using crystal violet [26].

4. References

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