

*ADVANCES IN GRAPHENE-BASED
MATERIALS AND THEIR COMPOSITES WITH
FOCUS ON BIOMEDICAL APPLICATIONS*

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To my family and everyone who supports me

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4. C. Gonçalves*, A.M. Pinto*, A.V. Machado, J.A. Moreira, I.C. Gonçalves, F.D. Magalhães, Biocompatible reinforcement of poly(lactic acid) with graphene nanoplatelets, *Polymer Composites* (2016) article in press, DOI: 10.1002/pc.24050. *equal contribution
5. A.M. Pinto, J.A. Moreira, F.D. Magalhães, I.C. Gonçalves, Polymer surface adsorption as a strategy to improve the biocompatibility of graphene nanoplatelets, *Colloids and Surfaces B: Biointerfaces* 146 (2016) 818-824.
6. A.M. Pinto, C. Gonçalves, I.C. Gonçalves, F.D. Magalhães, Effect of biodegradation on thermo-mechanical properties and biocompatibility of poly (lactic acid)/graphene nanoplatelets composites, *European Polymer Journal* 85 (2016) 431-444.

ABSTRACT

Graphene is the elementary structure of graphite, being a one carbon atom thick sheet, composed of sp^2 carbon atoms arranged in a flat honeycomb structure. Due to its unique characteristics, graphene has unmatched electronic, mechanical, optical, and thermal properties, amongst others. In 2010, Geim and Novoselov were awarded the Nobel prize "for groundbreaking experiments regarding the two-dimensional material graphene". Since then significant financial and human investment has been made on graphene applications in the most diverse forms. This means that graphene tends to become widespread. However, only a small fraction of the research focuses on the biological field.

Poly(lactic acid) (PLA) is an aliphatic polyester derived from renewable sources, which has several applications in various areas. In bioengineering, PLA is used for production of bioresorbable artificial ligaments, hernia repair meshes, scaffolds, screws, surgical plates, and suture yarns. To make this material more effective in several applications some properties can be improved, being mechanical performance usually the most relevant. Reinforcement with small amounts of nanofillers, as graphene/based materials (GBMs) is an interesting option because it allows improvement of target properties without changing PLA's main characteristics.

Since GBMs have several potential applications in biomedical engineering and biotechnology, this thesis is focused in understanding their biointeractions, and which sort of physical-chemical features have impact on their biocompatibility. It also studies GBMs as fillers for PLA, and the way by which they improve PLA properties and affect biocompatibility.

An extensive literature review is presented on the key issues regarding GBMs biocompatibility, namely production methods, physical-chemical properties, concentrations, time of exposure, and encapsulation in polymer matrices. In this thesis, the biological properties of GBMs dispersed in liquid media are studied in *in vitro* assays, as well as how they are affected by the materials' morphology, degree of oxidation, and surface modification with polymers. Human fibroblasts are used as an *in vitro* model to characterize GBMs biocompatibility, in terms of induction of reactive oxygen species production, metabolic activity changes, effect on cell morphology, evaluation of membrane damages, and particles internalization. Smaller graphene nanoplatelets (GNP) are observed to be generally more biocompatible than larger sized ones, which tend to cause membrane damages. Also, complete oxidation of GNP folds its sharp edges, therefore preventing toxicity. Surface adsorption of poly(vinyl alcohol) increases GNP size, preventing its internalization, therefore improving its biocompatibility. These results are relevant for the materials' use in biomedical applications.

Literature on composites of PLA with carbon-based nanomaterials is also reviewed in depth. Usually, GBMs are reported to improve several PLA properties, namely thermal, electrical, mechanical, and biological properties. However, GBMs physico-chemical properties, composites production methods, and ideal incorporation amounts vary between studies.

In this thesis, the production of PLA/GNP composites is explored by two different methods: solvent mixing followed by doctor blading, and melt-blending followed by compression moulding. Optimization of both methods is performed, in order to improve PLA physical-chemical properties. GNP improves the mechanical properties of PLA, and reduces the decay of its mechanical performance after 6 months degradation in physiological conditions. In addition, the composites have stable behaviour under cyclic creep-relaxation testing, while PLA exhibits significant cumulative permanent strain, and ruptures after a few cycles. This is particularly relevant in the context of PLA uses in orthopedics, or other implantable materials that need to have a suitable mechanical performance along time when inside the body.

Improvements in biological properties are also observed, namely increase in cell proliferation for PLA/graphene oxide composites, and decrease of platelets activation for PLA/graphene nanoplatelets composites, both produced by solvent mixing. Composites produced by melt-blending, and their degradations products, do not affect cell metabolic activity and morphology.

Finally, the above-mentioned results are discussed in the light of the most recently available literature reports, and new research perspectives are pointed out. *In vivo* characterization of the PLA/GNP composites is being performed, since they present outstanding mechanical performance after degradation in biological conditions, and lack of *in vitro* toxicity, which perspectives them as a promising material for biomedical applications.

Keywords

graphene; poly(lactic acid); biocompatibility; biomaterials; biodegradation; composites; mechanical properties.

RESUMO

O grafeno é a estrutura elementar da grafite, sendo uma folha com a espessura de um átomo de carbono, composta por átomos de carbono com hibridização sp^2 , ordenados numa estrutura de colmeia 2D. Devido a estas características únicas, o grafeno possui ímpares propriedades eletrônicas, mecânicas, óticas, térmicas, entre outras. Em 2010, o prémio Nobel da Física foi atribuído a Geim e Novoselov “por experiências revolucionárias com o material bidimensional grafeno”. Desde então, tem sido feito um significativo investimento financeiro e humano nas aplicações do grafeno nas mais diversas formas. Isto significa que o grafeno tende a estar disseminado, no entanto, apenas uma pequena parte da investigação na área está focada no campo biológico.

O ácido poliláctico (PLA) é um poliéster alifático derivado de fontes renováveis, que tem aplicações em diversas áreas. Em bioengenharia, o PLA é usado para produção de ligamentos artificiais bioabsorvíveis, redes para reparação de hérnias, estruturas para regeneração de tecidos, parafusos e placas cirúrgicas e fios de sutura. Para tornar este material mais eficaz para várias aplicações, algumas propriedades podem ser melhoradas, sendo o desempenho mecânico normalmente a mais relevante. O reforço com pequenas quantidades de nano-cargas, como os materiais com base em grafeno (GBMs) é uma opção interessante, pois permite a melhoria das propriedades alvo sem modificar as principais características do PLA.

Como os GBMs têm várias potenciais aplicações em engenharia biomédica e biotecnologia, esta tese está focada na compreensão das suas bio-interações e no tipo de características físico-químicas que têm impacto na sua biocompatibilidade. Também estuda os GBMs como cargas para incorporação em PLA e a forma como melhoram as propriedades do PLA e afetam a sua biocompatibilidade.

É apresentada uma extensa revisão bibliográfica dos pontos-chaves da biocompatibilidade dos GBMs, nomeadamente métodos de produção, propriedades físico-químicas, concentrações, tempos de exposição e encapsulação em matrizes poliméricas. Nesta tese, as propriedades biológicas dos GBMs quando dispersos em meio líquido, são estudadas em ensaios *in vitro*, assim como a forma como estas são afetadas pela morfologia dos materiais, grau de oxidação e modificação superficial com polímeros. Fibroblastos humanos são usados como modelo *in vitro* para caracterizar a biocompatibilidade dos GBMs, em termos de indução de espécies reativas de oxigénio, alterações na atividade metabólica, efeito na morfologia celular, avaliação de danos na membrana e internalização de partículas. Foi observado que nanoplaquetas de grafeno (GNP) mais pequenas, no geral são mais biocompatíveis que as de tamanho maior, que tendem a causar danos nas membranas celulares. Para além disso, a oxidação completa das GNP dobra as suas

extremidades afiadas, prevenindo assim a sua toxicidade. A adsorção superficial de álcool polivinílico aumenta o tamanho das GNP, impedindo a sua internalização e assim melhorando a sua biocompatibilidade. Estes resultados são relevantes tendo em conta as aplicações biomédicas destes materiais.

A literatura sobre materiais compósitos de PLA com nanomateriais com base em carbono também é revista em profundidade. É descrito que normalmente os GBMs melhoram várias propriedades do PLA, nomeadamente térmicas, elétricas, mecânicas e biológicas. No entanto, as propriedades físico-químicas dos GBMs, os métodos de produção dos compósitos e quantidades de GBMs incorporadas variam entre os estudos.

Nesta tese, a produção de compósitos de PLA/GBMs é explorada usando dois métodos diferentes: 1) mistura em solvente, seguida de doctor blading; 2) mistura em fundido, seguida de moldagem por compressão. É realizada otimização de ambos os métodos, de forma a melhorar as propriedades físico-químicas do PLA. As GNP melhoram as propriedades mecânicas do PLA e reduzem o decaimento do seu desempenho mecânico após degradação em condições fisiológicas durante 6 meses. Para além disso, os compósitos apresentam comportamento estável sob testes cíclicos de fluência-relaxamento, enquanto que o PLA apresenta deformação cumulativa constante e rompe após poucos ciclos. Isto é particularmente relevante tendo em conta as aplicações do PLA em ortopedia ou noutros materiais implantáveis que necessitem de ter um desempenho mecânico adequado ao longo do tempo quando no interior do corpo.

São observadas melhorias nas propriedades biológicas, nomeadamente aumento da proliferação celular à superfície de filmes compósitos de PLA/óxido de grafeno e decréscimo da ativação de plaquetas para PLA/nanoplaquetas de grafeno, ambos produzidos por mistura em solvente. É observado que os compósitos produzidos por mistura em fundido e os seus produtos de degradação não afetam a atividade metabólica nem a morfologia celular.

Finalmente, os resultados acima mencionados são discutidos à luz da literatura mais recente e novas perspetivas de investigação apontadas. Está a ser realizada caracterização *in vivo* dos compósitos de PLA/GNP, uma vez que apresentam um desempenho mecânico notável após degradação em condições biológicas e não são tóxicos *in vitro*, o que os torna um material promissor para aplicações biomédicas.

Palavras-chave

grafeno; ácido polilático; biocompatibilidade; biomateriais; biodegradação; compósitos; propriedades mecânicas.

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LIST OF ABBREVIATIONS AND ACRONYMS

- %ID/g** – percent injected dose per gram of wet tissue
- aG** – aggregated graphene
- APS** – ammonium persulfate
- at.%** – atomic percentage
- BB-rGO** – brilliant blue functionalized rGO
- BBS** – balanced buffer solution
- BSA** – bovine serum albumin
- C18PMHePEG** – poly(maleic anhydride-alt-1-octadecene)
- CB** – carbon black
- CBNs** – carbon-based nanomaterials
- CCCP** – carbonyl cyanide m-chlorophenylhydrazone
- CCVD** – catalytic carbon vapor deposition
- CL** – cellulose
- CM-H2DCFDA** – chloromethyl-2',7'-dichlorodihydrofluorescein diacetate
- CNT(s)** – carbon nanotube(s)
- CON** – chondroitin
- CPII** – cell proliferation inhibition index
- CS** – chitosan
- CVD** – chemical vapor deposition
- CXYG** – carboxyl graphene nanoplatelets
- d** – diameter
- DAPI** – 4',6-Diamidino-2-phenylindole dihydrochloride
- DCC** – *N,N'*-dicyclohexylcarbodiimide
- DD** – degree of deacetylation
- DLS** – dynamic light scattering
- DMA** – dynamic mechanical analysis
- DMEM** – Dulbecco's modified eagle's medium
- DMF** – dimethylformamide
- DNA** – deoxyribonucleic acid
- DSC** – differential scanning calorimetry
- EDC** – N-(3-dimethylamino-propyl-N'-ethylcarbodiimide)
- EDTA** – ethylenediaminetetraacetic acid

ERG – electroretinography
FBS – fetal bovine serum
FDA – food and drug administration
f-G – functionalized graphene
FTIR – Fourier transform infrared
G2xO – doubly-oxidized graphene oxide
GBM(s) – graphene-based material(s)
GLU – glucosamine
G-NH₂ – amine-modified graphene
GNP(s) – graphene nanoplatelets
GNP-ox – oxidized graphene nanoplatelets
GNSs – graphene nanosheets
GO – graphene oxide
GO-COOH – graphene oxide functionalized with carboxylic groups
GONP – graphene oxide nanoplatelets
GONSs – graphene oxide nanosheets
GPC-SEC – size exclusion chromatography
G-Pluronic – graphene dispersed in Pluronic aqueous solution
GS – graphene sheets
GSH – glutathione
Gt – graphite
GtO – graphite oxide
HA – hyaluronic acid
HA – hydroxyapatite
Hb – hemoglobin
HD – hydrodynamic diameter
HEC – hydroxyethyl cellulose
Hep/BSA-g-pRGO – heparin/bovine serum albumin grafted pRGO
hpf – hours post-fertilization
HPMEC – human pulmonary microvascular endothelial cells
Hr – Hydrazine reduction
HUVECS – human umbilical vein endothelial cells
i.v. – intravenous
IL – interleukin
IOP – intraocular pressure
IPTES – 3-isocyanatopropyl triethoxysilane

l – length

MA – maleic anhydride

MDG – methanol derived graphene

MFG – magnetic multifunctional graphene

MG – magnetic graphene

MHM – modified Hummers method

MMP – mitochondrial membrane potential

MPC – 2-(methacryloyloxy) ethyl phosphorylcholine

MPS – mononuclear phagocyte system

mt – mitochondria

MTT – 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan

M_w – molecular weight

MWCNT(s) – multi-walled carbon nanotube(s)

NA-rGO – 1-Naphthalenesulfonate functionalized rGO

nGO – ultra small graphene oxide

NGO-SS-mPEG – PEGylated nano-graphene oxide

PANI – poly(aniline)

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PC – human platelets concentrate

PDMS – polydimethylsiloxane

PEG – poly(ethylene glycol)

PEG – poly(ethylene glycol)

PEG-NGS – poly(ethylene glycol) functionalized nanographene sheets

PEI-g-GNR – poly(ethylenimine)-grafted graphene nanoribbon

PFA – paraformaldehyde

PFG – polymer-functionalized graphene nanoparticles

p-G – pristine graphene

PI – propidium iodide

PLA – poly(lactic acid)

PLGA – poly(lactide-co-glycolide acid)

PLL – poly(l-lysine)

pm – plasma membrane

PMMA – poly(methyl methacrylate)

pRGO – poly(dopamine) adhered RGO

PS – poly(styrene)

PU – poly(urethane)
PVA – poly(vinyl alcohol)
PVK – poly(N-vinylcarbazole)
PVP – poly(vinyl pyrrolidone)
Py – 1-Pyrenemethanol
RBC – red blood cells
RES – reticuloendothelial system
rGO/RGO – reduced graphene oxide
ROS – reactive oxygen species
rpm – revolutions per minute
Sa – arithmetic average height of the surface
SD – standard deviation
SEM – scanning electron microscopy
Sp – maximum peak height
SSLS – surface selective laser sintering
Sv – maximum valley depth
SWCNT(s) – single-walled carbon nanotubes
Sz – maximum height of the surface
t – thickness
t_{1/2} – area under the curve after
t_{1/2} – elimination half-life
T_c – cold crystallization temperature
T_{d max} – temperature of maximum degradation rate
T_{d5} – decomposition temperature for 5 wt.% loss
T_{d50} – decomposition temperature for 50% weight loss
T_{di} – beginning of thermal degradation
TEM – transmission electron microscopy
T_g – glass transition temperature
TGA – Thermogravimetric Analysis
THF – tetrahydrofuran
T_m – melting temperature
TMRE – tetramethylrhodamine, ethyl ester
trGO – thermally reduced Graphene Oxide
uCNT – unzipped Carbon Nanotubes
UHMWPE – ultra-high molecular weight polyethylene
vs – vesicle

wt.% – weight percentage

XPS – X-ray photoelectron spectroscopy

XRD – X-ray diffraction

ΔH_c – cold crystallization enthalpy

ΔH_m – melting enthalpy

ε_∞ – permanent creep strain

$\varepsilon_{\max}$ – maximum creep strain

χ_c – degree of crystallinity

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Chapter 1

Introduction

1 INTRODUCTION

1.1 Scope

The present chapter starts by introducing some of the key concepts intimately related to the work in the chapters presented later in this thesis.

Since historical basis usually provides the motivation to do research within a certain scope, sometimes it is hard to dissociate between the two. However, a separate section, entitled motivation and scope, was introduced to explain the events and conditions that made this work possible and that directed it along the last years.

Finally, an outline of the chapters in this thesis is presented, with the relations between them pointed out and their contents briefly explained.

1.2 Key concepts

1.2.1 Biomaterials and biocompatibility

The use of biomaterials can be traced back to the origins of civilization, in parallel with the invention and application of instruments and tools. Ranging from Mesoamerica to Asia, including Mexico, Rome, Athens, Egypt, and India, findings of antient artificial limbs, eyes, ears, teeth, and noses took place. This states the early concern of mankind to augment or repair the body, using materials available in nature such as wood, glue, rubber, manufactured materials such as iron, gold, zinc, and glass, and even tissues from living origins. ^[1-6] Materials were tested, selected, and modified for medical applications along the ages with early concepts starting to arise, however it was only in the late 17th century with advancements in the field of surgery, the usage of aseptic techniques, and radiography that a more accurate use and scientific understanding of biomaterials was possible. ^[2, 5, 7, 8] In the late 19th and early 20th centuries, previous knowledge and concepts resulted in the advent of today's routinely used biomaterials in for example, intraocular lenses, orthopedic prostheses, dental implants, kidney dialysis machines, catheters, pacemakers, heart

valves, stents, and breast implants. ^[1, 2, 9] Every day, advances on biomaterials field result in improvement of the quality of life and life expectancy. The refinement of the production methods, along with the improvement of existent materials, and the arising of new ones, as synthetic polymers, ceramics, and metal alloys, increased the application range of biomaterials. Also, the 1970s advances in molecular biology and 1990-2000s in genomics and proteomics, allowed the study and understanding of biological interactions at the interface with materials. The possibility of using methods or molecules to modify biomaterials interactions with the body or even using living tissue as a biomaterial itself, revolutionized the field to a point in which it is already hard to define the main terms commonly used. ^[1, 9] For that reason, the definition of “biomaterial” has been evolving and adapting to new insights along the past decades. One of the first definitions was resultant from the National Institutes of Health (NIH) consensus development conferences in 1982, and describes biomaterial as “any substance (other than a drug) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or part of a system which treats, augments, or replaces tissue, organ, or function of the body.” ^[2, 5, 10, 11] Some years later, at the 1986 consensus conference of the European Society for Biomaterials in Chester, England, several key definitions within the scope of biomaterials science were debated with some consistency being achieved. ^[8, 12, 13] Consensual definitions for artificial organ, bioactive material, bioadhesion, bioattachment, biocompatibility, biomaterial, bioprosthesis, host response, hybrid artificial organ, implant, medical device, prosthesis, and thrombogenicity were published in the proceedings. ^[14] The definition agreed for biomaterial was “a non viable material used in a medical device, intended to interact with biological systems.” ^[1, 8, 12, 13, 15] The success of the first consensus conference led to a second meeting in the same place in 1991, in which the past definitions were further discussed and perfected. For example, in the case of biomaterial definition, the reference to non-viability was removed. ^[13, 16] Further reflection and refinement of the concepts was published in a contextual dictionary of biomaterials science published in 1999. ^[17] The most recent refinement of the definition of biomaterial in literature was published in 2009, in which after a methodical discussion the following conclusion was stated, “a biomaterial is a substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure, in human or veterinary medicine.” ^[12]

Another key term that has been discussed along the years in biomaterials science is “biocompatibility”. ^[1, 8, 13, 18, 19] Despite papers studying tissue reaction to implanted biomaterials appearing since 1940s, only by 1970 the word “biocompatible” was used. ^[8] The most widespread concept for it was originated at the 1986 consensus conference on biomaterials held in Chester. There, biocompatibility is defined as “the ability of a material to perform with an appropriate host response in a specific application”. ^[1, 8, 18]

This simple and at the same time broad definition reminds us that a biomaterial should not elicit a deleterious host response, but leaves questions open regarding the criteria to evaluate if that response is acceptable or not. In a comprehensive leading opinion article, the previous definition was updated to, “biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimising the clinically relevant performance of that therapy.” This definition reaffirms the importance of biomaterials safety in a more specific manner, and adds that the material should exert beneficial effects to the host.^[18] However, it is still being a qualitative definition, because it doesn’t specify the criteria to judge the “undesirable local or systemic effects”. Absolute absence of undesirable effects with desirable biological actions can be considered the ultimate goal of biomaterials. However, while that is not achieved a risk/benefit approach on accessing biocompatibility can be taken. An interesting term found in literature is “biomaterial performance”. It consists of characterizing biomaterials using many qualitative, semi-quantitative, and/or quantitative observations or measurements. Some of these observations can be data in surface corrosion, changes in bulk and surface properties, and histological and biological analysis of inflammation.^[19] Here, physico-chemical characterization of the biomaterial is important because it can impact on its performance, both by leading to “undesirable effects” or changing/preventing “the clinically relevant performance”. Also, the application of this concept requires the support of well-documented reference standards of materials, methods, and responses.^[19] *In vitro* studies allow a first understanding of tissues and cells interactions with a biomaterial. Also, low levels of endotoxins and no measurable leachables are a predictor of better performance after implantation. *In vivo* studies allow the assessment of the body’s overall response towards a biomaterial, and allow the understanding of how the immune system reacts to it, which can be done by evaluating a possible foreign body reaction and its extent.^[8, 19] Usually, a biomaterial that elicits the formation of a thin, stable foreign body capsule and only low levels of cellular reaction at the implant site is accepted as biocompatible by physicians, regulatory agencies, and standards organizations. However, the evolution on materials science field led to “the ability of a material to locally trigger and guide non-fibrotic wound healing, reconstruction and tissue integration”, and this is the latest proposed definition for biocompatibility, regarding biomaterials to which it is applicable. Thus, “the ability of a material to reside in the body for long periods of time with only low degrees of inflammatory reaction” should be considered biotolerability.^[8] The definitions of biomaterial and biocompatibility have been evolving since they first started to be found in literature around 1970. Since then, their use has been growing as can be noticed in Figure 1.1. As the biomaterials science field expand new insights keep arising, that creates a pressing need for reflection and constant adjustment of the definitions, so those should be regarded as dynamic

instead of dogmatic. Also, the definitions can be subdivided according to the context, because for example the concept of biocompatibility regarding an implant is surely different from when considering a polymer-nucleotide conjugate, and the attempt to make a definition that comprises such different biomaterials, can result in a hollow one.

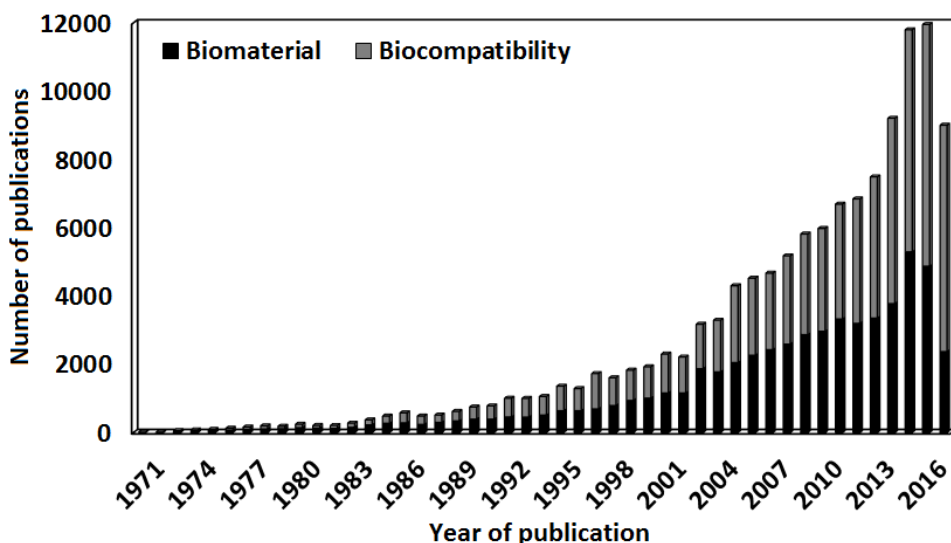


Figure 1.1: Evolution of the number of publications mentioning biomaterials and biocompatibility from 1970 to 2016. Source: Scopus; keywords searched: “biomaterial” - “biocompatibility”; search date: 30/12/2016.

1.2.2 Graphene-based materials

Carbon has been observed since prehistoric times in the form of soot, charcoal, graphite and diamond. For example, the usage of graphite has been traced to before 4000 BC, when the Marican, Boian, and Gumelnița cultures in Europe used it to paint pottery. ^[20-24] Obviously, ancient cultures did not realize, that these substances were different forms of the same element, ^[23, 24] because the identification of carbon as an element only millennia after was worked out step by step by R.-A.-F. de Reaumur, H.-L. Duhamel du Monceau, Torbern Bergman, C. W. Scheele, C.-L. Berthollet, A.-L. Lavoisier, and others. ^[24] In 1789, A.-L. Lavoisier listed “*Carbone*” in his “*Traité Élémentaire de Chimie*” as one of the newly identified chemical elements, whose versatility was already known since it had been shown that it was the elementary component of both diamond and graphite. ^[25, 26] Since then, more allotropes of carbon have been discovered and studied, ranging from amorphous carbon, to fullerenes (0 D), graphene (1D), and nanotubes (2 D). ^[26-28] Graphite ore has been found and mined in England since the 16th century. One of its

uses was to mark sheep, and because of that in 1789 A. G. Verner named it after the Greek word “graphein”, which means “to write”.^[21, 29] Since the development of the pencil industry in the 18th century, graphite has been one of the most widely used materials to write.^[21] Due to its layered structure when mechanical friction occurs between the paper and graphite in the pencil, graphite flakes are delaminated becoming attached to the paper. Thus by the simple act of writing, we have been on the way between graphite and graphene since centuries ago, but that only started to be considered recently, due to the importance that graphene has been achieving.^[30] Reviews on graphene history^[29, 31] report the firstly produced monolayer graphene-based material (GBM) to be graphene oxide (GO). Since around 1840, Schafhaeutl, Brodie, Staudenmaier, and others were studying the intercalation (insertion of small molecules in between the graphite layers) and exfoliation of graphite with strong oxidizing acids (Figure 1.2).^[29] In 1948, G. Ruess and F. Vogt, observed few nanometers flakes of GO by transmission electron microscopy (TEM), being these studies continued by the group of Ulrich Hofmann, which in 1962, together with Hanns-Peter Boehm identified some GO fragments as monolayers.^[31] The later, proposed the term graphene for the first time in 1962 in the following terms, “The ending -ene is used for fused polycyclic aromatic hydrocarbons, even when the root of the name is of trivial origin, *e.g.* naphthalene, anthracene, tetracene, coronene, ovalene. A single carbon layer of the graphitic structure would be the final member of infinite size of this series. The term graphene layer should be used for such a single carbon layer.”^[32] In 1997, IUPAC recognized graphene by including the following recommendations into their Compendium of Chemical Technology, “previously, descriptions such as graphite layers, carbon layers or carbon sheets have been used for the term graphene. Because graphite designates that modification of the chemical element carbon, in which planar sheets of carbon atoms, each atom bound to three neighbours in a honeycomb-like structure, are stacked in a three-dimensional regular order, it is not correct to use for a single layer a term which includes the term graphite, which would imply a three-dimensional structure. The term graphene should be used only when the reactions, structural relations or other properties of individual layers are discussed [emphasis added].” In 1999 Ruoff and co-workers micromechanically exfoliated graphite into thin lamellae comprising multiple graphene layers. In this method, lithographic patterning of highly ordered pyrolytic graphite was combined with oxygen-plasma etching to create pillars, which were converted into the thin lamellae by rubbing.^[29] In 2004, Geim, Novoselov, and co-workers using a similar micromechanical approach, followed by repeated peeling of flakes from graphite with scotch tape, finally achieved a single layer – graphene, which could be located by optical and electron microscopy and its electric-field effects characterized.^[33]

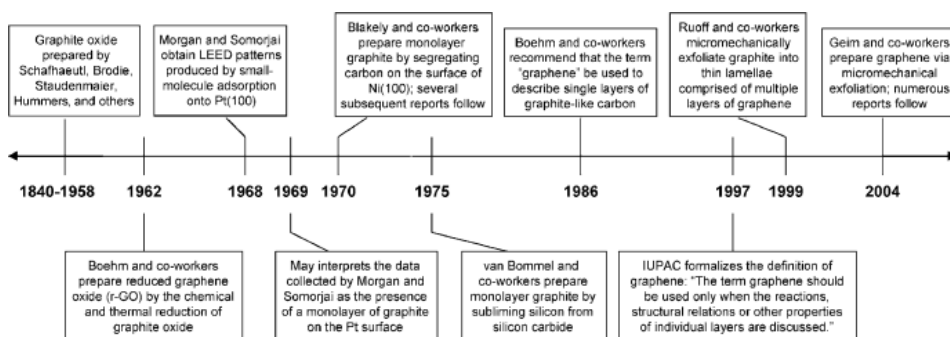


Figure 1.2: Timeline of selected events in the history of the preparation, isolation, and characterization of graphene. Reprinted from reference 29, with permission from Wiley, Copyright (2016).

Graphene's outstanding electronic, mechanical, and thermal properties, amongst others, surprised researchers and started a whole new field of research that has been growing ever since, due to its vast potential applications. ^[34-38] Around 2009 several technological companies, like IBM, Samsung, and Fujitsu, started investing on graphene-based technology. ^[21] In 2010, Geim and Novoselov were awarded the Nobel prize "for groundbreaking experiments regarding the two-dimensional material graphene". ^[39] Realizing the potential of graphene, several governmental agencies started investing in graphene from 2011 on. The outputs of industry and governmental investment have been arising in the most diverse forms up to date, as for example graphene-based inks, water desalinators, transistors, batteries, supercapacitors, sensors, and displays, amongst others. ^[21] This means that currently and each time more, graphene will be widespread in the environment, however, it can be observed from Figure 1.3, that only a tiny fraction of the research on graphene field is focused on the biological field. The first publication regarding graphene biocompatibility that could be traced, was from 2008, and shown that mouse fibroblasts presented a normal morphology and grew at the same rate on top of a graphene paper, as at the surface of tissue culture polystyrene. ^[40] However, graphene-based materials (GBMs) can also be dispersed in solution, present different dimensions, degree of oxidation and functionalizations. With the arise of new studies of graphene-based materials biological properties, it could be concluded that most studies report cell viability decreases inferior to 20% after exposure to GBMs concentrations around $10 \mu\text{g mL}^{-1}$ during 24 h or longer. Also, the *in vivo* effect of GBMs depends on their physical-chemical properties, concentration, time of exposure, and administration route, and also on the characteristics of the animals used. Most studies report no occurrence of adult animal death ^[41], with an exception for the death of a mice due to intravenous administration of 0.4 mg GO ^[42]. However, there are some reports of GBMs accumulation and histological findings associated with inflammation, and, more rarely, fibrosis. Small sized GBMs

present fast elimination. Existing information on *in vivo* toxicity mechanisms is still scarce, and more studies are needed to support safe biological applications of these materials. Little is known about long-term toxicity of GBMs. This is an issue that merits consideration in future studies. Also, the effect of GBMs on cell signaling is being studied, and more information should become available soon. ^[41] More detailed information regarding GBMs biological effects can be found on Chapter 2. However, in the scope of this thesis, it is important to note that, encapsulation of GBMs in a matrix reduces potential toxicity. In addition, incorporation of hydrophilic forms improves cell adhesion at the biomaterials surface. Also, some reports of antibacterial properties and improved hemocompatibility of GBMs-based composites offer interesting perspectives for future research and developments. ^[41]

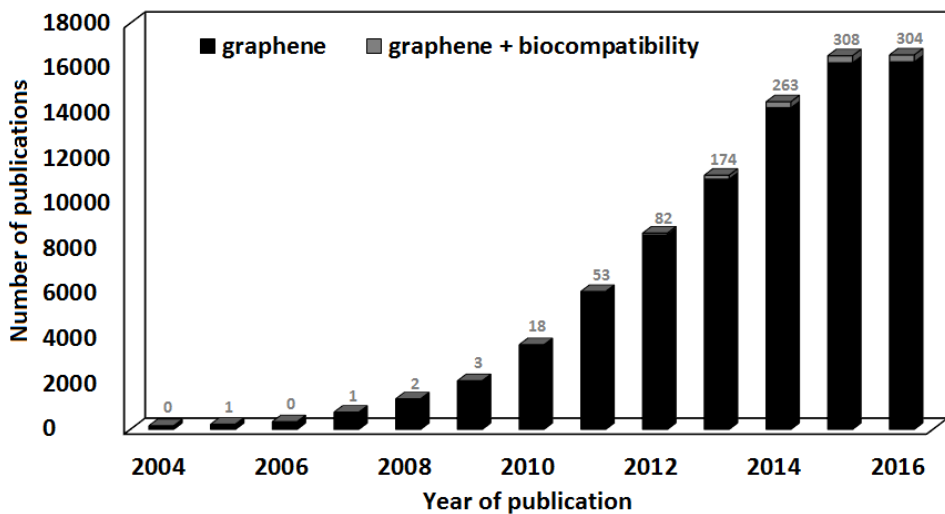


Figure 1.3: Evolution of the number of publications mentioning graphene and graphene + biocompatibility from 2004 to 2016. Source: Scopus; keywords searched: “graphene” - “graphene; biocompatibility”; search date: 31/12/2016. The number of “graphene; biocompatibility” publications was subtracted from “graphene” ones in the graph. Labels on top of the bars show the number of “graphene + biocompatibility” publications.

1.2.3 Poly(lactic acid)

The basic building block for poly(lactic acid) (PLA) is lactic acid, which was first isolated from sour milk by Scheele, in 1780, and started to be produced for commercial purposes in 1881. ^[43] PLA was firstly obtained in 1845 by Pelouze, which condensed lactic acid by distillation of water. ^[44, 45] However, because each step of polymerization forms water that degrades the polymer that is

being produced, and no method to remove it was known by then, only low molecular weight PLA was obtained, which had few or no commercial interest. ^[46] For that reason, for almost a century, research on PLA was focused in trying to produce a polymer with high molecular weight by a method with industrial applicability. ^[45] The most widely used method to obtain PLA, ring-opening polymerization (ROP) of lactide, was first demonstrated by Carothers in 1932, but high molecular weights were not obtained until in 1954, when the purification techniques of lactide were developed by Dupont. ^[44, 46, 47] Due to the high cost of high molecular weight PLA production, its use was limited to the biomedical field, as for example in the development of sutures, implants, and drug delivery systems. In 1990s, Cargill Inc. found a commercially viable lactide ring opening reaction and, in 1997, Cargill Dow LLC was formed between Cargill Inc. and The Dow Chemical Company, beginning to significant commercialize high molecular weight PLA under the trade name NatureWorks™. ^[44, 45, 47] Due to its suitable mechanical properties and biodegradability, PLA has been regarded as a promising material to replace non-degradable oil-based polymers. For that reason, it has been used to produce coatings, flexible packaging films, rigid packaging containers, cold drink cups, bottles, and injection- and extrusion-moulds, textiles, automotive materials, amongst others. ^[44]

Despite the existence of the abovementioned early research on PLA's medical applications, it was only after the year 2000, with the beginning of PLA's widespread commercialization, that the outputs of the research on this field increased considerably, as can be observed from Figure 1.4. Several PLA-based products are now approved for biomedical applications and contact with body fluids by Food and Drug Administration (FDA). This polymer has several applications in bioengineering, like the production of bioresorbable artificial ligaments, hernia repair meshes, scaffolds, screws, surgical plates, and suture yarns. PLA can also be used in the production of drug delivery systems, and in the packaging of pharmaceutical products. To make this material more effective in several applications, namely orthopaedic, some properties can be improved, being mechanical performance usually the most relevant. Approaches like adjustment of crystallinity, incorporation of plasticizers, blends with other polymers and addition of nanofillers have been tested. Reinforcement with small amounts of nanofillers is an interesting option because it allows improvement of target properties without changing PLA's main characteristics. ^[48, 49] More detailed information on the subject can be found on Chapters 5-8.

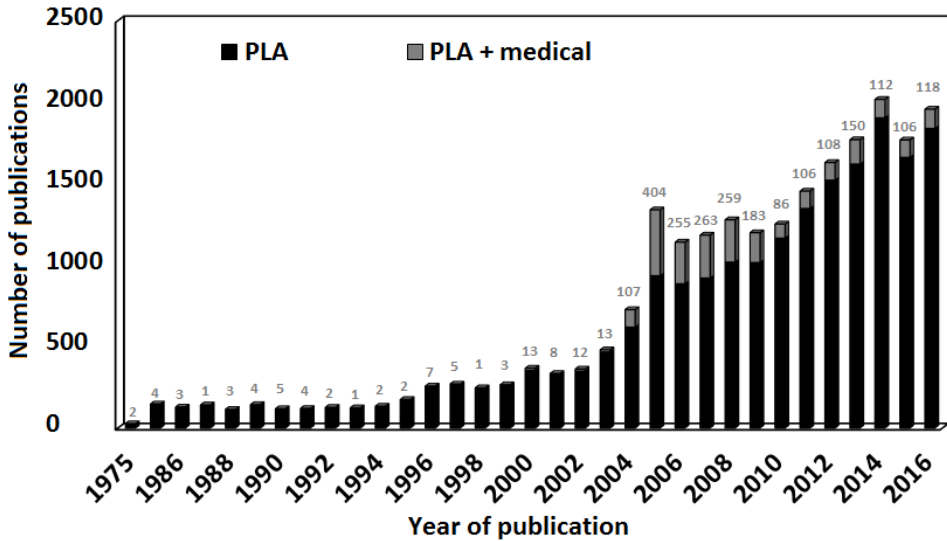


Figure 1.4: Evolution of the number of publications mentioning PLA and PLA + medical from 1975 to 2016. Source: Scopus; keywords searched: “PLA” - “PLA; medical”; search date: 31/12/2016. The number of “PLA; medical” publications was subtracted from “PLA” ones in the graph. Labels on top of the bars show the number of “PLA + medical” publications.

1.3 Motivation and Scope

In 2010, the same year in which Geim and Novoselov were awarded the Nobel prize "for groundbreaking experiments regarding the two-dimensional material graphene", we started our study on GBMs and GBMs composites, at the Faculty of Engineering of the University of Porto research unit LEPABE (Laboratory for Process Engineering, Environment, Biotechnology and Energy). The work was initially focused on understanding the physicochemical properties of the composites, in particular using PLA as the polymer matrix. About 1 year later, informal cooperation in this area started with INEB (National Institute of Biomedical Engineering), allowing to extend the work to the study of the materials biocompatibility. A joint application for a PhD grant to Foundation for Science and Technology (FCT) – Portuguese Ministry of Education and Science, was accepted and Artur Pinto became a PhD research fellow (SFRH/BD/86974/2012) since January 2013, with a working plan focused on "Production and characterization of composite bioabsorbable biomaterials".

When this PhD work started, little information was available regarding GBMs and GBMs/composites biocompatibility. However, observing the abovementioned recent findings on graphene production methods, properties and potential applications, and early investments of several companies on the field, there was no doubt that soon GBMs would play a key role on state of the art technology in the most diverse fields.

Within the scope of an on-going research project (PTDC/EME-PME/114808/2009), led by prof. Rui Guedes (FEUP), which goal was the production of bioabsorbable artificial ligaments, PLA was being studied as a biomaterial, but its excessive fatigue/creep that leads to laxity was a problem. ^[50] Previously, the base knowledge from work developed at LEPABE on GBMs for different applications, and on polymer/nanofillers composites, set the roots for preliminary testing of GBMs as fillers to improve the properties of PLA. These studies were regarded with broad scope due to this biodegradable polymer having several potential applications (see above). Results revealed that PLA performance was improved with incorporation of GBMs, namely in terms of mechanical, thermal and gas permeation properties. ^[51] Thus, our previously acquired knowledge on the potential of GBMs as fillers for PLA further strengthened the decision to continue working with these fillers to reinforce PLA for biomedical applications.

The lack of knowledge regarding GBMs biocompatibility, and reports on the toxicity of other nanomaterials, ^[52-54] including the most similar known structures, carbon nanotubes, ^[55, 56] motivated the study of GBMs biocompatibility, both when dispersed in culture media, to allow free contact with cells, and when integrated in a PLA matrix, to assure the safety of the biocomposite and its viability for biomedical applications.

For those reasons, this research was focused on developing original work that could contribute with pertinent advancements on the field. It started with the production and characterization of PLA/GBMs composites, whose methods were further explored and improved, in parallel with the characterization of GBMs biological properties when dispersed in liquid media, and the effect of their morphology, degree of oxidation, and surface modification with polymers. This work is presented in this dissertation, whose outline can be found below.

1.4 Dissertation Outline

This thesis is divided into 8 chapters. Chapter 1 is the present introduction, Chapters 2-4 and 6-8 are based on articles published in peer-reviewed international indexed journals, and Chapter 5 is

based on the content of a manuscript in preparation. Figure 1.5, shows a schematic diagram illustrating the different topics covered in this thesis and their articulation.

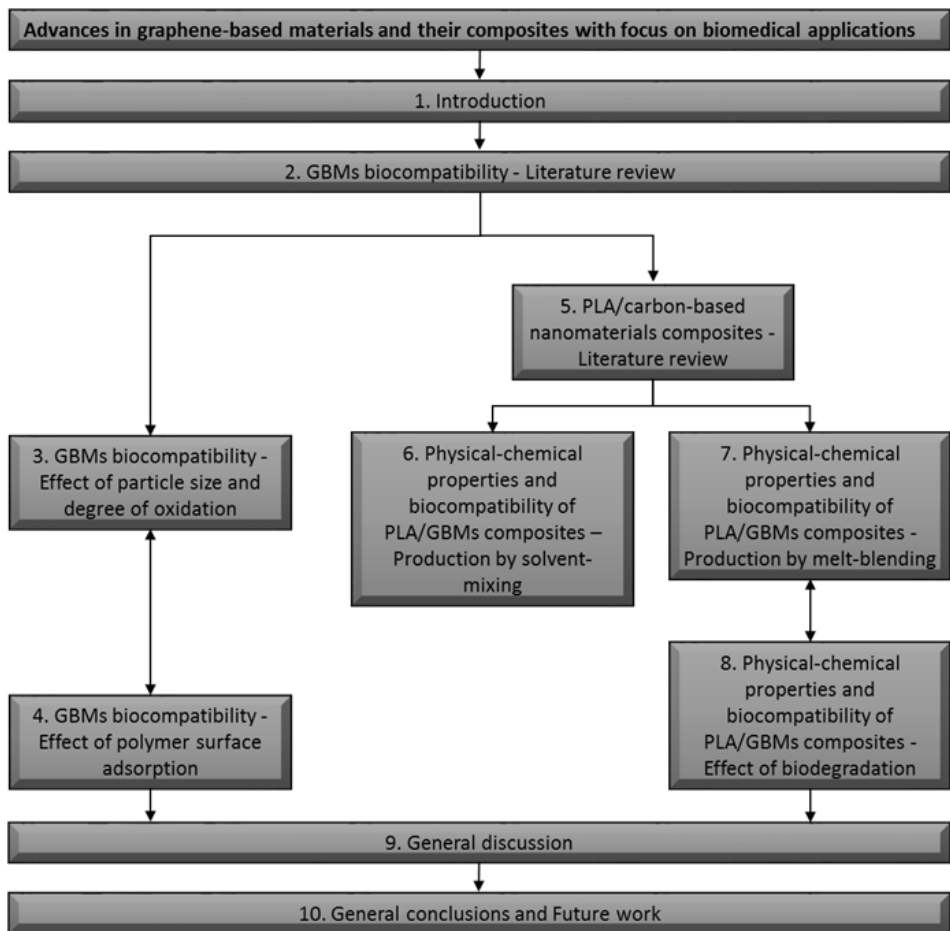


Figure 1.5: Schematic diagram illustrating the different topics covered in this thesis and their articulation.

- Chapter 2 presents a review of published data regarding the biocompatibility of GBMs in order to provide a critical overview of the state of the art. Firstly, the distinct physico-chemical nature of the GBMs available is clarified, as well as the production methods involved. This chapter also discusses the available *in vitro* (with bacterial and mammalian cells) and *in vivo* studies concerning evaluation of GBMs biocompatibility, as well as existing hemocompatibility studies. The biocompatibility issues concerning composite materials that incorporate GBMs are also addressed, since encapsulation in a polymer matrix modifies biological interactions. The most pertinent questions that

should be addressed in the scope of this work and regarding future works on the field are also highlighted.

- Information gathered on Chapter 2 shows that GBMs have recognized potential for biomedical applications, but also that different production methods and treatments originate divergent biocompatibility. For that reason, Chapter 3 reports the study of two commercially available graphene nanoplatelets (GNP), differing in platelet size, GNP-C (with 1–2 μm) and GNP-M (with 5 μm). Also, GNP-M was oxidized in different extents so that an *in vitro* study of the effect of oxidation and size on biocompatibility could be made.
- Chapter 4 reports further manipulation of GNP biocompatibility, by adsorbing different polymers onto its surface. Graphene nanoplatelets (GNP-C) were modified with poly(vinyl alcohol), hydroxyethyl cellulose, polyethylene glycol, poly(vinyl pyrrolidone), and glycosaminoglycans (chondroitin, glucosamine, and hyaluronic acid). The obtained materials were characterized in terms of physicochemical properties and their *in vitro* biocompatibility is studied.
- Chapter 5 reviews existing information on PLA reinforced with two types of carbon-based nanofillers (CBNs): carbon nanotubes (CNTs) and graphene-based materials (GBMs). It describes the composites production methods, and the effects of CBNs on PLA properties, namely mechanical, thermal, electrical and biological properties.
- Chapter 6 presents the production, by solvent casting followed by doctor blading, of thin PLA films and composite PLA films incorporating two GBMs – graphene oxide (GO) and GNP-M. These films were characterized regarding not only biocompatibility, but also mechanical performance, surface topography, chemistry, and wettability. Studies on platelet adhesion and activation on PLA and PLA/GBMs films surface are also presented.
- Chapter 7 explores a solvent free method (melt blending) to incorporate different loadings of GNP-C (0.1–0.5 wt%) in PLA. The effect of varying mixing time and mixing intensity is reported and the best conditions identified. An analysis of the

physical-chemical properties, with focus on mechanical performance is presented, along with the study of the PLA and PLA/GNP-C composites *in vitro* biocompatibility.

- Chapter 8 complements the work presented in chapter 7. It presents a study on the hydrolytic degradation along 6 months, in phosphate-buffered saline at 37 °C, of PLA/GNP-C and PLA/GNP-M composites produced by melt-blending at 0.25 wt.% loading. Physico-chemical characterization is performed before and after degradation, and biocompatibility of the degradation products tested *in vitro*.
- Finally, chapter 9 and 10 present a brief general discussion of the most relevant results, in the light of the most recently available literature reports, and the general conclusions withdrawn from the work presented in this thesis. Also, ongoing, and future work is presented and proposed.

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Chapter 2

GBMs biocompatibility:

Literature review

This chapter was based in the following published paper:

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Review

Graphene-based materials biocompatibility: A review

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2 GBMs BIOCOMPATIBILITY – LITERATURE REVIEW

2.1 Scope

The present chapter focuses on graphene-based materials (GBMs) potential applications in biomedical engineering and biotechnology. It presents a review of published data in order to provide a critical overview of the state of the art. Firstly, the distinct physical–chemical nature of the GBMs available is clarified, as well as the production methods involved. Then it discusses the available *in vitro* (with bacterial and mammalian cells) and *in vivo* studies concerning evaluation of GBMs biocompatibility, as well as existing hemocompatibility studies. The biocompatibility issues concerning composite materials that incorporate GBMs are addressed in a separate section, since encapsulation in a polymer matrix modifies biological interactions. The most pertinent questions that should be addressed in future works are also emphasized.

2.2 State of the art

2.2.1 Introduction

Graphene, the elementary structure of graphite, is an atomically thick sheet composed of sp^2 carbon atoms arranged in a flat honeycomb structure. It possesses very high mechanical strength, surface area and thermal and electrical conductivity.^[1-9] However, graphene is hydrophobic and, consequently, stable dispersions in polar solvents can only be obtained with addition of proper surfactants.^[10, 11] Graphene oxide (GO) is similar to graphene, but presents oxygen-containing functional groups.^[12-17] The presence of these polar and reactive groups reduces the thermal stability of the nanomaterial, but may be important to promote interaction and compatibility with polar solvents or with a particular polymer matrix.^[1, 2, 18-20] Both graphene and GO can be modified in order to obtain other graphene-based materials (GBMs) with suitable properties to

specific applications. [21-28] Some examples of chemical modifications, which will be mentioned in this review, are shown in Figure 2.1.

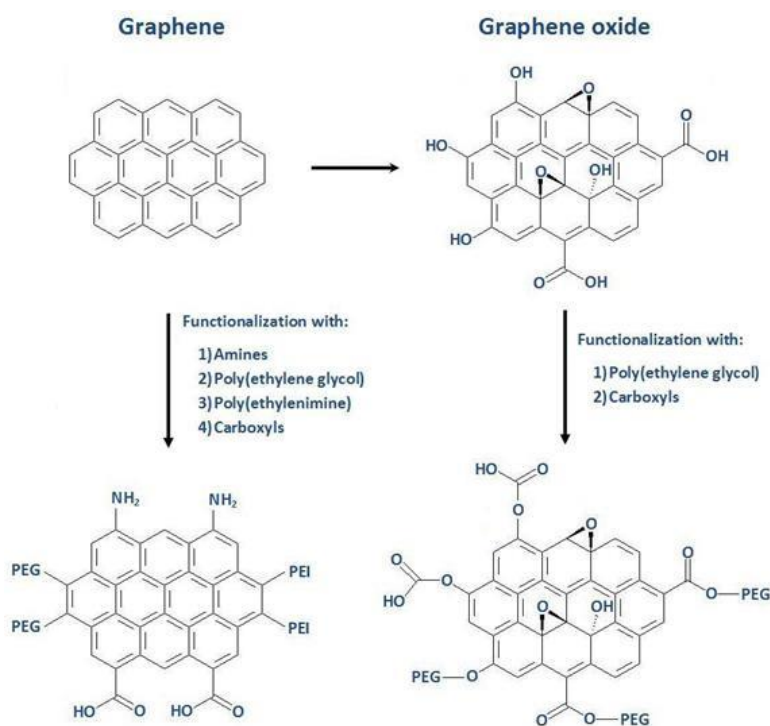


Figure 2.1: Simplified scheme showing graphene and graphene oxide structures and some examples of functionalization for both materials.

In the last years there has been a surge in research work on GBMs, with applications in fields as diverse as nanoelectronics [29-32], energy technology [33, 34], sensors [35, 36], and composite materials [37-39]. Several biomedical applications have also been studied, like biosensing/bioimaging [21], drug delivery [18], cancer photothermal therapy [40], and antibacterial materials [18]. Furthermore, approaches for applications in biomedical engineering [41, 42], regenerative medicine [41, 42], and biotechnology [43] are under study. Some examples of the reported advantages of GBMs are: (a) improvement of biomaterials mechanical/electrical properties, (b) increase of cellular attachment and growth at biomaterials surface, and (c) production of more efficient biosensors.

In view of the growing interest in using GBMs in medical applications, the issue of biocompatibility has gained relevance. This is evidenced in Figure 1.3, which shows the evolution in the number of scientific publications mentioning the term “graphene biocompatibility” between 2004 and 2016. It must be noted that existing information on other carbon-based materials is not

necessarily valid for GBMs, due to differences in production methodologies, particle morphology, and surface chemistry. For instance, contrary to carbon nanotubes, metal catalysis is not usually employed in the synthesis of GBMs, thus, cytotoxicity and inflammation caused by residual metals can be avoided. [44, 45]

GBMs biocompatibility depends on their intrinsic physical–chemical properties. These are dependent on the raw materials and production methods used. Sometimes GBMs obtained from different sources and/or methods are described using identical designations. [1, 2]

Table 2.1: GBMs and respective production methods.

Material	Production	Refs.
Graphite (Gt)	Raw material (commercial product) usually from different suppliers and with different characteristics (<i>e.g.</i> size, purity)	[54-56, 60-64]
Graphite oxide (GtO)	Modified Hummers Method (MHM)	[54]
Graphene oxide (GO)	MHM	[54, 55, 60, 61, 63-69, 73, 74, 77-79, 85]
	MHM + 7 days dialysis	[56]
	Refluxing with sulfuric and nitric acid mixture	[86]
	Ultrasonically separated from graphite and modified by chloroacetic acid	[87]
Ultra small (nGO)	MHM + functionalization with six-arm branched PEG – poly(ethylene glycol) (10 kDa)	[80]
(G2xO) Doubly-oxidized graphene oxide	GO lyophilization + Modified Hummers method	[64]
GO–Pluronic hydrogel	GO 0.5 wt.% solution (Angstrom Materials, OH, USA) (46 wt.% C and O)	[82]
	GO and Pluronic solutions were refrigerated to make sol-state solutions placed at 37 °C to make hydrogels	
GO functionalized with carboxylic groups (GO-COOH)	(information only available in Chinese)	[87]
PEGylated nano-graphene oxide (NGO-SS-mPEG)	MHM + conjugation of amino-terminated PEG to carboxylated NGO sheets <i>via</i> a disulfide bond	[70]
Reduced graphene oxide (rGO)	MHM + hydrazine reduction (Hr)	[54, 55]
GO nanosheets functionalized with fetal bovine serum (FBS)	MHM + 10% FBS in cell culture medium (adsorption by electrostatic and hydrophobic interactions)	[73]
Reduced graphene oxide (RGO)	GO rapid thermal heating (>2000 °C min ⁻¹ , to 1100 °C)	[85, 86]
RGO-PEG	GO + Hr + PEG-grafted poly (maleic anhydride-alt-1-octadecene) (C18PMHePEG) non-covalent functionalization	[80]
nRGO-PEG	nGO + Hr + (C18PMHePEG) non-covalent	[80]

functionalization		
Polydopamine adhered RGO (pRGO)	GO and dopamine hydrochloride were dispersed in PBS by sonication and stirred at 60 °C for 12 h, followed by centrifugation and dialysis	[74]
Heparin/bovine serum albumin grafted pRGO (Hep/BSA-g-pRGO)	pRGO was dispersed in PBS by sonication and pristine heparin or BSA was added and stirred at 25 °C, 24 h, followed by centrifugation and dialysis	[80]
1-Naphthalenesulfonate functionalized rGO (NA-rGO)	MHM + NA direct sonication + Hr	[57]
Brilliant blue functionalized rGO (BB-rGO)	MHM + BB direct sonication + Hr	[58]
Graphene sheets (GS)	Hydrothermal processing of GO in deionized water (pH ≈ 3)	[61]
Pristine graphene (p-G)	Chemical oxidation of graphite + thermal exfoliation	[62]
	Arc-Discharge Method [84]	[83]
COOH-functionalized graphene (f-G)	Chemical oxidation of p-G	[62]
Functionalized graphene (f-G)	Chemical oxidation of p-G	[83]
Graphene dispersed in Pluronic aqueous solution (G-Pluronic)	Natural graphite flakes ultrasonication in Pluronic aqueous solution followed by ultracentrifugation	[78]
Aggregated graphene (aG)	Flocculation with isopropyl alcohol 4:1 of G-Pluronic	[78]
Magnetic multifunctional graphene (MFG)	Magnetic graphene (MG) obtained by GO and ferrocene microwave irradiation MG modified with poly(acrylic acid) and fluorescein <i>o</i> -methacrylate (sonication + microwaves)	[66]
(PEG-NGS) Poly(ethylene glycol) functionalized nanographene sheets	MHM + conjugation <i>via</i> amide formation with amine terminated PEG	[76]
Amine-modified graphene (G-NH ₂)	GO stirring in SOCl ₂ and dimethylformamide (DMF), dispersing the product in sodium azide and DMF (40 h, room temperature)	[86]
Methanol derived graphene (MDG)	Sodium hydroxide and methanol supersaturated solution heating (under flow of argon)	[59]
(GNP) Graphene nanoplatelets	Graphite rapid microwave heating + ultrasonic treatment	[63]
Graphene oxide nanoplatelets (GONP)	MHM	[72]
Polyethylenimine-grafted graphene nanoribbon (PEI-g-GNR)	Unzipping of multiwalled carbon nanotubes by a MHM	[71]

For this reason, it is often difficult to extract general conclusions from existing literature. The present work presents a review of the literature available until the current date, regarding the evaluation of GBMs biocompatibility, taking care in distinguishing the particular types of

materials tested. Table 2.1 congregates the GBMs types mentioned along the text, together with a succinct description of the corresponding production methods.

This review focuses on *in vitro* studies performed with bacterial and mammalian cell models, and on *in vivo* studies performed with animals and embryos. The issue of GBMs hemocompatibility is also analyzed. Finally, the existing studies on biocompatibility of GBMs incorporated in composite materials are also presented.

It is relevant to mention that part of the available data regarding GBMs toxicity (mostly *in vitro*) is obtained from studies on its potential use as vehicle in drug delivery. In some cases the authors characterize the toxicity of GBMs in conjugation with the drug. ^[46-50] These studies were not included in this review, due to the impossibility in isolating the biological effect of the GBM material alone.

Some studies have been recently published concerning the effect of GBMs on cell signaling. ^[51-53] However, this subject is not within the scope of this work. We believe that due to the complexity of this topic, it should be reviewed at a later date, when more data are available.

2.2.2 *In vitro* biocompatibility studies

2.2.2.1 Bacterial cells

The effect of GBMs on bacteria structure, metabolism and viability has been shown to depend on the materials' concentration, time of exposure, and physical–chemical properties, as well as on the characteristics of bacteria used in the tests. ^[54-59] Table 2.2 presents the main results of current available studies regarding the effect of GBMs on bacterial cells. Most works report bacterial viability decrease after exposure to GBMs. ^[54-59] Moreover, viability was observed to decrease with increase of contact time ^[54] and GBMs concentration. ^[54, 55, 59] Some authors consider that viability decrease is associated with GBMs causing physical damage on bacterial membranes upon direct contact, resulting in the release of intracellular contents. This rupture is originated by the blade-like action of low thickness GBMs (a few nanometers) having sharp edges (like GO, rGO ^[54, 55], and BB-rGO ^[58]).

Table 2.2: Effect of GBMs on bacteria viability and membrane integrity.

Ref	Material	Particle size	Bacterial model	Cell viability	Membrane damage
Liu <i>et al.</i> [54]	GtO	Length (<i>l</i>) $\approx 6.28 \mu\text{m}$	<i>Escherichia coli</i> (<i>E. coli</i>)	GtO $\approx 85\%$, Gt $\approx 74\%$, rGO $\approx 54\%$, GO $\approx 31\%$ ($80 \mu\text{g mL}^{-1}$, 2 h)	Not studied (n/s)
	Gt	<i>l</i> $\approx 6.87 \mu\text{m}$		Decreases with incubation time (results not shown), concentration increase and size decrease	n/s
	rGO	<i>l</i> $\approx 2.75 \mu\text{m}$			Yes
	GO	Thickness (<i>t</i>) $\approx 0.31 \mu\text{m}$			Yes
Hu <i>et al.</i> [55]	GO	<i>t</i> $\approx 1.1 \text{ nm}$	<i>E. coli DH5α</i>	$\approx 70\%$ ($20 \mu\text{g mL}^{-1}$, 2 h) $\approx 13\%$ ($85 \mu\text{g mL}^{-1}$, 2 h)	Yes
	rGO	<i>t</i> $\approx 1 \text{ nm}$		$\approx 24\%$ ($85 \mu\text{g mL}^{-1}$, 2 h)	
Ruiz <i>et al.</i> [56]	GO	n/s	<i>E. coli</i>	Cell proliferation: 130% ($25 \mu\text{g mL}^{-1}$, 16 h)	n/s
Cai <i>et al.</i> [57]	NA-rGO	<i>t</i> $\approx 0.5 \text{ nm}$	<i>E. coli</i>	$\approx 7.5\%$ ($\approx 100 \mu\text{g mL}^{-1}$, 6 h)	n/s
			<i>Staphylococcus aureus</i> (<i>S. aureus</i>)	$\approx 11.2\%$ ($\approx 100 \mu\text{g mL}^{-1}$, 6 h)	
Cai <i>et al.</i> [58]	BB-rGO	n/s	<i>E. coli</i>	$\approx 50\%$ ($1.2 \mu\text{g mL}^{-1}$, 24 h)	Yes
			<i>S. aureus</i>	$\approx 50\%$ ($0.8 \mu\text{g mL}^{-1}$, 24 h)	
Pandey <i>et al.</i> [59]	MDG	<i>t</i> $\approx 3.5 \text{ nm}$	<i>E. coli</i>	$\approx 70\%$ ($20 \mu\text{g mL}^{-1}$, 2 h) $\approx 55\%$ ($40 \mu\text{g mL}^{-1}$, 2 h)	n/s

Additionally, Liu *et al.* [54] observed GBMs-induced oxidative stress, in terms of strong time and concentration dependent oxidization capacity toward glutathione (GSH), an endogenous antioxidant. They also found that when in direct contact with cells, reduced graphene oxide (rGO) and graphite (Gt) originate more intense oxidative stress than GO (graphene oxide) and GtO (graphite oxide). This is explained taking into account that the reduced forms of graphene are good electrical conductors. These authors also asserted that GBMs containing higher density of functional groups, and that are smaller in size, have more chances to interact with bacterial cells. This results in deposition on cellular surfaces, leading to more pronounced decreases in viability.

Contrasting to what is suggested in most studies, Ruiz *et al.* [56] reported increase of *E. coli* proliferation after exposure to GO. The GO used was dialyzed during 7 days after being produced by modified Hummer's method. Increase in proliferation was attributed to GO acting as a support for bacterial growth.

It must be noted that purification of the materials used is essential to assure removal of toxic contaminants. Information on the procedures adopted in this context is often absent from published works. This may mask the effect of contaminations on the results.

2.2.2.2 Mammalian cells

Table 2.3 presents the main results of currently available studies regarding the effect of GBMs on mammalian cell structure, metabolism and viability. Most studies report cell viability decreases inferior to 20% after exposure to GBMs concentrations around $10 \mu\text{g mL}^{-1}$ during 24 h or longer [57, 60-62, 64, 66, 69, 71-74]. Moreover, most of the studies that considered both the effects of contact time [60, 66, 73] and GBMs concentration [57, 60-64, 66, 68, 69, 72-74] conclude that increasing these variables leads to a decrease in cell viability.

Hydrophilic and small GBMs usually penetrate the cell membrane [60, 62, 71-73]. After internalization, these materials can lead to cytoplasmatic [60, 73], perinuclear [60, 62] or nuclear accumulation [60]. On the other hand, hydrophobic materials are hindered from penetrating into the cell membrane due to repulsive interactions or to presenting large hydrodynamic diameters, since they tend to agglomerate in physiological medium. [62] Despite not entering the cell, hydrophobic GBMs have been reported to cause membrane deformation, destabilize the cytoskeleton, and trigger intracellular stress, which may lead to apoptosis. [62] Hydrophilic materials may originate similar effects [57, 60], but no relevant toxicity was observed in some cases, even after internalization [62, 71].

It has been shown that both hydrophobic [61] and hydrophilic [66] GBMs generate reactive oxygen species (ROS). This is one of the major factors leading to lipid peroxidation, DNA damage, and caspase activation followed by chromatin condensation which eventually leads to cell death via apoptosis.

Additional studies are needed to clarify the effect of the GBMs degree of oxidation on biocompatibility. The tendency of hydrophilic (oxidized) materials to penetrate cell membranes can be counteracted by physical or chemical functionalization with polymers, in order to increase hydrophobicity. As abovementioned, this sort of modification might improve GBMs biocompatibility. [71, 74] This issue is discussed further in Section 2.2.5.

Peng *et al.* [65] observed that a small decrease in cell viability caused by GO was reversed by incubation with fresh medium. Further studies on the fate of GBMs after cell uptake, combined with the resultant long term effects of the materials internalization, should be performed. An interesting aspect that is lacking from existing studies has to do with the effect of particle size (length and thickness) on the biocompatibility of GBMs with similar chemical properties. In addition, there are no references in the literature regarding the effect of GBMs surface charge on biocompatibility. These have been shown to be relevant issues for cell adhesion and proliferation on biomaterial surfaces. [75]

Several authors ^[57, 64, 65-71] employ cancer cell lines to evaluate GBMs cytotoxicity. It is observed that in most cases cell viability decrease is lower for this type of cells (Table 2.3). This might occur due to cancer cell lines being more resistant to possible damages or metabolic impairment. This fact may imply some restraint regarding the significance of the obtained results and of the conclusions that may be withdrawn. Furthermore, studies by Rana *et al.* ^[68] revealed that GO caused different cell viability decreases in two different cancer cell models, for equal concentrations. Biocompatibility results may therefore be biased by the cell line used. Care should be taken in evaluating the suitability of the cell models.

2.2.3 *In vivo* biocompatibility studies

There are few *in vivo* studies concerning the biocompatibility of GBMs (Table 2.4). In the works available, radioactive labels are usually used to track GBMs *in vivo* and study its distribution in the body. Histological analysis of the tissues of most relevant organs is often performed, and in some cases inflammatory cells and cytokine levels are evaluated. The *in vivo* effect of GBMs was observed to depend on their physical–chemical properties, concentration, exposure time, administration route, and on the characteristics of the animals used in the assays ^[60, 66, 72, 76-82].

Table 2.3: Effect of GBMs on mammalian cell structure, metabolism and viability.

Ref	Material	Particle size	Cellular model	Cell viability	Cell Morphology/Integrity	GBMs location
Wang <i>et al.</i> [60]	GO	$t \approx 1$ nm	Human fibroblasts	$\approx 80\%$ ($<20 \mu\text{g mL}^{-1}$, 6d) $\approx 60\%$, cell floating and apoptosis ($>50 \mu\text{g mL}^{-1}$, 6d) Decreases with time	Cells rounded up, detached and displayed apoptotic morphology ($100 \mu\text{g mL}^{-1}$, 24 h) Membrane vesicles, fragmentation and unclear cell boundary ($20 \mu\text{g mL}^{-1}$, 72 h)	GO appeared in cell cytoplasm, around nucleus, and a few inside nucleus
Liao <i>et al.</i> [61]	GO	(HD) Hydrodynamic diameter (in PBS) $\approx 1.3 \mu\text{m}$	Human skin fibroblasts	$\approx 80\%$ ($200 \mu\text{g mL}^{-1}$, 24 h)	n/s	n/s
	GS	HD (in phosphate buffered saline – PBS) $\approx 4.3 \mu\text{m}$		$\approx 80\%$ ($12.5 \mu\text{g mL}^{-1}$, 24 h) $\approx 20\%$ ($200 \mu\text{g mL}^{-1}$, 24 h)		
Sasidharan <i>et al.</i> [62]	p-G	$t \approx 0.8$ nm	Vero cells	$\approx 90\%$ ($10 \mu\text{g mL}^{-1}$, 24 h) $\approx 40\%$ ($300 \mu\text{g mL}^{-1}$, 24 h)	Membrane deformation Destabilization of F-actin alignment	Aggregation outside membrane
	f-G			$\approx 100\%$ (until $300 \mu\text{g mL}^{-1}$, 24 h)	Normal	Perinuclear concentration
Pinto <i>et al.</i> [63]	GO (O at.% ≈ 22)	Diameter (d) ≈ 500 nm (wrinkled due to hydrogen bonds)	Mouse embryo fibroblasts 3T3	$\approx 70\%$ ($1 \mu\text{g mL}^{-1}$, 48 h)	n/s	n/s
	GNP (O at.% ≈ 8)	$t \approx 6\text{--}8$ nm $l \approx 5 \mu\text{m}$		$\approx 50\%$ ($10 \mu\text{g mL}^{-1}$, 48 h)		
Hong <i>et al.</i> [64]	GO (O at.% ≈ 38)	HD (75 mM NaCl , 48 h) ≈ 200 nm	Human cervical cancer cells Hela	$\approx 85\%$ ($10 \mu\text{g mL}^{-1}$, 48 h) $\approx 75\%$ ($100 \mu\text{g mL}^{-1}$, 48 h)	n/s	n/s
	G2xO (O at.% ≈ 54)			$\approx 95\%$ ($10 \mu\text{g mL}^{-1}$, 48 h) $\approx 75\%$ ($100 \mu\text{g mL}^{-1}$, 48 h)		
Peng <i>et al.</i> [65]	GO	$t \approx 0.9$ nm	HeLa cells	$\approx 80\%$ ($40 \mu\text{g mL}^{-1}$, 6 h) $\approx 95\%$ (refreshed medium, $40 \mu\text{g mL}^{-1}$, 24 h)	n/s	n/s
Gollavelli <i>et al.</i> [66]	GO	$t \approx 1\text{--}3$ nm $l \approx 40\text{--}60$ nm	HeLa cells	$\approx 85\%$ ($10 \mu\text{g mL}^{-1}$, 48 h) $\approx 65\%$ ($100 \mu\text{g mL}^{-1}$, 48 h) Decreases with time	Lactate dehydrogenase (LDH) release increased $\approx 80\%$ Apoptotic and necrotic cell death $<8\%$ ($200 \mu\text{g mL}^{-1}$, 24 h)	n/s
Yang <i>et al.</i> [67]	GO	n/s	HeLa cells	$\approx 90\%$ ($50 \mu\text{g mL}^{-1}$, 24 h)	n/s	n/s

Rana <i>et al.</i> [68]	GO	$t \approx 1$ nm	CEM (human lymphoblastic leukemia)	$\approx 80\%$ (10 $\mu\text{g mL}^{-1}$, 5 d) $\approx 30\%$ (400 $\mu\text{g mL}^{-1}$, 5d)	n/s	n/s
			MCF7-human breast cancer	$\approx 50\%$ (10 $\mu\text{g mL}^{-1}$, 5 d) $\approx 30\%$ (400 $\mu\text{g mL}^{-1}$, 5 d)		
Yang <i>et al.</i> [69]	GO	$t \approx 0.8\text{--}1$ nm $l < 200$ nm	HeLa cells	$\approx 100\%$ (10 $\mu\text{g mL}^{-1}$, 24 h) $\approx 80\%$ (500 $\mu\text{g mL}^{-1}$, 24 h)	n/s	n/s
Cai <i>et al.</i> [57]	NA-rGO	$t \approx 0.5$ nm	CNE1 cells	$\approx 90\%$ (10 $\mu\text{g mL}^{-1}$, 24 h) $\approx 80\%$ (100 $\mu\text{g mL}^{-1}$, 24 h)	Cell shape became irregular (500 $\mu\text{g mL}^{-1}$, 24 h)	n/s
Wen <i>et al.</i> [70]	NGO-SS-mPEG	$t \approx 0.87$ nm $d \approx 220$ nm	HeLa cells	$>100\%$ (50–1000 $\mu\text{g mL}^{-1}$, 24 h)	n/s	n/s
Dong <i>et al.</i> [71]	PEI-g-GNR	$l \approx 220\text{--}400$ nm	HeLa cells	$>95\%$ (24 $\mu\text{g mL}^{-1}$, 12 h)	Apoptotic ratio = 8.8%	Enter the cells
Zhan <i>et al.</i> [72]	GONP (labeled with $^{99\text{m}}\text{T}$)	Irregular shape (big particle size ≈ 200 nm)	ARPE-19 cells (human retinal pigment epithelium)	$>80\%$ (10 $\mu\text{g mL}^{-1}$, 72 h) $>60\%$ (100 $\mu\text{g mL}^{-1}$, 72 h)	LDH release $<5\%$ (100 $\mu\text{g mL}^{-1}$, 72 h) and decreases with time Apoptosis rate $\approx 1.5\%$ (similar to control)	Enter the cells (until 50 $\mu\text{g mL}^{-1}$, ≈ 50 $\mu\text{g mL}^{-1}$ is too aggregated)
Hu <i>et al.</i> [73]	GO nanosheets	$t \approx 1$ nm	A549 (adenocarcinomic human alveolar basal epithelial)	$\approx 80\%$, (20 $\mu\text{g mL}^{-1}$, 24 h) $\approx 50\%$ (100 $\mu\text{g mL}^{-1}$, 24 h) Decreases with time	Destroys cell membrane and directly induces cell death	Predominantly inside cell membrane
	GO nanosheets-FBS			Increased $\approx 30\%$ comparing to GO nanosheets (20 and 100 $\mu\text{g mL}^{-1}$, 24 h) FBS formed a protein corona on GO nanosheets surface preventing direct interaction with the cells	Normal cells	Few inside cell membrane
Cheng <i>et al.</i> [74]	GO	$t \approx 1.038$ nm d (PBS) ≈ 1429.1 nm	HUVECS (human umbilical vein endothelial cells)	$\approx 95\%$, (20 $\mu\text{g mL}^{-1}$, 24 h) $\approx 60\%$ (100 $\mu\text{g mL}^{-1}$, 24 h)	n/s	n/s
	pRGO	$t \approx 1$ nm d (PBS) ≈ 1596.8 nm		$\approx 100\%$, (20 $\mu\text{g mL}^{-1}$, 24 h) $\approx 70\%$ (100 $\mu\text{g mL}^{-1}$, 24 h)	Normal cells	n/s
	Hep-g-pRGO	$t \approx 1$ nm d (PBS) ≈ 1241.5 nm		$\approx 110\%$, (20 $\mu\text{g mL}^{-1}$, 24 h) $\approx 95\%$ (100 $\mu\text{g mL}^{-1}$, 24 h)	Normal cells	n/s
	BSA-g-pRGO	$t \approx 1$ nm d (PBS) ≈ 1131.7 nm		$\approx 105\%$, (20 $\mu\text{g mL}^{-1}$, 24 h) $\approx 75\%$ (100 $\mu\text{g mL}^{-1}$, 24 h)	Normal cells	n/s

Most studies report no occurrence of adult animal's death, even for GBMs doses up to 20 mg kg⁻¹.^[76-82] However, Wang *et al.*^[60] observed that almost half mice population died after intravenous (i.v.) administration of GO. When administered by i.v. route, GBMs generally accumulate in MPS (mononuclear phagocyte system) – mainly in liver and spleen – and in lung^[60, 72, 76-78]. These materials are reported to have mostly fecal elimination via bile when accumulated in MPS, and renal elimination when particles have sufficiently small sizes^[60, 76-78]. Small size GBMs (single sheets with diameter of 10 nm or less) present $t_{1/2}$ (elimination half-life) close to 0.4 h, while $t_{1/2}$ for GBMs with larger sizes can be superior to 5 h.^[76, 77] There is only one study that reports possible long term degradation, using PEG-NGS (PEG-functionalized nanographene sheets).^[76]

Some studies report dose and time dependent inflammation, usually in lung and liver, with cell infiltration and in few cases fibrosis^[60, 77, 78]. However, Yang *et al.*^[76] did not observe appreciable inflammation with PEG-NGS.

Yang *et al.*^[80] observed that few hours after being administered by oral route, nGO-PEG, RGO-PEG and nRGO-PEG were present in high quantities in stomach and intestine, but not in other major organs. However, after 1 day only low levels were detected in the organs, and no trace was detected after 1 week. Thus, these GBMs seem to be poorly adsorbed in gastrointestinal tract, presenting fast excretion. Yan *et al.*^[81] reported no clear clinical evidence of ocular changes caused by GO intravitreal injection. When subcutaneously injected, a GO-Pluronic hydrogel caused mild inflammation after 3 weeks, which reverted later^[82]. Finally, Dutch *et al.*^[78] tested three different GBMs (GO, G-Pluronic – graphene dispersed in Pluronic aqueous solution, and aG – aggregated graphene) by intratracheal administration. They observed that GO caused persistent lung inflammation, which was attributed to its ability to increase the rate of mitochondrial respiration, and the generation of reactive oxygen species in cells, activating inflammatory and apoptotic pathways. Opposingly, mice treated with hydrophobic G-Pluronic presented no signs of fibrosis. However, aG, which probably presented larger particle sizes, persisted in the medium or small airways of the lung, and induced peribronchial inflammation and mild fibrosis. Li *et al.*^[79] also observed that GO caused acute lung injury and mild to severe fibrosis.

Gollavelli and Ling^[66] evaluated the embryotoxicity of GO and MFG (magnetic multifunctional graphene) injected in the pole region of *Danio rerio* and observed minute drop in survival comparing to control. The authors also found that both GO and MFG distributed from head to tail, despite being observed mainly in the yolk sac region, where they caused edema. Additionally, mutations were observed, occurring probably due to interactions with some specific proteins and biomolecules.

Table 2.4: GBMs *in vivo* biocompatibility.

Ref	Material	Particle size	Animal model	Animals survival	Toxicokinetics	Histology
Wang <i>et al.</i> [60]	GO	$t \approx 1$ nm	Kunming mice (female, 28–30 g, 4–5 weeks old)	100% (0.1 and 0.25 mg per mouse) 66% (0.4 mg per mouse) (deaths occurred: 1, 7d)	(i.v. administration) Biodistribution: mainly to lungs, liver and spleen Not present in brain Few in kidney (probably excreted in bile)	Dose-dependent lung inflammatory response (neutrophils and foamy alveolar macrophage) After 7 d: lesions surrounded GO After 30 d: Multifocal macrophage-containing granulomas GO in hepatic macrophage phagosome
Yang <i>et al.</i> [76]	PEG-NGS (labeled with ^{125}I)	$t \approx 1$ nm $l \approx 10$ –30 nm	Balb/c mice (female)	100%, no significant body weight drop (20 mg kg $^{-1}$, 3–90 d)	(i.v. administration) $t_{1/2} \approx 0.4$ h (smaller particles) and 7 h $\text{AUC}_{0-\infty} \approx 4.6$ mg min mL $^{-1}$ Biodistribution: mainly accumulated on MPS (liver and spleen) Elimination: renal (small size – single sheets $d < 10$ nm), fecal via bile (accumulated in MPS), possible long term degradation by oxidative metabolic pathways	No obvious organ damage Liver and spleen turned brown (NGS accumulation) Reverted after 20 d
Zhang <i>et al.</i> [77]	GO (labeled with ^{188}R)	$t \approx 1$ nm $l = 10$ –800 nm	Kunming mice	100%, no body weight drop (1 and 10 mg kg $^{-1}$, 14 d)	(i.v. administration) $t_{1/2} \approx 5$ h Biodistribution: (48 h) mainly accumulated in lungs ($\approx 26\%$ injected dose per gram of tissue (%ID/g)) and MPS – liver ($\approx 3\%$ ID/g) and spleen ($\approx 2.5\%$ ID/g) Elimination: renal – small GO particles, large size GO accumulated in lungs	1 mg kg $^{-1}$, 14 days: n pathological changes (lungs, liver, spleen, kidney) 10 mg kg $^{-1}$, 14 days: granulomatous lesions, pulmonary edema, inflammatory cell infiltration, and fibrosis throughout the lung
Zhan <i>et al.</i> [72]	GONP (labeled with $^{99\text{m}}\text{T}$)	Irregular shape (big particle size ≈ 200 nm)	Kunming mice (female, 15–18 g)	100%, no body weight drop Biodistribution: 0.3 mL $^{99\text{m}}\text{T}$ -GONP Histology: 1 mg of GONP/mouse	(i.v. administration) Biodistribution: (24 h) Mainly accumulated in MPS – liver ($\approx 15\%$ ID/g) and spleen ($\approx 5\%$ ID/g) and lungs ($\approx 3\%$ ID/g) Elimination: $\approx 0.5\%$, 24 h (feces + urine)	Observed in stomach (by eyes) and in transmission electron microscopy (TEM) of chyme and gastric tissues 2 h post i.v.: accumulated in liver and phagocytosed by Kupffer cells, little observed in lung/spleen
Duch <i>et</i>	GO	$t = 0.5$ –2 nm	C57BL/6 mice (8–12 week)	100%, no body weight drop (50 μg /mouse in	(Intratracheal administration) Leakage of protein into the alveolar space,	After 21 d: Persistent lung inflammation

<i>al.</i> [78]		Area (90%) $\leq 25,000 \text{ nm}^2$	old, 20–25 g, male)	50 μL , 24 h)	bronchoalveolar lavage (BAL) fluid pleiocytosis, ↑pro inflammatory cytokines, hyaline membrane formation Induction of prothrombotic state Increase in apoptosis ROS after uptake into macrophages	Little evidence of lung fibrosis
	G-Pluronic	$t = 1.2\text{--}5 \text{ nm}$ Areas (90%) $\leq 25,000 \text{ nm}^2$			Contains more macrophages with a black cytoplasm than aG (better distribution in lung)	No evidence of fibrosis (21 days)
	aG	n/s			Persisted in medium/small airways Macrophages with black cytoplasm	Induced peribronchial inflammation and mild fibrosis (21 days)
<i>Li et al.</i> [79]	GO (labeled with ^{125}I)	$t \approx 1 \text{ nm}$ $d = 10\text{--}800 \text{ nm}$	KunMing mice	100%	(Intratracheal administration) (10 mg kg^{-1} , 24 h) Biodistribution: (10 min) $\approx 70\%$ remained in lung, (2 h) $\approx 20\%$ remained in lung, (6 h) NGO may pass through the air–blood barrier and be delivered to other organs mainly liver and intestine. Elimination: higher concentration in urine observed within 6 h Sheets with small sizes can penetrate the alveolar–capillary barrier and be quickly eliminated by a renal route	Acute lung injury with the greatest severity at 48 h being then alleviated 1 week: fibroproliferation and organization of lung tissue – emergence of lung fibrosis 1–3 months: consolidation of the lung and an accumulation of alveolar macrophages
	(labeled with ^{125}I) GO	$t \approx 0.94 \text{ nm}$ $d = 450 \text{ nm}$				
<i>Yang et al.</i> [80]	nGO-PEG	$t \approx 1.22 \text{ nm}$ $d = 25 \text{ nm}$			(Oral route) (4 mg kg^{-1}) 4 h: high levels of nGO-PEG, RGO-PEG and nRGO-PEG in stomach and intestine, but not in other major organs; 1 day: low levels in all organs; 1 week: undetectable.	(Oral route) (100 mg kg^{-1}) 30 days: PEGylated GO derivatives were not adsorbed by organs and were rapidly excreted (Intraperitoneal injection) (50 mg kg^{-1})
	RGO-PEG	$t \approx 4.43 \text{ nm}$ $d = 50 \text{ nm}$	Female balb/c mice	100%	(Intraperitoneal injection) (4 mg kg^{-1}) 1 and 7 days: high accumulation in liver and spleen, RGO-PEG with larger sizes showed nearly 2 fold RES uptake compared to smaller nGO-PEG and nRGO-PEG at day 7	30 days: PEGylated GO derivatives (but not GO) were not engulfed by phagocytes in the RES system (size and surface coating)
	nRGO-PEG	$t \approx 5.66 \text{ nm}$ $d = 27 \text{ nm}$				Long-term retention occurred for injected GO and PEGylated GO No significant toxicity was noticed
<i>Yan et al.</i> [81]	GO	$t \approx 1 \text{ nm}$	Japanese white rabbits	100%, no body weight drop (0.1, 0.2 and	(Intravitreal injection) No clear clinical evidence of ocular changes	Very small amount of residue in the eye

			(2–3 kg)	0.3 mg GO in 0.1 mL BBS)	No influence on IOP Negligible ERG changes	No apparent damage to retinal morphology
Sahu <i>et al.</i> [82]	GO-Pluronic hydrogel	Single layer $d < 500$ nm	Female BALB/c mice (female, 6–7 weeks)	100%, no body weight drop (do not refer which quantity is injected)	(Subcutaneous injection) No noticeable degradation was observed	No signs of acute inflammation, tissue necrosis, hemorrhaging or hyperemia After 3 weeks: Migration of a few macrophages into the gel – suggest mild chronic inflammation After 8 weeks: the number of macrophages indicated a time dependent healing of the early sign of inflammation
Gollavelli <i>et al.</i> [66]	GO	$t \approx 1\text{--}3$ nm $l \approx 40\text{--}60$ nm	Wild-type AB strains of zebrafish embryos	Drop in survival was relatively minute comparing to control ($n = 50$) Effects were evaluated until 72 h post-fertilization (hpf)	(10 nl volume was microinjected into the pole region, stages 1–3)	For 0.05 ng nl ⁻¹ : 4% yolk sac edema and 6% tail or spinal cord flexure For 0.1 ng nl ⁻¹ : 6% yolk sac edema and 2% of cardiac malfunction
	MFG	$t \approx 4\text{--}6$ nm $l \approx 40\text{--}60$ nm			(Same conditions as for GO) Distribution: from head to tail. Mainly observed in the yolk sac region, blood vessels, brain ventricle, and other organs	For 0.05 ng nl ⁻¹ : 9% yolk sac edema and 8% tail flexure For 0.1 ng nl ⁻¹ : 12% yolk sac edema and 6% of cardiac malfunction Mutations occur

i.v., intravenous; $t_{1/2}$, elimination half-life; $AUC_{0-\infty}$, area under the curve after a single dose; MPS, mononuclear phagocyte system; %ID/g, percent injected dose per gram of wet tissue; BBS, balanced buffer solution; IOP, intraocular pressure; ERG, electroretinography; hpf, hours post-fertilization; RES - reticuloendothelial system.

As abovementioned, there are not many studies concerning *in vivo* biocompatibility of GBMs. More detailed characterization and mechanistic toxicity studies are essential for safer design and production of GBMs, in order to optimize potential biological applications of these materials with minimal risks for health.

2.2.4 Hemocompatibility

Several biomedical applications are being studied for GBMs (*e.g.* drug delivery, contrast imaging, cancer thermal ablation, biosensors, implants).^[36, 37, 41-43] In this context, hemocompatibility studies are necessary to assess if intravenous administration or implantation of GBMs is safe. Even so, there is a relatively low number of studies on this subject (Table 2.5). Most of the available literature considers GBMs to be hemocompatible. These materials are described as not causing hemolysis, platelet activation, and changes in coagulation or hematological markers abnormalities.^[76, 77, 83, 87] However, Singh *et al.*^[85, 86] reported that GO caused strong platelet aggregation and extensive thromboembolism in mice. Interestingly, much less platelet aggregation occurred with RGO.^[86] Moreover, amine-modified graphene (G-NH₂) did not show stimulatory effects on human platelets or thromboembolism and did not induce erythrocyte lysis.^[86] Sasidaram *et al.*^[83] observed that peripheral blood mononuclear cells (PBMCs) treated with p-G expressed more IL-8 and IL-6 than PBMCs exposed to f-G. These results oppose those obtained by Singh *et al.*^[86], mentioned before, where GO revealed lower hemocompatibility than RGO. Additionally, high GO concentrations (>100 µg mL⁻¹) were shown to cause erythrocyte lysis, while with low concentrations (<10 µg mL⁻¹) no effect was observed.^[74, 77] In our unpublished work, GNP and GNP oxidized by modified Hummers method did not cause lysis of red blood cells when incubated in concentrations up to 200 µg mL⁻¹. The effect of GBMs physical-chemical properties and concentration on hemocompatibility deserves further attention, in order to clarify conflicting results and pending questions.

2.2.5 Biocompatibility of composite materials containing GBMs

GBMs can be incorporated in different polymer matrices in order to improve relevant properties, namely mechanical^[88-89], thermal^[88-89], and electrical^[90].

Table 2.5: GBMs hemocompatibility.

Reference	Material	Particle size	Hemocompatibility
Sasidharan et al. [83]	p-G	$t \approx 0.4$ nm	Hemolysis: both (p-G and f-G) did not interfere with RBC membrane integrity Platelet activation/aggregation: both did not interfere with natural platelet functioning Coagulation studies: both did not interfere with intrinsic or extrinsic pathways of coagulation
	f-G	(n/s)	Pro-inflammatory cytokines: p-G treated peripheral blood mononuclear cells (PBMCs) express relatively higher levels of IL-8 and IL-6, comparing to f-G treated PBMCs
Singh et al. [85]	GO	Single or few layer $d \approx 0.2\text{--}5$ μm	GO caused strong aggregatory response in platelets through activation of Src kinases and release of calcium from intracellular stores GO i.v. administration induced extensive pulmonary thromboembolism in mice (Swiss, 8–12 weeks, male)
	RGO	(n/s)	RGO was much less effective in aggregating platelets
Singh et al. [86]	G-NH ₂	Single and few layer $l \approx 2$ μm	No platelet aggregation ($2 \mu\text{g mL}^{-1}$) No effect on $[\text{Ca}^{2+}]_i$ (intracellular free calcium) ($10 \mu\text{g mL}^{-1}$) Did not evoke cytosolic proteins or tyrosine phosphorylation Did not increase ROS ($2 \mu\text{g mL}^{-1}$) Did not evoke significant lysis of erythrocytes ($50 \mu\text{g mL}^{-1}$)
			(i.v., $250 \mu\text{g kg}^{-1}$) No pulmonary thromboembolism in mice (Swiss male mice, 8–12 weeks old) $\approx 90\%$ platelet aggregation ($2 \mu\text{g mL}^{-1}$) $[\text{Ca}^{2+}]_i \uparrow 4\times$ ($2 \mu\text{g mL}^{-1}$)
	GO	Size distribution similar to G-NH ₂ and RGO (flow cytometry)	Evoked strong tyrosine phosphorylation ROS $\uparrow 2\times$ ($2 \mu\text{g mL}^{-1}$) Evoked significant concentration dependent lysis of erythrocytes ($2\text{--}10 \mu\text{g mL}^{-1}$) (i.v., $250 \mu\text{g kg}^{-1}$) Occlusion of large number of lung vessels with platelet thrombi
	RGO	Similar to G-NH ₂ and GO	$\approx 15\%$ platelet aggregation ($2 \mu\text{g mL}^{-1}$) $[\text{Ca}^{2+}]_i \uparrow 2\times$ ($2 \mu\text{g mL}^{-1}$)
Zhang et al. [77]	GO (labeled with ^{188}R)	$t \approx 1$ nm $l = 10\text{--}800$ nm	Little effect on erythrocyte morphology and membrane integrity ($10 \mu\text{g mL}^{-1}$ for 1 and 4 h) A part of erythrocyte membranes were ruptured and ghost cells were observed ($80 \mu\text{g mL}^{-1}$ for 4 h) No significant difference was found between RBC exposure to PBS and GO ($10\text{--}80 \mu\text{g mL}^{-1}$ for 1 h)
Yang et al. [76]	PEG-NGS (labeled with Cy7)	$t \approx 1$ nm $l \approx 10\text{--}30$ nm	Normal hematology markers (20 mg kg^{-1} , unlabeled, 3, 40, and 90 d p.i.)
Dong et al. [87]	GO	(information only available in Chinese)	GO-COOH recalcification time increased 11 min ($1.25 \mu\text{g mL}^{-1}$) GO-COOH hemolysis ratio was less than 5% ($0.5\text{--}100 \mu\text{g mL}^{-1}$) GO-COOH anti-clotting properties better than GO, due to direct complexation between carboxyl group and clotting factors
	GO-COOH		
Cheng et al. [74]	GO	$t \approx 1.038$ nm d (PBS) ≈ 1429.1 nm	Hemolysis: GO hemolysis ratio of 2.3% (5 mg mL^{-1}) and 78.5% (200 mg mL^{-1}) pRGO, Hep/BSA-g-pRGO hemolysis ratio $< 2\%$ (200 mg mL^{-1})
	pRGO	$t \approx 1$ nm d (PBS) ≈ 1596.8 nm	
	Hep-g-pRGO	$t \approx 1$ nm d (PBS) ≈ 1241.5 nm	Activated partial thromboplastin time (APTT): slightly increases in presence of GO (attributed to —COOH) gradually increases with Hep-g-pRGO concentration
	BSA-g-pRGO	$t \approx 1$ nm d (PBS) ≈ 1131.7 nm	

Table 2.6 introduces the composite types mentioned in the existing biocompatibility studies. In the case of biomedical applications, the incorporation of GBMs should not decrease

biocompatibility, and desirably would inhibit bacterial growth on the surfaces and reduce thrombogenicity. These issues are addressed below.

Table 2.6: Types of GBMs composites used in biocompatibility studies and respective production methods.

Type of composite	Matrix	GBM filler	GBM production method	Composite production method	Ref
PLGA/GO nanofibers	PLGA – Poly(D,L-lactic-co-glycolic acid, 1:1)	GO	MHM	Electrospinning	Yoon <i>et al.</i> [91]
PLGA/GO films	PLGA	GO	MHM	Spin coating	Yoon <i>et al.</i> [92]
PLA/HA/GO nanofibers	PLA – poly(lactic acid) $M_w = 26,000 \text{ g mol}^{-1}$	GO and hydroxyapatite (HA, 99% purity $d = 20\text{--}40 \text{ nm}$)	MHM	Electrospinning	HaiBi n <i>et al.</i> [93]
PLA/GO and GNP films	PLA (96% L-lactide) $M_w = 121,400 \text{ g mol}^{-1}$	GO (O at.% ≈ 22)	MHM	Solvent casting + doctor blading	Pinto <i>et al.</i> [63]
		GNP (O at.% ≈ 8)	XG Sciences, USA (commercial product)		
UHMWPE/G NP films	Ultra-high molecular weight poly (ethylene) – UHMWPE films $d = 20\text{--}30 \mu\text{m}$	GNP	XG Sciences, USA (commercial product)	Electrostatic powder coating	Lahiri <i>et al.</i> [94]
CS/N-GS films	Chitosan (CS) degree of deacetylation (DD) > 90%	N-doped graphene sheets (N-GS)	Arc-discharge [96] using NH_3 as one of the buffer gases	Solvent casting	Fan <i>et al.</i> [95]
CS/GO scaffolds	CS 50 kDa DD = 80%	GO	Not mentioned	Solvent casting (GO –COOH cross-linking with CS –NH ₂) + freeze-drying	Depan <i>et al.</i> [97]
PVA/GO scaffolds	PVA – Poly(vinyl alcohol)	GO	MHM	Solvent casting + freeze-drying	Zang <i>et al.</i> [98]
PVA/GO and R-PVA/GO films	PVA $M_w \approx 80,000$	GO	MHM	Solvent casting self assembling of PVA coated onto GO sheets	Li <i>et al.</i> [99]
		RGO	Films reduced with hydroiodic acid		
RGO/CL papers	Cellulose (CL)	GO	MHM	Solvent mixing of GO with cellulose; reduction of GO by cellulose with the assistance of 1-butyl-3-methylimidazolium chloride + filtration	Peng <i>et al.</i> [100]
GO and graphene/PAN	Polyaniline (PANI)	GO	Pre-oxidation of	Preparation of GO papers by	Yan <i>et al.</i>

I papers			graphite + MHM	filtration + immersion of GO papers in an aniline solution in which a polymerization reaction was recently induced	<i>al.</i> [101]
	Graphene		Hr of GO-PANI paper		
PVK/GNP composites and films	poly(<i>N</i> -vinylcarbazole) – PVK	GNP	XG Sciences, USA (commercial product)	Solvent mixing + PVK/GNP composites electrodeposition at indium tin oxide (ITO) surfaces Spin coating of ITO surfaces with pristine GNP (control)	Santos <i>et al.</i> [102]
GO/rGO-DS-PLL and PET-GO-DS-PLL films	(poly-L-lysine) – PLL <i>M_w</i> = 30,000–70,000	GO	MHM	Functionalization of GO and rGO with benzene diazonium salt (DS) to generate more -COOH groups + composites with PLL prepared by mixing in aqueous solution + spin coating of glass cover slips with 100 μL of 1 mg mL^{-1} dispersions	Some <i>et al.</i> [103]
		rGO	Not mentioned	Pre-plasma treated PET coated with 250 μL of 1 mg mL^{-1} GO-DS-PLL	
Solid culture mediums with GBMs composites	CS	Small area GO (sGO)	Obtained from graphite powder (MHM)	Solvent casting (1.0 wt% chitosan solution added with 0.3 wt% GBMs) + preparation of broth solutions with 2% GBMs Petri dish: 100 μL broth solution + 15 mL trypticase soy agar	Lim <i>et al.</i> [104]
		Large area GO (hGO)	Obtained from graphite flakes by a simplified hummers method [105]		
		rGO	NaOH, 80 °C, overnight		
Polyurethane (PU)/functional graphene oxide nanocomposite films (PU/GO-g-pMPC)	PU	Graphene oxide functionalized with 2-(methacryloyloxy) ethyl phosphorylcholine (GO-g-pMPC)	GO was obtained from graphite powder by Modified Brodie Method [107] and GO-g-pMPC was	GO-g-pMPC powders were dispersed in dimethylformamide (DMF) by sonication and then dispersed in a PU dispersion by the same method	Su-xing <i>et al.</i> [106]

			synthesized by reverse atom transfer radical polymerization in alcoholic media using peroxide groups as initiator	PU and PU/GO-g-pMPC films were obtained by solvent evaporation at 100 °C	
Cotton-GO fabrics	Cotton	GO	MHM	Adsorption (Cotton-GO), radiation-induced crosslinking (Cotton-rx-GO) and chemical crosslinking (Cotton-cx-GO)	Zhao <i>et al.</i> [108]

2.2.5.1 Mammalian cells

Table 2.7 presents several studies in which the biocompatibility of GBM-based composites is assessed. Most studies show that the toxic effect of the fillers is reduced when incorporated in biomaterials [63, 100-102], due to minimization of direct biological interactions with the encapsulated materials. GO usually increases surface hydrophilicity of the biocomposites [63, 91-93], therefore improving biocompatibility [63, 91, 92] by providing potential adhesion sites, like hydroxyl groups [97]. Additionally, incorporation of GO may create a more favorable surface topography toward cell adhesion [93]. Hydrophobic GBMs have been reported to not change [63] or cause reduction [96] of cell proliferation at the surface of biocomposites.

Table 2.7: Biocompatibility of GBMs composites.

Ref	Material	Size	Biocompatibility
Yoon <i>et al.</i> [91]	PLA/GO 1 and 2 wt.% nanofibers	GO ($t = 1.5$ nm, $l \approx 1$ μ m) Nanofibers (length = 11–14 μ m)	Cell proliferation: (PLGA = 100%, PLGA/GO 1 wt.% $\approx$ 102%, PLGA/GO 2 wt.% $\approx$ 108%, 48 h) (PC 12 cells)
Yoon <i>et al.</i> [92]	PLGA/GO films	GO (not mentioned) Films ($t \approx 5$ μ m)	Cell proliferation: small increase ($\approx$ 10%) comparing to PLGA for PLGA/GO 2 wt.% (48 h) (Hela cells)
HaiBin <i>et al.</i> [93]	PLA/HA (10 wt.%)/GO nanofibers	GO (few layer) PLA/HA/GO ($d = 0.3$ –1.3 μ m)	Cell proliferation: 1, 2 and 5 wt.% GO $\uparrow$ cell proliferation, comparing to PLA/HA (24 h) Only nanofibers with 5 wt.% GO presented higher proliferation than PLA/HA (48 h) (MC3T3-E1 cells)
Pinto <i>et al.</i> [63]	PLA/GO films (0.4 wt.%)	$d \approx 500$ nm Films ($t = 25$ –65 μ m)	Cell proliferation: no variations until 48 h, except for PLA/GO after 24 h (more 13% than pristine PLA) (mouse embryo fibroblasts 3T3 – ATCC CCL-164) Hemocompatibility: less platelets activated in
	PLA/GNP films (0.4 wt.%)	GNP ($t \approx 6$ –8 nm, $l \approx 5$ μ m) Films ($t = 25$ –65 μ m)	

			PLA/GNP comparing with PLA in presence of plasma proteins (human platelets concentrate)
Lahiri <i>et al.</i> [94]	UHMWPE/GNP films	GNP ($t = 6-8$ nm, $l = 5$ μ m) Films ($t = 200$ μ m)	Cell proliferation: UHMWPE/GNP 0.1 wt.% $\downarrow$ 6, 14 and 17%, UHMWPE/GNP 1 wt.% $\downarrow$ 43, 69 and 87% comparing to pristine polymer after, respectively, 1, 3 and 5 d. (osteoblasts, ATCC)
Fan <i>et al.</i> [95]	CS/N-GS films	Films ($t =$ not mentioned) GS (2–6 layers, $l \approx 500$ nm)	Cell proliferation: = for CS and CS/N-GS 0.1–0.6 wt.% (24 and 48 h) Cell adhesion: completed at 6 h for CS/N-GS 0–0.3 wt.%, at 9 h for 0.6 wt.%, at 12 h was similar for every concentration and reached 80% Morphology: no differences for every concentration (24 h) (L929 fibroblasts)
Depan <i>et al.</i> [97]	CS/GO 1 and 3 wt.% scaffolds	GO ($t = 1.3$ nm, $d \approx 2$ μ m) Pore diameter = 100–120 μ m Porosity 84–91%	Cell adhesion: $\uparrow$ CS/GO than CS (7 d) Cell viability: $\uparrow$ CS/GO than CS scaffolds (9 and 21d) Degradation products: cell viability was higher after 48 h (mouse pre-osteoblast MC3T3-E1, ATCC)
Zhang <i>et al.</i> [98]	PVA/GO 0–1 wt.% scaffolds	PVA (any information) GO ($t =$ several layers)	Cell adhesion: 1 d – most adhered to PVA and PVA/GO 0.4%, PVA/GO 1% – did not adhere and appear spherical; 3 d – almost completely flat and spread out and fewer cells are observed for all samples; 5 d – no differences in the cell morphology or the number of cells. (osteoblasts)
Li <i>et al.</i> [99]	PVA/GO films Reduced (R)-PVA/GO films	GO ($t = 1.3-1.7$ nm, $l =$ several hundred nm to several μ m) PVA/GO films ($t \approx 10$ μ m) R-PVA/GO films ($t \approx 7.5$ μ m) GO ($t = 1$ nm)	Cell adhesion: 2.5 h – 56% adhered to R-PVA/GO films, 49% adhered to common tissue culture polystyrene (TCPS) Cell viability: 72 h – $\uparrow$ PVA R-PVA/GO films than on TCPS (human umbilical vein endothelial cells – HUVECs)
Peng <i>et al.</i> [100]	RGO (24 wt.%)/CL papers	RGO/CL papers ($t = 1.9$ nm)	Cell viability: very few necrotic cells on RGO/CL, necrosis in RGO (24 h) (HeLa cells)
Yan <i>et al.</i> [101]	GO/PANI paper Graphene/PANI paper	Both papers ($t = 4-5$ μ m, distance between layers = 100–200 nm)	Cell viability: 2 d – 71% GO, 77% GO/PANI, 75% graphene, 84% graphene/PANI; 4 d: 92% GO, 97% GO/PANI, 92% graphene, 100% graphene-PANI. (Fibroblasts L-929 cells)
Santos <i>et al.</i> [102]	PVK/GNP composites PVK/GNP films PVK films GNP films	GNP ($t = 1.8$ nm, $l = 10-20$ μ) Films ($t = 150$ nm)	Cell viability: GNP $\approx$ 60%, PVK $\approx$ 90%, PVK/GNP 3 wt.% composites $\approx$ 80%; concentrations of 1.0 mg mL ⁻¹ in culture medium (NIH 3T3 Fibroblasts, Univ. Wisconsin-Madison) Antibacterial properties: (<i>E. coli</i> and <i>B. subtilis</i>) Cell viability: after 1 h – PVK > 85%, GNP < 30% (concentrations of 1.0 mg mL ⁻¹ in culture medium), PVK/GNP 3 wt.% composites <90%, <90%, <20%, <10%, respectively for 0.01, 0.05, 0.5 and 1 mg mL ⁻¹ in culture medium. Biofilm formation: inhibited at PVK/GNP and GNP films surfaces
Some <i>et al.</i>	GO/rGO-DS-PLL		Cell proliferation: $\uparrow$ GO-PLL, GO-DS-PLL

<i>al.</i> ^[103]	films PET-GO-DS-PLL films		and rGO-DS-PLL than PLL and GO (non-small cell lung carcinoma A549 and human adipose derived stem cells) Antibacterial properties (<i>E. coli</i> DH5- α): Live dead: control 4%, GO-DS-PLL 97% dead cells (25 $\mu\text{g mL}^{-1}$) Cell viability: <i>E. coli</i> = 100% (control), GO-PLL, rGO-DS-PLL, GO-DS-PLL $\approx$ 0; PLL $\approx$ 43%, rGO-PLL $\approx$ 52%, GO-DS 78%, rGO 87%, GO 96%, rGO-DS 104% (25 $\mu\text{g mL}^{-1}$) Biofilm formation: PET-PLL presented bacterial growth contrarily to PET-GO-DS-PLL.
Lim <i>et al.</i> ^[104]	Solid culture mediums with GBMs composites	sGO (l = 5 μm) hGO (l = 100 μm)	Cell viability: <i>P. aeruginosa</i> = 0% CS/srGO and hrGO; for CS/sGO and hGO growth was not suppressed
Su-xing <i>et al.</i> ^[106]	PU/GO-g-pMPC films	GO-g-pMPC ($t \approx$ 1 nm)	Hemocompatibility: PU/GO-g-pMPC films (in plasma) showed improved resistance to nonspecific protein adsorption and platelet adhesion (comparing with PU)
Zhao <i>et al.</i> ^[108]	Cotton-GO Cotton-rx-GO Cotton-cx-GO	$t \approx$ 1.1 nm	Bacteria: <i>E. coli</i> and <i>B. subtilis</i> $\approx$ 0% (4 h, 37 $^{\circ}\text{C}$), membrane damage occurred in <i>E. coli</i> , it was not studied for <i>B. cereus</i> Rabbits: no evidence of significant irritation in a skin irritation test until 72 h

The composite production method may influence biocompatibility. Cell proliferation at the surface of graphene nanoplatelets (GNP) composites produced by electrostatic powder coating decreased due to leaching of the filler ^[94], which was shown to be toxic ^[63]. However, for materials produced by solvent mixing no changes in cell proliferation were observed, since GNP was effectively embedded in the polymer matrix ^[63]. Hydrophilic GO released by degradation of chitosan (CS) scaffolds was found to be non-toxic ^[97].

2.2.5.2 Bacterial cells

Since it has been reported that some GBMs have antibacterial properties, it is relevant to address if incorporation of GBMs in polymer matrices decreases bacterial proliferation at the composite surface. This is an important issue because infection is frequent in biomaterial implantation procedures. ^[109]

In a few studies, the incorporation of both hydrophilic and hydrophobic GBMs (GO, rGO, GNP) in polymer matrixes [PLL – poly-l-lysine, CS, PVK – poly(N-vinylcarbazole)] was shown to decrease bacterial cell viability. ^[102-104] This was explained by GBMs causing direct damage ^[102, 104] on cell membrane and/or by oxidative stress. ^[104] This effect was enhanced in the reduced

form of the materials, due to stronger interaction between the more sharpened edges of the sheets and the bacteria's cell membrane and/or better charge transfer between the bacteria and the edge of the GBMs. ^[104]

The ideal biocomposite should inhibit bacterial growth at the surface, while promoting cell attachment and proliferation. ^[104] However, conjugation of these two effects is not evident and must be evaluated for each case. As an example, Santos *et al.* ^[102] observed that incorporation of GNP on PVK matrix decreased biofilm growth but simultaneously caused a slight decrease in cell viability. Additionally, Zhao *et al.* ^[108] observed that cotton fabrics modified with GO inhibit almost completely bacteria proliferation and that the fabrics caused no irritation to rabbit skin after 72 h contact.

2.2.5.3 Hemocompatibility

Hemocompatibility studies are relevant to assess whether implantation of composites with GBMs is safe (see Section 2.2.4). In our previous study [63] the hemocompatibility of PLA composite films was evaluated. Results showed that less adherent platelets were activated in PLA/GNP films comparing with pristine PLA films, in the presence of plasma proteins. Hydrophobic GNP seemed to favor albumin adsorption, which blocked platelet activation. Su-xing *et al.* ^[106], observed that PU/GO-g-pMPC films showed improved resistance to nonspecific protein adsorption and platelet adhesion, in the presence of plasma proteins, comparing with pristine PU films.

These results suggest that incorporation of GBMs in biomaterials may decrease their thrombogenicity. However, further studies are needed to clarify whether these fillers can indeed be used to prevent thrombus formation associated with implantation procedures.

2.3 Conclusions

Most existing works report bacterial viability decrease after exposure to GBMs. Often, physical damages occur upon direct contact of bacterial membranes with sharp edges of graphene sheets. Additionally, oxidative stress has been observed. GBMs with small particle size and in oxidized state seem to present higher antibacterial effect.

Studies frequently show that mammalian cell viability decreases slightly after exposure to GBMs. These materials were shown to induce oxidative stress and apoptosis. The most hydrophilic forms of GBMs were found to penetrate the cellular membrane. Even so, these materials were found to be generally less toxic than hydrophobic forms, which accumulate on cell membrane surfaces. One issue that has not yet been addressed is the effect of particle size on cell viability, for the same type of GBMs.

The *in vivo* effect of GBMs depends on their physical–chemical properties, concentration, time of exposure, and administration route, and also on the characteristics of the animals used. Most studies report no occurrence of adult animal death. However, there are some reports of GBMs accumulation and histological findings associated with inflammation, and, more rarely, fibrosis. Small sized GBMs present fast elimination. Existing information on *in vivo* toxicity mechanisms is still scarce, and more studies are needed to support safe biological applications of these materials.

Most of the available literature considers GBMs to be hemocompatible, but, again, further work is needed for confirmation.

Encapsulation of GBMs in a matrix reduces potential toxicity. In addition, incorporation of hydrophilic forms improves cell adhesion at the biomaterials surface. Some reports of antibacterial properties and improved hemocompatibility in GBM-based composites offer interesting perspectives for future research and developments.

Little is known about long-term toxicity of GBMs. This is an issue that merits consideration in future studies. Also, the effect of GBMs on cell signaling has just started being studied, and more information should become available soon.

2.4 References

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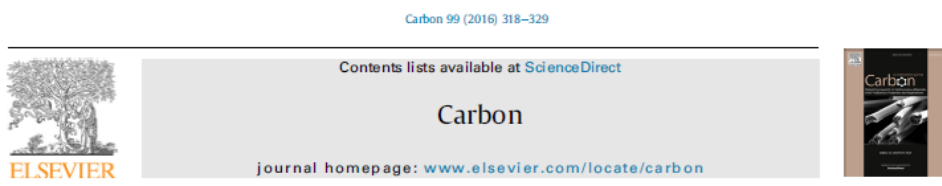
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Chapter 3

GBMs biocompatibility:

Effect of particle size and degree of oxidation

This chapter was based in the following published paper:



Smaller particle size and higher oxidation improves biocompatibility of graphene-based materials



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3 GBMs BIOCOMPATIBILITY – EFFECT OF PARTICLE SIZE AND DEGREE OF OXIDATION

3.1 Scope

Graphene-based materials (GBMs) have recognized potential for biomedical applications, however different production methods and treatments originate divergent biocompatibility. In this chapter, two commercially available graphene nanoplatelets (GNP) were studied, differing in platelet size. The effect of oxidation is known to change GBMs physico-chemical properties, thus affecting its biointeractions. For those reasons, the effect of oxidation and size on biocompatibility was evaluated *in vitro*. GBMs biocompatibility was studied in terms of hemolysis potential, induction of reactive oxygen species production, metabolic activity changes, effect on cell morphology, evaluation of membrane damages and particles internalization. The studies were performed using two different cell models, human fibroblasts, and pulmonary microvascular endothelial cells.

3.2 State of the art

Graphene is a single layer of sp^2 carbon atoms arranged in a honeycomb structure. It possesses very high mechanical strength, specific surface area and high thermal and electrical conductivity. [1-9] Strong oxidizing agents can be used to produce oxidized forms of graphene, designated as graphene oxide (GO). Also, graphite can be directly oxidized to produce graphite oxide, which can then be exfoliated originating GO. [4] The presence of polar and reactive oxygen-containing functional groups in graphene oxide [10-15] reduces its thermal stability, but may be important to promote interaction and compatibility with polar solvents and with some polymer matrices. [4, 8, 16-18]

Several biomedical applications have been studied for graphene-based materials (GBMs), like biosensing/bioimaging ^[19], drug delivery ^[18], cancer photothermal therapy [20], regenerative medicine ^[21-22], and antibacterial materials ^[18]. Examples of GBMs functions in some of the mentioned fields are: a) improvement of biomaterials mechanical/electrical properties, b) adsorption and targeted delivery of drugs, c) strong optical absorption of near infrared radiation, d) increase of cellular attachment and growth at biomaterials surface, and e) induction of selective damages in bacteria. In view of the growing interest in using GBMs in medical applications, it is relevant to evaluate their biocompatibility. From existent studies it can be concluded that generally GBMs induce a decrease on cell viability above a concentration of $10 \mu\text{g mL}^{-1}$ after 24 h incubation, with cell viability often decreasing with time and concentration. GBMs interaction with cells depends on their intrinsic physical—chemical properties (*e.g.* size, hydrophilicity), which are related to the raw materials and production methods used. However, few studies are available and some contradictory results can be found in literature. This subject was recently reviewed by Pinto *et al.* ^[23-24]. There is still the need to perform a comprehensive characterization for the different GBMs available, in terms of both physical—chemical properties and biocompatibility. Also, since several biomedical applications under study for GBMs imply contact with blood, hemocompatibility studies are necessary to assess the clinic safety of intravenous administration or implantation of GBMs ^[23-24]. Some GBMs have been reported to induce cell death by different mechanisms: a) generation of reactive oxygen species (ROS), which leads to lipid peroxidation, denaturation of proteins, DNA degradation, and activation of apoptosis, b) direct damage on cell membrane by a blade-like action of the particles sharp edges ^[25], c) decrease of free cell surface area modifying metabolic changes with the cell medium, leading to poor nutrient absorption and inefficient waste release, or affecting cell—cell interaction. ^[26] Thus, careful characterization of each particular GBM is needed to assure their biocompatibility.

The present work studies a commercially available product, with reduced cost comparing with single layer graphene: graphene nanoplatelets (GNP). Each particle is constituted by at least 2 stacked graphene layers, possessing oxygen-containing functional groups at the edges. Since this is a commercial product, there is the advantage of material consistency and availability. Moreover, GNP has been reported to display good results as biopolymers fillers, improving mechanical performance ^[24] and biocompatibility, namely reducing platelets activation without increasing toxicity. ^[24] In this work, the biocompatibility of GNP is studied for different sizes and oxidation states. Namely, evaluating hemolysis, effects on cell viability, proliferation, mitochondrial membrane potential and morphology, and ROS production.

3.3 Materials and methods

3.3.1 Graphene-based materials oxidation

Graphene nanoplatelets (GNP) grades M5 and C750, were acquired from XG Sciences (Lansing, USA), with the following characteristics: Grade M5 (GNP-M) – average thickness of 6–8 nm, maximum length 5 μm , and surface area between 120 and 150 $\text{m}^2 \text{g}^{-1}$; Grade C750 (GNP-C) – average thickness lower than 2 nm and surface area of 750 $\text{m}^2 \text{g}^{-1}$. The sizes of a typical grade C sample has a distribution that ranges from very small flakes (diameter below 100 nm) up to larger flakes (1–2 μm). According to the manufacturer, GNP production is based on exfoliation of sulphuric acid-based intercalated graphite by rapid microwave heating, followed by ultrasonic treatment. [27]

GNP-M was oxidized by modified Hummers method (MHM), as described in our previous work. [24] Briefly, 50 mL of H_2SO_4 were added to 2 g of GNP-M at room temperature and the solution was cooled using an ice bath, followed by gradual addition of KMnO_4 , 6 g for GNP-M-ox-1:3 and 12 g for GNP-M-ox-1:6. Then 300 mL of distilled water were added, followed by addition of H_2O_2 until oxygen release stopped. GNP-M-ox-1:3 and 1:6 were washed 5 times with water by centrifugation at 4000 rpm during 15 min. The solids were dispersed in 500 mL of water by sonication (Bandelin Sonorex R K512 H) during 5 h, frozen at -80°C and lyophilized for 72 h.

3.3.2 GBMs physical-chemical characterization

3.3.2.1 GBMs physical characterization

3.3.2.1.1 *Scanning electron microscopy (SEM)*

The morphology of GBMs was observed using SEM (FEI Quanta 400FEG, with acceleration voltage of 3 kV). Powders of the nanomaterials were applied on conductive carbon strips for visualization.

3.3.2.1.2 *Dynamic light scattering*

Particle sizes and distributions of the materials were determined with an LS230 laser particle analyzer (Coulter, USA). GBMs were dispersed in water at a concentration of 100 $\mu\text{g mL}^{-1}$ and sonicated for 1 h. Just before sample testing, 10 min sonication was performed to redisperse agglomerates using a Bandelin Sonorex R K512 H ultrasound bath. Data were collected performing 3 scans of 60 s, including polarization intensity differential scattering and using Fraunhofer's model. This model assumes spherical shape for the particles in suspension. The obtained size distributions must therefore be considered as relative evaluations of the degree of

deagglomeration of the different materials in water, and not as precise estimations of particle sizes. Li *et al.* [28] used a similar approach to evaluate graphene oxide particle size distribution in water by dynamic light scattering.

3.3.2.2 GBMs chemical characterization

3.3.2.2.1 X-ray photoelectron spectroscopy (XPS)

The XPS analysis of GBMs powders tablets ($d = 10$ mm) was performed at CEMUP (Centro de Materiais da Universidade do Porto) using a ESCALAB 200A, VG Scientific (UK) with PISCES software for data acquisition and analysis. For analysis, an achromatic Al ($K\alpha$) X-ray source (1486.6 eV) operating at 15 kV (300 W) was used, and the spectrometer, calibrated with reference to Ag 3d5/2 (368.27 eV), was operated in constant analyser energy mode with a pass energy of 20 eV for regions of interest, and 50 eV in survey. The core levels for O 1s and C 1s were analyzed. The photoelectron take-off angle (the angle between the surface of the sample and the axis of the energy analyzer) was 90° . The electron gun used focused on the specimen in an area close to 100 mm². Data acquisition was performed with a pressure lower than 1×10^{-6} Pa. The effect of the electric charge was corrected by the reference of the carbon peak (285 eV). The deconvolution of spectra was performed with the XPSPEAK41 program, in which a peak fitting was performed using Gaussian–Lorentzian peak shape and Shirley type background subtraction.

3.3.2.2.2 Raman spectroscopy

The unpolarized Raman spectra of GNP-C, GNP-M, GNP-M-ox-1:3 and GNP-M-ox-1:6 powders were obtained under ambient conditions, in several positions for each sample. The linear polarized 514.5 nm line of an Ar⁺ laser was used as excitation. The Raman spectra were recorded in a backscattering geometry by using a confocal Olympus BH-2 microscope with a 50× objective. The spatial resolution is about 2 μ m, and laser power was 15 mW. The scattered radiation was analysed using a Jobin–Yvon T64000 triple spectrometer, equipped with a charge-coupled device. The analysis diameter was 10 μ m and spectral resolution was better than 4 cm^{-1} .

The spectra were quantitatively analysed by fitting a sum of damped oscillator to the experimental data, according to the equation:

$$I(\omega, T) = B(\omega) + (1 + n(\omega, T)) \sum_{j=1}^N A_{oj} \frac{\omega \Omega_{oj}^2 \Gamma_{oj}}{(\Omega_{oj}^2 - \omega^2)^2 + \omega^2 \Gamma_{oj}^2} \quad (1)$$

Here $n(\omega, T)$ is the Bose-Einstein factor: A_{oj} , Ω_{oj} and Γ_{oj} are the strength, wave number and damping coefficient of the j -th oscillator, respectively, and $B(\omega)$ is the background. In this

work, the background was well simulated by a linear function of the frequency, which enable us to obtain reliable fits of equation (1) to the experimental data. The fitting procedure was performed for all Raman bands collected from the same sample, but in different positions. This procedure allows us to determine the average and standard deviation (SD) values of the phonon parameters, namely the wave number and intensity. [29]

3.3.2.2.3 Thermogravimetric analysis (TGA)

Thermal stability of samples was determined with a Netzsh STA 449 F3 Jupiter device. Sample amounts ranged from 10 to 12 mg. The thermograms were recorded between 50 and 800 °C at a heating rate of 10 °C min⁻¹ under nitrogen flow.

3.3.3 GBMs biocompatibility

GBMs were sterilized in ethanol by dispersion and sonication during 20 min (Bandelin Sonorex RK 512 H). Materials were dried in a vacuum oven overnight at 50 °C under sterile conditions. GBMs were redispersed in appropriate volume of phosphate buffered saline (PBS, pH 7.4) or cell culture medium.

3.3.3.1 Hemolysis assay

Red blood cells (RBCs) were isolated from buffy coats (obtained from Immunohemotherapy Service, Hospital S. João, Porto, Portugal), as described previously. [30] Briefly, RBCs were centrifuged over density gradient with Histopaque-1077 (Sigma–Aldrich) according to the manufacturer's instructions. After removal of the plasma upper layer, the lower layer containing RBC was washed three times in PBS. The purified RBCs were diluted to a concentration of 2×10^8 cells mL⁻¹ and 100 µL of RBCs were placed in round bottom 96 wells polypropylene microtiter plates. Next, 100 µL of GNP dispersions were added to the wells and incubated for 3 h at 37 °C and 80 rpm. Afterwards, 96-well plates were centrifuged at 4000 rpm for 15 min to collect supernatant, which was transferred (80 µL) from each well to black polypropylene 96 wells microtiter plates for absorbance reading. Absorbance was read at 380, 415, and 450 nm using a micro-plate reader spectrophotometer (Biotek Plate Reader, Synergy MX). The amount of hemoglobin (Hb) is calculated as follows: Hb value of sample (mg dL⁻¹) = $[(2 \times A_{415} - (A_{380} + A_{450})) \times 1000 \times \text{Dilution factor}]/(E)$, where A₄₁₅, A₃₈₀, A₄₅₀ are the absorbance values at 415, 380, and 450 nm, respectively. A₄₁₅ is the Soret band absorption of Hb and A₃₈₀, A₄₅₀ are correction factors of uroporphyrin whose absorption falls under the same wavelength range. E is the molar absorptivity of oxyhemoglobin at 415 nm, which is 79.46. The hemolytic potential of GNP was calculated as follows: Hemolysis (%) = Hb value of sample/Total Hb value × 100,

where total Hb value corresponds to 100% hemolysis with Triton 1% (Sigma Aldrich, X100).^[31] GBMs concentrations tested were 100, 200 and 500 $\mu\text{g mL}^{-1}$. Controls with PBS (lysis negative control) and Triton 1% in PBS (positive control for 100% hemolysis) were performed. Additionally, controls with only GBMs in PBS were performed for each concentration tested. All assays were performed in triplicate and repeated 3 times.

3.3.3.2 Biocompatibility with cell line

Human foreskin fibroblasts HFF-1 (from ATCC) were grown in DMEM+ (Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (V/V) foetal bovine serum (Gibco) and 1% (V/V) penicillin/streptomycin (biowest) at 37 °C, in a fully humidified air containing 5% CO₂. The media were replenished every 3 days. When reaching 90% confluence, cells were rinsed with PBS (37 °C) and detached from culture flasks (TPP®) using 0.25% (w/V) trypsin solution (Sigma Aldrich) in PBS. For all assays, HFF-1 cells were seeded at a density of 2×10^4 cells mL^{-1} in 96 well plates or in 8 chamber Lab-Tek-II. Upon subconfluence (24 h), DMEM+ was removed and cells incubated with increasing concentrations of GBMs in DMEM+ (1–100 $\mu\text{g mL}^{-1}$) for 24, 48, and 72 h. At indicated time-points, cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200) and phase contrast images were acquired. All experiments were performed using cells between passages 10 to 14.

HPMEC-ST1.6R cells (from ATCC) were cultured in culture flasks (TPP®) treated with 0.2% gelatine (Sigma) in medium M199+ (M199 medium (Sigma–Aldrich) supplemented (M199+) with 20% (V/V) foetal bovine serum (FBS), 2 mM Glutamax I, 100 U/100 $\mu\text{g mL}^{-1}$ Pen/Strep, (all Gibco Invitrogen), 25 $\mu\text{g mL}^{-1}$ sodium heparin (Sigma–Aldrich), 25 $\mu\text{g mL}^{-1}$ endothelial cell growth supplement (ECGS, Becton Dickinson), and 50 $\mu\text{g mL}^{-1}$ G-418 (Gibco, Invitrogen). For resazurin assay, HPMEC cells were seeded at a density of 2×10^4 cells mL^{-1} in 96 well plates coated with 0.2% gelatine. Upon subconfluence (24 h), M199+ was removed and cells incubated with increasing concentrations of GBMs in M199+ (10–100 $\mu\text{g mL}^{-1}$) for 24, and 72 h. At indicated time-points, cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200) and phase contrast images were acquired. All experiments were performed using cells between passages 26 to 37.

3.3.3.2.1 Cytotoxicity assays

Cell metabolic activity was quantified by resazurin assay at 24, 48, and 72 h of incubation. Briefly, 20 μL (1 mg mL^{-1}) resazurin (Sigma Aldrich) solution in PBS was added to each well. Cells were incubated for 3 h and fluorescence ($\lambda_{\text{ex/em}} = 530/590$ nm) read in a micro-plate reader spectrophotometer. Negative control for metabolic activity decrease was performed incubating

cells with DMEM+ and positive control incubating with Triton 0.1 wt.%. Cell metabolic activity (%) was calculated as follows: Fluorescence of sample/Fluorescence of negative control * 100. Controls were performed with materials only (without cells) dispersed in DMEM+, for all concentrations tested. All assays were performed in sextuplicate and repeated 3 times.

Cell viability/membrane integrity was evaluated by LIVE/DEAD assay. Hoechst 33342 permeates membrane and stains nucleic acids. Calcein also permeates membrane and is metabolized in cytoplasm by intracellular esterases originating a green highly fluorescent derivative. Cell membrane has residual permeability to propidium iodide, which only enters cells whose membrane integrity is compromised, staining nucleic acids. At 24, 48 and 72 h DMEM+ was removed and cells incubated with Hoechst 33342 (Molecular Probes) and Calcein (Molecular Probes), both at $2.5 \mu\text{g mL}^{-1}$ in PBS for 15 min, at 37°C in the dark. Then, propidium iodide (PI) (Sigma Aldrich) in PBS was added to each well to a final concentration of $1.25 \mu\text{g mL}^{-1}$ and images acquired in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200). Cell death (%) was calculated as follows: number of cells stained with PI/number of cells stained with Hoechst 33342 * 100. All assays were performed in triplicate and repeated 3 times.

TMRE (tetramethylrhodamine, ethyl ester) Mitochondrial Membrane Potential (MMP) Assay Kit (Abcam, ab113852) was used to measure MMP of HFF-1 cells according to manufacturer's instructions. HFF-1 cells were seeded in 96-well plates and exposed to GBMs at concentrations from 10 to $100 \mu\text{g mL}^{-1}$, for 48 h. Then, cells were rinsed $3\times$ with DMEM+ to assure no interference from materials in fluorescence determination and were loaded with 500 nM TMRE in DMEM+ for 30 min at 37°C . Cells were rinsed once with BSA 0.2 wt.% in PBS and fluorescence ($\lambda_{\text{ex/em}} = 549/575 \text{ nm}$) read in a micro-plate reader spectrophotometer. Negative control for MMP decrease was performed with cells cultured in culture medium (DMEM+), as positive control for MMP decrease cells were exposed to carbonyl cyanide m-chlorophenylhydrazone (CCCP) $20 \mu\text{M}$ for 15 min. MMT (%) was calculated as follows: Fluorescence of sample/Fluorescence of negative control for MMP decrease * 100. Controls were performed with materials only (without cells) dispersed in DMEM+ for all concentrations tested and revealed similar fluorescence to DMEM+. All assays were performed in triplicate and repeated 3 times. Cells were prepared for image acquisition in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200), following the same procedure, but incubating with 500 nM TMRE and Hoechst 33342 ($2.5 \mu\text{g mL}^{-1}$), to allow observation of cell nuclei on cells with decreased MMP.

3.3.3.2.2 Intracellular ROS evaluation

Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) is a chloromethyl derivative of H2DCFDA, useful as an indicator for reactive oxygen species (ROS) in cells. It passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases

originating a highly fluorescent derivative (DCF) and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. The fluorescence intensity is proportional to the ROS levels within the cell cytosol.

HFF-1 cells were seeded as previously described and, after reaching a state of subconfluence (24 h), washed with PBS at 37 °C and incubated 45 min at 37 °C with 95 µL CM-DCFH-DA (Molecular Probes) at a concentration of 2 µg mL⁻¹. The reagent was removed and cells incubated at 37 °C for 1 h, with 150 µL GBMs dispersed in PBS (1, 10, and 50 µg mL⁻¹). Finally, 100 µL Triton 1% in PBS was added to each well and fluorescence ($\lambda_{\text{ex/em}}$ = 480/530 nm) read in a micro-plate reader spectrophotometer. Controls were performed with GBMs dispersed in PBS, for all concentrations tested, incubated with the same amount of CM-H2DCFDA, in wells without cells. PBS was used as negative control and H2O2 (Merck) 100 mM as positive control for intracellular ROS levels increase. ROS levels were calculated as follows: ROS levels (%) = Fluorescence of the sample/Fluorescence of negative control * 100. All assays were performed in triplicate and repeated 3 times.

3.3.3.2.3 Immunocytochemistry

Cells in each well exposed to 50 µg mL⁻¹ of GBMs (at 100 µg mL⁻¹ GBMs interfere with staining and images had worse quality) were washed with PBS. Then, fixation was performed with paraformaldehyde (PFA – Merck) 4 wt.% in PBS for 15 min. PFA was removed, cells washed with PBS and stored at 4 °C. Cell cytoskeletal filamentous actin can be visualized by binding of fluorescent phalloidin and the nucleus can be stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) that intercalates with nucleic acids. Cell membrane was permeabilized with Triton 0.1 wt.% at 4 °C for 5 min. Washing was done with PBS and incubation performed with phalloidin (Alexa Fluor 488; Molecular Probes) solution in PBS in a 1:80 dilution for 20 min in the dark. After rinsing with PBS, DAPI (Sigma Aldrich) solution at 3 µg mL⁻¹ was added to each well and incubated for 15 min in the dark. Finally, cells were washed and kept in PBS to avoid drying. Plates with adherent cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200).

For evaluation of cell proliferation, Ki-67 immunocytochemistry was performed. Fixed and permeabilized cells were incubated with a blocking solution 4 wt.% BSA and 1% (V/V) FBS for 1 h, and subsequently incubated with rabbit anti-Ki67 primary antibody (1:100) from Abcam (ab15580) overnight. Cells were further incubated with the secondary antibody goat anti-rabbit IgG Alexa Fluor® 488 (1:800) from Invitrogen (a11070) for 1 h. Nuclei were stained with Nuclear Mask (Invitrogen, H10294). In order to identify possible nonspecific labelling, a negative control was performed excluding the incubation with primary antibody. Images were acquired using the In cell analyser 2000 6E Healthcare, equipped with an Nikon 10×/0.45 Plan Apo. Image analysis/Ki67 quantification was performed using the software In cell investigator developer toll

box (GE Healthcare). Ki67 positive (Ki67⁺) cells percentage were calculated as follows:

$$\text{Ki67}^{+}(\%) = \text{Ki67}^{+} \text{ cells} / \text{total cell number} \times 100.$$

3.3.3.2.4 Transmission electron microscopy (TEM)

After 72 h incubation, cells exposed to 100 $\mu\text{g mL}^{-1}$ GBMs in Lab-Tek-II were fixed in 2% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 4% PFA in cacodylate Buffer 0.1 M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–60 nm thickness) were prepared on a Leica Reichert SuperNOVA Ultramicrotome (Germany) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan) at the HEMS – Institute for Molecular and Cell Biology (IBMC) of the University of Porto.

3.3.4 Statistical analysis

Statistical analysis was performed using the software Statistical Package for the Social Sciences (SPSS 20, IBM, USA) performing Kruskal–Wallis one-way analysis of variance. Resazurin assays were analysed performing parametric analysis of variance (ANOVA) followed by post hoc tests Tamhane, Dunnett, and Games-Howell. Multiple means comparison were performed between samples to identify significant differences, which were considered for $p < 0.05$.

3.4 Results

3.4.1 GBMs physical-chemical characterization

3.4.1.1 Physical characterization

SEM imaging of the powders (Figure 3.1) shows that GNP-C is constituted by platelets with a length below 2 μm and small flakes ($<0.5 \mu\text{m}$) that form agglomerates (Fig. 3.1(A and B)). The larger platelets have planar conformation, being constituted by few single layer sheets, while small flakes are wrinkled and possess folded edges (Fig. 3.1(B)). This probably occurs due to the flakes short dimensions allowing the oxygen-containing functional groups at the edges to form hydrogen bonds, distorting the sheets. GNP-M (Fig. 3.1(C and D)) is also constituted by agglomerated platelets. However, some agglomerates can be redispersed in liquid medium. Each platelet is formed by stacks of few individual “graphene” sheets, assuming planar conformations and presenting sharp edges. These platelets have a length of about 5 μm (Fig. 3.1(E and F)).

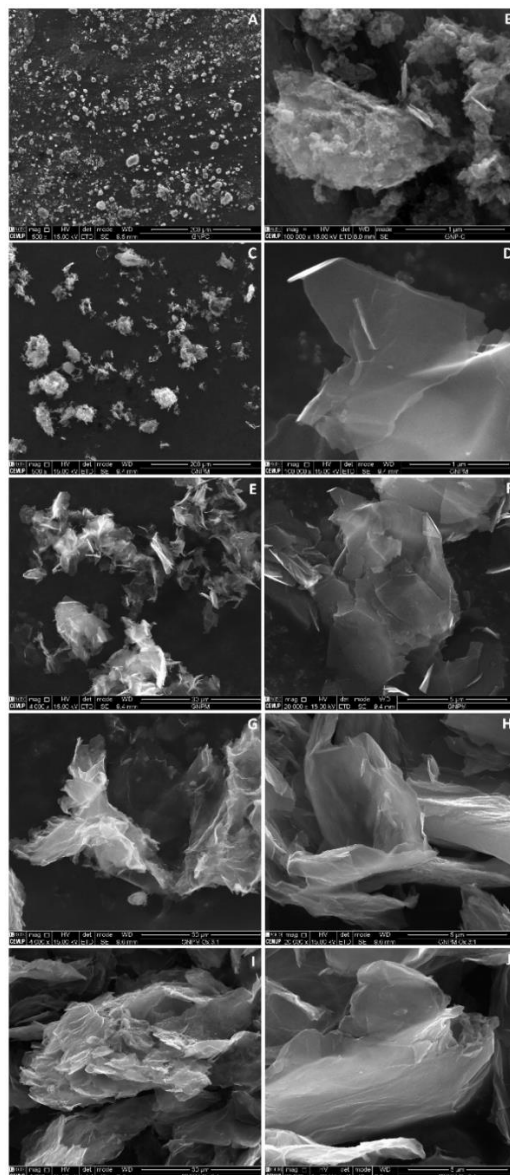


Figure 3.1: SEM images of dry powders of GNP-C at (A) 500 $\times$, (B) 100000 $\times$; GNP-M at (C) 500 $\times$, (E) 4000 $\times$, (F) 20000 $\times$, and (D) 100000 $\times$; GNP-M-ox-1:3 at (G) 4000 $\times$, and (H) 20000 $\times$; and of GNP-M-ox-1:6 at (I) 4000 $\times$, and (J) 20000 $\times$. Scale bars are 1 (B, D), 5 (F, H, J), 30 (E, G, I), and 200 μ m (A, C).

Oxidation of GNP-M changes its morphology. Both GNP-M-ox-1:3 (Fig. 3.1(G and H)) and GNP-M-ox-1:6 (Fig. 3.1(I and J)) powders have sizes similar to GNP-M, however oxidized platelets are more wrinkled and exhibit folded edges, due to formation of hydrogen bonds between intra-platelet oxygen-containing functional groups.

Figure 3.2 reveals that GNP-C is constituted by considerably smaller particle sizes than GNP-M, showing two narrow peaks at 0.5 and 2 μm , while GNP-M has broad peaks at 20, 35 and 60 μm , with a particle size distribution ranging from few μm to 70 μm . GNP-M-ox-1:3 and 1:6 display broad distributions, almost unimodal, with particle sizes ranging from few to about 100 μm in the first case and 70 μm in the second. These results suggest that GNP-C is effectively exfoliated in water, presenting two populations of platelets with sizes below 2 μm , which is consistent with the SEM images. GNP-M is significantly agglomerated in water. Both, GNP-M-ox-1:3 and 1:6 are also agglomerated, despite the oxidation treatment, which may be due to formation of inter-particle hydrogen bonds that do not dissociate in aqueous medium.

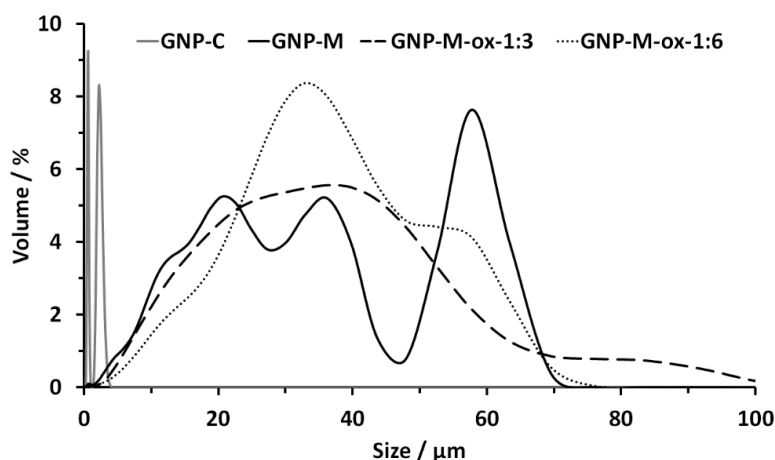


Figure 3.2: Particle size distributions of GNP-C, GNP-M and GNP-M-ox-1:3 and 1:6 dispersed in water at a concentration of 100 $\mu\text{g mL}^{-1}$.

3.4.1.1.1 Chemical characterization

XPS results (Figure 3.3(A)) show that both GNP-M and GNP-C have a low degree of oxidation (atomic percentage of oxygen – O 1s (at.%) < 4%). This was expected since GNP are obtained from graphite by microwave and ultrasonic treatment (see section 3.3.1), and its hexagonal carbon structure should unveil defects, in the form of oxygen-containing groups, mostly at the platelet edges. XPS data also reveal that oxidation of GNP-M by modified Hummer's method, to produce GNP-M-ox-1:3 and GNP-M-ox-1:6, increases the O 1s (at.%) in the final products by about 20% (Fig. 3.3(A)).

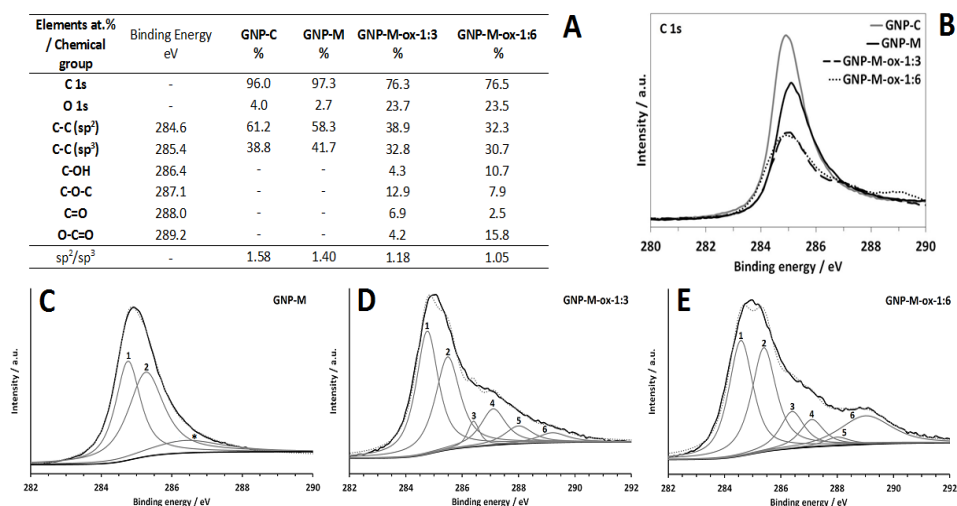


Figure 3.3: A) Table shows atomic composition of GBMs and content of C 1s chemical groups resulting from spectra fitting (*fitting for oxygen groups of GNP-C and M could not be performed due to having low content); B) XPS spectra for the core level C 1s for GNP-C, M, GNP-M-ox-1:3 and 1:6; C, D, E) spectra fitting for GNP-M, GNP-M-ox-1:3 and 1:6, respectively. Deconvoluted peaks shown correspond to: 1) C=C (sp²), 2) C-C (sp³), 3) C-OH, 4) C-O-C, 5) C=O, and 6) O-C=O.

Sulphuric acid (H₂SO₄) forms some epoxy groups (C–O–C) in alkenes double bonds in GNP honeycomb structure, mostly at the edges and surface defects.^[11] Potassium permanganate (KMnO₄) also reacts with alkene double bonds (C=C) forming diols (HO–C–C–OH). However, the principal mechanism proposed for graphene oxidation by modified Hummers method is through the formation of manganese heptoxide (Mn₂O₇), which is more reactive than potassium permanganate. This strong oxidant forms carbonyls (C=O) by cleavage of alkenes double bonds. Moreover, carboxyls (HO–C=O) can be formed by further oxidation of aldehydes (H–C=O).^[11] In the formation of manganese heptoxide, sulphuric acid was used in excess and potassium permanganate was the limiting reagent. GNP-M-ox-1:6 was oxidized in presence of 2 fold the KMnO₄ amount comparing to GNP-M-ox-1:3. This caused strong oxidation of GNP-M-ox-1:6, probably leading to preferential formation of carboxyls (O–C=O) and hydroxyls (C–OH) (Figure 3.3(A)). For GNP-M-ox-1:3, the oxidation was milder, with mostly epoxy (C–O–C) and carbonyl (C=O) groups being formed. XPS results also show that the total C–C (sp² + sp³) is lower for GNP-M-ox-1:6, despite the total C at.% being similar for both materials. This suggests that in GNP-M-ox-1:6 the oxygen-containing functional groups are distributed by more carbon atoms than in GNP-M-ox-1:3. This statement is in agreement with what is observed in Raman results (see below), which show that GNP-M-ox-1:6 is more homogeneously oxidized than GNP-M-ox-

1:3. This may be due to oxidation in GNP-M-ox-1:3 having occurred predominantly at the platelets edges. For GNP-M-ox-1:6, due to excess manganese heptoxide, oxygen-containing functional groups were also produced throughout the platelets surfaces, despite this being less sterically favourable than edge oxidation. ^[11]

The ratio between C sp^2 and sp^3 is lower for GNP-M than for GNP-C (Fig. 3.3(A)), because it has higher thickness, thus more electronic delocalization through parallel single carbon sheets. This ratio also decreases with oxidation ^[32], due to the induction of defects at platelets surface by cleavage of alkene double bonds and aldehydes oxidation to form carboxyls. Moreover, stronger oxidation, as observed for GNP-M-ox-1:6, leads to the lowest sp^2/sp^3 ratio. This is in agreement with this material being more homogeneously oxidized.

As seen in Figure 3.4(A), Raman spectra obtained for GBMs powders are similar. D-band, located between 1270 and 1450 cm^{-1} , is assigned to the carbon lattice disorder typical of crystal edges and defects in the graphene network. G-band close to 1580 cm^{-1} , is associated with the sp^2 hybridization. ^[33, 34] From the fitting procedure, we have calculated the intensity of the observed Raman bands. It is well established that the ratio between the intensities of the D- and G-bands, $I_{D/G}$, is widely used for characterizing the defects degree in graphene materials and to evaluate multilayered graphene crystallite size. ^[33] Usually, the D band is absent or weak in the surface of graphene sheets and intense in their edges. ^[34] For this reason, the ratio between the D- and G-bands is higher for GNP-C, because it exhibits a smaller mean diameter than GNP-M, having more edges in the analysis volume.

The oxidation of GNP-M lead to an increase of D-band intensity (Fig. 3.4(B)), due to disruption of sp^2 hybridization in the interior of graphene sheets due to introduction of oxygen containing functional groups and also due to weakening of π interactions between individual sheets and particles. ^[8-35] GNP-M-ox-1:3 has a lower $I_{D/G}$ ratio, comparing with GNP-M-ox-1:6, because in some spectra acquired, D-band was intense but in other cases, this band presented an intensity similar to non oxidized GNP-M. This points out for a non-uniform oxidization when a ratio of GNP-M 1:3 $KMnO_4$ is used, as it was discussed previously (see section 3.3.3.1.2). Oppositely, all spectra of GNP-M-ox-1:6 present an intense D-band and a broader G-band, evidencing both homogeneous and large degree of oxidation. Moreover, G band width slightly increases for both oxidized materials comparing with non-oxidized GNP-M and D band width is higher for the more oxidized GNP-M-ox-1:6 (Fig. 3.4(B)).

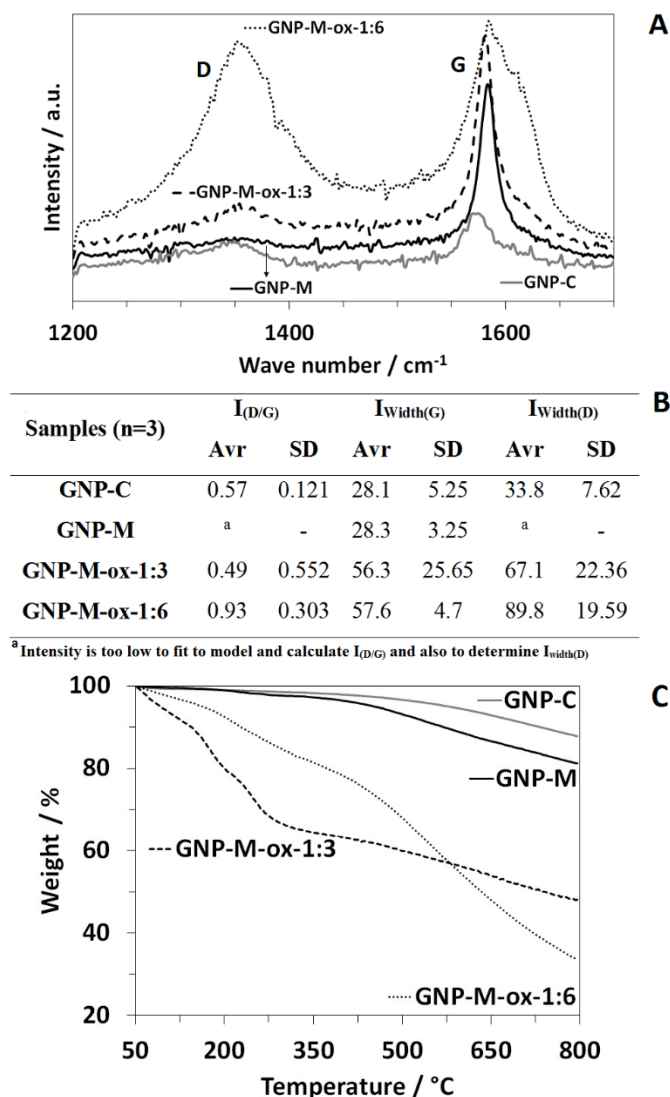


Figure 3.4: Representative unpolarized Raman spectra for GNP-C, GNP-M, GNP-M-ox-1:3, and GNP-M-ox-1:6 powders, acquired at ambient conditions. B) Intensity ratio of the (D/G) bands of GBMs powders. Results are presented as mean and standard deviation ($n = 3$). C) TGA curves and weight loss for GBMs powders.

Thermograms for GBMs are shown in Fig. 3.4(C). GNP-C and GNP-M display similar behaviour along heating ramp, having small weight losses (GNP-C = 13.8 wt.%, GNP-M = 20.7 wt.%) mostly above 400 °C. GNP-M-ox-1:3 shows 55.5 wt.% weight loss and GNP-M-ox-1:6 68.3 wt.% after heating up to 800 °C, more 34.8 and 47.6 wt.% than pristine GNP-M, respectively. This confirms that oxidation occurred to a higher degree in GNP-M-ox-1:6 than for GNP-M-ox-

1:3. Also, GNP-M-ox-1:3 earlier occurring weight loss points out the predominant location of thermolabile oxygen-containing groups at sheets edges. [36-37]

3.4.2 GBMs biocompatibility

3.4.2.1 Hemolysis evaluation

Figure 3.5 shows that both GNP-M (2.5%) and GNP-C (1.7%) induce low hemolysis even in concentrations up to $500 \mu\text{g mL}^{-1}$. Despite being dark-coloured, GBMs did not interfere with measurements in Hb absorption wavelength for all concentrations tested. Materials were washed or centrifuged in order to assure the removal of possible contaminants from production/storage, however these procedures revealed unnecessary, because hemolysis was not decreased (Figure A1 – see Appendix A).

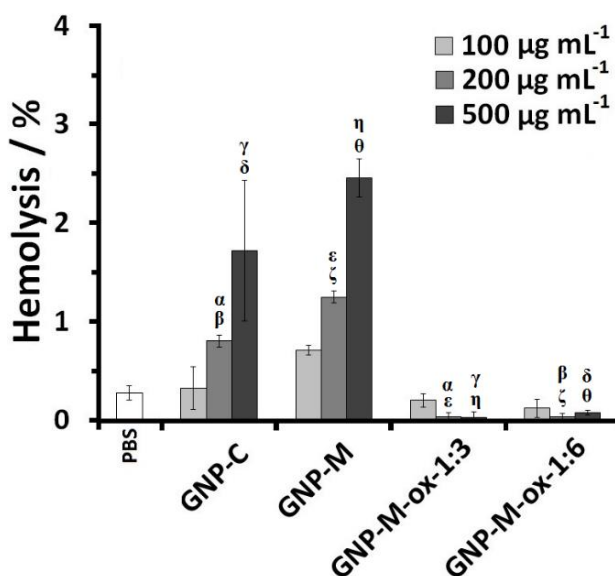


Figure 3.5: Percentage of RBCs lysis after 3 h incubation at 37 °C with PBS (negative control), and different concentrations of GBMs (100, 200 and $500 \mu\text{g mL}^{-1}$). Positive control (Triton 1% in PBS) resulted in 100% hemolysis. Results are presented as mean and standard deviation ($n = 3$). Greek symbols represent statistically significant differences between samples within the same concentrations ($p < 0.05$). No significant differences were observed between PBS and GBMs for all concentrations tested ($p > 0.05$).

No significant differences in hemolysis were observed between GNP-M and GNP-C for all concentrations tested ($p > 0.05$). The oxidation of GNP-M, significantly reduced hemolysis ratio for both GNP-M-ox-1:3 and 1:6 for the higher concentrations tested of 200 and 500 $\mu\text{g mL}^{-1}$ (Fig. 3.5). As an example, for 500 $\mu\text{g mL}^{-1}$, GNP-M hemolysis was 2.5%, GNP-M-ox-1:3 0.04% and 1:6 0.08%. This can be explained by the presence of oxygen-containing functional groups at sheets edges causing folding, therefore reducing the occurrence of physical damages to cells.

3.4.2.2 Biocompatibility with fibroblasts

After verifying that the materials do not show relevant toxic effects towards RBCs, further assays were performed to evaluate their effect when in direct contact with human foreskin fibroblasts (HFF-1). Phase contrast images were obtained for each time and concentration tested, representative images are shown in Figure A2. Further characterization was performed and described below.

3.4.2.2.1 Cytotoxicity

Considering that a sample has cytotoxic potential if its metabolic activity is reduced to less than 70% comparing to negative control for metabolic activity decrease (cells incubated with DMEM+) [ISO 10993-5:2009(E)], it can be observed from Figure 3.6(A) that both GNP-M and GNP-C are potentially non-toxic up to a concentration of 20 $\mu\text{g mL}^{-1}$, until 72 h. GNP-C is toxic at 24 h for concentrations above 20 $\mu\text{g mL}^{-1}$. However, cell metabolic activity recovered over time, and at 48 h toxicity is only present above 50 $\mu\text{g mL}^{-1}$. At 72 h the results show toxicity only slightly above the limit of 70% for 100 $\mu\text{g mL}^{-1}$. For GNP-M, toxicity occurs at 24 h above 50 $\mu\text{g mL}^{-1}$. At 48 and 72 h, toxicity is observed above 20 $\mu\text{g mL}^{-1}$. These results are in agreement with conclusions from our previous review on graphene-based materials (GBMs) biocompatibility, in which most analysed studies reported cell viability decreases inferior to 20% after exposure to GBMs concentrations around 10 $\mu\text{g mL}^{-1}$ during 24 h or longer. [23]

GNP-M-ox-1:3 unveils a toxicity profile similar to GNP-M, while GNP-M-ox-1:6 biocompatibility is greatly improved. This occurs because GNP-M-ox-1:3 oxidization was not as uniform as for GNP-M-ox-1:6, which is only slightly toxic (viability $\approx 69\%$, SD = 5.1%) at 24 h for 100 $\mu\text{g mL}^{-1}$, however at 48 and 72 h of incubation, no toxicity is observed. Controls performed with materials dispersed in DMEM+ without cells, for all concentrations tested,

line at 70%, marks the toxicity limit, according to ISO 10993-5:2009(E). Statistical analysis is presented in Table A1. B) Percentage of HFF-1 cell death after 72 h of incubation with GBMs. Cell death percentage was corrected by subtraction of the value for cells cultured in DMEM+ (negative control for cell death). Results are presented as mean and standard deviation ($n = 3$). Statistically significant differences, analysed within each concentration between all GBMs are represented by c – GNP-C, m – GNP-M, 1:3 – GNP-M-ox-1:3 (differences were only found comparing with GNP-M-ox-1:6); Differences comparing with DMEM+ are represented by Ø. Symbols not underlined represent $p < 0.05$, for $p < 0.01$ signs are underlined.

Results are also presented in terms of fluorescence intensity values, so that the increase of cell metabolic activity over time can be observed in Figure A3. It also provides images of the cells incubated with culture medium and GBMs for a concentration of $100 \mu\text{g mL}^{-1}$, showing an increase of cell density from 24 to 72 h.

For evaluation of cell proliferation, Ki-67 assay was performed. Ki-67 is a nuclear antigen present at all stages of the cell cycle, except G0 when cells are in the resting state. [38, 39] Results presented in Figure A5 revealed that there is significant decrease in cell proliferation for GNP-C $100 \mu\text{g mL}^{-1}$ at 24 h comparing with control ($p < 0.05$), followed by a recovery of the proliferative capacity of the cells at 48 h and 72 h. These results are in agreement with the metabolic activity assay obtained for this condition. Importantly, as for the other conditions tested, an increase of cell proliferation has been observed from 24 to 72 h for 50 and $100 \mu\text{g mL}^{-1}$ concentrations.

Resazurin assay was also performed with HPMEC (Human Pulmonary Microvascular Endothelial Cells). The results showed the same trend as for HFF-1, with GNP-M and GNP-M-ox-1:3 being the most toxic materials, while GNP-M-ox-1:6 showed no toxicity. Also, GNP-C presented initial mild toxicity which was recovered over time. Detailed results are presented as supplementary data in Figure A7.

Figure A4 show example images for HFF-1 cells fluorescently labelled for LIVE/DEAD assay. As expected, positive control of cell death (cells incubated with Triton 0.1 wt.% in DMEM+ for 72 h) unveils 100% cell death. Negative control was performed incubating cells with DMEM+, presenting mostly live cells with scarce dead cells.

Fig. 3.6(B) shows that cell death is below 11% for all materials tested. The resazurin assays showed larger metabolic activity decreases. A possible explanation for this difference is that metabolic activity decreased in some cells, despite possessing intact membranes, avoiding being stained by PI. GNP-M exhibits higher cell death values than GNP-C for all concentrations tested, but no significant differences were observed ($p > 0.05$). However, GNP-C only reveals significantly higher cell death than DMEM+ for $100 \mu\text{g mL}^{-1}$ ($p < 0.05$), while GNP-M presents it

for 20, 50 and 100 $\mu\text{g mL}^{-1}$ ($p < 0.01$), thus exhibiting higher toxicity. Also, GNP-M-ox-1:3 (8%) exhibits similar cell death ($p > 0.05$) to GNP-M (10%) for 100 $\mu\text{g mL}^{-1}$. However, GNP-M-ox-1:3 is equivalent to DMEM+ below this concentration ($p > 0.05$), contrary to GNP-M. GNP-M-ox-1:6 (0%) unveils no cell death for 100 $\mu\text{g mL}^{-1}$, being similar to DMEM+ for all concentrations tested ($p > 0.05$) and significantly less toxic than GNP-M for 20 $\mu\text{g mL}^{-1}$ ($p < 0.05$), 50 and 100 $\mu\text{g mL}^{-1}$ ($p < 0.01$). It is also less toxic than GNP-M-ox 1:3 ($p < 0.05$) and GNP-C ($p < 0.01$) for 50 $\mu\text{g mL}^{-1}$, and GNP-M-ox-1:3 for 100 $\mu\text{g mL}^{-1}$ ($p < 0.01$). These results are in agreement with those observed in the cell metabolic activity assays. The fact that GNP-M-ox-1:6 is homogeneously oxidized, presenting less sharp edges than GNP-M, suggests that less physical damages are induced by oxidized material, explaining the lower number of cell stained with PI and higher cell viability observed in resazurin assay.

Figure 3.7, shows that for 10 $\mu\text{g mL}^{-1}$ GNP-C increases ROS production by 2.8 fold, being significantly higher ($p < 0.01$) than GNP-M, GNP-M-ox-1:3 (1 fold) and GNP-M-ox-1:6 (1.6 fold, $p < 0.05$). Also, GNP-M-ox-1:6 is significantly higher than GNP-M and GNP-M-ox-1:3 ($p < 0.05$). For 50 $\mu\text{g mL}^{-1}$, GNP-C (4.4 fold) induces significantly more ROS production than GNP-M (1.5 fold, $p < 0.05$) and GNP-M-ox-1:6 (1.6 fold, $p < 0.01$).

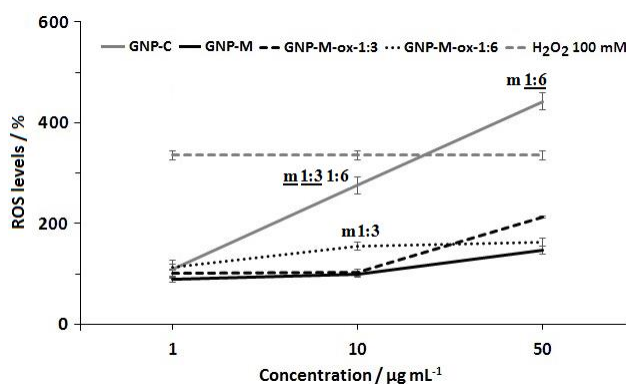


Figure 3.7: Intracellular reactive oxygen species levels (ROS) induced by GNP-C, GNP-M, GNP-M-ox-1:3, and GNP-M-ox-1:6 (1, 10, 50 $\mu\text{g mL}^{-1}$), when incubated with HFF-1 cells for 1 h. Negative control for ROS levels increase is PBS (considered 100%) and positive control H₂O₂ 100 mM. Results are presented as mean and standard deviation ($n = 3$). Statistically significant differences, analysed within each concentration are represented by c – GNP-C, m – GNP-M, 1:3 – GNP-M-ox-1:3, 1:6 – GNP-M-ox-1:6. Symbols represented above a sample concentration indicate that sample is different from samples represented by the symbols for that concentration. Symbols are not underlined when $p < 0.05$ and underlined when $p < 0.01$. Above 10 $\mu\text{g mL}^{-1}$ all samples are different from PBS ($p < 0.01$).

These results indicate that size is the main factor leading to ROS formation, because smaller GNP-C penetrates cell membrane more easily than GNP-M, GNP-M-ox-1:3, and GNP-M-ox-1:6, inducing higher intracellular ROS formation.

Controls performed with GBMs dispersed in PBS, incubated with CM-H2DCFHDA in wells without cells, present similar values to PBS only, incubated with the indicator. Thus, GBMs do not interfere in ROS quantification method used.

Mitochondrial membrane potential (MMP) was evaluated for GBMs at concentrations between 10 and 100 $\mu\text{g mL}^{-1}$, with 48 h incubation. It was observed to be decreased for both GNP-M and GNP-M-ox-1:3 for concentrations above 10 $\mu\text{g mL}^{-1}$ and for GNP-C above 50 $\mu\text{g mL}^{-1}$. For GNP-M-ox-1:6 no significant differences in MMP were perceptible ($p > 0.05$) comparing with cells cultured in DMEM+. Detailed results are presented in Figure A6.

3.4.2.2.2 Cell morphology and interaction with GBMs

After the resazurin assays, cells exposed to 50 $\mu\text{g mL}^{-1}$ of GBMs were stained and observed (at 100 $\mu\text{g mL}^{-1}$ GBMs interfere with staining and images had lower quality). Immunofluorescence images (Figure 3.8) show that for toxicity positive control (Triton 0.1% in DMEM+), cells cytoskeleton was disassembled. A control was performed with cells cultured in DMEM+, which exhibited the typical “spindle” like shape of fibroblasts. After 72 h of incubation with 50 $\mu\text{g mL}^{-1}$ GNP-C, cells exhibit a normal morphology, similar to negative control. For GNP-M, the cell density is lower, with some cells showing altered morphology and being weakly attached to the bottom of the well. Similar images were obtained for GNP-M-ox-1:3. However, for GNP-M-ox-1:6, cell morphology is normal and similar to cell cultured in DMEM+. As expected, these results are in agreement with those observed in cell viability assays results (see section 3.3.3.2.2.1) at 72 h for all materials.

TEM images (Figure 3.9(B)) show that GNP-C interacts with plasma membrane, being internalized without causing membrane damages, probably by pinocytosis. GNP-C is found often in cytoplasm, being able to interact with mitochondria (Fig. 3.9(C)), which may induce ROS production (see section 3.3.3.2.2.1). Sometimes, GNP-M causes cell membrane damage (Fig. 3.9(D)), entering cells and being found inside plasma membrane (Fig. 3.9(E)). However, this seems to be less frequent than for GNP-C. It is not clear if internalization always occurs by membrane rupture or other mechanism. GNP-M-ox-1:3 interacts with plasma membrane, being internalized and forming vesicles in cytoplasm. The internalization process occurs both with and without causing membrane damages (Fig. 3.9(F)). GNP-M-ox-1:6 was found not to cause membrane damages (Fig. 3.9(G)), probably do to having less sharp edges. However, it was found inside cells in some cases (Fig. 3.9(H)). For all materials, the internalized particles had sizes from hundreds of nanometres to 5 μm . Internalized agglomerated particles have therefore not been observed.

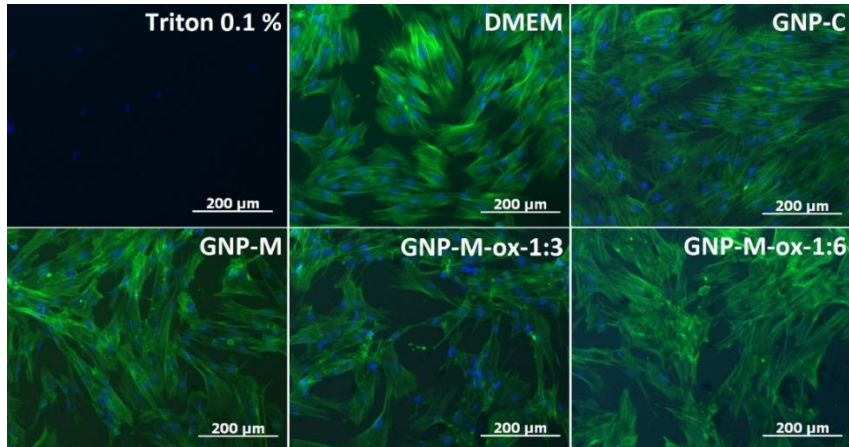


Figure 3.8: Representative immunofluorescence images of HFF-1 cells after 72 h incubation with $50 \mu\text{g mL}^{-1}$ of GNP-C, GNP-M, GNP-M-ox-1:3 and GNP-M-ox-1:6. Triton 0.1% in DMEM+ was used as positive control for changed morphology and cells grown in DMEM+ as negative control. Cells were stained with DAPI (nuclei) – blue and Phalloidin (F-actin in cytoskeleton) – green. Scale bar represents 200 μm .

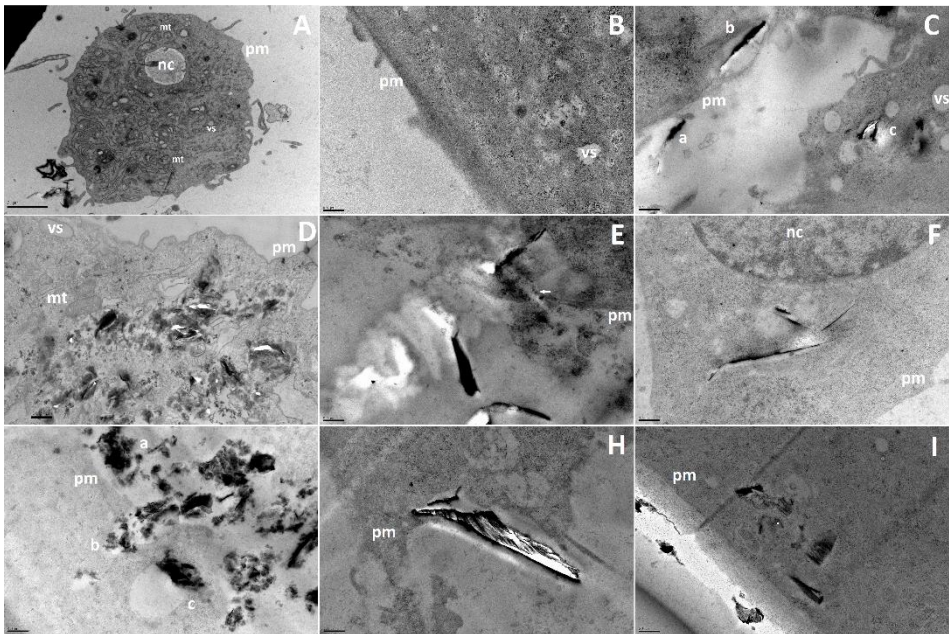


Figure 3.9: TEM images of HFF-1 cells incubated for 72 h with $100 \mu\text{g mL}^{-1}$ GBMs. A, B – DMEM+, C – GNP-C (a – particle interacting with plasma membrane (pm), b – particle internalized and in contact with plasma membrane, c – particle inside a vesicle (vs) in cytoplasm), D – GNP-C particles spread in cytoplasm and interaction with a mitochondria (mt), E – Membrane rupture (white arrow) and cytoplasmic content leakage caused by GNP-M particle, F – GNP-M in cytoplasm (nc – nucleus), G – GNP-M-ox-1:3 (a –

interacting with plasma membrane, b – entering through plasma membrane, c – vesicle containing an internalized particle), H – GNP-M-ox-1:6 particle in contact with plasma membrane causing no damages, I – GNP-M-ox-1:6 inside cytoplasm. Scale bar represents 0.5 μm for all images except for image A, in which it represents 2 μm .

3.5 Discussion

The particle size distribution of GBMs dispersed in water was determined to evaluate their agglomeration state in conditions similar to those used in the biocompatibility assays. After sonication, agglomerated fractions were identified for all materials except GNP-C. The oxidation of GNP-M to GNP-M-ox-1:3 and 1:6 slightly reduced agglomeration, as expected due to increased hydrophilicity. However, it was observed by optical microscopy that over time all materials sediment on the bottom of the wells contacting with the cells. This can be explained by the presence of more oxygen-containing functional groups, allowing hydrogen bonds between GNP particles. Despite agglomerates with considerable size being present, TEM images show that only small platelets and agglomerates, with diameters below 5 μm , are internalized.

To our knowledge, a complete characterization of the *in vitro* biological effect of materials oxidized to different degrees, comparing with the base GBM, has not been performed until date. For this reason, the oxidation degree of the materials and the functional groups present were studied with particular attention. GNP-M-ox-1:6 was oxidized in higher extension and more homogeneously than GNP-M-ox-1:3, because of the higher amount of KMnO_4 used. Also, more sp^2 disruption is observed for GNP-M-ox-1:6 due to more effective introduction of oxygen-containing functional groups in GNP-M surface. GNP-M-ox-1:3 was preferentially oxidized in platelets edges, because peripheral oxidation demands lower amount of KMnO_4 . Finally, different amounts of oxygen-containing functional groups were introduced in both materials, GNP-M-ox-1:3 presenting mostly epoxides and carbonyls and GNP-M-ox-1:6 carboxyls and hydroxyls.

No toxicity (0.1% lysis) was caused by both oxidized materials in RBCs, while for the original GNP-M, 2.5% hemolysis occurred after 3 h incubation with high concentrations of 500 $\mu\text{g mL}^{-1}$. The published literature on this topic is scarce and shows that hemocompatibility is dependent on the particular GBMs considered. Dong *et al.* observed that functionalization of GO with carboxylic groups improved its hemocompatibility. [40] Sasidharan *et al.* reported that both pristine bilayer graphene (p-G) obtained by chemical oxidation of graphite and thermal exfoliation, and carboxyl-functionalized graphene (f-G) by mild chemical oxidation were nonhemolytic (hemolysis $\approx 0.2\%$) up to a tested concentration of 75 $\mu\text{g mL}^{-1}$ with incubations of 3 h at room temperature. [31] Liao *et al.* [41] performed hemolysis assays for GO (C at.% 2:1 O

at.%) obtained by a MHM from graphite and GS – graphene sheets (C at.% 7:1 O at.%) obtained from GO by hydrothermal treatment. They observed that GO caused about 80% hemolysis and graphene sheets (GS) about 15%, for the higher concentration tested of $200 \mu\text{g mL}^{-1}$, for the same incubation time.

Metabolic activity of two cell lines (HFF-1 and HPMEC) exposed to different concentrations of GBMs over time was evaluated. These models were chosen because they are human non-cancerous cell lines, from tissues that may be exposed to GBMs in case of either dermal contact or inhalation, respectively for HFF-1 and HPMEC. To the best of our knowledge, biocompatibility of HPMEC exposed to GBMs has never been reported. The results show similar toxicological profile for GBMs in contact with both cell lines. GNP-M-ox-1:3 causes a decrease of HFF-1 cells metabolic activity similar to the base material GNP-M. However, the more homogeneously oxidized GNP-M-ox-1:6 is biocompatible up to the highest concentration tested ($100 \mu\text{g mL}^{-1}$). Hong *et al.* [42] observed that GO (produced by MHM from graphite (1:3 KMnO_4), O at.% = 38%, hydrodynamic diameter (HD) = 200 nm) caused a decrease in HeLa cells metabolic activity of about 15% and 25%, respectively for 48 h incubations with 10 and $100 \mu\text{g mL}^{-1}$. When this material was re-oxidized by the same method (G2xO – O at.% of 54%, HD = 200 nm), the metabolic activity decreased about 5% and 25% for 10 and $100 \mu\text{g mL}^{-1}$ (48 h), respectively. Comparing with our work, GO and G2xO are nanometric, while GNP-M, GNP-M-ox-1:3 and 1:6 are in the micrometer range. Thus, the biologic relevance of oxidation may be different.

GNP-M-ox-1:6 causes no metabolic activity decrease, contrary to GNP-M-ox-1:3. With a more extensive and homogeneous oxidation, GNP-M sharp edges fold, by formation of intra-platelet hydrogen bonds, causing much less damage to cell membrane and structures. However, GNP-M-ox-1:6 is also found inside cells, pointing out to a non-disruptive internalization mechanism. These results are in agreement with immunocytochemistry observations of normal cell morphology for GNP-M-ox-1:6, contrarily to GNP-M and GNP-M-ox-1:3. Also, LIVE/DEAD assay shows lower cell death for GNP-M-ox-1:6, comparing with GNP-M. The fact that GNP-M-ox-1:6 causes no damage on cell structure was confirmed by a non-reduced cell metabolic activity at all concentrations, comparing with cells cultured in DMEM+, contrary to GNP-M and GNP-M-ox-1:3, which revealed decreases for concentrations above $10 \mu\text{g mL}^{-1}$. Sasidharan *et al.* [43] observed Vero cells in contact with f-G to have higher cell metabolic activity (comparing to control with culture medium) than the originally toxic hydrophobic material (p-G), which presented a metabolic activity of 60% for a concentration $100 \mu\text{g mL}^{-1}$ and 24 h incubation. Also, Liao *et al.* [41] studied the biocompatibility of a hydrophilic and a hydrophobic GBM using human skin fibroblasts. They tested GO with an HD in deionized water of about $0.7 \mu\text{m}$ and GS with $3 \mu\text{m}$. It should be noted that as in our work, the same GBM presented larger HD in the reduced

form in relation to the oxidized material, due to agglomeration. Using the WST assay, the metabolic activity values for GO were of 88% and for GS of 20% for 24 h incubations with 50 $\mu\text{g mL}^{-1}$ concentrations. Thus, based on the current studies, it can be concluded that the oxidation of a hydrophobic GBM often improves its biocompatibility. Actually, GNP-M-ox-1:6 has increased metabolic activity, comparing with negative control (cells cultured in DMEM+) at 24 h for 100 $\mu\text{g mL}^{-1}$, and at 72 h for 50 $\mu\text{g mL}^{-1}$ and above. This suggests a favourable effect on cell proliferation. Such was confirmed by evaluating cell proliferation, which showed to significantly increase ($p < 0.05$), not only for GNP-M-ox-1:6, but for all GBMs at 72 h, for the higher concentration – 100 $\mu\text{g mL}^{-1}$, comparing with DMEM+. This points out that GNP-M-ox-1:6 is not toxic at higher concentrations, opposing to other GBMs. Interestingly, Ruiz *et al.* [44] observed that mammalian colorectal adenocarcinoma HT-29 cells attached and proliferated more efficiently in GO coated glass slides, than in control (glass slides).

An interesting aspect that is lacking from existing studies on GBMs biocompatibility has to do with the effect of particle size on the biocompatibility of hydrophobic GBMs. For this reason, biocompatibility tests were also performed for GNP-C, which exhibits smaller size than GNP-M and completely exfoliates in aqueous medium in particles with HDs of about 0.5 and 2 μm . GNP-C seems to lead to lower (1.7%) Hb leakage from RBCs than GNP-M (2.5%) for 3 h incubation with a high concentration of 500 $\mu\text{g mL}^{-1}$. However, these differences are not statistically significant ($p > 0.05$). Liao *et al.* [41] compared the hemolytic activity of GO with different sizes, produced by ultrasounds fragmentation of the base GO. They conclude that after 30 min sonication, GO decreased size by half to about 0.8 μm and caused higher hemolysis than the original material, which was already hemolytic. Liao *et al.* [41] also observed that no hemolysis was observed after coating the most hemolytic GO with chitosan, due to prevention of toxic interactions of RBCs with materials. Thus, polymer coating is a method that can be tried to improve GBMs biocompatibility.

Higher decreases on cell metabolic activity were observed for GNP-C at 24 h than for GNP-M. However, at 48 and 72 h GNP-M decreases metabolic activity more than GNP-C. This can be explained by GNP-C inducing more ROS production than GNP-M in the initial contact with cells (1 h incubation), causing early toxicity at 24 h, from which cells recover over time. Sasidharan *et al.* observed highly increased ROS levels in Vero cells for p-G, unfortunately f-G was not tested. However, Liao *et al.* [41] observed a 9-fold increase (control: cells with no GBMs) in ROS production for GO, comparing to GS (about 1.3 fold). For GNP-M, metabolic activity decreases with incubation time, because physical damages are caused on cell membranes, by the platelets' sharp edges. LIVE/DEAD results show that GNP-M causes 3-fold more cell death than GNP-C. Additionally, immunocytochemistry images show that cells incubated with GNP-C display normal cell morphology, as opposed to cells incubated with GNP-M. In fact, the latter particles

were found to cause cell membrane rupture by TEM. GNP-C particles, on the other hand, were found inside cells, in higher quantities, without evidence of damages, despite an observed occurrence of interaction with cell membrane. To our knowledge it is the first time different types of GBMs interaction, internalization and effects in non-phagocytic or cancer line cells is shown in TEM images. Lamel *et al.* [25] observed that graphene oxide (GO) and carboxyl graphene nanoplatelets (CXYG) penetrate through the plasma membrane of Hep G2 (human hepatocellular carcinoma) cells into the cytosol, are concentrated and encapsulated in intracellular vesicles. They also observed that exposure to GO (HD $\approx 0.4 \mu\text{m}$) and CXYG (HD $\approx 1.7 \mu\text{m}$) nanoplatelets results in high intracellular ROS levels by perturbation of mitochondrial structure and function. GNP-C (HD ≈ 0.5 and $2 \mu\text{m}$) caused high increases of ROS levels and was found spread in cytoplasm close to mitochondria. However, as mentioned above, this material did not cause membrane damages. GNP-M-ox-1:6, which was internalized, did not cause observable membrane damages, despite having a particle size distribution around $35 \mu\text{m}$. It must be noted, however, that only particles below $5 \mu\text{m}$ were found in cytoplasm for all materials tested.

Since GNP-C induces higher ROS production than the other materials and was found close to mitochondria, mitochondrial membrane potential (MMP) was evaluated. However, since a decrease on MMP was observed not only for GNP-C, but also for GNP-M and GNP-M-ox-1:3, another mechanism besides ROS formation is involved in MMP decrease, such as direct mitochondrial damage. Lamel *et al.* [25] also proposed that MMP disruption can be caused by physical interaction with mitochondria, besides being induced indirectly through ROS resultant from GBMs biointeraction. The collapse of the MMP coincides with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome c into the cytosol, which in turn triggers other downstream events in the apoptotic cascade. [45] MMP is decreased for both GNP-M and GNP-M-ox-1:3 for concentrations above $10 \mu\text{g mL}^{-1}$ and for GNP-C above $50 \mu\text{g mL}^{-1}$, pointing out apoptosis induced by these GBMs. For GNP-M-ox-1:6 no significant differences in MMP were perceptible ($p > 0.05$) comparing with cells cultured in DMEM+. These results are in agreement with above discussed data regarding cell death (LIVE/DEAD assay).

3.6 Conclusions

The biocompatibility of GNPs with different platelet sizes and oxidation degrees was evaluated *in vitro*. GNP-C, which exhibited smaller sizes, was shown to be generally more biocompatible than GNP-M. The sharper and longer edges of GNP-M platelets cause

membrane damages on cells being cytotoxic above $20\text{ }\mu\text{g mL}^{-1}$. GNP-C, despite being internalized without causing membrane damages, increases ROS levels being toxic above $50\text{ }\mu\text{g mL}^{-1}$.

The complete oxidation of GNP-M (GNP-M-ox-1:6) folds its sharp edges, assuring biocompatibility until the highest concentration tested ($100\text{ }\mu\text{g mL}^{-1}$). GNP-M-ox-1:6 has an oxygen content of 24% (mostly carboxyls and hydroxyls) and is dispersible in water by sonication, revealing potential to be used for biomedical purposes.

Generally, oxidation was found to be more important than size, since more oxidized GNP-M performed better than smaller GNP-C.

3.7 References

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Chapter 4

GBMs biocompatibility:

Effect of polymer surface adsorption

This chapter was based in the following published paper:



Polymer surface adsorption as a strategy to improve the biocompatibility of graphene nanoplatelets



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4 GBMs BIOCOMPATIBILITY – EFFECT OF POLYMER SURFACE ADSORPTION

4.1 Scope

The biointeractions of graphene-based materials depend on their physico-chemical properties. Thus, these properties can be manipulated in order to understand and improve GBMs biocompatibility. This chapter presents a study on the biocompatibility of graphene nanoplatelets modified by surface adsorption of several water-soluble polymers. In addition to biocompatibility, the materials produced were characterized by scanning electron microscopy, dynamic light scattering, X-ray photoelectron spectroscopy, Raman spectroscopy, and thermogravimetric analysis.

4.2 State of the art

Excellent physico-mechanical properties have been extensively reported in the literature concerning graphene and graphene-based materials (GBMs), such as high mechanical strength, specific surface area and high thermal and electrical conductivity.^[1-9] Several biomedical applications have been studied, including biosensing/bioimaging^[10], drug delivery^[11], cancer photothermal therapy^[12], regenerative medicine^[13-14], and antibacterial materials^[11]. In view of the growing interest in using GBMs in this context, it is paramount to evaluate their biocompatibility. Existing studies indicate that GBMs generally induce a decrease in cell viability above a concentration of $10\ \mu\text{g mL}^{-1}$ after 24 h of incubation, with cell viability often decreasing as time and concentration increase. However, it is expected that cell interactions will be dependent on the physical-chemical properties of GBMs (*e.g.*, size, hydrophilicity), which are related to the raw materials and production methods used. However, the number of available studies is still relatively low, and some contradictory results can be found. This subject was recently reviewed by Pinto *et al.*^[15]. The relationship between the physical-chemical properties of GBMs and their biocompatibility is still not clearly established and has to be evaluated in each

case. In addition, because intravenous administration or implantation of GBMs is often considered, haemocompatibility studies must be included.^[15-16]

Covalent and non-covalent surface modification with polymers is a strategy to overcome possible toxicity of GBMs. Regarding covalent modification, GBMs biocompatibility has been shown to be improved by functionalization with 2-methacryloyloxyethyl phosphorylcholine^[17], and crosslinked poly(*N*-isopropylacrylamide)-*g*-poly(ethylene oxide)^[18]. A limitation of some reported works that describe chemical functionalization in the context of drug delivery applications is that the biocompatibility of only the modified GBMs conjugated with drugs is reported, without comparison to the polymer-functionalized material.^[19-21] The approach of non-covalent modification has been less fully explored, with few biocompatibility studies available. The only such reports concern surface adsorption of polyethylene glycol (PEG)^[22] and poly(ethylene imine) – PEI^[23], with biocompatibility improvements being observed. A main advantage of this strategy is that it can usually be performed in aqueous medium. Covalent functionalization, on the other hand, often implies using toxic solvents as reaction medium. In addition, the procedures for non-covalent surface modification are simple and easily up-scaled.

The present work studies a commercially available product, graphene nanoplatelets (GNP), with reduced cost compared to single layer graphene. Each particle of GNP-C consists of 2 or more stacked graphene layers, possessing oxygen-containing functional groups at the edges, which may interact with solvent or polymers. Because this is a commercial product, it has the advantage of material consistency and availability. Moreover, GNP has been reported to display good results as a biopolymer filler, improving mechanical performance^[24] and biocompatibility, namely reducing platelet activation without increasing toxicity^[24]. In this work, the biocompatibility of GNP was studied, as were the effects of non-covalent surface modification with several biocompatible polymers, namely poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG), poly(vinyl pyrrolidone) (PVP), hydroxyethyl cellulose (HEC), and glycosaminoglycans: chondroitin (CON), glucosamine (GLU) and hyaluronic acid (HA).^[25-28] Polymers such as PVA, PEG, PVP, and HEC are often used in biomedical applications themselves or as modifying agents to improve the biocompatibility of materials.^[29] Glycosaminoglycans are a significant component of proteoglycans, and they do not elicit immune response and have been used extensively for tissue engineering applications.^[30] As an example, HA has been used for surface modification of carbon nanotubes and GO.^[31] Wu *et al.*^[32] observed that GO functionalized with adipic acid and covalently conjugated with HA was biocompatible with HeLa and L929 cells and non-toxic to mice up to high concentrations.

In the present work, biocompatibility was evaluated in terms of hemolysis, effects on cell viability, morphology and membranes, and ROS production.

4.3 Materials and methods

4.3.1 Materials preparation

Graphene nanoplatelets (GNP) grade C750 were acquired from XG Sciences (Lansing, USA), presenting an average thickness lower than 2 nm and surface area of $750 \text{ m}^2 \text{ g}^{-1}$. GNP-C has a size distribution that ranges from very small flakes (diameter below 100 nm) up to larger flakes (1–2 μm). According to the manufacturer, GNP production is based on exfoliation of sulphuric acid-based intercalated graphite by rapid microwave heating, followed by ultrasonic treatment. [39]

GNP-C were modified by surface adsorption with: a) poly(vinyl alcohol) – PVA, b) hydroxyethyl cellulose – HEC, c) poly(ethylene glycol) – PEG, d) poly(vinyl pyrrolidone) – PVP e) chondroitin sulfate potassium – CON, f) d-glucosamine sulfate potassium – GLU and g) hyaluronic acid – HA. HEC – WeKcelo 300 was acquired from WeiKem Chemical Co., China; according to manufacturer, the polymer has a particle size of 180 μm (98,5%) and $\bar{M}_w = 3000000 \text{ g mol}^{-1}$. PVA – *Mowiol 47-88*, $\bar{M}_w = 205000 \text{ g mol}^{-1}$, hydrolysis degree (HD) = 88% – was purchased from Clariant. CON ($\bar{M}_w = 463.3 \text{ g mol}^{-1}$), GLU ($\bar{M}_w = 277.2 \text{ g mol}^{-1}$) and HA ($\bar{M}_w = 1450000 \text{ g mol}^{-1}$) were acquired from PureBulk, USA. PVP-K30 ($\bar{M}_w = 40000\text{--}80000 \text{ g mol}^{-1}$) was purchased from Alfa Aesar. PEG-*bioultra* ($\bar{M}_w = 16000\text{--}24000 \text{ g mol}^{-1}$) was acquired from Sigma Aldrich. Polymers and GNP-C (1:1 w/w ratio) were dispersed in water, performing sonication with a Hielsher UIP 1000 probe for 5 min. Next, the dispersion was centrifuged at 4000 rpm for 15 min and supernatant discarded, assuring removal of excess polymer. The precipitate was dried at 60 °C for 1 week. Controls were performed by washing GNP-C with 5 L distilled water by filtration, and this material was designated GNP-C-w. GNP-C was centrifuged at 4000 rpm for 15 min, and the precipitate was recovered and tested. This material was designated GNP-C-c. [37]

4.3.2 Physical-chemical characterization

Dynamic light scattering particle sizes and distributions of the materials were determined with an LS230 laser particle analyzer (Coulter, USA). GBMs were dispersed in water at a concentration of $100 \mu\text{g mL}^{-1}$ and sonicated for 20 min. Just before sample testing, a 10 min sonication was performed to redisperse agglomerates. Data were collected performing 3 scans of 60 s, including

polarization intensity differential scattering and using Fraunhofer's model. This model assumes spherical shape for the particles in suspension. The obtained size distributions must therefore be considered as relative evaluations of the degree of deagglomeration of the different materials in water and not as precise estimations of particle sizes. Li *et al.* [40] used a similar approach to evaluate graphene oxide particle size distribution in water by dynamic light scattering.

4.3.3 Biocompatibility evaluation

GBMs were sterilized in ethanol by sonication for 20 min (Bandelin Sonorex RK512H). The materials were dried in a vacuum oven at 50 °C under sterile conditions. GBMs were redispersed in appropriate volume of phosphate buffered saline (PBS, pH 7.4) or Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (V/V) newborn calf serum (Gibco) and 1% (V/V) penicillin/streptomycin (biowest) (DMEM +).

4.3.3.1 Hemolysis assay

Red blood cells (RBCs) were isolated from buffy coats (obtained from Immunohemotherapy Service, Hospital S. João, Porto, Portugal) as described previously. [41] Briefly, RBCs were centrifuged over a density gradient with Histopaque-1077 (Sigma-Aldrich) according to manufacturer's instructions. The lower layer containing RBCs was washed three times in PBS. The purified RBCs were diluted to a concentration of 2×10^8 cells mL⁻¹, and 100 µL of RBCs were placed in round bottom 96-well polypropylene microtiter plates. Next, 100 µL of GBMs dispersion was added to the wells and incubated for 3 h at 37 °C and 80 rpm. Afterwards, the 96-well plates were centrifuged at 4000 rpm for 15 min to collect supernatant, which was transferred (80 µL) from each well to black polypropylene 96-well microtiter plates. Absorbance was read at 380, 415, and 450 nm using a micro-plate reader spectrophotometer (Bioteck Plate Reader, Synergy MX). The haemolytic potential of GBMs was calculated as follows: Hemolysis (%) = Hb value of sample/Total Hb value $\times$ 100, where total Hb value corresponds to 100% hemolysis with 1% Triton X-100. [34] The GBMs concentrations tested were 100, 200 and 500 µg mL⁻¹. Controls with PBS (lysis negative control) and 1% Triton (Sigma Aldrich, X100) in PBS (positive control for 100% hemolysis) were performed. Additionally, controls with only GBMs in PBS were performed for each concentration tested. All assays were performed in triplicate and repeated 3 times.

4.3.3.2 Biocompatibility with fibroblasts

In vitro assays were performed using HFF-1 (from ATCC), grown in DMEM+ at 37 °C, in fully humidified air containing 5% CO₂. The medium was replenished every 3 days and cells were detached from T75 flasks upon reaching 90% confluence. For all assays, HFF-1 cells were seeded at a density of 2×10^4 cells mL⁻¹ in 96-well plates or an 8-well chamber slide system Lab-Tek-II. Upon confluence (24 h), the DMEM+ was removed and cells were incubated with increasing concentrations of GBMs in DMEM+ (1–100 µg mL⁻¹) for 24, 48, and 72 h. At the indicated time-points, cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200) and phase contrast images were acquired. All experiments were performed using cells between passages 10 and 14.

4.3.3.3 Cytotoxicity assays

Cell mitochondrial metabolic activity was quantified by resazurin assay at 24, 48, and 72 h of incubation. Briefly, 20 µL (1 mg mL⁻¹) of resazurin (Sigma Aldrich) solution in PBS was added to each well. Cells were incubated for 3 h and fluorescence ($\lambda_{\text{ex/em}} = 530/590$ nm) read in a micro-plate reader spectrophotometer. The negative control for metabolic activity decrease was performed by incubating cells with DMEM+ and the positive control by incubating with triton 0.1 wt.%. Cell metabolic activity (%) was calculated as follows: Fluorescence of sample/Fluorescence of negative control x 100. Controls were performed with materials only (without cells) dispersed in DMEM+ for all concentrations tested. All assays were performed in sextuplicate and repeated 3 times.

Cell viability/membrane integrity was evaluated by LIVE/DEAD assay. At 72 h, the medium was removed and cells incubated with Hoechst 33342 (Molecular Probes) and Calcein (Molecular Probes), both at 2.5 µg mL⁻¹ in PBS for 15 min at 37 °C in the dark. Then, propidium iodide (PI) (Sigma Aldrich) in PBS was added to each well to a final concentration of 1.25 µg mL⁻¹ and images were acquired in a Carl Zeiss – Axiovert 200. Cell death (%) was calculated as follows: number of cells stained with PI/number of cells stained with Hoechst 33342 * 100. All assays were performed in triplicate and repeated 3 times.

4.3.3.4 Intracellular ROS evaluation

HFF-1 cells were seeded as previously described and, after reaching a state of subconfluence (24 h), washed with PBS at 37 °C and incubated 45 min at 37 °C with 95 µL chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probes) at a concentration of 2 µg mL⁻¹. The reagent was removed and cells incubated at 37 °C for 1 h with 150 µL GBMs

dispersed in PBS (1, 10, and 50 $\mu\text{g mL}^{-1}$). Finally, 100 μL Triton 1% in PBS was added to each well and fluorescence ($\lambda_{\text{ex/em}} = 480/530 \text{ nm}$) read in a micro-plate reader spectrophotometer. Controls were performed with GBMs dispersed in PBS, for all concentrations tested, and incubated with the same amount of CM-H₂DCFDA in wells without cells. PBS was used as a negative control and H₂O₂ (Merck) 100 mM as a positive control for intracellular ROS production. ROS levels were calculated as follows: ROS levels (%) = Fluorescence of the sample/Fluorescence of negative control x 100. All assays were performed in triplicate and repeated 3 times.

4.3.3.5 Transmission electron microscopy (TEM)

After 72 h of incubation, cells exposed to 100 $\mu\text{g mL}^{-1}$ GBMs in a Lab-Tek-II were fixed in 2% glutaraldehyde (Electron Microscopy sciences, Hatfield, USA) and 4% PFA in cacodylate Buffer 0.1 M (pH 7.4), dehydrated and embedded in Epon resin (TAAB, Berks, England). Ultrathin sections (40–60 nm thickness) were prepared on a Leica Reichert SuperNOVA Ultramicrotome (Germany) using diamond knives (DDK, Wilmington, DE, USA). The sections were mounted on 200 mesh copper or nickel grids, stained with uranyl acetate and lead citrate for 15 min each, and examined under a JEOL JEM 1400 TEM (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100 W (Tokyo, Japan) at the HEMS – Institute for Molecular and Cell Biology (IBMC) of the University of Porto.

4.3.4 Statistical analysis

Statistical analysis was performed using the software SPSS20 (IBM) performing Kruskal–Wallis one-way analysis of variance. The resazurin assays were analysed by performing parametric analysis of variance (ANOVA) followed by Tamhane, Dunnett, and Games-Howell post hoc tests. Multiple means comparisons were performed between samples to identify significant differences, which were considered for $p < 0.05$.

4.4 Results

4.4.1 Physical-chemical characterization

Figure 4.1 shows particle size distributions obtained after GBMs dispersion in water. GNP-C exfoliates effectively in water, originating two populations of platelets with sizes below 2 μm

(approximately 0.5 and 2 μm). Dispersion after treatment with PEG, PVP, CON, GLU and HA does not increase particle size. However, it increases for GNP-C-PVA (approximately 25 μm) and GNP-C-HEC (approximately 8 μm), indicating significant formation of platelet agglomerates due to interaction with these two polymers. GNP-C is composed of platelets with length below 2 μm and small wrinkled flakes with folded edges ($< 0.5 \mu\text{m}$) that tend to form agglomerates.

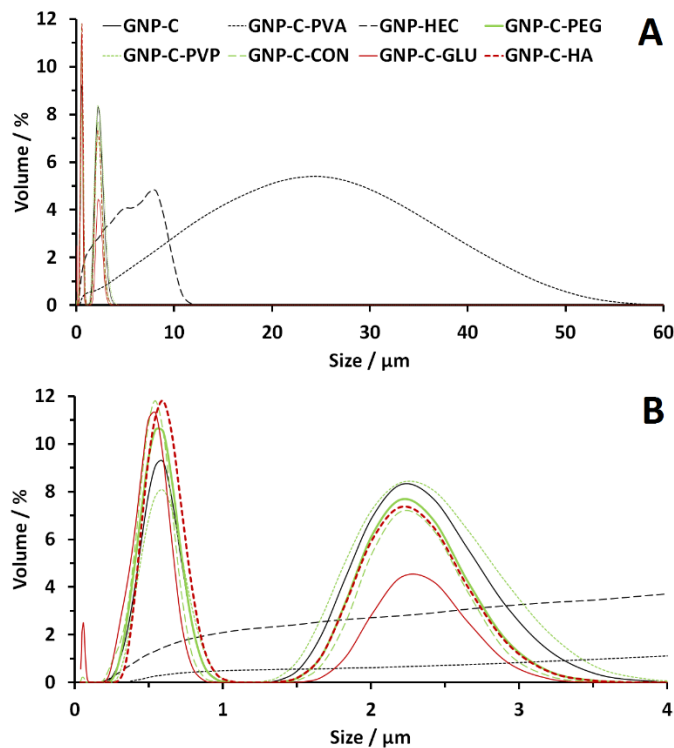


Figure 4.1: A) Particle size distributions of GBMs dispersed in water at a concentration of $100 \mu\text{g mL}^{-1}$. B) Particle size distributions for a narrower size range than in image A, to allow comparison between GBMs with smaller size distribution.

Polymer presence at the GNP-C surface was confirmed and quantified by XPS, Raman spectroscopy, and TGA (Figure B2 – Appendix B). The presence of these polymers increased O (at.%) in the final products in all cases, this increment being above 10% for GNP-C-PVA and HEC. The morphology of GNP-C before and after surface adsorption of the polymers was observed by SEM (Figure B1).

4.4.2 Biocompatibility evaluation

4.4.2.1 Hemolysis evaluation

Figure 4.2 shows that the hemolysis ratio seems to be decreased with surface adsorption of most polymers, except for HA, despite significant differences ($p < 0.05$) only being observed between GNP-C (1.7%), GNP-C-PVA (0.05%) and HEC (0%) at the higher concentration ($500 \mu\text{g mL}^{-1}$).

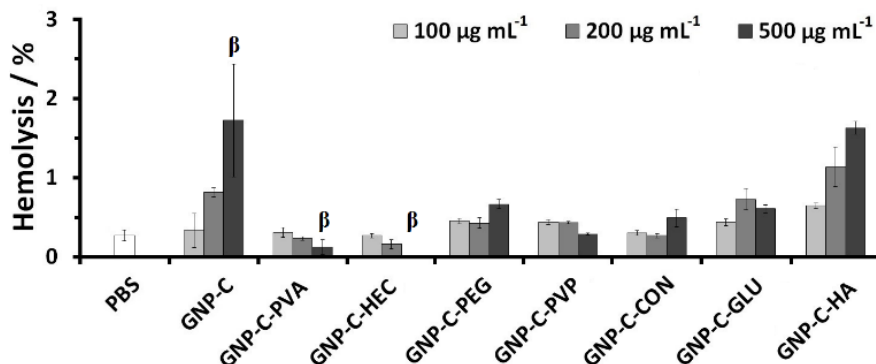


Figure 4.2: Percentage of RBCs lysis after 3 h incubation at 37 °C with PBS (negative control) and different concentrations of GBMs (100, 200 and $500 \mu\text{g mL}^{-1}$). The positive control (Triton 1% in PBS) resulted in 100% hemolysis (data not shown). The results are presented as the mean and standard deviation ($n = 3$). Greek symbols represent statistically significant differences between samples within the same concentration ($p < 0.05$).

Because PVA and HEC presented the best performance at reducing hemolysis, these polymers were selected for testing in further biocompatibility assays, presented next.

4.4.2.2 Biocompatibility with fibroblasts

After verifying that the materials do not show relevant toxic effects towards RBCs, further assays were performed to evaluate their effect when in direct contact with human foreskin fibroblasts (HFF-1). Phase contrast images were obtained for each time and concentration tested, and representative images are shown in (Fig. B1). Further characterization was performed and described below.

4.4.2.3 Cytotoxicity

Considering that, if metabolic activity is reduced to less than 70% compared to the negative control for mitochondrial metabolic activity decrease (cells incubated with DMEM+), a sample

has cytotoxic potential [ISO 10993-5:2009(E)], it can be observed from Figure 4.3 (A) that GNP-C is potentially non-toxic up to a concentration of $20 \mu\text{g mL}^{-1}$ until 72 h. GNP-C is toxic at 24 h at concentrations above $20 \mu\text{g mL}^{-1}$, however, the cell mitochondrial metabolic activity recovers over time until 72 h. For GNP-C-HEC, no improvements in biocompatibility are observed compared to pristine GNP-C. GNP-C-PVA is toxic at 24 h only above $50 \mu\text{g mL}^{-1}$, which constitutes an improvement in biocompatibility compared with GNP-C. Moreover, at 48 and 72 h, it also presents higher viability than GNP-C, revealing no toxicity until the highest concentration tested of $100 \mu\text{g mL}^{-1}$. Controls performed with materials dispersed in DMEM+ without cells, for all concentrations tested, presented similar values to DMEM+, suggesting that the materials tested did not interfere with fluorescence determination.

Figure B4 (Appendix B) shows representative images of HFF-1 cells, with and without contact with GBMs, after labelling for LIVE/DEAD assay. As expected, the positive control for cell death (cells incubated with 0.1 wt.% Triton in DMEM+ for 72 h) unveiled 100% cell death. A negative control was performed incubating cells with DMEM+, presenting mostly live cells with scarce dead fibroblasts. Fig. 4.3(B) shows that cell death was below 5% for all materials tested. At 20 and $50 \mu\text{g mL}^{-1}$, cell death was significantly lower ($p < 0.05$) for modified GNP-C-PVA compared to pristine GNP-C.

Fig. 4.3(C), shows that for $50 \mu\text{g mL}^{-1}$ at 1 h, GNP-C increases ROS production by 4.4-fold compared with the negative control (PBS). For GNP-C-PVA and HEC it also increases by 3.3- and 5.2-fold, respectively. Despite no significant differences being observed between materials ($p > 0.05$), there is a trend toward surface adsorption of HEC increasing and PVA reducing ROS production compared to unmodified GNP-C (Figure 4.3(C)). All materials are different from the negative control (PBS) at 10 and $50 \mu\text{g mL}^{-1}$ ($p < 0.05$). Controls performed with GBMs dispersed in PBS, incubated with CM-H₂DCFHDA in wells without cells, present similar values to PBS only, incubated with the indicator. Thus, GBMs do not interfere in the ROS quantification method used.

4.4.2.4 Cell interaction with GBMs

TEM images (Figure 4.4(C)) show that GNP-C is almost completely exfoliated, interacting with plasma membrane, being internalized without causing membrane damage, and being found inside vesicles in fibroblast cytoplasm (Fig. 4.4(D)). GNP-C is found often in cytoplasm, being able to interact with mitochondria (Fig. 4.4(C, D)), which may induce ROS production (Fig. 4.4(C)). GNP-C also contacts the nucleus (Fig. 4.4(C)), despite apparently causing no damage.

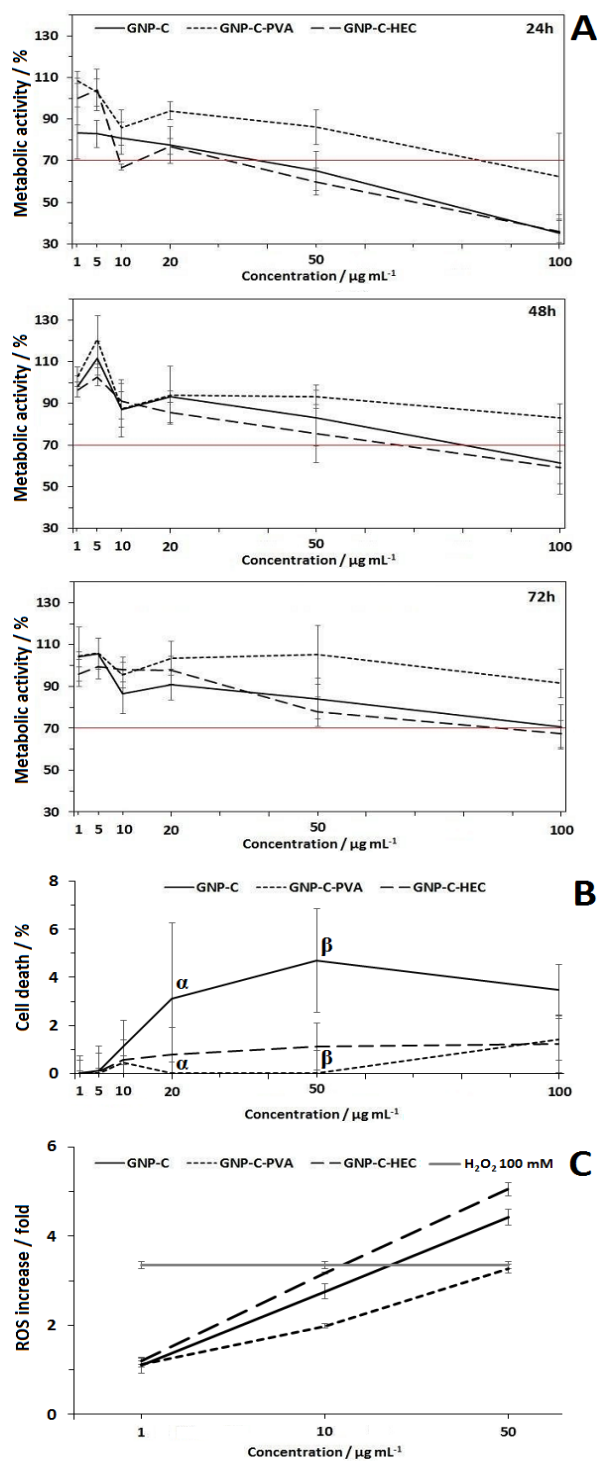


Figure 4.3: A) HFF-1 cell viability after incubation with GBMs in DMEM+, at 24, 48 and 72 h. Cell mitochondrial metabolic activity is represented as a percentage in comparison

with cells cultured in DMEM+ (100%). The results are presented as the mean and standard deviation ($n = 6$). The red line at 70% marks the toxicity limit according to ISO 10993-5:2009(E). The statistical analysis is presented in Table B1) Percentage of HFF-1 cell death after 72 h of incubation with GBMs. Cell death percentage was corrected by subtraction of the value for cells cultured in DMEM+ (negative control for cell death). The results are presented as the mean and standard deviation ($n = 3$). Statistically significant differences ($p < 0.05$) analysed within each concentration between all GBMs are represented by Greek symbols. C) Intracellular reactive oxygen species levels (ROS) induced by GBMs (1, 10, 50 $\mu\text{g mL}^{-1}$) when incubated with HFF-1 cells for 1 h. The negative control for ROS production is PBS (considered 100%) and the positive control is H_2O_2 100 mM. The results are presented as the mean and standard deviation ($n = 3$). No statistically significant differences ($p > 0.05$) were observed between samples within each concentration.

GNP-C-PVA was more agglomerated, presenting larger volume than GNP-C (Fig. 4.4(E)), being found more often outside the plasma membrane (Fig. 4.4(F)) than in cytoplasm. Internalized GNP-C-HEC particles presented smaller length (0.5–1.5 μm) than GNP-C-PVA or GNP-C (0.5–3 μm), being often found spread in cytoplasm (Fig. 4.4(G)). GNP-C-HEC was also observed in contact with mitochondria (Fig. 4.4(H)). For all materials, the internalized particles ranged in size from hundreds of nanometres to 3 μm .

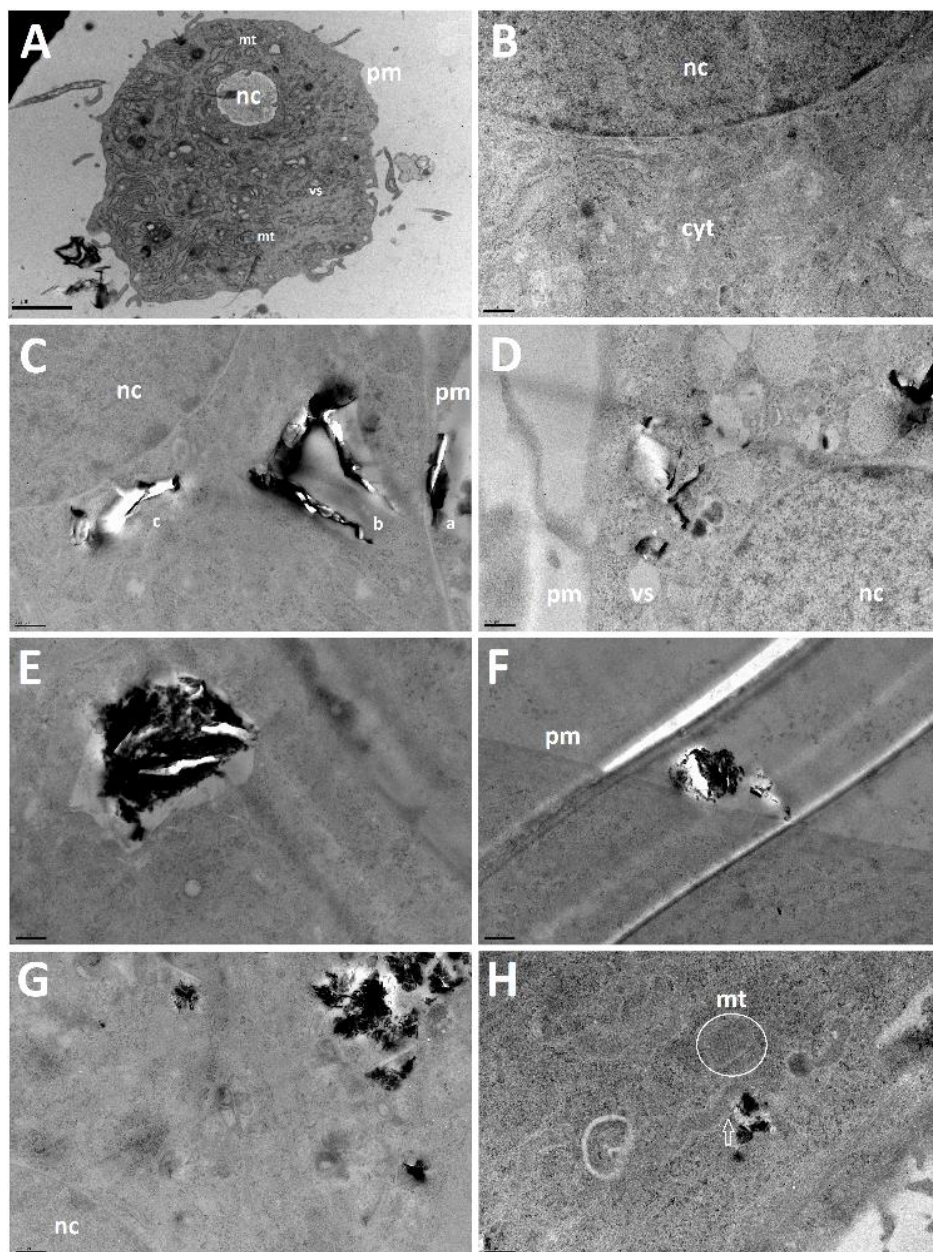


Figure 4.4: TEM images of HFF-1 cells incubated for 72 h with $100 \mu\text{g mL}^{-1}$ GBMs. DMEM+ – A $^{[33]}$, B (cyt – cytoplasm, nc – nucleus); GNP-C – C (a – particle interacting with pm – plasma membrane, b – particles inside cytoplasm, c – particle in contact with nucleus), D (particles inside cytoplasm, vs- vesicles); GNP-C-PVA – E (agglomerated particles inside cytoplasm), F (particle outside plasma membrane); GNP-C-HEC – G (particles spread in cytoplasm), H (particles interacting with mitochondria – mt). Scale bar represents $0.5 \mu\text{m}$, except for A – $2 \mu\text{m}$.

4.5 Discussion

To the best of our knowledge, the *in vitro* biological effects of GBMs modified by the polymers evaluated in this work have not been completely characterized to date. Polymer presence at the GNP-C surface was confirmed by several methods and quantified by TGA (Figure B2 – Appendix B). The presence of these polymers increased O (at.%) in the final products in all cases, this increment being above 10% for GNP-C-PVA and HEC. This higher hydrophilicity contributed to more stable dispersion in water and culture medium despite the increased particle size. The morphology of the GBMs was qualitatively evaluated by SEM (Fig. B1). Particle size distributions after dispersion in aqueous medium were determined by dynamic light scattering to evaluate the agglomeration state in conditions similar to those used in the biocompatibility assays. After sonication, GNP-C yields a bimodal distribution with narrow peaks at 0.5 and 2 μm , indicating very good deagglomeration into primary nanoplatelets.

Similar results are obtained when treating with the selected materials except for HEC and PVA. In the first case, the final hydrodynamic diameter (HD) is approximately 8 μm , while for PVA HD it is approximately 25 μm . The increased platelet agglomeration when combined with HEC or PVA can be explained by the affinity of this polymer towards the GNP-C surface together with an inter-particle bridging effect due to hydrogen bonding between the polymer's hydroxyl groups. This effect appears to be dominant over steric stabilization and therefore leads to agglomeration. As shown in supplemental material (Fig. B3), it was observed by optical microscopy that over time all the materials sediment on the bottom of the wells, contacting the cells. Despite agglomerates with considerable size being present, TEM images show that only small platelets and agglomerates with diameter below 3 μm are internalized into fibroblasts.

Hemolysis in the presence of GNP-C (1.7%) was significantly decreased with surface adsorption of PVA (0.05%) and HEC (0%) for the higher concentration tested (500 $\mu\text{g mL}^{-1}$) with 3 h of incubation. The published literature on this topic is scarce and shows that haemocompatibility is dependent on the particular GBMs considered because interactions depend on the materials' physicochemical properties (*e.g.*, size, shape, chemical composition).^[33] Due to having small size and sharp edges that may damage erythrocytes, GNP-C causes some hemolysis, but to a low extent (1.7%). Surface adsorption of PVA and HEC reduced this value even further, rendering the material almost completely nonhaemolytic. Adsorption of polymers that present low chemical affinity for GNP-C resulted in no significant differences ($p > 0.05$) in hemolysis, as for example HA, whose percentage in relation to polymer is only 5.1 wt.% and hemolysis 1.6%. For the purpose of discussion, comparisons can be made with Sasidharan *et al.*^[34], who reported pristine bilayer graphene (p-G) obtained by chemical oxidation of graphite and thermal exfoliation, to be nonhaemolytic (hemolysis $\approx 0.2\%$) up to a tested concentration of 75 $\mu\text{g mL}^{-1}$ with incubation of

3 h. However, Liao *et al.* [35] showed that GS – graphene sheets obtained from GO by hydrothermal treatment caused about 15% hemolysis at the higher concentration tested of $200\ \mu\text{g mL}^{-1}$ for the same incubation time. Because there are no *in vitro* studies regarding the haemocompatibility of GBMs modified with polymers, a comparison can be made with Yang *et al.* [36], who observed nanographene sheets (NGS) functionalized with polyethylene glycol (PEG) to be non-toxic to Balb/c mice over a period of 3 months at a concentration of $20\ \text{mg kg}^{-1}$ injected intravenously. Due to the reduced number of studies in this area, further testing is needed to better understand GBMs biocompatibility.

GNP-C for 24 h is toxic at concentrations above $20\ \mu\text{g mL}^{-1}$, however cell mitochondrial metabolic activity recovers over time until 72 h, in line with our previous work. [37] For GNP-C-HEC, no improvements in biocompatibility are observed compared to pristine GNP-C. GNP-C-PVA is toxic at 24 h only above $50\ \mu\text{g mL}^{-1}$, which constitutes an improvement in biocompatibility compared with GNP-C. Moreover, at 48 and 72 h, it also presents better performance than GNP-C, revealing no toxicity until the highest concentration tested of $100\ \mu\text{g mL}^{-1}$. Feng *et al.* [23] had previously modified GO by surface adsorption (ultrasounds + centrifugation) of PEI. GO (thickness $\approx 1\ \text{nm}$, length $< 100\ \text{nm}$) entered HeLa cells and caused a viability decrease of 32% (MTT assay) at a concentration of $100\ \mu\text{g mL}^{-1}$ for 24 h. GO-PEI (thickness $\approx 4\ \text{nm}$, length $< 100\ \text{nm}$) only reduced viability by 20% under the same conditions. Additionally, Wojtoniszak *et al.* [22] modified reduced GO – rGO (thickness ≈ 4 layers, length $\approx 100\ \text{nm}$) with PEG by simple mixing in PBS. rGO caused L929 fibroblast viability decreases (WST-1 test) of 22% at $12.5\ \mu\text{g mL}^{-1}$ for 24 h. For rGO-PEG, viability decreases were reduced to 14% employing equal circumstances. Interestingly, at higher concentrations, PEG was shown to not be as effective. Unfortunately, no further assays are available to unveil the toxicity mechanisms. For this reason, we performed complementary tests.

LIVE/DEAD assays revealed that cell death is lower for GNP-C-PVA compared to pristine GNP-C. It was already observed in resazurin assays that GNP-C-PVA presented better biocompatibility than pristine GNP-C. Additionally, cell death is below 5% for all materials. These values are inferior to the higher viability decreases observed in resazurin assays. A possible explanation for the difference is that metabolic activity is decreased in some cells despite presenting intact membranes and not being stained by propidium iodide, which only penetrates cells with compromised membrane integrity. For this reason, Fig. 4.3(B) is also an evaluation of membrane damage to HFF-1 cells. Generally, it shows that membrane damage increases with concentration being low for all materials. For GNP-C, the highest value was 4.7%, with PVA and HEC surface adsorption preventing membrane damage, presenting values of 1.4 and 1.2%, respectively. Because low membrane damage is observed, GNP-C probably has mainly a non-membrane disruptive internalization mechanism and intracellular toxicity. For this reason, ROS levels were

determined. Despite no significant differences being observed between materials ($p > 0.05$), there is a trend toward ROS production increasing with surface adsorption of HEC and decreasing with adsorption of PVA compared to unmodified GNP-C. These findings may explain the reduced membrane damage and early reversible metabolic activity decreases observed (respectively) in the LIVE/DEAD and resazurin assays. To clarify membrane and cell interactions with GBMs, imaging techniques were used.

GNP-C is almost completely exfoliated in liquid medium, interacting with plasma membrane and being internalized without causing membrane damage. TEM images show that GNP-C-PVA was more agglomerated, presenting larger size, and it was found more often outside the plasma membrane than in cytoplasm. Internalized GNP-C-HEC particles presented smaller length (0.5–1.5 μm) than GNP-C-PVA and GNP-C (0.5 – 3 μm), being often found spread in cytoplasm. Thus, we can assume that surface adsorption of PVA increases particle size, decreasing internalization, and HEC favours internalization of small particles compared with GNP-C. For these reasons, GNP-HEC could have had higher interaction with mitochondria, inducing higher ROS production and earlier toxicity. The same applies to GNP-C compared with GNP-C-PVA. These phenomena were already reported by Lamel *et al.* [38] They observed that graphene oxide (GO) and carboxyl graphene nanoplatelets (CXYG) penetrate through the plasma membrane of Hep G2 (human hepatocellular carcinoma) cells into the cytosol, concentrating and becoming encapsulated in intracellular vesicles. They also reported that exposure to GO (HD $\approx 0.4 \mu\text{m}$) and CXYG (HD $\approx 1.7 \mu\text{m}$) nanoplatelets results in high intracellular ROS levels from perturbation of mitochondrial structure and function.

4.6 Conclusions

GNP-C was modified by surface adsorption of PVA, HEC, PEG, PVP, chondroitin, glucosamine and hyaluronic acid, leading to an increase of oxygen content on the nanoplatelets' surface and, in the case of GNP-C-PVA and GNP-C-HEC, increasing apparent particle size in aqueous medium.

All of the materials caused low hemolysis ($<1.7\%$) up to $500 \mu\text{g mL}^{-1}$, usually decreasing after polymer adsorption, with PVA and HEC leading to the best results.

GNP-C-PVA is non-cytotoxic at concentrations up to $100 \mu\text{g mL}^{-1}$, whereas GNP-C can only be used at a maximum concentration of $50 \mu\text{g mL}^{-1}$. This difference can be explained by PVA encapsulating and agglomerating GNP-C platelets, thereby decreasing the internalization and interaction with HFF-1 cells that lead to ROS production.

HEC favoured internalization of small GNP-C particles, having the opposite effect regarding ROS levels. For these reasons, modification with HEC, in contrast to PVA, was counter-productive regarding increasing the biocompatibility of GNP-C with fibroblasts.

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Chapter 5

PLA/carbon-based nanomaterials composites:

Literature review

This chapter was based in a paper to be submitted:

Poly(lactic acid) composites reinforced with carbon-based nanomaterials: a review

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5 PLA/CARBON-BASED NANOMATERIALS COMPOSITES – LITERATURE REVIEW

5.1 Scope

Poly(lactic acid) (PLA) is a green alternative to petrochemical commodity plastics, used in packaging, agricultural products, disposable materials and textiles, and automotive. It also is approved by regulatory authorities for several biomedical applications. However, for some uses it is required that some of its properties are improved, namely thermo-mechanical and electrical properties. The incorporation of nanofillers is a common approach to attain this goal. The outstanding properties of carbon-based nanomaterials (CBNs), caused a surge on research works on PLA/CBNs composites. Since there is a great amount of information on these materials, it was gathered, compared and conclusions withdraw from it. This chapter is focused on PLA/CNTs (carbon nanotubes) and GBMs (graphene-based materials) composites production methods, and the effects of CBNs on PLA properties, namely mechanical, thermal, electrical and biological properties.

5.2 State of the art

5.2.1 Introduction

The growing environmental awareness and new rules and regulations are forcing the industries to seek more ecologically friendly materials for their products. ^[1] In the last two decades, industrial and academic research on polymer composites was pursued to provide added value properties to the neat polymer without sacrificing its processability or adding excessive weight. ^[2]

Poly(lactic acid) (PLA) has had significant demand due to its versatile applications in packaging, pharmaceutical, textiles, engineering, chemical industries, automotive, biomedical and tissue engineering fields. ^[3] However, the relatively low glass transition temperature, low thermal dimensional stability, and mechanical ductility limit the number of its applications. A significant

body of research has dealt with the use of fillers for improving the properties of PLA. ^[4-6] In this context, carbon based nanomaterials (CBNs), offer the potential to combine PLA properties with several of their unique features, such as high mechanical strength, electrical conductivity, thermal stability and bioactivity. ^[7-12] Carbon nanotubes (CNTs) and graphene-based materials (GBMs) are state of the art and very promising representatives of these materials. CNTs have exceptional mechanical properties, aspect ratio, electrical and thermal conductivities, and chemical stability. However, their production methods are usually complex and expensive, often leaving toxic metal residues. ^[13-16] Hence, GBMs provide an alternative option to produce functional composites due to their excellent properties and the natural abundance of their precursor, graphite. Moreover, GBMs can be produced by simple and inexpensive physico-chemical methods (Figure 5.1). ^[17-20]

In the last years there has been a surge of research works on PLA/CNTs and PLA/GBMs composites. Due to the large amount of information available, there is the need to congregate, compare and withdraw conclusions.

Several recent reviews have addressed PLA ^[3, 21-26] and CBNs ^[26-43] synthesis/production, applications and properties, however, there is no such work available focused on PLA/CBNs composites. This reason, this work presents some introductory information about PLA and CBNs, namely CNTs and GBMs, followed by a comprehensive comparison and discussion on the current progress regarding the production of PLA/CBNs composites and the resulting properties, namely mechanical, electrical, thermal and biological.

5.2.2 Materials

5.2.2.1 Poly (lactic acid)

PLA is a thermoplastic aliphatic polyester commonly produced by polymerization of lactic acid monomer. As lactic acid is a chiral molecule, existing in l and d isomers, the term “poly(lactic acid)” refers to a family of polymers: poly-l-lactic acid (PLLA), poly-d-lactic acid (PDLA), and poly-d,l-lactic acid (PDLLA). PLA can be polymerized by diverse methods, like polycondensation, ring opening polymerization, azeotropic dehydration condensation, and enzymatic polymerization. Direct polymerization and ring opening polymerization are the most used. Controlling polymerization parameters is important, since PLA properties vary with isomer composition, temperature, and reaction time used. ^[3, 21, 24, 25, 44-47]

The increasing interest on PLA has to do with aspects that lack in other polymers for the same applications, regarding renewability, biocompatibility, processability, and energy saving. ^[25] PLA

is derived from renewable and biodegradable resources such as corn and rice, and its degradation products are non-toxic. Thus, PLA finds application as biodegradable matrix for surgical implants and in drug delivery systems. [3] Furthermore, PLA is a green alternative to petrochemical commodity plastics, used in packaging, agricultural products, disposable materials and textiles, and automotive. [21]

The use of PLA has some shortcomings, related to poor chemical modifiability, mechanical ductility, slow degradation [45], and relatively high price [24]. To overcome some of these issues, some approaches are commonly used, like blending with other polymers [48-55], functionalization [56-60], and addition of nanofillers [5, 6, 47, 61-66]. The last is an interesting choice, since with small filler amounts it is possible to enhance desired features, keeping PLA's key properties intact. The most used nanofillers are nanoclays [4, 67-76], nanosilicas [5, 64, 65, 69, 77, 78], and carbon nanomaterials [6, 73, 79-84].

3.2.2.2. *Carbon-based nanomaterials*

There are several types of carbon-based nanomaterials (carbon nanotubes, graphene-based materials, fullerenes, nanodiamonds) and most have been tested to improve PLA properties. This review is focused on the most widely tested and available: CNTs and GBMs. The high specific area of these materials allows for low loadings to be sufficient to tune key properties concerning mechanical, thermal, and electrical performance.

3.2.2.2.1. *CBNs production methods and modifications*

Graphene is the elementary structure of graphite, being a one carbon atom thick sheet, composed of sp^2 carbon atoms arranged in a flat honeycomb structure composed of two equivalent sublattices of carbon atoms bonded together with σ bonds (in plane) and a π bond (out-of-plane), which contributes to a delocalized network of electrons. [36, 43, 85] These unique characteristics explain its unmatched electronic, mechanical, optical and thermal properties. For that reason, this material has been studied to be applied in many fields, such as electronics [86-91], energy [92-95], membrane [96-99], composite [17, 18, 20, 100], and biomedical technology [10, 101-103].

The intrinsic properties of graphene, and GBMs in general, are affected by the production or modification methods. For example, structural integrity of graphene sheets is disrupted by

oxidation and some other chemical modifications. The dimensions (diameter and thickness) of the final GBMs also depend on the raw materials and methods employed. [10, 30, 31, 43, 86] Thus, those should be chosen according to desired applications.

GBMs can be obtained by top-down and bottom-up approaches. [100] The first involves exfoliating graphite to obtain few or single layer graphene sheets. [35, 104] The second, consists in assembling graphene from deposition of carbon atoms from other sources. [105, 106] The main difficulty in top-down methods is to overcome the van der Waals forces that hold the graphene layers together in graphite, preventing reagglomeration and avoiding damages in the honeycomb carbon structure. [107, 108]

Some examples of such methods are micromechanical exfoliation, direct sonication, electrochemical exfoliation, and superacid dissolution. Figure 5.1 summarizes the paths for obtaining different GBMs from top-down approaches. Bottom-up methods include chemical vapor deposition (CVD), arc discharge, and epitaxial growth on silicon carbide. [100]

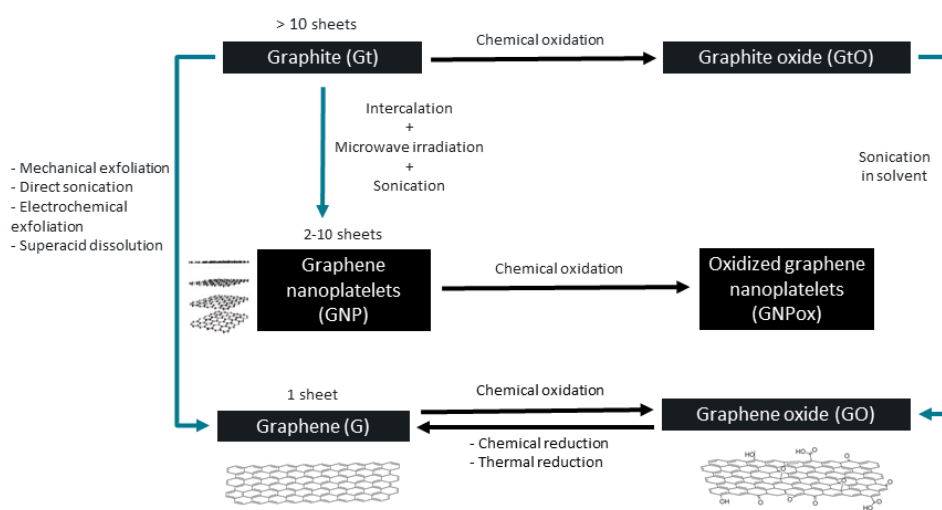


Figure 5.1: Evolution Scheme showing the relation between different types of GBMs and their top-down production methods.

The structure of CNTs can be conceptualized by wrapping graphene into a cylinder. Typically, CNTs are classified as either single-walled carbon nanotubes (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). SWCNTs exhibit better electrical properties, while MWCNTs display better resistance to chemicals. [109]

CNTs can be produced using different methods, which mainly involve gas phase processes [110, 111], like CVD, arc discharge, and laser ablation [112]. The most commonly used and efficient

methods are the ones involving CVD, in which a carbon source is deposited on a catalyst that causes it to decompose into carbon atoms forming CNTs. This method has the advantages of allowing mild and controllable synthesis in large scale, with reduced costs. ^[113] CNTs are strong, flexible, electrically conductive, and can be functionalized. ^[114] Potential applications of CNTs have been reported such as in composite materials ^[115], electrochemical devices ^[116], hydrogen storage ^[117], field emission devices ^[118], nanometer-sized electronic devices, sensors and probes ^[119]. Determining the toxicity of CNTs has been one of the most pressing questions in nanotechnology. ^[120] There is still some controversy on this subject, thus continued research is needed to assure that these materials are safe for biomedical applications. ^[121, 122] Parameters such as structure, size distribution, surface area, surface chemistry, surface charge and agglomeration state, as well as the sample purity, have considerable impact on CNTs properties. ^[114]

In the research works reported in this review, CBNs are both commercial products or lab-made by the authors. Most CNTs are commercial, because their main production method is CVD, which requires expensive equipment. The suppliers often make available the dimensions of the materials and sometimes the type of CVD used. On the other hand, GBMs are usually produced by researchers from graphitic precursors, using top-down methods involving chemical oxidation and exfoliation, namely the Soudenmaier and modified Hummers methods (Figure 5.2). Commercial GBMs are also used, with suppliers giving information about dimensions, and sometimes production methods. These involve chemical oxidation and exfoliation processes, using sonication and microwaves. Commercial products offer insured reproducibility and widespread availability. Moreover, with the optimization of the production processes, the costs of GBMs are coming closer to its precursor, graphite. ^[10]

CBNs have been extensively used in polymer composites. In order to take advantage of their large surface area maximizing its effectiveness as filler, the degree of dispersion must be the highest possible, leading to attainment of reasonable quantities of deagglomerated single units. Functionalization is often used to improve compatibility with the polymer matrix. However, this can disrupt the sp^2 hybridisation of the graphene sheets and subsequently hinder their properties. ^[123] Some examples of CBNs modifications used on the research works reported in this review are compiled in Figure 5.3. Some of these involve simple chemical oxidation, prior to surface modification with isocyanates, polymers (ethylene glycol, poly(caprolactone), methyl methacrylate, poly(vinyl pyrrolidone), and PLA)), polyols, silanes or amines. The impact of these on the composite properties is discussed in section 5.2.4.

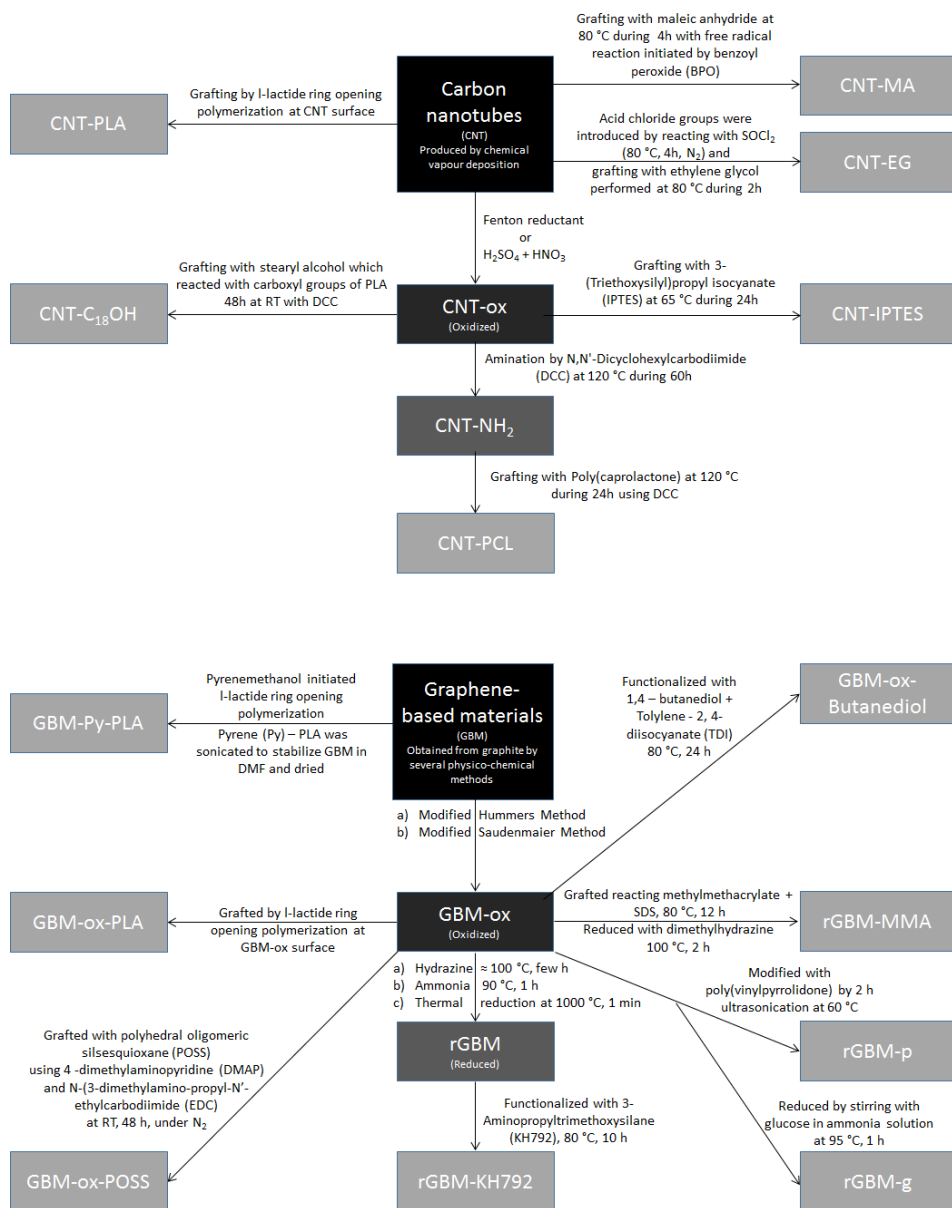


Figure 5.2: Scheme showing the different types of modifications performed on CBNs prior to incorporation in PLA.

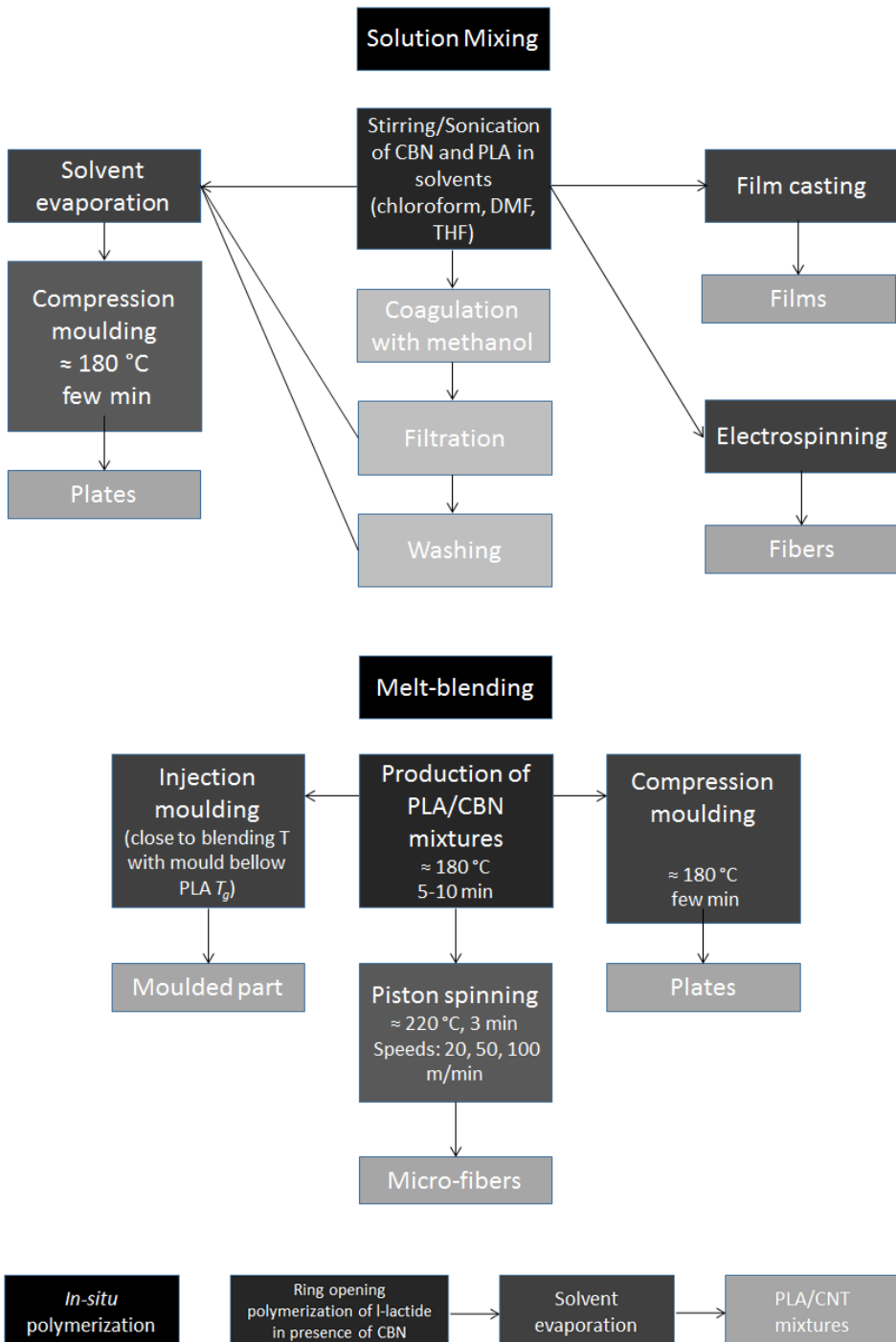


Figure 5.3: Scheme showing the different production methods of PLA/CBNs composites.

5.2.3 Production of PLA/CBNs composites

Three methods are most frequently used to obtain a dispersion of CBNs into a polymer matrix: solution mixing, melt blending, and *in situ* polymerization. [18, 100]

5.2.3.1 Solution mixing

Solution mixing is a simple procedure, requiring no special equipment, and allowing for straightforward scale-up. This method typically consists of three steps: i) dispersion of the nanomaterial in a suitable solvent using sonication or mechanical stirring, ii) dissolution of the polymer in the previous dispersion, under appropriate stirring, and iii) removal of the solvent by distillation or lyophilization. Often the dispersion is cast into a flat mold, and then the solvent is evaporated. Flat composite slabs are therefore obtained. For this reason, the procedure is often called “solvent casting”. As an alternative, the dispersion may be cast onto a low surface energy material (*e.g.* PTFE coated surface) using a blade applicator. After solvent evaporation, thin composite films are obtained. The viscosity of the dispersion needs to be adjusted for this procedure, which can be done by changing the concentration of polymer. [124] If production of fibers is desired, the third step can be replaced by electrospinning. This technique allows obtaining fibers that are much smaller in diameter (ranging from micrometers to nanometers) than those produced by conventional techniques. The basis of electrospinning is to charge the polymer solution in the spinneret tip with a high voltage, so that the induced charges cause the polymer solution to eject and travel towards the ground collector. [23]

Complete solvent removal is a critical issue when using solution mixing to prepare composites, since toxicity concerns may arise when organic solvents are used. In addition, presence of residual solvent induces plasticization of the polymer matrix, which may alter significantly its mechanical properties. [125-127]

PLA is soluble in organic solvents such as chlorinated solvents, benzene, tetrahydrofuran (THF), dimethyl formamide (DMF) and dioxane, but insoluble in ethanol, methanol, and aliphatic hydrocarbons. CBNs are hydrophobic, thus not being easily dispersed in polar solvents, however, they can be oxidized or modified with hydrophilic groups, in order to allow dispersion in this sort of solvents. Solubility limitations can also be overcome to a certain point by using ultrasonication to produce short-time metastable dispersions of CBNs in organic solvents, which can then be mixed with polymer solutions. [128]

Chloroform is the most used solvent to prepare PLA/CNTs composites. ^[129-134] McCullen *et al.* ^[135] concluded that a combination of chloroform and DMF is beneficial. Some authors obtained good results with THF ^[84, 136], and dichloromethane ^[137, 138]. Sometimes the introduction of new functional groups may originate incompatibility with the polymer matrix. To elude this problem, improvement of CNTs dispersion by surfactant addition (*e.g.*, polyoxyethylene 8 lauryl, dodecyl octaethylene) may be used, which allows preserving the chemical structure of the nanofiller. ^[139] GBMs have been often incorporated in PLA by solution mixing using chloroform ^[126, 140-142] or DMF ^[143-149] as solvents. Agglomeration of CBNs may take place during solvent evaporation. Composite formation by electrospinning allows minimizing this problem, but leads to formation of fibers and not films. ^[23, 135]

5.2.3.2 Melt-blending

Melt blending is an economically attractive, environmentally friendly and highly scalable method for preparing nanocomposites. This strategy involves direct addition of the nanomaterial into the molten polymer, allowing optimization of the state of dispersion by adjusting operating parameters such as mixing speed, time and temperature. Due to the absence of solvent, the only compatibility issue is placed in terms of the nanofiller towards the polymer matrix. ^[23, 47] The drawbacks of this procedure are the low bulk density of CBNs, that makes the feeding of the melt-mixer a troublesome task and the lower degree of dispersion that is usually attained when compared to solvent mixing. ^[128, 150]

Most published research works use a lab-scale melt mixer to melt PLA and mix it with the nanofillers. Typical processing conditions correspond to temperatures between 160 and 180 °C ^[151-157], mixing times of 5 to 10 minutes ^[151-153, 155, 156, 158], and rotation speeds between 50 and 100 rpm ^[151-155, 157-160]. After mixing, the composite materials are almost always moulded into flat sheets with controlled thickness in a hot press, however, other methods are also used (*e.g.*, injection moulding and piston spinning). Typically, the pressing is performed between 160 and 190 °C for 2 to 5 minutes, under 110 to 150 Kgf cm⁻² pressure. ^[151, 156-161]

In addition to melt blending not being as effective as the solution mixing method or *in situ* polymerization in terms of the ability to achieve good filler dispersion, damage to the nanofillers or polymer may occur under severe conditions. Some studies have shown that processing conditions can have an impact on the molecular weight of PLA. ^[162] This can be mainly attributed

to the presence of impurities such as acidic species, peroxide groups, metallic ions or other residual products that can increase the degradation of PLA during melt mixing.^[163]

5.2.3.3 *In situ* polymerization

In situ polymerization for production of polymer composites generally involves mixing the filler in neat monomer, or a solution of monomer, in the presence of catalysts and under proper reaction conditions.^[164] The polymer chains grow on the filler surface, forming covalently bonded grafts. *In situ* polymerization generally results in more homogeneous particle dispersion than melt blending.^[165] Contrary to CNTs, that usually are post-treated, graphene and GBMs already present some chemical groups that can be used in further functionalization, such as grafting polymer chains via atom transfer radical polymerization. Examples of *in situ* polymerization on GBMs include polymers such as polyaniline (PANI), polyurethane (PU), polystyrene (PS), poly(methyl methacrylate) (PMMA) and polydimethylsiloxane (PDMS).^[20]

Concerning PLA *in situ* polymerization, only a few approaches have been presented in the literature. Ring opening polymerization of l-lactide in presence of GBMs has been reported by Yang *et al.*^[166] and Promoda *et al.*^[167]. No significant studies were found for PLA/CNTs composites.

The abovementioned methods can be used both for GBMs and CNTs, for that reason, the methods used in the research works reported in this review were congregated in Figure 5.3. Since the methods were already discussed, further details are only given in Tables 5.1-4 and described on the discussion of the results in those presented, in section 5.2.4, for studies that show important achievements in PLA properties improvement.

5.2.4 Properties of PLA/CBNs Composites

As mentioned above, PLA has potential to replace petrochemical plastics if its mechanical and thermal properties are improved. Also, its bio-origins can be an advantage, when considering biomedical applications. For those reasons, numerous researchers have studied the different properties and applications of PLA alone and in combination with other materials that are able to tune key properties regarding its specific applications.^[47] Here the effect of the incorporation of some of those materials in PLA will be discussed, namely CNTs which are known for two

decades with established large scale production methods, and GBMs, which present a growing interest from scientific community, and are cheaper and comparable in properties to CNTs. ^[167]

5.2.4.1 Mechanical Properties

CBNs present remarkable mechanical properties and when well dispersed in polymer matrices often improve their mechanical performance. Physico-chemical interactions between fillers and polymer phase contribute to load transfer and distribution along the CBNs network. Table 5.1 shows that solution mixing is the most commonly reported method for incorporation of CBNs in PLA. The most frequently used solvents are chloroform, DMF and THF. The filler concentrations most often tested are between 0.1 – 2 wt.%. Maximum improvements in Young's modulus (E), storage modulus (E'), and tensile strength (σ_{max}) are found for concentrations between 0.25 - 5 wt.% for CNTs, and between 0.1 - 1 wt.% for GBMs. The larger observed improvement in E , relative to unfilled PLA, was of 372%, obtained with 0.25 wt.% MWCNTs incorporated by sonication in a PLA/chloroform dispersion, followed by compression moulding of the dried mixture. ^[129] For GBMs, the best performance was an increase of 156 % with incorporation of 0.4 wt.% GNP-M, also by sonication, but followed by film casting by doctor blading. ^[126] The maximum increase on E' was of 1500 %, and was achieved with incorporation of 0.5 wt.% rGO-KH792 in PLLA, by simple stirring, casting on PTFE mould, and vacuum drying the resultant films at 120 °C for 48h. ^[149] However, this increase only occurs around PLA transition temperature (60-65 °C). At ambient temperature, the best result was an increase of 67 % with incorporation of 3 wt.% A-SWCNTs-Si (acid treated and grafted with 3-isocyanatopropyl triethoxysilane) in PLA by sonication, followed by drying and compression moulding at 190 °C. ^[136] The maximum increase in σ was of 129 wt.%, obtained with incorporation of 0.4 wt.% GNP-M in PLA by sonication and film casting by doctor blading. ^[126] For CNTs the best result was an increase of 47 % obtained with MWCNT grafted with PLA, and then incorporated at a loading of 1 wt.% in PLA by sonication in chloroform, separation, drying and compression moulding at 180 °C. ^[132] When considering CNTs without modification, the best result reported is an increase of 9% for 1.2 wt.% MWCNTs incorporated in PLA by solution mixing, followed by drying and compression moulding at 180 °C with a pressure of 1000 Kg. ^[133]

Melt-blending is less frequently reported than solution mixing for production of PLA/CBNs composites, probably due to the lower availability of the necessary equipment. Results show that it tends to be not as effective improving mechanical properties, as solution mixing. The best performance in terms of E (↑88%) and E' (↑76%) was reported by Lin *et al.* ^[151] for an

incorporation of 3 wt.% MWCNT grafted with stearyl alcohol (MWCNT-C₁₈OH) in PLA by melt blending (180 °C, 5 min, 50 rpm), using Ti(OBu)₄ for transesterification, followed by compression moulding at the same temperature. When PLA was not transesterified, E and E' increases were of 74 and 44 %, respectively. The maximum increase in σ_{max} (40 %) was obtained incorporating 0.08 wt.% rGO using a twin-screw mixer (175 °C, 8 min, 60 rpm), followed by compression moulding at 180 °C. ^[159]

In-situ polymerization is the least used technique. It has been reported by Pramoda *et al.* ^[167], who performed PLA ring-opening polymerization in presence of 1 wt.% of GO functionalized with butanediol and GO modified with POSS silsesquioxane. In the first case, improvements of 1 % and 14 % in E and hardness were obtained, respectively. In the second, the performance was increased by 33 % and 45 %, in the same order.

Comparing the results for CNTs and GBMs, we can conclude that both can effectively improve PLA mechanical properties, whether by solution mixing and melt blending. However, use of GBMs usually implies lower amounts of GBMs than of CNTs. Several chemical modifications have been tried to improve compatibility with the polymer matrix, with ineffective results in some cases. Functionalization with carboxyl groups is the most common and effective procedure to improve CNTs compatibility with PLA matrix. ^[138] On the other hand, no relation has been observed between CNTs morphological properties (size, length, and diameter) and the mechanical performance of the composites.

Table 5.1: Mechanical properties of PLA/CBNs composites in comparison with non-modified PLA. Production methods and CBNs characteristics.

Method	Procedure for composite preparation	CBNs characteristics	CBNs loading (wt.%)	Mechanical properties relative to neat polymer	References
				ΔE : maximum Young's modulus improvement $\Delta E'$: maximum storage modulus improvement $\Delta \sigma_{max}$: maximum tensile strength improvement	
Solution mixing	Sonication in chloroform and DMF, electrospinning	Diameter (d) 15±5 nm Length (l) 5-20 µm 95 % purity Produced by plasma enhanced CVD	MWCNT: 0.25, 0.5, 1	$\Delta E \uparrow 372 \%$ (0.25 wt.%)	[135]
	Sonication in chloroform, drying and compression moulding (200 °C, 150 Kg/cm ² , 15 min)	d not given 1 ± 2000 µm	MWCNT: 0.5, 3, 5, 10	$\Delta E \uparrow 150 \%$ (5 wt.%)	[129]
	Sonication in chloroform, film casting.	Unzipped CNTs Diameter 30 nm l = 10 µm 95 % purity	uCNT: 1, 2, 3, 4, 5	$\Delta E' \uparrow 14 \%$ (3 wt.%)	[130]
	PLA was modified with benzoyl chloride and pyridine (PLAm), then acid chloride groups were added by reaction with thionyl chloride and triethylamine, then fMWCNTs were added and the mixture centrifuged and filtered to remove excess filler and salts. Finally, sonication in chloroform and film casting was performed.	MWCNT functionalized with COOH using Fenton reactant and then reacted with SOCl ₂ and ethylene glycol (fMWCNT). d = 9.5 nm l = 1.5 µm 95 % purity	Not clear	$\Delta E \uparrow 17 \%$, $\Delta \sigma_{max} \uparrow 8 \%$ (comparing to PLAm)	[131]
		MWCNT (thermal CVD, d = 10-15 nm, l = 10-20 µm, 95 % purity)			
	Sonication in chloroform, coagulation with methanol, filtration, vacuum drying, and compression moulding (180 °C)	MWCNT carboxyl-functionalized (MWCNT-COOH) by H ₂ SO ₄ 1:3 HNO ₃ , 3h, 120 °C MWCNT grafted with PLA (MWCNT-g-PLA): MWCNTs-COOH + l-lactide, 12 h, 150 °C, + tin(II) chloride, 20h, 180 °C, under vacuum, filtration,	MWCNT: 1 MWCNT-COOH: 1 MWCNT-g-PLA: 0.1, 0.2, 0.5, 1, 5	MWCNT-g-PLA: $\Delta E \uparrow 32 \%$, $\Delta \sigma_{max} \uparrow 47 \%$ (1 wt.%)	[132]

	Solution mixing in chloroform, drying and compression molding (180 °C).	vacuum drying MWCNT, MWCNT grafted with PLLA after reaction with SOCl ₂ and ethylene glycol (MWCNT-g-PLLA) Dimensions not given 95 % purity	MWCNT and MWCNT-g-PLLA: 0.1, 0.2, 0.4, 0.6, 0.8, 1.2	MWCNT: $\Delta E \uparrow 46 \%$, $\Delta \sigma_{max} \uparrow 9 \%$ (1.2 wt.%) MWCNT-g-PLLA: $\Delta E \uparrow 86 \%$, $\Delta \sigma_{max} \uparrow 13 \%$ (1.2 wt.%)	[133]
	Solution mixing in chloroform, filtered, washed, dried under vacuum, and compression moulded (180 °C, 500 psi).	MWCNT, MWCNT-COOH (both as in [101]), and MWCNT grafted with PLA chains of 122-530 g mol ⁻¹ by ring open polymerization (MWCNT-g-PLA530). d = 10-15 nm l = 10-20 μ m 95 % purity	MWCNT-COOH: 1 MWCNT-g-PLA530: 1	MWCNT-COOH: $\Delta E \uparrow 4 \%$, $\Delta \sigma_{max} = 9 \%$ MWCNT-g-PLA530: $\Delta E \uparrow 44 \%$, $\Delta \sigma_{max} = 44 \%$	[134]
	Solution mixing in THF, vacuum drying, thermal compression	SWCNT (d < 2 nm, l = 5-15 μ m, 95 % purity) treated with 3:1 H ₂ SO ₄ /HNO ₃ (A-SWCNT), and functionalized (1:2 v/v) with 3-isocyanatopropyl triethoxysilane (IPTES) - A-SWCNT-Si	SWCNT, A-SWCNT and A-SWCNT-Si: 0.1, 0.3, 0.5, 1, 3	SWCNT: $\Delta E' \uparrow 20 \%$ A-SWCNT: $\Delta E' \uparrow 33 \%$ A-SWCNT-Si: $\Delta E' \uparrow 67 \%$ (3 wt.% for all conditions)	[136]
	Sonication in dichloromethane and THF, vacuum drying, and compression molding (190 °C)	MWCNT (d = 9-20 nm, l = 5 μ m) functionalized with 3:1 H ₂ SO ₄ /HNO ₃ (MWCNT-COOH)	MWCNT-COOH: 0.5, 1, 2.5 MWCNT: 2.5	MWCNT-COOH: $\Delta E \uparrow 80 \%$, $\Delta E' \uparrow 35\%$, $\Delta \sigma_{max} \uparrow 28\%$ (2.5 wt.%) MWCNT: $\Delta E \uparrow 25 \%$, $\Delta E' \downarrow 6\%$, $\Delta \sigma_{max}$ (not reported) (2.5 wt.%) (3 wt.%)	[138]
	Internal mixer (180 °C, 50 rpm, 5 min) with and without transesterification with Ti(OBu) ₄ , compression molding (180 °C)	MWCNT (l = 1-10 μ m) functionalized with HNO ₃ (120 °C, 40 min) - MWCNT-COOH, and modified with DCC and stearyl alcohol (MWCNT-C ₁₈ OH)	PC: MWCNT/PLA PC-18: MWCNT-C ₁₈ OH/PLA PC-18T: MWCNT-C ₁₈ OH/PLA transesterified 0.5, 1.5, 3	PC: $\Delta E \uparrow 73\%$, $\Delta E' \uparrow 34\%$ PC-18: $\Delta E \uparrow 74\%$, $\Delta E' \uparrow 44\%$ PC-18T: $\Delta E \uparrow 88\%$, $\Delta E' \uparrow 76\%$	[151]
	Twin-screw extrusion (150-190 °C, 100 rpm), injection molding (160-190 °C) High-crystalline PLA (HC-PLA) and low-crystalline PLA (LC-PLA) were tested	MWCNT (l = 5-20 μ m, d = 40-60 nm) functionalized with maleic anhydride (MWCNT-g-MA) at 80 °C, 4h, + benzoyl peroxide	LC-PLA/MWCNT, HC-PLA/MWCNT and MWCNT-g-MA: 0.25, 0.5, 0.75, 1, 2, 4	LC-PLA/MWCNT: $\Delta \sigma_{max} \uparrow 23 \%$ HC-PLA/MWCNT: $\Delta \sigma_{max} \uparrow 13 \%$ MWCNT-g-MA: $\Delta \sigma_{max} \uparrow 27 \%$ (4 wt.% for all conditions)	[154]

Melt blending

	Twin-screw extrusion (180 °C, 150 rpm, 5 min), compression molding at 180 °C	d = 6-13 nm, l = 2.5-20 µm, specific surface area = 220 m ² g ⁻¹ produced by CVD	MWCNT: 1.5, 3, 5	MWCNT: $\Delta E' \uparrow 28 \%$, $\Delta \sigma_{max} \uparrow 27\%$ (5 wt.%)	[156]
	Twin-screw extrusion (160 °C-190 °C)	Carboxyl-functionalized d = 10-11 nm, l = 12-15 µm	MWCNT-COOH: 1	ΔE and $\Delta \sigma_{max} \uparrow 8 \%$ (1 wt.%)	[168]
Method	Procedure for composite preparation	CBNs characteristics	CBNs loading (wt.%)	Mechanical properties relative to neat polymer	
				ΔE : maximum Young's modulus improvement $\Delta E'$: maximum storage modulus improvement $\Delta \sigma_{max}$: maximum tensile strength improvement	References
Solution mixing	Sonication in chloroform, casting and doctor blading GO was pre-dispersed in acetone while GNP was directly dispersed in chloroform	GNP grade M (commercial product) t = 6-8 nm, d $\approx$ 5 µm. GO (MHM) d $\approx$ 100 nm	GO and GNP: 0.2, 0.4, 0.6	GO: $\Delta E \uparrow 115 \%$, $\Delta \sigma_{max} \uparrow 95 \%$ (0.3 wt.%) GNP: $\Delta E \uparrow 156 \%$, $\Delta \sigma_{max} \uparrow 129 \%$ (0.4 wt.%)	[126]
	Sonication in chloroform, filtration, vacuum drying, compression moulding (170 °C, 10 min)	GO (from natural graphite, MHM + lyophilization) d $\approx$ 300 nm GO-g-PLLA (GO + l-lactide (Sn(oct) ₂), filtration, vacuum drying)	GO and GO-g-PLLA: 0.5	GO: $\Delta \sigma_{max} \uparrow 51 \%$ GO-g-PLLA: $\Delta \sigma_{max} \uparrow 106 \%$	[141]
	Stirring and sonication in DMF, coagulation with methanol, filtration, and vacuum drying	GO (MHM) from expandable graphite, chemically reduced with hydrazine, and lyophilized (GNSs – solvent free graphene nanosheets) t < 1 nm, d < 50 nm.	GNSs: 0.2	$\Delta E' \uparrow 18 \%$, $\Delta \sigma_{max} \uparrow 26 \%$	[143]
	Sonication in DMF, coagulation with methanol, drying, compression moulding (185 °C)	TRG (commercial product, t = few layer, d = hundreds of nm) TRG/PLA/Py-PLA: Py-PLA-OH (1-Pyrenemethanol + l-lactide, Sn(oct) ₂) + TRG (10:1) – sonication + PLA – coagulation and drying	TRG and TRG/Py-PLA-OH: 0.25, 1	trGO: $\Delta E' \uparrow 1-3 \%$, $\Delta \sigma_{max} \uparrow 8 \%$ trGO/Py-PLA: $\Delta E' \uparrow 10-15 \%$, $\Delta \sigma_{max} \uparrow 19 \%$	[145]
	Solution mixing in DMF, film casting.	GO prepared according to MHM, reduced to rGO and functionalized with N-(aminoethyl)-aminopropyltrimethoxysilane (KH792).	rGO-KH792: 0.1, 0.2, 0.5	$\Delta E' \uparrow 1500 \%$ around the T_g (0.5 wt.%)	[149]

Melt blending	Twin-screw mixer (175 °C, 60 rpm, 8 min), compression molding at 180 °C	GO prepared by MHM and reduced with hydrazine and ammonia (rGO) t = 0.4-0.6 nm, d = 0.1-0.5 µm	rGO: 0.02, 0.04, 0.08, 0.2, 0.5, 1, 2	$\Delta E' \uparrow 27 \%$, $\Delta \sigma_{max} \uparrow 40 \%$ (0.08 wt.%) $\Delta E' \uparrow 54 \%$, $\Delta \sigma_{max} \downarrow 40 \%$ (2 wt.%)	[159]
	Internal mixer (160 °C, 25 rpm, 10 min), compression moulding (160 °C, 10 min) (Polymer was PLA/PEG 9:1 blend)	GNP grade M15 (commercial product) t = 6-8 nm, d ≈ 15 µm	GNP-M15: 0.1, 0.3, 0.5, 0.7, 1	$\Delta E' \uparrow 84$ and 70% , $\Delta \sigma_{max} \uparrow 20$ and 33% (0.1 and 0.3 wt.%) (relative to pristine PLA/PEG blend)	[158]
	Internal mixer (180 °C, 80 rpm, 10 min)	GO (MHM) + SDS, ultrasounds, stirring 12h, 25 °C Methylmethacrylate (MMA), stirring 12h + ammonium persulfate (APS) 12h, 80 °C + reduction with dimethyl hydrazine, 100 °C, 2h (PFG - polymer-functionalized graphene nanoparticles) t = 2.4 nm	PFG: 1, 2, 3, 4, 5	$\Delta E \uparrow 80 \%$, $\Delta \sigma_{max} \uparrow 10 \%$ (5 wt.%)	[155]
In-situ polymerization	Sonication of l-lactide + filler in toluene, addition of Tin(II)-2-ethylhexanoate under N ₂ , stirring at 110 °C, 3 days	Expanded graphite (MHM) to GO GO-fuctionalized: GO + TDI + 1,4-butanediol, 80 °C, 24h GO-g-POSS: GO + POSS – polyhedral oligomeric silsesquioxane + DMAP – 4-(dimethylaminopyridine) + EDC – N-(3-dimethylamino-propyl-N'-ethylcarbodiimide), 2 days, room temperature, N ₂ (dimensions not given)	GO-fuctionalized, GO-g-POSS, GO+POSS (physical mixture): 1	GO-fuctionalized: $\Delta E' \uparrow 1 \%$, $Hardness \uparrow 14 \%$ GO-g-POSS: $\Delta E' \uparrow 33 \%$, $Hardness \uparrow 45 \%$ GO+POSS: $\Delta E' \uparrow 29 \%$, $Hardness \uparrow 36 \%$	[167]

5.2.4.2 Electrical properties

Neat PLA is electrically insulating with a low electrical conductivity ($\sigma \approx 1 \times 10^{-16} \text{ S m}^{-1}$), and high sheet resistance $\rho_{\square} \approx 5 \times 10^{12} \Omega/\text{sq}$.^[136, 151] Since CNTs and reduced forms of GBMs present high electrical conductivity, they can be incorporated in PLA to improve its conductivity. These sort of composites have potential to be used as electrical stimulating implants, since PLA is used as a biodegradable matrix in orthopedic material. Other advantages of increasing PLA conductivity are the possibility of using it as antistatic coating/material or for electromagnetic shielding.^[100] The minimum amount of filler required to form a conductive network within the polymer is called percolation threshold, and should be as low as possible in order to keep processing simple (relatively low viscosity of the melt) and low costs. Table 5.2 shows that, once again, the most used method to incorporate CBNs on PLA for electrical properties evaluation is solution mixing. The amount of fillers ranges from 0.01 to 10 wt.%. The best result, considering electrical conductivity (σ) with CNTs was $3.5 \times 10^{-3} \text{ S m}^{-1}$, obtained incorporating 10 wt.% MWCNT in PLA by sonication in chloroform, followed by drying and compression moulding at 200 °C during 15 min.^[129] Results are also often presented in terms of sheet resistance ($\rho_{\square}$), being the lowest value reported by Shao *et al.*^[169], of $1 \times 10^2 \Omega/\text{sq}$ achieved incorporating 5 wt.% MWCNTs previously oxidized (treated with HCl and HNO₃) in PLA by solution mixing, followed by electrospinning of aligned nanofibers ($d \approx 250 \text{ nm}$). The alignment of the fibers slightly improved sheet resistance, comparing with random meshes. Interestingly, Yoon *et al.*^[134] observed a considerable sheet resistance of $1 \times 10^5 \Omega/\text{sq}$, with incorporation of 1 wt.% MWCNT-COOH, also oxidized by treatment with strong acids (H₂SO₄ and HNO₃). For GBMs, the maximum conductivity reported was 2.2 S m^{-1} , higher than for CNTs, obtained incorporating 1.25 wt.% rGO-g (reduced with ammonia) in PLA by sonication in DMF. Interestingly, the solvent used for dispersion of CNTs in PLA was always chloroform and for GBMs was always DMF. Melt-blending is the second most used approach to disperse CBNs in PLA in order to improve its electrical properties, being most often performed by twin-screw extrusion, followed by compression moulding. The highest σ considering CNTs was 50 S m^{-1} , which was reported by Pötschke *et al.*^[170]. These authors prepared MWCNTs mixtures by twin-screw extrusion, followed by piston spinning at different speeds. They concluded that non-spun mixtures with 5 wt.% MWCNTs in PLA presented the same conductivity as 3 wt.% mixtures after piston spinning at a speed of 20 m min^{-1} . Considering $\rho_{\square}$, the best performance was obtained incorporating 3 wt.% MWCNT-C₁₈OH (MWCNTs modified with DCC and stearyl alcohol) using an external mixer, followed by compression moulding at 180 °C during 5 min, resulting in a $\rho_{\square}$ of $1 \times 10^{-1} \Omega/\text{sq}$.^[151] This was the most effective modification performed, considering the sheet resistance values

obtained with incorporation of the same amount of non-modified MWCNT, which was $3 \times 10^5 \Omega/\text{sq}$. For GBMs, the higher σ was $2.6 \times 10^{-2} \text{ S m}^{-1}$, resultant from dispersion using an internal mixer at 180°C , of 5 wt.% PFG (graphene nanoparticles functionalized with methylmethacrylate).^[155] For rGO, a non-functionalized GBM, the best conductivity value was obtained for 2 wt.% incorporation in PLA using a twin-screw extruder and compression moulding. The value obtained was of $1 \times 10^{-9} \text{ S m}^{-1}$, being higher than for the other concentrations tested. It can be compared, for example, with a σ of $1 \times 10^{-13} \text{ S m}^{-1}$ for 0.2 wt.%.^[159] In most works evaluated, electrical properties improve with the increase of filler amount.

In-situ polymerization is the least explored technique, despite interesting results being obtained by Yang *et al.*^[166], which incorporated 0.01 – 2 wt.% trGO (thermally reduced) in PLA by ring-opening melt polymerization of l-lactide in presence of the filler. As example, σ obtained was 5×10^{-6} and $1.6 \times 10^{-2} \text{ S m}^{-1}$ for 1.5 and 2 wt.%, respectively.

An interesting study by Chiu *et al.*^[84], shows that purification of MWCNT by sonication with strong acids improved fillers compatibility and dispersibility in PLA, resulting in better electrical conductivity. The values of σ for incorporations of 7 wt.% were 5×10^{-8} and $2 \times 10^{-6} \text{ S m}^{-1}$, respectively for non-purified and purified MWCNT. Purification introduced polar functional groups on the CNTs surface, allowing better dispersion, which resulted in more deagglomerated particles that formed a wider conductive network on PLA matrix.

Table 5.2: Electrical properties of PLA/CBNs composites in comparison with non-modified PLA. Production methods and CBNs characteristics.

Method	Procedure	CBNs characteristics	CBNs Content (wt.%)	Electrical Properties	References
				σ : electrical conductivity $\rho_{\square}$: sheet resistance (PLA $\sigma \approx 1 \times 10^{-16} \text{ S m}^{-1}$, $\rho_{\square} \approx 5 \times 10^{12} \text{ }\Omega/\text{sq}$) [106, 122]	
Solution mixing	Sonication in chloroform, drying and compression moulding (200 °C, 150 Kg/cm ² , 15 min)	Diameter (d) not given Length (l) = $\pm 2000 \text{ }\mu\text{m}$	MWCNT: 0.5, 3, 5, 10	$\sigma = 1.8 \times 10^{-3}$ and $3.5 \times 10^{-3} \text{ S m}^{-1}$ (3 and 10 wt.%)	[129]
	Sonication in chloroform, coagulation with methanol, filtration, vacuum drying, and compression moulding (180 °C)	MWCNT (thermal CVD, d = 10-15 nm, l = 10-20 μm , 95 % purity) MWCNT carboxyl-functionalized (MWCNT-COOH) by H ₂ SO ₄ 1:3 HNO ₃ , 3h, 120 °C MWCNT grafted with PLA (MWCNT-g-PLA): MWCNTs-COOH + l-lactide, 12 h, 150 °C, + tin(II) chloride, 20h, 180 °C, under vacuum, filtration, vacuum drying	MWCNT: 1 MWCNT-COOH: 1 MWCNT-g-PLA: 0.1, 0.2, 0.5, 1, 5	MWCNT: $\rho_{\square} = 1 \times 10^{12} \text{ }\Omega/\text{sq}$ (for 0.1 and 0.2 wt.% is similar to PLA), 1×10^5 and $1 \times 10^4 \text{ }\Omega/\text{sq}$ (0.5 wt.%, and 1-5 wt.%) MWCNT-g-PLA: $\rho_{\square} = 1 \times 10^{12} \text{ }\Omega/\text{sq}$ (0.1-5 wt.% - always similar to PLA)	[132]
	Solution mixing in chloroform, drying and compression molding (180 °C).	MWCNT, MWCNT grafted with PLLA after reaction with SOCl ₂ and ethylene glycol (MWCNT-g-PLLA) Dimensions not given 95 % purity	MWCNT and MWCNT-g-PLLA: 0.1, 0.2, 0.4, 0.6, 0.8, 1.2	MWCNT: $\sigma = 2 \times 10^{-13} \text{ S m}^{-1}$ (0.1-0.4 wt.%), $3 \times 10^{-9} \text{ S m}^{-1}$ (0.6 wt.%), and $2 \times 10^{-5} \text{ S m}^{-1}$ (1.2 wt.%) MWCNT-g-PLLA: $\sigma = 2 \times 10^{-13} \text{ S m}^{-1}$ (0.1-0.4 wt.%), $5 \times 10^{-13} \text{ S m}^{-1}$ (0.6 wt.%), and $3 \times 10^{-8} \text{ S m}^{-1}$ (1.2 wt.%) Increases with filler amount	[133]
	Solution mixing in chloroform, filtered, washed, dried under vacuum, and compression moulded (180 °C, 500 psi).	MWCNT, MWCNT-COOH (both as in [101]), and MWCNT grafted with PLA chains of 122-530 g mol ⁻¹ by ring open polymerization (MWCNT-g-PLA530). d = 10-15 nm l = 10-20 μm	MWCNT-COOH: 1 MWCNT-g-PLA122-530: 1	MWCNT-COOH: $\rho_{\square} = 1 \times 10^5 \text{ }\Omega/\text{sq}$ MWCNT-g-PLA112-530: $\rho_{\square} = 2 \times 10^6$, 2×10^{12} , and $1 \times 10^{12} \text{ }\Omega/\text{sq}$ (122, 250, 530 g mol ⁻¹)	[134]

	Sonication in THF, vacuum drying, thermal compression	95 % purity MWCNTs (d = 8-15 nm, l = 50 µm) purified by sonication with H ₂ SO ₄ and HNO ₃ at 50 °C, filtration, and washing	MWCNT purified/non-purified: 1, 3, 5, 7	MWCNT purified: $\sigma = 4 \times 10^{-9}$, 1×10^{-9} , and 2×10^{-6} S m ⁻¹ (1, 5, and 7 wt.%) MWCNT non-purified: $\sigma = 7 \times 10^{-11}$, 2×10^{-8} , and 5×10^{-8} S m ⁻¹ (1, 5, and 7 wt.%) Increases with filler amount	[84]
	Solution mixing in THF, vacuum drying, thermal compression	SWCNT (d < 2 nm, l = 5-15 µm, 95 % purity) treated with 3:1 H ₂ SO ₄ /HNO ₃ (A-SWCNT), and functionalized (1:2 v/v) with 3-isocyanatopropyl triethoxysilane (IPTES) - A-SWCNT-Si	SWCNT, A-SWCNT and A-SWCNT-Si: 0.1, 0.3, 0.5, 1, 3	SWCNT: $\sigma = 2 \times 10^{-16}$, 3×10^{-9} , and 5×10^{-8} S m ⁻¹ (0.3, 1, 3 wt.%) A-SWCNT-Si: $\sigma = 5 \times 10^{-15}$, 5×10^{-8} , and 2×10^{-6} S m ⁻¹ (0.3, 1, 3 wt.%) Increases with filler amount	[136]
	MWCNTs-ox (HCl, 2h at 25°C + HNO ₃ , 4h at 110 °C) Nanofibers (MWCNTs-ox sonicated in DMF 2h + SDS, adding to PLA in dichloromethane, 1h sonication before electrospinning)	MWCNTs (l = 10–20 µm, d = 10–20 nm) Nanofibers (PLA ≈ 400 nm, PLA/MWCNTs-ox ≈ 250 nm)	PLA/MWCNTs-ox (3 wt.%) random (R) and aligned (A) nanofibers: 1, 2, 3, 4, 5 wt.%	PLA/MWCNTs-ox-R: $\rho_{\square} = 1 \times 10^4$, 5×10^2 Ω/sq (3 and 5 wt.%) PLA/MWCNTs-ox-A: $\rho_{\square} = 5 \times 10^3$, 1×10^2 Ω/sq (3 and 5 wt.%) Increases with both fillers amount	[169]
	Internal mixer (180 °C, 50 rpm, 5 min) with and without transesterification with Ti(OBu) ₄ , compression molding (180 °C)	MWCNT (l = 1-10 µm) functionalized with HNO ₃ (120 °C, 40 min) - MWCNT-COOH, and modified with DCC and stearyl alcohol (MWCNT-C ₁₈ OH)	PC: MWCNT/PLA PC-18: MWCNT-C ₁₈ OH/PLA PC-18T: MWCNT-C ₁₈ OH/PLA transesterified 0.5, 1.5, 3	PC: $\rho_{\square} = 2 \times 10^7$, 3×10^6 , and 3×10^5 Ω/sq (0.5, 1.5, 3 wt.%) PC-18: $\rho_{\square} = 8 \times 10^3$, 9×10^4 , and 1×10^{-1} Ω/sq (0.5, 1.5, 3 wt.%) PC-18T: $\rho_{\square} = 5 \times 10^{12}$, 9×10^5 , and 9×10^{-2} Ω/sq (0.5, 1.5, 3 wt.%)	[151]
	Twin-screw extruder (180, 215 and 250 °C; 100, 200 and 500 rpm; 5 min) 1 st – masterbatch production 2 nd – dilution of masterbatches and composites production	d = 9.5 nm l = 1.5 µm 90 % purity	MWCNT: 0.5, 0.75, 1, 2	σ is below 2.5×10^{-1} S m ⁻¹ (0.5-2 wt.%) slightly decreasing with filler wt.% increase	[153]
	Twin-screw extrusion (150-190 °C, 100 rpm), injection molding (160-190 °C) High-crystalline PLA (HC-PLA) and low-crystalline PLA (LC-PLA) were tested	MWCNT (l = 5-20 µm, d = 40-60 nm) functionalized with maleic anhydride (MWCNT-g-MA) at 80 °C, 4h, + benzoyl peroxide	LC-PLA/MWCNT, HC-PLA/MWCNT and MWCNT-g-MA: 0.25, 0.5, 0.75, 1, 2, 4	LC-PLA/MWCNT: $\rho_{\square} = 2 \times 10^{13}$, 5×10^3 , and 5×10^2 Ω/sq (0.5, 2, 4 wt.%) HC-PLA/MWCNT: $\rho_{\square} = 1 \times 10^{14}$, 9×10^{10} , and 8×10^{10} Ω/sq (0.5, 2, 4 wt.%) LC-PLA/MWCNT-g-MA: $\rho_{\square} = 3 \times 10^3$, 2×10^2 , and 7×10^1 Ω/sq (0.5, 2, 4 wt.%)	[154]
	Twin-screw extrusion (180 °C, 150 rpm, 5 min), compression molding at	d = 6-13 nm, l = 2.5-20 µm, specific surface area = 220	MWCNT: 1.5, 3, 5	$\sigma = 1 \times 10^{-9}$, 1×10^{-2} , and 1 S m ⁻¹ (1.5, 3, 5 wt.%)	[156]

180 °C		m^2g^{-1} produced by CVD		MWCNT: 0.5, 1, 2, 3, 5	Extruded composites: $\sigma = 4, 14$, and 50 S m^{-1} (2, 3, 5 wt.%) Fibres (3 wt.%): $\sigma = 50, 40$, and 1 S m^{-1} (spinning speeds of 20, 50, and 100 m min^{-1})	[170]
Twin-screw extruder (180-220 °C, 500 rpm) Piston spinning (20, 50, 100 m min^{-1}) to produce micro-fibres (220 °C, 3 min)		d = 9.5 nm l = 1.5 μm 90 % purity				
Method	Procedure	CBNs characteristics	CBNs Content (wt. %)	Electrical Properties ρ : electrical resistivity σ : electrical conductivity Rs: surface resistance R: surface resistivity		References
Solution mixing	Sonication in DMF, coagulation with methanol, drying, compression moulding (185 °C)	TRG (commercial product, t = few layer, d = hundreds of nm) TRG/PLA/Py-PLA: Py-PLA-OH (1-Pyrenemethanol + l-lactide, $\text{Sn}(\text{oct})_2$) + TRG (10:1) – sonication + PLA – coagulation and drying	TRG and TRG/Py-PLA-OH: 0.25, 1	TRG: $\sigma = 1 \times 10^{-16}$ and $1 \times 10^{-6} \text{ S m}^{-1}$ (0.25 and 1 wt.%) TRG/Py-PLA-OH: $\sigma = 1 \times 10^{-16}$ and $1 \times 10^{-7} \text{ S m}^{-1}$ (0.25 and 1 wt.%)		[145]
	Sonication in DMF, coagulation with methanol, drying, and compression molding (210 °C)	GO: from graphite flakes (modified Staudenmeier method) rGO-p: GO + Polyvinylpyrrolidone (1:5), sonication at 60 °C rGO-g: reduced by stirring with glucose in ammonia solution at 95 °C, 60 min Dimension not given.	GO rGO-p rGO-g (0.5-2.5 vol.%)	GO: $\sigma = \uparrow 6.5 \times 10^{-13} \text{ S m}^{-1}$ rGO-p: $\sigma = \uparrow 4.7 \times 10^{-8} \text{ S m}^{-1}$ rGO-g: $\sigma = 2.2 \text{ S m}^{-1}$ (for 1.25 vol.% for all) Increases with filler amount		[146]
Melt blending	Twin-screw mixer (175 °C, 60 rpm, 8 min), compression molding at 180 °C	GO prepared according to MHM and chemically reduced to rGO. Thickness 0.4-0.6 nm and lateral dimension 0.1-0.5 mm.	rGO: 0.02, 0.04, 0.06, 0.2, 0.5, 1, 2	$\sigma = 1 \times 10^{-13}$ and $1 \times 10^{-9} \text{ S m}^{-1}$ (0.2 and 2 wt.%) Increases with filler amount		[159]
	Internal mixer (180 °C, 80 rpm, 10 min)	GO (MHM) + SDS, ultrasounds, stirring 12h, 25 °C Methylmethacrylate (MMA), stirring 12h + ammonium persulfate (APS) 12h, 80 °C + reduction with dimethyl	PFG: 1, 2, 3, 4, 5	$\sigma = 2.6 \times 10^{-2}$ and $1 \times 10^{-12} \text{ S m}^{-1}$ (1 and 5 wt.%) Increases with filler amount		[155]

		hydrazine, 100 °C, 2h (PFG - polymer-functionalized graphene nanoparticles) t = 2.4 nm			
<i>In-situ</i> polymerization	Ring-opening melt polymerization of lactide in presence of trGO	GO prepared according to MHM and thermally reduced to trGO. Dimensions not given.	TrGO: 0.01, 0.1, 0.5, 1, 1.5, 2	$\sigma = 5 \times 10^{-6}$ and $1.6 \times 10^{-2} \text{ S m}^{-1}$ (1.5 and 2 wt.%) Increases with filler amount	[166]

5.2.4.3 Thermal properties

Several works report changes on thermal properties of PLA by incorporation of CBNs. CNTs incorporations range from 0.01 to 15 wt.%, while for GBMs lower amounts are needed 0.01 - 2 wt.% (Table 5.3). The most frequently used techniques to evaluate thermal properties in polymer composites are thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and dynamic mechanical analysis (DMA). TGA allows determination of thermal degradation temperatures, and DSC and DMA phase transition temperatures (T_g – glass transition temperature, T_m – melting temperature, and T_c – cold crystallization temperature).

When using solution mixing, the highest variation in terms of T_g was an increase of 10 °C, obtained using 1 wt.% MWCNT purified by treatment with strong acids. Comparing with non-purified filler at the same loading, the increase was 5 °C higher. This is explained by purified MWCNT having stronger interfacial interactions with PLA matrix, imposing increased restriction to the mobility of macromolecular chains, and therefore rising T_g . Also, T_d (decomposition temperature) presented an increase of 10 °C for purified materials. [84] For T_m , the higher increase was of 16 °C for 0.3 and 1 wt.% MWCNT-PCL (functionalized with poly(caprolactone)) incorporated in PLA aligned fibers by sonication in dichloromethane and electrospinning. Also, T_c decreased more than 10 °C, due to MWCNT inducing heterogeneous crystallization. [137] However, the higher decrease in T_c (< 20 °C), was obtained by Moon *et al.* [129], with the incorporation of 3 - 10 wt.% MWCNT, with a length of about 2000 µm. In literature, the degradation temperatures of the polymeric materials determined by TGA are presented in different terms. For example, as T_{di} (beginning of thermal degradation), T_{d5} (decomposition temperature for 5 wt.% loss), and T_{d50} (decomposition temperature for 50% weight loss). For T_{di} , the highest increase was of 20 °C, obtained incorporating 2.5 wt.% MWCNT-COOH (carboxylated with strong acids) by sonication in PLA dispersed in dichloromethane and THF, followed by vacuum drying and compression moulding. [138] Considering T_{d50} , the best result was an increase of 1 - 3 °C, in a work above described. [137]

GBMs incorporation also induces changes on thermal properties of PLA. For T_g , an increase of 7 °C was obtained sonicating 0.4 wt.% GNP in PLA films prepared by solvent mixing. [126] The highest increases in T_m have been of 5 °C, for samples obtained by compression moulding of PLA with 0.5 wt.% GO grafted with PLA, produced by vacuum drying a dispersion in chloroform. [141] Significant decrease in T_c , of 20 °C, was observed for PLA with 2 wt.% GO, obtained by solvent mixing. [144] Thermal stability of PLA has been shown to improve with addition of GBMs. 2 wt.% GONSs (graphene oxide nanosheets) increased T_{di} by 16 °C in samples produced by solvent mixing. [147] Also, T_{d5} was increased by 11 °C sonication of 0.2 wt.% GNSs (graphene nanosheets) in PLA dispersed in DMF, which was dried under vacuum. The authors explain these

increases by GNS forming a tortuous path, which delays the diffusion of oxygen and the escape of volatile degradation products. and also char formation. ^[143] Finally, $T_{d\ max}$ (T of maximum degradation rate) increased 33 °C PLA filled with TRG, produced by solution mixing. ^[145] Chemical modifications of MWCNT were reported to increase thermal properties of the composites. For example, directly comparing with PLA/MWCNT(non-modified), the incorporation of 1 wt.% MWCNT grafted with PLA in the same PLA matrix, resulted in increases of about 3 °C in T_g and decreases of 9 °C in T_c . ^[132] Treatment with strong acids followed by silanization of SWCNT, ^[136] which were incorporated in PLA at loading ranging from 0.1 and 3 wt.% resulted in increases of about 5 °C in T_g .

Concerning composites produced by melt-blending, the highest increases in T_g were of 5 - 6 °C, reported for PLA micro-fibers with 3 wt.% MWCNT to PLA. ^[170] Also, T_c was observed to decrease at most 12 °C with incorporation of 0.5 and 2 wt.% MWCNT. ^[161] Chieng *et al.* ^[158], studied the thermal properties of PLA/PEG (9:1) blends with addition of 0.1 – 1 wt.% GNP, which resulted in e variations on T_g , T_m , and T_c . However, T_{di} , T_{max} , and T_{50} , increased by 56, 53, and 44 °C, respectively, for 0.5 wt.% loadings.

In-situ polymerization of l-lactide in presence of TRG in amounts from 0.01 to 2 wt.% resulted in considerable increases on T_g , T_m , and T_{dmax} . For example, at 2 wt.% loading, increases of 5, 14, and 18 °C were obtained, respectively. ^[166] In a different work reporting in-situ polymerization of l-lactide, covalent functionalization of GO with both 1,4-butanediol, and polyhedral silsesquioxane resulted in increases in T_g (18, 20 °C), T_c (15, 8 °C), T_m (7, 5 °C), and T_{d5} (23, 11 °C) comparing with PLA/GO composites at 1 wt.% loadings. ^[167]

Table 5.3: Thermal properties of PLA/CBNs composites in comparison with non-modified PLA. Production methods and CBNs characteristics.

Method	Procedure	CBNs characteristics	CBNs content (wt.%)	Thermal properties relative to neat polymer	References
Solution mixing	Sonication in chloroform, drying and compression moulding (200 °C, 150 Kg/cm ² , 15 min).	Diameter (d) not given Length (l) ± 2000 µm	MWCNT: 0.5, 3, 5, 10	T_g (glass transition) ↓ 1-4 °C (3, 5 wt.%) and = (10 wt.%) T_c (crystallization) ↓ >20 °C (3, 5, 10 wt.%) T_m (melting) = (3, 5, 10 wt.%) T_d (degradation) ↑ 10-20 °C (3, 5, 10 wt.%)	[129]
	Sonication in chloroform, film casting.	Unzipped CNTs d = 30 nm l = 10 µm 95 % purity	uCNT: 1, 2, 3, 4, 5	T_g ↑ 7, 8 °C (3, 5 wt.%) T_m ↑ 5, 3 °C (3, 5 wt.%)	[130]
	PLA was modified with benzoyl chloride and pyridine (PLAm), then acid chloride groups were added by reaction with thionyl chloride and triethylamine, then fMWCNTs were added and the mixture centrifuged and filtered to remove excess filler and salts. Finally, sonication in chloroform and film casting was performed.	MWCNT functionalized with COOH using Fenton reactant and then reacted with SOCl ₂ and ethylene glycol (fMWCNT). d = 9.5 nm l = 1.5 µm 95 % purity	Not clear	T_g (tanδ) ↑ 9 °C T_{di} (beginning of thermal degradation) ↑ 80 °C	[131]
	Sonication in chloroform, coagulation with methanol, filtration, vacuum drying, and compression moulding (180 °C)	MWCNT (thermal CVD, d = 10-15 nm, l = 10-20 µm, 95 % purity) MWCNT carboxyl-functionalized (MWCNT-COOH) by H ₂ SO ₄ 1:3 HNO ₃ , 3h, 120 °C MWCNT grafted with PLA (MWCNT-g-PLA): MWCNTs-COOH + l-lactide, 12 h, 150 °C, + tin(II) chloride, 20h, 180 °C, under vacuum, filtration, vacuum drying	MWCNT: 1 MWCNT-COOH: 1 MWCNT-g-PLA: 0.1, 0.2, 0.5, 1, 5	No significant changes in T_m for all materials PLA/MWCNT: T_g ↑ 3, T_c ↓ 3 °C (1 wt.%) PLA/MWCNT-COOH: T_g ↑ 2, T_c ↓ 3 °C (1 wt.%) PLA/MWCNT-g-PLA: T_g ↑ 5-6 T_c ↑ 1 ↓ 2, 6, 12, 19 °C (0.1, 0.2, 0.5, 1, 5 wt.%)	[132]
	Sonication in dichloromethane, electrospinning.	MWCNTs (d = 8-15 nm, l - not given, 95 % purity) were functionalized with -COOH by H ₂ SO ₄ and HNO ₃ (3:1). Then, MWCNTs-NH ₂ were produced reacting MWCNTs-COOH with N,N'-dicyclohexylcarbodiimide (DCC). MWCNTs-PCL were produced reacting 1g MWCNTs-NH ₂ , 10 g PCL, and 20 g DCC.	MWCNT-PCL(0.3, 0.5, 1, 3)/PLA aligned composite fibers	T_{d50} (50% weight loss) ↑ 1-3 °C (0.3, 1 wt.%) T_g = (0.3, 1 wt.%) T_m ↑ 16 °C (0.3, 1 wt.%) T_c ↓ 13 °C and 12 °C (0.3, 1 wt.%)	[137]
	Sonication in THF, vacuum drying, thermal compression	MWCNTs (d = 8-15 nm, l = 50 µm) purified by sonication with H ₂ SO ₄ and HNO ₃ at 50 °C,	MWCNT purified/non-purified: 1, 3, 5, 7	MWCNT non-purified: T_g ↑ 5-6 °C (1, 3, 5, 7 wt.%) MWCNT purified: T_g ↑ 10,7,5,5 °C (1, 3, 5, 7 wt.%) MWCNT non-purified Vs purified: T_d ↑ 10, 11, 7, 8 °C	[84]

	Solution mixing in THF, vacuum drying, thermal compression	filtration, and washing SWCNT (d < 2 nm, l = 5-15 μ m, 95 % purity) treated with 3:1 H ₂ SO ₄ /HNO ₃ (A-SWCNT), and functionalized (1:2 v/v) with 3-isocyanatopropyl triethoxysilane (IPTES) - A-SWCNT-Si	SWCNT, A-SWCNT, and A-SWCNT-Si: 0.1, 0.3, 0.5, 1, 3	(1, 3, 5, 7 wt.%) T_{ds} (5 wt.% loss) ↓ for SWCNT (poor interfacial interaction), = for A-SWCNT, and A-SWCNT-Si T_g : (higher than pure PLA) SWCNT < A-SWCNT < A-SWCNT-Si (considering all loadings, increases are below 5 °C)	[136]
	Sonication in dichloromethane and THF, vacuum drying, and compression molding (190 °C)	MWCNT (d = 9-20 nm, l = 5 μ m) functionalized with 3:1 H ₂ SO ₄ /HNO ₃ (MWCNT-COOH)	MWCNT-COOH: 0.5, 1, 2.5	MWCNT-COOH: T_{di} ↑ 10-20 °C (0.5-2.5 wt.%) T_g ↑ 0, 1, 2 °C (0.5, 1, 2.5 wt.%) T_c ↑ 1, 2, 4 °C (0.5, 1, 2.5 wt.%) T_m ↑ 3, 4, 5 °C (0.5, 1, 2.5 wt.%)	[138]
Melt blending	Internal mixer (180 °C, 50 rpm, 5 min) with and without transesterification with Ti(OBu) ₄ , compression molding (180 °C)	MWCNT (l 1-10 μ m) functionalized with HNO ₃ (120 °C, 40 min) - MWCNT-COOH, and modified with DCC and stearyl alcohol (MWCNT-C ₁₈ OH)	PC: MWCNT/PLA PC-18: MWCNT-C ₁₈ OH/PLA PC-18T: MWCNT-C ₁₈ OH/PLA transesterified 0.5, 1.5, 3	PC, PC-18 - No change in T_m PC-18T – 2 melting peaks, 1 below T_m for pristine PLA (low M_w PLA from transesterification), other at the same T_m	[151]
	Sonication in THF, vacuum drying + Microextruder (180 °C, 50 rpm, 5 min)	MWCNTs (d = 9.5 nm, l = 1.5 μ m) produced by catalytic carbon vapor deposition (CCVD)	MWCNT: 0.1, 1	T_g ↑ 1 °C (0.1, 1 wt.%)	[152]
	Twin-screw extruder (180, 215 and 250 °C; 100, 200 and 500 rpm; 5 min) 1 st – masterbatch production 2 nd – dilution of masterbatches and composites production	d = 9.5 nm l = 1.5 μ m 90 % purity	MWCNT: 0.5, 0.75, 1, 2, 7.5, 15	Similar T_g (7.5, 15 wt.%)	[153]
	Twin-screw extruder (210 °C, 400 rpm), compression molding (210 °C)	d = 5-20 nm l = 10 μ m Specific surface area = 100-700 m ² g ⁻¹ CCVD	MWCNT: 0.5, 1, 2, 3, 5	T_g ↓ 1, 2 °C (0.5, 1-5 wt.%) T_c ↓ 12, 10, 12, 7, 6 °C (0.5, 1, 2, 3, 5 wt.%) T_m ↓ 1, 2 °C (0.5-3, 5 wt.%)	[161]
	Twin-screw extruder (180-220 °C, 500 rpm) Piston spinning to produce micro-fibres (220 °C, 3 min)	d = 9.5 nm l = 1.5 μ m 90 % purity	MWCNT: 0.5, 1, 2, 3, 5	T_g : pellet = (3 wt.%) Fibres ↑ 5-6 °C (3 wt.%)	[170]
Method	Procedure	CBNs characteristics	CBNs content (wt.%)	Thermal properties relative to neat polymer	References
Solution mixing	Sonication in chloroform, casting and doctor blading GO was pre-dispersed in acetone while GNP was directly dispersed in chloroform	GNP grade M (commercial product) t = 6-8 nm, d ≈ 5 μ m. GO (MHM) d ≈ 100 nm	GO and GNP: 0.2, 0.4, 0.6	GO: T_g ↑ 3, 4, 3 °C (0.2, 0.4, 0.6 wt.%) GNP: T_g ↑ 6, 7, 5 °C (0.2, 0.4, 0.6 wt.%) Similar T_m for both GO and GNP	[126]
	Sonication in chloroform, filtration, vacuum drying, compression moulding (170 °C, 10 min)	GO (from natural graphite, MHM + lyophilization) d ≈ 300 nm GO-g-PLLA (GO + l-lactide (Sn(oct) ₂), filtration, vacuum drying)	GO and GO-g-PLLA: 0.5	PLA/GO: T_g ↑ 6 °C T_m ↑ 3 °C PLA/GO-g-PLLA: T_g ↑ 6 °C T_m ↑ 5 °C	[141]

	Stirring and sonication in DMF, coagulation with methanol, filtration, and vacuum drying	GO (MHM) from expandable graphite, chemically reduced with hydrazine, and lyophilized (GNSs – solvent free graphene nanosheets) t < 1 nm, d < 50 nm.	GNSs: 0.2	$T_{d5} \uparrow 11$ °C.	[143]
	Sonication in DMF, film casting, vacuum drying	GO prepared according to Staudenmaier method (H ₂ SO ₄ + HNO ₃ + KClO ₃) (dimensions not given)	GO: 0.5, 1, 2	(0.5, 1, 2 wt.%) $T_c \downarrow 9, 15, 20$ °C T_g similar	[144]
	Sonication in DMF, coagulation with methanol, drying, compression moulding (185 °C)	TRG (commercial product, t = few layer, d = hundreds of nm) TRG/PLA/Py-PLA: Py-PLA-OH (1-Pyrenemethanol + l-lactide, Sn(oct) ₂) + TRG (10:1) – sonication + PLA – coagulation and drying	TRG and TRG/Py-PLA-OH: 0.25, 1	TRG/PLA: $T_{d5} \downarrow 32$ °C $T_{d\max}$ (max. degradation) $\uparrow 33$ °C TRG/PLA/Py-PLA: $T_{d5} \downarrow 2$ °C $T_{d\max} \uparrow 25$ °C (loadings not clear)	[145]
	Sonication in DMF, coagulation with water, vacuum drying, compression moulding (200 °C, 3 min)	Graphene oxide nanosheets - GONSs (MHM) from expandable graphite (t = few layer, d = 5-20 µm)	GONSs: 0.25, 0.5, 1, 2	(0.25, 0.5, 1, 2 wt.%) $T_{m1} \downarrow 1, 4, 0, 1$ °C $T_{m2} \downarrow 0, 1, 1, 1$ °C $T_c \downarrow 3, 6, 2, 4$ °C $T_{d1} \uparrow 2, 6, 11, 16$ °C	[147]
	Sonication in DMF, film casting, vacuum drying	GNS (commercial product) t = 5-25 nm, d = 0.5-20 µm, specific surface area = 50 m ² g ⁻¹	GNS: 1	Similar T_g and T_{m1} and 2 $T_c \uparrow 3$ °C	[148]
Melt blending	Internal mixer (160 °C, 25 rpm, 10 min), compression moulding (160 °C, 10 min) (Polymer was PLA/PEG 9:1 blend)	GNP grade M15 (commercial product) t = 6-8 nm, d ≈ 15 µm	GNP-M15: 0.1, 0.3, 0.5, 0.7, 1	(relative to pristine PLA/PEG blend) (0.1, 0.3, 0.5, 1 wt.%) $T_g \downarrow 0, 0, 1, 1$ $T_m \uparrow 2, 4 \downarrow 1, 1$ $T_c \uparrow 1, 2, 2, 1$ $T_{d1}, T_{d\max}, T_{50} \uparrow 56, 53, 44$ °C (0.5 wt.%)	[158]
In-situ polymerization	Melt ring-opening polymerization of l-lactide in presence of TRG (Sn(oct) ₂ , 170 °C, 4h), filtration, vacuum drying	Natural graphite (MHM + lyophilization) - GO GO thermal reduction (1000 °C, 1 min) to TRG t = few layers	TRG: 0.01, 0.1, 0.5, 1, 1.5, 2	(0.01, 0.1, 0.5, 1, 1.5, 2 wt.%) $T_g = \uparrow 9, 6, 6, 7, 8, 5$ °C $T_m = \uparrow 11, 12, 13, 14, 14, 14$ °C $T_{d\max} = \uparrow 4, 13, 10, 11, 16, 18$ °C	[166]
	Sonication of l-lactide + filler in toluene, addition of Tin(II)-2-ethylhexanoate under N ₂ , stirring at 110 °C, 3 days	Expanded graphite (MHM) to GO GO-functionalized: GO + TDI + 1,4-butanediol, 80 °C, 24h GO-g-POSS: GO + POSS – polyhedral oligomeric silsesquioxane + DMAP – 4-(dimethylaminopyridine) + EDC – N-(3-dimethylamino-propyl-N'-ethylcarbodiimide), 2 days, room temperature, N ₂ (dimensions not given)	GO-functionalized, GO-g-POSS, GO+POSS (physical mixture): 1	PLA/GO-functionalized: $T_{d5} \uparrow 8, T_g \downarrow 8, T_c \uparrow 14, T_m \downarrow 2$ °C PLA/ GO-g-POSS: $T_{d5} \uparrow 31, T_g \uparrow 10, T_c \uparrow 29, T_m \uparrow 5$ °C PLA/ GO+POSS: $T_{d5} \uparrow 19, T_g \uparrow 12, T_c \uparrow 22, T_m \uparrow 3$ °C	[167]

5.2.4.4 Biological properties

Since CBNs generally present toxicity when isolated, *i.e.* when not incorporated in a polymer matrix, ^[37, 171] biocompatibility of the composite must be tested when considering uses as biomaterials. Table 5.4 shows that PLA/CBNs composites (films and nanofibers) do not tend to decrease *in vitro* metabolic activity of several cell types, or cause increases up to 40% until 72 h incubations. Also, the selection of production method used (spin coating, electrospinning, and solvent mixing followed by casting or doctor blading), does not seem to influence cell proliferation. For long term incubations, McCullen *et al.* ^[172] showed that scaffolds of PLA with 1 wt.% MWNTs do not to influence metabolic activity of adipose-derived human mesenchymal stem cells (hMSCs) at 7 days. At 14 days, cells present increased metabolic activity and longitudinal alignment induced by the scaffolds. Sherrell *et al.* ^[173] reported that PLGA (1:1) with a surface layer of graphene applied by CVD to increase PC-12 cells average length of neurites by 2.5 fold when electrical stimulated. Also, hemocompatibility improvements were reported with both incorporation of 0.4 wt.% GNP by solvent mixing followed by doctor blading ^[140] and 4 wt.% MWCNTs by extrusion followed by injection moulding ^[174] in PLA. In the last case, MWCNTs alignment is associated with decreased platelet adhesion and activation. Thus, alignment seems to be generally benefic for biocompatibility. The effectiveness of electrical stimulation together with fiber alignment was confirmed by Shao *et al.* ^[169], which cultured osteoblasts at the surface of PLA/MWCNTs-ox (3 wt.%) produced by solution mixing followed by electrospinning. They observed maximum improvements, comparing with control PLA fibers ($d \approx 400$ nm), in cell elongation (190 %) and metabolic activity (20 %) for random nanofibers ($d \approx 250$ nm) under DC 100 μ A. For aligned fibers the previous values increased by 355 and 40 %, respectively. Finally, An *et al.* ^[175] found PLA/PU 3 wt.%/GO 5 wt.% films and nanofibers to completely suppress *Escherichia coli* and *Staphylococcus aureus* growth after 24h, not affecting MC3T3-E1 cells metabolic activity.

In an *in vivo* study, Kanczler *et al.* ^[176] observed that PLA-CB 0.1 wt.% scaffolds seeded or not with fetal femur-derived cells, when implanted in a murine critical-size femur segmental defect model aided the regeneration of bone defect.

Table 5.4: Biological properties of PLA/CBNs composites in comparison with non-modified PLA. Production methods and CBNs characteristics.

Production methods	Materials characteristics	CBNs content (wt.%)	Biocompatibility properties	References
GO - MHM Nanofibers - electrospinning	GO (thickness (t) = 1.5 nm, Length (l) $\approx$ 1 μ m) Nanofibers (l = 11-14 μ m)	PLGA (1:1)/GO 1 and 2 wt.% nanofibers	Cell metabolic activity (MA): (PLGA = 100%, PLGA/GO 1 wt.% $\approx$ 102%, PLGA/GO 2wt.% $\approx$ 108%, 48h) (PC 12 cells)	[177]
GO - MHM Films – spin coating	GO (not found) Films (t $\approx$ 5 μ m)	PLGA (1:1)/GO films	Cell MA: Small increase ($\approx$ 10%) comparing to PLGA for PLGA/GO 2wt.% (48h) (Hela cells)	[178]
GO - MHM Nanofibers - electrospinning	GO (few layer) PLA/HA/GO (diameter (d) = 0.3-1.3 μ m)	PLA/HA(10 wt.)/GO nanofibers	Cell MA: 1, 2 and 5 wt.% GO $\uparrow$, comparing to PLA/HA (24h) Only nanofibers with 5 wt.% GO presented higher MA than PLA/HA (48h) (MC3T3-E1 cells)	[179]
GO - MHM Films – solvent mixing + doctor blading	d $\approx$ 500 nm Films (t = 25-65 μ m)	PLA/GO films (0.4 wt.%)	Cell MA: No variations until 48h, except for PLA/GO after 24 h (more 13% than pristine PLA) (Mouse embryo fibroblasts 3T3 - ATCC CCL-164) Hemocompatibility: Less platelets activated in PLA/GNP comparing with PLA in presence of plasma proteins (Human platelets concentrate)	[140]
GNP–commercial product Films – solvent mixing + doctor blading	GNP (t $\approx$ 6-8 nm, l $\approx$ 5 μ m) Films (t = 25-65 μ m)	PLA/GNP films (0.4 wt.%)		
Graphene – CVD (chemical vapor deposition) Films – solvent casting over graphene	Graphene (t = 2 layers) Films (t = 25-65 μ m)	PLGA(1:1)/graphene surface layer	Cell MA: No significant changes until 4 days for PC-12 cells (rat adrenal gland pheochromocytoma). Cell differentiation: with electrical stimulation the average length of neurites increased 2.5 fold	[173]
GO - MHM Films – solvent mixing + solvent casting Nanofibers - electrospinning	GO (not found) Films (not found) Nanofibers (d $\approx$ 1 μ m)	PLA/PU (3 wt.)/GO (5 wt.%) films and nanofibers	Cell proliferation: not decreased (MC3T3-E1 cells) Antibacterial effect: <i>E. coli</i> and <i>S. aureus</i> growth 100% reduced at 24h	[175]
MWNTs - catalytic chemical vapor deposition Composites – extrusion + injection moulding Aligned composites – mechanical stretching at 90°C	MWNTs (l = 10–30 mm, d = 20–40 nm) Composites (not found)	PLA/MWNTs (5, 10, 15 wt.%) composites	Hemolysis: bellow standard permissible (5%) in all cases, decreases with MWNTs incorporation and alignment Kinetic clotting time: increases with MWNTs incorporation and alignment (best was PLA/MWNTs 5 wt.% which increased time by 480%) Platelet adhesion and activation: decreases with MWNTs incorporation and alignment	[174]
MWNTs - plasma chemical vapour deposition Scaffolds – electrospinning	MWNTs (l = 5–20 mm, d = 5–15 nm) Scaffolds (d = 0.7 μ m, average porosity = 87%, void space = 89%)	PLA/MWNTs (1 wt.%) scaffolds	Cell MA: equal until day 7 and increased with MWNTs at day 14 (hMSCs) Cell morphology: MWNTs induced longitudinal alignment on cells at day 14	[172]

MWCNTs-ox (HCl, 2h at 25°C + HNO ₃ , 4h at 110 °C) Nanofibers (MWCNTs-ox sonicated in DMF 2h + SDS, adding to PLA in dicloromethane, 1h sonication before electrospinning)	MWCNTs (l = 10–20 µm, d = 10–20 nm) Nanofibers (PLA ≈ 400 nm, PLA/MWCNTs-ox ≈ 250 nm)	PLA/MWCNTs-ox (3 wt.%) random (R) and aligned (A) nanofibers	Cell MA: increased for osteoblasts at day 3 for PLA/MWCNTs-ox (3 wt.%) R – 20 % and A – 40 %, under DC = 100 µA Cell morphology: induced osteoblasts alignment at day 3 for PLA/MWCNTs-ox (3 wt.%) R – 190 % and A – 355 %, under DC = 100 µA Both increased with electrical stimulation for DC = 50 and 100 µA	[169]
CB – not found Scaffolds - surface selective laser sintering	(CB) Carbon black (d = 360 nm, surface area = 100 m ² g ⁻¹) Scaffolds – several shapes	SSLS-PLA/CB 0.1 wt.% scaffolds	SSLS-PLA/CB 0.1 wt.% scaffolds seeded or not with fetal femur-derived cells aided regeneration of murine bone defect	[176]

5.3 Conclusions

Both CNTs and GBMs nanofillers are effective at improving PLA thermo-mechanical and electrical properties, whether produced by solution mixing or melt blending. However, lower amounts of GBMs (0.1-1 wt.%) are usually needed, comparing with CNTs (0.25-5 wt.%). Melt-blending is less frequently reported than solution mixing for production of PLA/CBNs composites, probably due to the lower availability of the necessary equipment. Moreover, results show that melt blending tends to be not as effective at improving PLA properties. *In-situ* polymerization is the least reported technique, with further research being needed to demonstrate possible advantages over the previous approaches.

Several chemical modifications can be tried to improve compatibility with a polymer matrix. Functionalization with carboxyls is the most common and effective procedure to improve CNTs dispersibility and compatibility with PLA. Some authors refer that purification with strong acids introduces polar groups in the carbon surface, which introduces positive interaction with PLA. Besides simple chemical oxidation of CBNs, other chemical modifications include reaction with isocyanates, polymers (ethylene glycol, poly(caprolactone), poly(methyl methacrylate), poly(vinyl pyrrolidone), and PLA)), polyols, silanes or amines.

No relation can be determined between CBNs morphological properties (size, length, and diameter) and the composites performance.

The alignment of PLA/CBNs fibres, was reported to improve their conductivity. Also, in most works evaluated, electrical properties improved with the increase of the amount of CBNs incorporated.

Production methods reported for PLA/CBNs composites for biomedical applications are spin coating, electrospinning (most used), and solvent mixing followed by casting or doctor blading. The process selection does not influence cell metabolic activity. Different studies reveal that PLA/GBM composites (films and nanofibers) do not decrease the metabolic activity of cell lines, and in some cases induce increases below 40% until 72 h incubations, comparing with non-filled PLA. Improvements in hemocompatibility were achieved with incorporation of both CNTs and GBMs. Also, both fiber alignment and electrical stimulation, improved cell metabolic activity and elongation. Incorporation of GO has lead to suppression of *Escherichia coli* and *Staphylococcus aureus* growth, without compromising the composite biocompatibility. However, there is still no information on antimicrobial activity of these composites on other types of microorganisms or with other types of GBMs.

Interesting fields for future work would be to further explore in-situ polymerization, since there are few studies focused on that approach. Also, it is important to better understand how the fillers physico-chemical properties, and their alignment inside a polymer matrix, affect the composites properties. Emerging technologies, like 3D printing, will surely contribute for the conception of materials with the desired properties for the broad potential applications of PLA/CBNs composites.

5.4 References

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Chapter 6

Physical-chemical properties and biocompatibility of PLA/GBMs composites:

Production by solvent-mixing

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Biocompatibility of poly(lactic acid) with incorporated graphene-based materials

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6 PHYSICAL-CHEMICAL PROPERTIES AND BIOCOMPATIBILITY OF PLA/GBMs COMPOSITES - PRODUCTION BY SOLVENT-MIXING

6.1 Scope

There is a need to improve some of poly(lactic acid) (PLA) properties for several biomedical applications, being mechanical performance the most pressing. Due to its particular physical-chemical properties, graphene-based materials (GBMs) are able to interact with polymer matrices when incorporated as fillers, even when used in low amounts, usually improving composites performance and leaving the base polymer key properties intact. This chapter, describes the preparation by solvent mixing followed by doctor blading, and characterization of PLA films and composite PLA films incorporating two GBMs – graphene oxide (GO) and graphene nanoplatelets (GNP). The materials were prepared and characterized regarding not only biocompatibility, but also surface topography, chemistry, and wettability.

6.2 State of the art

Aliphatic polyesters with reactive groups have attracted attention because of the demand of synthetic biopolymers with tuneable properties, including features such as hydrophilicity, biodegradation rates, bioadhesion, drug/targeting moiety attachment, etc. Poly(lactic acid) (PLA) has been widely investigated for biomedical applications because it is biodegradable, bioresorbable, and biocompatible.^[1, 2] This polymer has several applications in tissue and surgical implant engineering, like production of: bioresorbable artificial ligaments, hernia repair meshes, scaffolds, screws, surgical plates, and suture yarns.^[3, 4] PLA is also used in production of nano/microparticles for drug delivery, and in packaging of pharmaceutical products.^[5] To make this material more attractive for some applications, as an effective alternative to petrochemical

plastics, some properties should be improved, namely mechanical performance.^[6] To attain these objectives different approaches have been tried according to the required applications. Some commonly used strategies are adjustment of crystallinity^[7], incorporation of plasticizers^[8], blends with other polymers^[6] and addition of nanofillers. The latter is an interesting option, since with the addition of small weight percentages (wt.%) target properties can, in principle, be improved, while maintaining other key PLA properties intact. Good dispersion and interfacial interaction with the polymer matrix is paramount in order for these improvements to be significant. Most of the nanofillers reported are nanoclays^[9], carbon nanotubes^[10], and nanosilicas^[11].

Graphene, the elementary structure of graphite, is an atomically thick sheet composed of sp^2 carbon atoms arranged in a flat honeycomb structure. It possesses remarkable mechanical strength and an extremely high surface area.^[12] Since graphene is hydrophobic, stable dispersions in water can only be obtained with addition of proper surfactants.^[13] Graphene oxide (GO) is similar to graphene, but presents oxygen-containing functional groups. The presence of these polar groups reduces the thermal stability of the nanomaterial, but may be important to promote interaction and compatibility with a particular polymer matrix.^[12, 14]

GO and graphene have been reported as efficient drug carriers^[15, 16], as well as PLA^[17]. Development of hybrid vehicles for drug targeting can take advantage of both materials properties and originate synergistic effects.^[18] In addition, several graphene based biosensors are being developed.^[19] Recent studies show that graphene substrates promote adherence of human mesenchymal stromal cells and osteoblasts^[20], which can lead to better performance on tissues regeneration using scaffolds containing graphene and graphene oxide. Due to their great potential, several approaches are under study for future applications of these nanomaterials in biomedical engineering and biotechnology.^[21]

There are only a few studies regarding the biological effects of graphene and graphene derivatives.^[22-28] Moreover, in some cases contradictory results are reported.^[22, 28] Some of the materials tested have identical designations, but in fact are obtained from different products and by different methods, leading to divergent conclusions. Most studies refer concentration dependent toxicity.^[23-26] Effective mechanical reinforcement of polymeric materials using very small loadings of GO and graphene nanoplatelets (GNP) has been described by several authors.^[29-31] Therefore, toxicological effects may not occur if the amount of nanofillers exposed or released from the polymer is sufficiently low to avoid attaining toxic concentrations.

The synthesis of GNP and GO does not require metal catalysis, contrarily to the production of carbon nanotubes, a chemically similar material that has attracted significant interest. Thus, cytotoxicity and inflammation caused by residual metals does not occur for GNP and GO. [32]

An appropriate cellular response to implanted surfaces is essential for tissue regeneration and integration. It is well described that implanted materials are immediately coated with proteins from blood and interstitial fluids, and it is through this adsorbed layer that cells sense foreign surfaces. Although several studies have been made, it is not yet clear which material properties (e.g. topography, chemical composition, wettability, surface charge) favor *in vitro* protein adsorption and cell adhesion and proliferation. [33] Graphene and graphene oxide can affect protein adsorption and cell adhesion and proliferation, according to their intrinsic morphology and wettability. The adhesion and activation of platelets is also affected by the abovementioned factors. Presence of carbon nanotubes at the surface of PLA films was reported to decrease thrombogenicity. [34] In addition, the starting materials and methods used in the production of graphene-based materials, as well as the presence of toxic functional groups and contaminants, can affect biocompatibility.

In a previous study [35], we showed that incorporation of small amounts (0.4 wt.%) of GO and GNP in PLA significantly increases tensile strength and Young's modulus. Thus, this type of composites have a potential use in the production of surgical implants with improved mechanical performance. However, it is paramount to assure that these biomaterials do not present toxicity problems. In this work surface properties of PLA/GO and PLA/GNP thin films are characterized. Biocompatibility of the composite films is evaluated through cytotoxicity and cell proliferation assays. Platelet adhesion and activation studies are used to assess hemocompatibility of the films.

6.3 Materials and methods

6.3.1 Materials

Poly(lactic acid) (PLA) 2002 D (4% d-lactide, 96% l-lactide content, molecular weight 121,400 g mol⁻¹), was obtained from Natureworks (Minnetonka, USA). Graphene nanoplatelets (GNP) grade M5, were purchased from XG Sciences (Lansing, USA), with the following characteristics: average thickness of 6–8 nm, maximum length 5 µm, and surface area between 120 and 150 m² g⁻¹. GNP production is based on exfoliation of sulfuric acid-based intercalated graphite by rapid microwave heating, followed by ultrasonic treatment. [36, 37]

Carbon graphite micropowder, with purity above 99% and a diameter between 7 and 11 µm was purchased from American Elements, Los Angeles, USA.

6.3.2 Preparation of GO

Graphene oxide (GO) was prepared according to a modified Hummer's method. Briefly, 100 mL of H_2SO_4 were added to 3 g of graphite at room temperature and the solution was cooled using an ice bath, followed by gradual addition of 14 g of KMnO_4 . Then 300 mL of distilled water were added, followed by addition of H_2O_2 (to reduce KMnO_4 excess) until oxygen release stopped. The solid was filtered and washed with water. After overnight resting, the resultant solution was decanted and the remaining product was centrifuged at 2000 rpm, during 5 min (this process was repeated four times). The solid was recovered and dried at 110 °C for 48 h. [38]

6.3.3 Preparation of PLA/GO films

Nanocomposite thin films with GO and GNP were prepared by doctor blade casting of solvent dispersions, as described in our previous work. [35] GO was dispersed in acetone using an ultrasonic bath (Bandelin Sonorex RK 512 H) during 5 h and then added to a PLA/chloroform solution and again sonicated for 15 min. Concentration of GO relative to PLA was 0.4 wt.%, since in a previous work we have verified that this was the optimum loading for mechanical performance improvement in terms of tensile strength and Young's modulus. [35] Thin films (25–65 μm) were made by spreading the PLA/GO dispersion on a PTFE coated plate using a blade applicator. Solvent was completely removed by drying in a vacuum oven.

6.3.4 Preparation of PLA/GNP films

GNP were dispersed in chloroform using ultrasound sonication during 2 h and then dispersed in a PLA/chloroform solution. Concentration of GNP relative to PLA was 0.4 wt.%, for the abovementioned reason. Thin films (25–65 μm) were prepared and dried according to same procedures as the PLA/GO nanocomposites.

6.3.5 Films surface characterization

6.3.5.1 Contact angle and surface free energy measurements

A OCA 20 (Dataphysics) goniometer was used to measure the contact angles of ultrapure water, ethane-1,2-diol and hexadecane on pristine PLA, PLA/GO and PLA/GNP films, by sessile drop method. Data were collected with SCA 20 v2 software. Equilibrium contact angles (considered at 60 s) were measured for 5 μL droplet volumes. Determinations were made on 3 different locations for each condition. The total surface free energy and the polar and dispersive components of the films were evaluated by the OWRK Method using SCA 20 software. Polar and dispersive components of the surface tension of the liquids that were used are 46.80 and 26.00 mN m^{-1} for

water, 21.30 and 26.30 mN m⁻¹ for ethane-1,2-diol and 0.00 and 27.47 mN m⁻¹ for hexadecane, respectively.

6.3.5.2 Reflected light microscopy

Reflected light microscopy images of PLA, PLA/GO and PLA/GNP films were obtained with a Zeiss axiophot microscope, equipped with a Zeiss axiocam ICc 3. The specified spatial resolution is 370 nm.

6.3.5.3 X-ray photoelectron spectroscopy (XPS)

PLA, PLA/GO and PLA/GNP thin films and GNP, graphite and GO powders were analyzed with an Escalab 200 VG Scientific spectrometer working in ultra-high vacuum (1×10^{-6} Pa) and using achromatic Al K α radiation (1486.6 eV). The analyzer pass energy was 50 eV for survey spectra and 20 eV for high-resolution spectra. The spectrometer was calibrated using (Au 3d_{5/2} at 368.27 eV). The core levels for O 1s and C 1s were analyzed. The photoelectron take-off angle (the angle between the surface of the sample and the axis of the energy analyzer) was 90°. The electron gun used focused on the specimen in an area close to 100 mm². Analyzed samples were not conductive, for these reason spectra energy was displaced and a corrective shift based on the C 1s peak (285 eV) was performed. Curve fitting of the spectra was performed with the software XPS peak version 4.1.

6.3.5.4 Topography characterization

A stylus profilometer Hommel T8000, equipped with a pick-up-set taster TKL 300/17, was used to obtain a three-dimensional characterization of the topography of rectangular surface areas with 1.5 mm $\times$ 1.5 mm of PLA, PLA/GO and PLA/GNP films. Determinations were made on 3 different locations for each film. Roughness parameters determined were: S_a – arithmetic average height of the surface ($S_a = 1/A \iint_a |Z(x,y)| dx dy$, A – area) and S_p , S_v , and S_z which are parameters evaluated from the absolute highest and lowest points found on the surface, being: S_p – the maximum peak height, which is the height of the highest point; S_v – the maximum valley depth – which is the depth of the lowest point (expressed as a negative number); S_z – the maximum height of the surface. Thus, $S_z = S_p - S_v$ (ISO 25178-2).^[39, 40] The specified space resolution is 100 nm in x , y and z .

6.3.6 *In vitro* biocompatibility assays

In vitro assays were performed using mouse embryo fibroblasts 3T3 (ATCC CCL-164), grown in Dulbecco's modified Eagle's media supplemented with 10% newborn calf serum (Invitrogen) and

penicillin/streptomycin (1 mg mL^{-1}) (Sigma–Aldrich) [DMEM+], at 37.0°C , in a fully humidified air containing 5% CO_2 (Infrared auto Flow). The cells were fed every 2–3 days. The cells were detached when 90% confluence was reached using a 0.25% (w/v) trypsin-EDTA solution (Sigma) and resuspended in culture medium at cellular density according to the assay. All assays were performed in triplicate and repeated at least 3 times. Results are presented as mean and standard deviation (SD).

6.3.6.1 Cell adhesion and proliferation at the surface of PLA, PLA/GO, and PLA/GNP films

Films ($\varnothing$ 13 mm) constituted by PLA, PLA/GO and PLA/GNP were sterilized by immersion in ethanol (70%, v/v) and then washed with PBS (Phosphate Buffered Saline) before cells seeding at 5×10^4 cells/well and incubated. Polystyrene disc (PS) was used as positive control. Fibroblasts proliferation was evaluated using 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT, Sigma), a colorimetric assay that gives a measure of the mitochondrial metabolic activity. Before the addition of MTT solution (0.5 mg mL^{-1} in PBS) films were changed to a new plate containing new medium. MTT assays were performed at 24, 48 and 72 h after cell seeded. The absorbance was measured at 570 nm and the cell proliferation inhibition index (CPII) was calculated as follows: $\text{CPII} = 100 - (\text{OD } 570 \text{ nm of test culture} / \text{OD } 570 \text{ nm of control culture (PS)} \times 100)$.

6.3.6.2 Direct contact assay

A fibroblast suspension containing 3×10^4 cells mL^{-1} was plated into each well of a six-well plate. After reaching a state of subconfluence (after 24 h), samples of PLA, PLA/GO and PLA/GNP films ($\varnothing$ 13 mm) were placed on the wells, in direct contact with cells. After 48 h incubation, the cell morphology and viability was assessed, using the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells fluorescence (Invitrogen) labeling. Positive and negative controls (discs of latex and agar gel) were also used.

6.3.6.3 Platelet adhesion and activation

The adhesion of platelets to the surface of $\varnothing$ 14 mm disks of PLA, PLA/GO and PLA/GNP films were evaluated by counting and observation of morphological features using scanning electron microscopy (SEM). Wells of 24-well plates were blocked by adding BSA (bovine serum albumin) 1% (w/v) in each well followed by 1 h incubation at 37°C , and then rinsing with PBS (0.01 M, pH 7.4). Films were sterilized in ethanol 70% (v/v) for 20 min, and then rinsed with PBS. To evaluate the effect of serum proteins in platelet adhesion and activation, the samples, PS [poly(styrene)] – control, PLA, PLA/GO and PLA/GNP were first incubated with two different pre-immersion solutions: PBS or human plasma 1% (provided by the Portuguese Blood Institute) in a 24-well plate for 30 min at 37°C , and afterwards rinsed with PBS. The human platelets

concentrate (PC) (obtained from the Immunohemotherapy service – Hospital S. João, Porto, Portugal) was diluted in PBS to a concentration of 3×10^8 platelets mL^{-1} . Samples were incubated with the freshly prepared PC in the previously blocked 24-well plates for 30 min at 37 °C under 90 rpm. Finally, the samples were rinsed with PBS. Adherent platelets were fixed with freshly prepared solution of 1.5% glutaraldehyde (Merk) in 0.14 M sodium cacodylate buffer (Merk) for 30 min at room temperature and then rinsed with PBS. Afterwards, the samples were dehydrated with a growing ethanol/water gradient, for 10 min each: 50, 60, 70, 80, 90 and 99% (v/v). Next, 100 μL of hexamethyldisilazane (Sigma–Aldrich) were added to each well and the samples were left to dry in the hood overnight. Finally, the samples were sputtered with a conductive gold/palladium layer and observed by SEM (FEI Quanta 400FEG) at CEMUP – Centro de Materiais da Universidade do Porto. The degree of activation was evaluated by qualitative observation of the platelets morphology. Two degrees were considered: (a) non-activated and (b) activated (see Figure 6.1). Platelets were considered activated if they had more than one pseudopod or were fully spread ((Fig. 6.1(C, D)). Samples were pre-immersed in PBS or plasma. At least 10 samples were used to calculate mean and standard deviation for each material in each pre-immersion condition.

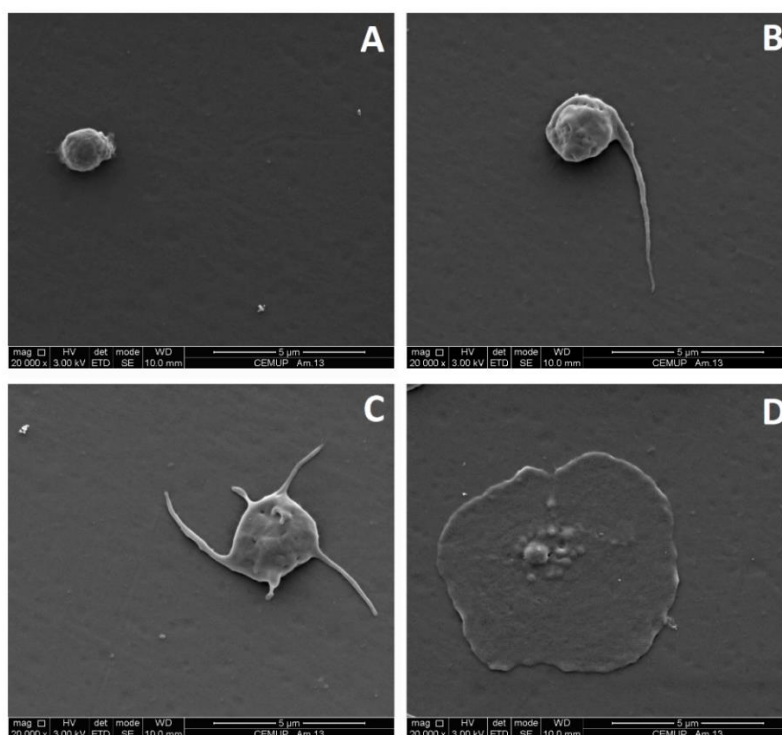


Figure 6.1: Activation degree of platelets at the surface of the films. Representative images of non-activated (A and B) and activated (C and D) platelets, at 20,000 $\times$ magnification.

6.3.7 Statistical analysis

Statistical analysis was made by analysis of variance (ANOVA). Multiple means comparison was performed between samples to identify significant differences, which were considered for $p < 0.05$. In suitable cases independent two samples Student's *t*-test was used. Significant differences were also considered for $p < 0.05$.

6.4 Results and discussion

6.4.1 Topographical characterization

The 3D topography images shown in Figure 6.2 compare the surfaces of pristine PLA films with PLA/GO and PLA/GNP composite films surfaces. The peaks seen in Fig. 6.2(B) correspond to agglomerates (1–2 μm), since individual GO sheets are too small to be detected by this technique (sizes in the order of tenths of micron).^[35] On the other hand, GNP particles have nominal lengths close to 5 μm , allowing for the observation of both individual and agglomerated particles Fig. 6.2(C). The peaks are distributed throughout the entire surface. A grooved pattern was observed on the surfaces of all samples. Reflected light microscopy was used to identify the cause of this pattern (discussed next).

Table 6.1 shows that films with GO and GNP incorporation present higher positive values of S_p and S_t than pristine PLA films, due to presence of fillers at the surface. PLA/GNP films presented negative values of S_v higher than PLA/GO films. This may be an indication of GNP having less compatibility with the polymer matrix than GO, thus being less embedded in it, existing more pronounced depressions surrounding GNP than GO particles. GO has more functional groups with oxygen than GNP (shown in Section 6.4.2), which form hydrogen bonds with similar groups in PLA. Differences in S_a between samples are not considerable because only a very small weight percentage of nanofillers are dispersed in the films.

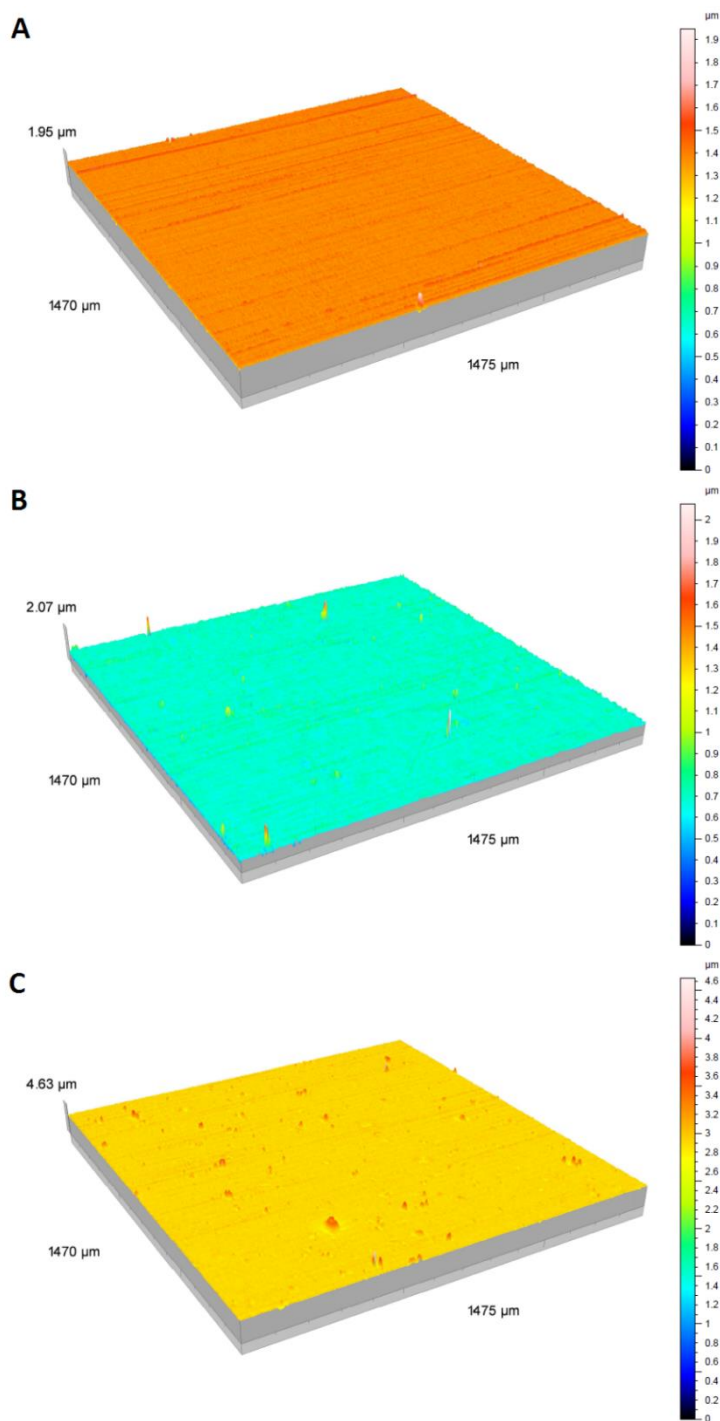


Figure 6.2: Representative 3D images of the topography of the surface of pristine PLA (A), PLA/GO (B) and PLA/GNP (C) films.

Table 6.1: Roughness parameters for PLA, PLA/GO and PLA/GNP films. S_a – arithmetic average height of the surface, S_p – maximum peak height, S_v – maximum valley depth, S_z – maximum height of the surface. Results are presented as mean and standard deviation (in parenthesis) for $n = 3$.

Roughness parameters				
Samples	S_a / nm	S_p / nm	S_v / nm	S_z / nm
PLA	43 (11)	730 (124)	-711 (427)	1441 (346)
PLA/GO	43 (6)	1623 (370)	-816 (203)	2439 (565)
PLA/GNP	37 (6)	1211 (476)	-1797 (963)	3008 (1424)

Reflected light microscopy images (Figure 6.3) show that the surface of the films presents grooves with pitches between 0.5 and 2 μm for all conditions. These grooves are in the same direction as the spreading of the films. This might occur due to imprinting of a micropattern present in the doctor blade during spreading. Rapid solvent evaporation in these thin films hinders surface leveling and originates this morphology. GO and GNP are visible in Fig. 6.3(B,C) as dark and bright spots, respectively.

6.4.2 Chemical characterization

Chemical properties of nanofillers used in a composite are relevant in two important contexts: (i) compatibility with the polymer matrix, and (ii) biological effects when exposed at the film surface or released due to matrix biodegradation. XPS results (Figure 6.4 and Table 6.2) show that, both graphite and GNP present a low degree of oxidation (atomic percentage of oxygen – O 1s (at.%) < 9%). This was expected since graphite is mainly constituted by carbon atoms and GNP is obtained from graphite by microwave and ultrasonic treatment (see Section 6.3.1). XPS data also reveal that oxidation of graphite by modified Hummer's method, to produce graphene oxide, increases the O 1s (at.%) in the final product (GO) by about 15%. The most ubiquitous oxygen functional groups identified in GO are ethers.

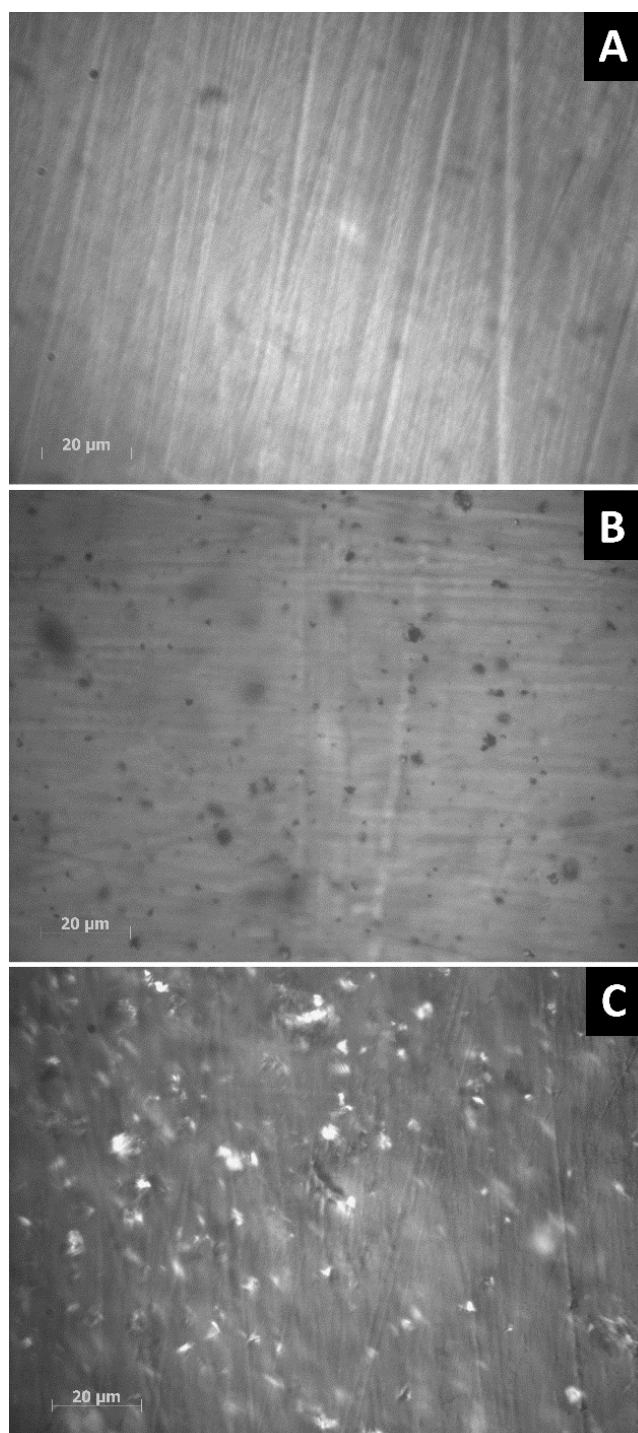


Figure 6.3: Reflected light microscopy of the surface of PLA (A), PLA/GO (B) and PLA/GNP (C) films.

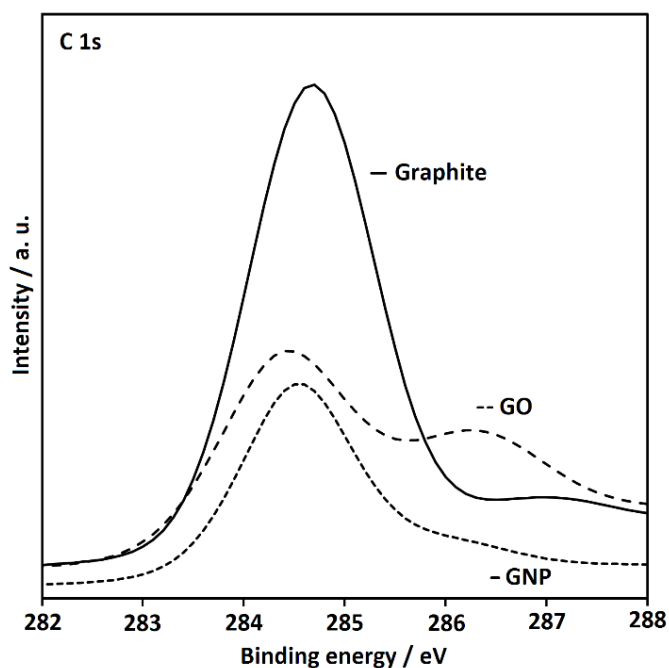


Figure 6.4: XPS spectra for the core level C 1s (after fitting) of graphite, GO and GNP powders.

Concerning the composite films, the C and O 1s (at.%) at the surface of PLA, PLA/GO and PLA/GNP films are similar for every condition (Table 6.3). Major oxygen containing functional groups identified for the three cases were ethers and carbonyls. Again there were no considerable differences between the films. This might happen because the contribution of the filler (at a loading of only 0.4 wt.%) to the final C and O 1s (at.%) cannot be detected by XPS.

Table 6.2: Atomic composition of graphite, GNP and GO, determined by XPS. Results are presented as mean and standard deviation (in parenthesis).

Sample	C 1s / at. %	O 1s / at. %
GNP	92.4	7.6
Graphite	91.7	8.3
GO	78.3	21.7

Table 6.3: Atomic composition analysis by XPS of the surface of PLA, PLA/GO and PLA/GNP films. Results are presented as mean and standard deviation (in parenthesis) for $n = 3$.

Sample	C 1s / at. %	O 1s / at. %
PLA	62.1 (2.36)	37.9 (2.36)
PLA/GNP	63.7 (2.73)	37.8 (2.63)
PLA/GO	61.7 (0.74)	38.3 (0.74)

6.4.3 Wettability of the films surface

Contact angle measurements (Table 6.4) show that the water contact angle of PLA/GO films decreased about 9° comparing with pristine PLA films. This shows that the presence of GO at the film surface increases its hydrophilicity. Hydrogen bond interactions between oxygen-containing groups in GO and water can explain this behavior. Hexadecane completely wetted the surface of PLA/GO films (Figure 6.5(E)), while at the surface of pristine PLA films it presented a contact angle close to 27°. Hydrophobic interactions with hexadecane might be established with the honeycomb sp^2 carbon atoms. This suggests that the presence of GO particles induces an amphiphilic behavior of the surface.

Table 6.4: Contact angles at 60 s of H₂O, ethane-1,2-diol and hexadecane on PLA, PLA/GO and PLA/GNP films. Results are presented as mean and standard deviation (in parenthesis) for $n = 3$.

Samples	Contact angles / °		
	H ₂ O	Ethane-1,2-diol	Hexadecane
PLA	87.2 (0.36)	56.9 (2.14)	26.7 (3.37)
PLA/GO	78.1 (2.06)	55.2 (3.38)	0 (0.00)
PLA/GNP	89.6 (0.97)	65.3 (0.94)	0 (0.00)

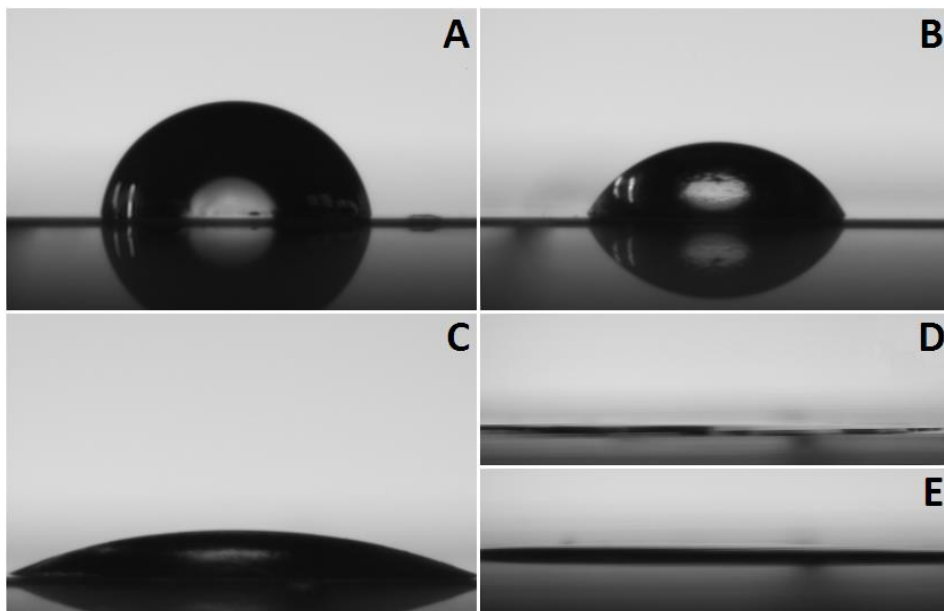


Figure 6.5: Contact angle images for: A – water on PLA, B – ethane-1,2-diol on PLA, C – hexadecane on PLA, D – hexadecane on PLA/GO and E – hexadecane on PLA/GNP film surface.

The water contact angle of PLA/GNP film increased 2.3°, comparing to pristine PLA films, showing a small hydrophobic effect. As occurred for PLA/GO films, hexadecane completely wetted the surface of PLA/GNP films (Fig. 6.5(D)). However, the contact angle for ethane-1,2-diol increased, probably due to the presence of much less oxygen functional groups in GNP particles than in GO sheets at the film surface.

All the above-mentioned findings suggest that the presence of the fillers at the film surface, despite not changing the surface composition significantly (shown in Section 6.4.2), affect its wettability. This might occur because of direct interaction of the liquids with partially exposed fillers at the PLA surface. Wang and co-workers measured water contact angles on poly(vinyl alcohol)/graphene dry films and observed that 0.5 wt.% graphene loading increased the contact angle from 36° to 93°. ^[41]

Figure 4.6 shows surface free energy values computed from the contact angle measurements. The total surface free energy of PLA increases about 12% with the incorporation of 0.4 wt.% GO. However, for the same incorporated amount of GNP, no changes are observed. Additionally, as expected, the polar component of PLA films increases about 59% with addition of GO and decreases 56% with incorporation of GNP. These results are in accordance with the discussion presented above for the contact angles.

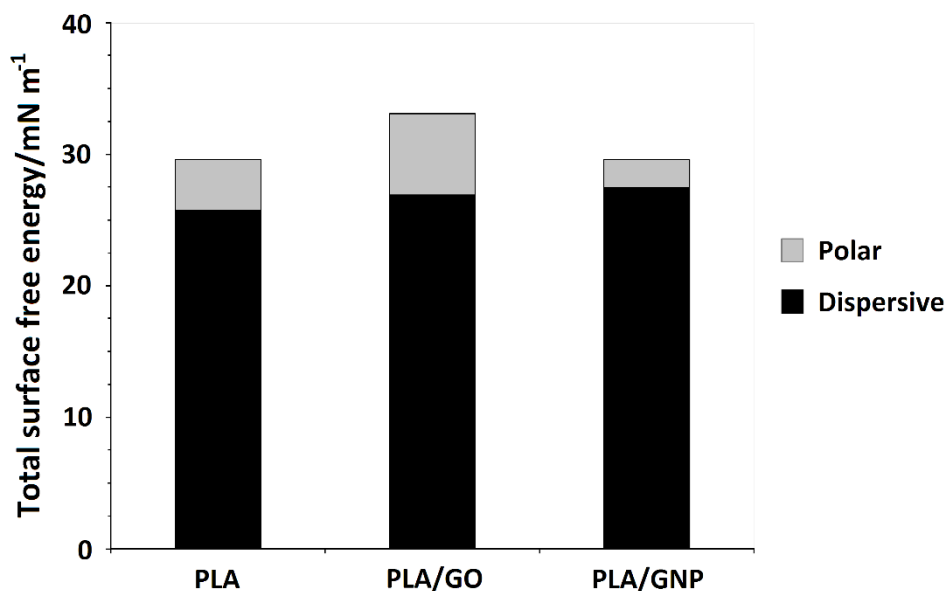


Figure 6.6: Dispersive and polar components of the total surface free energy of PLA, PLA/GO and PLA/GNP films.

6.4.4 *In vitro* biocompatibility assessment

In order to evaluate the cytotoxicity of the films, we have studied the effects of these materials in a mouse embryo fibroblast culture, namely in terms of cell adhesion and proliferation on the films, morphological features and cell death.

After 24 h of culture, fibroblasts adhesion and proliferation on the PLA/GO films (CPII ca. 17%) was significantly higher than for pristine PLA (CPII ca. 31%) ones ($p < 0.05$) (Figure 6.7). This can be due to the presence of GO at the surface increasing its hydrophilicity or creating a more suitable surface morphology for protein adsorption and cell adhesion. Higher surface hydrophilicity favors vitronectin adhesion and allows the maintenance of fibronectin functionality. Furthermore, cell proliferation requires the reorganization of surface-adsorbed fibronectin, which occurs in hydrophilic surfaces and is often impaired in more hydrophobic surfaces. Increase in surface roughness may lead to higher fibronectin adsorption due to the increase of surface area. Interestingly, Ruiz *et al.* [28] observed that mammalian colorectal adenocarcinoma HT-29 cells attached and proliferated more efficiently in GO coated glass slides, than in control (glass slides). After 48 and 72 h, the proliferation rate at the surface of PLA/GO

films seems to decrease and no significant differences are observed comparing to PLA films ($p > 0.05$). Also, no significant differences ($p > 0.05$) are observed in CPII between PLA and PLA/GO comparing to PLA/GNP films, until 72 h. Thus, the presence of GNP at films surface does not seem to affect cell adhesion and proliferation.

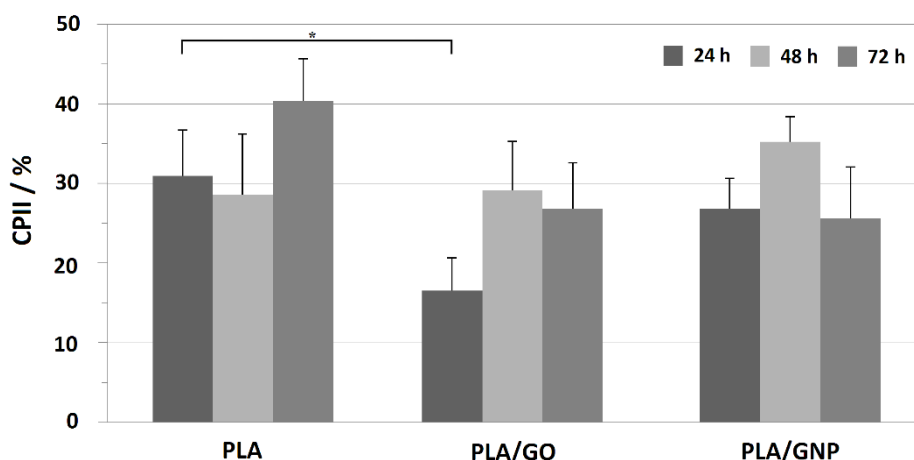


Figure 6.7: Cell proliferation inhibition index for mouse embryo fibroblasts, cultured on PLA, PLA/GO and PLA/GNP films. Results are presented as mean and error bars represent SD. *Significantly different ($p < 0.05$).

Yoon *et al.* ^[42] reported that the proliferation and viability of neuronal cells (PC 12) on poly(D,L-lactic-co-glycolic acid) [PLGA]/GO (2 wt.%) nanocomposite scaffolds increased by 8% in comparison to pristine PLGA scaffolds. However, Lahiri *et al.* ^[25], showed that the viability of osteoblasts (ATCC, Manassas, VA, USA) grown at the surface of ultra-high molecular weight poly (ethylene)–GNP nanocomposite films (0.1 wt.%) decreased about 6, 14 and 17% comparing to pristine polymer after, respectively, 1, 3 and 5 days of incubation.

The cytotoxicity of the films was also assessed using a direct contact method. For that purpose, the films were used together with a positive and negative control (latex rubber and agar, respectively). Each sample was placed on top of a sub-confluent cellular layer on a six-well plate, as described in the material and methods section.

Figure 6.8 shows the results obtained with the fluorescence labeling of the cell layers. As expected, the latex rubber is cytotoxic. Thus, the majority of cells were floating in the medium and some of those that remained attached were also dead (red labeled – Fig. 6.8(B)). No differences in morphology were found for the cells on the films surface and on the negative

control (agar – Fig. 6.8(A)). Moreover, all of the cells remained alive, as shown by the green fluorescence (Fig. 6.8(A, C–F)). These results suggest that films can be considered non-toxic.

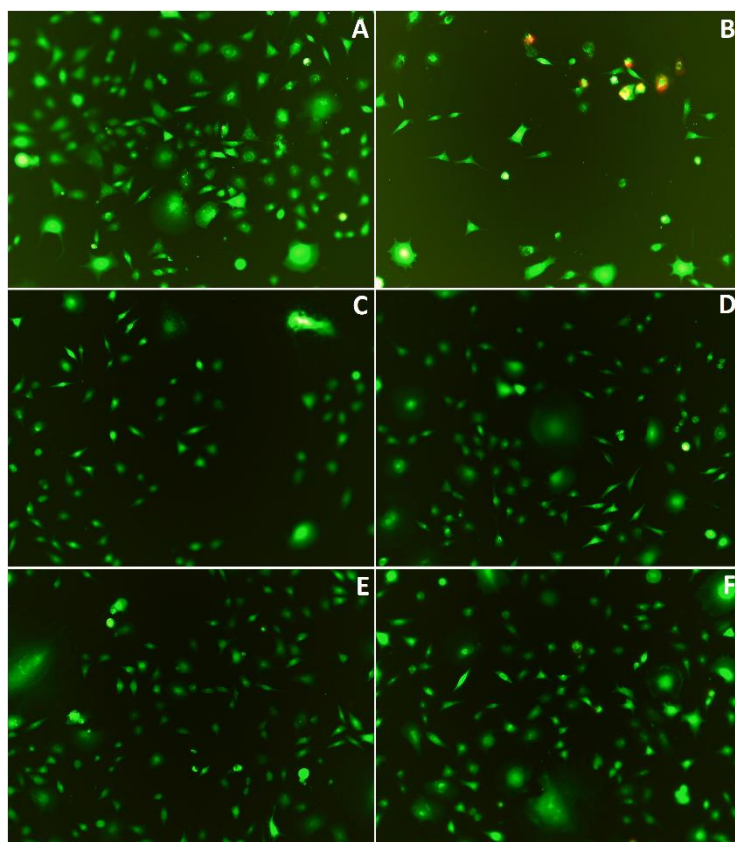


Figure 6.8: Fluorescence microscopy of mouse embryo fibroblasts after 48 h incubation in the direct contact assay: A – Agar (negative control); B – positive control (latex rubber); C and D – PLA; E – PLA/GO and F – PLA/GNP.

6.4.5 Platelet adhesion and activation

Since PLA is a biomaterial commonly used in surgery (*e.g.* orthopedy, dental medicine, hernia repair meshes), it should present low thrombogenicity in order to prevent the formation of post-operative blood clots.^[5] Adhesion and activation of platelets at the surface of PLA, PLA/GO and PLA/GNP films were evaluated by counting and qualitative observation of morphological features using SEM (see Section 6.3.6.4). To evaluate the effect of serum proteins on platelet

adhesion and activation the samples were pre-immersed either in PBS or human plasma 1%. Figure 6.9 shows that significantly less ($p < 0.05$) platelets adhered to PLA and PLA/GNP when samples were previously treated with human plasma comparing to those pre-immersed in PBS. This may occur due to adsorption of non-thrombogenic proteins, such as albumin, at the surface of the films preventing platelet adhesion. Moreover, when in presence of plasma proteins, the number of activated platelets in PLA/GNP films is significantly lower ($p < 0.05$) than that in PLA and PLA/GO films (Figure 6.9 and 6.10). This can be explained by the presence of hydrophobic GNP (see Section 6.4.3) at the surface of the films, favoring protein adsorption at the material surface. This blocks platelet activation in case of these proteins being albumin, which is generally the first to adhere because of its abundance in plasma and its small size. ^[44]

A decrease of platelet adhesion and activation after pre immersion in plasma has been previously described for surfaces that preferentially adsorb albumin over other plasma proteins. ^[45, 46] Also, Koh *et al.* observed decreases in platelet adhesion and activation in (PLGA) poly(lactic-co-glycolic-acid) films pre-immersed in fibrinogen and non-stimulated rich plasma, whose surface was coated with multi-walled nanotubes, comparing to PLGA films without MWCNTs at the surface.

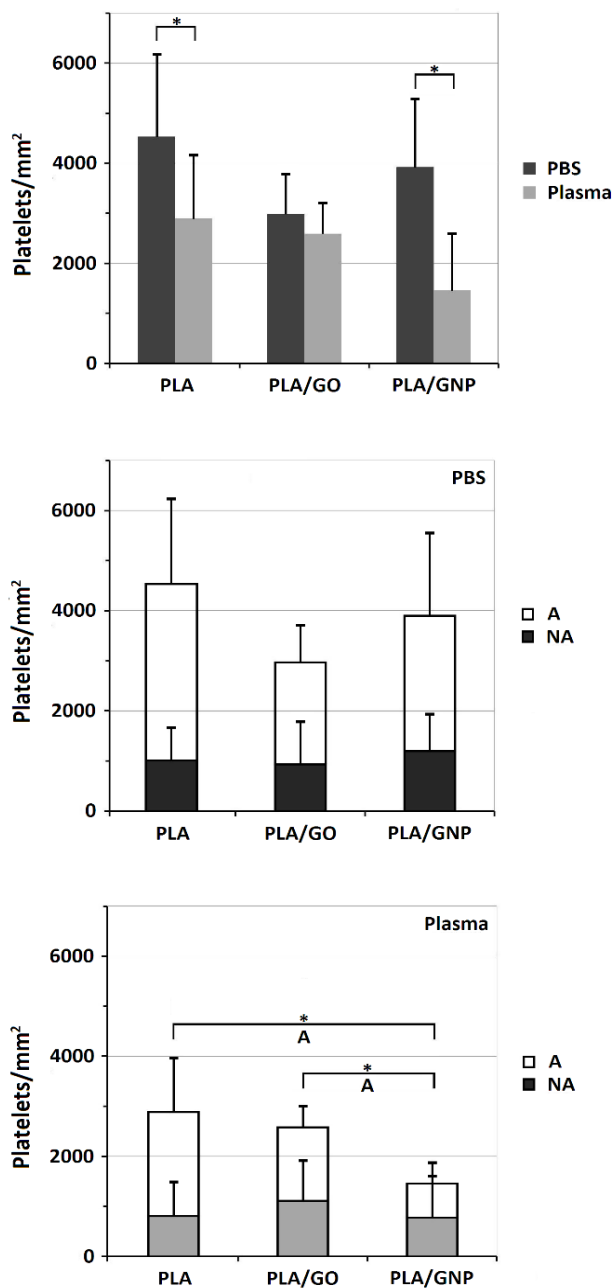


Figure 6.9: Platelet adhesion on PLA, PLA/GO and PLA/GNP films surface, pre-immersed in PBS or plasma. Degree of activation of the platelets adhered to the surface of the films pre-immersed in PBS or in plasma. A – activated, NA – non activated. Results are presented as mean and error bars represent standard deviation. *Significantly different ($p < 0.05$).

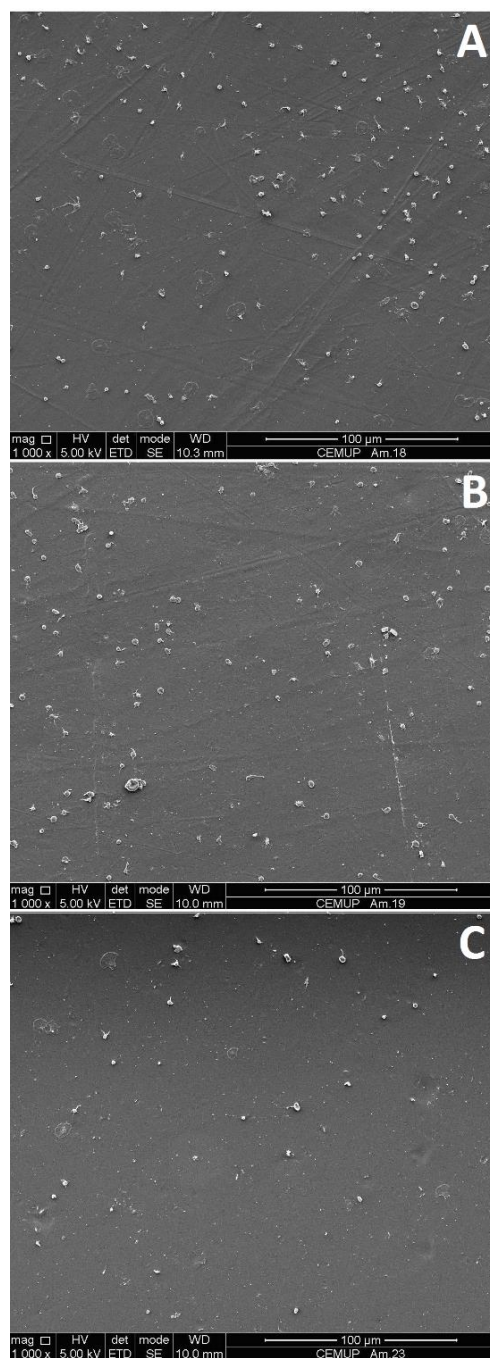


Figure 6.10: Platelets adherent on the surface of PLA (A), PLA/GO and (B) PLA/GNP films pre-immersed in plasma (images of the films pre-immersed in PBS are not shown).

6.5 Conclusions

Incorporation of 0.4 wt.% loadings of GO and GNP changed the surface topography and wettability of PLA composite films. However, no considerable variation in cell proliferation at the surface of the films was observed, except for those containing GO after 24 h incubation, which presented a CPII inferior to pristine PLA films ($p < 0.05$).

The presence of GO on the films surface may favor cell adhesion and proliferation due to creation of a more suitable surface morphology or to the increase in surface hydrophilicity. In the direct contact assay differences in morphology were not found for cells on the films surfaces or on the negative control (agar) surfaces. Moreover, all cells remained alive. Thus, it can be concluded that the films showed no cytotoxicity.

The number of activated platelets in PLA/GNP films is significantly lower than for pristine PLA films ($p < 0.05$) in the presence of plasma.

These results indicate that small amounts of GO and GNP can be safely incorporated in PLA to improve its mechanical properties for biomedical applications. Additionally, GO has apparently a positive effect on cell adhesion and proliferation, leading to faster tissue regeneration. GNP incorporation, on the other hand, decreases thrombogenicity, which might reduce post operative complications caused by blood clots formation.

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Chapter 7

Physical-chemical properties and biocompatibility of PLA/GBMs composites:

Production by melt-blending

This chapter was based in the following published paper:



Article

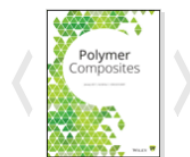
Biocompatible reinforcement of poly(Lactic acid) with graphene nanoplatelets

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7 PHYSICAL-CHEMICAL PROPERTIES AND BIOCOMPATIBILITY OF PLA/GBMs COMPOSITES - PRODUCTION BY MELT-BLENDING

7.1 Scope

Melt-blending is a commonly used method for polymer processing and fillers incorporation in polymer matrices. Comparing with solvent mixing followed by doctor blading, this method presents the advantage of not requiring the use of potentially toxic solvents. As mentioned in the previous chapter, there is the need to improve PLA mechanical properties for biomedical applications, as for example orthopaedical uses. In this chapter, graphene nanoplatelets are investigated as reinforcement filler for PLA. The parameters for production of PLA/GNP composites by melt-blending were optimized regarding mechanical performance, namely the effect of varying filler loading, mixing temperature, time, and intensity. Electron microscopy observations and Raman spectroscopy studies were used to understand why changing those variables affect mechanical properties. The biocompatibility of the composites was studied using human fibroblasts as an *in vitro* model.

7.2 State of the art

The biointeractions of graphene-based materials depend on their physico-chemical properties. In the last two decades, polymer composites have been studied as a strategy to provide added value properties to neat polymer without sacrificing its processability or adding excessive weight. Particular attention has been given to reinforcement with nanosized materials, which have the potential to present improved or even new properties when compared to conventional filled polymers. ^[1]

Poly(lactic acid) (PLA) has great worldwide demand due to versatile applicability in packaging, pharmaceutical, textiles, automotive, biomedical, and tissue engineering. [2] It has been widely investigated for biomedical applications due to its biodegradability, bioresorbability, and biocompatibility. [3, 4] Several applications have been described in tissue and surgical implant engineering, for production of bioresorbable artificial ligaments, hernia repair meshes, scaffolds, screws, surgical plates, and suture yarns. [5] PLA is also used in production of nano/microcapsules for drug delivery, and in packaging of pharmaceutical products. [6] Improvement and tuning of its properties has been reported by incorporation of plasticizers, blending with other polymers, and addition of nanofillers. [7-9]

Carbon-based fillers offer the potential to combine several unique properties, such as mechanical strength, electrical conductivity, thermal stability, and physical and optical properties, required for a spectrum of applications. [10-12] Graphene, in particular, has been playing a key role in modern science and technology. Its remarkable properties and the natural abundance of its precursor, graphite, make it an interesting option for production of functional composites. [13] Graphene is a one-atom-thick planar sheet of sp^2 -bonded carbon atoms densely packed in a honeycomb crystal lattice. It possesses very high mechanical strength, surface area per unit mass, and thermal and electrical conductivities. In the last years, there has been a surge in research work involving this material, with reported applications in diverse fields, including biomedical engineering and biotechnology. [14-20] On the other hand, not many studies are yet available concerning biocompatibility of graphene and graphene-based materials (GBM). These often show contradictory results. [21-26] In our recent study, graphene nanoplatelets with smaller size (GNP-C) revealed to be biocompatible with human fibroblasts (HFF-1) until a concentration of $50 \mu\text{g mL}^{-1}$, opposing to larger GNP-M, which are toxic above $20 \mu\text{g mL}^{-1}$. [27] Since several authors have shown that effective reinforcement of polymeric matrices can be obtained with small loadings of GBM [28-32], toxic concentrations achievement can be prevented. Additionally, graphene oxide (GO) and graphene nanoplatelets (GNP), grade M (GNP-M), have shown not to affect mouse embryo fibroblasts metabolic activity when incorporated in PLA at a loading of 0.4 wt.%. [33]

Many graphene-related materials have been reported in literature as potentially interesting fillers for polymer reinforcement. One commercial product that has been receiving particular attention, and is the object of study in this paper, is GNP: stacks of few graphene layers obtained by rapid heating of intercalated graphite. The platelets surface consists of mostly defect-free graphene, while oxygen is present in the sheet edges, in the form of, for instance, hydroxyl or carboxyl groups. Even though different grades are available for this material, grade M, with average platelet thickness of 6–8 nm and maximum length of 5 μm , is the most often tested in the available literature. [31]

Several studies exist on reinforcement of PLA with GBM. Even though performance improvements are reported, quantitative results are usually distinct, owing mainly to differences in chemical and morphological nature of GBM, filler incorporation and processing methods, and filler loadings. Cao *et al.* observed an 18% increase in Young's modulus with addition of only 0.2 wt.% of reduced graphene oxide. [30] The composite was prepared by solution mixing, followed by flocculation and drying. Pinto *et al.* showed that small loadings of GO and GNP-M (0.4 wt.%) in PLA thin films produced by solvent evaporation significantly improved mechanical and gas permeation barrier properties. [31] Tensile strength and Young's modulus were increased by about 15% and 85%, respectively. Chieng *et al.* investigated PLA/PEG melt blends with GNP-M loadings of 0.3 wt.%, obtaining increases of 33%, 69%, and 22% in tensile strength, Young's modulus, and elongation at break. [34] Bao *et al.* prepared PLA/graphene composites by melt blending and observed 58% improvement in storage modulus at 0.2 wt.% loading. [35] Yang *et al.* prepared poly(l-lactic acid) (PLLA)/thermally reduced graphene oxide composites via *in situ* ring-opening polymerization of lactide. The composite materials obtained, with loadings up to 2 wt.%, showed improved thermal stability, electrical conductivity, and crystallization rate. [36] In the work of Kim and Jeong, exfoliated graphite was incorporated in PLLA at different loadings by melt blending. [37] At 2 wt.% loading, tensile strength increased by about 13% and Young's modulus by 33%. Wenxiao *et al.* studied PLLA composites containing different low-dimensional carbonaceous fillers, with constant filler content of 0.5 wt.%. The fillers were pristine and silanized multiwalled carbon nanotubes (MWNTs) and exfoliated graphene. [38] All composites were prepared by solution mixing. It was found that tensile strength, elongation at break, and Young's modulus showed similar improvements when using carbon nanotubes or graphene (about 20%, 39%, and 33%, respectively). Silane modification of both fillers further improved elongation at break and Young's modulus, without sacrificing tensile strength. More recently, Wenxiao *et al.* successfully grafted GO with PLLA by *in situ* polycondensation. [39] This material was incorporated in PLLA by solution mixing, and the composite with 0.5 wt.% loading showed improvements in flexural and tensile strengths of 114% and 106%, respectively.

In this study, a recently made available commercial grade of GNP (grade C), made of thinner and shorter platelets than existing grades, is investigated for the first time as reinforcement filler for PLA. Melt blending is used for preparing the composite material as this is an economically attractive and industrially scalable method for efficiently dispersing nanofillers in thermoplastic polymers. The effects of blending conditions (mixing time, intensity, and temperature) and filler loading on the composite properties are analyzed, and the best conditions identified. This is an important aspect since results are often dependent on processing conditions, and this type of analysis is often absent from the literature. Melt blending is an environmentally friendly method

for filler incorporation that does not involve use of solvents. It avoids concerns with human health during processing and with toxicity of remaining solvent residues.

The quality of filler dispersion in the PLA matrix is investigated by SEM and Raman spectroscopy, and is seen to have a major influence on mechanical properties. For the first time, the biocompatibility of PLA/GNP-C composites is studied, namely, in terms of effects on cell metabolic activity and morphology.

7.3 Materials and methods

7.3.1 Materials

Poly(lactic acid) (PLA) 2003D (4% d-lactide, 96% l-lactide content) was purchased from Natureworks (Minnetonka, USA).

GNP, grade C750 (GNP-C), was acquired from XG Sciences (Lansing, USA), with the following characteristics, according to the manufacturer: average thickness lower than 2 nm and surface area of 750 m²g⁻¹. The platelet diameters have a distribution that ranges from tenths of micrometer up to 1–2 µm. GNP production is based on exfoliation of sulphuric acid-based intercalated graphite by rapid microwave heating, followed by ultrasonic treatment. ^[64]

7.3.2 Preparation of PLA/GNP composites

The PLA/GNP composites were prepared by melt blending in a Thermo Haake PolyLab internal mixer (internal mixing volume 60 cm³) at different temperatures (180, 200, 225, and 250 °C) mixing times (10, 15, and 20 min) and rotor speeds (25, 50, and 75 rpm). The GNP contents tested were 0.1, 0.25, and 0.5 wt.%. After removal from the mixer, the composites were molded in a hot press at 190°C for 2 min, under a pressure of 150 kg cm⁻², into sheets with approximately 0.5 mm thickness. After pressing, the sheets were rapidly cooled in water at room temperature. Samples with different dimensions were cut from these sheets, depending on the characterization test.

7.3.3 X-ray photoelectron spectroscopy (XPS)

GNP-C powder was analyzed with an Escalab 200 VG Scientific spectrometer working in ultrahigh vacuum (1×10^{-6} Pa) and using achromatic Al K α radiation (1486.6 eV). The analyzer pass energy was 50 eV for survey spectra and 20 eV for high-resolution spectra. The spectrometer

was calibrated using (Au 3d5/2 at 368.27 eV). The core levels for O 1s and C 1s were analyzed. The photoelectron takeoff angle (the angle between the surface of the sample and the axis of the energy analyzer) was 90°. The electron gun focused on the specimen in an area close to 100 mm².

7.3.4 Fourier transform Infrared Spectroscopy (FTIR)

PLA and PLA/GNP-C FTIR spectra were obtained between 600 and 4000 cm⁻¹, with 100 scans and a resolution of 4 cm⁻¹, using a spectrometer ABB MB3000 (ABB, Switzerland) equipped with a deuterated triglycine sulphate detector and using a MIRacle single reflection horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate.

7.3.5 Tensile properties

Tensile properties of the composites (dimensions of 60 × 15 mm, thickness of 300–500 µm) were measured using a Mecmesin Multitest-1d motorized test frame, at room temperature. Loadings were recorded with a Mecmesin BF 1000N digital dynamometer at a strain rate of 10 mm min⁻¹. The test parameters were in agreement with ASTM D 882-02. At least 10 samples were tested for each composite.

7.3.6 Thermal analysis

Glass transition temperatures (T_g) and melting temperatures (T_m) of samples were determined with a Setaram DSC 131 device. The thermograms were recorded between 30 and 200 °C at a heating range of 10°C min⁻¹ under nitrogen flow. Only the second heating thermograms were collected. Sample amounts ranged from 10 to 12 mg.

Thermal stability of samples was determined with a Netzsh STA 449 F3 Jupiter simultaneous thermal analysis device. Sample amounts ranged from 10 to 12 mg. The thermograms were recorded between 25 and 800 °C at a heating rate of 10°C min⁻¹ under nitrogen flow.

The degree of crystallinity was determined as follows: $\chi_c(\%) = 100 \times \frac{(\Delta H_c + \Delta H_m)}{\Delta H_m^c}$, where ΔH_c is the cold crystallization enthalpy, ΔH_m is the melting enthalpy, and ΔH_m^c is the melting enthalpy of purely crystalline poly(l-lactide).^[65]

7.3.7 Scanning Electron Microscopy (SEM)

The morphology of the PLA/GNP composites was observed using SEM (FEI Quanta 400FEG, with acceleration voltage of 3 kV) at Centro de Materiais da Universidade do Porto. Composites selected for SEM analysis were fractured transversely under liquid nitrogen, applied on carbon tape, and sputtered with Au/Pa (10 nm film). The number of agglomerates per unit of area (mm²) as a function of agglomerate length, for different GNP-C loadings, was evaluated by direct measurements from 5 SEM images collected for each material, using ImageJ 1.45 software.

7.3.8 Raman spectroscopy

The unpolarized Raman spectra of GNP-C powder, PLA, and PLA/GNP-C composites were obtained under ambient conditions, in several positions for each sample. The linear polarized 514.5 nm line of an Ar⁺ laser was used as excitation. The Raman spectra were recorded in a backscattering geometry by using a confocal Olympus BH-2 microscope with a 50× objective in a volume of 10 μm³. The spatial resolution is about 2 μm. The laser power was kept below 15 mW on the sample to avoid heating. The scattered radiation was analyzed using a Jobin–Yvon T64000 triple spectrometer, equipped with a charge-coupled device. The spectral resolution was better than 4 cm⁻¹.

The spectra were quantitatively analyzed by fitting a sum of damped oscillator to the experimental data, according to the equation ^[66]:

$$(1) \quad I(\omega, T) = B(\omega) + (1 + n(\omega, T)) \sum_{j=1}^N A_{oj} \frac{\omega \Omega_{oj}^2 \Gamma_{oj}}{(\Omega_{oj}^2 - \omega^2)^2 + \omega^2 \Gamma_{oj}^2}$$

Here $n(\omega, T)$ is the Bose-Einstein factor: A_{oj} , Ω_{oj} , and τ_{oj} are the strength, wave number, and damping coefficient of the j th oscillator, respectively, and $B(\omega)$ is the background. In this work, the background was well simulated by a linear function of the frequency, which enables us to obtain reliable fits of Eq. (1) to the experimental data.

The fitting procedure was performed for all Raman bands collected from the same sample, but in different positions. This procedure allows us to determine the average and standard deviation (SD) values of the phonon parameters, namely, the wave number and intensity.

7.3.9 Biocompatibility assays

Human foreskin fibroblasts HFF-1 (from ATCC) were grown in DMEM+ (Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (V/V) newborn calf serum (Gibco) and 1% (V/V) penicillin/streptomycin (biowest) at 37 °C, in a fully humidified air containing 5% CO₂. The media were replenished every 3 days. When reaching 90% confluence, cells were rinsed with PBS (37°C) and detached from culture flasks (TPP®) using 0.25% (w/V) trypsin solution (Sigma Aldrich) in PBS. All experiments were performed using cells between passages 10–14. Biocompatibility of the materials was evaluated using HFF-1 cells cultured at the surface of PLA and PLA/GNP-C 0.25 wt.% films ($\varnothing = 5.5$ mm). Cells were seeded at a density of 2×10^4 cells mL⁻¹. Resazurin (20 μ L) solution was added at 24, 48, and 72 h and incubated for 3 h, fluorescence ($\lambda_{ex/em} = 530/590$ nm) read and metabolic activity evaluated (metabolic activity (%) = $F_{\text{sample}}/F_{\text{PLA}} \times 100$). All assays were performed in sextuplicate and repeated 3 times. Cell morphology was evaluated by immunocytochemistry at 72 h. Cells were washed with PBS and fixation was performed with paraformaldehyde (PFA, Merck) 4 wt.% in PBS for 15 min. PFA was removed, cells were washed with PBS, and stored at 4 °C. Cell membrane was permeabilized with Triton X-100 0.1 wt.% at 4°C for 5 min. Washing was performed with PBS and incubation performed with phalloidin (Alexa Fluor 488; Molecular Probes) solution in PBS in a 1:80 dilution for 20 min in the dark, to stain cell cytoskeletal filamentous actin. After rinsing with PBS, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) solution at 3 μ g mL⁻¹ was added to each well and incubated for 15 min in the dark to stain the cell nucleus. Finally, cells were washed and kept in PBS to avoid drying. Plates with adherent cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200). For both assays, negative control for toxicity were cells cultured at the surface of PLA incubated in DMEM+, for positive control cells at PLA surface were incubated in DMEM+ with Triton 0.1 wt.%.

7.4 Results and discussion

7.4.1 GNP-C physico-chemical characterization

XPS results (Figure 7.1(a)) show that GNP-C presents a low degree of oxidation (atomic percentage of oxygen, O 1s = 4%), as expected for a graphene-based material that should present oxygen-containing functional groups mostly at the platelet edges. Thermogravimetry results (Fig. 7.1(b)) show that most of the thermal degradation of GNP-C occurs above 450 °C, the initial slight decrease in weight being associated with desorption of impurities. About 9% weight decrease is observed between 450 and 800 °C, probably due to loss of oxygen-containing groups. In many works, GBM with single or few layers are obtained by exfoliation of graphite through oxidative processes, which lead to obtainment of materials with high oxygen content. [40] On the

other hand, GNP-C is exfoliated by rapid microwave heating, followed by ultrasonic treatment; therefore, oxygen content is only associated to structural defects at the edges of basal planes. For comparison, Haubner *et al.* observed that pristine graphite presents an oxygen content of 1.4% and weight loss below 5% when heated until 800°C. [41]

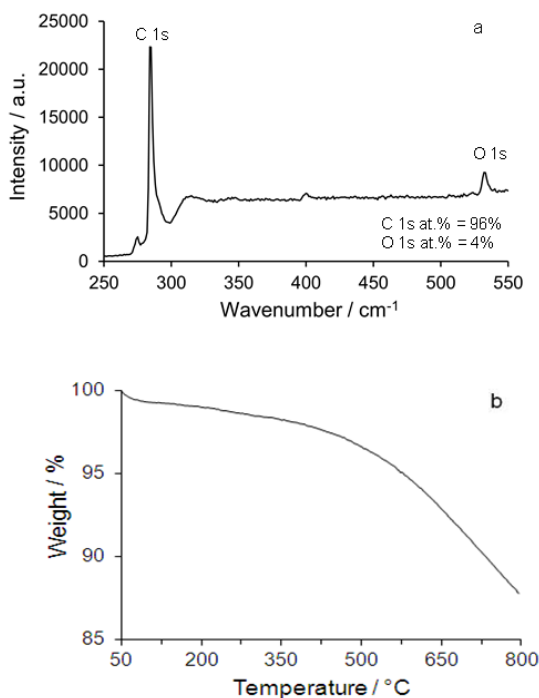


Figure 7.1: (a) XPS spectrum for atomic composition of GNP-C powder; (b) TGA curve for GNP-C powder.

7.4.2 FTIR analysis

Figure 7.2 shows the spectra typical for PLA presenting peaks from 3000 to 2850 cm⁻¹, correspondent to alkyl C-H stretches. The C=O stretching region appears around 1750 cm⁻¹. A band at 1450 cm⁻¹ attributed to CH₃ is found. The C-H deformation and asymmetric bands are present at 1380 and 1355 cm⁻¹. The C-O stretching modes of the ester groups are present around 1178 cm⁻¹. A band correspondent to C-O-C asymmetric stretching is present around 1078 cm⁻¹, and C-O alkoxy stretching vibration mode is at 1060 cm⁻¹. Also, a band correspondent to C-CH₃ vibrations is present around 1041 cm⁻¹. Bands at 865 and 754 cm⁻¹ are attributed to the amorphous and crystalline phase of PLA, respectively. [42, 43]

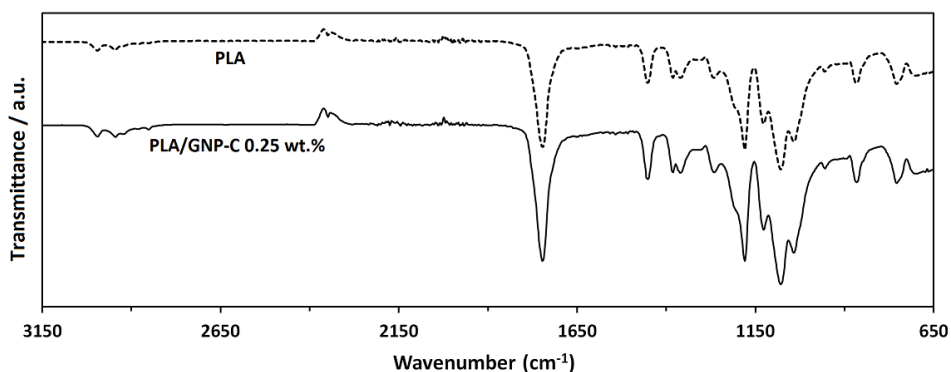


Figure 7.2: FTIR spectra for PLA and PLA/GNP-C 0.25 wt.% (180 °C, 20 min, and 50 rpm).

FTIR spectra for PLA and PLA/GNP-C 0.25 wt.% are similar. The low filler content makes it difficult to detect characteristic bands. Kong *et al.* also observed that incorporation of small amounts (0.3–3 wt.%) of carbon fillers (MWNTs) in the polyester poly(caprolactone) did not change the pristine polymer FTIR spectra.^[44]

7.4.3 Mechanical characterization

By adjusting operation parameters in melt blending, one can try to obtain a compromise between maximizing filler exfoliation and minimizing thermal/oxidative PLA degradation. In the literature, typical conditions for PLA melt processing correspond to temperatures of 160–180 °C, mixing times of 10–20 min, and rotation speeds around 50 rpm.^[3, 34, 45] In this work, PLA/GNP-C blends were initially prepared by mixing at 180°C during 20 min and at 50 rpm. The resulting composites were characterized in terms of Young's modulus, tensile strength, and toughness (area under stress–strain curve, AUC). Figure 7.3 shows that Young's modulus and tensile strength are maximum for a loading of 0.25 wt.%, with 20% increase in tensile strength, 12% increase in Young's modulus, and 16% increase in toughness. In comparison, Chartarrayawadee *et al.*^[46] observed an increase of 32% in tensile strength with the incorporation of 1 wt.% graphene oxide and stearic acid (1:1 ratio) in PLA. Also, Li *et al.*^[47] reported an increase in tensile strength of 39% with the incorporation of 1 wt.% graphene sheets in PLA. On the other hand, Narimissa *et al.*^[48] observed PLA/GNP-M 1 wt.% composites to have similar tensile strength and Young's modulus as pristine PLA, becoming brittle at 3 wt.% loading.

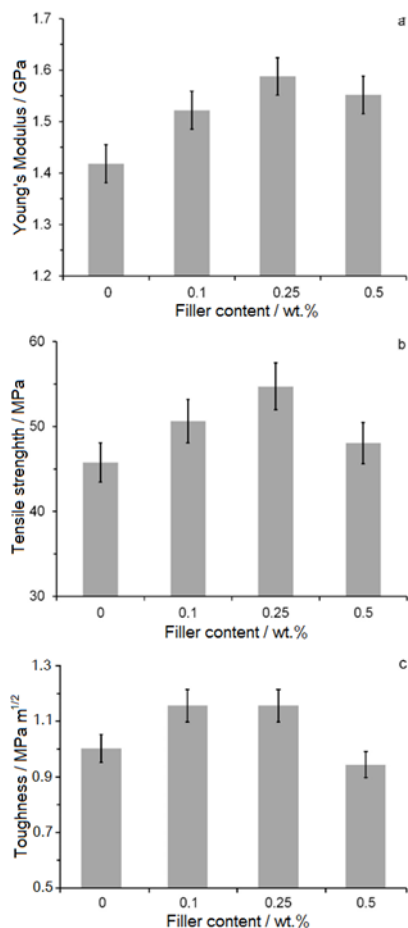


Figure 7.3: Effect of increasing nanofiller content on mechanical properties of PLA/GNP-C composites under the same processing conditions (180 °C, 20 min, and 50 rpm): (a) Young's modulus; (b) tensile strength; (c) toughness. Error bars represent standard deviations computed from measurements on at least 10 samples.

Figure 7.3 shows the decrease in mechanical performance when loading is raised to 0.5 wt.%, which can be attributed to increased platelet agglomeration introducing defects in the polymer matrix. The relation between agglomeration and loading level will be discussed further below. Toughness improves for 0.1 and 0.25 wt.% loadings, when compared to pristine PLA, but the two results are undistinguishable. Elongation at break results was of about 3.8%, with no significant differences observed between PLA and its composites.

The effects of varying mixing time and rotation speed were analyzed for the optimal loading of 0.25 wt.%. Figure 7.4 compares the results obtained for mixing times of 10, 15, and 20 min, and rotation speeds of 25 and 50 rpm. At 75 rpm, a brittle material was obtained, probably due to PLA degradation under high shear. The results show that the best processing conditions correspond to

20 min and 50 rpm. Lower mixing time or rotation speed probably yield worse GNP dispersion and hence lower mechanical performances. A higher mixing temperature of 200 °C was tested, but yielded very brittle materials, probably due to thermoxidative degradation of PLA. These results show that tuning of melt blending operation conditions can have an impact on the final properties of the nanocomposite, as it directly affects the quality of nanofiller dispersion and then possibility of polymer degradation.

Since the best processing conditions for PLA and PLA/GNP-C were determined to be 180 °C, 20 min, and 50 rpm, only the materials produced using these conditions were further characterized on physicochemical and biological studies.

7.4.4 Thermal analysis

Differential scanning calorimetry (DSC) was performed for all PLA/GNP-C loadings tested. The results are shown in Fig. 7.5. Glass transition occurs in the range 60–65 °C, followed by a small hysteresis peak, associated with physical relaxation. Melting takes place at around 155 °C, preceded by a broad cold-crystallization peak. It is interesting to note that PLA presents two combined melting peaks, which can be ascribed to differences in crystal morphology (*e.g.*, lamellar thickness).^[49] As GNP-C content increases, the higher temperature peak diminishes in intensity, indicating that polymer–nanoplatelet interaction leads to crystallinity uniformization.

Table 7.1 shows the estimated values of glass transition temperature (T_g) and melting temperature (T_m) for all samples tested. As the material is loaded with GNP-C, both T_g and T_m do not change significantly in relation to pristine PLA. An increase in T_g is often expected as a consequence of segment mobility being restricted due to filler-induced chain confinement. However, other studies have reported similar behavior for PLA loaded with nanofillers, concomitantly with observation of mechanical reinforcement.^[35, 39] A decrease in T_m would be observed if phase separation had occurred.^[50, 51]

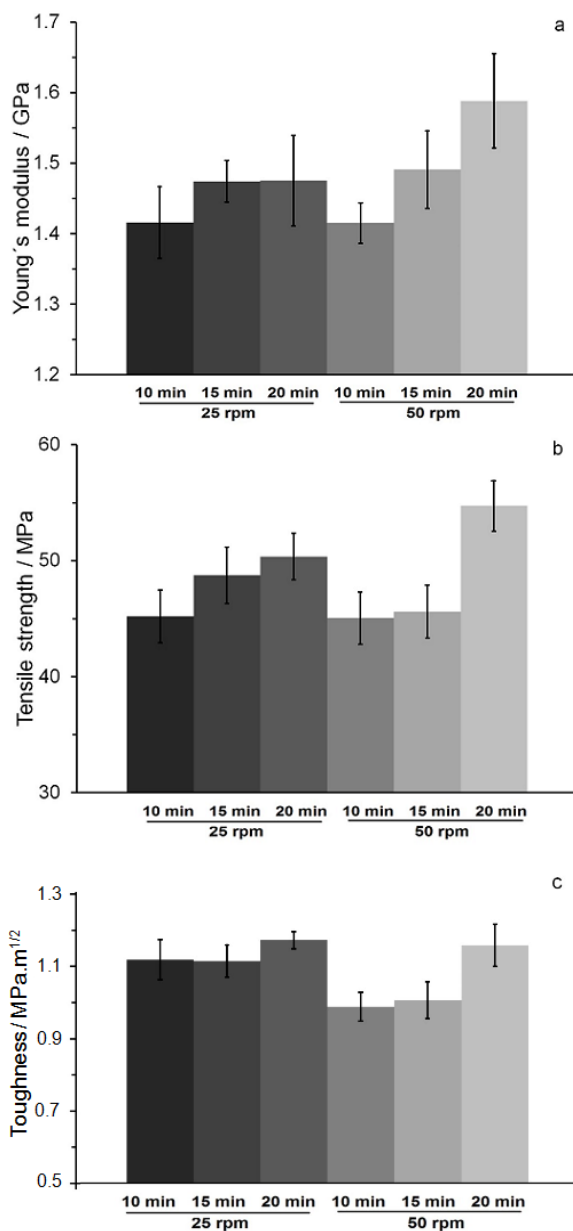


Figure 7.4: Effect of mixing time and rotation speed on mechanical properties of PLA/GNP-C composites processed at 180 °C, for a filler content of 0.25 wt. %: (a) Young's modulus; (b) tensile strength; (c) toughness. Error bars represent standard deviations computed from measurements on at least 10 samples.

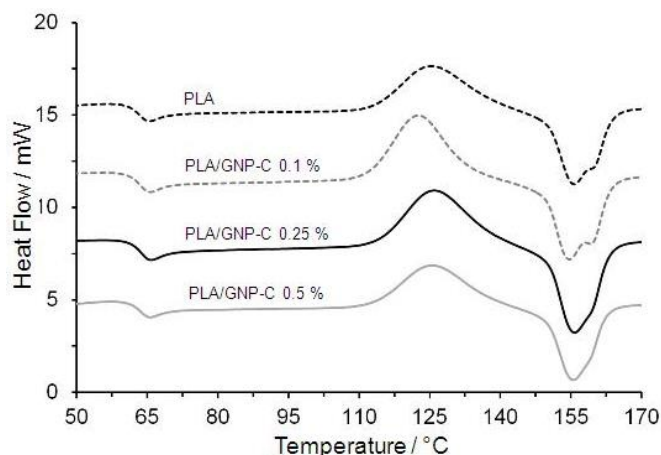


Figure 7.5: DSC thermograms for PLA and PLA/GNP-C (180 °C, 20 min, and 50 rpm) composites with different filler contents.

The computed degree of crystallinity (χ_c) is also shown in Table 7.1. Crystallinity seems to increase with GNP addition, but the differences are very small and are not expected to have a significant influence on the mechanical properties of the material. Interestingly, for the condition that resulted in better dispersion and mechanical performance (PLA/GNP-C 0.25 wt.%, 180 °C, 20 min, and 50 rpm), T_c is decreased by 3 °C, ΔH_c increased by 3 J g⁻¹, and crystallinity increased by 3%. This probably occurs because GNP-C particles cause heterogeneous nucleation, anticipating and increasing crystallization, as proposed by Kong *et al.* [52] for MWNTs, and similarly observed by Wang *et al.* [53] for GO.

Figure 7.6(a) shows the thermogravimetric curves obtained for pristine PLA and PLA/GNP-C. Thermal degradation is very similar for all samples: a single step between 300 °C and 370 °C, as expected for PLA. [54]

Table 7.1: Glass transition temperature (T_g) and melting temperature (T_m) for PLA and PLA/GNP-C composites (180 °C, 20 min, and 50 rpm) with different filler contents.

Samples (wt.%)	T_g (°C)	T_m (°C)	T_c (°C)	ΔH_c (J/g)	ΔH_m (J/g)	X_c (%)
PLA	63.2	155.7	125.4	25.3	22.0	3.57
GNP-C 0.10	63.6	155.5	126.0	25.4	22.1	3.50
GNP-C 0.25	63.4	154.8	122.5	28.2	22.1	6.42
GNP-C 0.50	63.6	155.9	126.1	26.0	21.6	4.75

Fig. 7.6(b), which represents the weight loss derivative (dTG) curves, allows a better differentiation between the results. The peak maximum values increase with graphene loading, indicating faster degradation rates. Similar behavior has been reported by Bao *et al.* [35] for PLA/graphene composites, which was attributed to the high thermal conductivity of graphene being the dominant contribution at low loadings. As a consequence, facilitated heat transfer overcomes the mass transfer barrier effect that often leads to improved thermal stabilities when lamellar fillers are used. In our particular case, the relatively small diameter of the GNP-C platelets may contribute to it being a less effective barrier, as the effect of path tortuosity is small. For a loading of 0.5 wt.%, the onset of degradation is shifted toward higher temperatures, which may indicate that at this concentration, diffusion of pyrolysis products is more effectively restrained.

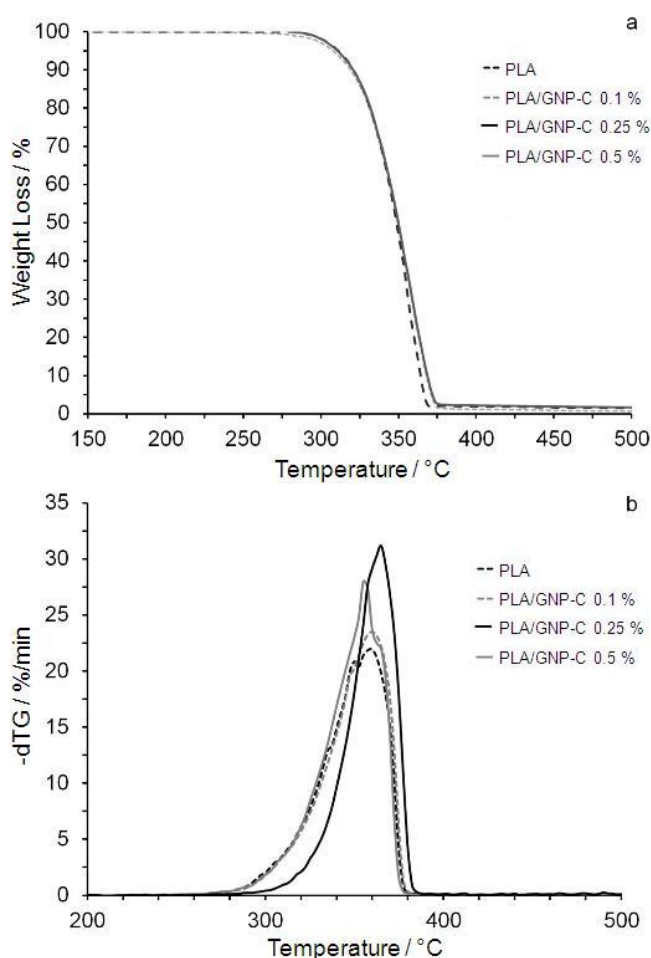


Figure 7.6: (a) TGA; (b) -dTG curves for PLA and PLA/GNP-C composites with different filler contents (180 °C, 20 min, and 50 rpm).

7.4.5 Scanning electron microscopy

SEM imaging was performed on PLA/GNP-C composites fractured under liquid nitrogen. Figure 7.7 shows the original GNP-C powder (Fig. 7.7(a)) and platelets found in fracture surfaces (Fig. 7.7(b–d)). Figure 7.7(a) shows that the powder is composed of flat platelets, lower than 2 μm in length, and smaller flake agglomerates. Figure 7.7(b) displays one of the largest agglomerates found in the fractured PLA matrix, which is seen to be composed of small aggregated flakes. Planar GNP can also be found embedded in the matrix (Fig. 7.7 (c, d)), showing that platelet individualization was achieved in the melt dispersion process.

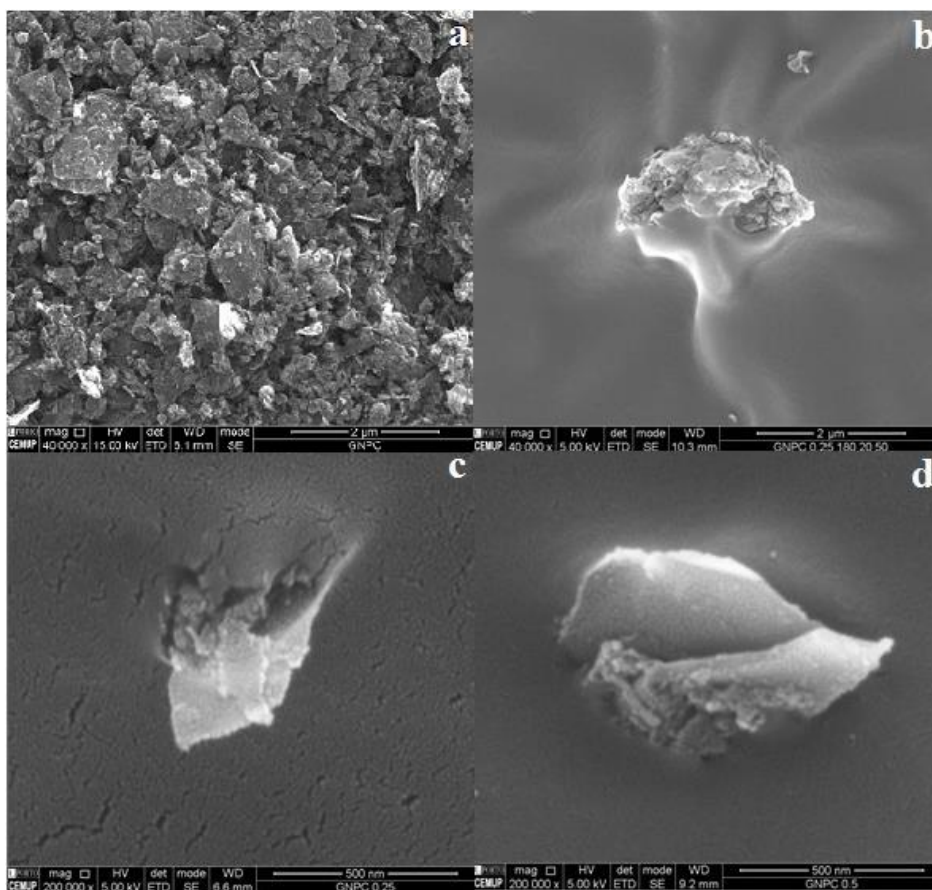


Figure 7.7: (a) SEM images of GNP-C powder at 100,000 $\times$ magnification; (b) fracture surfaces (under liquid nitrogen) of PLA/GNP-C composites (180 $^{\circ}\text{C}$, 20 min, and 50 rpm), showing GNP-C agglomerates at 40,000 $\times$ magnification; (c, d) individualized platelets at 200,000 $\times$ magnification, for loadings of 0.25 and 0.5 wt.% in PLA (180 $^{\circ}\text{C}$, 20 min, and 50 rpm), respectively.

As previously discussed, agglomeration at the higher loadings is the probable cause for the observed degradation of mechanical properties above 0.25 wt.% GNP-C content. To obtain a better notion of the incidence of agglomeration in these composites, different SEM images of fracture surfaces (at 5,000 $\times$ magnification) were inspected, and the number of agglomerates with different average sizes was computed per unit area of sample section, for the three loadings tested. Figure 7.8 shows the cumulative plots obtained and representative images. Very few agglomerates were found with sizes above 0.8 μm , and therefore these were ignored in the calculations. From Fig. 7.8, the composites with 0.1 and 0.25 wt.% loadings show similar results. However, for 0.5 wt.%, there is a noticeably higher concentration of agglomerates of all sizes. This is consistent with the observed decrease in mechanical performance. At this loading interplatelet interactions promote higher agglomeration, which cannot be efficiently overcome by melt mixing. The agglomerates work as defects in the polymer matrix, facilitating crack initiation during mechanical deformation. [55]

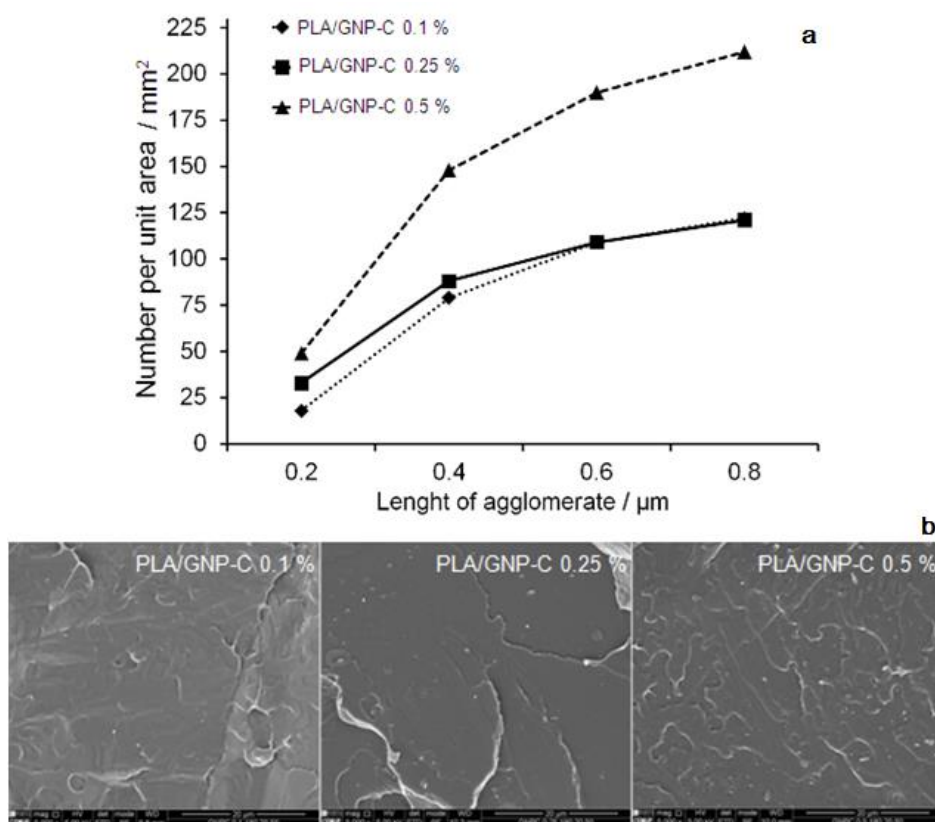


Figure 7.8: (a) Cumulative plots of number of agglomerates per unit of area (mm^2) as a function of agglomerate length, for different GNP-C loadings (180 °C, 20 min, and 50 rpm); (b) SEM images of fracture of surfaces for 5,000 $\times$ magnification.

7.4.6 Raman spectroscopy

As observed in Figure 7.9, the Raman spectrum of pristine PLA exhibits a well-defined band at 1458 cm^{-1} , in the $1200\text{--}1700\text{ cm}^{-1}$ spectral range. The Raman spectrum of the “as-received” GNP-C powder exhibits the strong D- and G-bands, located at 1345 cm^{-1} and at 1575 cm^{-1} , respectively, as well as the weak D'-band at 1615 cm^{-1} .^[56, 57] The G-band is assigned to the in-plane TO and LO vibrations of the carbon lattice, while the D and D' bands arise from double resonance processes involving the in-plane TO phonon with defects and edge structure of the graphene sheets. The Raman spectrum of PLA/GNP-C 0.5 wt.%, presented here as representative example, exhibits a band at 1458 cm^{-1} , from PLA matrix, and two sets of bands located at the left and right sides of this one, which deserve detailed attention.

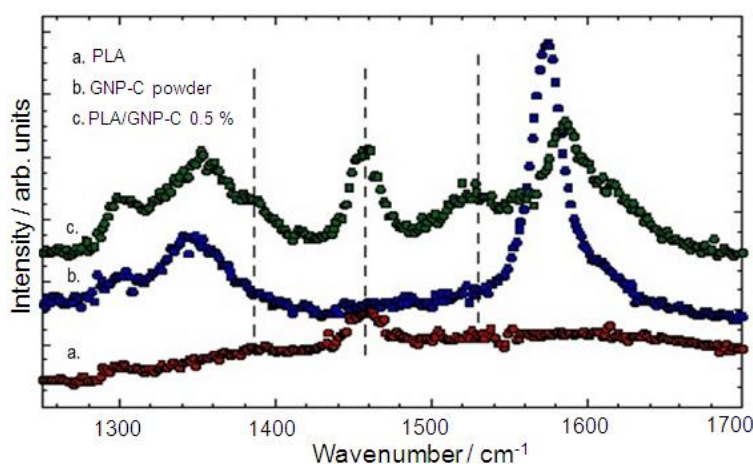


Figure 7.9: Representative unpolarized Raman spectra for PLA, GNP-C powder, and PLA/GNP-C 0.5 wt.% 20 min, recorded at ambient conditions.

Figure 7.10 shows the best fit of Eq. (1) to the spectrum of PLA/GNP-C 0.5 wt.%. Both low and high frequency sets can be deconvoluted into three main bands. The existence of several bands in the spectral range where the D-band is expected and where the PLA matrix does not exhibit any Raman band, evidencing structural disorder in the samples due to the exfoliation degree of GNP. In fact, the width of the D bands increases in the composites, evidencing disorder, as reported by Ramirez *et al.*^[56] The two bands, not presented in pristine GNP-C or PLA matrix spectra, appear in the composite sample: peak 3 at 1390 cm^{-1} and peak 5 at 1525 cm^{-1} . The existence of these bands is an evidence of physical interaction between polymer matrix and GNP-C, originating new modes in the system. Moreover, the frequency upshifts, of about 5 and 10 cm^{-1} observed for

peaks 2 and 6, respectively, when GNP-C is incorporated in PLA, corroborates the filler–matrix interaction and compression of the filler by the polymer matrix upon cooling. [58]

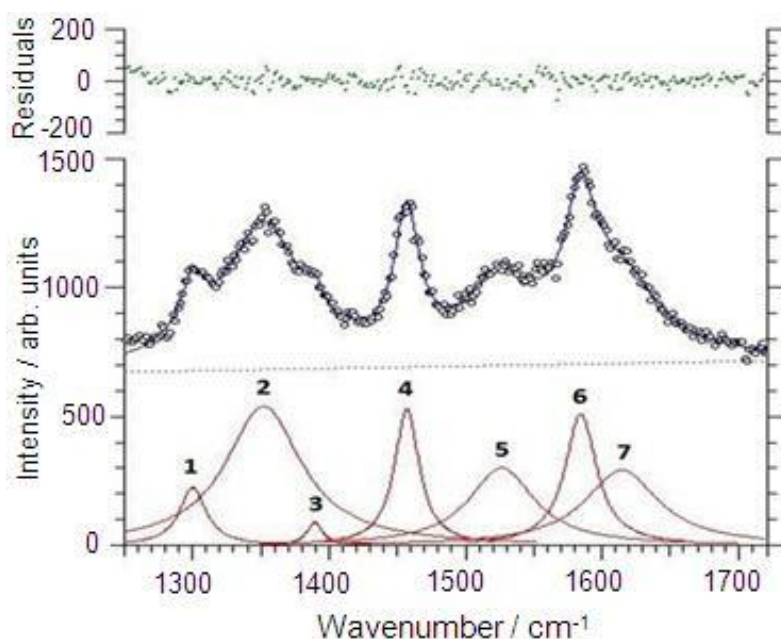


Figure 7.10: Example of Raman spectrum fitting according to Eq. (1) to PLA/GNP-C 0.5 wt.% 20 min. Bands 1–3 are attributed to D band and 5–6 to G band of GNP-C, while band 4 arises from PLA matrix.

Figure 7.11 shows the representative Raman spectra of PLA and PLA/GNP-C composites with different filler amounts, mixed during 10 (Fig. 7.11 (a)) and 20 min (Fig. 7.11 (b)). In both cases, as the graphene concentration increases, the intensity of Raman bands assigned to GNP-C increases. The Raman signals recorded for different sample positions did not show significant differences, pointing out the homogeneity of the graphene distribution within the polymer matrix, considering both individualized platelets and agglomerates.

From the fitting procedure, we have calculated the intensity of the observed Raman bands. It is well established that the ratio between the intensities of the D- and G-bands, I_D/I_G , is widely used for characterizing the level of defect in graphene. [59] Moreover, the intensity of a Raman band depends on the scattering cross-section of the corresponding mode and on the number of scatters per unit volume. Therefore, the ratio between the intensities of two aforementioned modes gives qualitative information regarding the ratio between the corresponding scatters concentration. [60]

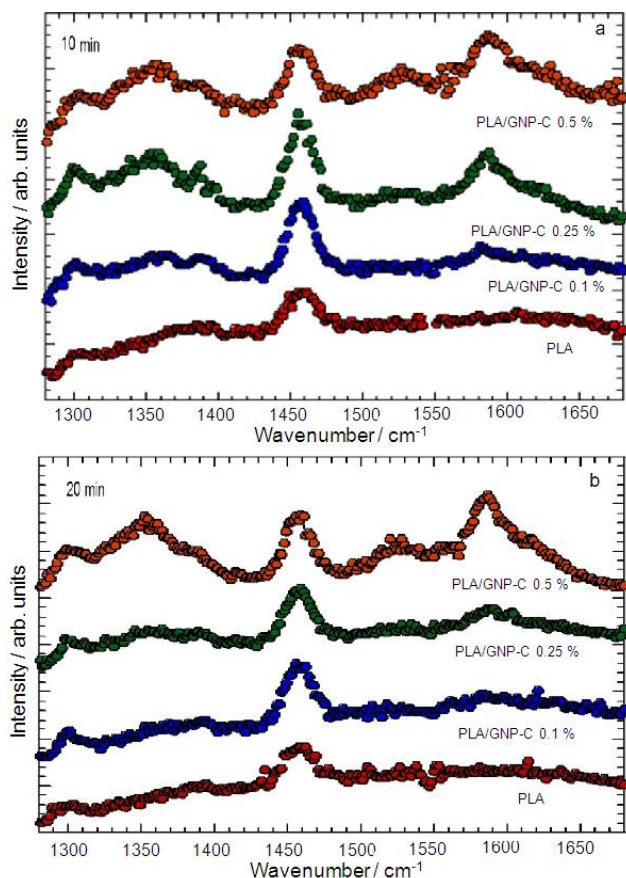


Figure 7.11: Unpolarized Raman spectra of PLA and PLA/GNP-C 0.1, 0.25, and 0.5 wt.% for (a) 10 and (b) 20 min mixing times.

Figure 7.12 shows the ratio between the intensities relative to the D and G bands (peaks 2 and 6), for the as-received GNP-C powder and to the PLA/GNP-C composites, for 10 and 20 min mixing times. In both cases, as the GNP-C concentration increases, the ratio I_D/I_G tends to increase, which is interpreted as an evidence for the increasing disorder in graphene, associated with defects arising from the interaction between graphene sheets and the polymeric matrix, and from the agglomerates of graphene in the matrix, as it is well evidenced by the SEM images. For a sufficiently high filler loading (0.5 wt.%), the content of agglomerated material introduces defects that can have a negative impact on the mechanical performance of the composite. This detrimental action surpasses the reinforcement effect, in agreement with the results of mechanical testing. This interpretation of the I_D/I_G ratio is consistent with the fact that, for the same GNP-C initial concentration, the ratio decreases with increasing mixing time, evidencing that longer mixing induces more efficient deagglomeration of the nanoplatelets within the PLA matrix.

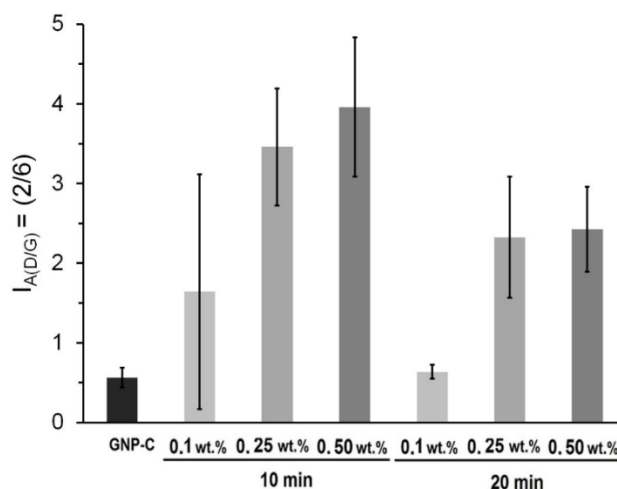


Figure 7.12: Intensity ratios of the D and G bands of monolayer GNP-C (peak 2/peak 6) for GNP-C powder and PLA/GNP-C 0.1, 0.2, and 0.5 wt.% for 10 and 20 min mixing times. Results are presented as average values and error bars represent standard deviation ($n > 3$).

7.4.7 Biocompatibility with fibroblasts

Biocompatibility was studied by culturing cells at materials' surface, evaluating cell metabolic activity and morphology. For providing a negative control for cell death, cells were cultured at the surface of PLA, presenting the typical “spindle”-like shape of fibroblasts. This was expected, as PLA is generally a biocompatible material.^[61] For positive control of cell death, PLA cultured in DMEM+ with Triton 0.1%, metabolic activity was close to 0% and cytoskeleton was disassembled. Since the best mechanical results were obtained for PLA/GNP-C 0.25 wt.% (180 °C, 20 min, and 50 rpm), only these materials were tested on biological assays.

HFF-1 cell metabolic activity at PLA/GNP-C 0.25 wt.% surface never decreased below 97%, in comparison with PLA (Figure 7.13). Also, immunocytochemistry images show no morphological differences between PLA and PLA/GNP-C 0.25 wt.%. Thus, filler incorporation has no impact on cell growth at materials surface. The fact that a small amount of GNP-C is used, together with the platelets being well encapsulated in the polymer may be on the base of the lack of toxicity observed.^[26] Yoon *et al.*^[62] observed that the incorporation of 2 wt.% GO in poly(d, l-lactic-co-glycolic acid) matrix by solvent mixing improved neuronal cell metabolic activity. However, Lahiri *et al.*^[63] observed ultrahigh molecular weight polyethylene/GNP-M 1 wt.% composites produced by electrostatic spraying to be toxic to osteoblasts. Toxicity can be caused by filler leaching^[26], which suggests that electrostatic spraying may not promote effective embedding of GBM in polymer matrix. Solvent mixing and melt blending seem to be more effective in this sense, therefore, avoiding toxicity effects.

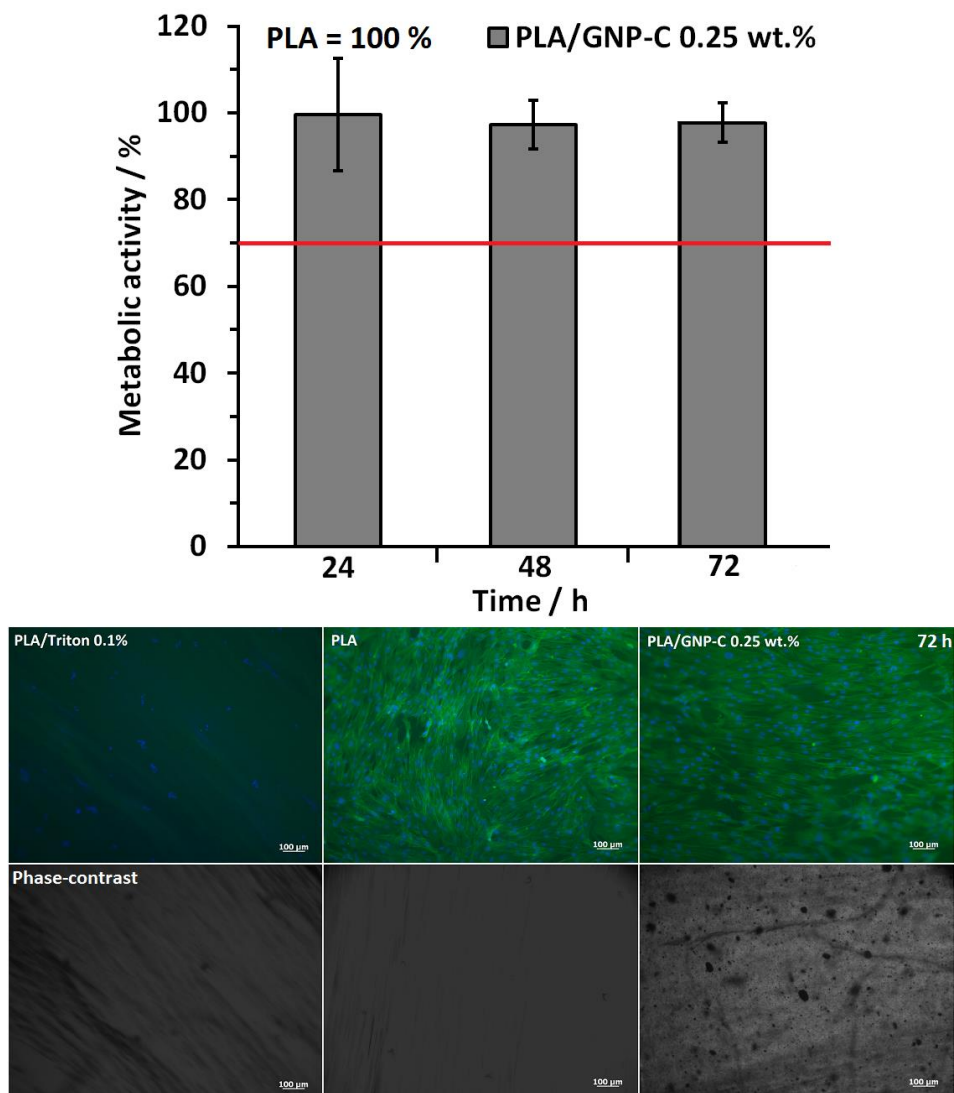


Figure 7.13: Metabolic activity of HFF-1 cells cultured at the surface of PLA/GNP-C 0.25 wt.% (180 °C, 20 min, and 50 rpm) in DMEM+, at 24, 48, and 72 h. Cell metabolic activity is represented as percentage in comparison with cells cultured at PLA surface in DMEM+ (100%). Results are presented as mean and standard deviation ($n = 6$). The red line at 70% marks the toxicity limit, according to ISO 10993-5:2009(E). For positive control of cell death, cells were cultured at PLA surface in DMEM+/Triton 0.1%, with metabolic activity being close to 0% (data not shown). For representative immunofluorescence images of HFF-1 at 72 h, cells were stained with DAPI (nuclei) blue and phalloidin (F-actin in cytoskeleton) green. Bottom line presents the phase-contrast images of materials surface. Scale bar represents 100 μm .

7.5 Conclusions

The purpose of this chapter was to evaluate the mechanical and thermal properties of PLA filled with GNP-C, which presents particularly small platelet diameters and thicknesses. The effects of blending conditions (mixing time, intensity, and temperature) and nanofiller loading on the composite properties were analyzed. Both factors were observed to have a major effect on the material's performance.

The best processing conditions were found to be mixing for 20 min at 50 rpm and 180 °C. The optimum loading was 0.25 wt.%, resulting in 20% increase in tensile strength, 12% increase in Young's modulus, and 16% increase in toughness. At higher loadings, defects due to filler agglomeration cause decay in mechanical performance. The higher incidence of agglomeration at 0.5 wt.% loading was demonstrated by SEM and Raman analysis, in what we believe are novel approaches for the use of these techniques in composite characterization.

Thermal analysis (DSC and TGA) showed no differences in glass transition or degradation temperature between pristine PLA and the composites. However, an increase in rate of thermal degradation with GNP-C loading was identified, which was interpreted in terms of a dominant effect of enhanced heat transfer over mass transfer barrier, in agreement with other reported works.

Melt mixing intensity and duration were found to have an impact on the mechanical properties of PLA/GNP-C composites.

Raman spectroscopy analysis, based on intensity ratios of D and G bands, confirmed that longer mixing times yield better dispersion of GNP. Evidence of effective interaction between the nanofiller and the polymer matrix was found in the form of frequency shifts and appearance of new Raman bands.

HFF-1 cells metabolic activity and morphology were not affected by the incorporation of 0.25 wt.% GNP-C in PLA.

The increased mechanical performance of these composites, achieved at low filler loadings, associated with their biocompatibility, provides interesting perspectives for use in biomaterial applications.

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Chapter 8

Physical-chemical properties and biocompatibility of PLA/GBMs composites:

Effect of biodegradation

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Macromolecular Nanotechnology

Effect of biodegradation on thermo-mechanical properties and biocompatibility of poly(lactic acid)/graphene nanoplatelets composites



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8 PHYSICAL-CHEMICAL PROPERTIES AND BIOCOMPATIBILITY OF PLA/GBMs COMPOSITES - EFFECT OF BIODEGRADATION

8.1 Scope

Physico-chemical characterization of biomaterials is important because it can impact on its performance, both by leading to “undesirable effects” or changing/preventing “the clinically relevant performance”. In the case of poly(lactic acid) PLA, this characterization is of outmost relevance, since it is a biodegradable polymer, so its properties considerably change after implantation. PLA has potential to be used for several biomedical applications, as for example orthopaedical uses, in which mechanical properties are relevant. For that reason, it is important to assure that they do not decrease at an excessive rate, hampering the implants clinical function. Graphene nanoplatelets (GNP) potential to be used as fillers to improve PLA mechanical performance has already been demonstrated in the previous chapters and their lack of adverse effects on cell lines proliferation at PLA/GNP composites surface shown. However, along biodegradation toxic products can be released, as well as GNP particles. The last were shown to have potential to be toxic in high concentrations, which will be hardly achieved by leaching from the polymer matrix, since the filler loading required to improve mechanical properties is very low. Despite that, two different grades of GNP were incorporated in PLA using the conditions that resulted in better mechanical performance, as described in Chapter 7. The produced composites were characterized after being subject to hydrolytic degradation in physiological conditions. Materials were analyzed by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), size exclusion chromatography (GPC-SEC), scanning electron microscopy (SEM), thermogravimetric analysis (TGA), tensile testing, creep-recovery testing, and biocompatibility assays.

8.2 State of the art

Poly(lactic acid) (PLA) has been widely investigated for biomedical applications because it is biodegradable, bioresorbable, biocompatible and easily processable.^[1,3] In addition, PLA-based products are approved for biomedical applications and contact with body fluids by Food and Drug Administration (FDA).^[3] This polymer has several applications in bioengineering, like the production of bioresorbable artificial ligaments, hernia repair meshes, scaffolds, screws, surgical plates, and suture yarns.^[4, 5] PLA can also be used in the production of drug delivery systems, and in the packaging of pharmaceutical products.^[6] To make this material more effective in several applications, namely orthopaedic, some properties can be improved, being mechanical performance usually the most relevant.^[7] Approaches like adjustment of crystallinity^[8], incorporation of plasticizers^[9], blends with other polymers^[7] and addition of nanofillers have been tested. Reinforcement with small amounts of nanofillers is an interesting option because it allows improvement of target properties without changing PLA's main characteristics. The most frequently reported nanofillers are nanoclays^[10], carbon nanotubes^[11], and nanosilicas^[12].

Graphene is a single layer of sp^2 carbon atoms arranged in a honeycomb structure and possesses extraordinary mechanical strength and an extremely high specific surface area.^[13] Graphene nanoplatelets (GNPs) are a commercial product with reduced cost when compared to single layer graphene. GNPs are constituted by few stacked graphene layers, possessing oxygen-containing functional groups along the edges.^[14] They present high aspect ratio, thus being able to form a percolated network with large interfacial interaction between nanoplatelets and polymer matrix, resulting in effective load transfer and increased strength.^[15] In a previous study, we have shown that GNPs, at loadings below 1 wt.%, can provide effective mechanical reinforcement of PLA.^[16] However, in that work the composite films were prepared by solvent-mixing technique, which may raise issues related to the toxicity of solvent residues that may remain in the materials, which are often difficult to remove, and to occupational health concerns in industrial production.^[17] Melt blending, on the other hand, is a solvent-free and easily up-scalable processing method, which has been adopted for the current work. Several authors have used this technique for production of PLA composites filled with GNP or other graphene-related materials in recent years, reporting improvements in mechanical performance at loadings below 1 wt.%.^[18-21] Narimissa *et al.*^[22] observed that higher loadings are unbeneficial and the composites become brittle.

To our knowledge, there are no studies regarding the behaviour of PLA filled with graphene-based materials along biodegradation time. This is a pertinent issue when use as implanted

medical devices is intended. PLA biodegrades mainly by simple hydrolysis of the ester linkages, forming monomeric lactic acid that is eliminated through the Krebs cycle.^[23] Poly (l-lactide) acid (PLLA), which presents better mechanical properties, degrades more slowly than the d-lactide, with complete degradation occurring between 10 months to 4 years, depending on factors like molecular weight, crystallinity, material geometry and location in the body. Degradation leads to gradual decreases in molecular weight and mechanical performance.^[24] For some orthopaedic applications, like prosthetic ligaments, one important concern regarding PLA is to maintain mechanical properties for long time periods, preventing rupture by fatigue and excessive laxity due to creep, while the natural ligament is rebuilding itself.^[25]

A study by Wan *et al.* compared PLA loaded with carbon fibre to pristine PLA, and reported that the composite material had better mechanical properties along degradation than the unloaded polymer.^[26] However, not all fillers are able to improve the mechanical performance of PLA along degradation time.^[27] Since this subject has not been addressed for graphene-based materials (GBMs), the current work focuses on characterization of the mechanical and thermal behaviour of PLA filled with two grades of graphene nanoplatelets (GNP-M and GNP-C) along 6 months degradation. In addition, the biocompatibility of the composites and of their degradation products is studied in terms of effects on metabolic activity, cell death and morphology of human fibroblasts (HFF-1).

8.3 Materials and methods

8.3.1 Materials

Poly(lactic acid) (PLA) 2003D (4% d-lactide, 96% l-lactide content), was purchased from Natureworks (Minnetonka, USA).

Graphene nanoplatelets (GNP), grade C750 (GNP-C) and M-5 (GNP-M) were acquired from XG Sciences (Lansing, USA). The manufacturer reports the following characteristics. GNP-C: average thickness lower than 2 nm and surface area of 750 m² g⁻¹; the platelet diameters have a distribution that ranges from tenths of micrometer up to 1–2 µm. GNP-M: average thickness of 6–8 nm, diameter of about 5 µm and surface area between 120 and 150 m² g⁻¹. Production of these GNP grades is based on exfoliation of sulphuric acid-based intercalated graphite by rapid microwave heating, followed by ultrasonic treatment.^[28]

8.3.2 Preparation and degradation of PLA and PLA/GNP composites

Composites of PLA filled with GNP-M and GNP-C at 0.25 wt.% loadings were prepared by melt blending in a Thermo Haake PolyLab internal mixer (internal mixing volume 60 cm³) at 180 °C, with 20 min mixing time and with a rotor speed of 50 rpm. The conditions used had been previously optimized in terms of filler loading, mixing temperature and duration, and rotor speed.^[29] After removal from the mixer, the composites were moulded in a hot press at 190 °C for 2 min, under a pressure of 150 kg cm⁻², into sheets with approximately 0.5 mm thickness. After pressing, the sheets were rapidly cooled in water at room temperature. PLA without GNP addition was processed in the same way to be used as a control. Samples with different dimensions were cut from these sheets, depending on the characterization test.

Samples with dimensions of 60 × 15 mm and thickness of approximately 0.5 mm, were immersed in 50 mL PBS in sterile conditions in plastic flasks (3 per flask) and incubated for 2, 4 and 6 months at 37 °C under 100 rpm.

8.3.3 Physico-chemical characterization

8.3.3.1 Scanning Electron Microscopy (SEM)

The morphology of the samples was observed using SEM (FEI Quanta 400FEG, with an acceleration voltage of 3 kV) at Centro de Materiais da Universidade do Porto. Composites selected for SEM analysis were fractured transversely under liquid nitrogen, applied on carbon tape and sputtered with Au/Pa (10 nm film).

8.3.3.2 Fourier transform infrared spectroscopy (FTIR)

FTIR was used to evaluate the effect of fillers incorporation and biodegradation on materials' chemical composition. Spectra were obtained between 600 and 4000 cm⁻¹, with 100 scans and a resolution of 4 cm⁻¹, using a spectrometer ABB MB3000 (ABB, Switzerland) equipped with a deuterated triglycine sulphate detector and using a MIRacle single reflection horizontal attenuated total reflectance (ATR) accessory (PIKE Technologies, USA) with a diamond/Se crystal plate.

8.3.3.3 X-ray diffraction analysis (XRD)

XRD was used to evaluate the effect of fillers incorporation and biodegradation on materials' crystalline structure. Samples were scanned using a Philips X'Pert diffractometer in the range of diffraction angle 2θ , according to a Bragg-Brentano geometry. The anticathode used was cobalt with a wavelength of 0.178896 nm. Samples in the form of films were analysed as they were, and GNP-M and C powders were gently placed in an aluminium sample holder.

8.3.3.4 Gel permeation chromatography and size exclusion chromatography (GPC-SEC)

In order to evaluate materials degradation along 6 months in PBS, samples were dried in an oven at 50 °C during 48 h and stored in a desiccator. Samples were dispersed in chloroform during 24 h (in a fridge at 4 °C), at a concentration of 5 mg mL⁻¹, sonicated for 5 min, filtered and 300 µL injected for GPC-SEC analysis. Calibration was performed with Shodex SM-105 set of polystyrene standards ($1.27 \times 10^3 < M_w < 3.04 \times 10^6$), at the same concentration as the studied samples. Data were processed using the program Multioffline (Polymer Laboratories) and molecular weight (M_w) determined. At least 3 replicates were performed for each sample. The equipment used was the PLA-GPC 50 Plus with a chloroform flow of 1 mL min⁻¹, oven temperature of 30 °C, using a column Viscotek T4000 Org GPC/SEC (300 × 8.0 mm) and a pre-column Viscotek Tguard Org Guard (10 × 4.6 mm).

8.3.3.5 Thermal analysis

Thermal stability of samples was determined with a Netzsh STA 449 F3 Jupiter device. Sample amounts ranged from 10 to 12 mg. The thermograms were recorded between 30 and 800 °C at a heating rate of 10 °C min⁻¹ under nitrogen flow.

8.3.4 Mechanical properties characterization

Tensile properties of the samples (60 × 15 × 0.5 mm) were measured using a Mecmesin Multitest-1d motorized test frame, at room temperature. Loadings were recorded with a Mecmesin BF

1000 N digital dynamometer at a strain rate of 10 mm min^{-1} . The test parameters were in agreement with ASTM D 882-02. At least five samples were tested for each composite.

Creep/recovery tests were performed with a DMA 242 E Artemis (Netzsch) equipment in tension mode at 37°C . Samples ($12 \times 6 \times 0.5 \text{ mm}$) were subjected to a static force of 6.0 N during 10 min with a recovery mode also lasting 10 min. Multi-cycle measurements were carried out using the same conditions for 10 cycles of creep/recovery, each with the duration of 10 min.

8.3.5 Biocompatibility evaluation

Human foreskin fibroblasts HFF-1 (from ATCC) were grown in DMEM (Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% (V/V) newborn calf serum (Gibco) and 1% (V/V) penicillin/streptomycin (biowest) at 37°C , in a fully humidified air containing 5% CO_2 . The media were replenished every 3 days. When reaching 90% confluence, cells were rinsed with PBS (37°C) and detached from culture flasks (TPP®) using 0.25% (w/V) trypsin solution (Sigma Aldrich) in PBS.

Biocompatibility of the materials was evaluated by the direct contact test, and by the elution test method. In the direct contact test, cells were cultured on the surface of PLA, PLA/GNP-M and C 0.25 wt.% films ($\varnothing = 5.5 \text{ mm}$). For the elution test method a monolayer of cells, after 24 h cell growth, was exposed to materials' extracts obtained after 6 months incubation in PBS (50 μL in 150 μL DMEM) at 37°C and 100 rpm, as described in 8.3.2. In both assays, cells were seeded in 96 well plates (7500 cells per well). All experiments were performed using cells between passages 10–14.

Negative control (DMEM) for metabolic activity decrease, toxicity or changed morphology, for all biocompatibility assays was performed incubating cells in DMEM. Positive control for metabolic activity decrease, toxicity or changed morphology, was performed incubating cells in DMEM with 0.1% Triton X-100 (Triton 0.1%). For the elution test method, performed to test degradation products biocompatibility, DMEM control consists in replacing culture media by 50 μL PBS incubated 6 months in the same conditions as other samples (PBS 6 M), in 150 μL DMEM+.

8.3.5.1 Cytotoxicity

For cell metabolic activity evaluation, at 24, 48, and 72 h incubation with materials or materials extracts, 20 μL resazurin solution were added and incubated for 3 h, fluorescence ($\lambda_{\text{ex/em}} = 530/590 \text{ nm}$) read, and values determined as follows: Metabolic activity (%) = $F_{\text{sample}}/F_{\text{DMEM}} \times 100$. All assays were performed in sextuplicate and repeated 3 times.

Cell viability/membrane integrity was evaluated by LIVE/DEAD assay. Hoechst 33342 permeates membrane and stains nucleic acids. Calcein also permeates the membrane and is metabolized in the cytoplasm by intracellular esterases originating a green highly fluorescent derivative. The cell membrane has a residual permeability to propidium iodide, which only enters cells whose membrane integrity is compromised, staining nucleic acids. After cells being incubated for 72 h on the surface of the materials or in presence of extracts from the materials, DMEM was removed and cells incubated with Hoechst 33342 (Molecular Probes) and Calcein (Molecular Probes), both at $2.5 \mu\text{g mL}^{-1}$ in PBS for 15 min, at 37°C in the dark. Then, propidium iodide (PI) (Sigma Aldrich) in PBS was added to each well to a final concentration of $1.25 \mu\text{g mL}^{-1}$ and images acquired in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200).

8.3.5.2 Immunocytochemistry

Cell morphology was evaluated by immunocytochemistry after cells being incubated for 72 h on the surface of the materials or in presence of extracts from the materials. Cells were washed with PBS and fixation was performed with paraformaldehyde (PFA - Merck) 4 wt.% in PBS for 15 min. PFA was removed, cells washed with PBS and stored at 4°C . Cell cytoskeletal filamentous actin can be visualized by binding of fluorescent phalloidin and the nucleus can be stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) that intercalates with nucleic acids. Cell membrane was permeabilized with triton X-100 0.1 wt.% at 4°C for 5 min. Washing was performed with PBS and incubation performed with phalloidin (Alexa Fluor 488; Molecular Probes) solution in PBS in a 1:80 dilution for 20 min in the dark. After rinsing with PBS, DAPI (Sigma Aldrich) solution at $3 \mu\text{g mL}^{-1}$ was added to each well and incubated for 15 min in the dark. Finally, cells were washed and kept in PBS to avoid drying. Plates with adherent cells were observed in the Carl Zeiss – Axiovert 200.

8.3.6 Statistical analysis

Statistical analysis was performed using the software SPSS20 (IBM) performing Kruskal–Wallis one-way analysis of variance. Multiple means comparison were performed between samples to identify significant differences, which were considered for $p < 0.05$.

8.4 Results

The main goal of this work was to investigate the effect of biodegradation on the mechanical and biological properties of PLA filled with low amounts (0.25 wt.%) of GNP-M and C. For that reason, materials were characterized by several techniques and results are presented next.

8.4.1 Physical-chemical characterization

8.4.1.1 Scanning electron microscopy

Figure 8.1 shows that after 6 months degradation in PBS at 37 °C and 100 rpm, PLA and PLA/GNP-C present evidence of surface erosion, shown in the images within elliptical contours. In PLA/GNP-M 6 M, surface erosion does not seem to be so evident in SEM images. However, some voids are observed, mainly around nanoplatelets (Fig. 8.1(D)), which are not seen in the non-degraded sample and in PLA/GNP-C before and after degradation.

Both GNP-M and GNP-C are well dispersed in PLA matrix, presenting mostly completely exfoliated particles and small agglomerates, as shown by SEM images and agglomerate size analysis in Figure C1 (Appendix C).

8.4.1.2 FTIR and XRD

FTIR and XRD spectra were performed and are presented in Appendix C. No differences were observed between samples in FTIR analysis, due to the low amount of fillers present (0.25 wt.%). However, XRD analysis, allowed identification of the fillers characteristic diffraction bands in the composites. No differences were seen between samples before and after 6 months degradation, indicating permanence of the fillers.

8.4.1.3 GPC-SEC

Figure 8.2 presents average molecular weight ($\bar{M}_w$) evolution along 6 months degradation for PLA, and for PLA/GNP-M and PLA/GNP-C at 0.25 wt.% loading.

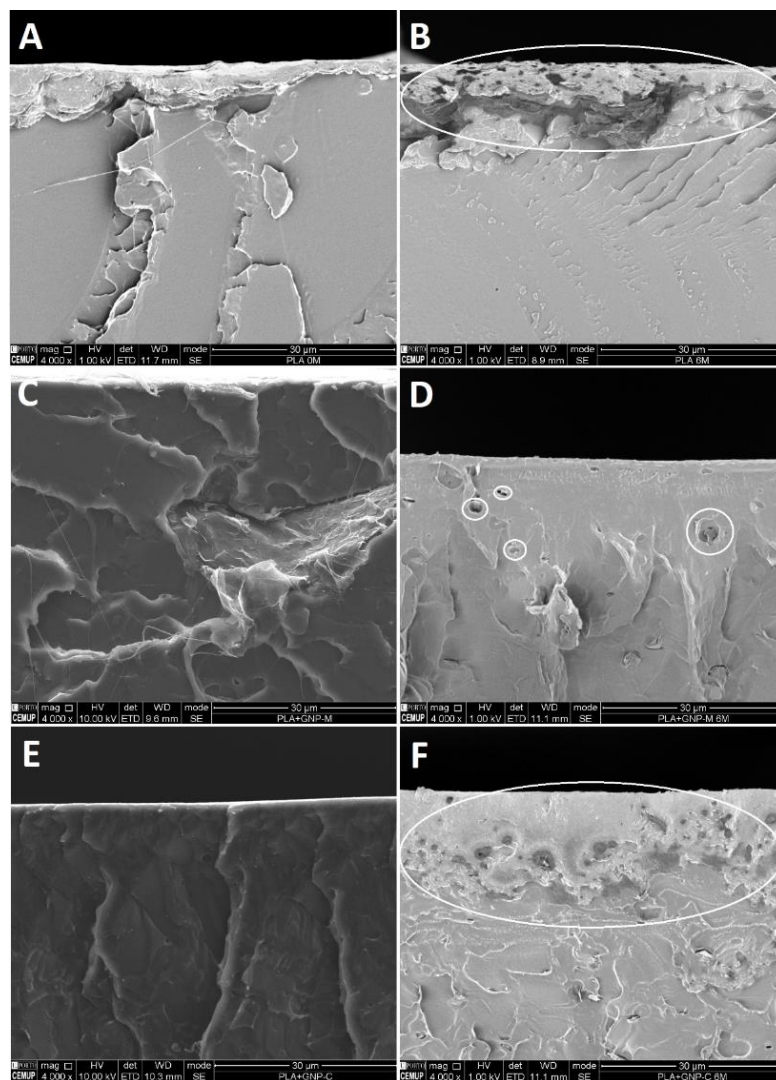


Figure 8.1: SEM images of PLA 0 M (A), PLA 6 M (B), PLA/GNP-M 0.25 wt.% 0 M (C), PLA/GNP-M 0.25 wt.% 6 M (D), PLA/GNP-C 0.25 wt.% 0 M (E), and PLA/GNP-C 0.25 wt.% 6 M (F), broken under liquid nitrogen. Magnification is 4000×. Scale bar represents 30 μm. Elliptical contours point out surface erosion and possible bulk degradation.

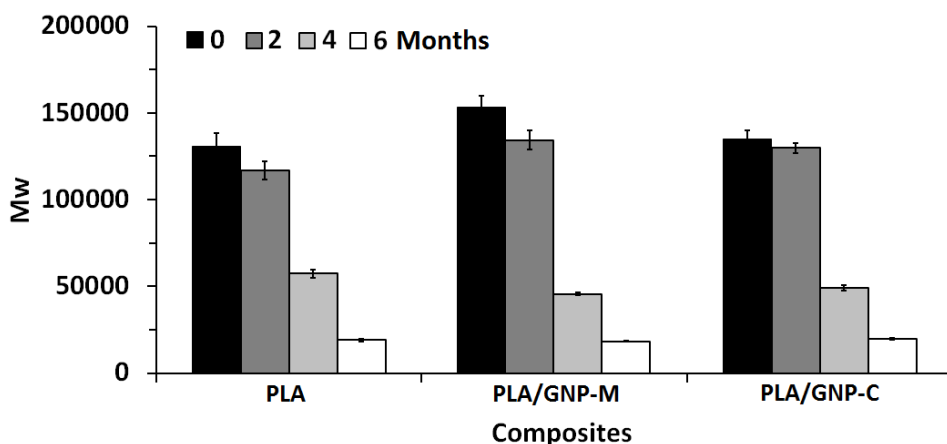


Figure 8.2: Average $\bar{M}_w$ evolution for PLA, PLA/GNP-M and C 0.25 wt.% along degradation time (0, 2, 4 and 6 months). Results are presented as mean and standard deviation ($n = 3$).

8.4.1.4 Thermogravimetric analysis

Figure 8.3 shows that thermogravimetric curves for PLA, PLA/GNP-M and C 0.25 wt.% 0 M and 6 M are very similar, consisting of a single step between 300 °C and 370 °C, as expected for PLA.^[30] Temperatures of onset of intense thermal degradation, T_d (shown in a figure inset), are similar for undegraded samples, PLA/GNP-M showing a slightly higher value (2.6 °C more than PLA). After biodegradation, T_d decreases by about 10 °C in all samples.

8.4.2 Mechanical characterization

8.4.2.1 Tensile tests

Figure 8.4 shows that incorporation of 0.25 wt.% of GNP-C and M in PLA increased its Young's modulus by 14%. Also, tensile strength is increased by 20% with GNP-C incorporation and by 6% with GNP-M. Only with GNP-C is observed a considerable improvement in toughness (20%). Best performance of GNP-C probably arises from its smaller dimensions. This may allow for more effective dispersion and interaction with the polymer matrix.

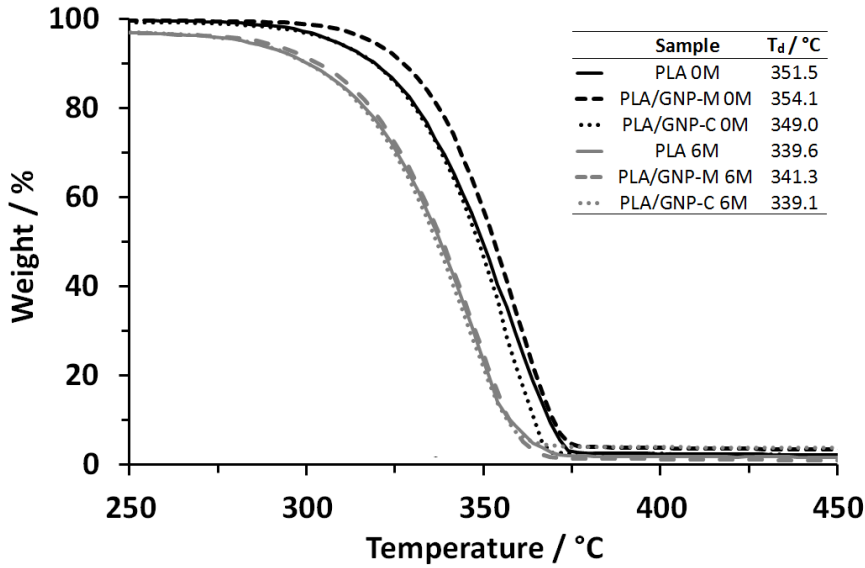
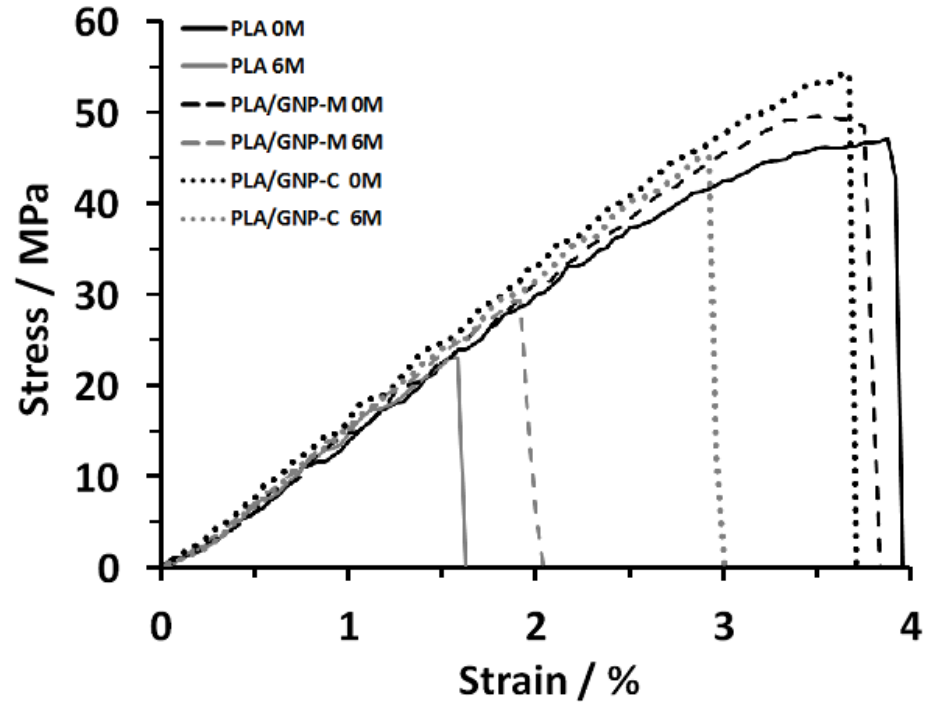


Figure 8.3: TGA curves and temperatures of onset of intense thermal degradation (T_d) for PLA and composites before and after 6 months degradation in PBS at 37 °C and 100 rpm.

After 6 months degradation in PBS, no significant changes are observed in Young's modulus for all materials tested. However, decreases in tensile strength, elongation at break, and toughness are considerably higher for PLA (2.6, 2.5, and 10-fold, respectively) comparing with PLA/GNP-M (1.6, 1.8, and 3.3-fold), and PLA/GNP-C (1.4, 1.4 and 1.7-fold). Thus, the incorporation of both fillers is improving PLA mechanical properties after 6 months degradation, being GNP-C the most effective material in this sense.

8.4.2.2 Creep and recovery tests

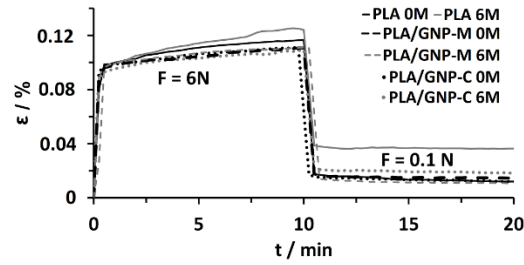
Figure 8.5 shows that the materials present similar behaviour, with a relatively large instantaneous increase of the strain upon load application, followed by a steady creep, and rapid strain recovery after load removal. PLA after 6 months biodegradation presents the highest permanent creep strain (ϵ_∞), with a value of 0.029%, comparing with 0.016% for the non-degraded polymer. The GNP-filled samples display similar ϵ_∞ before and after biodegradation, varying between 0.01 and 0.014%.



Sample (n = 5)	Young's Modulus Gpa		Elongation at break %		Tensile Strength MPa		Toughness (AUC) MPa m ^{1/2}	
	Avr	SD	Avr	SD	Avr	SD	Avr	SD
PLA 0M	1.4	0.13	3.8	0.51	45.8	3.38	1.0	0.16
PLA+GNP-M 0M	1.6	0.00	3.8	0.55	48.5	4.11	1.0	0.29
PLA+GNP-C 0M	1.6	0.01	3.8	0.12	54.7	3.64	1.2	0.10
PLA 6M	1.4	0.31	1.5	0.51	17.6	9.37	0.1	0.01
PLA+GNP-M 6M	1.7	0.02	2.1	0.28	30.2	2.64	0.3	0.05
PLA+GNP-C 6M	1.6	0.13	2.7	0.68	39.4	8.29	0.6	0.24

Figure 8.4: Stress-strain curves and mechanical parameters for PLA and composites before and after 6 months degradation in PBS at 37 °C and 100 rpm.

Figure 8.6 shows that, after 10 creep/recovery cycles, all non-degraded materials (0 M) show similar maximum creep strain - $\epsilon_{\max}$ (at 6 N), being of 0.118, 0.103, and 0.104% for PLA, PLA/GNP-M, and C 0.25 wt.%, respectively. After 6 months degradation in PBS, the PLA samples ruptured after 4 cycles (Fig. 8.6(A)), showing a very intense cumulative increase in permanent creep strain. Remarkably, on the other hand, PLA/GNP-M and C did not rupture after 10 cycles (Fig. 8.6(B, C)) and presented only a slight increase in ϵ_{∞} .



Sample (n=3)	$\varepsilon_{\max} / \%$ (max. creep strain)	SD / %	$\varepsilon_{\infty} / \%$ (permanent creep strain)	SD / %
PLA 0M	0.1153	0.00197	0.0154	0.00515
PLA 6M	0.1249	0.01728	0.0290	0.00939
PLA/GNP-C 0M	0.1119	0.00729	0.0114	0.00139
PLA/GNP-C 6M	0.1025	0.00630	0.0123	0.00758
PLA/GNP-M 0M	0.1102	0.00684	0.0137	0.00171
PLA/GNP-M 6M	0.1047	0.00974	0.0104	0.00793

Figure 8.5: Creep and recovery curves for PLA, PLA/GNP-M and C 0 and 6 M at 37 °C.

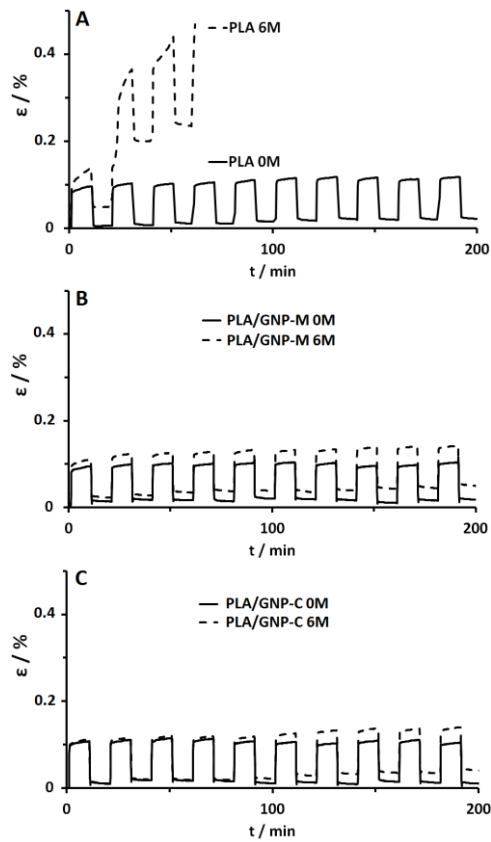


Figure 8.6: Multicycle creep-relaxation curves for PLA and composites before (0 M) and after 6 months (6 M) degradation in PBS at 37 °C. ε – strain.

8.4.3 Biocompatibility with human fibroblasts

Biocompatibility of PLA, PLA/GNP-M, and C 0.25 wt.% composites, and of their degradation products after 6 months biodegradation in PBS at 37 °C at 100 rpm, was studied in terms of effects on metabolic activity, cell death and morphology of human fibroblasts (HFF-1). Results are presented below.

8.4.3.1 Biocompatibility of PLA and PLA/GNP materials

As presented in Figure 8.7(A), HFF-1 cells metabolic activity at PLA surface was 75% at 24 and 48 h, and 94% at 72 h, comparing with negative control for metabolic activity decrease - cells incubated in DMEM. These differences are statistically significant ($p < 0.05$) at 24 and 48 h. Also, positive control for metabolic activity decrease - cells incubated with Triton 0.1% in DMEM, as expected presented almost no metabolic activity ($< 5\%$ - data not shown). In comparison with PLA, cell metabolic activity was maintained for PLA/GNP-M and PLA/GNP-C ($p > 0.05$). Considering that a sample has cytotoxic potential if its metabolic activity is reduced to less than 70% comparing to negative control for metabolic activity ISO 10993-5:2009(E) [32], it can be observed from Fig. 8.7(A), that the incorporation of both fillers has no significant toxic impact on metabolic activity of cells cultured at material's surface, because it is always higher than 70%.

Fig. 8.7(B) shows representative images for HFF-1 cells fluorescently labelled for LIVE/DEAD assay. As expected, positive control of cell death (cells incubated with PLA/Triton 0.1 wt.% in DMEM + for 72 h) unveils cell death close to 100%. Negative control was performed incubating cells with DMEM, presenting mostly live cells with scarce dead cells. In agreement with metabolic activity results, no considerable differences are observed between DMEM and PLA at 72 h (Fig. 8.7(B)). Also, there are no more dead cells at the surface of PLA/GNP-M and C than at PLA surface.

Cells cultured at material's surface were stained and observed after 72 h incubation in DMEM. Immunofluorescence images (Fig. 8.7(C)) show that toxicity positive control cells, which were incubated with triton 0.1%, presented disassembled cytoskeleton. Negative toxicity control, performed with cells cultured in DMEM, exhibiting the typical "spindle" like shape of fibroblasts. Also, cells incubated at the surface of PLA, PLA/GNP-M and C 0.25 wt.% had a normal morphology, similar to negative control.

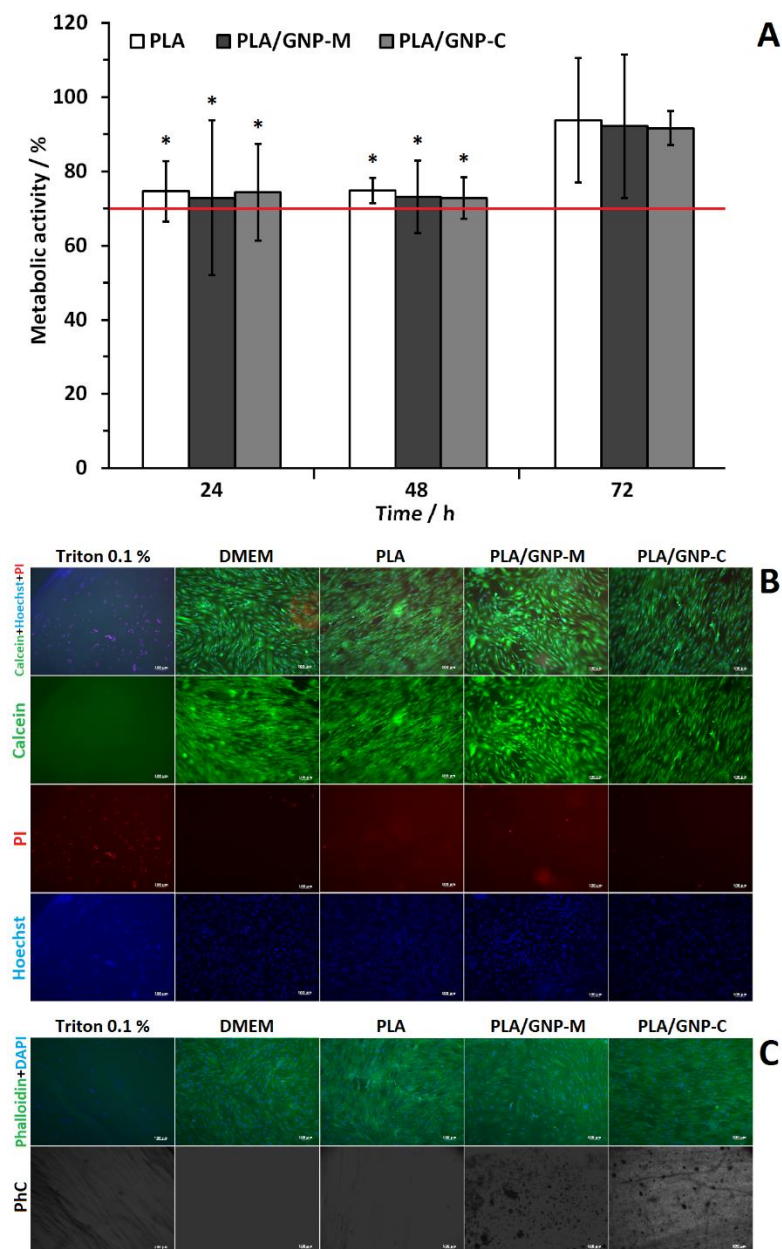


Figure 8.7: (A) HFF-1 cells viability at the surface of PLA, PLA/GNP-M and C 0.25 wt.% cultured in DMEM, at 24, 48 and 72 h. Cell metabolic activity is represented as a percentage in comparison with negative control for metabolic activity decrease - DMEM (100%). Positive control for metabolic activity decrease – Triton 0.1% presented metabolic activity below 5% (data not shown). Results are presented as the mean and standard deviation ($n = 6$). The red line at 70%, marks the toxicity limit, according to ISO 10993-5:2009(E). ^[31] * Statistically significantly different from the negative control ($p < 0.05$). All samples were different from positive control – Triton 0.1% ($p < 0.05$). (B) LIVE/DEAD

staining of HFF-1 cells incubated at the surface PLA, PLA/GNP-M and C 0.25 wt.% for 72 h. Triton 0.1% in DMEM was used as toxicity positive control and DMEM as negative control. Cytoplasm is stained with calcein – green, all nuclei with Hoechst 33342, and cells with membrane integrity compromised were stained in the nucleus with propidium iodide (PI) – red. The bottom line presents a phase-contrast image of the materials surface. Scale bar represents 100 μm . (C) Immunofluorescence images of HFF-1 cells after 72 h culture at the surface of PLA, PLA/GNP-M and C 0.25 wt.%. Triton 0.1% in DMEM was used as positive control for changed morphology, and cells grown in DMEM as negative control. Cells were stained with DAPI (nuclei) - blue and Phalloidin (F-actin in cytoskeleton) - green. Scale bar represents 100 μm .

8.4.3.2 Biocompatibility of the degradation products

Negative control for metabolic activity decrease (DMEM 6 M) was performed with 50 μL PBS 6 M (37 $^{\circ}\text{C}$, 100 rpm, 6 months) in 150 μL DMEM presenting similar cell metabolic activity to degradation products of PLA after 6 months in PBS (PLA 6 M), tested in the same conditions, which shows that those are not toxic. Metabolic activity of cells exposed to PLA 6 M comparing with DMEM 6 M, were of 105, 96, and 100%, for 24, 48 and 72 h, respectively (Figure 8.8(A)), with no significant differences being found overtime ($p > 0.05$). Also, degradation products of PLA/GNP-M and C 0.25 wt.% 6 M are not toxic, comparing with DMEM 6 M ($p > 0.05$). For PLA/GNP-M and C 6 M, respectively, metabolic activities comparing with PLA 6 M were of 84 and 93, 92 and 104, and 88 and 120%, for 24, 48 and 72 h, in the previous order.

Fig. 8.8(B) shows example images for HFF-1 cells fluorescently labelled for LIVE/DEAD assay. Negative control for cell death (DMEM 6 M) was performed as described for metabolic activity assay and presented mostly live cells with few red stained dead cells (Fig. 8.8(B)). Comparing with cells incubated with 6 months degradation products of PLA 6 M, PLA/GNP-M and C 6 M 0.25 wt.%, no more dead cells were observed.

Cells morphology was evaluated by immunocytochemistry (Fig. 8(C)). Toxicity positive control cells (Triton 0.1%) cytoskeleton was disassembled (results not shown - similar to Fig. 8.8(C)). Negative control (DMEM 6 M), PLA 6 M, PLA/GNP-M and C 6 M 0.25 wt.% exhibited the typical “spindle” like shape of fibroblasts.

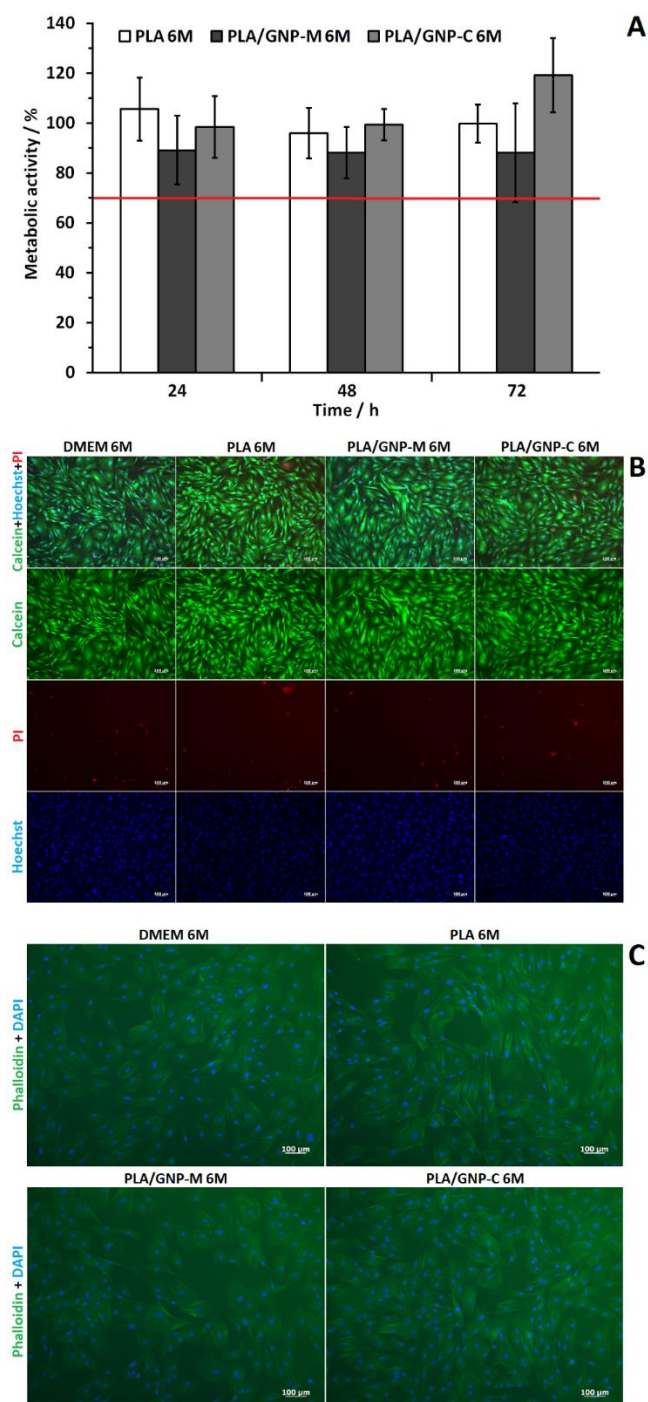


Figure 8.8: (A) HFF-1 cells viability after incubation with 6 months degradation products of PLA 6 M, PLA/GNP-M and C 6 M 0.25 wt.% cultured in DMEM+, at 24, 48 and 72 h. Cell metabolic activity is represented as percentage in comparison with negative control for

metabolic activity decrease (100%) – DMEM 6 M – cells exposed to 50 μ L PBS 6 M (incubated 6 months at 37 °C at 100 rpm) in 150 μ L DMEM. Positive control for metabolic activity decrease – Triton 0.1% presented a metabolic activity below 5% (data not shown). Results are presented as the mean and standard deviation ($n = 6$). The red line at 70%, marks the toxicity limit, according to ISO 10993-5:2009(E).^[31] There are no statistically significant differences ($p > 0.05$) between samples, and between samples and negative control (DMEM). (B) LIVE/DEAD staining of HFF-1 cells incubated with 6 months degradation products of PLA 6 M, PLA/GNP-M and C 6 M 0.25 wt.% for 72 h. Triton 0.1% was used as toxicity positive control (images not shown – similar to Fig. 8.7(B)). Negative control was DMEM 6 M (50 μ L PBS 6 M + 150 μ L DMEM). Cytoplasm is stained with calcein – green, all nuclei with Hoechst 33342, and cells with membrane integrity compromised were stained in the nucleus with propidium iodide (PI) – red. Scale bar represents 100 μ m. (C) Representative immunofluorescence images of HFF-1 cells after incubation for 72 h with PLA 6 M and PLA/GNP-M and C 6 M 0.25 wt.% degradation products. Triton 0.1% was used as positive control for changed morphology (images not shown – similar to Fig. 8.7(C)). Negative control was DMEM 6 M (50 μ L PBS 6 M + 150 μ L DMEM). Cells were stained with DAPI (nuclei) - blue and Phalloidin (F-actin in cytoskeleton) - green. Scale bar represents 100 μ m.

8.5 Discussion

PLA and PLA/GNP-M and C 0.25 wt.% composites were studied in terms of chemical, physical, mechanical and biocompatibility characteristics, before and after 6 months degradation. This time period was considered because PLA is known to show an appreciable loss in strength during the first 6 months of hydrolytic degradation, even though significant changes in mass only are observed for much longer stages.^[32] Maintaining the integrity of PLA mechanical properties within the first 6 months of degradation is important regarding its biomedical applications, namely orthopaedic, as discussed in the introduction.

FTIR spectra for PLA and composites (shown in Appendix C), before and after degradation, were similar, indicating unchanged chemical structures. It must be noted that the low filler content does not allow the detection of characteristic bands of GNP. Kong *et al.* also observed that incorporation of small amounts (0.3–3 wt.%) of multiwalled carbon nanotubes in polyester poly(caprolactone) did not change the pristine polymer FTIR spectra.^[33] On the other hand, XRD analysis of the composites revealed characteristic peaks corresponding to GNP-M and C. No differences were observed after degradation, indicating that the fillers remained in the material.

SEM imaging of samples biodegraded for 6 months indicated evidence of surface erosion for PLA and PLA/GNP-C. PLA samples with very low thickness (*e.g.* fibres) are expected to exhibit

bulk hydrolytic erosion due to fast water diffusion within the material compared to water-mediated hydrolysis.^[24] However, for the 500 μm thickness of the slabs tested in our work, hydrolysis seems to have occurred more intensely near the surface. Effective polymer biodegradation in all materials was confirmed by GPC-SEC, in terms of $\bar{M}_w$ decreased along time. The intense decrease in molecular weight observed between 2 and 4 months may be due to bulk erosion becoming more intense after water penetration in the material core. From this time range, samples containing GNP-M and C presented higher decreases in molecular weight than pristine PLA, which can be explained by defects caused by larger GNP agglomerates facilitating water diffusion within PLA and subsequent degradation. The fact that only GNP-M samples showed evidence of bulk degradation can be due to GNP-M agglomerates having larger sizes, resulting in larger voids inside PLA matrix. Kontou *et al.* observed that after 3 months biodegradation, PLA/montmorillonite and PLA/silica nanocomposites presented higher degradation rates than pristine PLA.^[34] They also propose that filler agglomerates create voids and cracks in PLA where degradation can start. In contrast, Fukushima *et al.*^[35, 36] observed that the addition of nanoclays Cloisite 30B and Sepiolite S9 delayed the degradation of PLA at 37 °C due to induction of polymer crystallization and/or by uptaking water, therefore preventing hydrolysis of the polymer matrix.

Thermal properties of the materials are often influenced by fillers addition. It is interesting to note that, before degradation, PLA/GNP-M yielded slightly higher molecular weight and higher T_d than PLA and PLA/GNP-C. One may hypothesize that this is due to GNP-M improving thermal homogenization of the melt, minimizing the occurrence of hot-spots and consequent polymer degradation during melt-blending and/or compression moulding. Liu *et al.*^[37] reported a similar effect with the incorporation of hydroxyapatite in PLA.

Tensile tests on the non-degraded materials revealed that the presence of the fillers improved mechanical properties: Young's modulus increased by about 14% with both fillers, while tensile strength increased by 20% using GNP-C and by 6% using GNP-M. The smaller particle size of GNP-C in relation to GNP-M, and consequent higher concentration of peripheral oxygen-containing groups per surface area, may play a key role in improving filler/matrix compatibility and thus providing the better mechanical performance observed at the same low loading. Reinforcement of PLA with small amounts (<1 wt.%) of GNP-M15, using melt-blending and adding epoxidized palm oil as a plasticizer, had been shown by Chieng *et al.*^[38] to lead to increases of about 27% in tensile strength. In a previous work, we have reinforced thin films of PLA with GNP-M5. For a loading of 0.2 wt.%, close to the one used in the current work, Young's modulus and tensile strength increased by 6% and 5%, respectively, in unplasticized films, and by 7% and 21% in plasticized films. The optimal improvement was observed for 0.4 wt.%

loading.^[17] However, solvent-mixing was used for preparing the composite, while melt-blending was used here, taking into account the focus on the development of biocompatible materials.

An important finding in the current work is that incorporation of both GNP materials, at low loadings, prevents significant decrease of PLA mechanical properties after 6 months degradation, namely in terms of tensile strength, elongation at break and toughness. In addition, GNP-C incorporation seems to have a more beneficial effect than GNP-M, especially in terms of toughness.

Materials under cyclic loading typically fail at lower stress than under static conditions. Also, this can induce physical ageing in viscoelastic materials like PLA.^[39] Evaluation of mechanical properties under these circumstances may be particularly important when orthopaedic applications are considered, like bioabsorbable artificial ligaments.^[25] The cyclic creep/recovery tests performed in this work showed a significant decay in PLA performance after 6 months degradation, while PLA/GNP-M and C displayed behaviours equivalent to the non-degraded samples. The GNP fillers are therefore significantly counteracting the very negative effect of biodegradation on PLA's creep stability. This remarkable effect can be attributed to the fillers' interaction with the PLA matrix, since the molecular weights have been observed to not be significantly different for all materials after degradation. This may provide a strategy for tuning PLA's creep stability in biosorbable bioimplants. The effect of platelet-like fillers on improving polymer creep stability has already been described by Faraz *et al.*^[39] for clay platelets. Zandiatashbar *et al.*^[40] have also observed improvement in epoxy creep behaviour for graphene loadings between 0.1–0.5 wt.%. In addition, Rafiee *et al.*^[41] observed that low loadings (0.05–0.5 wt.%) of graphene prevented crack propagation in epoxy polymers, increasing fracture toughness. Interestingly, for the maximum number of cycles considered in our work, the beneficial effect of GNP addition is only evident after biodegradation. It is possible that a difference would be observable in non-degraded samples for a higher number of cycles.

Often, when encapsulated in a polymer matrix, graphene-based materials are not toxic.^[42] However, their biological effects should be studied to ensure safe use. Also, as the matrix degrades, filler leaching takes place. Since GNP-M and GNP-C can be toxic at high concentrations, respectively of 20 and 50 $\mu\text{g mL}^{-1}$ ^[43], it is relevant to assure that no toxicity occurs in biological degradation conditions, despite the fact that the amount of GNP present in the composites is quite low (0.25 wt.%). The interaction of human fibroblasts with the materials' surfaces was studied. In addition, cells were incubated with the degradation products resultant from 6 months incubation in PBS at 37 °C and 100 rpm. To our knowledge, there are no studies available simulating a prolonged extraction of these materials in physiological conditions, mimicking what occurs during clinical use, providing an assessment of the hazard potential. No

toxicity was observed since there are no statistically significant differences ($p > 0.05$) between samples (PLA 6 M, PLA/GNP-M and C 6 M), and between samples and negative control (DMEM 6 M). Moreover, for all conditions, cells present low cell death and normal morphology, when in contact with the degradation products. The same is observed for direct contact assay, in which cells incubated at the surface of non-degraded materials. PLA at 24 and 48 h, presented lower metabolic activity ($p < 0.05$) than negative control (DMEM), however, at 72 h both present similar values ($p > 0.05$). This can occur due to PLA surface presenting less favourable physicochemical properties (e.g. too hydrophobic)^[44] than control surface, which is optimized for cell culture, allowing faster cell attachment and growth in the first 48 h. Since our goal is to compare PLA biological properties, before and after incorporation of the fillers, more important was the fact that no significant differences ($p > 0.05$) were found between PLA and PLA/GNP-M and C 0.25 wt.%. These results are similar to those we obtained for thinner PLA/GNP-M 0.4 wt.% films produced by solvent mixing followed by doctor blading^[17], except that in that case the effect of degradation was not studied. Yoon^[45, 46] and Ma^[47] *et al.* had already shown incorporation of graphene oxide in low amounts (<2 wt.%) not to decrease PLA or PLGA, and PLA/hydroxyapatite biocompatibility. Both latter authors used solvents to produce the composites. On the other hand, Lahiri *et al.*^[15] used electrostatic deposition to produce ultra-high molecular weight polyethylene/GNP-M composites. Filler incorporation resulted in viability decreases of 14% at 72 h with the addition of 0.1 wt.% GNP-M5, and of 69% with the incorporation of 1 wt.%. The observed toxicity may have been caused by excessive GNP concentration at the surface and/or rapid leaching, as a result of the production method used.

8.6 Conclusions

It was shown that addition of graphene nanoplatelets (grades GNP-M and GNP-C) to PLA at low loadings (0.25 wt.%) by melt-mixing, not only produces composites with improved mechanical properties, but also leads to a much lower decay in performance after 6 months hydrolytic degradation when compared to unfilled PLA.

GNP-C incorporation seems to have a more beneficial effect than GNP-M, especially when concerning the toughness of the composites after the degradation period. In addition, the composites revealed high stability under cyclic creep-recovery testing, while unfilled PLA exhibited a significant cumulative increase in permanent creep strain and ruptured after 4 cycles. This result is particularly relevant in the context of use in orthopaedic implants and

suture yarns. The nano-fillers were shown not to impair the polymer degradation process, actually evidencing some acceleration of the molecular weight decrease.

Comparing with unfilled PLA, PLA/GNP-M and PLA/GNP-C composites allow similar HFF-1 cell adhesion and growth at the surface before degradation, and after 6 months degradation in physiological conditions, do not release toxic products towards human fibroblasts.

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Chapter 9

General discussion

9 GENERAL DISCUSSION

9.1 Scope

Despite results already have been discussed in each chapter of this thesis, a brief general discussion of the most relevant results is performed in this section, in the light of the most recently available literature reports. The points discussed are the effects of GBMs physical-chemical properties on biocompatibility, the effect of their surface modification with polymers, and the effect of encapsulating them in a polymer matrix. The production methods, mechanical performance before and after degradation, and biocompatibility of PLA/GBMs composites are also discussed. Finally, pertinent questions, potential applications, and promising approaches are discussed taking into account the most recent reports found in literature.

9.2 Discussion

From the results presented on chapter 3 and 4, we can conclude that all GBMs (in powder form) tested caused low hemolysis ($< 2.5\%$) even when considering very high concentrations as $500\ \mu\text{g mL}^{-1}$. Smaller size, oxidation, and surface adsorption of PVA and HEC further reduced the hemolysis ratio. This can be explained by those materials presenting less sharp edges to cause membrane damages on erythrocytes. Comparison was already performed with works that also state the low hemolytic potential of some GBMs ^[1] and its further reduction by introduction of oxygen-containing functional groups ^[2] or surface adsorption of a polymer ^[3] at their surface. A recent study presents an interesting approach to further evaluation of GBMs hemocompatibility. It reports the use of a microfluidic system for the evaluation of blood clot formation under dynamic flow. It reveals that both albumin and fibrinogen adsorb well on the surface of GO, and that GO can be functionalized with albumin to enhance its antithrombotic effect. ^[4] On chapter 6, it is demonstrated that when in presence of plasma proteins, the number of activated platelets in PLA/GNP-M films is significantly lower than that in PLA and PLA/GO films. This can be explained by the presence of hydrophobic GNP at the surface of the films, favoring protein adsorption at the material surface. This blocks platelet activation in case of these proteins being

albumin, which is generally the first to adhere because of its abundance in plasma and its small size. These results are supported by a recent study that states and demonstrates that mono or few layer graphene coated nitinol substrates, present a reduced fibrinogen/albumin ratio relative to pristine nitinol, which is correlated with low platelet adhesion and thrombus formation. [5] A different work reports that a mixed matrix membrane of GO-polyvinylpyrrolidone in a polyetherimide matrix, greatly enhances protein adsorption resistance, suppressing platelet adhesion, lowering complement activation, and prolonging clotting time. [6] It was also reports that the incorporation of GO-g-2-methacryloyloxyethyl phosphorylcholine in polyurethane results in improved resistance to nonspecific protein adsorption and platelet adhesion. [7] These later works point out interesting strategies to further improve the antithrombotic properties of the PLA/GNP composites that we produced.

Chapters 3 and 4 also show that GNP-C, which exhibits smaller sizes, is generally more biocompatible *in vitro* than GNP-M. The sharper and longer edges of GNP-M platelets cause membrane damages on cells, being cytotoxic above $20 \mu\text{g mL}^{-1}$. GNP-C, despite being internalized without causing membrane damages, increases ROS levels, being toxic above $50 \mu\text{g mL}^{-1}$. The complete oxidation of GNP-M (GNP-M-ox-1:6) folds its sharp edges, assuring biocompatibility until the highest concentration tested ($100 \mu\text{g mL}^{-1}$). GNP-M-ox-1:6 has an oxygen content of 24% (attributed mostly to carboxyls and hydroxyls) and is dispersible in water by sonication, revealing potential to be used for biomedical purposes. Generally, oxidation is more important than size, since more oxidized GNP-M performs better than smaller GNP-C. GNP-C-PVA can be used at concentrations up to $100 \mu\text{g mL}^{-1}$, whereas GNP-C can only be used up to concentrations of $50 \mu\text{g mL}^{-1}$. This difference can be explained by PVA encapsulating and agglomerating GNP-C platelets, thereby decreasing the internalization and interaction with HFF-1 cells that lead to ROS production. HEC favours internalization of small GNP-C particles, having the opposite effect regarding ROS levels. For these reasons, modification with HEC, in contrast to PVA, is counter-productive regarding increasing the biocompatibility of GNP-C with fibroblasts. Comparison was already performed with related works in which it was also observed that oxidation [3] and adsorption of polymers [8, 9] at the surface of GBMs usually improve their biocompatibility. In Chapter 2, it was stated that “an interesting aspect that is lacking from existing studies has to do with the effect of particle size (length and thickness) on the biocompatibility of GBMs with similar chemical properties. In addition, there are no references in the literature regarding the effect of GBMs surface charge on biocompatibility.” Fortunately, new data has arisen more recently. A comprehensive study reports preparation of small (HD = 330 nm), larger GO (HD = 2007 nm), and graphene coated with BSA (HD = 1179 nm) or pluronic (PF108) (HD = 2007 nm) to enable dispersion in aqueous solution. *In vitro* characterization revealed that none of the GBMs caused toxicity (reduction of cell viability below 70%), but both GOs triggered the production of inflammation mediators in cell lines. Also, all GBMs, except

graphene coated with PF108, induce pulmonary fibrosis when administered by oropharyngeal aspiration in mice at 2 mg Kg^{-1} , with large GO providing the strongest response.^[10] A different study also shows that small sized GO (hydrodynamic diameter (HD) = 243 nm) presents lower toxicity in mice for intravenous multiple-low-dose exposure, than large GO (HD = 914 nm).^[11] *In vitro* studies revealed that GO with different sizes and thickness caused no toxicity when in contact with cells, up to the highest concentrations tested ($80 \text{ } \mu\text{g mL}^{-1}$ or higher).^[12, 13] However, GNP with larger diameter, thickness, and lower surface charge, caused toxicity above $40 \text{ } \mu\text{g mL}^{-1}$. Also, considerable increases were observed in ROS productions, being those higher for GOs, which presented smaller sizes than GNP.^[12] The observations from abovementioned works, regarding lower toxicity of smaller GBMs, are consistent with what is observed in Chapter 3 and 4, regarding lower toxicity of smaller sized GNP-C, in comparison with GNP-M. The finding that GBMs that are non-toxic to cell lines can induce inflammation, both *in vitro* and *in vivo*,^[10] indicates that the GBMs that we tested should be evaluated in similar assays.

Chapter 2 states that “encapsulation [of GBMs] in a polymer matrix modifies biological interactions”. This was studied on Chapter 6 by incorporating GO and GNP-M in PLA by solvent casting, with improvement of mechanical and biological properties of the polymer matrix being achieved, and no toxicity caused by the embed fillers being observed. Chapters 7 and 8 explore a melt-blending approach for production of PLA/GBMs composites, which does not require the use of potentially toxic solvents. Firstly, the best filler loading, mixing time, and mixing intensity are identified. Then, physical-chemical characterization of PLA/GNP-C and M 0.25 wt.% composites is performed, with focus on mechanical performance, before and after degradation in PBS at 37°C during 6 months, and biocompatibility of the biomaterials and their degradation products tested *in vitro*. It is observed that GBMs incorporation in PLA not only produces composites with improved mechanical properties, but also leads to a much lower decay in performance after 6 months hydrolytic degradation when compared to unfilled PLA. GNP-C incorporation seems to have a more beneficial effect than GNP-M, especially when concerning the toughness of the composites after the degradation period. In addition, the composites reveal high mechanical stability under cyclic creep-recovery testing, while unfilled PLA exhibits a significant cumulative increase in permanent creep strain and ruptures after only 4 cycles. This result is particularly relevant in the context of use in orthopaedic implants and suture yarns. The nano-fillers are shown not to impair the polymer degradation process, actually evidencing some acceleration of the molecular weight decrease. Comparing with unfilled PLA, PLA/GNP-M and PLA/GNP-C composites allow similar HFF-1 cell adhesion and growth at the surface before degradation, and after 6 months degradation in PBS, do not release toxic products towards human fibroblasts. A recent study confirms that GNP-C improves PLA stiffness and elasticity. However, the composites are produced by solution mixing followed by compression moulding, and the amounts of fillers tested are much higher than those we used: 3 and 6 wt.%. Also, surface modification of

GNP-C with 12-aminododecanoic acid, a zwitterionic surfactant, enhances the composites performance by improving GNP-C dispersion and interaction with PLA matrix. ^[14] Thus, this is an approach to take in consideration for further improvement of the mechanical performance of our materials. Another work uses GNP-C, at 1 and 5 wt.% loadings, to improve the stiffness of Bioflex™, a commercial biodegradable polymer-blend based on PLA and a copolyester. ^[15] A different study shows that with the incorporation of low amounts of GNP-M (0.3 wt.%), the tensile strength of PLA plasticized with epoxidized palm oil (EPO) is increased. This occurs because GNP-M is uniformly dispersed in the PLA/EPO matrix, with no aggregation being observed. ^[16] The same authors show a slight reduction of the number of gram positive and negative bacteria at the composites surface, comparing with PLA. ^[17] This underlines the potential of GBMs as antibacterial fillers for PLA and other biopolymers, and reinforces the idea that we can do further work in this field. State of the art approaches report the production of polymer/graphene 3D printable composites. ^[18-20] One of those studies shows that 3D printed PLA/graphene 10 wt.% composites show improved mechanical and thermomechanical properties compared to PLA. This technology is promising regarding production of materials with tailored properties for several applications, including orthopedics and tissue engineering. ^[18] A comprehensive study demonstrates that a polylactide-co-glycolide/graphene (d = 5-20 μm , t = 3-8 layers) 3D composite material is mechanically robust and flexible, presenting considerable electrical conductivity. These scaffolds support human mesenchymal stem cell (hMSC) adhesion, viability, proliferation, and neurogenic differentiation with significant upregulation of glial and neuronal genes. Also, *in vivo* experiments indicate that the materials are biocompatible until 30 days implantation, with no evidence of graphene particles presence in the kidney, liver, or spleen. Surgical tests using a human cadaver nerve model revealed that the materials present exceptional handling characteristics, can be intraoperatively manipulated, and applied to fine surgical procedures. ^[19] An interesting question regarding biodegradable polymer/GBMs composites is the fate that GBMs might have after the polymer matrix is degraded. An *in vivo* study in mouse, developed until 3 months, reports biodegradation by macrophages of carboxyl-functionalized single layer graphene with a lateral size between 150 and 200 nm, which aggregates, 24 hours post injection, into larger clusters up to 10 μm . Despite this positive insight regarding the possibility of some GBMs being naturally degraded by the body, further studies are needed to understand the mechanisms that lead to biodegradation, which characteristics of the GBMs make them more prone to it, like for example, surface properties, dimensions, and concentrations. This knowledge would allow the selection and modification of GBMs properties to maximize their long-term biocompatibility, which would surely result in valuable outcomes, both regarding their potential applications as fillers of composite materials or when freely dispersed as particles.

Previously it was mentioned that GNP-C particles present less toxicity than GNP-M to human cells. Also, oxidation and surface modification of GNP-C with PVA was proposed for further reduction of its toxicity. Since PLA/GNP-C composites perform better in terms of mechanical properties than PLA/GNP-M ones, testing GNP-C-ox, and GNP-C-ox-PVA as fillers for PLA may be an interesting approach. Despite there being no evidence of *in vitro* toxicity of the PLA/GBMs composites that we produced, it is uncertain if the GBMs would trigger *in vitro* or *in vivo* inflammation after being released, while the matrix degrades. For that reason, it is always important to further improve the biocompatibility/biotolerability of the GBMs, while simultaneously conferring surface properties that allow good dispersibility within the polymer matrix.

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Chapter 10

General conclusions and

Future work

10 CONCLUSIONS AND FUTURE WORK

10.1 General conclusions

- The effect of size and thickness on GBMs *in vitro* biocompatibility was evaluated using two different GNP grades. GNP-M platelets were larger and sharper, causing membrane damages on cells in concentrations higher than 20 mg mL⁻¹. GNP-C is internalized due to its small size, inducing ROS production that leads to mild toxicity in concentrations higher than 50 mg mL⁻¹, from which the cells can recover along time. GNP-M toxic effects can be avoided by extensive oxidation (GNP-M-ox-1:6), which folds its sharp edges, preventing membrane damages. GNP-M-ox-1:6 has an oxygen content of 24% (mostly carboxyls and hydroxyls) and is dispersible in water by sonication, revealing potential to be used for biomedical purposes.
- All the GBMs tested cause low haemolysis (<2.5%) up to 500 µg mL⁻¹. This decreases further after oxidation and polymer adsorption, the best results being obtained with PVA and HEC.
- Surface adsorption of PVA to GNP-C platelets encapsulates and agglomerates them, thereby decreasing the internalization and interaction with cells that lead to ROS production and toxicity. When coated with HEC, small GNP-C particles present higher internalization, which increases ROS levels and toxicity. For these reasons, PVA surface adsorption improves GNP-C biocompatibility, while the use of HEC is counter-productive.
- A method for evaluation of GBMs degree of dispersion in PLA matrix by Raman spectroscopy was developed and implemented.
- Incorporation of 0.4 wt.% loadings of GO and GNP-M in PLA by solvent mixing followed by doctor blading changes the surface topography, and wettability of the

composite films. Additionally, the incorporation of GO in PLA films, slightly increases the metabolic activity of cells adhered at their surface. GNP-M incorporation, on the other hand, decreases platelet activation at the films surface. This might be relevant for bioapplications that involve contact with blood.

- The dispersion of GNP-M and C in PLA by melt-blending, in amounts as low as 0.25 wt.%, improves its mechanical properties under cyclic creep-recovery testing after degradation under physiological conditions during 6 months. This is relevant for several PLA applications, as for example production of orthopaedic implants and suture yarns. PLA/GNP-C composites present a general better mechanical performance than PLA/GNP-M composites.
- The incorporation of 0.25 wt.% loadings of GNP-M and C in PLA films, produced by melt-blending, does not cause toxicity to HFF-1 cells in direct contact with their surfaces. Both before degradation, and after 6 months degradation in physiological conditions. Also, the degradation products of those composites are not toxic for human fibroblasts.

10.2 Ongoing and Future work

- Following the promising results obtained with PLA/GNP-M composites, *in vivo* biocompatibility studies in BALB/c mice have already been initiated at University of Washington in Prof. Buddy Ratner's Lab. PLA, PLA/GNP-M and C 0.25 wt.% composites were implanted subcutaneously in mice during 3 weeks. The materials were explanted and embed in paraffin. This work was not included in this thesis since the histological analysis is not yet available. After analyzing these results it will be decided if long term assays are needed to access biocompatibility of the composites, and particularly of GBMs that may eventually be released from PLA matrix as it degrades.
- Since GNP-M complete oxidation improves its biocompatibility, it would be an interesting approach to apply on GNP-C. Also, modification with PVA, which is effective for GNP-C, could be tried after its oxidation for further improvement of its biocompatibility. If those materials prove non-toxic and non-inflammatory, they can be

tested as fillers of PLA. Despite there being no evidence of *in vitro* toxicity of the PLA/GBMs composites that we produced, it is uncertain if the GBMs would trigger *in vitro* or *in vivo* inflammation after being released, while the matrix degrades. For that reason, it is always important to further improve the biocompatibility/biotolerability of the GBMs, and if possible simultaneously conferring them surface properties that allow better dispersion and compatibility with the polymer matrix, which would reflect on increased mechanical performance.

- GBMs have been described to have antibacterial effect, and a study reports that PLA/GNP-M plasticized with epoxidized palm oil has antibacterial activity against both gram negative and gram positive bacteria. Thus, testing the antibacterial properties of GBMs, and modifying them to increase it, as well as producing and optimizing PLA/GBMs composites for the same effect is one of our future goals.
- Recent studies report the production of polymer/GBMs composite 3D printed structures. This technology is promising regarding production of materials with tailored properties for several applications, including in orthopedics and tissue engineering.
- Since incorporation of GBMs has been described to confer electrical conductivity to PLA, development of electrically conductive PLA composites materials to stimulate tissue regeneration is an interesting perspective, even more so when associated with 3D printing of biomaterials.

11 APPENDICES

APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

SMALLER PARTICLE SIZE AND HIGHER OXIDATION IMPROVES BIOCOMPATIBILITY OF GRAPHENE-BASED MATERIALS

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APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

POLYMER SURFACE ADSORPTION AS A STRATEGY TO IMPROVE THE BIOCOMPATIBILITY OF GRAPHENE NANOPATELETS

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APPENDIX C

SUPPLEMENTARY MATERIAL FOR CHAPTER 8

EFFECT OF BIODEGRADATION ON THERMO-MECHANICAL PROPERTIES AND BIOCOMPATIBILITY OF POLY(LACTIC ACID)/GRAPHENE NANOPATELETS COMPOSITES

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APPENDIX A

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

GBMs BIOCOMPATIBILITY - EFFECT OF PARTICLE SIZE AND DEGREE OF OXIDATION

A1. Hemolysis

GNP-M and GNP-C were washed with 5 L distilled water by filtration, and these materials were designated as GNP-M-w and GNP-C-w. GNP-M and GNP-C were centrifuged at 4000 rpm during 15 min, the precipitate was recovered and tested. These materials are designated as GNP-M-c and GNP-C-c.

Figure A1 shows that both GNP-M (2.5 %) and GNP-C (1.7 %) induce low hemolysis up to concentrations of 500 $\mu\text{g mL}^{-1}$. Materials were washed in order to assure removal of possible contaminants from production/storage. However, hemolysis is similar for “as received” and washed materials. Thus, washing is not necessary to reduce toxicity. Centrifugation of both materials and recovery of the precipitate was also tested to remove possible contaminants present. This treatment did not reduce hemolysis for both materials at 500 $\mu\text{g mL}^{-1}$.

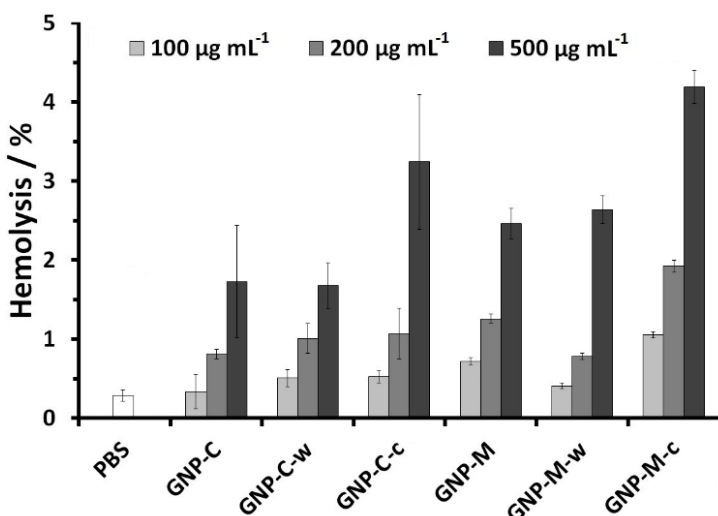


Figure A1: Percentage of RBCs lysis after 3h incubation at 37°C with PBS (negative control), GNP-M and GNP-C “as received”, after washing (GNP-M-w and GNP-C-w), and after centrifugation (GNP-M-c and GNP-C-c). GBMs concentrations tested were 100, 200 and 500 $\mu\text{g mL}^{-1}$. Positive control for 100 % hemolysis was Triton 1% in PBS. Results are presented as mean and standard deviation (n=3).

A2. Cell morphology

Phase contrast images were obtained for each incubation time and concentration. Examples images for 24, 48, 72h for 10 and 50 $\mu\text{g mL}^{-1}$ are shown in Figure A2. Materials deposited in wells bottom and are in contact with the cells, however it is imperceptible whether they penetrate cell membrane or not. To clarify this question, HFF-1 cells incubated with materials were analysed by TEM (see section 3.4.1.2.2.2). The increase of materials concentration, leads to more accumulation at cell membrane (Fig. A2). GNP-C, possesses agglomerates with smaller size after sedimentation, than GNP-M. Also, GNP-M-ox-1:3 and GNP-M-ox-1:6 have larger agglomerates in well bottom and in contact with cells than GNP-M, with less isolated particles being observed.

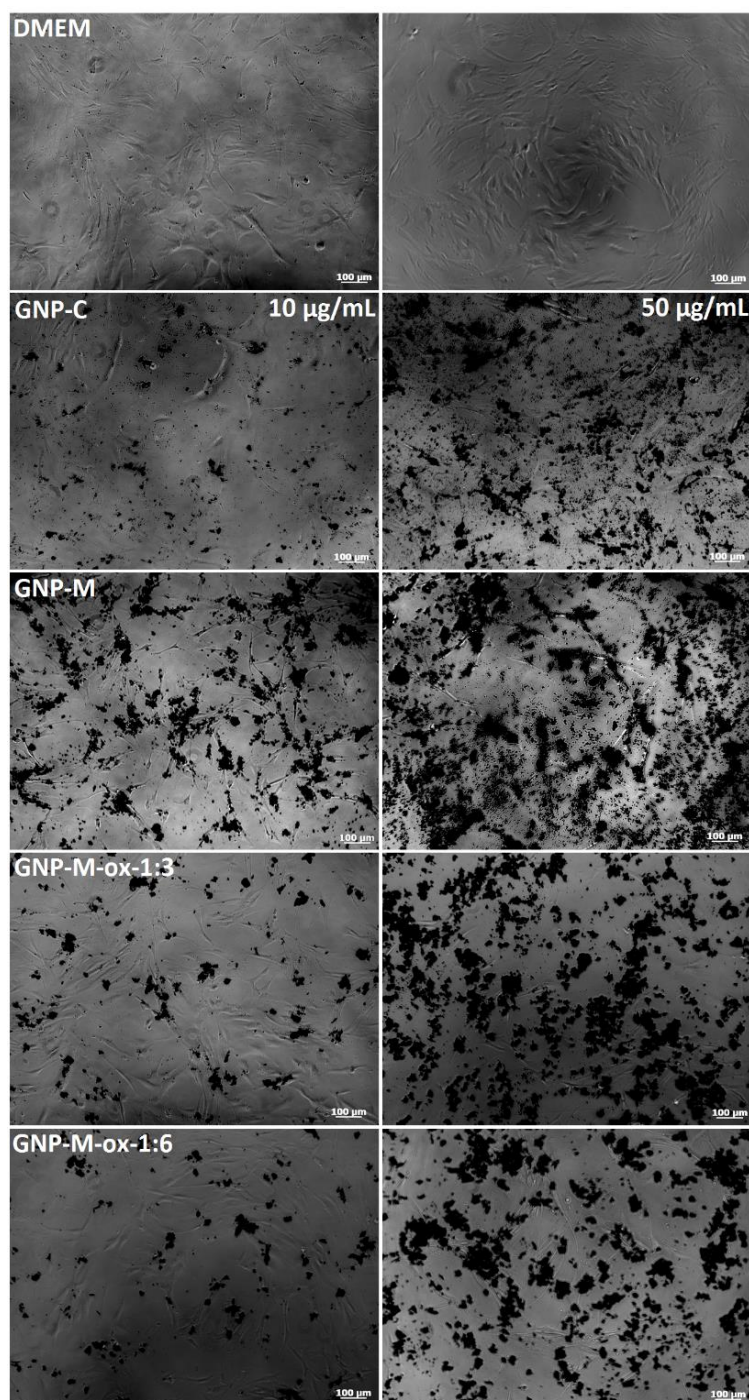


Figure A2: Optical microscopy images of HFF-1 cells in DMEM+ and incubated with 10 and 50 µg mL⁻¹ (left and right column, respectively) of GNP-C, GNP-M, GNP-M-ox-1:3 and 1:6 for 72h. Scale bar represents 100 µm.

A3. Cytotoxicity

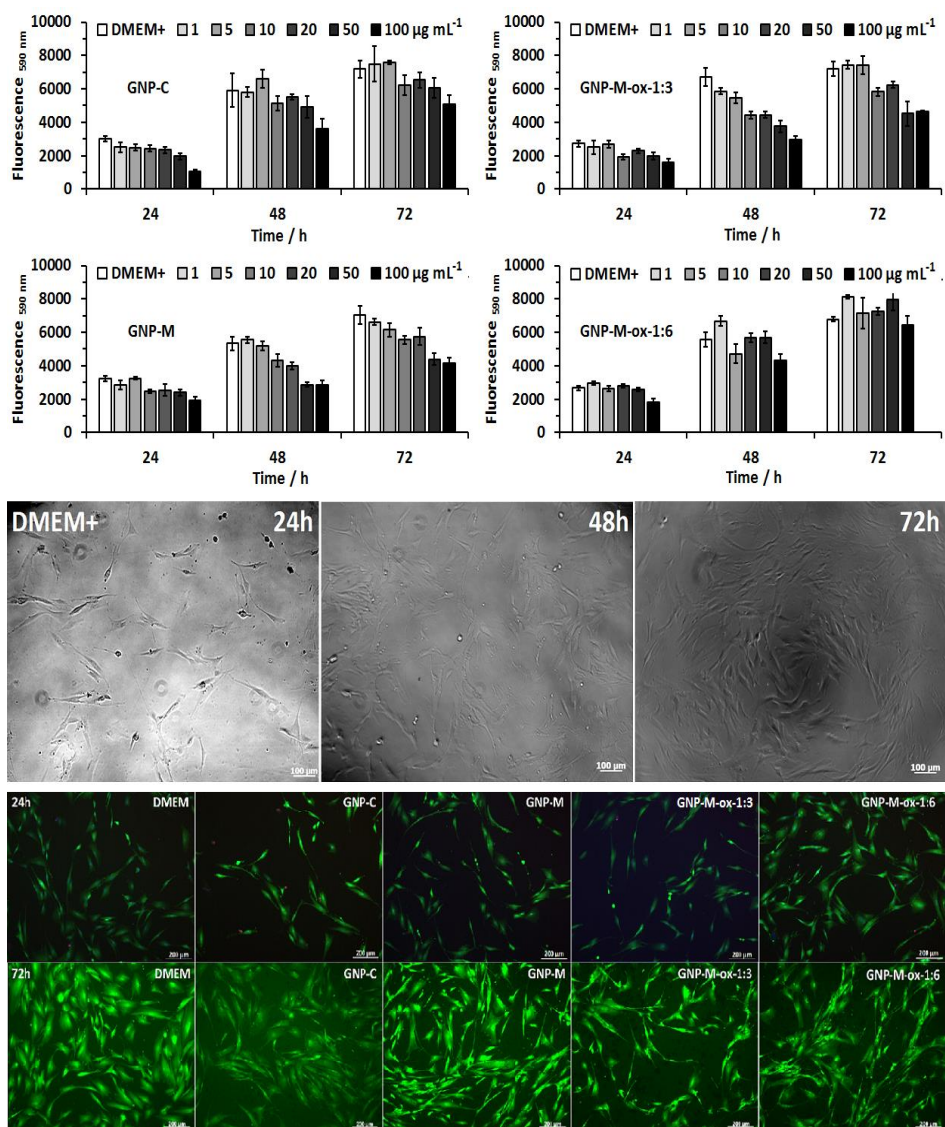


Figure A3: Fluorescence intensities from resazurin assay for HFF-1 cells incubated with DMEM+ (control) and GBMs from 1-100 µg mL⁻¹ for 24, 48 and 72h. Results are presented as mean and standard deviation (n=6). PhC images show the density of cells incubated in DMEM+ at 24, 48, and 72h. Scale bar represents 100 µm. Immunofluorescence images show cell density for cells incubated with DMEM+ and 100 µg mL⁻¹ GBMs at 24 and 72h. Scale bar represents 200 µm.

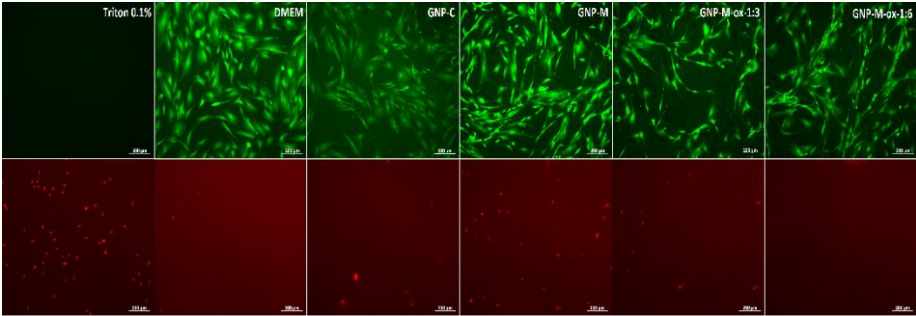


Figure A4: LIVE/DEAD staining of HFF-1 cells incubated with $100 \mu\text{g mL}^{-1}$ GBMs for 72h. Triton 0.1 wt.% in DMEM+ was used as toxicity positive control and cells cultured in DMEM+ as negative control. Cytoplasm is stained with calcein – green. Cells with membrane integrity compromised were stained in the nucleus with propidium iodide – red. Scale bar represents $200 \mu\text{m}$.

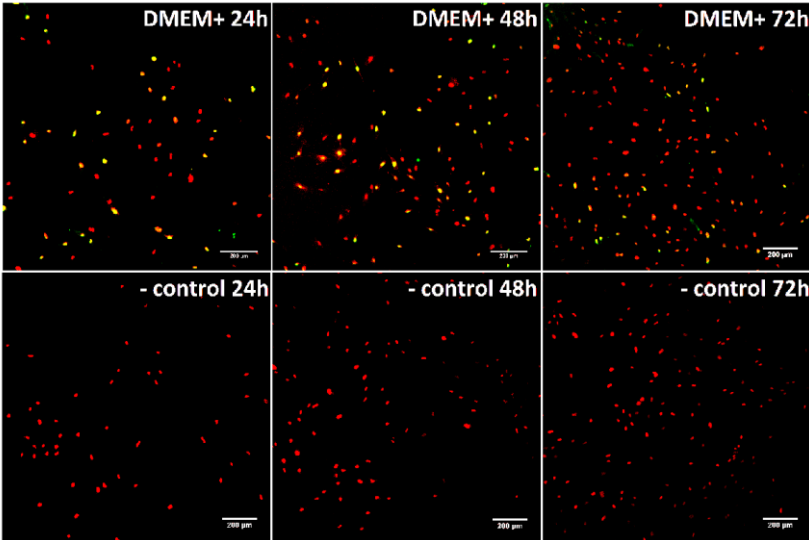
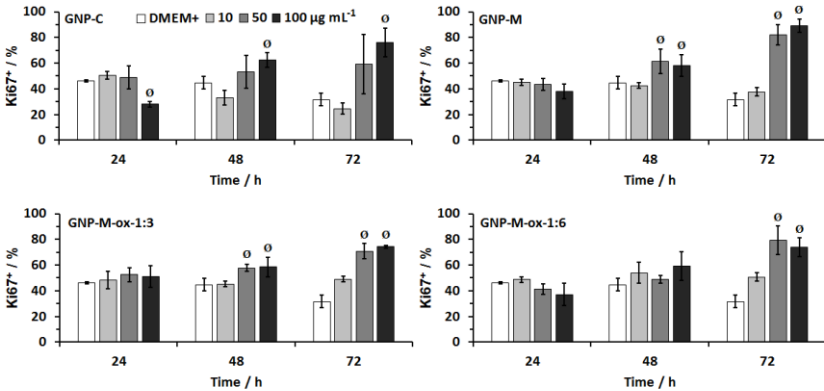


Figure A5: Ki-67 expression (Ki-67⁺ - positive) for HFF-1 cells incubated with GBMs in DMEM+ at 24, 48, and 72h. Results are presented as mean and standard deviation (n=3).

Significant differences ($p < 0.05$) comparing with control without GBMs (DMEM+) are represent by Ø. Example of the images used to calculate Ki-67 expression (Ki-67⁺ - green, all nuclei - red) for HFF-1 cells incubated with DMEM+ and respective negative control without primary antibody (- control) at 24, 48 and 72h. Scale bar represents 200 μm .

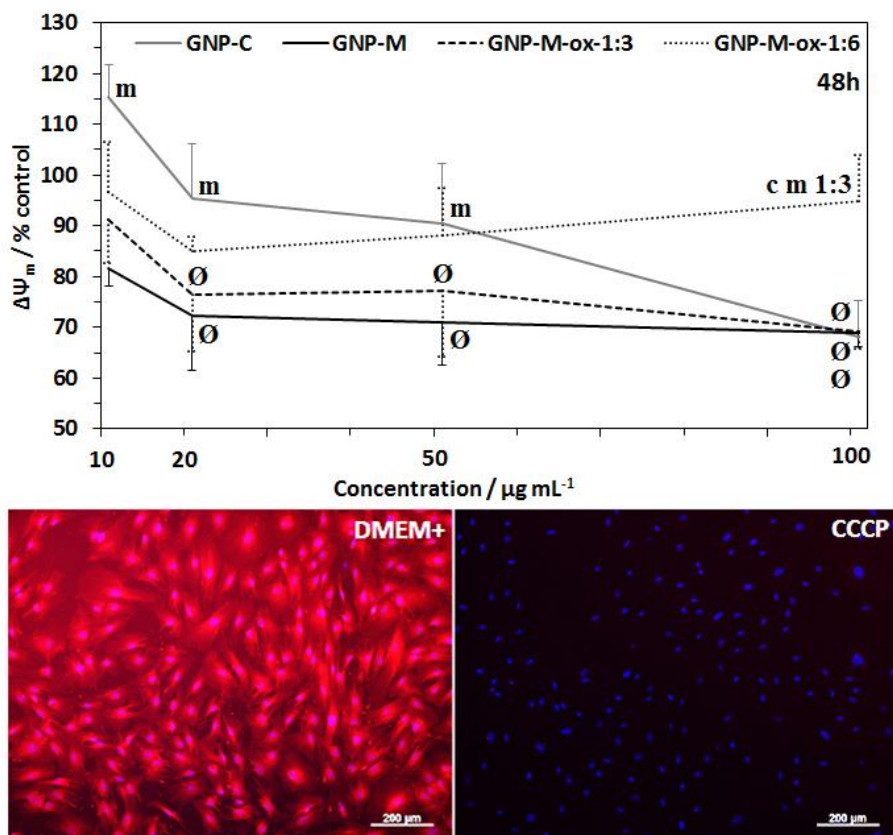


Figure A6: Mitochondrial membrane potential ($\Delta\Psi_m$ - MMP) as % of negative control for MMP decrease – HFF-1 cells cultured in DMEM+, for positive control for MMP decrease - cells exposed to CCCP 20 μM for 15 min ($\Delta\Psi_m = 19.7\%$), and GBMs for concentrations between 10-100 $\mu\text{g mL}^{-1}$, for 48h. Results are presented as mean and standard deviation ($n=3$). Statistically significant differences, analysed within each concentration are represented by c – GNP-C, m – GNP-M, 1:3 – GNP-M-ox-1:3, 1:6 – GNP-M-ox-1:6. Symbols represented above a sample concentration indicate that sample is different ($p < 0.05$) from samples represented by the symbols for that concentration. Samples different from DMEM+ are signalled for each concentration as Ø ($p < 0.05$). Immunofluorescence images of HFF-1 cells incubated with controls performed with culture medium (DMEM+, 48h) and CCCP 20 μM for 15 min. Nuclei are stained with Hoechst 33342.

Figure A6, shows that only cells incubated with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) 20 μM for 15 min, present very low red staining, because CCCP uncouples mitochondrial electron transfer decreasing membrane potential. This compound

decreases mitochondrial membrane potential (MMP) to 19.7 % comparing with cells cultured with DMEM+. Both GNP-M and GNP-M-ox-1:3 present decreased MMP comparing with DMEM+ above a concentration of 10 $\mu\text{g mL}^{-1}$ ($p > 0.05$). For GNP-C MMP only decreases above 50 $\mu\text{g mL}^{-1}$. GNP-M-ox-1:6 show no significant differences in MMP comparing with cells cultured in DMEM+ ($p > 0.05$).

A4. Statistical analysis

Table A1: Statistical analysis for resazurin assay (see section 3.4.1.2.2.1). p values are shown when differences were found between samples.

Time	Conc.	Samples	DMEM+	GNP-C	GNP-M	GNP-M-ox-1:3	GNP-M-ox-1:6
24h	5 $\mu\text{g mL}^{-1}$	DMEM+	x				
		GNP-C		x	$p < 0.01$		
		GNP-M		$p < 0.01$	x		
		GNP-M-ox-1:3				x	
		GNP-M-ox-1:6					x
	10 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.05$	$p < 0.05$	$p < 0.05$	
		GNP-C	$p < 0.05$	x			
		GNP-M	$p < 0.05$		x		$p < 0.01$
		GNP-M-ox-1:3	$p < 0.05$			x	$p < 0.05$
		GNP-M-ox-1:6			$p < 0.01$	$p < 0.05$	x
	20 $\mu\text{g mL}^{-1}$	DMEM+	x				
		GNP-C		x			
		GNP-M			x		
		GNP-M-ox-1:3				x	$p < 0.05$
		GNP-M-ox-1:6				$p < 0.05$	x
	50 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.01$	$p < 0.05$		
		GNP-C	$p < 0.01$	x			$p < 0.05$
		GNP-M	$p < 0.05$		x		
		GNP-M-ox-1:3				x	
		GNP-M-ox-1:6		$p < 0.05$			x
	100 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.01$	$p < 0.01$	$p < 0.05$	$p < 0.05$
		GNP-C	$p < 0.01$	x	$p < 0.05$		
		GNP-M	$p < 0.01$	$p < 0.05$	x		
		GNP-M-ox-1:3	$p < 0.05$			x	
		GNP-M-ox-1:6	$p < 0.05$				x
48h	1 $\mu\text{g mL}^{-1}$	DMEM+	x				
		GNP-C		x			
		GNP-M			x	$p < 0.01$	
		GNP-M-ox-1:3			$p < 0.01$	x	
		GNP-M-ox-1:6					x
	5 $\mu\text{g mL}^{-1}$	DMEM+	x			$p < 0.05$	
		GNP-C		x		$p < 0.01$	
		GNP-M			x		
		GNP-M-ox-1:3	$p < 0.05$	$p < 0.01$		x	$p < 0.01$
		GNP-M-ox-1:6				$p < 0.01$	x
	10 $\mu\text{g mL}^{-1}$	DMEM+	x			$p < 0.01$	
		GNP-C		x			
		GNP-M			x		
		GNP-M-ox-1:3	$p < 0.01$			x	$p < 0.01$
		GNP-M-ox-1:6				$p < 0.01$	x
	20 $\mu\text{g mL}^{-1}$	DMEM+	x		$p < 0.05$	$p < 0.01$	
		GNP-C		x	$p < 0.01$		
		GNP-M	$p < 0.05$	$p < 0.01$	x		$p < 0.01$
		GNP-M-ox-1:3	$p < 0.01$			x	

72h	50 $\mu\text{g mL}^{-1}$	GNP-M-ox-1:6		$p < 0.01$		x
		DMEM+	x	$p < 0.01$	$p < 0.01$	
		GNP-C		x	$p < 0.01$	
		GNP-M	$p < 0.01$	$p < 0.01$	x	$p < 0.01$
		GNP-M-ox-1:3	$p < 0.01$		x	
		GNP-M-ox-1:6		$p < 0.01$		x
	100 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.05$	$p < 0.01$	
		GNP-C	$p < 0.05$	x		
		GNP-M	$p < 0.01$		x	$p < 0.01$
		GNP-M-ox-1:3	$p < 0.01$		x	$p < 0.01$
		GNP-M-ox-1:6		$p < 0.01$	$p < 0.01$	x
	1 $\mu\text{g mL}^{-1}$	DMEM+	x			
		GNP-C		x	$p < 0.05$	
		GNP-M		$p < 0.05$	x	$p < 0.05$
		GNP-M-ox-1:3		$p < 0.05$	x	$p < 0.01$
		GNP-M-ox-1:6		$p < 0.01$		x
	5 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.05$		
		GNP-C	$p < 0.05$	x	$p < 0.01$	
		GNP-M		$p < 0.01$	x	$p < 0.05$
		GNP-M-ox-1:3			x	
		GNP-M-ox-1:6		$p < 0.05$		x
	10 $\mu\text{g mL}^{-1}$	DMEM+	x		$p < 0.05$	
		GNP-C		x	$p < 0.05$	
		GNP-M	$p < 0.05$	$p < 0.05$	x	$p < 0.01$
		GNP-M-ox-1:3	$p < 0.05$		x	$p < 0.01$
		GNP-M-ox-1:6		$p < 0.01$	$p < 0.01$	x
	20 $\mu\text{g mL}^{-1}$	DMEM+	x			
		GNP-C		x		
		GNP-M			x	$p < 0.05$
		GNP-M-ox-1:3			x	
		GNP-M-ox-1:6		$p < 0.05$		x
	50 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.01$	$p < 0.01$	$p < 0.05$
		GNP-C		x	$p < 0.01$	$p < 0.01$
		GNP-M	$p < 0.01$	$p < 0.01$	x	$p < 0.01$
		GNP-M-ox-1:3	$p < 0.01$	$p < 0.05$	x	$p < 0.01$
		GNP-M-ox-1:6	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.01$
	100 $\mu\text{g mL}^{-1}$	DMEM+	x	$p < 0.01$	$p < 0.01$	$p < 0.05$
		GNP-C		x	$p < 0.01$	$p < 0.05$
		GNP-M	$p < 0.01$	$p < 0.01$	x	$p < 0.01$
		GNP-M-ox-1:3	$p < 0.01$	$p < 0.05$	x	$p < 0.01$
		GNP-M-ox-1:6	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.01$

A5. Biocompatibility with HPMEC cells

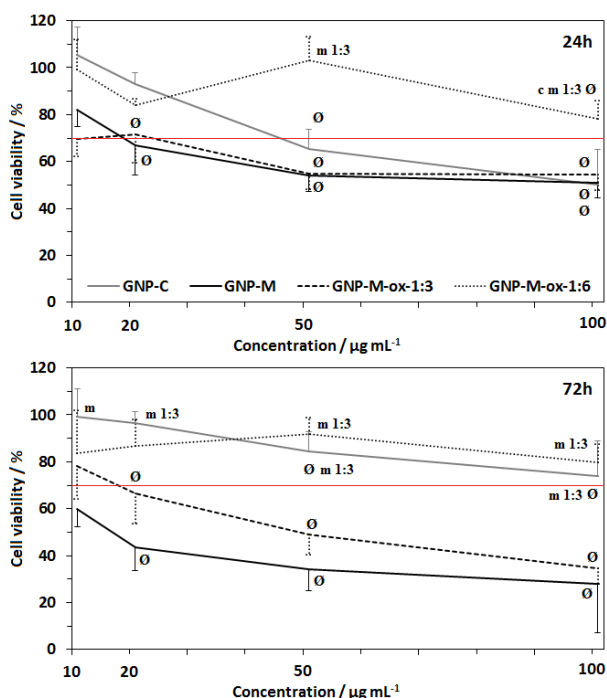


Figure A7: HPMEC cells viability after incubation with GBMs in M199+, at 24, and 72 h. Cell metabolic activity is represented as percentage in comparison with cells cultured in M199+ (100 %). Results are presented as mean and standard deviation ($n=6$). The red line at 70%, marks the toxicity limit, according to ISO 10993-5:2009(E). Statistically significant differences, analysed within each concentration between all GBMs are represented by c – GNP-C, m – GNP-M, 1:3 – GNP-M-ox-1:3 (differences were only found comparing with GNP-M-ox-1:6); Differences comparing with M199+ are represented by Ø. Significant differences were considered for $p < 0.05$.

Considering that a sample has cytotoxic potential if its metabolic activity is reduced to less than 70 % comparing to negative control for metabolic activity decrease - cells incubated with M199+ [ISO 10993-5:2009(E)], it can be observed from Figure A7 that GNP-C is toxic at 24 h for concentrations above $20 \mu\text{g mL}^{-1}$. However, cell metabolic activity recovered over time, and at 72 h toxicity is only observed above $50 \mu\text{g mL}^{-1}$. For GNP-M and GNP-M-ox-1:3, toxicity occurs at 24 h above $10 \mu\text{g mL}^{-1}$. At 72 h, toxicity is observed above $20 \mu\text{g mL}^{-1}$ for GNP-M-ox-1:3 and at $10 \mu\text{g mL}^{-1}$ for GNP-M. GNP-M-ox-1:6 reveals no toxicity until the higher concentration tested of $100 \mu\text{g mL}^{-1}$ ($p < 0.05$). These results are in agreement with results from resazurin assay for HHF-1 cells, since GNP-M and GNP-M-ox-1:3 are the most toxic materials, while GNP-M-ox-1:6 shows no toxicity. Also, GNP-C shows initial mild toxicity which is almost recovered over time.

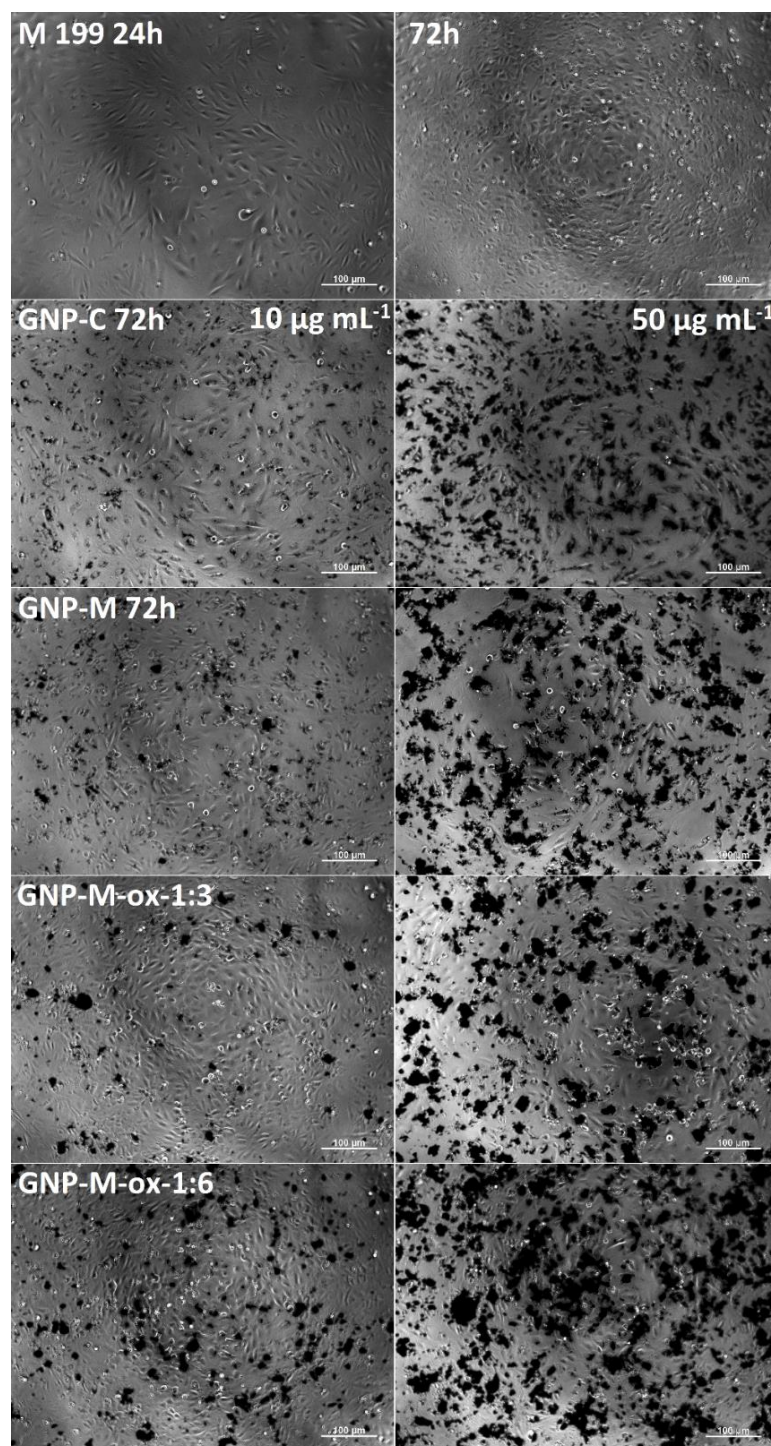


Figure A8: Optical microscopy images of HPMEC cells in culture medium (M199+) at 24 and 72h, and incubated for 72h with 10 and 50 µg mL⁻¹ (left and right column, respectively) of GNP-C, GNP-M, GNP-M-ox-1:3 and 1:6. Scale bar represents 100 µm.

APPENDIX B

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

GBMs BIOCOMPATIBILITY - EFFECT OF POLYMER SURFACE ADSORPTION

B1. Materials and methods

X-ray photoelectron spectroscopy (XPS) The XPS analysis of GBMs powders tablets ($d = 10$ mm) was performed at CEMUP (Centro de Materiais da Universidade do Porto) using a ESCALAB 200A, VG Scientific (UK) with PISCES software for data acquisition and analysis. For analysis, an achromatic Al ($K\alpha$) X-ray source (1486.6 eV) operating at 15kV (300 W) was used, and the spectrometer, calibrated with reference to Ag $3d_{5/2}$ (368.27 eV), was operated in constant analyser energy mode with a pass energy of 20 eV for regions of interest, and 50 eV in survey. The core levels for O 1s and C 1s were analysed. The photoelectron take-off angle (the angle between the surface of the sample and the axis of the energy analyser) was 90° . The electron gun used focused on the specimen in an area close to 100 mm^2 . Data acquisition was performed with a pressure lower than 1×10^{-6} Pa. The effect of the electric charge was corrected by the reference of the carbon peak (285 eV). The deconvolution of spectra was performed with the XPSPEAK41 program, in which a peak fitting was performed using Gaussian-Lorentzian peak shape and Shirley type background subtraction.

Raman spectroscopy The unpolarized Raman spectra of GBMs powders were obtained under ambient conditions, in several positions for each sample. The linear polarized 514.5 nm line of an Ar^+ laser was used as excitation. The Raman spectra were recorded in a backscattering geometry by using a confocal Olympus BH-2 microscope with a 50x objective. The spatial resolution is about $2 \text{ }\mu\text{m}$, and laser power was 15 mW. The scattered radiation was analysed using a Jobin–Yvon T64000 triple spectrometer, equipped with a charge-coupled device. The diameter of analysis was $10 \text{ }\mu\text{m}$ and the spectral resolution better than 4 cm^{-1} .

The spectra were quantitatively analysed by fitting a sum of damped oscillator to the experimental data, according to the equation:

$$I(\omega, T) = B(\omega) + (1 + n(\omega, T)) \sum_{j=1}^N A_{oj} \frac{\omega \Omega_{oj}^2 \Gamma_{oj}}{(\Omega_{oj}^2 - \omega^2)^2 + \omega^2 \Gamma_{oj}^2} \quad (1)$$

Here $n(\omega, T)$ is the Bose-Einstein factor: A_{oj} , Ω_{oj} , and τ_{oj} are the strength, wave number and damping coefficient of the j -th oscillator, respectively, and $B(\omega)$ is the background. In this work, the background was well simulated by a linear function of the frequency, which enable us to obtain reliable fits of the equation (1) to the experimental data. The fitting procedure was performed for all Raman bands collected from the same sample, but in different positions. This procedure allows us to determine the average and standard deviation (SD) values of the phonon parameters, namely the wave number and intensity. ^[1]

Thermogravimetric analysis (TGA) The thermal stability of the samples was determined with a Netzsh STA 449 F3 Jupiter device. Sample amounts ranged from 10 to 12 mg. The thermograms were recorded between 50 and 800 °C at a heating rate of 10 °C min⁻¹ under nitrogen flow.

Scanning electron microscopy (SEM) The morphology of GBMs was observed at CEMUP using SEM (FEI Quanta 400FEG, with an acceleration voltage of 3 kV). Powders of the nanomaterials were applied on conductive carbon strips for visualization.

Immunocytochemistry Cells in each well exposed to 50 µg mL⁻¹ of GBMs (at 100 µg mL⁻¹ GBMs interfere with staining and images had worse quality) were washed with PBS. Then, fixation was performed with paraformaldehyde (PFA - Merck) 4 wt.% in PBS for 15 minutes. PFA was removed, the cells washed with PBS and stored at 4 °C. Cell cytoskeletal filamentous actin can be visualized by binding of fluorescent phalloidin and the nucleus can be stained with 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) that intercalates with nucleic acids. Cell membrane was permeabilized with triton X-100 0.1 wt.% at 4 °C for 5 min. Washing was performed with PBS and incubation performed with a phalloidin (Alexa Fluor 488; Molecular Probes) solution in PBS in a 1:80 dilution, during 20 min in the dark. After rinsing with PBS, a DAPI (Sigma Aldrich) solution at a concentration of 3 µg mL⁻¹ was added to each well and incubated for 15 min in the dark. Finally, the cells were washed and kept in PBS to avoid drying. Plates with adherent cells were observed in an inverted fluorescence microscope (Carl Zeiss – Axiovert 200).

B2. Results

B2.1. Physical-chemical characterization

Figure B1(A) shows a nanoplatelet of pristine GNP-C. After adsorption of PVA or HEC, platelet agglomerates become more evident, as shown in Fig. B1(B, C), for GNP-C-PVA and GNP-C-HEC, respectively. Polymer presence on the GNP-C surface was confirmed by other methods as shown below.

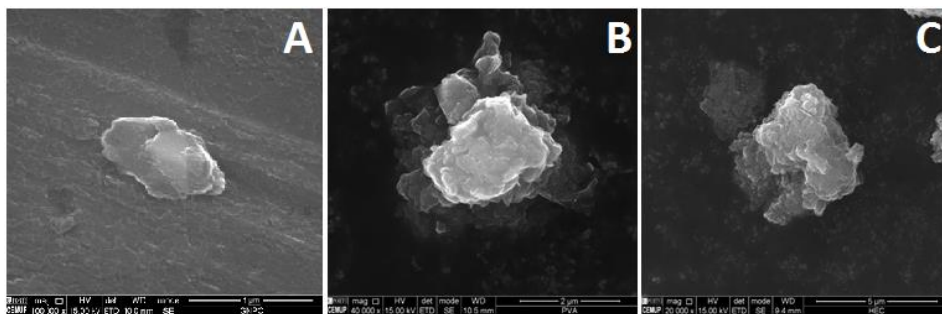


Figure B1: SEM images of dry powder of GNP-C (A – 100 000x), GNP C-PVA (B – 40 000x), and GNP-C-HEC (C – 20 000x).

XPS results (Figure B2(A)) reveal that treatment of GNP-C (which has atomic percentage of oxygen – O (at.%) < 4 %) with polymers increases the final O (at.%). The highest increases are for GNP-C-PVA and HEC, being above 10 %. These results confirm the adsorption of all polymers onto the GNP-C surface.

As seen in Figure B2(B), the Raman spectra obtained for the GBMs powders are similar. The D-band, located between $1270\text{--}1450\text{ cm}^{-1}$, is assigned to the carbon lattice disorder typical of crystal edges and defects in the graphene network. The G-band close to 1580 cm^{-1} is associated with sp^2 hybridization. [2, 3] From the fitting procedure, we have calculated the intensity of the observed Raman bands. It is well established that the ratio between the intensities of the D- and G-bands, $I_{\text{D/G}}$, can be used to characterize the defect degree in graphene materials and to evaluate multilayered graphene crystallite size. [2] Usually, the D band is absent or weak on the surface of graphene sheets and intense at their edges. [3] GNP modified with all polymers presents a less intense D-band than pristine GNP-C. This occurs because the latter is less agglomerated and is not covered with polymer, thus, more edges are present in the analysis site, which leads to a more intense signal. [3] This result further confirms the presence of polymers at the GNP-C surface, already evidenced before by laser diffraction, SEM and XPS.

Thermograms for the GBMs are shown in Figure B2(C). GNP-C modified with polymers starts to lose weight at lower temperatures (approximately $250\text{ }^{\circ}\text{C}$) than pristine GNP-C, which presents little weight loss (GNP-C = 13.8 wt.%), mostly above $400\text{ }^{\circ}\text{C}$. These temperatures seem to be in the range of the temperature of onset of intense degradation for the polymers used. [4, 5] As an example, GNP-C-PVA and HEC lose, respectively, approximately 35 and 29 wt.% until $800\text{ }^{\circ}\text{C}$. Taking into account the mass lost by pristine GNP-C up to that temperature, it can be concluded that the total amount of PVA and HEC polymers on the GNP-C surface is 21 % and 15 %, respectively.

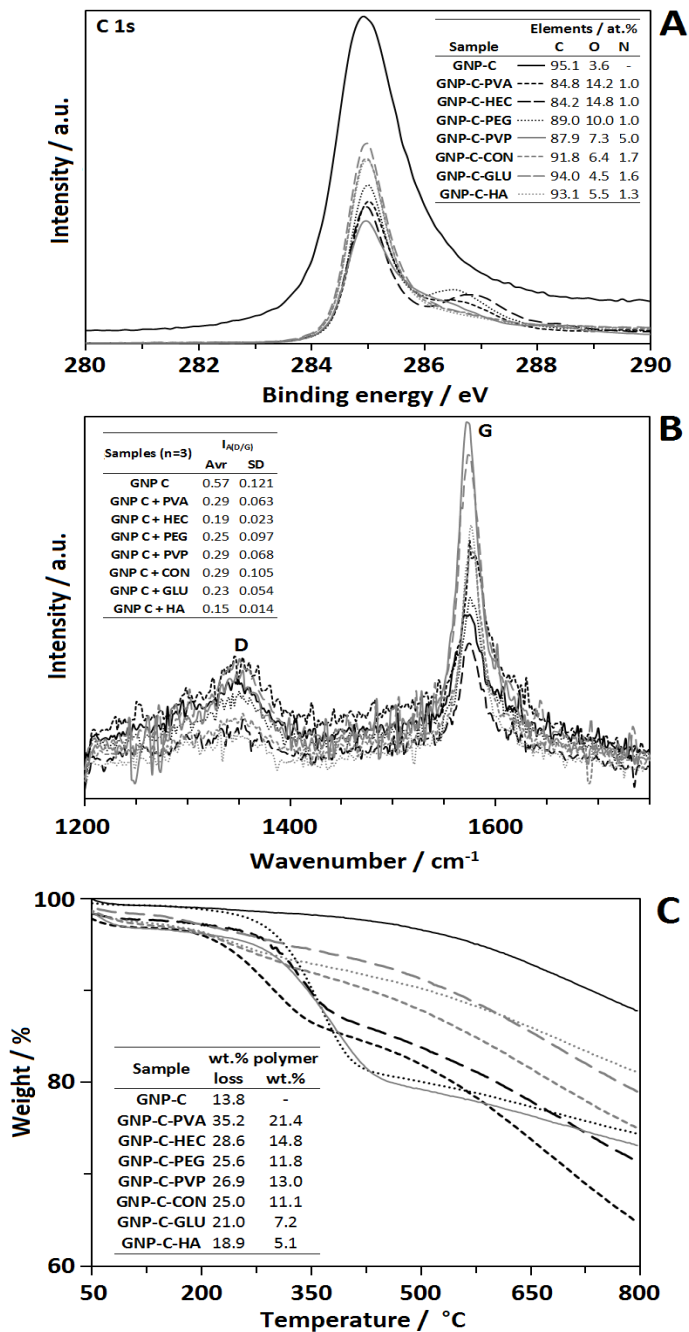


Figure B2: A) XPS spectra and atomic composition of GBMs powders for the core level C 1s. B) Representative unpolarized Raman spectra and intensity ratio of the (D/G) bands for GBMs powders. The results are presented as the mean and standard deviation (n=3). C) TGA curves and weight loss for GBMs powders.

B2.2. GBMs dispersion in culture medium

Phase contrast images were obtained for each incubation time and concentration tested. Example images for DMEM+ at 24 and 72 h, and for all GBMs at 72 h for concentrations of 10 and 50 $\mu\text{g mL}^{-1}$ are shown in Figure B3. The materials deposited in well bottoms and are in contact with the cells, however it is imperceptible whether they penetrate the cell membrane or not. To clarify this question, HFF-1 cells incubated with materials were analyzed by TEM (Figure 4.4 – Chapter 4). An increase of materials concentration leads to more accumulation at the cell membrane (Fig). GNP-C possesses agglomerates with smaller size after sedimentation than GNP-C-PVA or HEC. Moreover, GNP-C-PVA has larger agglomerates than GNP-C-HEC, and both results are in agreement with laser scattering results (Fig. 4.1 – Chapter 4).

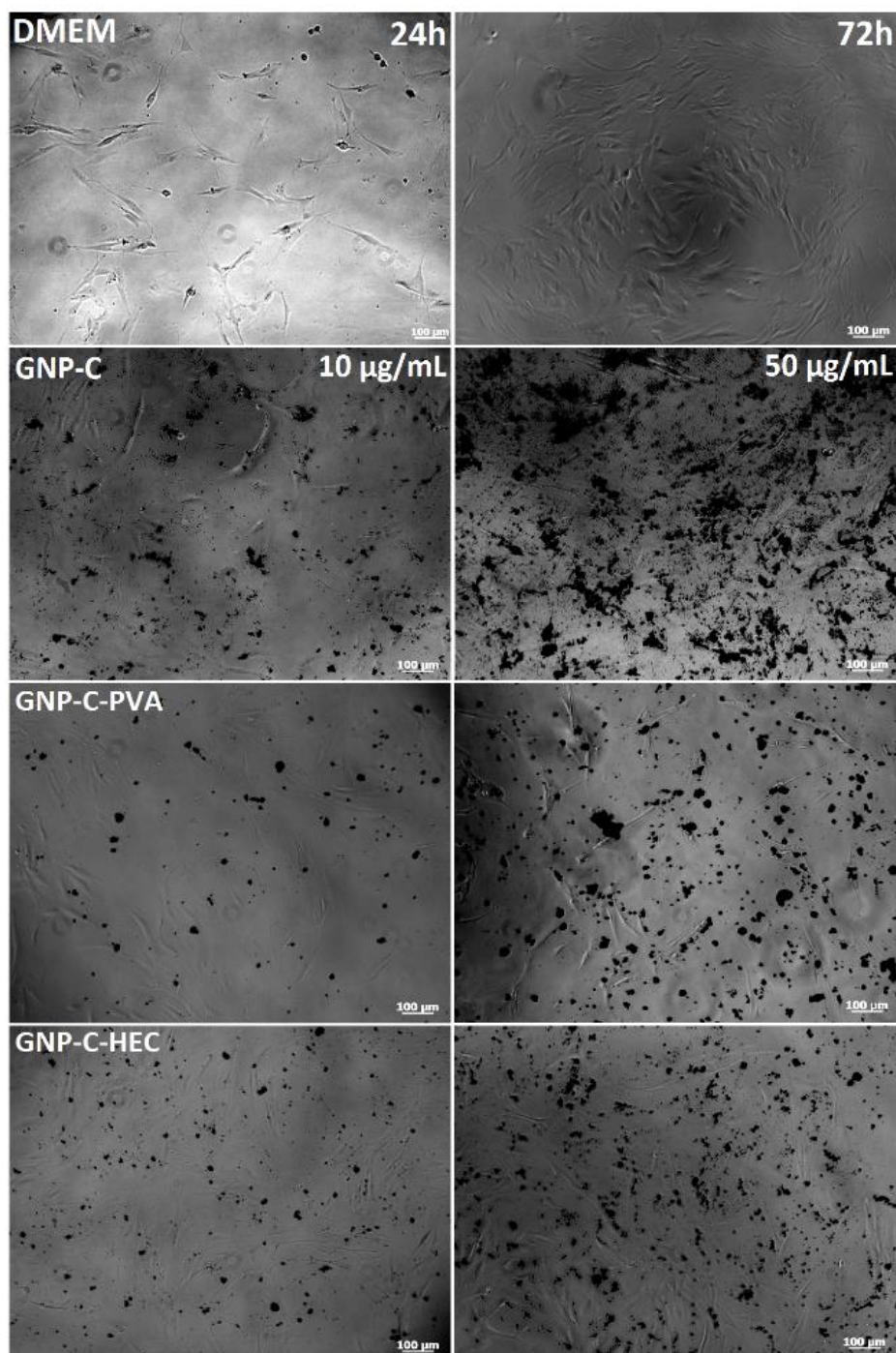


Figure B3: Optical microscopy images of HFF-1 cells in culture medium (DMEM+) at 24 and 72 h, and incubated with GBMs at concentrations of 10 and 50 $\mu\text{g mL}^{-1}$ (left and right column, respectively) for 72 h. The scale bar represents 100 μm .

S 3. Cytotoxicity

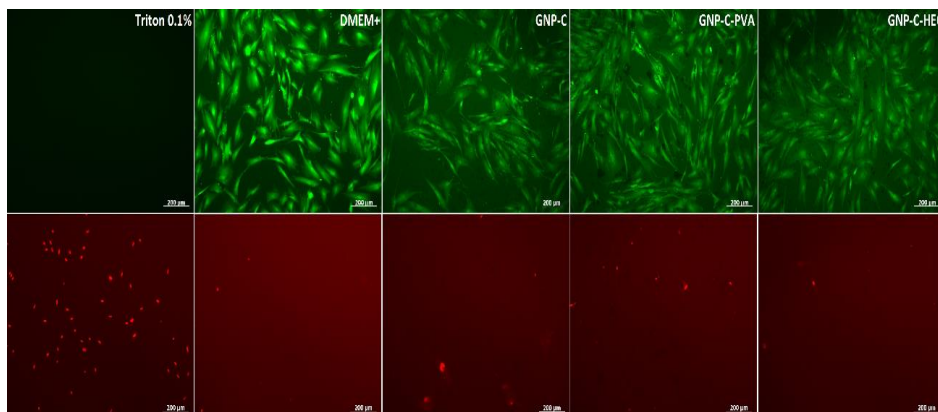


Figure B4: LIVE/DEAD staining of HFF-1 cells incubated with $100 \mu\text{g mL}^{-1}$ GBMs for 72 h. Triton 0.1 wt.% in DMEM+ was used as a toxicity positive control and cells cultured in DMEM+ as a negative control. Cytoplasm is stained with calcein – green. Cells with compromised membrane integrity were stained in the nucleus with propidium iodide – red. The scale bar represents 200 μm .

B2.4 Cell morphology

After the resazurin assays, cells in each well exposed to $50 \mu\text{g mL}^{-1}$ of GBMs were stained and observed (at $100 \mu\text{g mL}^{-1}$ GBMs interfere with staining and images had lower quality). Immunofluorescence images (Figure B5) show that for toxicity positive controls (triton 0.1 % and DMSO 10 % in DMEM+), the cell cytoskeleton was disassembled. A control was performed with cells cultured in DMEM+, which exhibited typical “spindle” like shape of fibroblasts. After 72 h of incubation with $50 \mu\text{g mL}^{-1}$ GNP-C, GNP-C-PVA or HEC, cells exhibit normal morphology similar to the negative control. These results are in agreement with those observed in the cytotoxicity assays (see Chapter 4) at 72 h for all materials because in these conditions, no considerable metabolic decrease or cell death is observed.

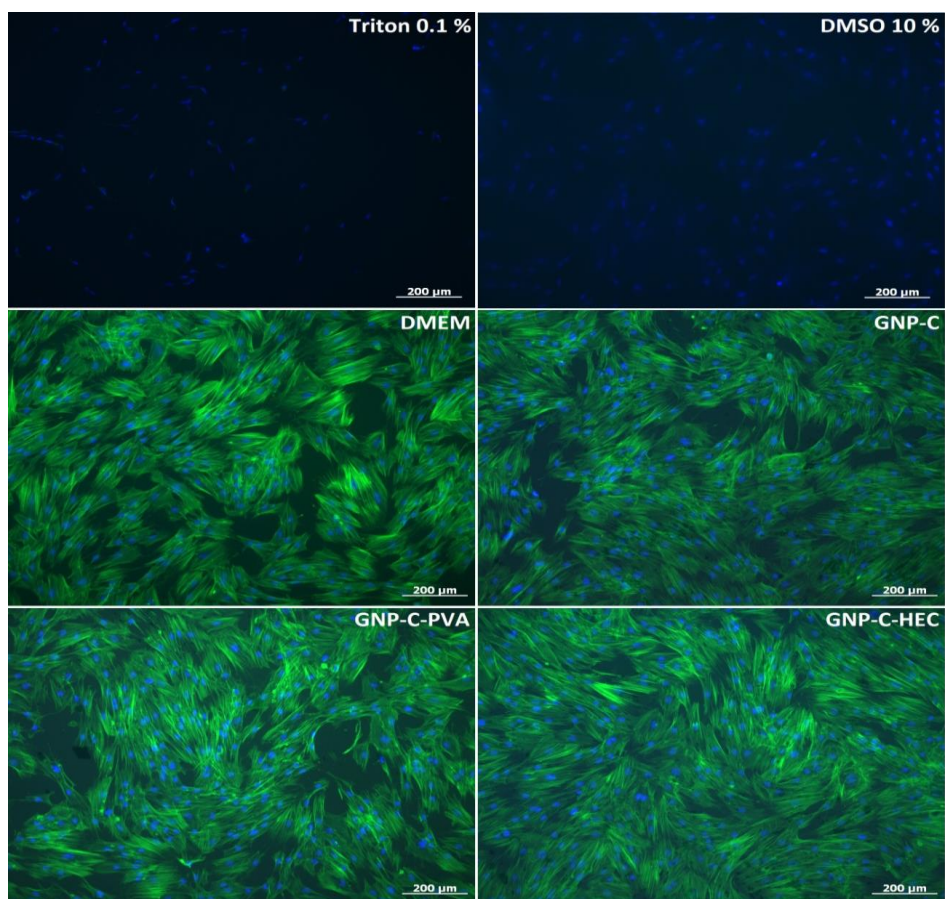


Figure B5: Representative immunofluorescence images of HFF-1 cells after 72 h incubation with $50 \mu\text{g mL}^{-1}$ GBMs. Triton 0.1 % and DMSO 10 % in DMEM+ were used as a positive control and cells grown in DMEM+ as a negative control. Cells were stained with DAPI (nucleus) blue and Phalloidin (F-actin in cytoskeleton) - green. The scale bar represents 200 μm .

B2.5. Statistical analysis

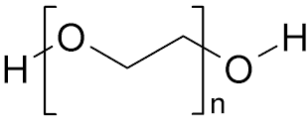
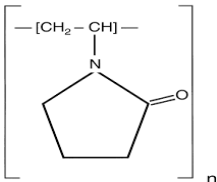
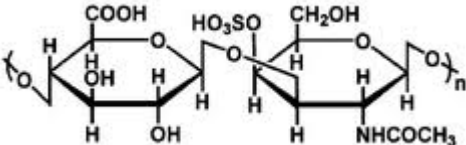
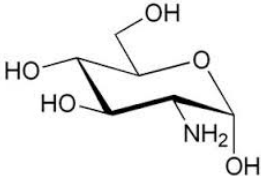
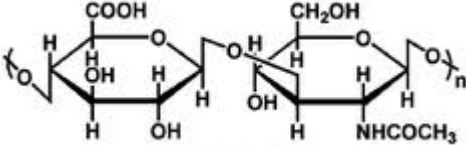
Table B1: Statistical analysis for resazurin assay. p values are shown when differences were found between samples.

Time	Conc.	Samples	DMEM+	GNP-C	GNP-C-PVA	GNP-C-HEC
24h	10 µg mL ⁻¹	DMEM+	x	p < 0.05		p < 0.01
		GNP-C	p < 0.05	x		
		GNP-C-PVA			x	
		GNP-C-HEC	p < 0.01			x
	20 µg mL ⁻¹	DMEM+	x			
		GNP-C		x		
		GNP-C-PVA			x	p < 0.05
		GNP-C-HEC			p < 0.05	x
	50 µg mL ⁻¹	DMEM+	x	p < 0.01		p < 0.01
		GNP-C	p < 0.01	x	p < 0.05	
		GNP-C-PVA		p < 0.05	x	p < 0.01
		GNP-C-HEC	p < 0.01		p < 0.01	x
	100 µg mL ⁻¹	DMEM+	x	p < 0.01	p < 0.05	p < 0.01
		GNP-C	p < 0.01	x	p < 0.01	
		GNP-C-PVA	p < 0.05	p < 0.01	x	p < 0.01
		GNP-C-HEC	p < 0.01		p < 0.01	x
48h	100 µg mL ⁻¹	DMEM+	x	p < 0.05		p < 0.01
		GNP-C	p < 0.05	x		
		GNP-C-PVA			x	p < 0.01
		GNP-C-HEC	p < 0.01		p < 0.01	x
72h	50 µg mL ⁻¹	DMEM+	x			p < 0.05
		GNP-C		x		p < 0.05
		GNP-C-PVA			x	
		GNP-C-HEC	p < 0.05	p < 0.05		x
	100 µg mL ⁻¹	DMEM+	x			p < 0.05
		GNP-C		x		p < 0.05
		GNP-C-PVA			x	
		GNP-C-HEC	p < 0.05	p < 0.05		x

B3. Polymer’s chemical structure

Table B2: Chemical structure of the polymers studied for GBMs modification.

Polymer	Chemical Structure
PVA	$\left[\begin{array}{cc} \text{H} & \text{H} \\ & \\ -\text{C} & - & \text{C}- \\ & \\ \text{H} & \text{OH} \end{array} \right]_n$
HEC	$\left(\begin{array}{c} \text{RO} \quad \text{OR} \\ \diagdown \quad \diagup \\ \text{O} \quad \text{O} \\ \diagup \quad \diagdown \\ \text{OR} \end{array} \right)_n$ R = H or CH₂CH₂OH

PEG	
PVP	
CON	
GLU	
HA	

B4. References

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APPENDIX C

SUPPLEMENTARY MATERIAL FOR CHAPTER 8

PHYSICAL-CHEMICAL PROPERTIES AND

BIOCOMPATIBILITY OF PLA/GBMs COMPOSITES -

EFFECT OF BIODEGRADATION

C1. Fillers distribution in PLA matrix

Figure C1 shows the number of agglomerates per unit of area (mm^2) as a function of apparent particle sizes, for different PLA/GNP-M and C 0.25 wt.%, evaluated by direct measurements on several SEM images collected for each material, using ImageJ software. ^[1] Both GNP-C and M are well exfoliated in PLA matrix. GNP-C presents apparent particle sizes mostly bellow 2 μm , in agreement with the maximum nanoplatelet diameters reported by the manufacturer. GNP-M presents a fraction of material with sizes above 2.5 μm , corresponding to the visible nanoplatelets with lateral dimensions of about 5 μm and some small agglomerates.

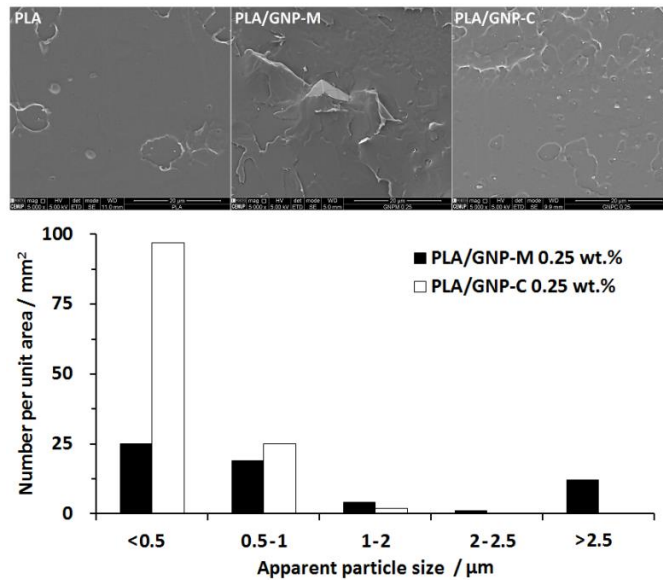


Figure C1. Example of SEM images of samples broken under liquid nitrogen, showing fillers distribution on PLA matrix. Number of agglomerates per unit of area (mm^2) as a function of agglomerate length, for PLA/GNP-C and M 0.25 wt.%.

C2. Fourier Transform Infrared Spectroscopy

Figure C2, shows spectra typical for PLA, with peaks correspondent to alkyl C-H stretches being observed from 3000-2850 cm^{-1} , and the C=O stretching region appearing around 1750 cm^{-1} . A band at 1450 cm^{-1} attributed to CH_3 is found. The C-H deformation and asymmetric bands are present at 1380, and 1355 cm^{-1} . The C-O stretching modes of the ester groups are present around 1178 cm^{-1} . A band correspondent to C-O-C asymmetric stretching is present around 1078 cm^{-1} , and C-O alkoxy stretching vibration mode is at 1060 cm^{-1} . Also, a band correspondent to C- CH_3 vibrations is present around 1041 cm^{-1} . Bands at 865, and 754 cm^{-1} are attributed to the amorphous and crystalline phase of PLA, respectively. [2] FTIR spectra for PLA, and composites before and after degradation are similar.

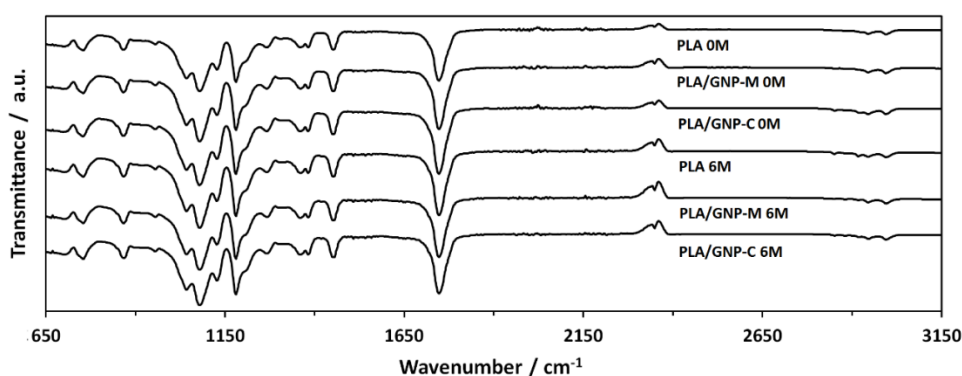


Figure C2: FTIR spectra for PLA and composites before and after degradation during 6 months in PBS at 37 °C and 100 rpm.

C3. X-ray Diffraction

X-ray diffraction analysis, confirmed the presence of GNP in the PLA matrix. Figure C3 shows that GNP-M and C powders present similar XRD spectra, typical of graphitic materials [3], with an intense peak around 31°, and two broad peaks around 50° and 65°. GNP-M presents a more intense peak at 31° because it has a higher number of stacked graphene sheets per platelet than GNP-C, thus having different planes contributing to the same diffraction. [4] PLA, before (0M) and after the 6 months (6M) biodegradation period, presents similar typical spectra, with two broad peaks. The first, around 20°, is more intense than the second, around 35°. [5] PLA/GNP-M 0.25 wt.% 0M and 6M present similar spectra, with PLA and GNP-M peaks being observed,

which confirms the presence of filler in the polymer matrix. For PLA/GNP-C 0.25 wt.% 0M and 6M, the two spectra are also similar, however the GNP-C peak is much less intense than GNP-M peak, as expected since the pristine powders also show the same intensity disparity.

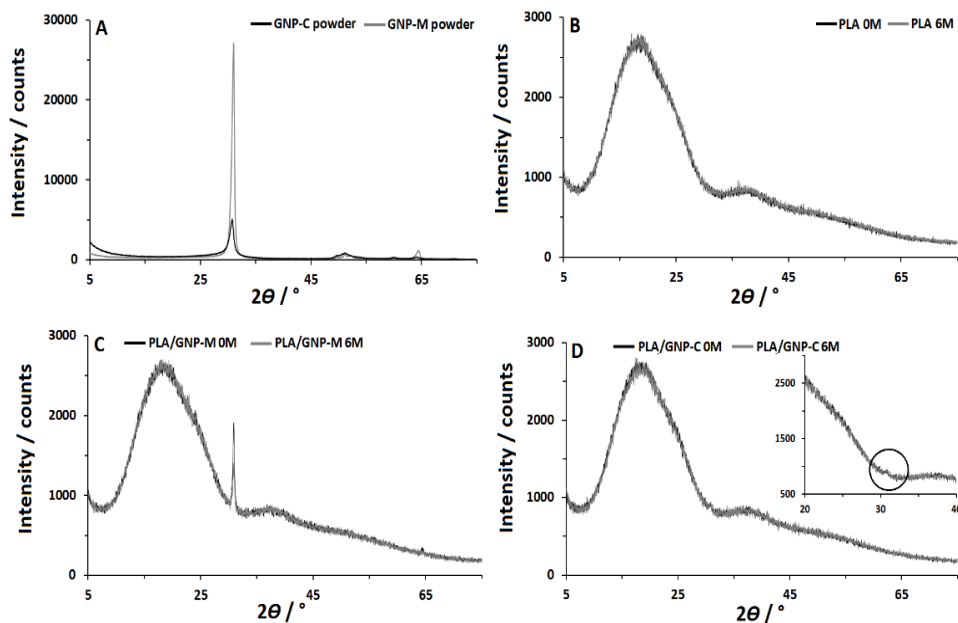


Figure C3. XRD spectra for GNP-M and C powders (A), PLA 0M and 6M (B), PLA/GNP-M 0.25 wt.% 0M and 6M (C) and PLA/GNP-C 0.25 wt.% 0 and 6M (D).

C5. References

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