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Graphene-containing dECM-based Hydrogels for Cardiovascular Applications

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DISSERTATION

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Resumo

As doenças cardiovasculares (DCVs) são a principal causa de morte no mundo, afetando cerca de 523 milhões e matando mais de 18 milhões de pessoas anualmente. O principal impulsionador destes números é o enfarte do miocárdio, geralmente designado como ataque cardíaco, que ocorre quando o fluxo sanguíneo no coração é comprometido, causando isquemia e levando a danos irreversíveis do miocárdio devido à morte dos cardiomiócitos. As terapias atuais focam-se apenas no restabelecimento do fluxo sanguíneo recorrendo a estratégias farmacológicas, à angioplastia com ou sem recurso ao uso de stents ou ao *bypass* coronário. No entanto, mesmo quando o fluxo sanguíneo é restabelecido com sucesso, o dano causado no tecido cardíaco é permanente, deixando os pacientes com sequelas ao longo da vida que, em última instância, culminam com a insuficiência cardíaca e morte do paciente. Este desfecho está intimamente relacionado com a falta de capacidade regenerativa dos cardiomiócitos. Consequentemente, um dos desafios atuais da bioengenharia é desenvolver novas estratégias que permitam a regeneração do tecido cardíaco.

O uso de hidrogéis derivados da matriz extracelular descelularizada (dECM) tem sido descrito como uma abordagem promissora para promover a regeneração cardíaca. Estes hidrogéis preservam os sinais bioquímicos que controlam várias funções celulares, incluindo adesão celular, interações com fatores de crescimento e capacidade de remodelação. Embora sejam utilizados em estratégias injetáveis, estes hidrogéis apresentam propriedades mecânicas fracas, o que impede o seu uso na criação de *scaffolds* 3D para suportar a regeneração cardíaca.

O objetivo desta dissertação é superar esta limitação dos hidrogéis de dECM, combinando-os com óxido de grafeno (GO), um dos materiais mais fortes da Terra, para produzir um hidrogel mecanicamente reforçado que possa suportar uma estrutura criada por impressão 3D, mantendo os sinais bioquímicos para apoiar a adesão celular.

Hidrogéis de dECM provenientes da placenta humana, especificamente do córion, foram combinados com dois tipos de GO disponíveis comercialmente (dispersão aquosa (GO-WD), 4% (p/v) e dispersão extremamente concentrada (GO-HC), 25% (p/v)). Apesar de apresentarem a mesma composição atômica (70,3% de carbono e 29,7% de oxigénio), o GO-WD apresenta partículas mais agregadas e com um tamanho lateral inferior, $11,9 \pm 4,0 \mu\text{m}$, do que o GO-HC, $22,4 \pm 9,5 \mu\text{m}$. A esterilização do GO foi efetuada em autoclave, não se verificando alterações significativas na sua composição química e estrutural. Diferentes concentrações de hidrogel de

dECM (3-4% (p/v)) e GO (1% a 100% (p/p de dECM)) foram testadas para obter uma *ink* imprimível. A incorporação do GO-HC em hidrogéis dECM mostrou ser capaz de produzir uma *ink* mais homogênea do que GO-WD. De todas as formulações testadas, a dECM 4% (p/v) com 25% GO (p/p) mostrou-se capaz de produzir um filamento com uma forma mais definida e estável, e com módulos elástico (G') e plástico (G''), e viscosidade superiores à dECM atingindo valores de 1280 Pa, 535,2 Pa e 550,1 Pa.s, respetivamente. Apesar da *ink* de dECM/GO apresentar uma capacidade de ser impressa superior, tanto dECM como dECM/GO apresentam comportamento reofluidificante.

Além da otimização da *ink*, foram avaliadas diversas plataformas e parâmetros de impressão para atingir uma impressão eficiente. Como plataforma de impressão, foram testados dois materiais (vidro e plástico) e dois revestimentos (plurónico e *sigmacote*), sendo o vidro revestido com *sigmacote* a melhor plataforma de impressão para produzir filamentos que mantêm a forma e se desprendem facilmente. Considerando que a gelificação dos hidrogéis dECM é desencadeada pela temperatura (a 37 °C), avaliou-se a influência da temperatura da plataforma (temperatura ambiente (RT) ou 37 °C), sendo a RT a melhor condição. Dois banhos de suporte diferentes, dextrano e água, também foram testados como plataformas de impressão. Embora fosse possível imprimir um filamento de alta resolução em água, não foi possível obter uma estrutura tridimensional porque as camadas de impressão não interagiam umas com as outras. No dextrano, o filamento desintegrou-se.

Em relação aos parâmetros de impressão foram testados diferentes tamanhos de agulha (22 G e 24 G) e velocidades de fluxo (0,8 - 2,3 mm/s), sendo que as condições que proporcionaram os melhores resultados foram a agulha 22 G e velocidades de fluxo entre 2,0 mm/s e 2,3 mm/s.

Como prova de conceito, foram impressas *scaffolds* com 12 x 12 mm, uma ou duas camadas e dois tipos de malha, com tamanhos de poros entre 1,8 - 2,0 mm, usando os parâmetros otimizados. Apesar disto, é importante salientar que as propriedades dos hidrogéis de dECM variam muito dependendo do lote de produção, o que pode, por vezes, afetar a estrutura final, exigindo pequenos ajustes nas condições de impressão dependendo do lote.

Para avaliar a capacidade destes materiais aderirem células, hidrogéis de dECM estéreis e GO-HC autoclavado foram usados para criar filmes dECM e dECM/GO. Ambos os hidrogéis, dECM e dECM/GO, mostraram capacidade de aderir e manter fibroblastos (linha celular HFF-1) durante 7 dias, mostrando que a incorporação de GO não alterou os sinais bioquímicos presentes na dECM.

No geral, este trabalho revelou que o GO é capaz de criar *inks* baseadas em dECM com propriedades mecânicas e de impressão superiores sem afetar os sinais bioquímicos. Estas descobertas contribuirão para o avanço da impressão 3D de dECM, permitindo a construção de um *scaffold* com alta resolução e precisão que pode ser usado na regeneração cardíaca, bem como numa infinidade de aplicações.

Abstract

Cardiovascular diseases (CVDs) are the leading cause of death worldwide, affecting around 523M and killing more than 18M people each year. The main driver for these numbers is the myocardial infarction, commonly known as heart attack, which occurs when the blood flow to the heart is compromised, causing ischemia, and leading to irreversible damage of the myocardium due to the cardiomyocyte's death. The current therapies focus on restoring blood flow through medication, angioplasty, stenting, or bypass surgery. However, even when the flow is successfully re-established, the damage in the cardiac tissue is permanent, leaving patients with lifelong effects that ultimately result in heart failure and death. This severity is deeply connected to the cardiomyocytes lack in regenerative properties. Consequently, one of the current challenges in bioengineering is to develop new strategies that enable the regeneration of cardiac tissue.

The use of hydrogels derived from decellularized extracellular matrix (dECM) has been described as a promising approach to enable cardiac regeneration. These hydrogels preserve the biochemical cues that control a number of cellular functions, including cell adhesion, interactions with growth factors, and capacity for remodelling. Despite being used in injectable strategies, they have poor mechanical properties, which impair the creation of refined 3D - scaffolds to support cardiac regeneration.

The aim of this dissertation is to overcome this limitation of dECM hydrogels, by combining them with graphene oxide, one of the strongest materials on earth, to produce a mechanically reinforced hydrogel that could support a 3D-structure created by 3D-printing while retaining biochemical cues to support cell adhesion.

ECM-hydrogels derived from human placental chorionic tissues were combined with two types of commercially available GO (water dispersion (GO-WD), 4% (w/v), and highly concentrated (GO-HC), 25% (w/v)). Despite exhibiting the same atomic composition (70.3% carbon and 29.7% oxygen), GO-WD was shown to be more aggregated and have smaller lateral size, $11.9 \pm 4.0 \mu\text{m}$, than GO-HC, $22.4 \pm 9.5 \mu\text{m}$. Autoclaving was performed for GO sterilization, showing no significant alterations on its chemical composition and structure. Different amounts of dECM hydrogel (3-4% w/v) and GO (1% to 100% (w/w of dECM)) were tested to achieve a printable ink. The incorporation of GO-HC in dECM hydrogels showed to be able to produce a

more homogeneous ink than GO-WD. From all tested formulation the 4% dECM (w/v) with 25% GO (w/w) showed to be able to produce a filament with better shape fidelity and stability, and to increase the dECM elastic (G') and viscous modulus (G''), and viscosity to 1280 Pa, 535.2 Pa and 550.1 Pa.s, respectively. Despite the dECM/GO ink presenting superior printability, both, dECM and dECM/GO present shear-thinning behaviour.

Besides ink optimization, several printing platform conditions and printing parameters were assessed to achieve an effective printing. As printing platform, two materials (glass and plastic) and two coatings (pluronic and sigmacote) were tested, being the glass coated with sigmacote the best printing platform to produce filaments which keep the shape and are easily detached. Considering that the gelation of dECM hydrogels is triggered by temperature (at 37 °C), the influence of platform temperature (room temperature (RT) or 37 °C) was evaluated, being the RT the best condition. Two different support baths, dextran and water, were also tested as printing platforms. Although a high-resolution filament could be printed in water, a three-dimensional structure could not be built because the printing layers did not interact with each other. In dextran, the filament disintegrated.

Regarding the printing parameters, different needle sizes (22 G and 24 G) and flow speeds (0.8 - 2.3 mm/s) were tested with 22 G needle and flow rates between 2 mm/s and 2.3 mm/s providing the best results.

As a proof of concept, constructs with 12 x 12 mm, one or two layers and two types of mesh grids, with pore sizes between 1.8 - 2.0 mm, were printed using the optimized parameters. Despite this, it is important to point out that dECM hydrogels properties vary greatly depending on the production batch, which can sometimes affect the final structure, requiring slight adjusts in printing conditions depending on the batch.

In order to assess the ability of these materials to adhere cells, sterile dECM hydrogels and autoclaved GO-HC were used to create dECM and dECM/GO films. Both hydrogels, dECM and dECM/GO, show ability to adhere and keep fibroblasts (HFF-1 cell line), for 7 days, showing that GO incorporation did not change the biochemical cues present in the dECM.

Overall, this study demonstrates that GO is suitable to create dECM-based ink with superior mechanical and printable properties without affecting its biochemical cues. These findings will contribute to the advancement of dECM 3D printing, enabling the construction of a scaffold with high resolution and precision that can be used in cardiac regeneration as well as in a plethora of applications.

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Table of Contents

Resumo	v
Abstract.....	vii
Agradecimentos	ix
Table of Contents.....	xi
List of figures	xiii
List of tables	xvii
Abbreviations and Symbols	xix
Chapter 1	23
Introduction.....	23
1.1 - Motivation	23
1.2 - Goal	25
1.3 - Dissertation Outline	26
Chapter 2.....	27
Literature Review	27
2.1 - Hydrogels and Extracellular Matrix	28
2.2 - Graphene-Based Materials	33
2.3 - dECM Hydrogels Containing GBMs.....	39
2.4 - 3D Printing	46
Chapter 3.....	51
Materials and Methods	51
3.1 - Graphene Oxide (GO).....	51
3.2 - Decellularized Extracellular Matrix (dECM) Gels	52
3.3 - dECM/GO Scaffolds	53
3.4 - <i>In vitro</i> biocompatibility of dECM/GO.....	55
Chapter 4.....	57
Results and Discussion	57
4.1 - GO characterization	57
4.2 - dECM/GO Scaffolds	59
4.3 - 3D Printing	71

4.4 - Biological Assessment	75
Chapter 5.....	77
Conclusion and Future Perspectives	77
5.1 - Conclusion.....	77
5.2 - Future Perspectives.....	78
References	81
Annex A.....	89
Annex B.....	91
Annex C.....	93

List of figures

Figure 1 - Death rate from cardiovascular disease in 2019 [6] and leading causes of death globally according to the World's Health Organization (WHO) [7] .	23
Figure 2 - Engineering human tissue equivalents in vitro [2].	27
Figure 3 - Extracellular matrix (ECM) compartments. Schematic representation of the main components of the two ECM compartments: basement membrane (BM) and interstitial matrix (IM) [41].	29
Figure 4 - Histological assessment of different chemical-based decellularization methods attempted for human cardiac tissue from 3 different donors. (a) Chemical-based decellularization methods tested. For each donor is shown: (b), (e), (h) Gross tissue morphology. Scale bars: 1 mm; (c), (f), (i) Hematoxylin & Eosin staining; (d), (g), (j) Masson's Trichome staining. Scale bars: 100 μ m. [50].	31
Figure 5 - Properties of GBMs (G: graphene; GO: graphene oxide; rGO: reduced graphene oxide) and a few of their current biomedical applications [35].	34
Figure 6 - The crystal structure of graphene, GO and rGO and the process used to obtain GO and rGO from graphene [98].	35
Figure 7 - Nanomaterials for the selective control of stem cell growth and differentiation. a: Different nanoscale materials for stem cell control and differentiation. b: Graphene and graphene oxide (GO) used as scaffold materials for the culture of various stem [1].	36
Figure 8 - (A) SEM images of SISMA/GO hydrogels crosslinked with temperature and with blue-light irradiation. (B) Temperature sweep experiments in SISMA-GO and SISMA hydrogels. (C) Time sweeps before and after irradiation [123].	40
Figure 9 - SEM images of dECM-rGO hydrogels. Deposition of rGO flakes (white arrows) on the pore walls can be observed. Adapted from [55], with permission from Elsevier.	42
Figure 10 - Sarcomere development in engineered heart tissues. Representative fluorescent images of Day 35 tissues generated with the different hydrogels stained for sarcomeric α -actinin (red) and nuclei (blue). Scale bar = 10 μ m. Adapted from [55] , with permission from Elsevier	43
Figure 11 - Macroscopic images of hydrogels with different concentrations of GO (A4G0, A4G25, A4G50). Adapted from [124], with permission from Elsevier.	45

Figure 12 - Process from 3D model to 3D printable model. Adapted from Pham, G. N., Lee, S.-H., Kwon, O.-H. & Kwon, K.-R. A 3D Printing Model Watermarking Algorithm Based on 3D Slicing and Feature Points. Electronics 7 (2018), with permission.	47
Figure 13 - Definition of extrusion-based biomaterial inks [3].	48
Figure 14 - Effect of CMs in hydrogels bioprinting [129].	49
Figure 15 - A: Atomic percentages (at %) of carbon 1s and oxygen 1s and carbon high-resolution spectrum acquires by XPS. B: TEM images and particles lateral size (n=14) of GO-WD, GO-HC, and autoclaved GO-HC.....	58
Figure 16 - dECM pre-gel.	59
Figure 17 - Printing simulation of dECM pre-gel with 3% and 4% concentration. The printing surface is plastic.....	59
Figure 18 - dECM 4% hydrogels with different concentrations of GO-WD and GO-HC suspensions.	60
Figure 19 - Comparison of a dECM 4% formulation with 10% GO using GO-WD and GO-HC.	61
Figure 20 - Comparison of the same formulations extruded onto plastic and glass surfaces...	62
Figure 21 - Extrusion onto pluronic coated surfaces.	62
Figure 22 - Extrusion of dECM/GO-WD onto Sigmacote coated surfaces.	63
Figure 23 - Comparison of the same formulations extruded for platform at room temperature and 37 °C	64
Figure 24 - Printing of the ink into dH ₂ O or Dextran 5% at room temperature.....	64
Figure 25 - dECM-i3S and dECM-MUV pre-gels.....	66
Figure 26 - Strain sweep assays for dECM and dECM/GO inks.....	67
Figure 27 - Frequency sweep assays for dECM and dECM/GO inks.	68
Figure 28 - Temperature Sweep assays for dECM and dECM/GO inks.....	68
Figure 29 - Time Sweep assays for dECM and dECM/GO inks.	69
Figure 30 - Shear Rate Ramp assays to determine if the dECM and dECM/GO inks present shear-thinning behaviour.	70
Figure 31 - Shear viscosity in function of shear rate to determine if dECM and dECM/GO inks present thixotropic behaviour.	70
Figure 32 - 3D printing of single-layered squares using different needles size and flow velocities.	71
Figure 33 - 3D printing of three-layered square.	71
Figure 34 - 3D printing of mesh grid square.	71
Figure 35 - Schematics followed for the printing of the scaffolds. Yellow arrow: needle path; dt: thickness; dp: pore size; dl: lateral size; dh: height.....	72
Figure 36 - Photographic images of the printed scaffolds using four different inks: dECM, dECM/GO, dECM/GO _{autoclave} and dECM/GO _{ethanol} and respective SEM imaging. Scale: 150 µm (left) and 15 µm (right).	73

Figure 37 - Photographic images of the printed scaffolds using two different inks: dECM/GO and dECM/GO _{autoclave} , with two different methods of homogenization: vortex and magnetic stirring.....	74
Figure 38 - Confocal images of cells seeding on dECM and dECM/GO films at day 1 and 7. Green fluorescence: phalloidin; cyan fluorescence: DAPI. Scale bar = 100 μ m.....	75

List of tables

Table 1 - Important aspects of the published works about dECM hydrogels reinforced with GBMs.	41
Table 2 - Main advantages and limitations of the different bioprinting techniques. Adapted from [129].	47
Table 3 - Gelation strategies and scaffolds features.	65
Table 4 - Ink Formulations, Printing Platform and Strategy. The parameters chosen are highlighted in blue.	66

Abbreviations and Symbols

List of abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
Amine-rGO	Amine-Functionalized Reduced Graphene Oxide
CAD	Computer-Aided Design
CEMUP	Centro de Materiais da Universidade do Porto
CM	Carbon Material
CN	Carbon Nanotubes
CSMA	Chitosan Methacryloyl
CVD	Cardiovascular Disease
Cx43	Connexin 43
DAPI	4',6-Diamidino-2-Phenylindole
dECM	Decellularized Extracellular Matrix
dECM-i3S	dECM digested at i3S
dECM-MUV	dECM digested at MUV
dECM-OA	dECM Containing Oxidized Alginate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
ds-DNA	Double-stranded DNA
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EHT	Heart Tissue Construct
EtOH	Ethanol
FBS	Foetal Bovine Serum
GAG	Glycosaminoglycan
GBM	Graphene Based Materials
GF	Graphene Foam

GFAP	Glial Fibrillary Acidic Protein
GO	Graphene Oxide
GO-HC	Graphene Oxide Highly Concentrated
GO-HC autoclave	Graphene Oxide Highly Concentrated Autoclaved
GO-WD	Graphene Oxide Water Dispersion
H&E	Haematoxylin And Eosin
hAD-MSC	Human Amnion-derived MSC
HCl	Hydrochloric Acid
HFF-1	Human Foreskin Fibroblast
hiPSC	Human Induced Pluripotent Stem Cells
hMSCs	Human Mesenchymal Stem Cell
IHD	Ischemic Heart Disease
iPSC	Induced Pluripotent Stem Cell
LF	Lorentzian Function
LVE	Linear-Viscoelastic
LVER	Linear-Viscoelastic Regime
MA	Methacryloyl
MSC	Mesenchymal Stem Cell
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NIR	Near-infrared
NSC	Neural Stem Cell
OA	Oxidized Alginate
PAA	Peracetic Acid
PBS	Phosphate-buffered Saline
PCL	Polycaprolactone
PEG	Poly(ethylene glycol)
Pen/Strep	Penicillin-Streptomycin
PFA	Paraformaldehyde
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly(lactic Acid)
PPG	Poly(propylene glycol)
PPT	Photothermal Therapy
rGO	Reduced Graphene Oxide
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT	Room Temperature
SEM	Scanning Electron Microscopy

Si-HPMC	Silated Hydroxypropylmethyl Cellulose
SIS	Small Intestine Submucosa
ss-DNA	Single-stranded DNA
TEM	Transmission Electron Microscopy
TG	Transglutaminase
TUJ1	Beta Tubulin III
XPS	X-ray Photoelectron Spectroscopy
B-TCP	B-tricalcium PhosphateXPS

List of symbols

γ	Shear Strain
$\eta(\dot{\gamma})$	Shear Viscosity
$\dot{\gamma}$	Shear Rate
G'	Elastic Modulus
G''	Viscous Modulus

Chapter 1

Introduction

1.1 - Motivation

Cardiovascular diseases (CVDs) are the primary cause of death in the world (**Figure 1**) leading to more than 18 million fatalities each year [4]. Furthermore, it is estimated that around 523 million people live with a CVD that forces the patients to adjust daily activities so as to accommodate this disability and, at the same time, increases the risk factor for severe cardiac events [5].

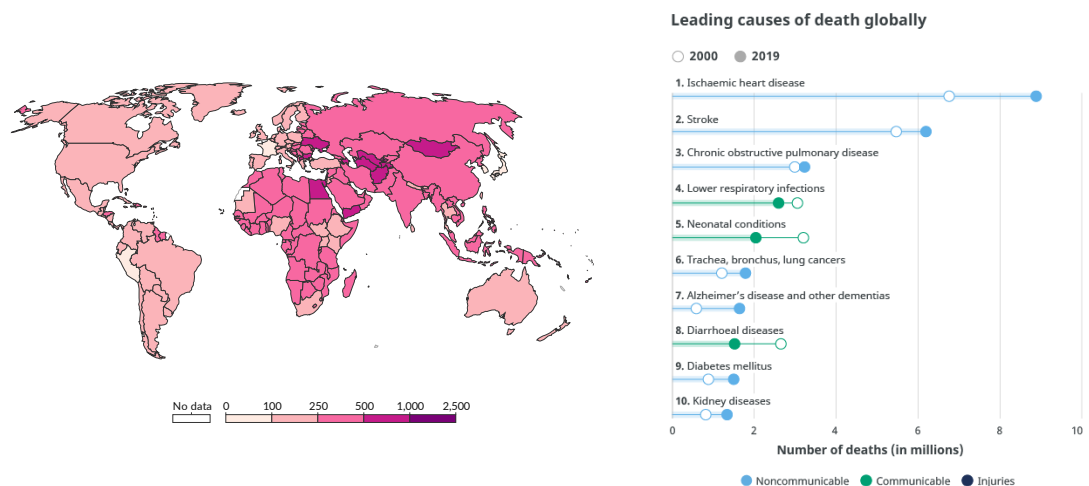


Figure 1 - Death rate from cardiovascular disease in 2019 [6] and leading causes of death globally according to the World's Health Organization (WHO) [7] .

Myocardial infarctions are one of the major contributors to the high mortality and morbidity of CVDs and manifest, in the majority of cases, as a consequence of ischemic heart disease (IHD), the most common type of CVD (11.7 million) [8, 9]. With such incidence, IHDs also present an enormous economic impact and health care costs are predicted to increase from \$126.2 billion in 2010 to \$177.5 billion in 2040, in the United States alone [10].

In myocardial infarction, the blood flow to the heart is restricted due to an obstruction in the coronary arteries leading to ischemia of the cardiac tissue. The oxygen deprivation on the myocardium results in cell death and necrosis. The current treatment of myocardial infarction is done with therapies that aim to re-establish blood flow. The implemented strategies include the administration of antiplatelet and anticoagulant drugs that inhibit the clot expansion, and fibrinolytics, also known as thrombolytics, to dissolve the obstruction [11]. When these are not sufficient, invasive procedures such as percutaneous coronary intervention (angioplasty with stent) and coronary artery bypass grafting (heart bypass surgery) are performed in efforts to achieve reperfusion [12]. Nevertheless, even if blood flow is successfully restored preventing the patient's death, the lack of oxygen results in a portion of heart tissue that is irreversibly damaged. In these cases, the patient's heart function is diminished, as the functioning cardiomyocytes are replaced with scar tissue, leading to a reduction in quality of life and a multitude of additional health problems, including heart failure [13].

In this regard and even though the current procedures in clinics are satisfactory to re-establish the blood flow to the heart, there is a need for new strategies that can regenerate the cardiomyocytes after myocardial infarction to promote the normal heart function, preventing the disease's progression to critical and deadly outcomes and alleviate the burden of CVDs [9]. One of the current challenges in biomedical research is the regeneration and proliferation of cardiomyocytes to replace the damaged tissue [14, 15]. However, contrary to other human tissues and organs such as skin, liver, skeletal muscles, and blood, the myocardium lacks a regenerative capacity requiring innovative therapies designed by exploring areas such as stem cell engineering, functional biomaterials, and biomimetic scaffolds. Although a vast number of papers have been published highlighting new advances in this field, it is still not possible to create fully matured and functional cardiac tissue [16].

Recently, the extracellular matrix (ECM), the acellular component of tissues, has been described as having a great impact on cardiomyocytes by modelling the cellular behaviour. The microenvironment created by the ECM is capable of providing foetal cardiomyocytes a very plastic and pro-regenerative nature while, after birth and in adults, changes occur that lead to the reduced capacity for regeneration typical of these cells [17]. By using ECM-based strategies to create scaffolds that modulate cellular performance, it is expected that ECM and decellularized ECM (dECM) can play a part both on the repairing of the injured myocardium and the inducing of cardiac regeneration. One of these strategies is the creation of dECM-based hydrogels with properties capable of mimicking the native tissues and high biocompatibility. However, dECM hydrogels lack mechanical properties that can lead to challenges in supporting cellular behaviour and creating structures that resemble the native morphology of the tissues [18]. To overcome the low mechanical features of biomaterials, it is common to supplement them with a higher performing material that can provide mechanical strength (e.g. carbon-based materials and nano-clays) [19]. Additionally, to provide the scaffolds the adequate

physical structure, manufacturing techniques such as 3D printing and electrospinning, allow the creation of high-resolution macroporous structures that facilitate cell loading and survival [18].

In particular, 3D printing has risen as a tool to revolutionize the biomedical field by allowing not only the creation of scaffolds for tissue engineering but also the printing of complete organs and models for disease study. Recently, this trend has exploded with the decrease in costs associated with the manufacturing and an increase in resolution and printing speed [20]. The global market of 3D printing for biomedical applications devices was evaluated in 1.27 billion euros in 2023 and is poised to reach 5.50 billion euros in 2028 [21].

1.2 - Goal

Considering the current biomedical status, socioeconomic impact of myocardial infarction, and the promising features of dECM in cardiac regeneration, the main aim of this dissertation is to create a 3D printed scaffold based on dECM hydrogels, that could be applied in the future to support cardiac regeneration. To achieve this, it was herein proposed the incorporation of graphene oxide (GO) to improve the printability and mechanical performance of dECM hydrogels. For this, three different goals were established:

- i) Obtain a printable ink based on dECM hydrogels by incorporating GO to enhance printability and mechanical properties.
- ii) Print a scaffold with reliable and adequate shape.
- iii) Achieve dECM/GO composites that can be cytocompatible and support cell adhesion

To fulfil our goals, we used decellularized extracellular matrix (dECM) hydrogels derived from human placental tissue and graphene oxide commercially available. First, the dECM/GO ink was optimized using an easy technique to simulate printing, which involves pipetting different inks to the printing platforms with a gel pipette. The influence of different parameters on the filament's consistency, fidelity, and capacity to be detached from the platform, was assessed. These parameters included the production method and concentration of dECM pre-gels, type and amount of GO, material and temperature of the printing platform and the use of support baths.

By using scanning electron microscopy and rheological assays, the surface topography and mechanical properties of the best formulation were assessed. As a proof of concept, the feasibility of printing a 3D structure with optimized dECM/GO ink with dimensions of 12 mm x 12 mm, two and three layers and a mesh grid was tested.

Finally, biological assays were conducted to assess biocompatibility, and capacity of dECM/GO to adhere cells on its surface.

1.3 - Dissertation Outline

Chapter 1 presents the motivation, the goal, and the outline of this dissertation.

Chapter 2 shows a literature review for the topics covered in this dissertation. Namely, the first section delves into the extracellular matrix, its properties, decellularization processes, and derived hydrogels. The second section gives an overview of the graphene-based materials (GBMs) that are currently used in biomedical applications and their mechanical, chemical, and biological properties. Lastly, a section about additive manufacturing in biomedicine including the 3D printing of dECM and hydrogels containing GBMs is provided.

Chapter 3 comprises the materials and methodology employed in this dissertation. This chapter provides a detailed description regarding the preparation of the dECM inks, their combination with the graphene oxide, the 3D printing of the scaffolds along with the adopted assays to characterize physically and biologically the dECM/GO hydrogels.

In Chapter 4, the obtained results are presented along with the discussion where a correlation with literature is established. Mainly, we confirm that the addition of GO in the dECM inks enhances its mechanical properties and printability. Additionally, we established the best formulation and parameters for 3D printing of dECM/GO scaffolds and printed scaffolds with different designs. Lastly, the biocompatibility of the dECM/GO hydrogels was proved by seeding assays using human fibroblasts.

Finally, Chapter 5 highlights the main conclusions of the work developed for this dissertation and provides insights on the future directions of this field.

Chapter 2

Literature Review

Tissue engineering is a multidisciplinary field that applies principles of biology and engineering with the purposes of i) understanding structure-function relationships in normal and diseased tissues [22], ii) developing functional biological substitutes that restore, maintain, or improve tissue function of damaged tissue [23], or iii) creating entire tissue substitutes/organs [22]. To regenerate new tissues we need cells, scaffolds, and growth factors although it is not all always necessary to use them simultaneously (**Figure 2**). The scaffold provides a similar environment to that of the native extracellular matrix (ECM) and

thus is responsible for assisting cell proliferation, differentiation, and biosynthesis. Moreover, a scaffold will also protect the new tissue formation by preventing disturbing cells from invading into the site of action. For this reasons, natural or synthetic scaffolds should closely resemble the natural cell environment so the best outcomes might be achieved [1, 24, 25].

In order to fulfil their purpose in tissue engineering, the scaffolds must meet certain mechanical, chemical, and biological criteria. These structures must be robust to physically support cell growth and proliferation but also dynamic enough to be able to transmit the biophysical and biochemical cues necessary for proper self-renewal and differentiation of stem cells [1, 26, 27]. The specific characteristics necessary for an efficient process will correlate with the tissue that is under study and will profoundly affect the success rate of tissue engineering [28]. Therefore, considering the high complexity of native tissues, one of the

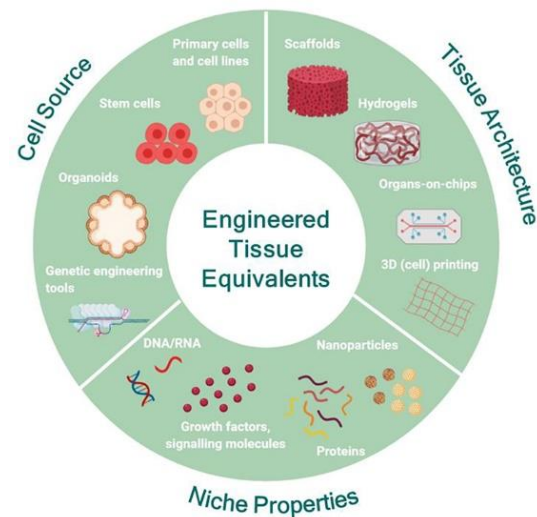


Figure 2 - Engineering human tissue equivalents in vitro [2].

biggest difficulties in tissue regeneration is the design of scaffolds that present a suitable architecture, adequate mechanical properties and the ability to deliver the molecular cues that promote efficient creation of new tissue [1, 29]. To overcome this, researchers have been exploring the use of biomaterials derived from ECM for the development of scaffolds. Typically, tissue sourced from a donor or species is used with all the cells being removed to avoid immunogenic reactions, thus obtaining the decellularized ECM (dECM). dECM based scaffolds can be used directly in biomedical interventions or can be further tuned and personalized, by repopulating them with cells for example, depending on the purpose of the application [30]. Additionally, dECM can be used itself without further modifications, if the 3D structure of decellularized tissue is similar to the one intended to be regenerated [31], or dECM can be digested to create hydrogels (dECM hydrogels) that can be extruded or moulded to obtain a desired 3D form [32]. In the case of dECM hydrogels, just the same as the majority of hydrogels, due to their high-water content, they are mechanically weak and exhibit poor conductivity, which impairs their application in regeneration of load-bearing or electrically stimulated tissues [33, 34]. One of the current strategies to overcome this undesired property of hydrogels while simultaneously making use of the advantageous ones is the hybridization with conductive nanomaterials, such as the case of graphene and related materials [35]. Graphene was firstly isolated in 2004, and due to its promising mechanical and antimicrobial properties as well as electrical and thermal conductivity, a family of materials has emerged, the graphene-based materials (GBMs), that are widely explored including in the biomedical field. Depending on the number of layers, oxidation degree and lateral size, GBMs can improve the mechanical and/or conductive properties of polymers, or promote stem cell differentiation, including in dECM derived biomaterials [1, 31].

2.1 - Hydrogels and Extracellular Matrix

2.1.1 ECM Features

The ECM is a complex network of non-cellular components present in all tissues and organs. The ECM provides physical scaffolding for the cellular constituents and allows the presentation of biochemical and biophysical cues which regulate diverse cellular functions such as cell adhesion, mechanotransduction, growth factor interactions, and remodelling capacity, and are, therefore, essential for tissue growth, migration, survival, morphogenesis, differentiation, and homeostasis [36-38].

Each tissue has a distinct and heterogeneous ECM composition (

Figure 3) and topology that results in a unique tissue specific microenvironment for resident cells. These characteristics are initially defined during tissue development through dynamic interactions between the cellular components and the surrounding microenvironment. In adult tissues, the ECM remains highly dynamic and its structural network constantly experiences

remodelling with both cellular and acellular components being subjected to a multitude of post-translational modifications. The ECM is thus accountable for the biochemical and mechanical properties that are required for the function of different tissues. Therefore, tensile and compressive strength, elasticity, and water retention will all be distinct to each tissue according to the composition of the matrix [37, 38]. In contrast, the variability and complexity of biochemical cues and architecture also means that it can be difficult to attribute a certain cell behaviour to a specific component. In the end, the architecture of the tissue together with the biochemical stimuli will play a significant role in conducting cell fate and, thus, it is believed that reseeding cells onto these decellularized scaffolds would enable improved regeneration [39, 40].

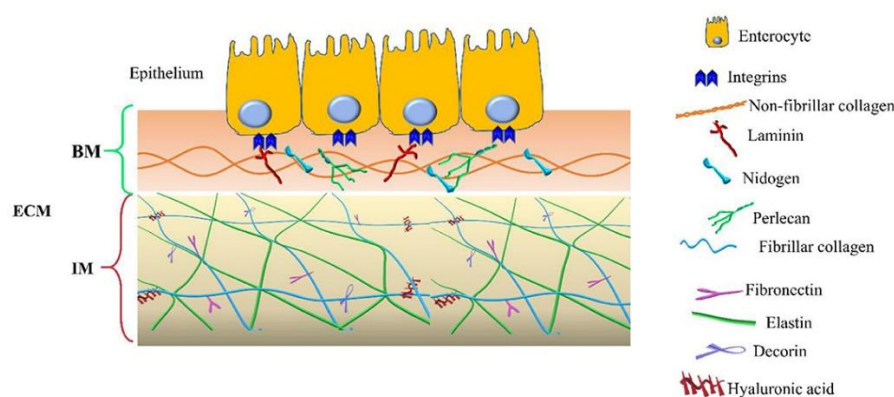


Figure 3 - Extracellular matrix (ECM) compartments. Schematic representation of the main components of the two ECM compartments: basement membrane (BM) and interstitial matrix (IM) [41].

In general, the main components of ECM are water, proteoglycans, and fibrous proteins that include collagen, elastin, fibronectin, and laminin [42, 43]. Proteoglycans are accountable for the sequester of water and divalent cations, due to their negative charge, thus conferring the gel distinctive buffering, hydration, binding, and force-resistance properties [42]. They also interact with growth factors, cell surface receptors, signalling proteins and other ECM molecules contributing to numerous cell functional properties, such as cell signalling, proliferation, migration, differentiation, apoptosis, and adhesion [44]. Collagen is the most abundant fibrous protein within the interstitial being responsible for maintaining the structural integrity of the tissue by providing tensile strength and regulating cell adhesion. Collagen is also fundamental in supporting chemotaxis and migration and directing tissue development [37]. Elastins are large fibres that provide recoil to tissues that endure constant stretch for instance in large blood vessels, heart, elastic ligaments, skin, and elastic cartilage [37, 38]. Fibronectins concurrently attach cell surface receptors, collagen, proteoglycans, and other fibronectin molecules [45] being directly involved in guiding the organization of the ECM components and, furthermore, have a very important role in mediating cell attachment and function [37]. Laminins are cell adhesion molecules vital for early embryonic development and

organogenesis holding critical functions in tissues such as muscle, nerve, skin, kidney, lung, and the vasculature [46].

In order to make use of the ECM acquired from a donor, we have to guarantee that no cells remain in the material in order to avoid immune responses [47]. The procedure to isolate the ECM is called decellularization and involves processing a donor organ or partial tissue in order to remove the cells and cellular debris. The decellularized ECM (dECM) is thus freed from most of the components present in the native tissue that are capable of eliciting an immunogenic response while its native non-immunogenic and desirable components remain [30].

2.1.2 Decellularized ECM

When it comes to choosing a tissue source, researchers usually opt for the same type of tissue that is planned to regenerate, especially if the purpose involves the use of the dECM as a scaffold without further digestion, since architecture and/or composition are naturally able to maintain selected cell functions and phenotypes for that tissue or organ [30, 48].

Despite the fact that, for biomedical applications, the ideal source for tissues would be human, it is understandable that the supply is limited. Nevertheless, some soft tissues, such as the birth-associated tissues including the placenta, umbilical cord, and foetal membranes are readily available. When human sources are not available, decellularized tissues derived from bovine, porcine, ovine, and equine donors can be used [49]. Additionally, AlloDerm®, TissueMend®, CuffPatch®, MatriStem®, and Lyoplast®, for example, are commercially available in the form of decellularized scaffolds, derived from tissues like dermis, small intestinal submucosa, urinary bladder, and pericardium sourced from both humans, and other animals like cows, and pigs [40].

There is a broad number of methods for decellularization that can be separated into three categories: biological, chemical, and physical decellularization. Biological methods work by exposing the tissue to enzymes, like proteases, that cleave adhesive proteins (e.g., trypsin and dispases) and digest residual genetic material (e.g., DNase and RNase). Chemical decellularization methods are based in solutions that contain an acid, alcohol, alkaline base, chelating agent, or detergent in which the tissues are immersed. Detergents, the most common method of chemical decellularization, work by disrupting the interactions between lipids and proteins that maintain membrane integrity. Physical decellularization techniques tend to be used as a complement to other methods as they are usually not able to obtain high degrees of cellular removal when used alone but tend to improve the efficiency of the other two methods. For example, mechanical agitation can help the penetration of biological/chemical agents into the tissue and osmotic pressure or freeze/thaw cycles can cause disruptions in the cells preparing them for additional agents [30, 48]. The optimal combination or sequence of decellularization methods is always conditioned by the tissue itself. Properties such as thickness and cellular density, the characteristics of the decellularizing agents, and the planned

application of the tissue will all dictate the steps needed for a correct and efficient process. Crapo *et. al* established the minimum criteria for the safety of a decellularized tissue as having less than 50 ng double-stranded DNA/mg in dry dECM, less than 200 base pair DNA fragment length, and no visible nuclear material in tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) or haematoxylin and eosin (H&E) [48].

As a result of the variation concerning tissue composition between donors [50] and the multitude of decellularization techniques described, one of the main drawbacks for using dECM materials is that there is no established protocol that will undoubtedly result in optimal decellularization of a specific tissue or organ [51]. In **Figure 4**, for example, we can see the differences in histological images of cardiac tissue from the same donor that was decellularized using different methods (A-D) and the difference in the decellularized tissues from different donors obtained by the same decellularization protocol (a-j). This signifies that there is often

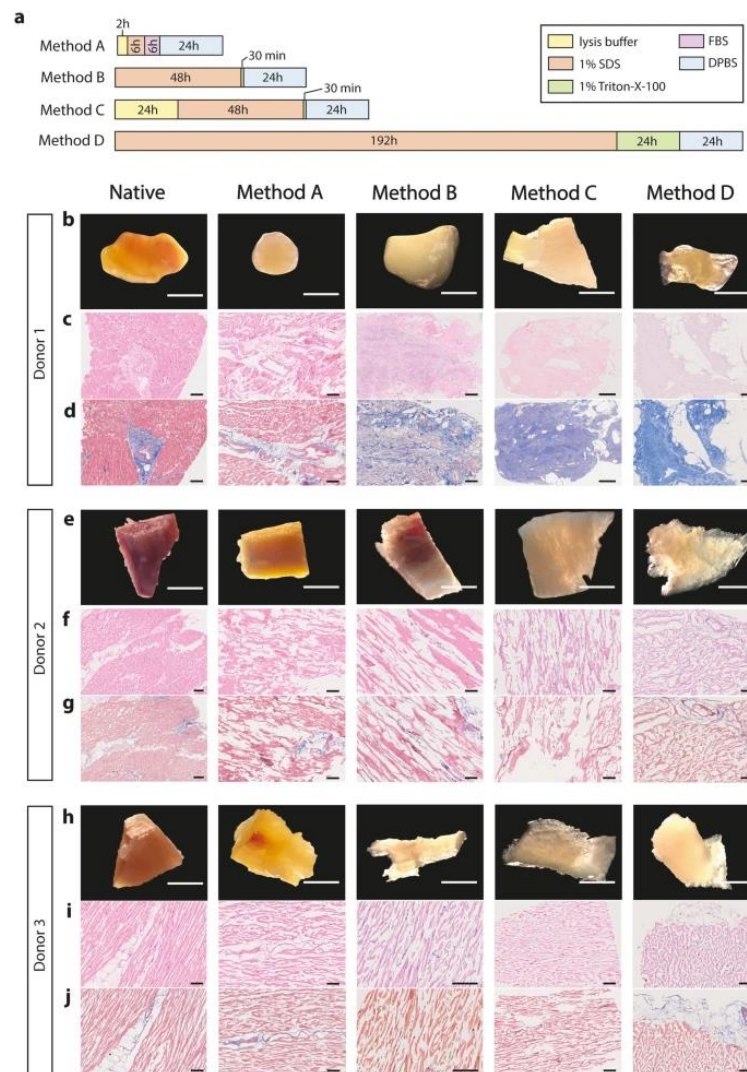


Figure 4 - Histological assessment of different chemical-based decellularization methods attempted for human cardiac tissue from 3 different donors. (a) Chemical-based decellularization methods tested. For each donor is shown: (b), (e), (h) Gross tissue morphology. Scale bars: 1 mm; (c), (f), (i) Hematoxylin & Eosin staining; (d), (g), (j) Masson's Trichrome staining. Scale bars: 100 μ m. [50].

a need for experimentation and process optimization and the subsequent protocols can be very distinct even for the same type of tissue and organ. Likewise, certain tissues have different “optimal” protocols throughout the literature and many of these vary significantly from each other [52, 53].

Another significant disadvantage of using dECM is that decellularization techniques can negatively impact the dECM that is left behind [53, 54] and, ultimately, every cell decellularizing agent and method will change the dECM composition and lead to disruptions of the native structure [48].

To this extent, decellularized scaffolds created from the same tissue types both via the same or different decellularization techniques are very likely to contain different types and amounts of native components [55], hence, the performance of a decellularized scaffold will be defined by the source tissue and the decellularization method used and both can alter the outcomes of an experiment and hinder the feasibility of the biomedical application intended.

2.1.3 dECM Hydrogels

Hydrogels are three-dimensional (3D) crosslinked networks formed by hydrophilic polymeric chains that can absorb and retain large amounts of water creating a water-rich environment oftentimes used to mimic natural tissues [34, 56].

To prepare dECM hydrogels, decellularized tissues typically undergo processes of lyophilization and milling generating a dECM powder. The dECM powder can then be further processed through digestion with an enzymatic agent, most often pepsin, resulting in thermoreversible solution that naturally self-assembles at 37 °C, the physiological body temperature, forming a hydrogel [57]. The digestion step is crucial since the enzymes will cleave the collagen triple helix telopeptide bonds unravelling the collagen fibril aggregates, the building blocks for the hydrogel [58]. Different digestion times will impact the bioactive and mechanical properties of the hydrogels, suggesting that the optimal digestion time needs to be tuned for each tissue and subsequent application [58, 59]. Additionally, the digestion allows the material to become malleable enough to acquire changes in its shape, which is important if the source tissue structure does not correspond to the desired engineered tissue. The hydrogels can be used for a wide variety of applications including the development of bioactive 2D coatings [60, 61], in conjunction with cells or growth factors [62, 63], and, more recently, as inks in 3D printing [64, 65]. Such hydrogels have been developed from a wide variety of tissue sources including cardiac [66, 67], bone [68, 69], and nervous [32, 70].

dECM derived hydrogels do not possess high mechanical strength, which results in a mismatch between the characteristics of the produced scaffolds and the native tissue. Since the stiffness of the matrix has been found to be a critical factor for tissue engineering, it is very important to design new strategies that allow the enhancement of the hydrogel strength [55]. Some strategies commonly used to increase the hydrogel mechanical properties rely on

the use of crosslinking agents that help to improve the stiffness of the material and increase degradation times. Another approach is to further manipulate the hydrogel by adding different materials, either relying on self-assembly or by promoting photo or chemical crosslinking, thus creating a composite material [39]. For example, Zhang, Li *et al.* developed an β -tricalcium phosphate (β -TCP) and chitosan methacryloyl (CSMA) composite hydrogel for *in situ* bone repair in which the β -TCP undesirable mechanical properties such as brittleness and degradation rate were improved by the multifunctional CSMA [71]. Boyer, Figueiredo *et al.* produced a silylated hydroxypropylmethyl cellulose (Si-HPMC) hydrogel reinforced with laponites (nano-clays) for cartilage tissue engineering. The resulting hydrogel presented enhanced mechanical properties while retaining its cytocompatibility [72]

2.2 - Graphene-Based Materials

2.2.1 Graphene

Graphene is a two-dimensional (2D) nanomaterial composed of sp^2 -bonded carbon atoms [73]. Firstly isolated in 2004 by Geim and Novoselov, graphene is a stable carbon allotrope where carbon atoms are tightly packed to form a single layer of six-atom rings in a 2D honeycomb lattice [1, 74]. The bonding between the adjacent carbons and the delocalization of the bonding electrons throughout the lattice contribute to a number of extraordinary electronic, optical, thermal, and mechanical properties [73]. Graphene was presumed not to exist in the free state - since strictly 2D crystals were considered thermodynamically unstable, - being initially described as an 'academic' or theoretical material [74].

Graphene is one of the strongest materials known to man, with a mechanical strength of 130 GPa, which is 325x higher than that of steel [75]. Although a very strong material, graphene is, in contrast, light weight and has a high specific surface area ($2630 \text{ m}^2\cdot\text{g}^{-1}$) [74] coupled with a robust surface chemistry [76]. The theoretical mobility of charge carriers in graphene due to the conjugated π -electrons is $200,000 \text{ cm}^2/\text{V}\cdot\text{s}$ [77] which translates to exceptional electronic conductivity [76]. Graphene also shows high Young's modulus with values around 1.0 TPa [75]. The thermal conductivity of graphene is also noticeable high with values of around $5000 \text{ W}/\text{m}\cdot\text{K}$ reported [78].

Graphene is thus an extremely strong material with excellent electrical and thermal conductivity, light weight, and elastic properties making it very unique and very attractive for diverse areas in many science fields [35, 79] from flexible electronics to biomedical applications. In the later, a lot of interesting work has been carried out to explore the use of this material for drug/gene delivery [80, 81], biosensing and molecular imaging [82, 83], antibacterial materials [84, 85], cancer therapy [86, 87], and for tissue engineering and regenerative medicine [31, 35] (Figure 5).

The most common method to obtain defect-free graphene is through chemical vapor deposition. During the process, hydrocarbon precursors are decomposed to carbon radicals and

single-layer and few-layers graphene is formed on a metal substrate that works as a catalyst to lower the energy barrier of the reaction and determine the graphene deposition mechanism, thus affecting the quality of the graphene. Although capable of obtaining pristine graphene, the industrial scale-up of the process is not yet possible due to its difficulty and high cost [88].

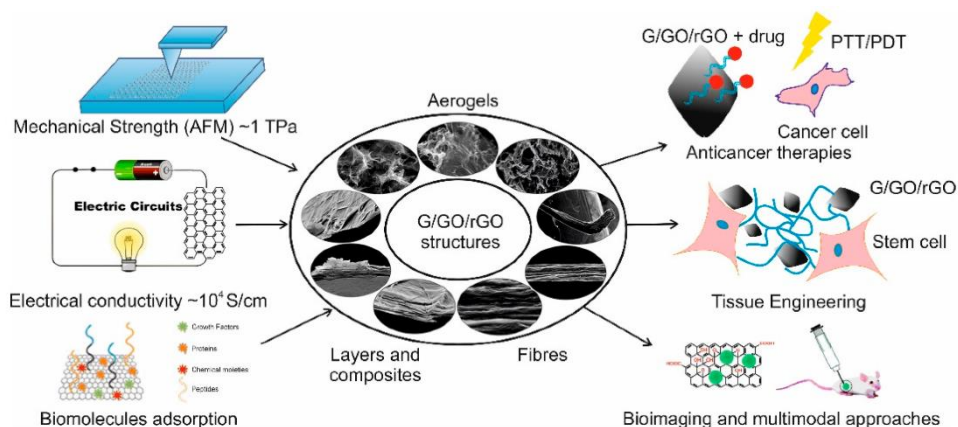


Figure 5 - Properties of GBMs (G: graphene; GO: graphene oxide; rGO: reduced graphene oxide) and a few of their current biomedical applications [35].

However, it is also possible to obtain graphene, through top-down strategies, from graphite, which is both abundant and inexpensive. Nevertheless, it is difficult to isolate a single layer graphene using these strategies, being most times obtained multi-layers or few-layers graphene, which exhibit similar but not the exact same properties of pristine, single-layer, graphene.

2.2.2 Graphene Oxide

Graphene oxide (GO) is the oxidized derivative of graphene, which is obtained by the oxidation of graphite using strong oxidants [89] followed by its exfoliation (**Figure 6**). The golden method for graphite oxidation is the modified Hummers' method. The protocol, initially reported in 1958 by Hummers and Offeman [90] and later improved in 2010 [91], can be accomplished in large scale production and the resulting graphene oxide has numerous defects in the form of functional groups that provide reactive sites for surface-modification reactions [79, 92].

Pure graphene is hydrophobic [93] but, in contrast, due to the presence of oxygen containing groups, the GO surface is partially hydrophilic, with different oxidization levels corresponding to different levels of water affinity [93, 94]. These functional groups also facilitate the processing of GO in dispersions and reveal a higher colloidal stability in water [95]. In contrast, the presence of these groups, breaks the conjugated structure and localizes π -electrons, resulting in a decrease of electrical and mechanical properties [96]. Ultimately, GO has a higher intrinsic biocompatibility, lower cost and scalable production while the oxygen groups enable biological and chemical functionalization of GO [73].

2.2.3 Reduced Graphene Oxide

To decrease the defect sites and thus grant the GO a structure and properties, such as electrical conductivity, more similar to those of graphene, GO can be reduced by chemical or thermal treatments, which results in partially deoxygenated sheets, called reduced graphene oxide (rGO) (

Figure 6) [96, 97].

Making use of a variety of methods, including electro, chemical, microwave- and photo-assisted, and thermal, rGO is thus an easier and more affordable approach to prepare graphene in industrial quantities from the more widely and easily produced GO. Different methods will lead to different degrees of reduction with some rGOs having properties closer to pristine graphene and other a combination of pristine graphene and GO [95]. It is important to noticed that, as a consequence of the incomplete reduction capacity of some methods, it is also possible to make use of the oxygen containing groups in rGO for functionalization, giving this material an advantage over pristine graphene for biomedical applications.

The electronic properties of rGO, such as conductivity, are improved when compared to that of GO, but a lower dispersion stability limits the rGO processability in aqueous solutions due to the reduction in number of hydrophilic groups present in the structure [79].



Figure 6 - The crystal structure of graphene, GO and rGO and the process used to obtain GO and rGO from graphene [98].

2.2.4 Biological Features

Notwithstanding the fact that GBMs are fairly new, several studies have revealed a few unique mechanisms of interaction with biological molecules, tissues, and cells. These materials have been shown to both benefit or harm the biological systems with the response differing according to the material's physicochemical properties and effective dose [99]. Nevertheless, a number of findings show that graphene nanomaterials are able to promote the growth, proliferation, and differentiation of mesenchymal stem cells (MSCs), neural stem cells (NSCs), and induced pluripotent stem cells (iPSCs) into tissues of various lineages [1] (**Figure 7**).

Graphene nanosheets, due to their strength and flexibility, can be used to coat different substrates and the resulting products can then be used as 2D surfaces in several tissue

engineered applications. Graphene films were shown to facilitate culture of osteoblasts and MSCs while also exhibiting excellent biocompatibility as the cells maintained their viability, normal adhesion, and migration characteristics [100] and accelerate the specific differentiation of human MSCs (hMSCs) into osteoblasts in the absence of additional growth factors [101]. A possible explanation is that graphene serves as a concentration platform for various growth factors and signalling molecules through its unique non-covalent π - π binding ability leading to certain serum proteins and osteogenic inducers to accumulate on graphene and consequently, improve the adhesion, proliferation, and bone differentiation of stem cells. This seems to indeed work for bone differentiation, however, the adipogenic differentiation of hMSCs was shown to be significantly suppressed. This happens because the same π - π interactions that are capable of binding the osteogenic inducers denature insulin which is an important regulator in the synthesis of fatty acids. On the contrary, when the graphene was substituted by GO the differentiation of stem cells towards adipogenic lineage occurred. This is due to the oxygen groups in GO that disrupt the extensive sp^2 conjugation and the π - π interactions and thus insulin is not denatured upon adsorption. This way, GBMs are able to play crucial roles in inducing hMSC differentiation towards different tissue lineages according to their ability to establish π - π interactions.

The capacity of graphene to adsorb molecules has been reported before and it seems to be favoured for molecules with low solubility, partial hydrophobicity, or positive charge, and for molecules with conjugated π bonds that intrinsically allow π - π interactions with the particular carbon surface of graphene, supporting the theory mentioned above [99]. Besides granting graphene the capacity to directly affect differentiation, this phenomenon has additional biological consequences such as micronutrient depletion from cell culture media leading, in turn, to cell growth inhibition [102], emergence of artifacts in toxicology assays due to adsorption of probe dyes [103], the capacity to carry small-molecules as drug delivery systems [104], and potentiating or reducing noxious effects of toxic

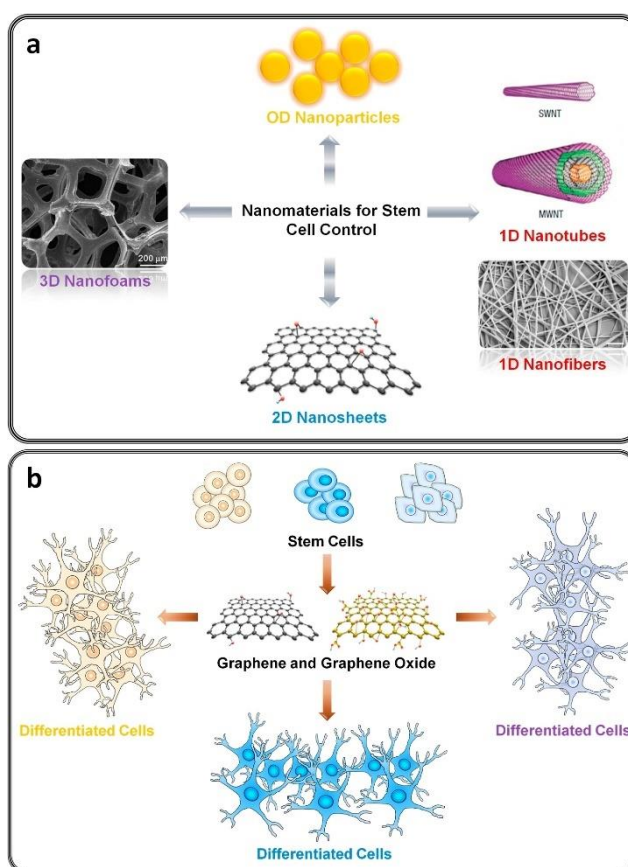


Figure 7 - Nanomaterials for the selective control of stem cell growth and differentiation. a: Different nanoscale materials for stem cell control and differentiation. b: Graphene and graphene oxide (GO) used as scaffold materials for the culture of various stem [1].

molecules whose bioavailability can be altered as they adsorb to the GBM surface. Overall, these findings seem to be consistent with the idea that the graphene surface is a dynamic platform of interactions which, in itself, might explain part of the capacity these materials have to conduct cellular behaviour when in contact with biological systems.

Cardiac applications have been amongst the most researched with reports that considerable enhancement in the cardiomyogenic differentiation of hMSCs can be achieved with the use of graphene [105]. The improved cardiomyogenic differentiation was attained due to a higher amount of ECM proteins and cell signalling molecules caused by up-regulation of the respective gene expression triggered by graphene. GMBs have also been explored for neuro tissue applications. In this case, human NSCs, when cultured on graphene, undergo enhanced differentiation than those grown on glass substrates and exhibit an elongated morphological structure with unique neurite outgrowths and formation of neural networks apparent on the graphene film [106]. Furthermore, graphene also seems to be capable of accelerating the development of NSCs and their progeny by interfering with the active and passive bioelectric properties of the cells' membranes, as they displayed enhanced action potential firing and overall synaptic activity during their maturation process ultimately resulting in improved differentiation towards the neural lineage [107].

Notwithstanding the promising results obtained when using 2D graphene-based films, the scientific community has long acknowledged the benefits of using 3D structures for cell culture and growth given that they better replicate the native biochemical and biomechanical microenvironments [108]. Following this notion, 3D graphene-based scaffolds have received a great amount of attention and have been developed for applications in culture and differentiation of stem cells and tissue engineering.

In this matter, a recent report has demonstrated that the neuronal differentiation of NSCs could be improved by using a 3D graphene foam (GF) scaffold. In comparison to cells differentiated on 2D graphene films, the cells differentiated on these 3D scaffolds showed a higher expression of TUJ1 and GFAP proteins but a much lower expression of the Nestin protein which suggests that the use of the 3D-GF scaffold might enhance the neuronal differentiation of NSCs towards astrocytes and neurons due to the presence of microscale topographical features and interconnected pores not present in the 2D films [109].

Another study has shown that rGO can effectively improve the therapeutic efficacy of MSCs for ischemic heart diseases by stimulating the expression of angiogenic growth factors and gap junction protein [110]. MSCs, when implanted into the ischemic myocardium, secrete reparative factors that result in improvement of the cardiac function by inducing angiogenesis and attenuating cardiac remodelling. Additionally, MSCs also express connexin 43 (Cx43) in low amounts. This gap junction protein can lessen post-infarct arrhythmia that commonly happens after myocardial infarction due to cardiac fibrosis and gap junction remodelling as a consequence of the loss of cardiac cells that express these proteins. Authors suggested that,

by incorporating rGO into MSC spheroids and implanting them into the infarcted myocardium enhancement of cardiac repair and restauration of cardiac function could be achieved. This hypothesis was supported by the improvement of the expression of Cx43, which normally happens in levels that are not sufficient to cause the regenerative effect, due to the cellular interactions with the electroconductive rGO and the capacity of rGO to adsorb ECM proteins present in the cell culture medium serum which in turn provides additional cell-ECM interactions capable of enhancing the expression of this protein and other beneficial paracrine factors.

Despite several studies demonstrating the great potential of GBMs in promoting and supporting tissue regeneration, they have some drawbacks. One of the major concerns is cytotoxicity that has been reported for some carbon based materials, including graphene and its derivatives [111]. The toxicity of graphene materials depends on several factors such their form (colloids, on surface or embed in polymer), concentration, lateral size, number of layers, surface topography, functionalization, oxidation degree, charge, presence of impurities, among others [112]. For example, GO has been shown to be susceptible to degradation in the presence of low amounts of horseradish peroxidase, while rGO is not affected [113]. In the same manner, GBMs were shown to degraded in blood after 14 days, with the degradation process being highly dependent on their oxidization degree [114]. GBMs containing low oxygen amounts are less affected due to the lower number of active sites, since active substances in blood plasma attack carbon atoms connected with oxygen-containing groups in their basal planes, leading in turn, to the breakage of C-C and C=C bonds thus forming holey sheets and aromatic hydrocarbons, which exhibit higher biocompatibility than pristine material [114]. Additionally, in one study, thinner oxidized (single layer) GBMs were completely degraded by myeloperoxidase upon 24 h, while thicker GBMs remain almost intact [113] and in another study, GO with different lateral sizes was shown to exhibit a high degradation potential by neutrophils [115].

Graphene can interact with DNA and RNA by intercalating into DNA strands [116], by inducing preferential adsorption of single-stranded (ss) over double-stranded (ds) forms [117], and by protecting adsorbed nucleotides from attack by nuclease enzymes [118]. While the first case might represent a setback for the use of these materials, the latter two make GBMs an attractive option for DNA or RNA delivery and biosensing, especially in the form of GO [99], since it can prevent degradation of DNA/RNA, increasing their stability. Besides interacting with genetic information, as noted with several materials from the carbon nanomaterial family such as nanotubes and carbon black, GBMs have been reported to deactivate the antioxidant glutathione and consequently cause an increase of intracellular oxidative stress, leading to cytotoxicity and inhibition of cell proliferation [119]. Furthermore, due to its favourable surface curvature, GBMs can bind the α -helical structures of peptides present in cell membranes leading to loss of membrane integrity [112]. These phenomena are the major causes of graphene cytotoxicity but, conversely, together with the sharp edges that physically damage

the membrane [120], provide graphene with antimicrobial properties that can be advantageously and conveniently used against bacteria, fungi and viruses [121]. This antimicrobial mechanism is considered to be similar to the previously reported for carbon nanotubes and consists on initial cell deposition on GMBs followed by membrane stress caused by direct contact and subsequent oxidative stress and genetic material interference that leads to cells death [122].

In conclusion, we can recognize that in order to efficiently mimic the natural cell microenvironment *in vivo*, graphene-based scaffolds should be manufactured as 3D structures instead of cell culture in 2D settings. Additionally, the combination of topographical, chemical, and electrical cues in one integrated 3D environment seems to be the most suitable approach to build stem cell scaffolds for tissue regeneration. However, it is also noteworthy that the fabrication and functionalization of the complex 3D graphene scaffolds is not yet a very explored area. Besides, potential negative effects of graphene and its derivatives in stem cells, such as oxidative stress, cell membrane damage, DNA fragmentation, and chromosomal aberration have been reported with the cytotoxicity being influenced by numerous factors, such as shape, size, dispersibility, and surface functionalization [1].

2.3 - dECM Hydrogels Containing GBMs

Considering all the promising features of GBMs, namely strong mechanical properties and high conductivity, they are ideally suited to overcome the limitations of dECM hydrogels, enabling a broader application of these material. As such, a new and upcoming area of bioengineering is growing: the production of dECM hydrogels containing GBMs. Due to the novelty of the field, there is not a substantial amount of published works, nevertheless, **Table 1** resumes the published works of dECM hydrogels containing GBMs.

Rueda-Gensini, Serna *et al.* produced an extrudable bioink for 3D printing prepared with methacryloyl-modified dECM hydrogels embedded with GO nanosheets with the purpose of manufacturing tissue constructs that respond to electrostimulation [123].

The ECM was sourced from porcine small intestine submucosa (SIS) and modified with methacryloyl (MA) groups to improve mechanical resistance (SISMA) while the GO was added to improve the electrostimulation of the constructs (SISMA-GO).

To improve dispersion and lessen the possibility of GO agglomerates in the hydrogel, GO was added to a working solution containing foetal bovine serum (FBS) to promote protein binding to the GO sheets. SEM analysis showed that SISMA-GO presents a low number of aggregates and a general homogeneous dispersion of the GO within the hydrogel confirming that GO dispersion was facilitated by protein adsorption. By doing so, this strategy seems to be advantageous as it does not require any alteration to GO's chemical structure with complex surface modification as some previous methods do.

As for mechanical behaviour, both SISMA and SISMA-GO exhibit a predominantly elastic behaviour over time with no crossover between G' and G'' curves during crosslinking. Besides composition, the authors also explored how thermal (typical of collagen hydrogels), and photo (using the MA functionalization) crosslinking affected the constructs observing that the two methods resulted in structural differences in the constructs (**Figure 8A**). Thermal crosslinking showed fibrillar patterns similar to the natural organization of collagen fibrils in the native tissue with the hydrogel being highly porous. Photo crosslinking produced a much more disorganized and much less porous structure. However, photo-stimulation was confirmed to yield faster and stronger crosslinking dynamics as the temperature stimulus increased G' values of SISMA-GO to 300 Pa after 5 minutes while blue-light irradiation led to a value of about 400 Pa after 1 minute (**Figure 8B,C**). Moreover, SISMA-GO constructs that underwent photo stimulated crosslinking seem to be more stable with $90.39 \pm 1.92\%$ of gel fraction still present after 24h while thermal-crosslinked SISMA-GO constructs only swell up to $27.90 \pm 4.88\%$. This happens due to the presence of covalent bonds in irradiated SISMA-GO that end up restricting extreme hydrogel swelling thus allowing these hydrogels to be more durable. Nevertheless, G' increased for both stimuli which translates to an increase in the elastic response of the hydrogels and, therefore, their structural stability, while G'' remained unchanged. Ultimately, GO was shown to have no harmful impact on the crosslinking dynamic of the collagen and on the rheological response of the produced bioinks, improving the mechanical stability of the material and producing hydrogels with shear-thinning behaviour.

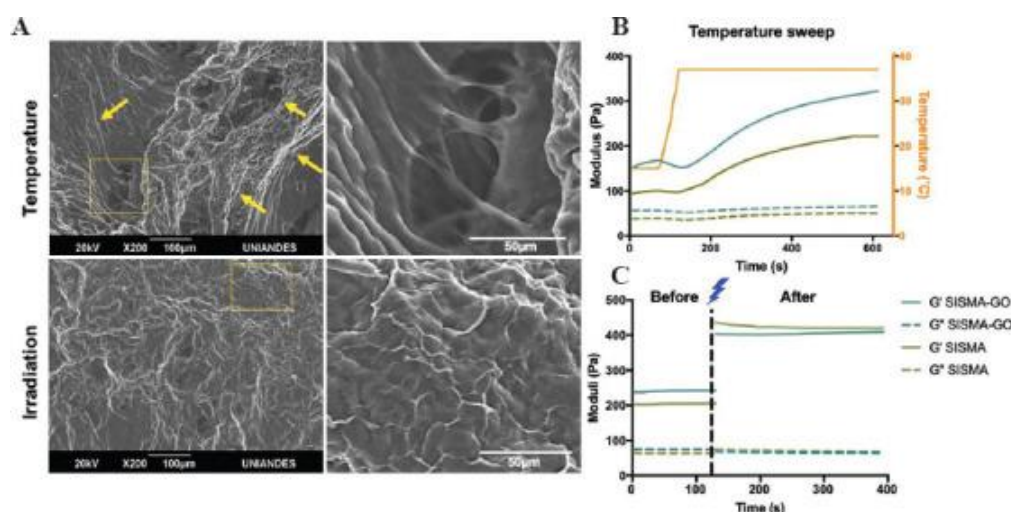


Figure 8 - (A) SEM images of SISMA/GO hydrogels crosslinked with temperature and with blue-light irradiation. (B) Temperature sweep experiments in SISMA-GO and SISMA hydrogels. (C) Time sweeps before and after irradiation [123].

To increase the conductivity of the scaffolds, GO was reduced *in situ*, after printing, with ascorbic acid. The process resulted in a 6124% increase in electroconductivity reaching values of 30.59 mS/m.

Biocompatibility assessed using hAD-MSCs showed high cell viability ($96.9 \pm 0.9\%$) for both

Table 1 - Important aspects of the published works about dECM hydrogels reinforced with GBMs.

APPLICATION	dECM GELS		GBM			dECM GELS + GBMS				REFERENCE
	Source	Production	Type & amount	Purpose	Incorporation method	Characterization	Electroconductivity (Four-point probe)	Mechanical performance	Biological performance	
3D Bioprinting	Small intestine submucosa (pig)	Decellularization - H ₂ O ₂ and NaOCl in dH ₂ O 15 minute - dECM powders obtained by cryogenic mill Hydrogel production - 4 mg/mL of dECM powder + 1 mg/mL pepsin (48 h) - dECM pre-gel was chemically modified with methacrylic acid (MA)	GO 0.25 mg/mL	↑ response to electro-stimulation	<i>SISMA-GO</i> - 40 mg/mL <i>SISMA</i> + GO in DMEM, FBS, P/S, RF, Tris-HCl + 4.5 × 10 ⁶ hAD-MSCs/mL - Thermal (37 °C) or photo-crosslinking (5 min, 62 mW/cm ² , 405 nm) <i>SISMA-rGO</i> - <i>SISMA-GO</i> reduced with ascorbic acid.	<i>SISMA-GO</i> Confocal microscopy - GO is well dispersed in dECM gel <i>SISMA-GO and SISMA-rGO</i> Raman spectra - G and D peaks FT-IR spectroscopy <i>SISMA-rGO</i> ↓ COOH (1721 cm ⁻¹) and OH (1364 cm ⁻¹) comparing to <i>SISMA-GO</i>	<i>SISMA-rGO</i> ↑ 6124% conductivity (30.59±1.27 mS/m) comparing to <i>SISMA-GO</i> photo-crosslinked (0.49±0.04 mS/m)	<i>SISMA-GO</i> Flow sweep Does not affect rheological response of bioink Time sweep (both crosslinking mechanisms) ↑ G' (300 Pa (↑ ~52%) thermal; 400 Pa (↑ ~40%) photo) = G'' (~50 Pa)	<i>In vitro</i> * <i>SISMA-GO</i> photo-crosslinked Viability 96.9 ± 0.9% Morphology ↑ amount of filopodia comparing to <i>SISMA</i> Proliferation ↑ proliferation rate	[123]
<i>In vitro</i> model to mimic native heart tissue	Myocardium (pig)	Decellularization - 1% w/v SDS for 36h, - 1% w/v Triton-X100 for 1h - 0.1% w/v peracetic acid in 4% w/v ethanol Hydrogel production - Lyophilized dECM was mixed with pepsin at a 10:1 mass ratio - 2% w/v dECM solution was used for all experiments	rGO 1-3 mg/mL	↑ electro-conductivity	rGO was added at same time of pepsin before digestion	SEM - rGO does not affect porous network - rGO aggregates with 300 ± 79 µm were deposited on the porous walls	↑ 600 000% (3.07 ± 0.12 S/m) comparing to dECM	Shear rate sweep ↑ ~1400% viscosity (30 Pa.s) comparing with dECM Compressive Young's modulus ↑ 70% (17.5 ± 0.5 kPa) comparing to dECM	<i>In vitro</i> Viability High cell viability (>90% , 35 days) Heart tissues constructs with cardiomyocytes <i>Comparing with dECM</i> ↑ 83% twitch forces (23.61 ± 2.62 µN) ↑ ~10% Sarcome length (~2.11 µm) ↑ Ca ²⁺ flux ↑ 42% action potential time (488 ± 21 ms) ↑ 75% conduction velocity (35.4 ± 2.3 cm/s) - Improved response to ciproasine (day 14: similar to native tissue)	[55]
Injectable Hydrogels	Myocardium (bovine)	Decellularization - 1% (w/v) SDS for 4-5 days - 1% (v/v) Triton X-100 for 30 min - Lyophilization and cryo-milling Hydrogel production - dECM powder + 1 mg/mL pepsin (72h) - 10 mg/mL of dECM + (3 or 4% w/v) of OA	NH ₃ -rGO 25&50 µg/mL	↑ electro-mechanical properties	- Mix NH ₃ -rGO + CaCO ₃ + dECM/OA - GDL solution + mixture transferred into a syringe and injected into a mold	FE-SEM No significant changes in the porous structure Gelation time - ↑ 5% (88.4 s) Swelling ↓ 10% (210% of swelling) Cross-linking density ↑ degree of crosslinking Degradation No significant changes	↑ 163 000 % (0.28 mS/m) comparing to dECM/OA	Young's modulus ↑ 14 % (41.1 kPa) Frequency sweep tests ↑ G' and G'' of hydrogels**	Viability (Human umbilical vein endothelial cells) No differences	[124]

AFM - Atomic Force Microscopy; DMEM - Dulbecco's Modified Eagle Medium; EDAX - Energy Dispersive X-Ray Analysis.; FBS - Fetal bovine serum; FE-SEM - Field Emission Scanning Electron Microscopy; FTIR - Fourier-Transform Infrared Spectroscopy; GDL - Glucono-Delta-Lactone; OA - Oxidized Alginate; SDS - Sodium Dodecyl Sulfate; SEM - Scanning Electron Microscopy.

SISMA-GO and SISMA-rGO scaffolds. Furthermore, a transition from rounded to elongated morphology related to the formation of cell-matrix interactions that mediate cell adhesion was noted after 6 days of incubation. Moreover, hAD-MSCs cultured in SISMA-GO present a superior amount of filopodia than those cultured in SISMA indicating that cell adhesion is facilitated by the presence of GO. Lastly, proliferation seems to be increased in SISMA-GO constructs which is likely due to the interactions with the proteins adsorbed on the nanosheets.

All things considered, the SISMA-GO bioink showed great promise for 3D bioprinting applications, confirming that GO incorporation and its consequent *in situ* reduction can be used to improve the colloidal stability of the graphene making use of the bioactivity and hydrophilicity of GO, and once it is stably dispersed, the reduction of GO into rGO greatly improves its electroconductive properties. In essence, its bioactive, mechanically stable, and electroconductive properties are a clear advantage over previously reported bioink formulations.

Tsui, Leonard *et al.* incorporated rGO into dECM hydrogels derived from pig myocardium to overcome the poor mechanical and electrical properties of scaffolds aimed to create an *in vitro* model to mimic native heart tissue [55].

SEM images (**Figure 9**) showed that resulting dECM-rGO hydrogels network was porous like that of decellularized native porcine myocardium while the rGO flakes were found to be deposited on the porous walls and not impact the porous network.

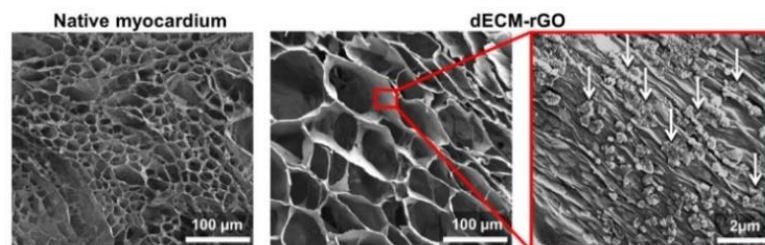


Figure 9 - SEM images of dECM-rGO hydrogels. Deposition of rGO flakes (white arrows) on the pore walls can be observed. Adapted from [55], with permission from Elsevier.

Regarding mechanical performance, the viscosity and compressive modulus increased ~1400% and 70% reaching values of 30 Pa.s and 17.5 kPa, respectively. The average compressive moduli measured is comparable to the range for native myocardium (Berry *et al.*, 2006). Additionally, the viscosity decreased as shear rate increased meaning that the dECM-based hydrogels tested exhibited shear-thinning properties. dECM-only hydrogels presented very low conductivity, as expected, while an increase in rGO reduction and content led to an increase of 600 000% in hydrogel electroconductivity reaching values of 3.07 ± 0.12 S/m. To guarantee that the improvement in conductivity was related to the presence of rGO and not the increase in stiffness, the authors used a stiffness control by crosslinking the matrix collagens with transglutaminase (TG). The resulting dECM-TG hydrogels presented a similar compressive modulus to that of the stiffest formulation of dECM-rGO but exhibited no enhanced electroconductivity, thus confirming that it is conveyed by the rGO.

Cell viability tests were performed with human induced pluripotent stem cells (hiPSC) and stromal cells and the presence of rGO was found to have no negative effects on either cell type with >90% viability assessed after 35 days.

The formulation with highest stiffness and electroconductivity, dECM with 0.3% w/v rGO, was used to produce 3D engineered heart tissue constructs (EHTs), by mixing cardiomyocytes differentiated from human induced pluripotent stem cells (hiPSC-CMs) and human bone marrow-derived (HS-27A) stromal cells to assess the effect of rGO on the function of the cells. Both types of cells were shown to be homogeneously distributed within the EHTs.

The contractile function and rate of the cardiomyocytes increased substantially when compared to cardiomyocytes grown on collagen tissues demonstrating that the rGO induces a better cardiac function on the cells. Additionally, the sarcomeres of cardiomyocytes differentiated in collagen tissues were comparable to the ones found in immature cardiomyocytes cultured on standard 2D substrates while the length of the sarcomeres in dECM-rGO increased progressively until they reached lengths similar to those found in adult human cardiomyocytes (**Figure 10**). Moreover, upregulation of the genes that code for cardiomyocyte contraction-mediating proteins (Troponin I), proteins responsible for muscle elasticity (Titin), calcium channels (CACNA1C and ATP2A2), and potassium and sodium channels (KCNH2 and KCNE1, and SCN5A, respectively), show that rGO promotes better maturation of these tissues, improvement in the development of cardiomyocytes, increase in action potential duration, and enhancement in conduction velocity (electrophysiological function). Additionally, proteins related to pre-cardiac mesoderm and cardiac progenitor cells are initially highly expressed (NKX2.5 and GATA4), but decrease significantly over time, showing that dECM-rGO does positively influence the maturation of cardiomyocytes.

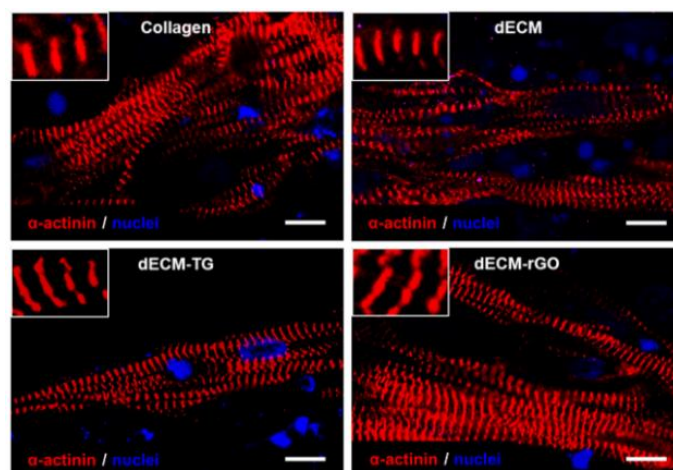


Figure 10 - Sarcomere development in engineered heart tissues. Representative fluorescent images of Day 35 tissues generated with the different hydrogels stained for sarcomeric α -actinin (red) and nuclei (blue). Scale bar = 10 μ m. Adapted from [55] , with permission from Elsevier

After biocompatibility was ascertained, dECM and dECM-rGO hydrogels were extruded to create a 3D scaffold, as *in vitro* systems, since they allow a more reliable mimicry of the native

tissue. When printed, both dECM and dECM-rGO hydrogels displayed shear-thinning behaviour with the material also retaining its capacity to reform after extrusion and then hold its final structure.

Considering the results presented it is evident that the presence of rGO leads to the improvement of contractibility and electrophysiological function in EHTs. The noticeable increase in expression markers associated with the development of cardiomyocytes within tissues demonstrates the benefit of these constructs for hiPSC-derived cardiomyocyte development. Additionally, the increase in matrix stiffness and the presence of hydrophilic oxygen-containing groups from the rGO seem to enhance cellular adhesion and the formation of focal adhesions. In this degree, the dECM-rGO hydrogels present a combination of biochemical, mechanical, and electroconductive cues that make a possibility to achieve a more evident maturation than that obtained with tissues cultured with only collagen or dECM for cardiomyocytes.

Mousavi, Mashayekhan *et al.* produced an injectable hybrid *in situ* forming hydrogel containing dECM, oxidized alginate (dECM-OA), and amine-functionalized rGO (Amine-rGO), aiming to mimic the physicochemical and electromechanical properties of cardiac tissue [124].

The ECM was sourced from bovine myocardium ventricular tissue and supplemented with partially oxidized alginate (OA) to prepare dECM-OA hydrogels. Alginate is an anionic linear polysaccharide, naturally derived from algae, with great biocompatibility, exceptional mechanical properties, non-thrombogenicity and angiogenic properties making it a very interesting biomaterial to be used in injectable therapies for cardiac tissue engineering. Due to its structure, in the presence of divalent cations, alginate promptly forms hydrogels making it idyllic for applications that require *in situ* hydrogel reticulation. Despite its advantages, alginate-based hydrogels present a very slow *in vivo* biodegradation. Contrarily, OA is more susceptible to *in vivo* hydrolysis and degradation and allows a better control of gelation time.

The use of rGO was intended to increase the conductivity of the hydrogel while the amine groups were introduced to mitigate the cytotoxicity of graphene-based nanosheets. However, the functionalization of the rGO sheets with amine cause them to fold on each other and form multi-layered tangled patches with many ripples and crosswise edges and, while the atomic content of carbon and nitrogen increased, the oxygen content was reduced. Moreover, the interlayer space decreased due to the decrease in oxygen containing groups and the zeta potential increased leading to higher propension for agglomerates.

Nevertheless, the crosslinking of dECM components by OA was successful and, together with Amine-rGO, the hydrogels originate completely uniform constructs with highly porous microstructures and randomly oriented, interconnected, uniform sized, and smooth walled pores. Some hydrogels presented pores with sizes similar to the desirable for vascularization, mass transport of nutrients and prolonged survival and growth of cardiac cells. Although amine-rGO nanosheets seem to cause no meaningful changes or disruptions in the structure they cause

a slight decreased in pore size. The same happens with the increase of OA concentration due to higher viscosity and higher crosslinking density of the hydrogel (**Figure 11**).

Gelation time tends to increase as the Amine-rGO concentration increases and, by contrast, decreases with the increase of OA. Besides altering the structure and the gelation kinetics, changing the concentration of OA and Amine-rGO, also allows for the tensile modulus of hydrogels to be tailored. Hydrogels prepared with 4% (w/v) showed similar Young's tensile modulus to the native myocardium, making this formulation the ideal hydrogel concentration to simulate the properties of cardiac tissue. All hydrogels showed values of ultimate breaking strain and tensile strength similar to those of the native myocardium.



Figure 11 - Macroscopic images of hydrogels with different concentrations of GO (A4G0, A4G25, A4G50). Adapted from [124], with permission from Elsevier.

Regarding rheological properties, all samples demonstrated gel-like or solid-like behaviour evident by the storage modulus (G') being higher than loss modulus (G'') and both values independent of frequency. All the hydrogels exhibited high flexibility with a ductile stress-strain behaviour. The improved values of G' indicate that the network structures were successfully formed and were stable under large deformations.

The hydrogels presented conductivities within the range shown to be enough to cause spontaneous beating in cardiomyocytes and increased with the amount of Amine-rGO. All the hydrogels exhibited reasonably high swelling ratios, with a reduction of around 5% observed when the concentration of Amine-rGO was increased. With the increase of amine-rGO content the degree of crosslinking increased, as measured using the Flory-Rehner equation [125], while the degradation rate for hydrogels with distinctive Amine-rGO concentrations was almost the same with all the hydrogels losing almost 50% of their weight in the first 2 weeks.

Human umbilical vein endothelial cells, cultured within the prepared hydrogels, were used to evaluate the cytotoxicity of the hydrogels and no major changes in cell viability were noted after 48h between the different groups, nevertheless, the lowest cell viability was detected for the hydrogel containing the highest amount of Amine-rGO. Biocompatibility of the products released due to the degradation of the hydrogel was proved by the absence of noteworthy differences between the 10-day extract of the prepared hydrogels and TCP. Even so, cell aggregations were noticeable with 86% viable cells after 96h of culturing.

All things considered, the results show that the electroactive, *in situ* forming hydrogel derived from dECM, OA, and Amine-rGO presents improved physicochemical,

electromechanical, and biological properties whilst not affecting cytotoxicity which could originate a new platform for upcoming tissue engineering applications.

Lastly, following a different approach where, instead of incorporating graphene into a dECM matrix, the dECM is itself incorporated in graphene-based scaffolds Serna *et al.* prepared a bioink for the treatment of volumetric muscle loss [126].

The dECM was sourced from mouse pup and crosslinked with PLGA. After 3D printing of the graphene structures, with and without dECM, using an extrusion-based system under ambient conditions, the constructs showed no differences in surface topography obtained with SEM. In terms of conductivity a reduction was noted on graphene scaffolds from 286.4 S/m to 74.4 S/m when 2.5% dECM was added and zeta potential values of -17.9 ± 5.14 mV for graphene containing scaffolds and -22.7 ± 5.76 mV for dECM only scaffolds.

Biological performance *in vitro* experiments showed that both scaffolds were capable of supporting glia and motor neurons growth and that both scaffold variants were indeed capable of supporting muscle myoblast adhesion, alignment, viability, proliferation, and differentiation. Additionally, confocal imaging allowed the observation of neural network interconnectivity in the motor neurons seeded onto graphene scaffolds containing dECM meaning that this might be a good strategy for the treatment of volumetric muscle loss.

Overall, the results of dECM incorporation into graphene constructs seem to produce similar outcomes as incorporation of graphene into dECM.

As the best of our knowledge, these are all the published works that contain dECM hydrogels with GO and/or rGO. Nevertheless, we can see a trend followed by the presented papers in which authors establish the production an ink, characterized its mechanical and rheological properties and the interaction between the dECM fibres and the graphene and finally test the biocompatibility with relevant cellular types. Additional efforts are taken to print high resolution structures by using 3D printing techniques. However, differences are also present namely in the native tissue from which the dECM is sourced, the decellularization and digestion processes and the incorporation of GO/rGO within the dECM. These highlight the novelty of this field and therefore lack of standardization.

2.4 - 3D Printing

To build completely new organs, a lot of information must be taken into consideration: different organs have different types of cells, most organs have more than one type of cell, vascularization needs to be guaranteed, etc. 3D printing technologies can be used to obtain simplified and complicated structures easily and using suitable materials. Additionally, by using additive manufacturing techniques, we can directly print cells into their corresponding places in the general design of the organ and thus facilitate the entire process [20, 127].

3D printing or additive manufacturing is a technique in which materials are stacked layer by layer to form constructs with diverse and complex shapes following a computer-aided design (CAD) model [20]. The 3D design model is decomposed, a process also called slicing, shown in **Figure 12**, into a series of parallel slices that allow the complex shapes to be fabricated one layer at a time, giving rise to the structure [128]. Additive manufacturing is expected to bloom in the (bio)medical field as a tool that allows to rapidly obtain general and personalized medical equipment and permanent implants but also for *in vitro* models, organ engineering and tissue regeneration [20].



Figure 12 - Process from 3D model to 3D printable model. Adapted from Pham, G. N., Lee, S.-H., Kwon, O.-H. & Kwon, K.-R. A 3D Printing Model Watermarking Algorithm Based on 3D Slicing and Feature Points. *Electronics* 7 (2018), with permission.

Additive manufacturing can be based in different techniques and using a variety of polymers and other materials. Not all materials can be successfully printed using all techniques so there is a need to understand which technique is more appropriated to the ink properties and which combination will produce the optimal results. For hydrogels, the most common methods of 3D printing are resumed in the table in **Table 2**, along with their advantages and limitations. In this section we will focus on extrusion-based bioprinting since it is the most suitable for dECM based hydrogels [129].

Table 2 - Main advantages and limitations of the different bioprinting techniques. Adapted from [129].

Bioprinting techniques		Advantages	Limitations
Inkjet-based bioprinting		Low cost High printing speed High cellular viability High resolution (10-50 μm)	Requires low ink viscosity (10 mPa/s ⁻¹) Prone to clogging Requires fast gelation Prone to cell sedimentation
	Laser-based bioprinting	High printing speed Orifice free (no clogging) High resolution (1-100 μm)	Expensive Ink viscosity 1 mPa/s ⁻¹ -300 mPa/s ⁻¹
	Stereolithography	High printing speed Orifice free (no clogging) High resolution (10-150 μm) Allows complex structures	Requires photosensitive polymers Requires optimization (e.g., PI, bioresin functional groups, light intensity, exposure time)
	Digital light processing	High printing speed (cures a whole layer in seconds) Orifice free (no clogging) High resolution (10-150 μm) Allows fabrication of complex structures	Requires photosensitive polymers Ink viscosity < 5 Pa/s ⁻¹ Prone to cytotoxic effects
Extrusion-based bioprinting		Low cost Wide range of ink viscosities Fabrication of large constructs	Low resolution (>100 μm) Prone to cellular death

Two strategies can be followed when printing hydrogel scaffolds for tissue regeneration (**Figure 13**): i) print biomaterials (biomaterial ink) to obtain a 3D structure and then cells are seeded on top or ii) directly print the biomaterial embedded with cells and bioactive molecules,

bioink [20]. Biomaterial inks must meet certain criteria when used in extrusion-based printing. Besides being biocompatible, biodegradable, bioactive, and allow molecular permeability, properties intrinsically connected to the nature of the scaffolds, they must also present appropriate mechanical strength, printability, and be capable of solidify while maintaining its shape [3].

The rheological properties of biomaterials thoroughly affect its printability as the ink should have the characteristics of non-Newtonian fluids with a certain viscosity and viscoelastic behaviour. Non-Newtonian fluids

do not follow Newton's Law of Viscosity, meaning that their viscosity is dependent on shear, most commonly, it changes when a shear stress or shear rate is applied [130]. For a material ink to be successfully printed, its viscosity should decrease when shear stress is applied leading thus to an increase in fluidity during the process. This behaviour is known as shear-thinning and it's the basis of extrusion 3D printing. Viscoelasticity can be divided into two components: the elastic modulus, also called the storage modulus (G') that represents the solid property of a fluid and the viscous modulus, or the loss modulus (G''), that represents the liquid property. This way, to guarantee that the print will hold its shape, we must also ensure that the ink viscosity modulus is never lower than its elastic modulus and that the ink presents shear thinning behaviour [3].

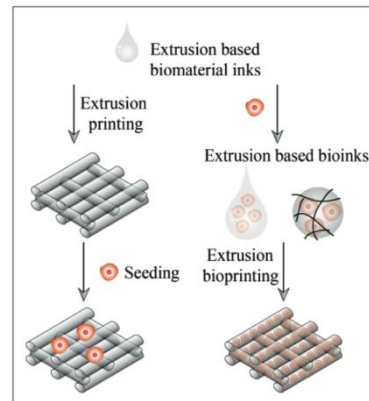


Figure 13 - Definition of extrusion-based biomaterial inks [3].

2.4.1 3D Printing of dECM

Since dECM based inks possess low mechanical properties they are often supplemented or conjugated with other materials to enhance printability [131, 132] or printed together with a supporting/sacrificial material whose mechanical characteristics are stronger and therefore offer physical support to the construct [133]. dECM is, in most cases, used as a main component of a bioink, being printed with cells. In these cases, the presence of the cells might be enough to improve the ink's properties to adequately form the constructs or, the ink might still require additional improvement steps like those before mentioned [133]. Conversely, by having cells inside the ink, it becomes more difficult to choose a printing method and define parameters since we must ensure that the cells are not stressed, survive and are apt to proliferate after printing [134] (for example, the needle gauge used for printing cannot be too thin or the cells will experience compressive forces that might compromise cellular integrity). Additionally, it is very common to fabricate inks and bioinks using only certain components of the ECM, like collagen and GAGs [127].

2.4.2 3D Printing of hydrogels containing GBMs

GBMs 3D printing has been explored for a diverse range of applications. For example, the native properties of graphene and the possibility of printing strategies to produce very intricate constructs have resulted in extensive investigation in energy storage with the production of supercapacitors and batteries [135]. Although GBMs present superior mechanical properties, their texture limits its direct use in 3D printing. To overcome this challenge, GBMs are commonly incorporated into polymers, such as alginate, gelatin, PLGA, PLLA or PCL [136]. Specifically for hydrogels, several types of carbon materials (CMs), from GO to carbon nanotubes (CNs) have been explored for tissue engineering and drug delivery [129]. As previously reviewed by our group [129], these CMs are versatile and have been used to 1) modify the rheological properties of biomaterial inks, 2) enhance the mechanical properties of hydrogels, and/or 3) add new physical characteristics to hydrogels, such as shape memory, roughness, and thermal and electrical conductivity, which have been shown to modify the biological response of bioprinted constructs (**Figure 14**).

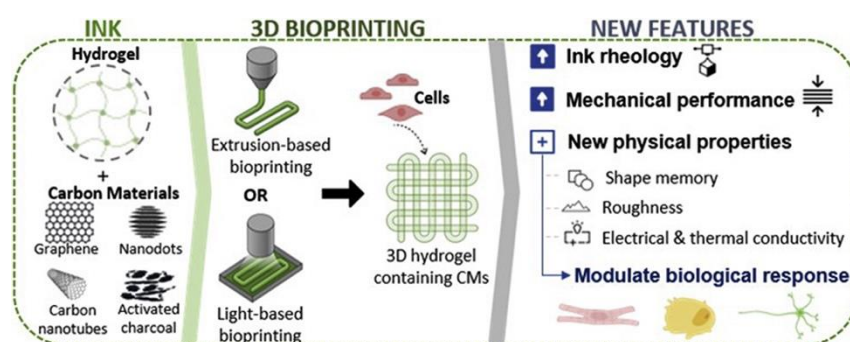


Figure 14 - Effect of CMs in hydrogels bioprinting [129].

Specifically for GBMs, they were combined with a wide variety of hydrogels, namely alginate, polyacrylic acid, polyacrylamide, poly(N-isopropylacrylamide). GBMs play a variety of roles in hydrogels, including enhancing their printability, conductive and mechanical properties, and promoting shape memory features. Several applications for these hydrogels were explored namely for tissue engineering, bone repair and cartilage replacement. Moura, Pereira *et al.* conducted an extensive research about bioprinting of hydrogels containing carbon materials. Some examples provided by them are summarized on Table 1 in **Annex A** [129].

In this manner, 3D printing of GMBs seems to allow the exploitation of graphene's biological properties and enhance them to build high resolution scaffolds that better interact with cells [137]. Despite the recent advances in 3D printing of the CMs containing hydrogels, there are some specific features that should be further explored, including other intrinsic properties of GBMs (e.g., antimicrobial properties), the hydrogels' architecture, and their translation to clinics. The majority of bioprinted structures have promising properties in terms of hydrogel architecture, but their structure and architecture are simple because they are frequently made

of filaments or films. Consequently, there have been no reports regarding more complex and bio-inspired architectures, which are frequently required for biomedical applications.

Chapter 3

Materials and Methods

3.1 - Graphene Oxide (GO)

Two commercially available GO were acquired from Graphenea Inc.: Graphene Oxide Water Dispersion (GO-WD) at 0.4% (w/v) concentration [138], and Highly Concentrated Graphene Oxide (GO-HC) at 2% (w/v) concentration [139].

3.1.1 Sterilization

GO sterilization was performed following two different approaches: autoclaving or washing in 70% ethanol. For the autoclaving approach, 40 mL of GO-HC was added to a falcon tube and autoclaved for 20 minutes at 120 °C and 1 Bar. After this, the dispersion was left to cool down until it reached room temperature (RT). For simplicity, autoclaved GO-HC is referred as $GO_{\text{autoclave}}$ when incorporated into the dECM ink. For the washing in 70% ethanol, 40 mL of GO-HC was centrifuged at 14 000 rpm for 10 minutes, the supernatant, which contains water, was removed, and 40 mL of 70% ethanol was added, and the dispersion was vortexed for 30 seconds to re-disperse GO particles. The dispersion was left to rest for 5 minutes to promote the contact between ethanol and GO particles. Then the ethanol was removed by centrifugation at 14 000 rpm for 10 minutes, and sterile dH_2O was added to the GO pellet. The process was repeated twice to remove all the ethanol that could be in the GO particles. GO-WD washed in ethanol is referred as GO_{ethanol} when incorporated into the dECM ink.

3.1.2 Characterization

3.1.2.1 X-ray photoelectron spectroscopy (XPS)

XPS analysis was performed to assess the elemental composition of GO-WD and GO-HC suspensions and to assess the effect of autoclaving in GO-HC composition.

Samples were prepared by adding 3 mL of each suspension (GO-WD, GO-HC, and autoclaved GO-HC) to propylene containers and left to dry at 37 °C for 2 days to create a GO film. After, each film was assembled in copper samples holders using a double face carbon tape. XPS analysis was carried-out at CEMUP (Centro de Materiais da Universidade do Porto), with the equipment Kratos Axis Ultra has. A monochromatic Al X-ray source (anode) operating at 15 kV (90 W) was used. A 300 μm $\times$ 700 μm square was the analysed area. 160 eV was the energy used for survey and 40 eV for high resolution carbon spectra. The effect of the electric charge was corrected by setting the reference of the C 1s peak to 284.6 eV. The high-resolution C 1s spectra were fitted into seven peaks for the following binding energies: sp^2 C=C (284.3-284.5 eV), sp^3 C-C (284.8-284.9 eV), C-OH (285.3-286.0 eV), C-O-C (286.1-286.6 eV), C=O (287.5-287.9), O-C=O (288.8-288.9 eV), as previously described [140]. The Gaussian-Lorentzian (70 : 30) function used to fit all the peaks with the exception of the sp^2 carbon peak, which is due to its asymmetric nature, was fitted using an asymmetric Lorentzian function (LF) with an asymmetry parameter of 0.14.

3.1.2.2 Transmission electron microscopy (TEM)

TEM images of GO-WD, GO-HC, and autoclaved GO-HC dispersions were acquired to assess the dispersibility in water and the lateral side of GO particles. For this, the dispersions were diluted with water until reaching a concentration of 0.4 mg/mL and 1 μL of each was added to a TEM grid and left for 30 minutes at RT to dry. The images were acquired with Jeol JEM-1400 Transmission Electron Microscope.

3.2 - Decellularized Extracellular Matrix (dECM) Gels

Two different dECM materials derived from human placenta chorion were kindly provided by the Centre for Biomedical Research and Translational Surgery from the Medical University of Vienna, Austria. Both dECM materials were decellularized as previously reported by them [141] and briefly described in section 3.2.1. However, one of them was provided in the form of dECM powder that requires a digestions step to obtain dECM pre-gels (herein called dECM-i3S powder, for simplicity), described in Section 3.2.2, while the other, is a dECM powder already digested (herein called dECM-MUV powder).

3.2.1 Decellularization Process

Chorion were isolated from the placenta in the operating room, washed with dH_2O and stored at -80°C for a minimum of 24h.

The frozen samples were chopped into smaller pieces with an approximate size of 2 mm, then washed with dH_2O and stirred at room temperature for 1 hour, with a change of water halfway through (after 30 minutes). After, the samples were again stirred for 1 hour in a 1.4%

NaCl solution at RT. The tissue was then strained and rinsed with dH₂O and stirred in a 2% Triton X-100 in PBS + 0.02% EDTA solution for 24h at RT.

At day 1, the tissue was rinsed with dH₂O, changed to 1X PBS, and stirred for 1 hour at RT before being stirred in a 2% Triton X-100 in PBS + 0.02% EDTA solution for 24 hours at RT. The same process was repeated every day from day 2 to 4.

At day 5, after rinsing with dH₂O for 1 hour, the tissue was transferred to a PBS + Pen/Strep solution and stirred for 48 hours at 4 °C.

At day 8, samples were added to PBS, transferred to a PBS + Calcium + Magnesium solution with Pen/Strep and finally transferred to DNase I (12 mg/ml) in PBS supplemented with calcium where it was stirred for 4 hours at RT and then stirred overnight at 4 °C.

At day 9, the tissue was again rinsed with dH₂O and transfer to a 4.8% EtOH + 0.02 PAA solution where it was left for 2.5 hours at RT. This step works as sterilization, so from here on, the process was done under sterile environment. Samples were washed and stirred with dH₂O + Pen/Strep overnight at 4 °C and at day 10 transferred to petri dishes for lyophilization.

After lyophilization, samples were milled to obtain the dECM powder.

3.2.2 Digestion of dECM

dECM-i3S powder was digested in HCl-pepsin solution, as previously described [141]. Briefly, 150 mg, 200 mg or 300 mg of dECM-i3S powder, for dECM 3%, dECM 4% and dECM 6%, respectively was added to a 5 mL solution with 7.5 mg, 10 mg and 15 mg of pepsin (Sigma P6887) dissolved in 0.1 M HCl solution. The digestion was performed under magnetic stirring for 48 hours at room temperature. After 48 hours of digestion, the dECM-i3S pre-gel solutions was neutralized with 10 M NaOH solution until pH reaches 7.4.

The dECM-MUV powder was already provided with the digestion completed using a confidential method optimized by our partners from the Medical University of Vienna.

3.3 - dECM/GO Scaffolds

3.3.1 Ink

For ink optimization, it was used dECM-i3S pre-gel. Different amounts of GO-WD (1-100% (w/w_{ECM})) and GO-HC (1-25% (w/w_{ECM})) were incorporated in dECM-i3S pre-gels with 3% and 4% dECM concentration. To incorporate GO-WD into the pre-gel, the suspension was centrifuged at 14000 rpm for 10 minutes to deposit the amount of GO-WD needed to achieve the different concentration (see Annex B). The supernatant was removed, and the GO-WD pellet re-suspended with dECM pre-gel. Contrarily to GO-WD, GO particles in GO-HC do not deposit to form a pellet upon centrifugation at 14 000 rpm for 10 minutes. As such, dECM pre-gels with higher concentration, 6% (w/v) were prepared to be diluted with GO-HC (see Annex B). For both approaches, different strategies were tested to achieve a homogenised ink, namely 30 seconds vortex or 24 hours magnetic stirring.

For the inks composed of dECM-MUV pre-gels, water dispersion of GO-HC at desired concentration and 4 °C were added to dECM-MUV in powder form. The mixture was vigorously vortexed.

All formulations were centrifuged to remove bubbles, for periods between 30 seconds to 1 minute at 2000 g.

3.3.2 Printing platform

Printing platforms of different materials (glass and plastic), with or without coatings (pluronic 127 or Sigmacote) and at different temperatures (room temperature and 37 °C) were tested to achieve a printed filament with high stability, shape fidelity, and that can be easily detached from it. As such glass and plastic petri dishes were used as printing platform. In the plastic petri dishes, it was performed a coating with 1% (w/v) Pluronic 127 overnight. After that, the solution was removed, and the petri dishes were left upside down to dry. A coating with sigmacote was performed in glass microscope slides. Briefly glass microscope slides were cleaned using ethanol 70% and left in the fume hood to dry. After, some drops of sigmacote solution we put on the slide surface and another slide was used to cover it. After making sure that there were no bubbles between the slips, we separated them and left for a few minutes inside the fume hood, to dry. To change the surface temperature, we placed the petri dishes inside water bath at 37 °C.

Besides this, two different support baths, water and 5% (w/v) dextran, were also tested as printing platforms. For this, water was added to a petri dish and the ink was printed into it. For the 5% (w/v) dextran, 2.5 mg of dextran (Sigma 31390) were dissolved in 50 mL of H₂O at 37 °C. The solution was poured onto a petri dish and the ink was printed into the material.

For all the tested conditions, the printing filament was mimicked by hand using a positive displacement pipette.

3.3.3 Rheological Properties

The rheological properties of dECM and dECM/GO inks were characterized [142] using a Kinexus Rheometer.

Firstly, an amplitude sweep test, from 0.1% to 100% strain, was done to determine the linear-viscoelastic (LVE) regime. For this, 60 µL of pre-gels were loaded into the rheometer plate, with a geometry of 8 mm diameter. The defined gap was 1 mm and frequency were set at 0.1 Hz with the tests being conducted at 10 °C, since the inks are kept at 4 °C but, as the printing is done at RT, the temperature of the ink is assumed to rise during the printing. Next, frequency sweeps were also conducted. The samples were loaded in the same manner as for the amplitude sweep tests but this time, strain was fixed at 0.1% with the frequencies varying from 0.01 to 10 Hz. As dECM gelation is temperature-controlled process, temperature sweep tests were conducted using the following settings: 2 °C/minute from 4 °C to 37 °C using LVE

values for strain and frequency. A time sweep was done, right after the temperature sweep, maintaining the same parameters and lasting 5 minutes.

Finally, to measure shear thinning properties, the samples were loaded into canonical 25 mm plates and a shear rate ramp was created by measuring the shear viscosity in an interval of shear rates between 0.1 and 100 s⁻¹. To assess thixotropic behaviour shear viscosity was measured in 3 intervals of 2 minutes. For the first 2 minutes, the sample was subjected to a low shear rate (0.1 s⁻¹), afterwards the shear rate was increased to 100 s⁻¹, and lastly decreased again to 0.1 s⁻¹. These measurements allow us to know if the ink recovers its viscosity after shear rate is applied during printing.

3.3.4 3D Printing

Before printing, the flow speeds and needles gauge were tested manually using a syringe pump (NE-1000 Programmable Single Syringe Pump).

3D printing was performed using a REGEMAT BIO V1 extrusion printer. Syringes were loaded with the ink and centrifuged to remove the bubbles at 2000 g for 1 minute. The designed was made and controlled using the REGEMAT 3D Designer.

To print our scaffolds, two different needles gauges of 22 G (0.41 mm) and 20G (0.58 mm) and flow speeds between 0.8 mm/s and 2 mm/s were tested. The printing velocity was constant and set at 3 mm/s.

As proof of concept, two different two-layered square mesh grid scaffolds with 12 mm x 12 mm, filament thickness of 0.41 mm and a pore size between 1.8 mm and 2 mm were idealized and printed.

3.3.4.1 SEM

SEM images of dECM/GO scaffolds were acquired in order to assess the interaction between the dECM and the GO particles. Scaffolds cross-sections were prepared by cryo-fracturing, which was done by freezing them with liquid nitrogen and using tweezers to break them. Samples were further freeze-dried for 3 days to remove the water. Samples were fixed to SEM stubs using a conductive carbon tape. Sputtering with a thin layer of gold was done to improve the samples conductivity. The samples were visualized with a Phenom XL Desktop SEM.

3.4 - *In vitro* biocompatibility of dECM/GO

In vitro biocompatibility assays were performed using a human foreskin fibroblast (HFF-1) cell line (ATCC, SCRC1041).

3.4.1 HFF-1 Culture

HFF-1 cells were grown at 37 °C, in a fully humidified atmosphere with 5% CO₂, using DMEM+ (Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX, Gibco, Thermo Fisher Scientific, USA

supplemented with 10% v/v foetal bovine serum (FBS, Gibco, Thermo Fisher Scientific, USA) and 1% v/v penicillin/streptomycin (Pen/Strep, Biowest, France)). Media was changed every 3 days.

When approximately 90% confluence was reached, cells were rinsed with 5 mL of PBS (37 °C) and detached from culture flasks using 2 mL of 0.25% w/v trypsin solution (Sigma Aldrich, USA) in PBS. DMEM+ was used to neutralize the trypsin and cells were then re-seeded at a density of around 15 000 cells/cm², as recommended by the American Type Culture Collection.

3.4.2 Cell adhesion assay

The ability of dECM and dECM/GO films to adhere cells was tested as previously described [143]. dECM and dECM/GO films were produced by pipetting 50 µL of hydrogel into the bottom of wells in 96-well plates. The films were left at 37 °C for 2h and then covered with PBS and left at 37 °C overnight. For the cell seeding PBS was removed before adding the cells. A cell suspension with 1×10^5 cells/mL was prepared in DMEM+. Cells were then seeded by placing 100 µL of the prepared cell suspension (10,000 cells) on top of each hydrogel film.

Cells were visualized in two different time points, day 1 (after 24 hours) and day 7, being for the last time point the medium changed every 3 days. For each time point, the medium was removed, films were rinsed with PBS and the cells were fixated with paraformaldehyde (PFA, Merck, USA) 4 wt.% in PBS for 15 minutes. PFA was removed, the cells were washed with PBS (3x) and stored at 4 °C until further use.

3.4.3 Cell morphology

For cell morphology observation, fibroblasts were stained with phalloidin (Alexa Fluor 488; Thermo Fisher Scientific, USA), which binds to cytoskeletal filamentous actin and with 6-diamidino-2-phenylindole dihydrochloride (DAPI, Merck, USA), which intercalates with nucleic acids, staining the nuclei of cells. For this, cells were permeabilized with Triton x-100 0.1 wt% at 4 °C for 5 min. After washing with H₂O, films with cells were incubated with 100 µL of phalloidin in 1:100 dilution was performed for 30 minutes for the films, in the dark. After, the films with cells were again washed with H₂O and 100 µL for the films of DAPI at 3 µL/mL was added and incubated for 15 minutes, in the dark.

After staining, scaffolds were transferred to an Ibidi dish and observed using a Laser Scanning Confocal Microscope Leica TCS SP5 II (Leica Microsystems, Germany) with an HC PL APO CS 40x/1.10 objective. Diode 405nm and Argon 488nm lasers were used to detect DAPI stained nuclei (blue) and phalloidin stained cytoskeleton (green). Confocal microscopy imaging files were processed either using ImageJ (Fiji) or the equivalent LAS X processing software.

Chapter 4

Results and Discussion

4.1 - GO characterization

The two commercially available GOs used in this study, water dispersion (GO-WD) and highly concentrated (GO-HC), were characterized regarding their oxidation degree, by XPS, lateral size and dispersibility in water, by TEM. XPS analysis shows that both GOs exhibit a similar atomic composition, being constituted by 70.3% of carbon and 29.7% of oxygen atoms (**Figure 15A**). Despite this, the carbon high resolution spectrum reveals that abundance of the oxygen containing groups present in these materials are slightly different with GO-WD presenting more C=O and less C-O-C than GO-HD. TEM imaging reveals that the GO-WD suspension is more prone to aggregation than GO-HC, as for the same concentration, more clusters of GO particles can be observed. Additionally, TEM images also reveal the particle size of GO-HC is higher than GO-WD, exhibiting $22.4 \pm 9.5 \mu\text{m}$ and $11.9 \pm 4.0 \mu\text{m}$, respectively, being both slightly above the values given by the supplier ($14 - 17 \mu\text{m}$ and $<10 \mu\text{m}$, respectively) (**Figure 15B**) [138, 139].

Since the created dECM/GO scaffolds will be applied in biomedical settings, it is essential to ensure that they are sterile. It has been reported that sterilization processes after printing can result in shape deformation in hydrogel-based 3D-printed scaffolds [52, 144]. To overcome this, in this study, the ink was prepared with sterile components (dECM and GO) and then printed under sterile conditions (in a laminar flow hood). Regarding GO sterilization, there is controversy over the ideal sterilization method [145]. As such, in this study we adopted two approaches to perform GO sterilization: sequential ethanol washing and autoclaving. Sequential ethanol washing caused significant GO loss during the process, not being a suitable method for large scale application. In the case of autoclaving, XPS demonstrated that it resulted in a 1% decrease in oxygen amount, which means that GO-HC was slightly reduced. Despite being performed at higher temperatures, optimal temperature $\sim 300^\circ\text{C}$ [146], the thermal reduction of GO, in which oxygen groups are removed from GO to form H_2O , CO, and CO_2 , is well described

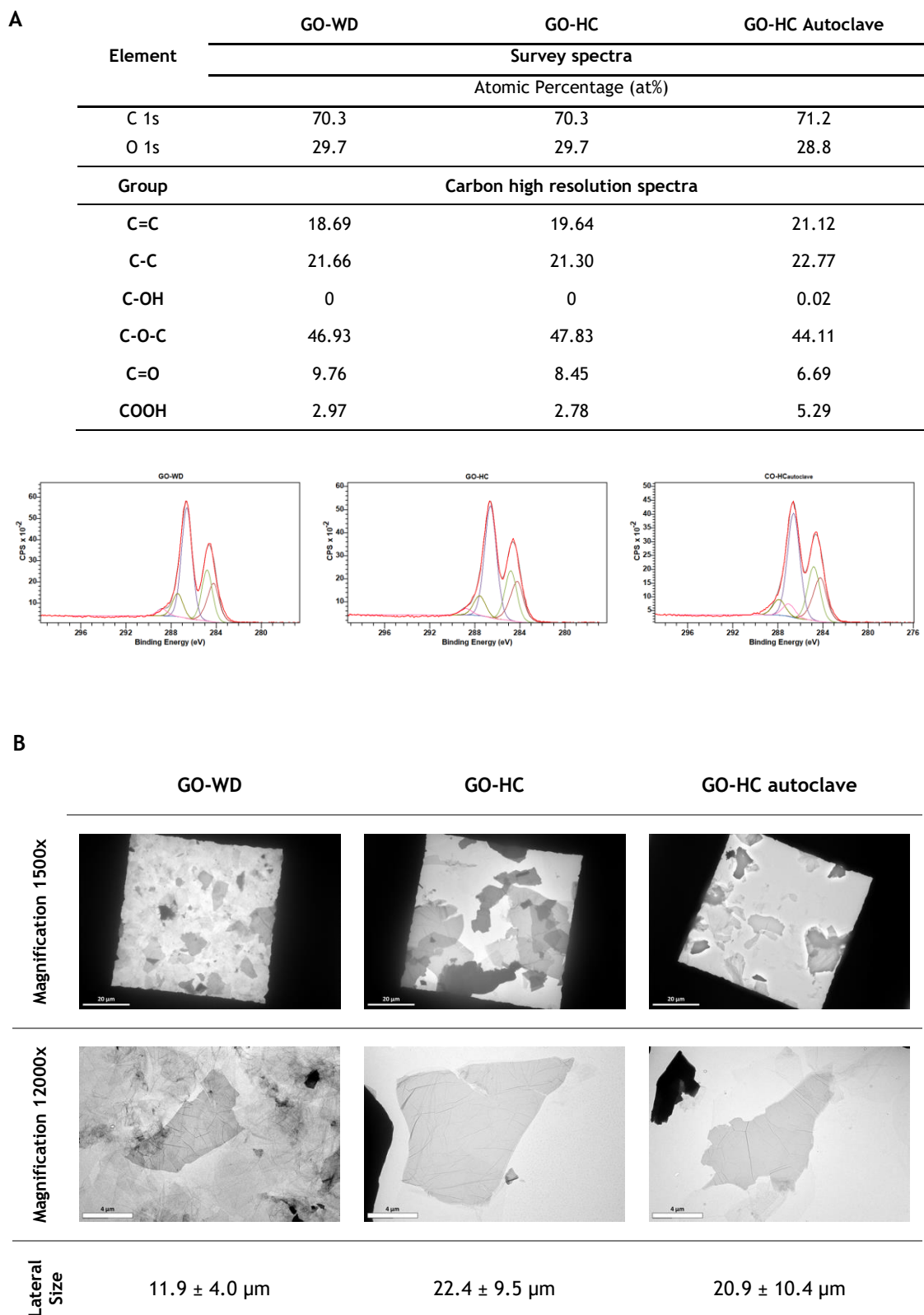


Figure 15 - A: Atomic percentages (at %) of carbon 1s and oxygen 1s and carbon high-resolution spectrum acquires by XPS. B: TEM images and particles lateral size (n=14) of GO-WD, GO-HC, and autoclaved GO-HC.

In literature [146], which could justify the observed decreased in oxygen content in GO (**Figure 15A**). TEM images show that autoclaving did not influence GO lateral side exhibiting 20.9 ± 10.4

μm (**Figure 15D**). These results are consistent with the literature, as sterilization by autoclave has been shown to not significantly change the GO's lateral size and atomic composition [147].

4.2 - dECM/GO Scaffolds

All the ink optimization process was done using dECM-i3S pre-gels and using a simple procedure to simulate the printing that involves pipetting the ink to the printing platform with a gel pipette to assess filament's consistency, shape fidelity, and detachment from the platform. The influence of different parameters was evaluated namely in the ink: the dECM or GO concentration and GO type and in the printing platform: the materials, coatings, temperature, and the use of support baths.

4.2.1 dECM Ink

The preparation of dECM pre-gel resulted in a viscous substance, shown in **Figure 16**, that could be manipulated to certain shapes.

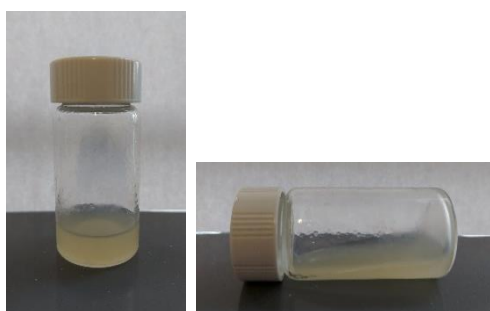


Figure 16 - dECM pre-gel.

4.2.1.1 dECM Concentration

Two concentrations of dECM: 3% and 4% (w/v) were tested being observed that, when printing, the 3% pre-gel was less viscous and thus the filament tended to spread out more and, consequently, seemed to lose its shape (**Figure 17**) resulting in constructs with low-definition.

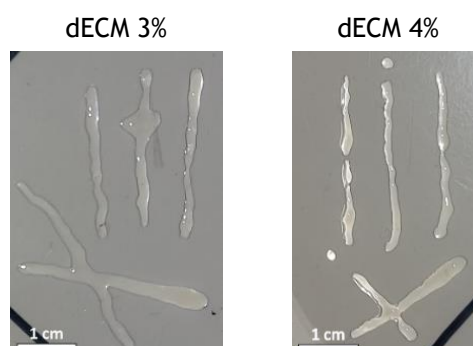


Figure 17 - Printing simulation of dECM pre-gel with 3% and 4% concentration. The printing surface is plastic.

On the contrary, dECM 4% pre-gel seemed to be more viscous and, as consequence, more stable, being capable to hold its shape (**Figure 17**). It is worth mentioning that it is not

recommended to prepare dECM hydrogels with concentrations higher than 5%/6% as the components will be very difficult to dissolve, and the pre-gel will have a very viscous consistency that will highly hinder the handling of the material (data not shown).

As such, we chose 4% dECM pre-gel as optimal concentration to proceed for the following experiments.

4.2.2 dECM/GO Inks

4.2.2.1 GO Concentration

Different concentrations of GO-WD (1-100% (w/w_{ECM})) and GO-HC (1-10%(w/w_{ECM})) were tested to achieve the best condition which will allow an enhancement in mechanical properties and printability while not interfering with the dECM gelation at 37 °C.

As can be seen in **Figure 18**, concentrations below 5% would not be suitable for our application since the amount of GO present within the hydrogel is not enough to constitute a uniform dispersion. The lack of a homogeneous distribution of GO can result in the emergence of regions that have different mechanical properties which will induce the collapse of 3D printed scaffolds not being possible to retain the shape.

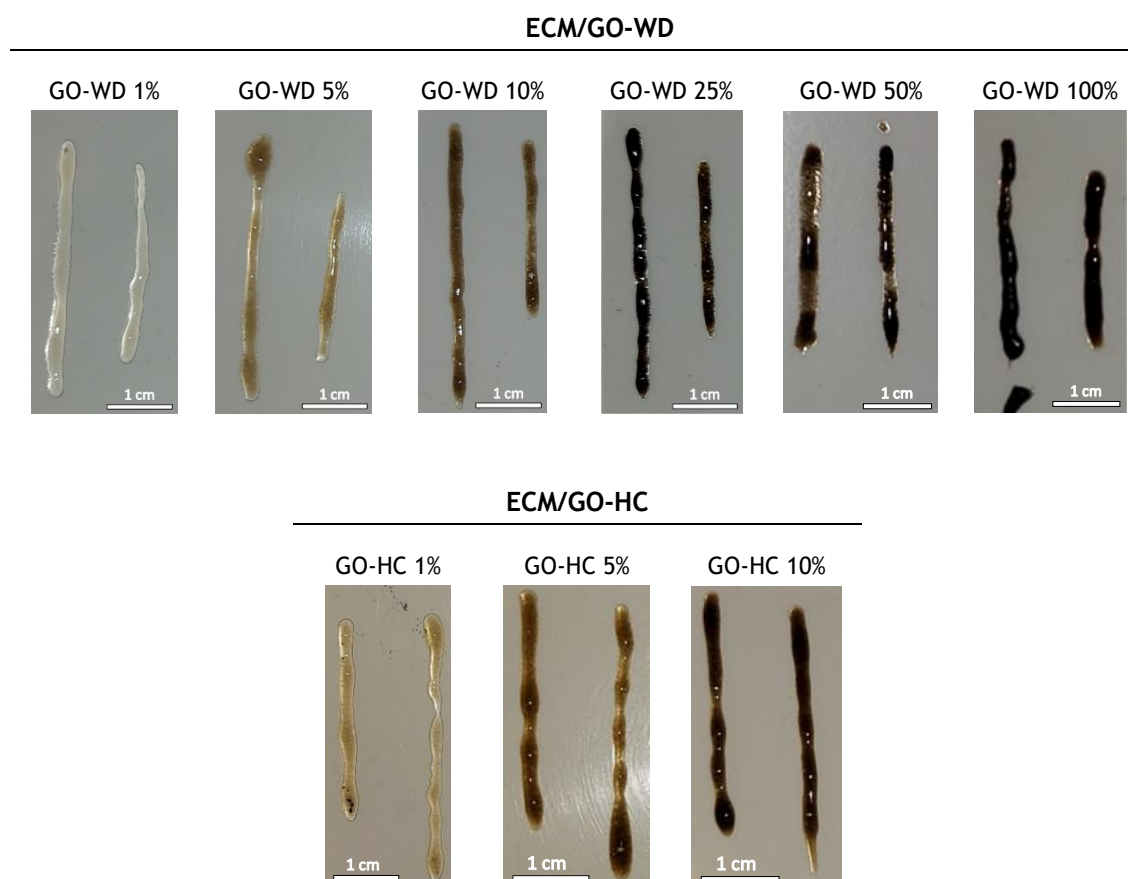


Figure 18 - dECM 4% hydrogels with different concentrations of GO-WD and GO-HC suspensions.

On the other end, concentrations higher than 50% reveal that the amount of GO is too high and interferes with the gelation of the dECM proteins resulting in an unstable hydrogel.

Ultimately, 10% and 25% of GO seemed to be the more suitable to guarantee an enhancement in the mechanical properties and printability of the dECM without interfering with its gelation and, as consequence, stability. The results presented here consider the choice of dECM 4% based on the last section. However, similar results were obtained for dECM 3% (see Annex C).

4.2.2.2 GO Type

When comparing the two types of GO, it was noticeable that GO-WD was more difficult to incorporate than GO-HC (**Figure 19**). This might be related to the fact that GO-WD seems to be more prone to agglomerations as shown in TEM images (**Figure 15**) and thus does not allow the achievement of a complete homogeneous hydrogel.

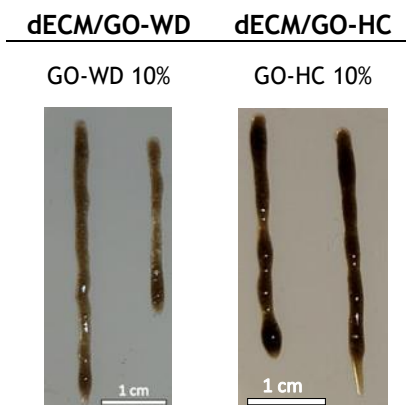


Figure 19 - Comparison of a dECM 4% formulation with 10% GO using GO-WD and GO-HC.

Additionally, it was also noted that, during extrusion the GO and the dECM underwent a separation phenomenon in which a part of the hydrogel separated from the mixture resulting in uneven prints. This phenomenon was more evident in formulations that contained GO-WD.

Bearing in mind these facts, we opted to use GO-HC for our formulation since it resulted in more stable hydrogels.

4.2.3 Printing Platform

4.2.3.1 Plastic VS Glass

All the results presented in section 4.2.2 were achieved using a plastic surface at RT as printing platform. However, the nature of printing platform is crucial for printing output [148].

Figure 20 shows that when extruding the ink into a plastic surface, the filament's shape was maintained with minimal spreading due to the hydrophobic nature of the plastic. On the contrary, since glass is hydrophilic and thus has a higher affinity for the hydrogel, the spreading was more perceptible, and the shape of the filament was not well maintained.

Additionally, after the hydrogel's gelation, we tested if it was possible to lift the prints from the platform. In both materials, the filaments could not be separated from the printing platform.

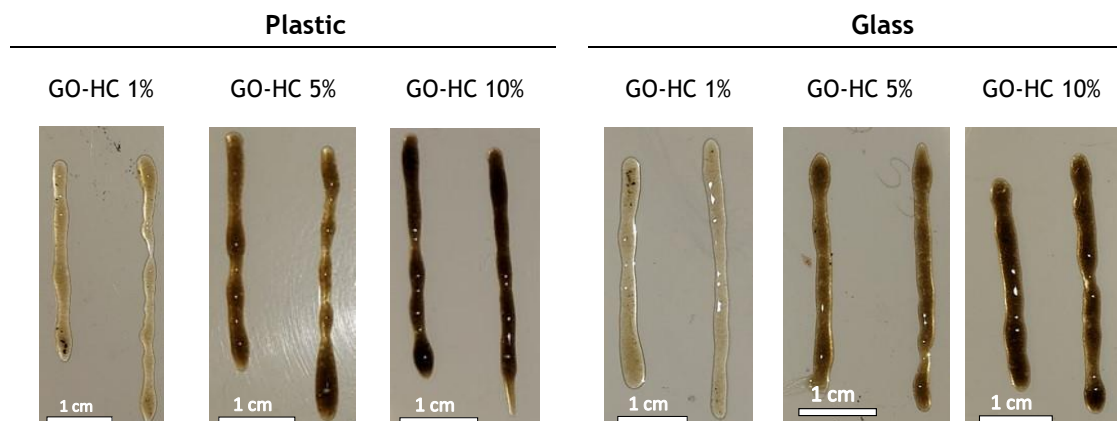


Figure 20 - Comparison of the same formulations extruded onto plastic and glass surfaces.

From these results, we learnt that the printing surface should be similar to plastic, ideally with hydrophobic properties to prevent the spreading of the material but also with capability to allow the fibres detachment from platform. These tests were performed for all ink formulations with similar results obtained (**Annex C**).

4.2.3.2 Pluronic VS Sigmacote coatings

To improve the printing platforms, we modified their features by coating them with pluronic or Sigmacote. The first tested coating was pluronic F-127 (**Figure 21**). Pluronics or poloxamers, are a class of synthetic block copolymers arranged in an A-B-A triblock structure with a central chain of hydrophobic poly(propylene glycol) (PPG) and flanked by the hydrophilic chains of poly(ethylene glycol) (PEG) [149]. By varying the number of repeated units, different polymers with different properties can be made. The amphiphilic behaviour conveys differences in solubility between the blocks and, thus, the ability of the hydrophobic block to adsorb to a hydrophobic surface (such as the polystyrene of the plastic surfaces we used) through hydrophobic interactions while the hydrophilic PEG chains extend away from the surface as well-solvated brushes, which sterically hinder protein adsorption [149].

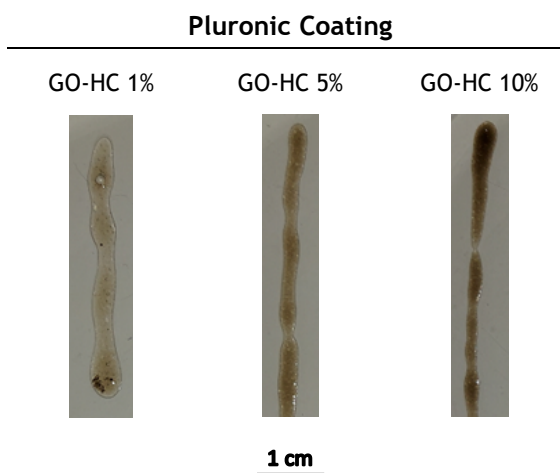


Figure 21 - Extrusion onto pluronic coated surfaces.

Pluronic 127 is the trade name of poloxamer 407, a type of poloxamer that has around 101 PEG and 56 PPG block repeats. This way, and as expected, the surface coated by the Pluronic behaved similarly to the glass, with the hydrogel having a high affinity for the hydrophilic PEG and thus resulting in very low fidelity for the filaments (**Figure 21**). The detachment was improved especially when, after the gelation was finalized, the prints were submerged in water, resulting in almost instant lifting. Nevertheless, this strategy was abandoned as the spreading would result in very low-resolution filaments.

Sigmacote is a chlorinated organopolysiloxane (silicone) solution in heptane that forms a covalent, microscopically thin film on glass that can repel water, retard the clotting of blood/plasma, and prevent surface adsorption of many proteins [150].

Due to the Sigmacote's hydrophobic properties the filament's shape was maintained as we can observe on **Figure 22**.

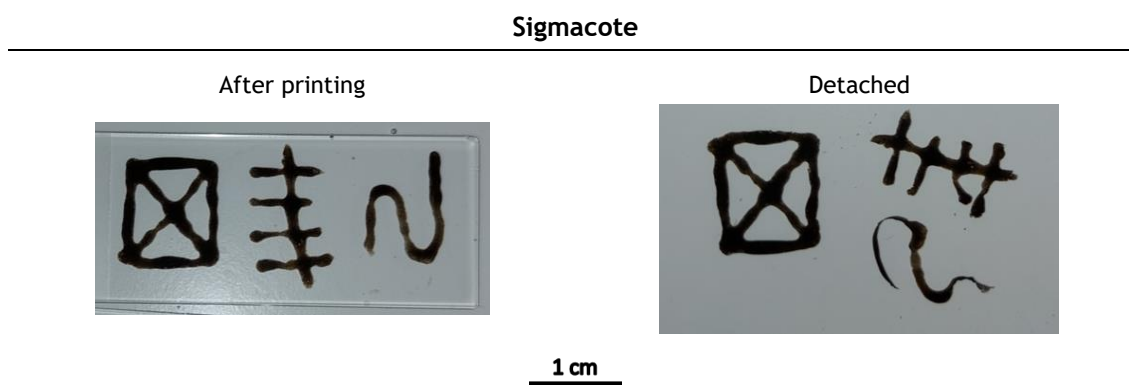


Figure 22 - Extrusion of dECM/GO-WD onto Sigmacote coated surfaces.

Additionally, following the strategy that proved good results for the detachment of the Pluronic F-127 coating, we left the prints until gelation was completed and then we submerged them in water. Similar to pluronic F-127, when in water, the prints extruded onto the Sigmacote treated surface lifted instantly without leaving noticeable residue on the printing surface.

4.2.3.3 Room Temperature VS 37 °C

The dECM hydrogels gelation is a process triggered by the temperature, more specifically, it occurs at 37 °C with some articles reporting that it starts around 15 °C to 20 °C [151, 152]. To try and boost the gelation, we extruded our hydrogel formulations into heated surfaces and compared the results with platforms at room temperature. Initially, just after printing, there were no major visible changes in the prints (**Figure 23**). However, after few minutes, the hydrogel started to dry out in the platform at 37 °C. This phenomenon is a major hindrance since the printing process of more complex structures may take more time than threshold at which the material starts to dry and thus cause defects in the structure. It is inviable to have a structure being printed while at the same time the bottom is drying out, and the hydrogel is being damaged.

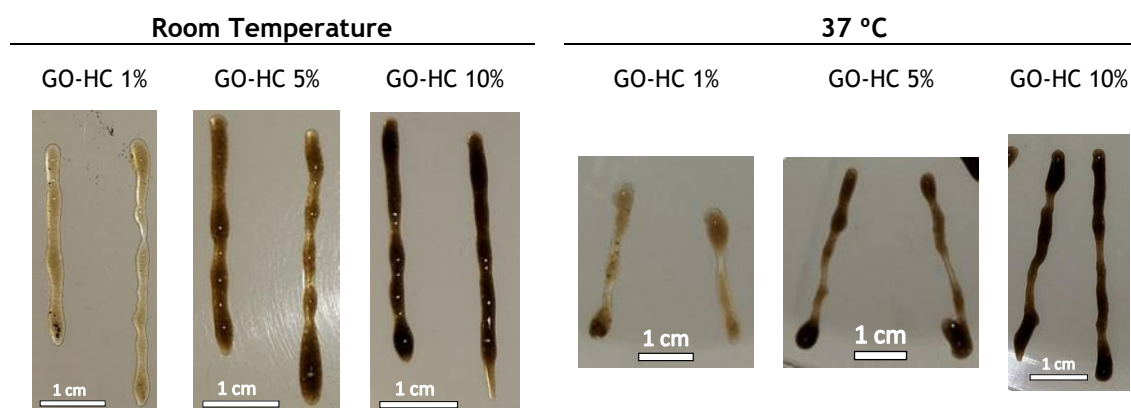


Figure 23 - Comparison of the same formulations extruded for platform at room temperature and 37 °C

This way, our strategy will rely on printing at room temperature.

4.2.3.4 Support Baths

A strategy commonly used when printing materials that lack mechanical strength or that cannot be easily printed is using support baths [153]. Support or fluid baths are materials capable of supporting the ink deposited in its liquid state for long periods of time while maintaining its structure/shape. They are most commonly used when inks do not possess rapid solidification properties or when the structure has “hanging” parts in order to avoid the need to create auxiliary supporting structures [154]. It is important to note that, although these support baths can physically help the structure resolution, they might also prove a challenge since complete removal from the final structure might be difficult and finding the ideal support material might also prove to be challenging [155]. This way, to avoid any possible contamination of the supporting bath in our dECM/GO scaffold or changes in surface topography when removing the support bath, we firstly tried printing the ink directly into water to help form the filament but, and although we could form the filament quite well, we could only form a continuous filament and not build an 3D structure (**Figure 24A**).



Figure 24 - Printing of the ink into dH₂O or Dextran 5% at room temperature.

After printing in water bath, we theorized that a denser material would help in keeping the printed structure. For this, we prepared a 5% dextran solution. Dextran is a polysaccharide derived from D-glucose. It has a diverse range of clinical applications from anaesthesiology and surgery to radiology and ophthalmology and can be administered both osmotically and intravenously [156]. As it is possible to observe in **Figure 24B**, the dextran enabled the extrusion and design of sharp edges however, due to its chemical composition the printed hydrogel dissolved.

Thus, printing on air (without a support bath) allow to obtain filaments with better resolution then when using the tested support baths.

4.3.2.5 Gelation Strategy

Since the prints need to be incubated at 37 °C and then covered with water, we tested three different strategies, summarized in **Table 3**, to understand the best time periods for each step.

Table 3 - Gelation strategies and scaffolds features.

Gelation Strategy	Strategy 1	Strategy 2	Strategy 3
	37 °C for 30 minutes ↓ cover with water	37 °C for 2 hours ↓ cover with water	37 °C overnight ↓ cover with water
Scaffolds	• Not stable • Tend to dissolve	• Stable • Hold their shape	• Dried out
Features	• Not dried	• Not dried	

In strategy 1, the prints were incubated at 37 °C for 30 minutes and then covered with water. By observing the prints, it was possible to find out that the incubation time was not long enough, since the gelation was not complete and some of the dECM material starts to dissolve and as such the filaments were not stable. In strategy 3, the prints were incubated overnight (approximately 18h), which turned out to be too long as they dried out. The best gelation strategy reveals to be the 2, where the prints were incubated for 2h at 37 °C following its immersion in water, which resulted in stable scaffolds that could easily be lifted from the printing platform.

For further printings, strategy 2 was the adopted procedure. The prints can then be detached and left in water until further use. It was observed that printed filaments start to degrade after around 2 weeks (data not shown).

4.3.3 Printing Strategy

Overall, the formulation with the best characteristics would be dECM 4% (w/v) with GO-HC 25% (w/w). The best printing platform is sigmacote-treated glass at RT with gelation being

optimized by incubation in a humid environment for 2 hours at 37 °C followed by water immersion (Table 4).

Table 4 - Ink Formulations, Printing Platform and Strategy. The parameters chosen are highlighted in blue.

Ink Formulation

dECM

3% vs 4% (w/v)

Graphene Oxide

Type

GO-WD vs GO-HC

Concentration

1%, 5%, 10%, 25%, 50% vs 100% (w/w_{ECM})

Printing Platform

Material

Glass, plastic, pluronic coated plastic vs sigmacote-treated glass

Temperature

RT vs 37 °C

Gelation Strategy

Strategy 1 (37 °C for 30 minutes + water immersion)

Strategy 2 (37 °C for 2 hours + water immersion)

Strategy 3 (37 °C for 24 hours + water immersion)

However, a new type of dECM, dECM-MUV, was made available to us after the optimization steps were concluded. When compared, dECM-MUV presented better viscosity than dECM-i3S. While the preparation of both dECM and dECM-MUV pre-gels resulted in a viscous substance, shown in Figure 25, that could be manipulated to certain shapes, the dECM-i3S pre-gel appeared to be less homogeneous than the dECM-MUV pre-gel whilst the later required less preparation time.

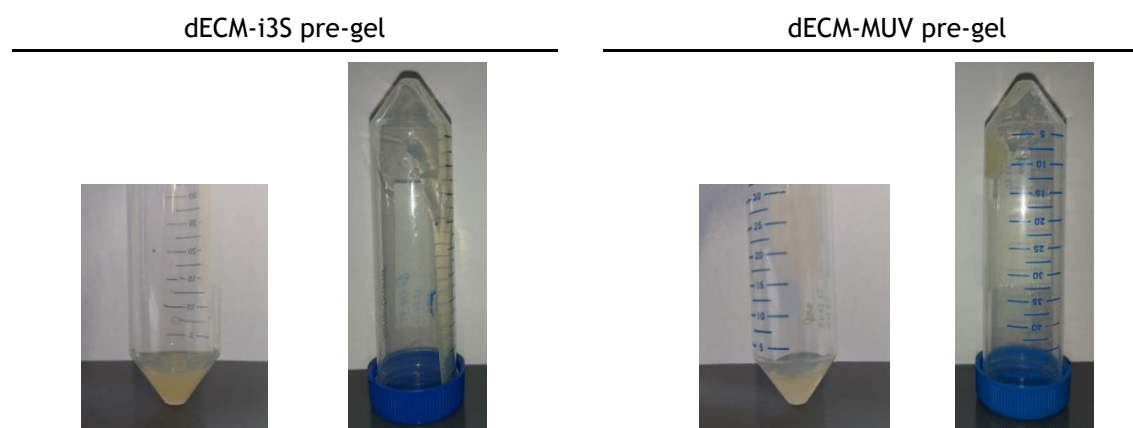


Figure 25 - dECM-i3S and dECM-MUV pre-gels.

The differences between the two pre-gels can be attributed to the differences in the decellularization and digestion steps (which are confidential due to patentability reasons) since it is described that different methods will produce dECMs with different properties.

Additionally, due to its already digested form, dECM-would allow us to increase the quantity of GO that is possible to incorporate, being possible to incorporate 25% (w/w) of GO-HC into this formulation.

This way, going forward in this work, the herein mentioned dECM/GO ink is composed by dECM-MUV at 4% (w/v) and GO-HC 25% (w/w) unless mentioned otherwise.

4.3.4 Rheological properties

To understand if the GO enhances the mechanical properties of the inks, we conducted temperature and time sweeps to observe the ink behaviour through a range of temperatures and at a set temperature for a specific period of time, respectively. To properly conduct the temperature and time sweeps we must first determine the linear viscoelastic region (LVER). The LVER will establish the range in which the assays can be conducted without destroying the sample and can be discovered by an amplitude sweep.

4.3.4.1 Amplitude Sweep and Frequency Sweep

From the amplitude sweep we observe that the dECM ink mechanical properties are stable for a broader range of applied strains. The dECM/GO ink will start to lose its mechanical properties after a shear strain of about 1% while the dECM ink G' and G'' remain constant (Figure 26). Nevertheless, we should use the same strain for both inks when conducting further assays and, as so, the LVER value chosen was 0.1% strain.

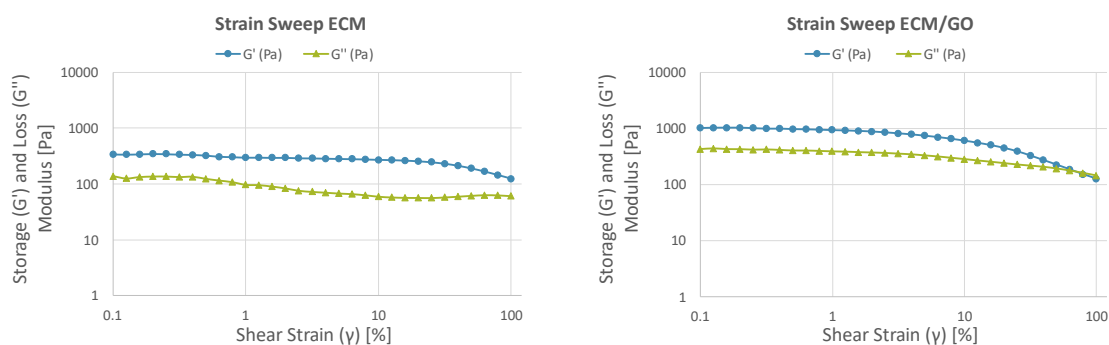


Figure 26 - Strain sweep assays for dECM and dECM/GO inks.

Frequency sweeps are useful to understand how the material behaves when under constant and increasing frequencies. Comparing the curves, we noticed that G' tends to increase abruptly when frequencies reach values close to 10 Hz for both inks, although the registered G' values are higher for dECM/GO inks (Figure 27). The increase in stored energy is related to the inability of the collagen fibres to expand due to the higher frequency of the applied strain.

This means that, since the frequency at which the determined strain applied is increasing, the fibres have less time to “decompress” and thus are able to accumulate more energy which in turn results in a higher storage modulus [157].

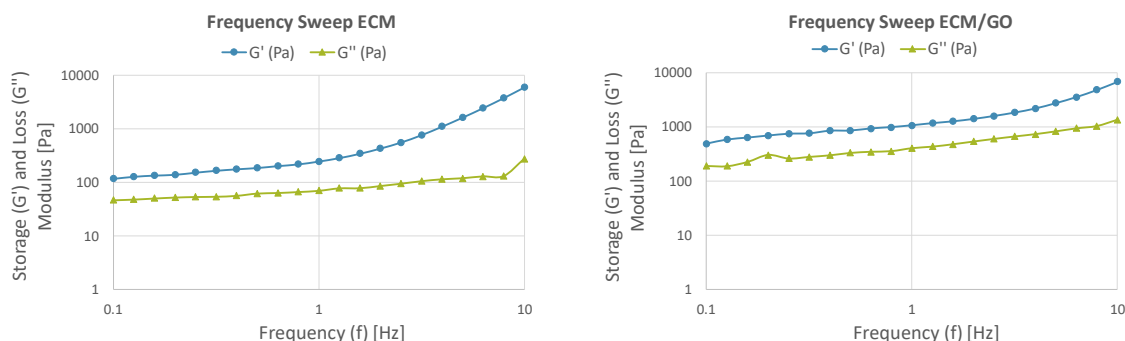


Figure 27 - Frequency sweep assays for dECM and dECM/GO inks.

Considering both graphs we chose to go forward with a frequency of 1Hz.

4.3.4.2 Temperature Sweep and Time Sweep

The gelation process of dECM hydrogels is based on a thermal-induced crosslinking reaction of the collagen fibrils. The crosslinking is reported to begin at 15 °C and reach its peak at 37 °C [151, 152]. This way, to guarantee that we could obtain a curve that would show us the crosslinking behaviour of our hydrogel we started the temperature sweep assays at 4 °C and increased the temperature to 37 °C in a rate of 2 °C/min.

Contrary to what we expected, for the dECM ink, G' is higher than G'' even when the temperature is lower than crosslinking temperature (**Figure 28**). We were not expecting this result as the elastic modulus being higher than the viscous modulus means that the material is behaving as a hydrogel instead of a pre-gel. However, and although the values are very similar and do not increase as they should with the gelation, there is a slight increase in G' at -20 °C and 30 °C. These values are close to the temperature at which the collagen starts to crosslink and the temperature of dECM hydrogels gelation, respectively. dECM/GO ink temperature sweep curves show the same profile of dECM hydrogels (**Figure 28**).

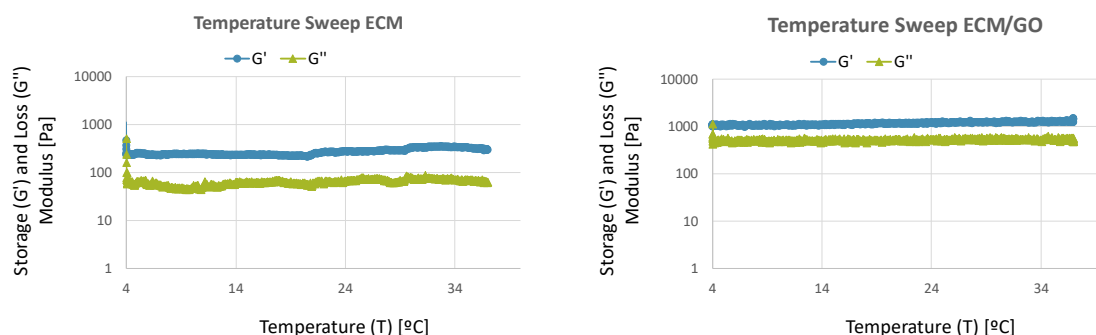


Figure 28 - Temperature Sweep assays for dECM and dECM/GO inks.

Additionally, to verify if the increase in modulus was not being observed due to lack of time (i.e., the hydrogel proteins needed more time at their optimal gelation temperature - 37 °C - to reach maximum mechanical properties) we conducted time sweeps of the same samples that were subjected to the temperature sweep without retrieving them from the rheometer, maintaining their shape and the temperature stable. Previous studies done by us (data not published) and described in the literature show that the dECM hydrogels gelation is fast and the final modulus can be reached in 5 minutes [158].

The results obtained showed that G' and G'' (**Figure 29**) are constant throughout the duration of the assay and values are similar to the ones acquired using the temperature sweep.

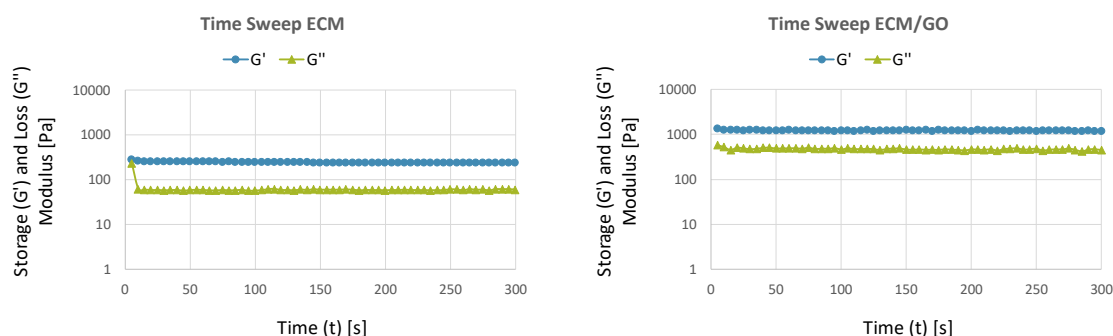


Figure 29 - Time Sweep assays for dECM and dECM/GO inks.

Although it was not possible to establish a gel-solid transition temperature, it was evident from the temperature sweep assays that the addition of GO improved the mechanical properties of the ink, with G' increasing from 304.7 Pa to 1280 Pa and G'' from 64.9 Pa to 535.2 Pa, corresponding to 320% and 725% increment, respectively. Additionally, analysing the time sweep results, we reach the same conclusion with increases of 408% for G' (from 239.4 Pa to 1216.6 Pa) and 675% for G'' (from 60.8 Pa to 470.8 Pa), when GO is added to the dECM ink.

4.3.4.3 Shear Thinning and Thixotropy

Shear thinning is a property of non-Newtonian fluids in which the viscosity of the material will decrease when the applied shear rate increases. This property is fundamental for 3D extrusion-based printing since the material's viscosity must decrease when the ink is driven through the needle in order to form the filaments. By measuring this property, we confirm that both the dECM and dECM/GO inks present shear-thinning behaviour with the viscosity significantly decreasing with the increase of the shear rate (**Figure 30**).

Besides shear thinning behaviour, optimal inks present thixotropic behaviour. Thixotropy, although related to shear thinning (all thixotropic materials present shear thinning behaviour), is the ability of a material to decrease the viscosity when the shear rate increases and then, when shear rate returns to the initial value, to increase the viscosity to the same or similar initial values. This property can be assessed by conducting viscosity measurements through a

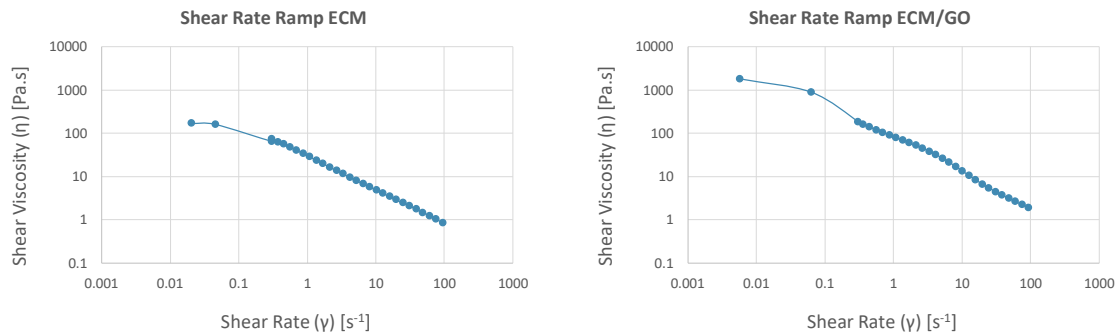


Figure 30 - Shear Rate Ramp assays to determine if the dECM and dECM/GO inks present shear-thinning behaviour.

period of time with an initial low shear rate (0.1 s^{-1}), increasing the shear rate to a high value (100 s^{-1}) and then reducing the shear rate back to the initial value. When subjected to this assay, both dECM and dECM/GO inks both do not presented thixotropic behaviour (**Figure 31**).

Additionally, by analysing the viscosity values for the first interval of low shear rate we noted that the presence of GO increased the viscosity of the ink from 226.8 Pa.s to 550.1 Pa.s.

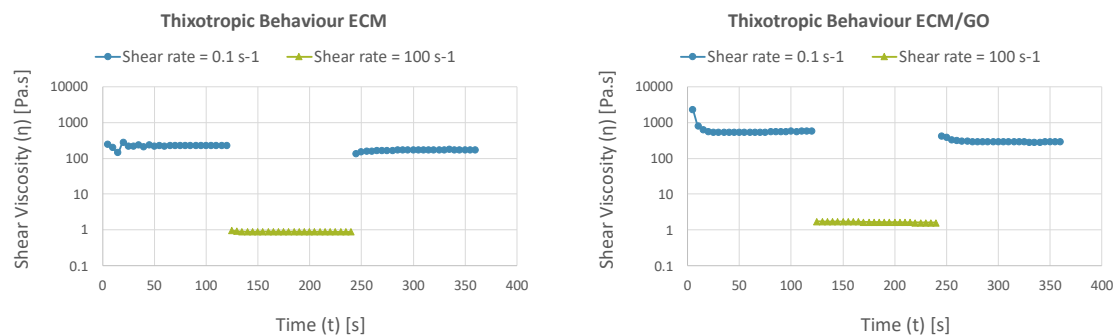


Figure 31 - Shear viscosity in function of shear rate to determine if dECM and dECM/GO inks present thixotropic behaviour.

As we can observe from the graphs, both inks viscosity decreases significantly when high shear rate is applied, confirming the shear thinning behaviour, and increases again when the shear rate returns to the initial low values. However, the dECM ink recovers 77% of its initial viscosity values, to 174.2 Pa.s and the dECM/GO becomes significantly less viscous by only recuperating about 52% of the initial viscosity, corresponding to 290.5 Pa.s, indicating that these materials do not possess thixotropic behaviour. This might be caused by the interference of the GO with the collagen and other dECM protein fibres that do not allow them to return to their initial stage.

4.3 - 3D Printing

4.3.1 Printing Parameters

For 3D printing, besides optimization of ink and printing platform, the printing conditions, namely needle size and flow velocities should be optimized. In this study were tested two needle sizes, 20 G (inner diameter of 0.58 mm) and 22 G (inner diameter of 0.41 mm).

We started by testing the 20 G needle with different flow velocities (0.8 mm/s to 1.5 mm/s) to understand, which would produce a better filament. As shown in **Figure 32**, the flow velocity of 1 mm/s seems to produce a filament with continuous thickness. Allowing to define a shape with a better fidelity.

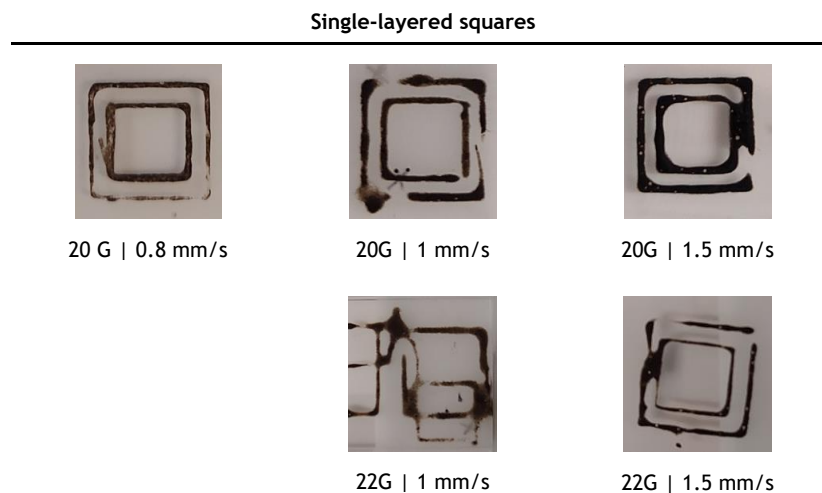


Figure 32 - 3D printing of single-layered squares using different needles size and flow velocities.

With 1 mm/s a 3 layered structure was printed to evaluate the capacity of the ink to be stacked. In **Figure 33**, we can observe that the ink can be layered and does not collapse when printed in stacks.

After this, a mesh grid shape was optimized. Considering that the pore size will be affected by both the needle and the viscosity of the ink, for this print, the printing velocity needed to be increased to maintain a consistent flow. Ultimately, a CAD design with pore sizes smaller than 1.9 mm (**Figure 34**) resulted in a very low number of pores with reduced size as the filament is too thick due to spreading of the ink.

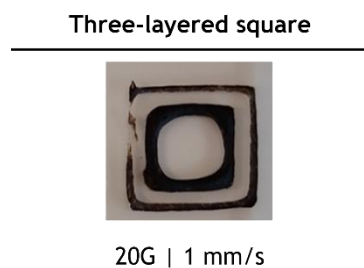


Figure 33 - 3D printing of three-layered square.

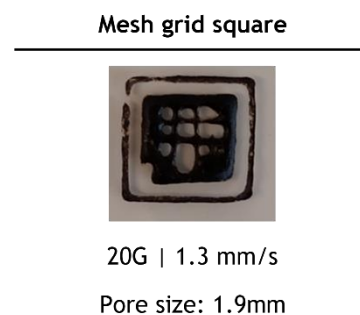


Figure 34 - 3D printing of mesh grid square.

As such, we tried decreasing the needle diameter to see if it would be possible to enhance the printing resolution. For the 22 G needle, a flow speed of 1 mm/s was not enough to produce a continuous filament causing a need to increase to 1.5 mm/s.

This way, we theorized that, printing a square mesh grid with 3 layers using a 22G needle and a flow speed of 1 to 1.3 mm/s would allow us to print the final design we purposed in **Figure 35**.

4.1.2 3D Printing of Scaffolds

Two different printing designs were tested. Initially, the printing was done following the scheme represented in **Figure 35A**, with the final design expected to look similar to **Figure 35B**. The parameters related to thickness of the filament are associated with needle diameter while the pore size, lateral size and height are determined from a combination of the input during the CAD design. Sometimes the material itself (if it spreads on the surface) and printing conditions (printing flow) can also have an influence in the achieved structure. The CAD design was established to achieve scaffolds to have a lateral size of 12 mm x 12 mm with a 2 mm pore size.

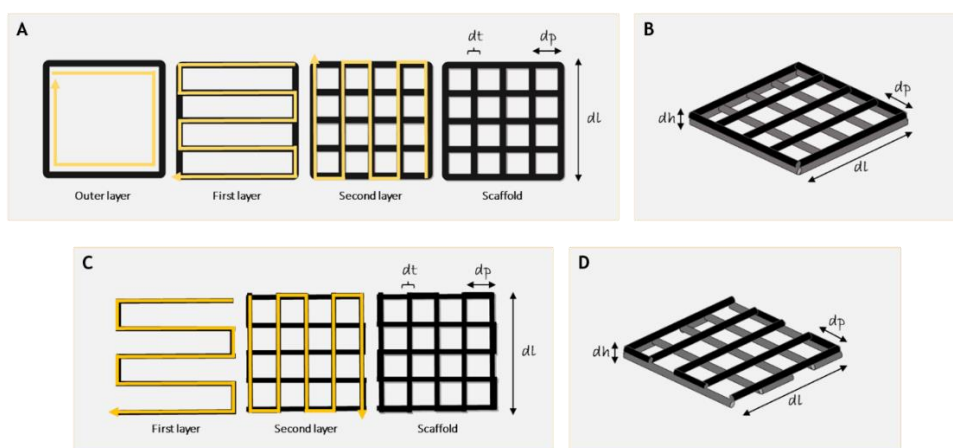


Figure 35 - Schematics followed for the printing of the scaffolds. Yellow arrow: needle path; dt : thickness; dp : pore size; dl : lateral size; dh : height.

Scaffolds were printed using 4 different ink to compare resolution, namely dECM, and dECM/GO as well as the scaffolds obtained using sterile GO, dECM/GO_{autoclave} and dECM/GO_{ethanol}.

In **Figure 36**, are present the scaffolds that resulted from the printing of the aforementioned inks with the specified parameters. By comparison of the dECM with the dECM/GO scaffold, we can conclude that the presence of GO improves the printability of the dECM as almost no pores are present in the dECM scaffold. This can be explained by the low viscosity of dECM ink which leads to high degree of filament spreading resulting in low shape fidelity. Additionally, from the similarity between the scaffold produced with the non-autoclaved and autoclaved GO, including SEM imaging and pore size, as well as the physical

handling of the construct, we can conclude that GO autoclaving does not interfere with produced scaffolds.

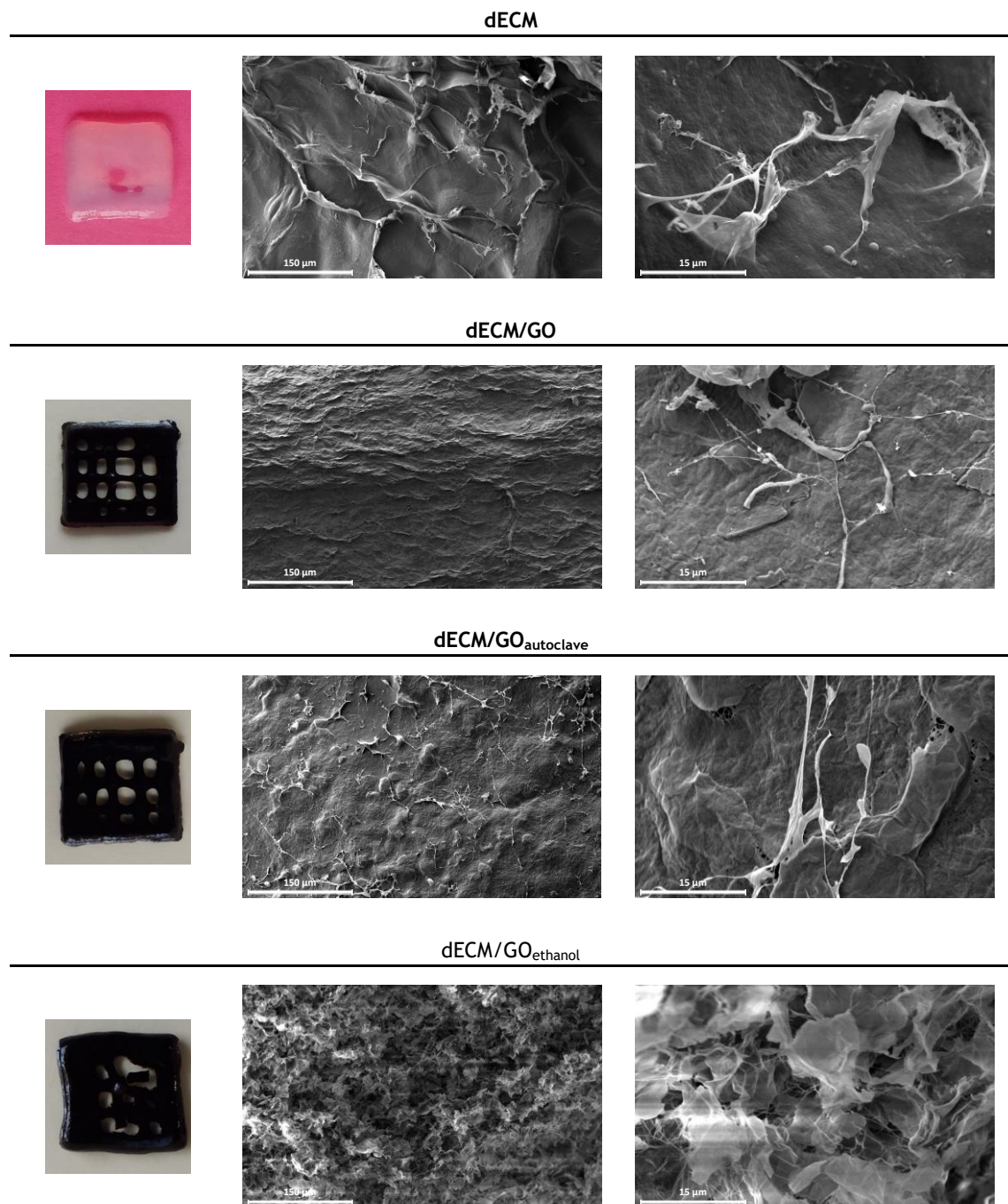


Figure 36 - Photographic images of the printed scaffolds using four different inks: dECM, dECM/GO, dECM/GO_{autoclave} and dECM/GO_{ethanol} and respective SEM imaging. Scale: 150 μm (left) and 15 μm (right).

However, when GO_{ethanol} was incorporated into the dECM, the resulting scaffold was not as stable as the others as we can see by the loss of integrity of the mesh in the construct. We theorize that this might result due to ethanol residues that negatively interact with dECM proteins and hinder polymerization or due to the presence of higher number of aggregated GO particles due to the removal of single layer particles during washes.

After obtaining a successful scaffold using the parameters and model design, we sought to improve the shape of our print. The prints obtained until now possessed an outer border that was thicker than the inside mesh leading to a collapsed effect in the centre of the scaffold. This phenomenon happens because after the frame is printed, the layers are done firstly horizontally and then vertically, meaning that a set of parallel lines is one layer, and the crossing lines correspond to another layer. In between these two layers, an additional border is printed resulting in a thickness mismatch (see **Figure 35A** and **Figure 35B** for the schematic representation). To avoid this type of structure, we prepared a new design, **Figure 35C** and **Figure 35D**, by removing the outer layer entirely.

To test the new design, we firstly tried the inks with better results before, dECM/GO and dECM/GO_{autoclave} and we noticed some issues with these formulations. Firstly, the dECM/GO ink produced very heterogeneous scaffolds. As we can see in **Figure 37**, some parts of the construct lack GO with the filament being only composed by dECM. As for the dECM/GO_{autoclave}, the new scaffolds appear to be more fragile and not so stable.

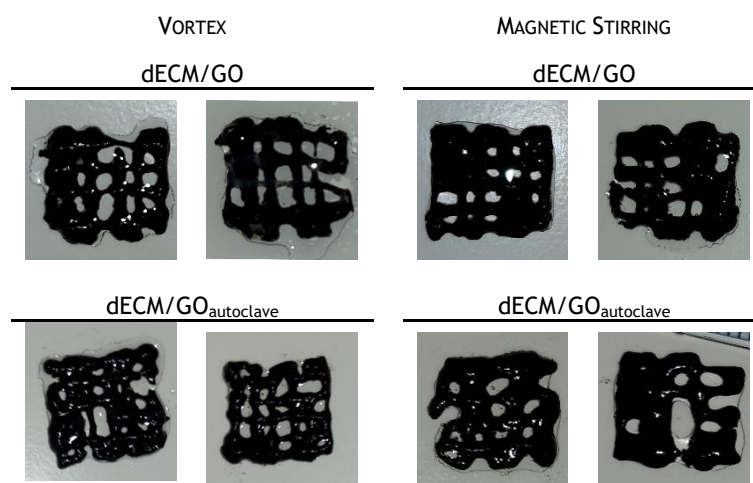


Figure 37 - Photographic images of the printed scaffolds using two different inks: dECM/GO and dECM/GO_{autoclave}, with two different methods of homogenization: vortex and magnetic stirring.

Considering that the protocol followed was the same as before, we hypothesize that the changes might have happened due to variabilities in the inks, or that the design is not suitable for the properties of our ink. The variability of the ink is related to different dECM batches from which the inks were prepared and to differences in the quantity of proteins present that, overall, do not affect the mechanical properties of the hydrogel but, for printing, require slight increases/decreases in flow speed.

To try and overcome the heterogeneity of the inks, we tried an additional method of homogenization. The ink was mixed using a magnetic stirrer for 12 hours at 4 °C. After examining the ink, we could observe that both the GO and GO_{autoclave} were better incorporated. However, when printing the ink proved to have a very low viscosity and thus, we could not print

with an adequate resolution (**Figure 37**). This correlates with the lack of thixotropic behaviour of the ink reported earlier.

In the end, despite GO improving the dECM printability, we were only able to produce a scaffold as represent in **Figure 37** without being able to produce the scaffolds schematized in **Figure 35D**. However, we could confirm that the presence of GO does increase the printability of the dECM inks and there is now greater leeway for additional manipulation of the ink to improve its properties.

4.4 - Biological Assessment

The ability of dECM/GO_{autoclave} composites to adhere cells on their surface was tested using 2D-films with a diameter of 6 mm and a height of 2.5 mm in order to simplify cell seeding and visualization (**Figure 38**).

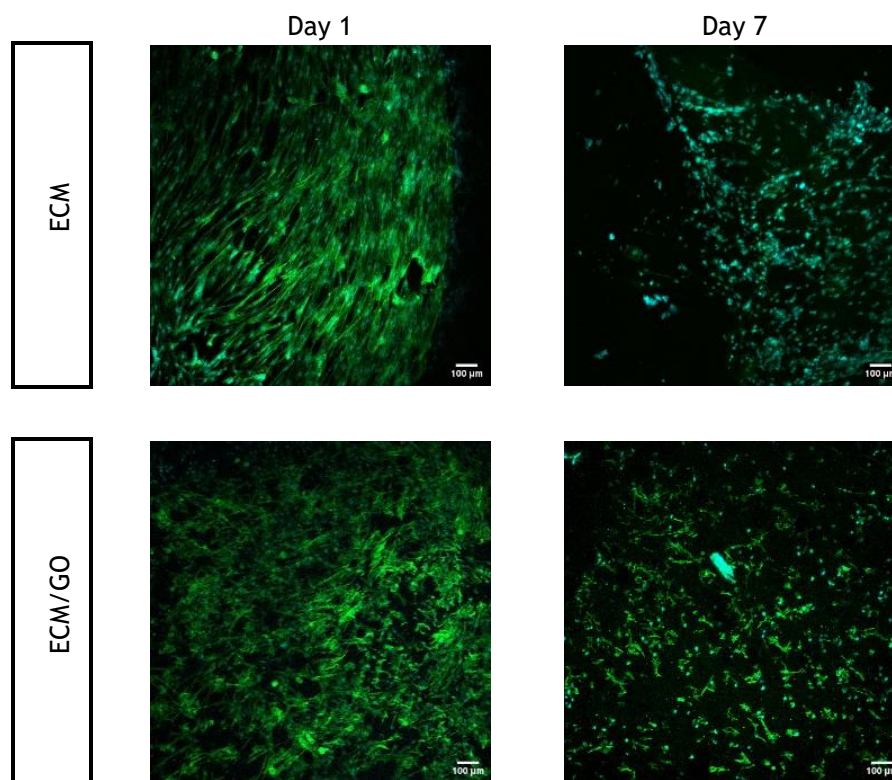


Figure 38 - Confocal images of cells seeding on dECM and dECM/GO films at day 1 and 7. Green fluorescence: phalloidin; cyan fluorescence: DAPI. Scale bar = 100 µm.

dECM and dECM/GO_{autoclave} films were able to adhere fibroblasts (HFF-1), being visible at day 1 a high number of cells on their surface. The observed morphology, with cells spread out evidenced by the phalloidin staining, suggests that cells easily adhere to the dECM proteins by recognizing their binding sites. At day 7, a smaller number of cells are detected in the scaffolds. Cell migration within the film could be the reason for this, and we were unable to acquire

images that fully covered all of the locations where the cells are because we are constrained by the confocal microscope's maximum depth (50 to 300 μm).

Considering these findings, we theorize that the adhesion of the cells is not affected by GO and thus the GO concentration used does not affect the interaction between the cells and the dECM.

Chapter 5

Conclusion and Future Perspectives

5.1 - Conclusion

Following an in-depth assessment of the literature, we were able to recognize the potential of using therapies based on dECM-hydrogels in regeneration of cardiac tissue. Moreover, literature also suggests that 3D printing of dECM hydrogels will pave the way for the creation of complex structures, allowing their successful and boarder application. However, further development on the dECM mechanical properties would be necessary for an effective application. The incorporation of GO in hydrogels showed to be a viable strategy to enhance their mechanical properties while preserving the biological cues. This dissertation's work was motivated by these maxims to provide solutions for an efficient printing of dECM-hydrogels.

In the course of the experimental work, it was possible to find that the homogeneity of dECM/GO composites is highly dependent on the type of GO, being possible to obtain better results when GO-HC is incorporated than GO-WD. Regarding the amount of dECM and GO that should be adopted to achieve a printable ink, our results showed that formulation containing 4% (w/v) of dECM and 25% (w/w) of GO retains the best features to be printed. The mechanical properties, namely G' and G'' , of dECM inks were shown to improve with the incorporation of GO from 304.7 to 1280.0 Pa and 64.9 to 535.2 Pa, respectively. Although the presence of GO resulted in an increase in viscosity, it did not affect the shear-thinning behaviour of the ink.

Regarding the printing platform, it was shown that the printing in air at RT onto a sigmacote treated glass, allows to obtain filaments with improved definition and that can be easily detached from substrate. After the printing, the best strategy to obtain a stable scaffold is by leaving them at 37 °C for 2 hours followed by submersion in water at 37 °C for 24 hours. Using these printing conditions and using a 22 G needle tip with flow speed around 2 mm/s, it was possible to print a 12 mm x 12 mm dECM/GO scaffold with 2 layers and a mesh grid with 2 mm

pore size. Even though the definition still not perfect, the grid shape can be recognized, unlike in scaffolds printed with dECM without GO.

It is important to ensure that the produced scaffolds are sterile since it will be used in biomedical settings. Accordingly with our results, it is possible to conclude that a sterile scaffold can be achieved by combining, under sterile conditions, autoclaved GO with sterile dECM powder, since GO autoclaving did not significantly affect its atomic composition, lateral size, and water dispersibility. Additionally, the printing dECM/GO_{autoclave} did not show significant differences, comparing to non-sterile formulations, while printing with dECM/GO_{ethanol} resulted in brittle and less stable scaffolds, confirming the unsuitable nature of this sterilization technique.

Despite the great insights of this work regarding the 3D printing of dECM, it is important to note that the variability of the dECM hydrogel batches stills poses a challenge because differences in batches have a significant impact on the ink (even in the presence of GO), which reflects extra challenges in establishing a scaffold design, since parameters need to be adjusted.

Biocompatibility assays show a similar profile in number of cells and cellular adhesion for dECM and dECM/GO inks, indicating that GO does not seem to interfere with the biochemical cues present in the hydrogel.

Overall, the presence of GO improved the mechanical and printable properties of the dECM hydrogels, resulting in the production of scaffolds with higher definition. Additionally, the cellular adhesion capacity of the dECM was not changed. As such, dECM/GO inks retain promising features to achieve dECM based hydrogel scaffolds by 3D printing.

5.2 - Future Perspectives

One of the biggest challenges that was encountered was printing scaffold with high-resolution, since the printing filament that we were able to produce was limited by the ink viscosity. To achieve this, we might need to consider the use of support baths. With their use, the viscosity of the material loses some significance, and we remove the hindrance of having a too thin but well homogeneous inks as we reported. A few support baths, like gelatin-based ones [153], can work in our favour since its gelation is thermal like dECM, but happens with opposite temperatures: at 4 °C the gelatin is solid while the dECM is in pre-gel state, and by rising the temperature to 37 °C gelation of dECM is induced, but the gelatin will melt. This behaviour might facilitate the removal of the sacrificial material without damaging the prints. One other strategy to improve our scaffolds is the possibility of functionalization of both the dECM and the GMBs that could promote a further enhancement on the ink's mechanical properties and as such promote the development of scaffolds with higher resolution.

Moreover, the variability of the batches can also be addressed and improved by establishing an optimized protocol for the decellularization of the tissues. However, since the source is

human there is a limit on how much we can equate different batches in terms of chemical/protein composition.

Additionally, considering a future application of this scaffold as a cardiac patch, it might be of interest to provide conductivity to the scaffolds as it has been shown that conductive materials improve cardiac regeneration [159]. GO itself is not conductive, but rGO has been described as a nanomaterial that can increase the conductivity of polymers. One strategy that can be adopted to make these scaffolds conductive can be achieved by reducing the GO present in the dECM/GO inks with a biocompatible reduction agent, such as ascorbic acid [96]. This will avoid a new optimization step for an ink containing dECM/rGO, that will be hard to achieve specially due to the hydrophobic nature of rGO and its low colloidal stability in hydrophilic environments such as dECM hydrogels [147].

Furthermore, due to the well-described antimicrobial properties of graphene-based materials, the antimicrobial potential of dECM/GO based scaffolds should be assessed to establish their potential application for battling infections and microbial propagation, while promoting regeneration. This could be helpful, for instance, in the healing of skin wounds.

In a different perspective, smart biomaterials that can be manipulated to respond to certain stimuli and consequently alter certain physiological properties are being highly explored to create biomimetic systems that simulate the physiological microenvironment [160]. GBMs can conduct electricity and dECM, due to its high content in proteins, can be functionalized and used as a bio-responsive platforms that, together with the GBMs, adapts to the surrounding environment. For example, graphene containing biomaterials have emerged as promising materials for photothermal therapy (PTT). These therapies rely on the capacity of graphene to convert near-infrared (NIR) light to thermal energy causing a raise in temperature that can be used for targeted drug delivery, cancer therapy, tissue engineering, etc, by inducing the thermal gelation of the dECM with the photothermal effect of graphene [161]. Likewise, the capability of these materials to support cell growth and induce differentiation while also sustaining the ability to respond to both internal and external stimuli make them interesting resources for technologies like microfluidics and biosensors. In these cases, the hydrogel can be used as an immobilization material for the graphene while maintaining enough yield to originate soft and flexible platforms. Graphene materials act as the sensors by, for instance, displaying a decrease in conductivity when a specific molecule is linked to it [162].

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Annex A

Table 1 - Hydrogels loaded with graphene produced by extrusion-based bioprinting and classified according to the cross-linking method. Adapted from [129].

Cross-linking method		Hydrogel matrix	Filler	Initiators	Graphene role	Application
Radical polymerization	Chemically induced	PNIPAAm	GO (1.77 wt%)	Initiator: KPS Catalyst: TEMED	Achieve a shape memory hydrogel	Hydrogel actuators (hydrogel with shape memory form upon NIR application)
		Pluronic F127-DMA	GO (0.2 wt%) MWCNTs (2 wt%)	Initiator: APS Catalyst: TEMED	Improve hydrogel mechanical properties	Several applications from robots to tissue engineering
	UV light induced	PNIPAAm	GO (0.44% (w/v)) + laponite nanoclay (6% (w/v))	Photoinitiator: Irgacure 2959 Exposure time = 15 min	Improve ink rheological properties	Biomedical and engineering applications
		PNIPAAm	GO (N.S.) + laponite nanoclay (6% (w/v))	Photoinitiator: Irgacure 2959 Exposure time = 15 min	Achieve a shape memory hydrogel	Drug delivery systems, actuators, and artificial muscles
		Pluronic F127DA/PLGA	GO (0.1% (w/v))	Photoinitiator: DMPA Exposure time = 2 min	Achieve a shape memory hydrogel	Bone repair: shape memory hydrogels
Radical polymerization and ionic interaction		PAAm Sodium alginate	GO (0–0.8 wt%)	Sodium alginate cross-linker: CaCl ₂ PAAm cross-linker: MBA Photoinitiator: Irgacure 2959 Exposure time = 60 min	Improve hydrogel mechanical properties	Tissue engineering
Physical interaction	Ionic interaction	Alginate	GO (0.005–0.025% (w/v))	Cross-linker: CaCl ₂	Improve ink rheological properties	N.S.
		PAA	GO (0.4% (w/v)) + amorphous calcium carbonate (0.2 mM)	—	Improve hydrogel mechanical properties	N.S.
		Collagen type I Chitosan	GO (N.S.)	Cross-linker: CaCl ₂	Drug loading system	Cartilage repair: influencing on Rank/Rank1/OPC pathway. Promoting chondrocyte proliferation/differentiation
	Other physical interactions	PLA	GO (PLA:GO = 9:1–1:9)	—	Achieve thermal distribution	Several applications from architecture to electronic engineering
		PU functionalized with PCL and PDLLA PU	GO (0.024% (w/v)) Graphene in pluronic (0.024% (w/v))	—	Improve hydrogel mechanical and electrical properties; modulate biological response	Neural tissue engineering: promote neuronal stem cells proliferation and differentiation
		PLGA	GO + hydroxyapatite (1:1 HA:GO)	—	Improve hydrogel mechanical and electrical properties	Tissue engineering

Annex B

CALCULATIONS

Example for dECM 4%/GO 25%

GO-WD	
$V_{\text{dECM}} = 0.5\text{mL}$ $[\text{dECM}] = 4\% \text{ (w/v)} = 40 \text{ mg/mL}$ $[\text{GO}] = 25\% \text{ (w/w)} = 10 \text{ mg/mL}$ $[\text{GO-WD}] = 0.4\% \text{ (w/v)} = 4 \text{ mg/mL}$	Calculate the mass of GO in an dECM 4%/GO 25% formulation: $C = \frac{m}{V} \Leftrightarrow m_{\text{GO}} = [\text{GO}] \times V_{\text{GO}} = 10 \times 0.5 = 5 \text{ mg}$ Calculate the volume of GO-WD that has the m_{GO} calculated before: $C = \frac{m}{V} \Leftrightarrow V_{\text{GO-WD}} = \frac{m_{\text{GO}}}{[\text{GO-WD}]} = \frac{5}{4} = 1.25 \text{ mL}$ Calculate the volume of water needed to balance the eppendorf in the centrifuge: <i>Usually, the volume chosen will be the round up of the highest volume solution that needs to be centrifuged.</i> $V_{\text{H}_2\text{O}} = V_{\text{Total}} - V_{\text{GO-WD}} = 2 - 1.25 \text{ mL} = 0,75 \text{ mL}$

Example for dECM 4%/GO 10%

GO-HC	
$V_{\text{dECM}} = 0.5\text{mL}$ $[\text{dECM}] = 4\% \text{ (w/v)} = 40 \text{ mg/mL}$ $[\text{GO}] = 10\% \text{ (w/w)} = 4 \text{ mg/mL}$ $[\text{dECM}]_i = 6\% \text{ (w/v)} = 60 \text{ mg/mL}$ $[\text{GO-HC}] = 2.5\% \text{ (w/v)} = 25 \text{ mg/mL}$	Calculate the mass of GO in an dECM 4%/GO 10% formulation: $C = \frac{m}{V} \Leftrightarrow m_{\text{GO}} = [\text{GO}] \times V_{\text{GO}} = 4 \times 0.5 = 2 \text{ mg}$ Calculate the volume of GO paste that has the m_{GO} calculated before: $C = \frac{m}{V} \Leftrightarrow V_{\text{GO-HC}} = \frac{m_{\text{GO}}}{[\text{GO-HC}]} = \frac{2}{25} = 0.08 \text{ mL}$ Calculate the volume of dECM prepared that is needed to obtain the desired concentration of dECM in the gel: $C_{\text{inicial}} \times V_{\text{inicial}} = C_{\text{final}} \times V_{\text{final}}$ $\Leftrightarrow V_{\text{dECM}_i} = \frac{[\text{dECM}] \times V_{\text{dECM}}}{[\text{dECM}]_i} = \frac{40 \times 0.5}{60} = 0.33 \text{ mL}$ Calculate the volume of water needed to finalize the formula: <i>The water will completely dilute the GO paste and dECM.</i> $\begin{aligned} V_{\text{H}_2\text{O}} &= V_{\text{dECM}} - V_{\text{dECM}_i} - V_{\text{GO-HC}} \\ &= 0.5 - 0.33 - 0.08 \\ &= 0.09 \text{ mL} \end{aligned}$

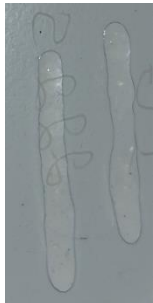
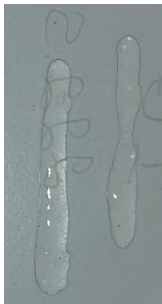
Annex C

dECM 3% vs dECM 4%

- Plastic

		Time (min)	ECM 3%	ECM 4%
PLASTIC		0		
		15		
		20		

- Glass

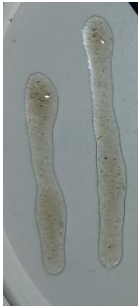
		Time (min)	ECM 3%	ECM 4%
GLASS	0			
	15			
	20			

ECM 3% with Highly Concentrated (HC) GO Suspension (1%, 5%, 10%, 25%)

- Plastic

TIME (MIN)		ECM 3%/GO 1%	ECM 3%/GO 5%	ECM 3%/GO 10%	ECM 3%/GO 25%
PLASTIC	0				
	5				
	10				
	15				

- Glass

Time (min)		ECM 3%/GO 1%	ECM 3%/GO 5%	ECM 3%/GO 10%	ECM 3%/GO 25%
GLASS	0				
	5				
	10				
	15				

ECM 4% with Highly Concentrated (HC) GO Suspension (1%, 5%, 10%,)

- Plastic

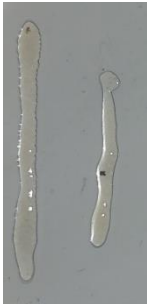
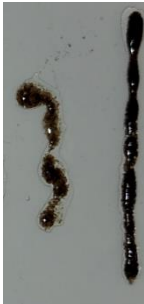
Time (min)		ECM 4%/GO 1%	ECM 4%/GO 5%	ECM 4%/GO 10%
PLASTIC	0			
	5			
	10			
	15			

- Glass

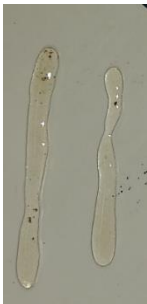
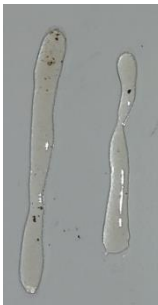
Time (min)		ECM 4%/GO 1%	ECM 4%/GO 5%	ECM 4%/GO 10%
GLASS	0			
	5			
	10			
	15			

ECM 4% with GO Suspension (1%, 5%, 10%, 25%, 50%, 100%)

- Plastic

		Time (min)	ECM 4%/GO 1%	ECM 4%/GO 5%	ECM 4%/GO 10%	ECM 4%/GO 25%	ECM 4%/GO 50%	ECM 4%/GO 100%
PLASTIC	0							
	5							
	10							
	15							

- Glass

TIME (MIN)		ECM 4%/GO 1%	ECM 4%/GO 5%	ECM 4%/GO 10%	ECM 4%/GO 25%	ECM 4%/GO 50%	ECM 4%/GO 100%
GLASS	0						
	5						
	10						
	15						

ECM 3% with Highly Concentrated (HC) GO Suspension (1%, 5%, 10%, 25%)

- Plastic at 37 °C

		GO 1%	GO 5%	GO 10%	GO 25%
GO DISPERSION 25% (GRAPHENE)	3% dECM				
	Few seconds in the bath				

ECM 4% with Highly Concentrated (HC) GO Suspension (1%, 5%, 10%)

- Plastic at 37 °C

		GO 1%	GO 5%	GO 10%
GO DISPERSION 25% (GRAPHENE)	4% dECM			
	Few seconds in the bath			

ECM 4% with GO Suspension (1%, 5%, 10%, 25%, 50%, 100%)

- Plastic at 37 °C

		GO 1%	GO 5%	GO 10%	GO 25%	GO 50%	GO 100%
GO SUSPENSION	4% dECM						
	After printing						
	Few seconds in the bath						

ECM 4% with Highly Concentrated (HC) GO Suspension (1%, 5%, 10%, 25%, 50%, 100%)

- Plastic with Pluronic Coating

		GO 1%	GO 5%	GO 10%	GO 25%	GO 50%	GO 100%
GO SUSPENSION	4% dECM						
	After 15 minutes at 37 °C						

ECM 4% with GO Suspension (1%, 5%, 10%)

- Plastic with Pluronic Coating

		GO 1%	GO 5%	GO 10%
GO SUSPENSION	4% dECM			
	After 15 minutes at 37 °C			

ECM 3% with GO Suspension (1%, 5%, 10%, 25%)

- Plastic with Pluronic Coating

		GO 1%	GO 5%	GO 10%	GO 25%
GO SUSPENSION	3% dECM				
	After printing				
	After 15 minutes at 37 °C				