

Faculdade de Engenharia da Universidade do Porto



**Nanomedicines-based phototherapy with
bioengineered hydrogels for skin cancer treatment**

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WORKING VERSION

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Resumo

Segundo estudos, o cancro da pele não melanoma é o cancro mais frequente no mundo, com mais de 3 milhões de diagnósticos feitos a cada ano nos EUA. Cerca de 80% das malignidades da pele que não o melanoma são carcinomas basocelulares (BCC). Terapias alternativas são necessárias porque as atuais baseadas em cirurgia ou quimioterapia ainda apresentam resultados clínicos desfavoráveis ou efeitos colaterais relacionados. A fototerapia é um tratamento médico baseado na exposição à luz e tem sido estudada como uma abordagem promissora para várias doenças, incluindo cancro da pele. A terapia fototérmica (PTT) é uma forma minimamente invasiva de fototerapia que usa substâncias que podem transformar a luz infravermelha próxima (NIR) em calor. Isso causa a morte das células cancerígenas enquanto causa a menor quantidade de danos aos tecidos saudáveis ao redor. Uma vez que vários nanomateriais de carbono (CNM) apresentam alta absorbância NIR e alta área superficial, estes têm sido investigados como agentes fototerapêuticos. Os materiais à base de grafeno (GBM) destacam-se entre os CNM pelas suas excelentes qualidades óticas e elétricas, bem como pela sua alta biocompatibilidade.

O objetivo principal desta dissertação é utilizar hidrogéis de pectina/GBM como agentes de terapia fototérmica para o tratamento do cancro da pele. Para isso, devem ser atendidos os seguintes objetivos específicos: i) produção e caracterização dos GBM, especificamente óxido de nanografeno (GOn) e óxido de nanografeno parcialmente reduzido (p-rGOn); ii) produção e caracterização de hidrogéis à base de pectina carregados com GBM; iii) avaliação da sua biocompatibilidade e impacto fototerapêutico numa linha celular representativa de cancro da pele e uma linha celular de pele normal, com e sem radiação NIR.

O método modificado de Hummers foi usado para criar o GOn, que foi então circulado por meio de uma sonda de ultrassom de alta potência, enquanto o p-rGOn foi parcialmente foto-reduzido pela luz ultravioleta (UV). As folhas de grafeno obtidas com tamanhos nanométricos são ideais para aplicações biomédicas. Para GOn e p-rGOn, os tamanhos de partículas determinados por análise de imagem de microscopia eletrônica de transmissão (TEM) foram 207 ± 80 nm e 199 ± 81 nm., respetivamente. Ambos os materiais demonstraram excelente estabilidade em água, com leituras de potencial zeta inferiores a -30 mV. A redução parcial de GOn em p-rGOn foi confirmada por espectroscopia no infravermelho por transformada de Fourier (FTIR), termogravimetria (TGA), espectroscopia UV/visível (UV-Vis) e espectroscopia de fotoelétrons excitados por raios X (XPS). Ensaio de irradiação NIR realizados com um sistema de LED personalizado (810 nm, 150 mW/cm², 30 min) demonstraram o efeito fototérmico do p-rGOn, atingindo temperaturas acima de 50 °C para uma dispersão de 500 µg/mL. Como o p-rGOn foi o GBM produzido com maior absorbância NIR, apresentando boa conversão de luz em temperatura e estabilidade térmica superior, foi o material escolhido para produzir hidrogéis de GBM e consequentes estudos *in vitro*.

Os hidrogéis foram reticulados internamente usando uma concentração otimizada de 2,5% de polímero, com 15 mM e 30 mM de CaCO₃ e GDL, respetivamente. Para alcançar a capacidade de impressão, uma solução precursora de hidrogel com 2% de polímero, 25 mM de CaCO₃ e 50 mM de GDL

pode ser usada. Hidrogéis com diferentes concentrações de GBM (0, 0,09 e 0,18% em peso) foram testados por compressão, e o aumento do teor de p-rGOn nos hidrogéis levou a um módulo de Young mais alto em todas as circunstâncias, atingindo um valor máximo de 26,73 kPa. Quando a capacidade de gerar calor a partir da luz NIR dos hidrogéis foi investigada, descobriu-se que os hidrogéis contendo 0,18% em peso de p-rGOn poderiam fazê-lo alcançando uma temperatura máxima de 48,6°C.

A citotoxicidade dos hidrogéis foi investigada por estudos de exposição a extratos, e verificou-se que nem as células HDFn nem as células A-431 variaram substancialmente em termos de viabilidade celular. Isso implicaria que os extratos de hidrogel não são citotóxicos. A citotoxicidade e o efeito fototérmico dos hidrogéis foram avaliados usando um ensaio de contato direto no qual as células foram semeadas na superfície do hidrogel. A adesão celular também foi avaliada. Ambos os hidrogéis de pectina e GBM apresentaram menor viabilidade celular, de acordo com os resultados. Esses resultados podem estar ligados à aderência das células à superfície do hidrogel. Imagens de microscopia de fluorescência demonstraram ainda que a morfologia das células A-431 não foi afetada pela irradiação NIR, hidrogéis de pectina ou hidrogéis GBM. Quando os resultados foram normalizados para o controle positivo (hidrogéis de pectina) foi visto que os hidrogéis de GBM reduziram ligeiramente a viabilidade celular quando expostos à radiação, o que pode indicar que eles têm algum efeito fototérmico. Já as células HFF-1 eram capazes de aderir aos hidrogéis, porém a sua viabilidade celular mostrou-se muito baixa, o que pode ser causado pela alteração de morfologia celular vista aquando da exposição das células a hidrogéis de pectina e hidrogéis GBM.

Trabalhos futuros concentrar-se-ão em otimizar a adesão celular e as doses de irradiação necessárias para alcançar um tratamento seletivo.

Abstract

According to reports, non-melanoma skin cancer is the most frequent cancer in the world, with over 3 million diagnoses made each year in the USA. About 80% of skin malignancies other than melanoma are basal cell carcinomas (BCC). Alternative medicines are required because current therapies based on surgery or chemotherapy still have unfavorable clinical outcomes or related side effects.

Phototherapy is a medical treatment based on light exposure and it has been studied as a promising approach for multiple diseases, including skin cancer. Photothermal therapy (PTT) is a minimally invasive form of phototherapy that employs substances that can turn near-infrared (NIR) light into heat. This causes the death of cancer cells while causing the least amount of damage to the surrounding healthy tissues. Since several carbon nanomaterials (CNM) exhibit high NIR absorbance and high surface area, they have been investigated as phototherapeutic agents. Graphene-based materials (GBM) stand out among CNM for their excellent optical and electrical qualities as well as their high biocompatibility.

This dissertation's major objective is to use pectin/GBM hydrogels as photothermal therapy agents for the treatment of skin cancer. To do this, the following specific goals must be met: i) production and characterization of GBM, specifically nanographene oxide (GOn) and partially reduced nanographene oxide (p-rGOn); ii) production and characterization of pectin-based hydrogels loaded with GBM; iii) assessment of their biocompatibility and phototherapeutic impact on a skin cancer cell line and a normal skin cell line with and without NIR radiation.

The modified Hummers method was used to create GOn, which was then circulated via a custom built high-power ultrasonication probe while p-rGOn was partially photo-reduced by ultraviolet (UV) light. Achieved graphene sheets of nanometric sizes are ideal for biomedical applications. For GOn and p-rGOn, the particle sizes determined by transmission electron microscopy (TEM) image analysis were 207 ± 80 nm and 199 ± 81 nm., respectively. Both materials demonstrated excellent water stability, with zeta potential readings less than -30 mV. The partial reduction of GOn into p-rGOn was confirmed by Fourier-transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), UV-Visible spectroscopy (UV-Vis) and X-ray photoelectron spectroscopy (XPS). NIR irradiation assays performed with a custom-build LED system (810 nm, 150 mW/cm², 30 min) demonstrated the photothermal effect of p-rGOn, achieving temperatures over 50 °C for a dispersion at 500 µg/mL. Since p-rGOn was the produced GBM with higher NIR absorbance, having both good conversion of light to temperature and superior thermal stability, it was the material chosen to produce GBM hydrogels and consequent *in vitro* studies.

Hydrogels were crosslinked internally using an optimized concentration of 2.5% polymer, with 15 mM and 30 mM of CaCO_3 and GDL, respectively. To achieve printability, a hydrogel precursor solution with 2% polymer, 25 mM CaCO_3 and 50 mM GDL could be used. Hydrogels with different concentrations of GBM (0, 0.09 and 0.18 wt.%) were compression tested, and increased p-rGOn content in the hydrogels led to higher Young's modulus under all circumstances, reaching a maximum value of 26.73 kPa. When composite hydrogels' ability to generate heat from NIR light was investigated, it was discovered that hydrogels containing 0.18 wt.% p-rGOn could do so to a maximum temperature of 48.6 °C.

The cytotoxicity of hydrogels was investigated by extract exposure studies, and it was found that neither HDFn nor A-431 cells substantially varied in terms of cell viability. This would imply that the hydrogel extracts are not cytotoxic. The cytotoxicity and photothermal effect of the hydrogels were assessed using a direct contact assay in which cells were seeded on the hydrogel surface. Cell adhesion was also evaluated. Both pectin and GBM hydrogels had lower cell viability, according to the results. These outcomes might be connected to the cells' adherence to the hydrogel's surface. Fluorescence microscopy images further demonstrated that the morphology of A-431 cells was unaffected by NIR irradiation, pectin hydrogels, or GBM hydrogels. When results were normalized for the positive control (pectin hydrogels) it was seen that GBM hydrogels slightly reduced cell viability when exposed to radiation which may indicate they have a some photothermal effect. On the other hand, HFF-1 cells were able to adhere to hydrogels, but their cell viability was very low, which may be caused by the change in cell morphology seen when cells were exposed to pectin hydrogels and GBM hydrogels.

Future work will focus on optimizing the cell adhesion and irradiation doses needed to achieve a selective treatment.

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Contents

Chapter 1: Introduction	1
1.1 Context.....	1
1.2 Motivation.....	5
1.3 Goals.....	6
1.4 Structure.....	6
Chapter 2: Carbon nanomaterials for 3D-Printing of Biomedical Devices	7
2.1 Introduction.....	7
2.2 Carbon Materials.....	7
2.2.1 Carbon Nanofibers	8
2.2.2 Carbon Nanotubes.....	8
2.2.3 Graphene	9
2.2.4 Graphene oxide	9
2.2.5 Reduced graphene oxide	9
2.3 Bioprinting Techniques.....	10
2.3.1 Inkjet-based bioprinting	11
2.3.2 Light-based bioprinting.....	11
2.3.2.1 <i>Laser-assisted bioprinting</i>	11
2.3.2.2 <i>Stereolithography</i>	12
2.3.3 Extrusion-based bioprinting	12
2.4 Applications	13
2.4.1 Scaffolds for tissue engineering	13
2.4.2 Sensors and biosensors.....	29
2.5 Conclusions and Outlook	32
Chapter 3: Materials and Methods	34
3.1 GBM Production.....	34
3.2 GBM Characterization.....	35
3.2.1 Physical Characterization.....	35
3.2.1.1 <i>Transmission electron microscopy (TEM)</i>	35
3.2.1.2 <i>Particle size measurements by dynamic light scattering</i>	35
3.2.1.3 <i>Zeta potential measurements</i>	35
3.2.2 Chemical Characterization.....	36
3.2.2.1 <i>Fourier transform infrared spectroscopy (FTIR)</i>	36
3.2.2.2 <i>Thermogravimetric analysis (TGA)</i>	36
3.2.2.3 <i>Ultraviolet-visible spectroscopy (UV-Vis)</i>	36
3.2.2.4 <i>X-ray Photoelectron Spectroscopy (XPS)</i>	36
3.2.3 Photothermal Properties	37
3.2.3.1 <i>NIR Irradiation</i>	37
3.3 Hydrogels Production	37

3.3.1 Pectin purification	37
3.3.2 Pectin biofunctionalization	38
3.3.3 Preparation of hydrogels	38
3.3.3.1 Pectin hydrogels.....	39
3.3.3.2 Pectin/p-rGOn hydrogels.....	39
3.3.3.3 Pectin/p-rGOn printable hydrogel	39
3.4 Hydrogels Characterization.....	40
3.4.1 Physical Characterization	40
3.4.2 Photothermal Properties	42
3.5 <i>In vitro</i> biological studies.....	43
3.5.1 Cell culture	43
3.5.2 Cytotoxicity of hydrogels.....	43
3.5.2.1 Preparation of hydrogels for <i>in vitro</i> assays.....	43
3.5.2.2 Extract exposure assays	43
3.5.2.2 Direct contact assays	44
3.5.3 Cell Morphology	44
3.6 Statistical Analysis.....	45
Chapter 4: Results.....	46
4.1 GBM Characterization	46
4.1.1 Physical Characterization	46
4.1.2 Chemical characterization	48
4.1.3 Photothermal Properties	52
4.2 Hydrogels Characterization.....	56
4.2.1 Physical Characterization	57
4.2.2 Photothermal Properties	60
4.2.3 Printability	63
4.3 <i>In vitro</i> biological studies.....	65
4.3.1 Extract exposure.....	65
4.3.2 Direct contact – Preliminary tests	66
Chapter 5: Discussion	70
Chapter 6: Conclusions and future work.....	77
Appendix A	87
Supplementary Information for Chapter 4	87
Appendix B.....	92
List of presentations.....	92

List of figures

Figure 2.1 – Representation of the structural characteristics of different carbon materials – graphene, multi-walled carbon nanotubes (MWCNT), single-walled carbon nanotubes (SWCNT), and carbon nanofibers (CNF) and classification according to their dimensions and carbon hybridization. Created with BioRender.com and adapted from [41].	10
Figure 2.2 – Bioprinting techniques: Inkjet-based, Laser-assisted, Stereolithography (A) Top-down system with scanning laser on top (SLA); B) bottom-up system with digital light projection (DLP), and Extrusion-based bioprinting. Adapted from [44, 45].	13
Figure 2.3 – Schematic of the preparation process of electroconductive hydrogels based on alginate/gelatin/CNFs, printed with extrusion-based bioprinting for neuronal TE (from [49]).	14
Figure 2.4 – Diagram of a 3D-printed GelMA-PEGDA-GO scaffold for encouraging hMSC chondrogenic differentiation from for cartilage TE (from [58]).	23
Figure 2.5 – Production of sensors/biosensors from the selected recent studies. A.a) Scheme of the procedure for obtaining the G-PLA 3D-printed working, counter, and reference electrodes; A.b) Scheme of the steps involved in the production of the biosensor, including the mixture of DHP and tyrosinase (from [65]); B) Schematic representation of DET bio-nanoink fabrication processes (from [65]).	32
Figure 3.1 – Production method of nanographene oxide (GOn) and partially reduced nanographene oxide (p-rGOn). Created with Biorender.com.	35
Figure 4.1 – Morphology and lateral dimensions of GOn and p-rGO aqueous dispersions. A, D) Representative TEM images of GOn (A) and p-rGOn (D) at $40 \mu\text{g mL}^{-1}$. Scale bar represents 200 nm. B, E) Cumulative percentage of size distribution of GOn (B) and p-rGO (E). C, F) Size distribution of GOn (C) and p-rGO (F) in percentage.	46
Figure 4.2 – Surface charge of GOn and p-rGOn aqueous dispersions at a concentration of $10 \mu\text{g/mL}$, determined by Zetasizer equipment ($n = 3$). The dashed line indicates the good water stability limit set at - 30 mV.	47
Figure 4.3 – FTIR spectra of GOn and p-rGOn. The lines indicate the contribution of different surface functionalities.	48
Figure 4.4 – UV-Vis absorption curves of GOn and p-rGOn. The inset shows a zoom-in view of the curves in the NIR range (750-850 nm).	49
Figure 4.5 – Thermal decomposition of GOn and p-rGOn. TGA curves and weight loss values for GOn (grey line) and p-rGOn (black line), under a nitrogen atmosphere.	50
Figure 4.6 – XPS analysis of GOn and p-rGOn. A, D) Atomic composition and content of C 1s and O 1s chemical functional groups resulting from spectra fitting of GOn (A) and p-rGOn (D); B, E) Deconvolution of high-resolution C 1s of GOn (B) and p-rGOn (E); C, F) Deconvolution of high-resolution O 1s XPS spectra for GOn (C) and p-rGOn (F).	52
Figure 4.7 – Photothermal heating curves of water, p-rGOn and GOn aqueous dispersions at $250 \mu\text{g/mL}$.	53
Figure 4.8 – Photothermal heating curves of water, p-rGOn and GOn aqueous dispersions at $500 \mu\text{g/mL}$.	54
Figure 4.9 – Thermal camera images and temperature profiles of represented lines of the heating of GBM dispersions. A) Thermal image before irradiation; B) Temperature profile of line	

connecting samples before irradiation; C) Thermal image after 30 minutes of NIR irradiation; D) Temperature profile of line connecting samples after irradiation.	55
Figure 4.10 – Disposition of GBM dispersions and water control in 48 well-plate to be NIR irradiated. From top to bottom: p-rGOn and GOn at a concentration of 250 µg/mL, water, p-rGOn and GOn at a concentration of 500 µg/mL.....	56
Figure 4.11 – Production of cylindrical pectin hydrogels using QGel® 3D disc caster. A) Deposition of pre-crosslinked hydrogel; B) Internally cross-linked produced hydrogels; C) CaCl ₂ bath (external gelation) for hydrogel reinforcement.	57
Figure 4.12 – Compression test using the texture analyzer for A) GBM hydrogel; B) pectin hydrogel; C) pectin incubated in DMEM hydrogel samples.....	57
Figure 4.13 – Effects on Young’s modulus of all conditions. The conditions are hydrogels as prepared (a.p.) with and without CaCl ₂ bath and hydrogels incubated in DMEM overnight with and without CaCl ₂ bath. Statistically significant differences are present as *, p<0.05 ; **, p< 0.01 ; ***, p< 0.001.	59
Figure 4.14 – Photothermal heating curves of DMEM and hydrogels with 0, 0.09 and 0.18 wt.% GBM.	61
Figure 4.15 – Thermal camera image and temperature profile of represented line after 30 minutes of NIR irradiation of hydrogel samples. A) Thermal image of hydrogels and control after 30 minutes of irradiation; B) Temperature profile of line connecting samples after irradiation.	62
Figure 4.16 – Disposition of produced hydrogels and DMEM control in 48 well-plate to be NIR irradiated. From top to bottom: DMEM; pectin hydrogel (0 wt.% GBM); 0.09 wt.% GBM hydrogel and 0.18 wt.% GBM hydrogel.	62
Figure 4.17 – Evaluation of the printability of pectin/GBM composite hydrogels through the modulation of polymer, CaCO ₃ and GDL concentrations.	64
Figure 4.18 – Deposition of hydrogel filaments, with 2% polymer concentration, and 25:50 mM of CaCO ₃ :GDL.	64
Figure 4.19 – Optical images of tested cell lines in culture. A) A-431 cell line; B) HFF-1 cell line. Magnification used was 5x.	65
Figure 4.20 – Cell viability after 24 hours of indirect contact as evaluated by resazurin assay for A) HDFn cells; B) A-431 cells. Results are normalized concerning values of the control incubated in complete DMEM. Results are presented as average and standard deviation. Dashed line marks the toxicity limit at 70 % (ISO10993-5).	66
Figure 4.21 – Cytotoxicity of pectin and p-rGOn hydrogels on A-431 cells, with and without 30 min irradiation. A) Cell viability of A-431 cells, normalized to cell only control; B) Cell viability of A-431 cells, normalized to pectin hydrogel. Statistically significant differences are present as ****, p<0.0001; C) Motorized inverted epifluorescence microscope images (10x magnification) of DAPI/Phalloidin stain of A-431 cells for cell only control, pectin and GBM hydrogels. Scale bar represents 50 µm.....	68
Figure 4.22 – Cytotoxicity of pectin and p-rGOn hydrogels on HFF-1 cells. A) Cell viability of HFF-1 cells, normalized to cell only control; B) Motorized inverted epifluorescence microscope images (10x magnification) of DAPI/Phalloidin stain of HFF-1 cells for cell only control, pectin and GBM hydrogels. Scale bar represents 50 µm.....	69

Figure A.1 – FTIR spectra of GOn and p-rGOn (from batch 027). The lines indicate the contribution of different surface functionalities.....	88
Figure A.2 – UV-Vis absorption curves of GOn and p-rGOn (from batch 027). The inset shows a zoom-in view of the curves in the NIR range (750-850 nm).....	89
Figure A.3 – Thermogravimetric analysis of GOn and p-rGOn (from batch 027) under a nitrogen atmosphere.	90
Figure 4.4 – Photothermal heating curves of water, p-rGOn and GOn (from batch 027) aqueous dispersions at 500 µg/mL.	90
Figure 4.5 – Photothermal heating curves of water control and hydrogels with 0, 0.09 and 0.18 wt.% GBM, using a laser as NIR light source.	91

List of tables

Table 2.1 – Selected recent studies on carbon nanomaterials for 3D printing of scaffolds for tissue engineering.	26
Table 3.1 – Settings used in the software for compression tests.	40
Table 3.2 – Different conditions on hydrogel fabrication to be tested.	42
Table 4.1 – Particle size and polydispersity index of GOn and p-rGOn aqueous dispersions at a concentration of 10 µg/mL, determined by DLS with a Zetasizer equipment (n = 3).	47
Table 4.2 – Polymer and CaCO ₃ :GDL concentrations used in hydrogel production optimization. The highlighted combination was used for further production.	56
Table 4.3 – Average Young’s modulus (E), standard deviation (SD) and average R ² of each tested condition.	58
Table 4.4 – Polymer and CaCO ₃ :GDL concentrations used in hydrogel printability optimization. The highlighted combinations showed potential for future hydrogel bioprinting.	63
Table A.1 – Particle size, polydispersity index and zeta potential of GOn and p-rGOn (from batch 027) aqueous dispersions at a concentration of 30 µg/mL, determined by DLS with a Zetasizer equipment (n = 3).	87

Abbreviations, Acronyms and Symbols

2D	Two-dimensional
3D	Three-dimensional
5-FU	5-fluorouracil
5-Ala	Aminolevulinic acid
ALP	Alkaline phosphatase
AM	Additive manufacturing
BCC	Basal cell carcinoma
BSA	Bovine serum albumin
CH	Chitosan
CM	Carbon materials
CNM	Carbon nanomaterials
CNF	Carbon nanofibers
CNT	Carbon nanotubes
COL	Collagen
CVD	Chemical vapor deposition
DAPI	4',6'-diamino-2-fenil-indol
DCM	Dichloromethane
DET	Direct-electron-transfer
DLP	Digital light processing
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
ECM	Extracelular matrix
IDE	Interdigitated electrode
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GBM	Graphene-based materials
GDL	Glucono delta-lactone
Gel	Gelatin
GelMA	Gelatin methacrylate
GO	Graphene oxide
GOn	Nanographene oxide
HA	Hydroxyapatite

HBSS	Hanks' balanced salt solution
KC	Keratinocyte carcinoma
LAA	L-Ascorbic acid
LAB	Laser-assisted bioprinting
MWCNT	Multi-walled carbon nanotubes
NIR	Near-infrared radiation
nHA	Nanohydroxyapatite
NMSC	Non-melanoma skin cancer
OCN	Osteocalcin
PBS	Phosphate-buffered saline
PCL	Polycaprolactone
PDI	Polydispersity index
PDT	Photodynamic therapy
PEGDA	Poly(ethylene glycol) diacrylate
PFA	Paraformaldehyde
PIC	Polyion complex
PLA	Poly(lactic acid)
PLG	Poly(lactide-co-glycolide)
p-rGOn	Partially reduced nanographene oxide
PpIX	protoporphyrin IX
PS	Photosensitizer
PTT	Photothermal therapy
PURpec	Purified pectin
RAWpec	Raw pectin
RGD	Arginylglycylaspartic acid
RGDpec	RGD-biofunctionalized pectin
rGO	Reduced graphene oxide
rGOn-PEG	PEGylated reduced nanographene oxide
ROS	Reactive oxygen species
RSF	Regenerated silk fibroin
RT	Room temperature
SCC	Squamous cell carcinoma
SLA	Stereolithography
SWCNT	Single-walled carbon nanotubes
TCP	Tricalcium phosphate
TE	Tissue engineering
TEM	Transmission electron spectroscopy
TGA	Thermogravimetric analysis

TPU	Thermoplastic polyurethane
UV	Ultraviolet
XPS	X-ray Photoelectron Spectroscopy

Chapter 1: Introduction

1.1 Context

Skin cancer is the result of abnormal cells of the skin uncontrollably developing. An excessive exposure to UV radiation from sun or from tanning beds is the main risk factor for all skin cancer types since the radiation can cause DNA damage in skin cells, leading to cell mutation and consequent growth [1, 2]. Other possible risk factors are light skin colour, age and hereditary predisposition [2, 3]. Skin cancer accounts for about 40% of all cancer cases [4] and it can be divided into two main categories: non-melanoma skin cancer (NMSC) and melanoma. NMSC, also typically called keratinocyte carcinomas (KC), can be divided into basal cell carcinoma (BCC) and squamous-cell carcinoma (SCC). NMSC have been reported as the most common cancer type worldwide, resulting in about 3 million new cases each year [5]. So many reported cases carry a substantial economic burden, leading to yearly treatment costs of about 4.8 billion dollars, in the United States [6]. About 80% of all NMSC cases correspond to BCC. Typically, the mortality and metastasis rate of BCC is low, but the risk of recurrence is substantial [7, 8]. The treatment assigned depends on the type, location, and progression of the cancer. For BCC, the first line treatment is surgical excision with margin evaluation. Other treatment options are Mohs micrographic surgery, radiation therapy, curettage and electrodesiccation, cryosurgery, photodynamic therapy, and topical chemotherapy with 5-fluorouracil (5-FU) or imiquimod. Treatments like surgical excision, Mohs surgery or curettage and electrodesiccation are invasive, and can cause poor cosmetic and functional outcomes [7]. Even with such a wide variety of possible treatments, the recurrence rates are high, and so are the side effects, therefore, novel approaches for skin cancer treatment must be developed [3, 9].

Phototherapy has been greatly investigated by the scientific community since it has low invasiveness and has reduced side effects. Phototherapy has long been used as a form of treatment, with records dating back as ancient Egypt, where sunlight exposure was used to treat skin conditions like vitiligo [10]. Modern phototherapy, using artificial light sources started in the 70's. There are two main phototherapy modalities: photodynamic therapy (PDT) and photothermal therapy (PTT). PDT relies on the use of photosensitizer (PS) agents, that can be

applied in topical form and get absorbed into the skin. They can also be administered through oral or intravenous route. These PS agents can generate reactive oxygen species (ROS) that can destroy cancer cells when a light source is applied. Some components of the tumour cells, like the membrane, proteins or DNA are harmed by the ROS [11]. Using PDT allows for a selective procedure, since the light source applied can be limited to the area of the cancer, and the ROS will accumulate on the cancer cells only [12]. Because of its low formula weight, 5-Aminolevulinic acid (5-Ala) is one type of modern PS that can penetrate the stratum corneum into the skin. It can be applied topically, and the abnormal cells can absorb it. Through a series of reactions, protoporphyrin IX (PpIX) is generated in the mitochondria. The actual photosensitizer is PpIX, which absorbs light energy and converts it to oxygen to produce ROS that damage nearby organelles such as mitochondria, endoplasmic reticulum, cell membranes, and other macromolecules. Cancerous or abnormal cells experience necrosis and apoptosis as a result of the oxidative damage [13]. Another advantage of PDT is the possibility of performing multiple cycles of treatment with minimal side effects [14]. PTT agents can absorb visible or near-infrared radiation (NIR). These agents will convert the energy from the light into heat, which will cause local temperature increase. Just like PDT, this process is selective since the agents tend to accumulate in the tumour [15]. NIR (700 – 1350 nm) has the advantage of penetrating tissues in a more effective way than visible light (400 – 700 nm) [10, 16, 17], so, together with a PTT agent, it can be used in cancer phototherapy to provoke mild hyperthermia or photothermal ablation. Mild hyperthermia happens when the temperature increases up to 42 – 52 °C, with consequent unrecoverable damages to the tumour cells taking place at 42 °C, like enhanced membrane permeability, cellular uptake, dysfunctional membrane transport and, metabolic suppression [10], [18]. At 46 – 52 °C, cell death occurs because of microvascular thrombosis and ischemia. Photothermal ablation, at temperatures higher than 60 °C, causes protein denaturation and plasma membrane destruction which will lead to instantaneous cell death [10]. The employment of combined phototherapy is also possible, using both PDT and PTT.

Whilst PDT has been approved by the Food and Drug Administration (FDA) for about 40 years [17], for treatment of other skin diseases, only more recently it has been applied to NMSC treatment [10]. The first approved PS for PDT was Photofrin®, but it has low tissue penetration and, is activated by light with low tissue penetration. The next approved PS was Foscan®, with a target application in advanced head and neck SCC. Other PS, like Levulan®, Metviz®, and Radachlorin® are also FDA-approved [19]. The use of PTT agents for cancer treatment is being investigated and reported as promising, but its use is still rather behind PDT [10]. Ideal agents for phototherapy should present high radiation absorbance and need to be able to convert light into ROS and/or heat. The main advantage of phototherapy is that the lesions are selectively destroyed while there is minimal damage to the surrounding healthy tissue. This is the case because PTT agents can be administered in the tumour sites only. Then, the light source can be focused on that

area and intensity and time of exposure to light can be modulated to maximize the efficacy and selectivity of the treatment. Several nanomaterials have the capability of absorbing light and producing ROS and/or raising the temperature.

Carbon nanomaterials (CNM) are recognized by their high absorption capability in the visible light and NIR regions, so their use with lower energy systems for phototherapy is an interesting approach [14]. Graphene-based materials (GBM) have been eyed by scientific community for their potential applications in several areas, namely the biomedical field. Graphene consists of two-dimensional (2D) crystals, comprised by an atom thick sheet of sp^2 hybridized atoms. In this single layer, the carbon atoms are arranged in an hexagonal shape [20, 21]. This material is the elementary structure of graphite and it can be produced in mass quantities, by the exfoliation of graphite in an environmentally-friendly way, which could result in a material with very small lateral dimensions [21–23]. Graphene has an array of functional characteristics that make this material widely investigated and used in biomedical applications, such as its chemical and physical properties, remarkable thermal and electrical conductivity, high surface area and small particle size [21]. Graphene oxide (GO), with its oxygen-containing functional groups, has improved polarity and solubility, allows a wide range of modifications and surface functionalization, and has reduced cytotoxicity [21, 24]. However, the polarity and reactivity of these oxygen groups lessens the optical absorption properties and the thermal stability of GO [23]. By reducing GO, it is possible to restore some of the original properties of graphene, like its thermal and electrical conductivity and its optical absorbance, whilst maintaining some polar groups [24]. Reduced graphene oxide (rGO) can be obtained by methods such as thermal-, chemical- or photo-reduction of GO. By having higher absorption in the NIR region, rGO can be used for PTT, but its hydrophobicity presents a challenge for its use in biomedical applications [16]. An innovative way to have the improved absorption in the NIR regions (to have higher phototherapeutic effect) of rGO, whilst maintain the aqueous stability of GO is the ultimate goal for a GBM to use in PTT.

Hydrogels have long been looked at as a versatile class of materials for a wide range of biomedical purposes, being mostly used as drug delivery systems and scaffolds for tissue regeneration with the ability to promote cell growth and proliferation [25]. Hydrogels are hydrophilic three-dimensional (3D) polymeric structures from either natural or synthetic origin. These crosslinked structures are able to expand and accumulate large quantities of water. In addition, hydrogels can be formed using physical or chemical reactions as well as combinations thereof, leading to the formation of physically-, chemically- or dual-crosslinked gel networks, respectively [25]. Chemical cross-linking of hydrogels happens when there are covalent bonds between the polymer chains, which result in a hydrogel with superior mechanical strength and stability. These often have extended degradation times, requiring the cleavage or degradation of the covalent bonds [27]. When reaction schemes such as ionic interactions and hydrogen bonding

are used, a physically-crosslinked hydrogel is achieved. In this case, the physical interactions between the polymer chains prevent hydrogel dissolution and this cross-linking method is easier to achieve and requires less potentially toxic agents [28]. One advantage of hydrogels is their viscoelasticity, which is soft and tissue-like, and that their mechanical properties and degradation can be tuned, making them ideal candidates in certain biomedical applications. Natural polymers, like alginate, pectin, hyaluronic acid and chitosan, are regarded as biocompatible and have been widely used since they share some resemblances to extracellular matrix (ECM) components and functions. The challenges of natural polymers when compared to synthetic ones, are the weaker mechanical properties and the difficulty to control the batch-to-batch variation [29, 30]. Pectin is a natural and biocompatible polymer that can be tuned in regards to mechanical and rheological properties, as well as cell-interaction [31, 32]. Since in native form pectin does not possess cell-adhesive or protease-cleavage sites, the cell response can be tuned via chemical modification and biofunctionalization. As an example, pectin can be biofunctionalized with a peptide containing the arginyl-glycyl-aspartic acid (RGD) sequence, which is one common motif responsible for cell adhesion to the ECM [33]. To modulate the rheological behaviour of pectin, the addition of controlled concentrations of common divalent or trivalent ions, like Ca^{2+} , Sr^{2+} , or Al^{3+} , can be explored, taking advantage of the inherent ionic gelation ability of pectin [31]. Furthermore, it is possible to add functional groups into the pectin backbone, like methacrylate or norbornene, allowing for UV crosslinking reactions, with the use of photo-initiator, ultimately changing the mechanical properties and stability of resulting hydrogels [32, 33]. Pectin remains a less mechanically strong material, regardless of the modifications, but the incorporation of GBM has been known to improve the mechanical behaviour substantially, whilst granting the possibility of their use in PTT [34].

Additive manufacturing (AM), commonly referred to as 3D-printing, encompasses a group of technologies where 3D objects are fabricated via the deposition of materials in a layer-by-layer fashion. AM technologies have been employed in the biomedical field, having the possibility of creating biomimetic constructs partially mimicking the architecture and features of certain tissues and organs [35]. Bioprinting is a specific branch of AM, where biomaterials, cells and/or bioactive molecules are bioprinted towards the fabrication of tissue and organ analogues with biological function. For bioprinting, the deposited formulation can be a biomaterial ink, without the use of cells, or a bioink, a cell-containing formulation usually made of hydrogel precursors. Despite of the application, each bioprinting technique has a set of properties that the ink must have in order to achieve printability [26]. Several biomaterials have been applied in bioprinting, such as hydrogels, resins, thermoplastic polymers, or a combination thereof, but at times, these biomaterials cannot sustain the assigned biological function and the printability without the addition of another material. CNM have been used to complement the materials used for

bioprinting, since they have the capability of tuning multiple key properties that can make an ink more suitable for a wide range of applications [36].

1.2 Motivation

Non-melanoma skin cancer has a high recurrence rate, and the most common treatments are invasive, and at times result in poor aesthetic outcomes. Alternative treatment approaches, like PTT have been investigated. Graphene-based materials possess unique characteristics, like nanometric sizes, allowing for accumulation in a lesion site, high surface area, making GBM usable for drug-delivery, high NIR absorption, improving the phototherapeutic potential, and biocompatibility [14]. The objective of this dissertation was to develop a biomaterial-based approach based on an improved GBM and pectin and evaluate its properties in PTT for skin cancer treatment.

The improved GBM has higher NIR absorption properties compared to graphene oxide, whilst remaining stable in water. The incorporation of this GBM in pectin hydrogels with LED-based systems for PTT constitutes a novel approach. Multiple works have used GBM for the treatment of cancer with phototherapy [16]. As an example, Costa-Almeida *et al.* developed PEGylated reduced nanographene oxide (rGOn-PEG), with nanometric sizes and high absorption in the NIR region, to reach mild hyperthermia as a NMSC treatment. After 30 min of NIR irradiation, nanographene oxide (GOn) reached a temperature of 34 °C whilst rGOn-PEG had a temperature increase up to 47 °C. When A-431 human epidermoid carcinoma cells were incubated with both GBM, rGOn-PEG elicited superior viability than GOn. After irradiation for 30 min, there was a 38% decrease of cell viability on cells incubated with 250 µg/mL of rGOn-PEG [24]. In other work, Lima-Sousa *et al.* produced injectable graphene-based hydrogels for cancer chemo-photothermal therapy. The hydrogels were of a natural polymeric origin, with chitosan being used due to its antibacterial activity and agarose because of its capability to mediate a thermos-responsive gelation. The thermo-hydrogels were incorporated with modified GO and rGO and when applied to MCF-7 human breast cancer cells and irradiated with NIR for 10 minutes, there was a decrease in cell viability of 73% and 60%, respectively [37].

Abovementioned results have motivated the use of GBM as effective agents for PTT in this thesis. However, the use of hydrogels with GBM for PTT is lacking in the treatment of skin cancer. Also, selectivity of the treatment regarding effectively killing only cancer cells, without affecting healthy ones is yet to be shown on the same study. Moreover, even the possibility of printing GBM-hydrogels to be administered for localized PTT, allowing for selective cancer eradication, while lessening the eventual damages to surrounding healthy tissues is lacking from literature. As briefly mentioned before, there is no current reduced graphene oxide that has water stability without any modification. Therefore, the development of water stable rGO without any

surface modifications would be practical since those modifications could lead to a deterioration of the optical and biological properties.

1.3 Goals

The main goal of this dissertation work was to develop innovative GBM-containing pectin hydrogels that can be 3D-printed for localized photothermal therapy of skin cancer. The main specific goals of this work were:

- Collection of information and organization of a literature review regarding state-of-the-art studies on the use of carbon nanomaterials in 3D-printed biomedical devices.
- Development and characterization of GBM with suitable physicochemical properties and photothermal effect.
- Optimization and production of pectin hydrogels loaded with GBM.
- Optimization of the pectin/GBM hydrogel bioink printability.
- Characterization of the physical and photothermal properties of the produced hydrogels.
- Understanding the effect and phototherapeutic potential of pectin/GBM hydrogels on healthy human skin fibroblasts and on skin cancer cells, in the presence or absence of NIR irradiation.

1.4 Structure

This dissertation is divided in six chapters. Besides this introduction, Chapter 2 reviews the state-of-the-art on carbon nanomaterials in 3D-printed biomedical devices, presenting the major features of the aforementioned CNM, bioprinting techniques, and different applications of such biomedical devices. Chapter 3 presents a description of the materials and methods used in the presented experimental work, whilst Chapter 4 describes the obtained results. Chapter 5 consists of a discussion of the most relevant results, in the light of current literature. Chapter 6 presents the main conclusions withdrawn out from the work performed.

Chapter 2: Carbon nanomaterials for 3D-Printing of Biomedical Devices

2.1 Introduction

Carbon materials (CM) are materials with remarkable properties that have been studied for several biomedical applications throughout the years. Carbon materials have been widely explored as a matrix or additional filler of different polymeric composites. CM have large surface area, outstanding mechanical properties, inherent antimicrobial activity, great electrical and thermal conductivity, and responsive behaviour to a variety of biofunctionalization strategies. All these properties allow for the design of biomaterials that have superior control over cell-material interactions, having unique cell-instructive properties [38]. With this in mind, CM have been used in scaffolds for tissue engineering (TE) to restore, replace or regenerate a tissue or organ [25]. The bioprinting community has been investigating the incorporation of carbon materials into biomaterial inks and bioinks, resulting in materials with improved rheological, mechanical, electrical, biosensing, shape memory and self-healing properties [39]. These properties can be modulated according to what carbon material is used, making them suitable for a wide range of applications [36]. With CM the printability of biomaterial inks can be tuned, whilst also originating constructs with enhanced mechanical properties, electrical and/or thermal conductivity and capability to modulate cell responses.

2.2 Carbon Materials

Carbon is an atom with six electrons, arranged in the $1s^2 2s^2 2p^2$ electronic configuration, with noteworthy properties and high plasticity. These features support the formation of covalent bonds to other atoms, being those carbon or other elements, resulting in more complex structures with different properties.

Carbon-nanomaterials (CNM) have been used for multiple biomedical applications thanks to their unique physico-chemical properties, which allow them to be used for bioapplications, such as phototherapy, drug delivery, or tissue engineering [14, 36, 40]. There is a wide range of carbon

materials, which present very varied properties, for example their dimensions. CNM can be zero-dimensional (0D), such as fullerenes; one-dimensional (1D), for instance, carbon nanotubes (CNT); two-dimensional (2D), like graphene; or three-dimensional, for instance, graphite [20]. In this chapter, mainly 1D and 2D carbon nanomaterials will be addressed, since literature shows that they are used the most in 3D-printing devices with biomedical purposes. Figure 2.1 organizes CNM according to their dimensions, and hybridization.

2.2.1 Carbon Nanofibers

Carbon nanofibers (CNF) are discontinuous 1D materials with sp^2 hybridized carbon atoms in a crystalline form. CNF are highly graphitic, constituted by stacked and curved graphene in cylindrical or conical shape, absent of a hollow cavity [41]. There are different synthesis techniques of CNF, resulting in materials with different carbon structures and morphologies. The main synthesis route of these materials are chemical vapor deposition (CVD), electrospinning, templating, drawing, or phase separation [42]. CNF are highly compatible with most polymer processing techniques, have remarkable mechanical properties, and high electrical and thermal conductivity. The surface state of CNF encourages functionalization or other surface modifications in order to adapt the nanofibers to the desired application. Their general application areas are tissue engineering, wound healing, electronic and optical nanodevices and sensors.

2.2.2 Carbon Nanotubes

As briefly mentioned, carbon nanotubes are 1D materials, mostly composed by sp^2 hybridized carbon atoms in a hexagonal lattice crystalline structure. CNT are hollow cylinders with diameters varying from 1 to 100 nm. These CNM can be single-walled carbon nanotubes (SWCNT), having just one layer of carbon atoms in the wall, or multi-walled carbon nanotubes (MWCNT), with two or more layers [20]. CNT have several possible production methods, like catalytic chemical vapour deposition, catalytic plasma-enhanced CVD, template-based CVD, laser ablation, or electric arc discharge. They can be several micrometres long, with low diameters, presenting a high aspect ratio. CNT are renowned for their mechanically strong properties, high electrical and thermal conductivity, and unique optical properties. Varying their dimensions, number of walls and chirality, their properties can be modulated. CNT are hydrophobic, so in order to improve dispersibility in the body and improve biological properties, they can be modified. Because of their shape, amphiphilic surface upon functionalization and possible different sizes, CNT can navigate through biological barriers or membranes [43].

2.2.3 Graphene

Graphene is a 2D material, with a crystalline form of carbon containing sp^2 hybridized atoms. This material is one atom thick sheet, composed by hexagons, being the basic building block of carbon materials that are hexagonally bonded. It possesses a 6-ring honeycomb lattice structure, where every atom is bonded to three others [41]. Graphene is the elementary structure of graphite, and it presents high specific surface area, remarkable mechanical, electrical, thermal and optical properties, which allow them to be such an important material in nanotechnology [23]. Graphene can be originated by top-down approaches, with graphite as the base material, or by bottom-up approaches, using alternative carbon sources. One major disadvantage of graphene in biomedical applications is its hydrophobicity, which makes it so difficult to disperse in aqueous medium [16].

2.2.4 Graphene oxide

To overcome the difficulties of graphene use in the biomedical field, graphene derivatives, usually named graphene-based materials (GBM) have been produced. GBM can be oxidized, resulting in graphene oxide (GO). GO is similar to graphene, but with the introduction of oxygen-containing functional groups, like carboxy and hydroxyl groups [16]. GBM oxidation disrupts the aromatic system, and the polarity and reactivity of oxygen-containing functional groups all lead to a decrease in conductivity, thermal stability, and optical properties. However, due to the increased number of oxygen-containing groups in GO, this material presents high hydrophilicity, being more stable in aqueous solutions and more versatile for surface functionalization, presenting good biocompatibility [16].

2.2.5 Reduced graphene oxide

The aromatic system can be partially re-established by chemical, thermal, or electrochemical reduction of GO, resulting in reduced graphene oxide (rGO). rGO is a derivative of GO, containing less oxygen-containing groups. With the restoration of the aromatic system, there is a restoration of the optical absorbance, and electrical and thermal conductivity. Notwithstanding, rGO is a more hydrophobic material, with less aqueous stability than GO. To further improve it for biomedical applications, additional surface functionalization or using stabilizing agents are common strategies [16].

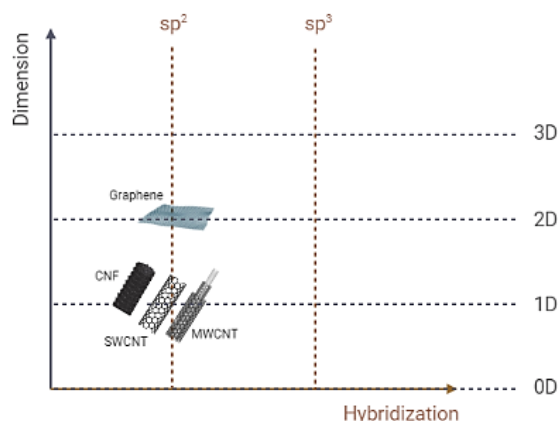


Figure 2.1 – Representation of the structural characteristics of different carbon materials – graphene, multi-walled carbon nanotubes (MWCNT), single-walled carbon nanotubes (SWCNT), and carbon nanofibers (CNF) and classification according to their dimensions and carbon hybridization. Created with BioRender.com and adapted from [41].

2.3 Bioprinting Techniques

Additive manufacturing (AM), commonly referred to as 3D-printing, encompasses a group of technologies where 3D objects are fabricated via the deposition of materials in a layer-by-layer fashion. AM technologies have been employed in the biomedical field, having the possibility of, for example, creating biomimetic constructs partially mimicking the architecture and features of certain tissues and organs [35]. Bioprinting is a specific branch of AM, where biomaterials, cells and/or bioactive molecules are deposited towards the fabrication of tissue and organ analogues with biological function. For this purpose, bioprinting can use either a bioink, which comprises a printable formulation containing cells eventually combined with materials and bioactive molecules, or a biomaterial ink, in which a printed cell-free material has the capacity to modulate the behaviour of seeded cells.

When bioinks are used, the requirements are even more complex since the chosen cells must agree with the chosen material.

Different bioprinting techniques have their own printability requirements. To achieve printability, there is a set of specific rheological properties that must be achieved [26]. Biomaterial inks and bioinks with suitable printability and biological function must be researched. Several biomaterials have been applied in bioprinting, like hydrogels, bioresins, thermoplastic polymers, or a combination thereof, but at times, these biomaterials cannot sustain the assigned biological function and the printability without the addition of another material. CNM have been used to improve the properties of materials used for bioprinting, since they have the capability of tuning multiple properties that can make an ink more desirable for a wide range of applications [36].

Regardless of the goal, the major 3D bioprinting techniques belong to three major categories, being inkjet-, light-, and extrusion-based bioprinting (Figure 2.2).

2.3.1 Inkjet-based bioprinting

With inkjet bioprinting, small droplets of a material are deposited onto a substrate with micrometer resolution, following a droplet-on-demand technology [44]. The 1-100 pL droplets have an average diameter of 10 – 50 μm and are formed by thermal or piezoelectric effect. These droplets are then ejected through a small orifice. Thermal inkjet uses a heating element, that will cause the vaporization of a small ink volume, that will consequently be ejected out of the printing head as a small droplet. The high temperatures reach by vaporization (300 $^{\circ}\text{C}$) do not tend to affect cell viability in bioinks as the bioink temperature usually increases only a few degrees for very short time periods [45]. In piezoelectric inkjet, the mechanical deformation of a piezoelectric transducer due to external voltage application, allows the ejection of small droplets of biomaterial ink/bioink [44].

The main advantages of inkjet-based bioprinting are its low cost, the high printing speed, and the high cellular viability in the printing process. Nonetheless, some setbacks still need to be overcome. The orifice of the printing head is prone to clogging, so the printable materials must have shear thinning behaviour, low viscosity (1 - 10 mPa.s) and limited cell density (<106 cells/mL) [44, 46]. With this technique it is challenging to obtain large and complex structures.

2.3.2 Light-based bioprinting

Light-based bioprinting techniques do not require an orifice for ink ejection, using laser or other light source systems to assist on the deposition of the ink. These techniques also allow for the selective photocrosslinking of photosensitive biomaterials termed as bioresins.

2.3.2.1 *Laser-assisted bioprinting*

With laser-assisted bioprinting (LAB), a high-energetic, fast-pulse laser beam (usually a NIR laser) is used to eject a bioink from a glass to a receiving substrate. This technique has a donor layer, fabricated in an energy-absorbing material, like gold or titanium, coated in the bioink. When the energy-absorbing layer gets irradiated by the laser, a small amount of the donor layer is vaporized, so the energy gets transferred to the bioink at the bottom of the layer. The energy transfer generates a high-pressure bubble that will result in the ejection of a small bioink droplet onto a hydrogel-coated substrate for subsequent crosslinking. Since this is an orifice-free technique, it can bioprint heterogeneous tissue constructs with high cell densities (10^8 cells/mL).

and with high resolution (1-100 μm) without clogging. For LAB, the bioink viscosity should range between 1 and 300 mPa.s [44].

2.3.2.2 Stereolithography

The stereolithography (SLA) technique uses a light source (visible, NIR, UV) to selectively photopolymerize a bioresin. The bioresin can be cell-laden, with the fabrication of constructs via chain-growth or step-growth mechanisms [47]. SLA is an orifice-free, fast technique with high resolution ($<100\ \mu\text{m}$). Since SLA bioprinting tends to use UV as the light source, it can induce cell damage during the printing process [45]. Two separate irradiation methods can be used in SLA: (1) laser writing, where a focused laser is used to trigger the selective photocrosslinking of the bioresin; and (2) mask-based method or digital light processing (DLP), which utilizes a digital mirror device that will map the regions were to project light onto photopolymerized materials, crosslinking the entire layer at a time as opposed to just one point as happens in laser writing method [48]. DLP bioprinting is similar to SLA, but has a higher print speed, especially when printing larger objects. However, because SLA is constrained by the use of digital light mirrors, it has a greater printable area and can therefore print smaller objects [45].

2.3.3 Extrusion-based bioprinting

The most common bioprinting technique is extrusion-based printing. This technology is so used due to its relatively low cost, availability, and versatility. Typically, these systems use multiple disposable medical grade syringes or reservoirs, loaded with the biomaterial ink or bioink [44]. These bioinks/biomaterial inks are then deposited through a thin nozzle, forming a filament that is collected in a platform or receiving substrate. The extrusion can be assisted by pneumatic, piston-, or screw- driven systems. In extrusion-based printing, the pressure of the extrusion process can be controlled [45]. Piston-driven systems can provide improved control over the material deposition, but screw-based systems provide higher spatial control and have the capacity to extrude bioinks with higher viscosities ($6 - 30 \times 10^7\ \text{mPa.s}$)[48]. Using extrusion-based bioprinting is prone to cellular death due to compression forces and shear stresses generated during the printing process, which requires optimization of bioink properties and processing parameters. Compared to other technologies, extrusion bioprinting has lower resolution ($>100\ \mu\text{m}$) and low printing speed due to the size of the needles used for extrusion [45].

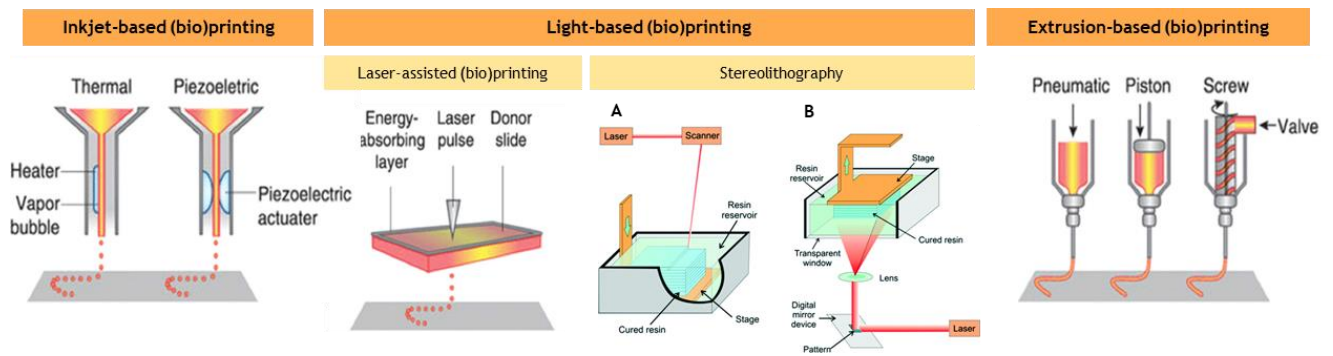


Figure 2.2 – Bioprinting techniques: Inkjet-based, Laser-assisted, Stereolithography (A) Top-down system with scanning laser on top (SLA); B) bottom-up system with digital light projection (DLP), and Extrusion-based bioprinting. Adapted from [44, 45].

2.4 Applications

2.4.1 Scaffolds for tissue engineering

The main application of 3D-printed devices with carbon materials in their matrix is scaffolds for tissue engineering. In table 2.1, the selected recent studies on carbon nanomaterials for 3D printing of scaffolds for tissue engineering are present.

A. Serafin *et al.* produced a novel electroconductive hydrogel based on alginate/gelatin with different concentrations of CNFs and characterized said hydrogels in terms of mechanical, structural, electrical, viscoelastic and cell biocompatibility. Hydrogels were prepared with 5% w/v of gelatin and alginate with concentrations of CNF varying between 0 and 5%. Stable CNF Hydrogels were printed through extrusion-based printing, at a speed of 4 mm/s, resulting in structures with two 2 mm layers, with a total height of 4 mm and width of 9 mm (Figure 2.3). NIH/3T3 fibroblasts were seeded onto the hydrogels and were allowed to proliferate during 96h. To evaluate the mechanical properties, compressive tests were performed, obtaining an increased Young's modulus for hydrogels with the highest CNF content (534.75 ± 0.68 kPa). One of the main aims in using CNFs was the improvement of electroconductive properties, which was achieved. Hydrogels without CNFs exhibited electrical conductivity of just $5.6 \times 10^{-5} \pm 3 \times 10^{-6}$ S/cm. Similar values were obtained for hydrogels with CNF concentrations of 0.5, 1 and 2% w/v, but for hydrogels with 5% w/v of CNFs, there is an increase in electrical conductivity to $4.1 \times 10^{-4} \pm 2 \times 10^{-5}$ S/cm. The material showed shear thinning behaviour, important for 3D printing, with increased viscosity for samples with higher CNF concentration. Hydrogels with 5% w/v CNFs were too viscous, so no scaffolds could be printed with those hydrogels. After 24h of proliferation of NIH/3T3 cells on the scaffolds, hydrogels without CNFs had the highest spike in proliferation ($75.96 \pm 36.03\%$), whilst all CNF hydrogels had moderate proliferation (between $30.49 \pm 5.3\%$ and $47.12 \pm 15.86\%$). At the 48h time point, there was a decrease in proliferation in all groups, but at the 96h time point it increased again, suggesting that the cells were adjusting to the presence

of the hydrogel. At 96h, proliferation was highest for hydrogels without CNFs, with $110.43 \pm 56.5\%$, followed by hydrogels with 0.5 and 5% w/v ($82.83 \pm 23.9\%$ and $75.6 \pm 25.1\%$, respectively). The control (cells without hydrogel) showed the smallest numbers in cell proliferation. After 96h, cell viability was analysed, with values over 88% for all groups, proving the biocompatibility of CNF hydrogels. These hydrogels have potential for a lot of TE applications, particularly those with interest in electroconductive materials, such as cardiac or neuronal TE [49].

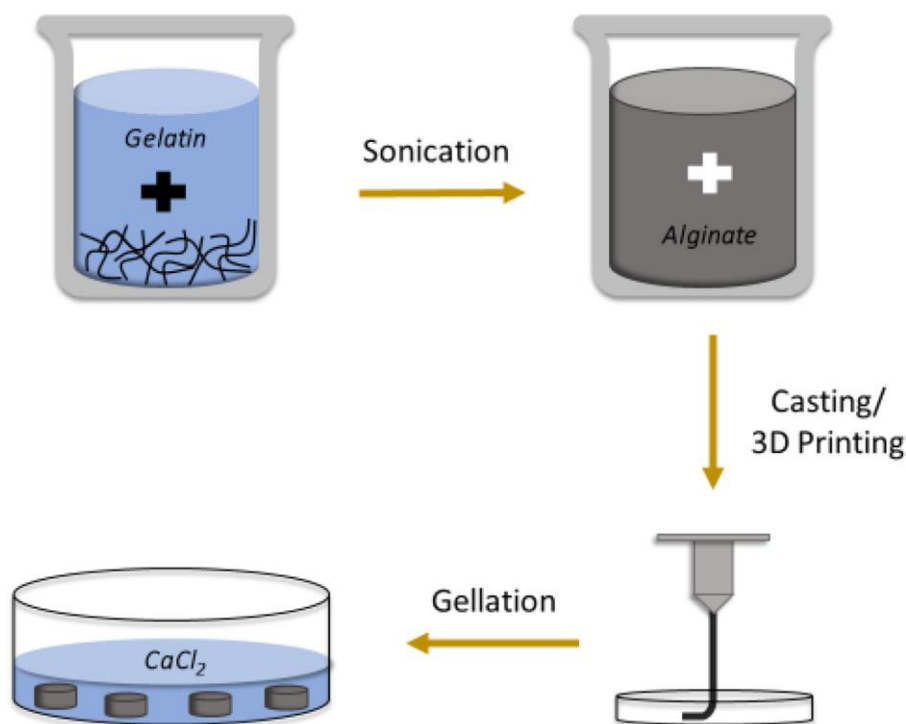


Figure 2.3 – Schematic of the preparation process of electroconductive hydrogels based on alginate/gelatin/CNFs, printed with extrusion-based bioprinting for neuronal TE (from [49]).

B. Huang, *et al.* produced 3D-printed porous scaffolds with aligned multi-walled carbon nanotubes and nanohydroxyapatite (nHA) in a polycaprolactone (PCL) matrix. Scaffolds were characterized morphological-, mechanical- and biologically. MWCNTs were fabricated with a 20 nm diameter and 10 μm length, through a CVD method. Three compositions were considered: PCL/MWCNT (99.25/0.75 wt.%), PCL/HA (80/20 wt.%), and PCL/HA/MWCNT (79.25/20/0.75 wt.%). Scaffolds were produced by a screw-assisted extrusion-based process, with air pressure of 6 bar, melting temperature of 90 $^{\circ}\text{C}$, deposition velocity of 20 mm/s and screw rotational velocity of 12 rpm. Scaffolds had a 0/90 $^{\circ}$ lay-down pattern, 330 μm filament diameter, and 350 μm pore size. By compressive tests, it could be seen that PCL/MWCNTs (about 80 MPa) show a smaller compressive modulus than PCL/HA (about 85 MPa) but still higher than PCL scaffolds (about 55 MPa). The addition of MWCNTs causes a slight increase in the yield strength of the scaffold. hADSCs, commonly used multipotent mesenchymal stem cells were seeded onto the scaffolds and at day 7 there is higher fluorescence intensities for PCL/HA, PCL/MWCNTs and

PCL/HA/MWCNTs, proving that cells proliferate faster in the scaffolds. The highest value is obtained for PCL/HA/MWCNTs scaffolds. By the quantification of alkaline phosphatase (ALP), it was seen that PCL/MWCNTs scaffolds show higher normalised ALP values compared to other scaffolds at day 7, suggesting that the presence of MWCNTs improves cell differentiation. HA containing scaffolds show lower values for ALP, indicating that HA might inhibit ALP secretion. On day 14, all scaffolds show high ALP values, showing that hADSCs are differentiating towards an osteogenic lineage. Collagen (COL) was quantified at days 14, 21 and 28 as a measure of mid-late stage of osteogenesis differentiation. At day 14, PCL/HA/MWCNTs present statistically higher values of COL. The same trend is observed for days 21 and 28. Osteocalcin (OCN) production is measured at days 1, 7, 14, and 28. At day 28, PCL/HA/MWCNTs and PCL/HA shows statistically higher values than just PCL scaffolds. These results suggest that, with time, the hADSCs are differentiating into osteoblasts. Results show that HA presence promotes OCN secretion from cells, and the combination with MWCNTs further enhance it. Analysing the mineralisation process, it could be mostly attributed to the addition of HA. At day 7, cells are producing calcium, contributing to the mineralisation of scaffolds. Removing scaffold calcium content and self-mineralisation effect showed that PCL and PCL/MWCNTs scaffolds had higher ARS values in comparison to PCL/HA and PCL/HA/MWCNTs scaffolds. This proves that MWCNTs stimulate the differentiated cells to induce mineralisation of the scaffold. MWCNTs bundles proved to act as nucleating sites, allowing the recrystallisation of PCL chains in the wall of the CNTs. These scaffolds presented analogue nanostructure to natural bone, showing their potential in bone regeneration [50].

E. M. Gonçalves, *et al.* mixed silicon-doped nanocrystalline HA, CNTs and PCL to produce a 3D printed scaffold with bioactivity, biocompatibility, porosity, pore size and mechanical properties compatible with bone tissue, as well as electrical conductivity for inducing the healing process of bone. The used HA is a ceramic with chemical composition comparable to the inorganic part of bone, achieving better biological responses than conventional HA. The composite scaffolds were characterized in a compositional, structural, morphological, and physical properties, as well as mechanical behavior, bioactivity and cell attachment. Scaffolds were prepared with 50 wt.% PCL, and CNT concentrations of 0, 0.75, 2, 4.5, and 10 wt.%. HA concentration was adapted accordingly to the CNT concentration. Extrusion-based printing was used to obtain scaffolds, using a 0.45 mm diameter nozzle. The produced scaffolds had seven layers, with 6 mm diameter and 3 mm height. The 10CNT scaffolds showed pore sizes around 21.5 μm , with BET specific surface area of m^2/g . The 0CNT scaffolds showed high electrical resistivity, similar to insulating materials, whilst scaffolds with small CNT content already showed electrical behavior. Scaffolds with low CNT content (0, 0.75%) showed higher compressive resistance, whilst 10CNT scaffolds would deform easier. The compressive strength of composites is in agreement with trabecular bone values (2 to 12 MPa). Experiments proved that the materials could develop an apatite layer on the surface, which is relevant for future

bonding ability to host bone and conditions for attachment of osteoblasts to the scaffolds. After 7 days, there was a reduction in Ca^{2+} and P^{4+} concentrations, however scaffolds without CNT showed slower a decrease, resulting in a formation of an apatitic layer on the surface of the material. MG63 osteoblast-like cells were seeded onto scaffolds, and, for all compositions, cells could attach to the surface. Cell adhesion proved more significant in 10CNT scaffolds. By the 6th day, the 10CNT scaffolds was already covered by a cellular layer, confirming that the higher the CNT amount, the better the cell adhesion and spreading. These scaffolds should allow for the application of electrical stimuli for bone healing applications [51].

H. Cui *et al.* created a hybrid hydrogel that combined tough polyion complex (PIC) hydrogel with MWCNTs, obtaining a material with good mechanical properties, as well as great biocompatibility, stimulating the osteogenic differentiation and the proliferation of bone marrow mesenchymal stem cells (BMSCs). The PIC/MWCNT precursor solution was prepared with a 0.3% (w/v) of MWCNT in PIC. 3D constructs of PIC/MWCNT hydrogels were created using an extrusion-based method, where the PIC/MWCNT precursor solution was loaded into a needle with 311 μm inner diameter and maintaining a homogenous printing temperature of 25 °C. The solutions were extruded pneumatically into deionized water with a pressure between 0.3 and 0.5 MPa, causing the precursor solution to rapidly gelate into solid hydrogels. Then, PIC and PIC/MWCNT hydrogel single and cross-bonded fibers were subjected to tensile tests, resulting in a 4.47 ± 0.50 MPa Young's modulus, 1.69 ± 0.11 MPa tensile strength, and $475 \pm 19\%$ breaking strain for the single strains of PIC/MWCNT, which are very close to the mechanical properties of the single fibers of PIC. The strain-stress curve of the cross-bonded fibers was also similar to the curve of single fibers, showing that they have similar properties. More precisely, the cross-bonded fibers sustained more than 450% strain and had a breaking strength above 1.6 MPa, which signifies that the bonded PIC/MWCNT fibers have enough interfacial strength to keep the mechanical properties of the scaffolds. Compressive tests were also done with PIC and PIC/MWCNT matrixes, showing that both scaffolds did not break and recovered their initial state when the compressive strain reached 90%. Live/dead assays and confocal laser scanning microscope images with the PIC and PIC/MWCNT scaffolds showed that both matrixes, especially the PIC/MWCNT, were cytocompatible and promoted the adhesion of the BMSCs. It was also shown using cell counting kit-8 reagent (CCK-8) that although BMSCs proliferated in both PIC and PIC/MWCNT scaffolds, the BMSCs grew faster on the PIC/MWCNT scaffolds than on PIC scaffolds after incubating for 3, 5 and 7 days. The calcium deposition of BMSCs and the expression of osteogenesis-related genes was also upregulated when the BMSCs were cultured with the PIC/MWCNT, showing that the PIC/MWCNT hybrid promotes osteogenic differentiation of the BMSCs. In vivo tests with rats with calvarial bone defect were also done, where microcomputed tomography showed that both PIC and PIC/MWCNT scaffolds promoted bone regeneration, but the PIC/MWCNT caused a more effective and faster bone repair, showing higher bone volume / total volume ratio and bone mineral density than the PIC group from 2 to 8

weeks. PIC/MWCNT also showed better osteoconductivity due to the large amount of mineralized tissue development and cell infiltration inside the scaffolds. Finally, CD31 immunohistochemical staining was used, showing that both PIC and PIC/MWCNT scaffolds promoted angiogenesis, but the PIC/MWCNT group had a higher neovascularization. The obtained results proved the potential of using PIC/MWCNT for bone tissue engineering [52].

C. M. B. Ho *et al.* fabricated different PCL and PCL-CNT scaffolds with 3D printing, studying the mechanical, thermal, and biological properties of the obtained scaffolds. The PCL solution contained a PCL/chloroform ratio of 8% (w/v) and was compared with 3 different compositions for the PCL-CNT composite: PCL-CNT-1, PCL-CNT-3, and PCL-CNT-5 corresponding to 1%, 3%, and 5% CNT, respectively. When it came to the 3D printing, the components were charged into 5 mL syringes connected to a gauge 21 nozzle tip. The pressure was initially set to 1 bar for the extrusion of the PCL and feed rates of 800, 1000, and 1200 were used while keeping the other parameters constant, resulting in the discovery of the optimised conditions for the different feed rates, while maintaining constant needle size, temperature, and pressure. After printing the scaffolds, it was possible to see that increasing the percentage of CNT made the scaffold structure more defined, having strips with smaller width. Differential scanning calorimetry showed that the heat of fusion increased with the percentage of CNT (from 73.4 J/g in PCL to 79.6 J/g in PCL-CNT-5). The crystallization temperature also increased with the percentage of CNT (from 25 °C in PCL to 35 °C in PCL-CNT-5), showing the restricted mobility of the polymer chain in the PCL-CNT composites. Nanoindentation tests showed elastic behaviour both in PCL and PCL-CNT, although a lower indentation depth with the higher peak load was shown for higher percentages of CNT, possibly due to increase in strength caused by the CNT reinforcement. An increased average load and maximum peak load with the increase in CNT percentage also highlighted the reinforcement effect of the CNT. CNT content also resulted in a bigger Young Modulus, with the PCL-CNT-5 having a value 70% higher than PCL. The hardness also increased with the CNT percentage (from 0.057 GPa in PCL to 0.072 GPa in PCL-CNT-5). Furthermore, the conductivity became bigger with the percentage of CNT, having an increase of nine orders of magnitude from PCL to PCL-CNT-5. MTT assay was performed to test the compatibility and toxicity of H9c2 mouse myoblast cells on PCL and PCL-CNT. This test showed that these cells were viable in both PCL and PCL-CNT. Although cells adhered and were growing in all the scaffolds, the PCL-CNT-1 was the one where there was more cell proliferation. LIVE/DEAD assays also proved that the cells were growing in all the scaffolds. In conclusion, the PCL-CNT-1 had the best properties to allow the proliferation of H9c2 cells. PCL and its CNT composites have the potential to be employed in cardiac tissue engineering because they can demonstrate cell proliferation [53].

F. Dolati *et al.* intended to print vascular conduits where carbon nanotubes were used to strengthen conduits in order to improve their mechanical characteristics and suitability for bioprinting. For the first four composite samples, MWCNT concentrations of 1% (w/v) and 0.5%

(w/v) were dissolved in alginate solutions of 3% and 4% concentrations, and each sample was placed on a stirrer for a day to ensure that the CNTs were dissolved uniformly. Additionally, two samples of alginate alone at 3% and 4% (w/v) concentrations were created. Using extrusion-based bioprinting, with CaCl_2 solution as the crosslinker, the conduits were printed. The inner tube was used to supply the crosslinker solution while the feed tube fed the composite solution. The coaxial assembly employed in this investigation included an exterior needle that was 14 gauge (1600 μm (I.D.), 2110 μm (O.D.)) and an inner needle that was 25 gauge (260 μm I.D., 510 μm O.D.). For 4% and 3% plain alginate, respectively, the CaCl_2 solution was dispensed at a rate of 16 ml/min and 12 ml/min, respectively. For 4% and 3% alginate, respectively, the dispensing pressure for the composite solution was 20.7 kPa and 13.8 kPa. The average inner and outer diameters of the fabricated vascular conduits were $461 \pm 68 \mu\text{m}$ and $930 \pm 43 \mu\text{m}$, respectively. Vascular conduits that were more than a meter long and capable of successful perfusion were created, proving their usefulness. Through printing, conduits may be produced in any desired length and pattern without causing any blockage or rupture that led to a leak or burst. The elastic modulus of the reinforced alginate with varied MWCNT concentrations for 4% did not significantly differ from that of the plain alginate, although there was a significant difference between them. In comparison to samples with lower concentrations of these ingredients, samples with higher concentrations of alginate and MWCNT showed greater tensile strength, elastic modulus, and lower ultimate strain. Because there were fewer polymer chain entanglements in the lower concentration alginate solutions, the integration of MWCNT probably had a greater impact on the conduits' mechanical characteristics. CNT reinforcement increased the ultimate tensile stress by 11% and the modulus of elasticity by 94%. It decreased the ultimate strain by 18%. The printing process caused some cell damage. Compared with the initial control, cell viability was decreased from $75.6 \pm 0.04\%$ to $53.3 \pm 0.01\%$ for plain alginate vascular conduits, and from $72.2 \pm 0.02\%$ to $53.9 \pm 0.01\%$ for MWCNT-reinforced upon printing. However, following printing, cells were able to repair damage sustained during *in vitro* culture. Due to the study's sparse use of MWCNT, reinforcing didn't have an impact on the survival of human coronary artery smooth muscle cells (HCASMC) in a short-term *in vitro* culture. The tissue histology study revealed distinct traits between CNT-reinforced conduits and CNT-free (positive control group). Most of the cells in CNT-free conduits were undamaged and had spherical nuclei that were evenly distributed across the conduit wall, whilst in CNT-reinforced conduits, damaged cells can be identified by their nuclei. A few cells moved to the farthest part of the conduit wall and multiplied, generating a well-aligned cellular layer with significant ECM production in CNT-free conduits, but there was essentially no ECM development seen in CNT-reinforced conduits. This study lays the groundwork for direct printing of mechanically reinforced vascular conduits, in which natural polymer nanofibers might take the place of CNTs for other applications including scaled-up tissue creation or blood vessel development [54].

W. Wang *et al.* developed poly(ϵ -caprolactone)(PCL)/graphene scaffolds for bone tissue engineering. Graphene nanosheets were produced and melt blending was used to create PCL/graphene pellets with three distinct graphene concentrations (0.25, 0.50, and 0.75 wt%). After blending, it was found that the graphene concentrations are 0.13, 0.50, and 0.78 wt%. The scaffolds were created using an additive manufacturing process based on extrusion. To create pores with an uniform geometry, a constant filament diameter of 330 μ m, and a filament distance of 680 μ m, the 0°/90° lay-down pattern was used. The best set of processing parameters for creating scaffolds with similar geometric properties to the ones that were intended were 90 °C for melting, 220 μ m for slice thickness, 22 rpm for screw rotation, and 20 mm/s for deposition. The manufacturing process was carried out at room temperature. It was possible to see that protein might be absorbed by all scaffolds, and that the amount of protein adsorption grows over time. In comparison to PCL scaffolds, PCL/graphene scaffolds (0.50 and 0.78 wt%) demonstrate statistically increased protein adsorption after 12 hours, although there is no discernible difference between them. All scaffolds displayed comparable cell attachment and biological performance on day 1. After day 3, the inclusion of graphene became significant since 0.50 and 0.78 wt% PCL/graphene scaffolds showed statistically higher cell viability/proliferation rates than PCL scaffolds. At days 3, 7, and 14, a difference between scaffolds with 0.78 weight percent of graphene and PCL scaffolds is discernible. According to the results, cell viability/proliferation rate likewise rises as graphene concentration does. After 14 days, extensive cell attachment and cell spreading was observed. One could see that PCL/graphene scaffolds had more cells than PCL scaffolds did. Additionally, better cell attachment and dispersion were seen in PCL/graphene scaffolds. At day 3, all scaffolds exhibit much lower relative expression values for TNF and IL-1 than the clinical suture material utilized as the positive control. As they cause a lesser inflammatory response than clinical suture material, the results imply that the various PCL/graphene scaffolds have a great potential for *in vivo* applications. The research presented in this paper demonstrates that the most effective method for promoting the formation of new tissues *in vivo* is the combination of electrical stimulation (microcurrent therapy) and electro-active scaffolds containing graphene, particularly at day 120 when the effect of bone remodeling becomes dominant, and the effect of new tissue formation is less obvious. The *in vivo* application of PCL/graphene electro-active scaffolds induced by exogenous microcurrent therapy demonstrated increased new tissue creation and altered bone remodeling phase, resulting in well-organized tissue deposition, which could be a potential strategy to apply in clinical trials [55].

A. E. Jakus *et al.* produced a 3D printed graphene composite (3DG), with graphene and polylactide-co-glycolide (PLG), a biocompatible elastomer. Firstly, PLG was dissolved in dichloromethane (DCM). Graphene powder is dispersed in a mixture of DCM and smaller relative proportions of 2-butoxyethanol, a typical surfactant, and dibutyl phthalate, an efficient plasticizer, in a solvent system previously employed for printing particle-filled inks. Then, the PLG solution and graphene dispersion are combined and thoroughly mixed until homogeneous. The 3DG

composites are printed with a 60 and 40 vol.% of graphene and PLG, respectively. The ink was printed with an extrusion-based technique. The 3DG ink could be extruded from tip diameters as small as 100 μm and at speeds greater than 40mm/s to produce highly uniform, multilayered structures. The printed composites are mechanically stable and versatile. Thinner printed structures can be rolled, folded, sliced, and even welded together with 3DG ink thanks to its special qualities. Composites were compressive and tensile tested. 3D-printed graphene-PLG composites can be stressed to greater than 210% for 20 vol.% (of solid) graphene loading and 81% for 60 vol.% graphene loading before failing, despite not being nearly as elastic as their pure PLG equivalent. The electrical characteristics of the 3D-printed graphene fibres and constructions because graphene is widely used in electronic and electrochemical devices was evaluated. The resistivity measured along the fiber length decreases to $0.114 \pm 0.002 \text{ } \Omega\cdot\text{cm}$ (conductivity of approximately 875 S/m). The loading of graphene also affects the material's electrical characteristics. Even with a lower dosage of 20 vol.% graphene, conductivity is still noticeable. By simply changing the particle content in the initial inks, it may be possible to adapt the 3D-printed graphene system to certain applications given the range of mechanical and electrical properties it exhibits. hMSCs are still alive after two weeks on scaffolds made of 20 and 60 vol.% graphene and proliferate, coating the construct. Compared to the scaffolds containing graphene, PLG had much decreased viability and proliferation. Both 20 and 60 vol % graphene scaffolds supported hMSC viability and proliferation to a similar degree. By day 14, changes in phenotypic response can be seen. Over the course of two weeks, cells on 20 and 60 vol.% graphene scaffolds exhibit upregulation of glial and neurogenic relevant genes, including glial fibrillary acidic protein (GFAP), neuron-specific class III α -tubulin (Tuj1), nestin (Nes), and microtubule-associated protein 2 (MAP2), compared to the initial expression of unseeded day 0 hMSCs (Figure 5c). But by day 14, cells on 60 vol.% graphene scaffolds show 6, 3, 2.5, and 2 times more GFAP, TujI, Nes, and MAP2 expression than cells on the 20 vol.% graphene scaffolds. hMSCs on 20 vol.% graphene exhibit a confluent sheet-like morphology, which is typical of adherent cell types like fibroblasts and related hMSC lineages. This is a notable variation in cell morphology at this time. On 60 vol.% graphene, however, hMSCs grow into very elongated morphologies and do not produce confluent sheets. A closer look at cells from day 14 reveals that a portion of the population displays morphologies resembling those of uni- or multipolar neurons. *In vivo* biocompatibility of 3DG was also evaluated in a mouse subcutaneous implant model. There is no evidence of graphene flakes accumulating in the kidney, liver, or spleen, according to *in vivo* investigations with 3D-printed graphene. The improvements in electrical, mechanical, biological, and handling properties, along with 3DG's simplicity in processing and fabrication into intricate, user-defined structures, position it to hasten the design and production of new functional electronic and medical devices [56].

Q. Chen *et al.* showed the potential applications as biocompatible materials of 3D printed nanocomposites made of thermoplastic polyurethane (TPU) with poly(lactic acid) (PLA) and GO.

Graphene oxide was produced following the modified Hummer's method. 150 mg/mL of TPU were dissolved in dimethylformamide (DMF), and 100 mg/mL of PLA were dissolved in Dichloromethane. GO was diluted in DMF at a rate of 5 mg/mL. TPU to PLA ratio was set to 7:3 to produce an elastic polymer nanocomposite. The reference sample was a TPU/PLA blend with no GO loading, and the GO loadings were adjusted to 0, 2, and 5 weight percent. Nanocomposites were 3D printed through an extrusion-based approach, with a nozzle size of 0.4 mm and a printing speed of 20 mm/s. The infill density was 100%, and the layer thickness was 0.1 mm. The buildplate temperature was set at 60 °C, while the nozzle temperature was set at 210 °C. Objects of various shapes were created with TPU/PLA at various GO loadings to show the high flexibility of structure design in 3D printing. Compression and tensile testing was performed, and it was obvious that when GO is loaded, the compression modulus continuously increases, indicating that the compression strength of the TPU/ PLA matrix has been greatly improved by the addition of GO. With a 90 °C rise in the degradation temperature and the creation of superior crystalline structures, thermal stability has also been increased. At a 0.5 wt% loading of GO, the tensile modulus and yield point increased by 75.50% and 69.17%, respectively, showing that the addition of GO greatly boosted the tensile strength. The yield point and tensile modulus, however, both fell as GO loading was raised further. NIH3T3 mouse embryonic fibroblast cells were seeded onto the scaffolds, and LIVE/DEAD results show no dead cells in any scaffold. The shape of the NIH3T3 cells further suggested that they were spreading and multiplying on all scaffolds. All scaffolds encouraged the growth and division of cells, however the TPU/PLA with 0.5% GO exhibited the highest cell density across all GO loadings, even when compared to the TPU/PLA control. The TPU/PLA/GO 3D printable nanocomposite had excellent mechanical, thermal, and cell viability characteristics that make it useful in a variety of applications, particularly as a possible scaffold material for tissue engineering [57].

X. Zhou *et al.* produced 3D printed graphene oxide-doped gelatin-based scaffolds to encourage chondrogenic development of human bone marrow mesenchymal stem cells (MSCs) (Figure 2.4). As a biocompatible photopolymerizable bioink, GO-gelatin methacrylate (GelMA)-poly (ethylene glycol) diacrylate (PEGDA) was created. To prepare GelMA-PEGDA ink, 15% GelMA PBS solution with 0.5% (w/v) 2-hydroxy-4'-(2-hydroxyethoxy)-2- methylpropiophenone as a photoinitiator was combined with 20% PEGDA. In order to assess the impact of concentration on scaffold performance, several concentrations of GO (0, 0.05, 0.1, 0.25, 0.5, and 1 mg/mL) were thoroughly mixed into the GelMA-PEGDA bioink solution. The cartilage scaffolds were printed with a stereolithography technique, in a wood-pile geometric pattern. The printing specifications were a laser beam with a diameter of 200 μ m, a 25 mJ output of UV radiation with a frequency of 20 kHz, and a printing speed of 10 mm/s. Scaffolds were mechanically compressed, at a crosshead speed of 8mm/min. According to the findings, PEGDA inclusion significantly improved compressive modulus, which also rose with GelMA concentration. The scaffold's compressive modulus was unaffected by the GO incorporation as

well, with the exception of the maximum GO concentration of 1 mg/mL. For 5 days, the proliferation of MSCs was assessed. On day 5, MSCs proliferated effectively on the GelMA and GelMA-PEGDA hydrogels compared to the PEGDA hydrogel. The MSC proliferation on the 10% and 15% GelMA groups was greater than the corresponding 10% and 15% GelMA 20% PEGDA groups, respectively. Additionally, 15% GelMA hydrogels had stronger MSC proliferation than 10% GelMA hydrogels did. Although there was a minor reduction in MSC proliferation due to the PEGDA blending when compared to GelMA only samples, 15% GelMA 20% PEGDA were chosen as the best ink composition taking into account the printability of inks and balancing compatibility and mechanical performance. The highest proliferation of MSCs was discovered in the 0.1 mg/mL incorporation group on day 5, an increase of 22% compared to that without GO incorporation. MSCs expanded more quickly in all groups throughout time. After 5 days of culture, MSCs expanded and spread out perfectly on scaffolds that contained GO (0.25 mg/mL). It was possible to encourage MSC adherence, development, and differentiation by properly incorporating GO. The 15% GelMA-PEGDA with GO incorporated at 0.1 mg/mL was ultimately tested as the best combination for the following MSC differentiation investigation. GelMA-PEGDA scaffolds with and without GO were tested for protein affinities using BSA as a model protein. After 24 hours, BSA levels on GelMA-PEGDA-GO scaffolds were 763% higher than those on GelMA-PEGDA scaffolds, according to the adsorption profiles. The outcomes showed that scaffolds with GO incorporation could adsorb considerably more protein than scaffolds without GO. MSCs can be induced into chondrogenesis and produce cartilage for tissue regeneration under specific culture conditions. On the GelMA-PEGDA-GO scaffolds, a three-week chondrogenic differentiation investigation was carried out. After 3 weeks, the GelMA-PEGDA-GO scaffold had significantly higher levels of total collagen, GAG secretion, and collagen II synthesis (66%, 71%, and 43% higher, respectively) than the GelMA-PEGDA scaffold. According to these results, GO inclusion significantly improves chondrogenic differentiation. Type II collagen and GAG are crucial ECM elements of articular cartilage, and the amount of chondrogenesis they undergo directly affects how much of each is produced. The tissue-specific gene expression of type II collagen, SOX-9, and aggrecan was assessed at the gene level in order to further investigate the impact of GO on the chondrogenic differentiation of MSCs on the scaffold. After three weeks, the GelMA-PEGDA-GO scaffold considerably outperformed the GelMA-PEGDA scaffold in terms of gene marker expression for collagen II, SOX-9, and aggrecan by 83%, 44%, and 101%, respectively. This study showed that adaptable GelMA-PEGDA-GO scaffolds created in 3D are ideal choices for encouraging hMSCs to differentiate into chondroblasts, which holds significant promise for future cartilage regeneration [58].

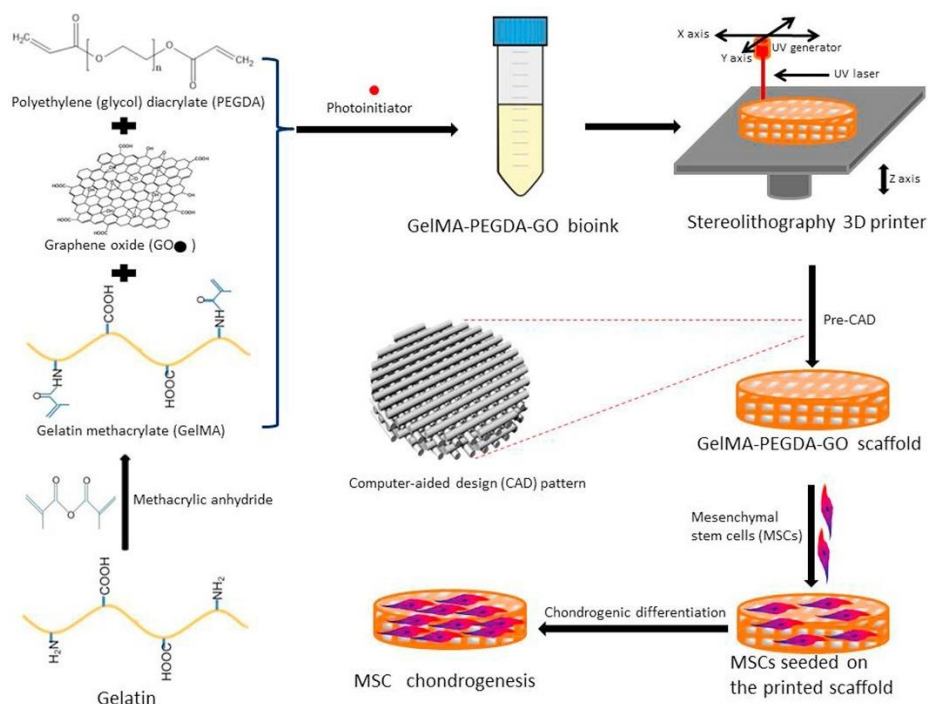


Figure 2.4 – Diagram of a 3D-printed GelMA-PEGDA-GO scaffold for encouraging hMSC chondrogenic differentiation from for cartilage TE (from [58]).

H. Belaid *et al.* developed a bioresorbable, biodegradable, and biocompatible synthetic polymer for bone tissue engineering. Polylactic acid (PLA) scaffolds were printed with extrusion-based bioprinting and reinforced by the incorporation of graphene oxide. GO was prepared by the modified Hummer's method and added to the PLA polymeric solution (10 mL of 10% (w/v)) in different concentrations (0.1 to 0.3 wt.%). Material was printed at 200 C, obtaining 1.75 mm filaments. Mechanical properties of the printed scaffolds were analyzed, obtaining 2.6 GPa Young's modulus after addition of 0.3% GO, whilst 2 GPa was achieved for not reinforced sampled. MC3T3-E1 preosteoblast cells were cultured on the scaffolds for 7 days, showing higher viability of the cells in PLA/GO scaffolds (especially for 0.2 and 0.3% GO). MG-63 cell proliferation was also assessed, and it was seen that cells readily proliferate on the scaffolds at 24h, reaching confluency at the 7th day, with higher values present for scaffolds incorporated with GO. MG-63 cell mineralization after differentiation was induced and on day 21, the calcium deposition was increased by 2.2-fold in GO containing samples compared to PLA scaffolds. The results prove that PLA reinforced with 0.2% GO is biocompatible and can improve MG-63 cell activity. The proposed solution promoted bone cell attachment, proliferation and differentiation, showing potential for bone tissue engineering [59].

S. R. Das *et al.* used ink-jet printing to produce graphene interdigitated electrode (IDE) circuit, that is highly conductive and can be used for electrical stimulation of cells, turning mesenchymal stem cells (MSCs) into Schwann cell (SC)-like phenotypes. To produce the ink, reduced graphene oxide was mixed with cyclohexane, terpineol, and ethyl cellulose. The rGO

ink was printed through 10 pL nominal drop nozzles onto polyimide substrates. Printing happened at 30 °C and 30 µm drop spacing. The graphene fingers had a 400 µm width, with a pitch of 250 µm and average thickness of 5 to 7 µm, and the printed electrodes were processed with a Nd:YAG pulsed laser (355 nm beam, 15 ns pulse width, 85 mJ/cm² energy density), increasing their electrical conductivity. The electrical conductivity of the obtained electrode was approximately 143 S/m. Brown Norway rat MSCs, isolated from bone marrow, were seeded onto the printed graphene IDEs to analyze the attachment, proliferation, and differentiation into SC-like phenotypes upon application of electrical or chemical stimuli. After 24h, the undifferentiated MSCs (uMSCs) were distributed and adhered over the IDEs. To promote differentiation of the cells, chemical (ctMSCs) and electrical (etMSCs) stimuli were conducted. MSCs were electrically differentiated on the graphene IDE with a 100 mV signal (50 Hz frequency) applied for 10 min per day for 15 days. The degree of differentiation into SCs was investigated using glial markers. More than ≈85% of the etMSCs on graphene displayed efficient staining for SC markers indicating successful electro-differentiation, whilst ctMSCs attached to the graphene IDE depicted more than ≈75% immunolabeling. There was no significant differentiation in IDEs not stimulated. The produced graphene IDEs can be implanted as a neural network circuit stimulated electrically for stem cell differentiation for neuroregeneration [60].

C.S.D. Cabral *et al.* 3D printed scaffolds with graphene oxide, tricalcium phosphate (TCP), gelatin (Gel) and chitosan (CH). Using L-Ascorbic acid (LAA), an *in situ* reduction of the scaffolds happened, resulting in scaffolds with rGO. To produce said scaffolds, TCP and Gel/CH were combined in a 80/20 (w/w) ratio. Then, 16 mg of GO was added to that solution. The scaffolds were extrusion-based bioprinted and then treated with LAA (10 mM, 6mL) at 40 °C for 24h, resulting in a *in situ* green reduction of GO containing scaffolds. Finally, scaffolds were crosslinked in sodium tripolyphosphate (TPP) solution (10% (w/v)) for 48 h, having a final product referred to as TGC_irGO. Normal human osteoblast (hOB) cells were seeded in the scaffolds and the viability and proliferation was evaluated. Scaffolds with GO or rGO presented higher phosphorus and calcium content, which can result in improved mineralization capacity of these materials. TGC_irGO scaffolds showed a higher compressive strength than TGC_GO and TGC scaffolds, with values within the range displayed by trabecular bone (2 – 20 MPa). Scaffolds also had higher elasticity (lower Young's modulus) than what is found in native bone (100 – 2000 MPa). Due to the intrinsic ability of TCP to induce mineralization, after a 21-day period, all scaffolds showed an apatite layer. After 7 days, hOB cells remained viable, proliferated, and exhibited elongated and flattened morphology, showing typical osteoblastic morphology. The cells were adhered to the scaffolds, and the cell number increased overtime, emphasizing the good biocompatibility of the produced constructs. More mineralization could be seen for TGC_irGO scaffolds compared to the other compositions (27 mg/mL after 21 days of incubation). Scaffolds with GO and rGO had higher ALP activity. Another advantage of the produced scaffolds was their antibacterial properties against *S. aureus* and *E. coli*, without compromising the viability

and proliferation of osteoblasts. This is a very important feature for future implantation of the scaffolds. These scaffolds showed remarkable features and high potential for bone tissue engineering applications [61].

H. Golzar *et al.* developed hybrid constructs with Beta-tricalcium-phosphate (β -TCP) to which gelatin/reduced graphene oxide-magnesium-arginine (GRMA) was added. The effect of different concentrations of reduced graphene oxide-Magnesium-Arginine (RMA) on morphology, mechanical properties, and biological activity of the 3D scaffolds was assessed. Firstly, graphene oxide was prepared using a modified Hummer's method to produce the RMA hybrid solution. RMA was synthesized with a hydrothermal reaction, where NaOH was dispersed in GO, forming a 0.5 mg/mL solution. To that solution, 36 mg of MgCl_2 and 10 mg Arg were added and left to incubate for 24h at 130 °C. The β -TCP was prepared, and ceramic-based scaffolds were printed with extrusion-based technique. The printing happened at 26 °C, at pressures between 1.5 and 1.7 bar and a printing speed ranging from 3 to 5 mm/s. The printed discs had 15 mm diameter, thickness of 2mm, 90° lay down patterns, and 0.6–0.8 mm distance between strands. To obtain β -TCP-GRMA constructs, the pores of the printed β -TCP scaffolds were filled with the RMA solution (at concentrations of 0, 0.25 and 0.75 wt.%) synthesized previously. Postnatal human dental pulp stem cells (DPSCs) were seeded onto the scaffolds. It was seen that, by increasing the concentration of GRMA, there is an increase in Young's modulus, enhancing the mechanical properties of the constructs. The addition of RMA also improved the cell proliferation, with the highest proliferation at the 7th day happening for β -TCP/0.75GRMA. In the 14th day, β -TCP/0.25GRMA was the one that exhibited the best results. It was also seen that the cells could attach and grow better on the surface of β -TCP/0.25GRMA compared to other scaffolds. The promoted adhesion and proliferation of cells can be attributed to the high biocompatibility of a suitable dose of RMA and gelatin. To evaluate osteoinductive capability of the produced constructs, ALP activity of DPSCs was analyzed. After 7 and 14 days it was seen that the ALP activity was higher on β -TCP/0.25GRMA. To further confirm cell differentiation, the mRNA expressions of cells was evaluated and after 7 and 14 days, DPSCs on β -TCP/0.25GRMA expressed an obviously higher level of the osteogenic gene (COL-I, Runx2, OCN). All results support the differentiation of DPSCs into osteoblasts due to the synergetic effect of gelatin and RMA. The produced scaffolds, especially β -TCP/0.25GRMA can be employed in bone tissue engineering due to its potential for cell viability, proliferation and differentiation [62].

P. Dorishetty, R. Balu, A. Gelmi *et al.* produced regenerated silk fibroin (RSF) and rGO hybrid ink. The hydrogels were fabricated with a ruthenium-mediated photocrosslinking method, where ruthenium-tris(2,2-bipyridyl)dichlororuthenium(II)hexahydrate [$\text{RuII}(\text{bpy}_3)$] and ammonium persulfate (APS) were added and mixed to RSF and RSF/rGO solutions. The final concentrations were 15 wt.% RSF, 5 mM $\text{RuII}(\text{bpy}_3)$ and 28 mM APS. For RSF/rGO hydrogels, three concentrations of rGO were used (0.05, 0.09 and 0.13 wt.%). The hybrid inks were printed

with extrusion-based technique, with a printing speed of 20 mm/s and pressures varying from 20 to 40 kPa. The printed filaments were then photocured for 40 s. L929 mouse fibroblasts were seeded onto gels to assess cell-gel interactions. The RSF/rGO hydrogels with 0.13 wt% rGO showed around 21% decrease in water uptake capacity, 2% increase in crosslink density, 26% decrease in micropore size, and 44% increase in β -sheet content. This combination also presented a 548% increase in storage modulus, and 556% increase in Young's modulus, but that implied a 63% decrease in viscosity (at printing shear rate), 31% decrease in contact angle, and 25% decrease in printing accuracy. The mechanical values of the produced hydrogels are in line with articular cartilage tissue, so the hydrogels show potential for load bearing tissue engineering applications. Hydrogels containing rGO had a decrease in cell attachment and proliferation, with a decrease in cell viability with the increase of rGO content but no affect in biocompatibility (after 7 days, the hydrogels presented more than 86% cell viability). The decrease in cell proliferation could be caused by the increase in stiffness or decrease in available sites for the attachment of the cells when rGO is incorporated in hydrogels. Nonetheless, these hydrogels demonstrated suitability for cartilage tissue engineering applications [63].

Table 2.1 – Selected recent studies on carbon nanomaterials for 3D printing of scaffolds for tissue engineering.

<i>Carbon material</i>	<i>Biomaterial ink/bioink</i>	<i>Bioprinting technique</i>	<i>Carbon material role</i>	<i>Application</i>	<i>Biocompatibility</i>	<i>Ref.</i>
CNFs	CNFs-Alg-Gel	Extrusion-based printing	Improve electroconductivity and mechanical properties	Tissue engineering – bone or cardiac tissue	NIH/3T3 fibroblasts cell viability for all hydrogel groups ranged above 88% Increased cell proliferation on hydrogels compared to control	[49]
MWCNTs	MWCNTs-nHA-PCL	Extrusion-based printing (screw-assisted)	Improve mechanical properties, osteogenic differentiation and promote ALP, COL and OCN expressions	Tissue regeneration - bone	The addition of MWCNTs improves hADSCs differentiation	[50]

CNTs	CNTs-HA-PCL	Extrusion-based printing	Improve mechanical and electrical properties, cell adhesion and cell spreading	Tissue regeneration - bone	MG63 osteoblast-like cells attach more to scaffolds with higher CNT % By 6 th day material is covered with cellular layer	[51]
MWCNTs	MWCNTs-PIC	Extrusion-based printing (pneumatic)	Improve bone regenerating capacity, better osteoconductivity, higher vascularization	Tissue regeneration - bone	BMSCs grew faster on the PIC/ MWCNT scaffolds after culturing for 3, 5 and 7 days. More expression of osteogenesis-related genes. Achieved bone repair in 2 to 8 weeks, with neovascularization.	[52]
MWCNTs	MWCNTs-PCL	Stereolithography	Improve physical properties, conductivity, biodegradation, and cell proliferation	Tissue engineering - cardiac	Optimal conductivity and stiffness for the proliferation of H9c2 cells	[53]
MWCNTs	MWCNTs-Alg	Extrusion-based printing	Improve mechanical properties, and perfusion	Vascular conduits	The printing caused HCASMC cell death, but cells were able to recover with a cell viability in day 3 of 68.8% Almost no ECM formation	[54]

Graphene	Graphene-PCL	Extrusion-based printing	Increased protein adsorption, cell attachment and proliferation;	Tissue engineering - bone	After 14 days, extensive hADSCs cell attachment and spreading Greater tissue formation after 120 days	[55]
Graphene	Graphene-PLG	Extrusion-based printing	Improve mechanical, electrical, and biological properties	Wearable and implantable electronics, sensors, and tissue engineering (neuronal)	hMSCs remain viable over the course of 2 weeks and proliferated. After 2 weeks, cells start to differentiate into neuron-like cells	[56]
GO	GO-TPU-PLA	Extrusion-based printing	Improve tensile strength, thermal stability, and supporting cell growth	Tissue engineering	NIH3T3 cells appear to be spreading and proliferating on scaffolds	[57]
GO	GO-GelMA-PEGDA	Stereolithography	Improve cell differentiation, and protein adsorption	Tissue regeneration – cartilage	GO induced chondrogenic differentiation of hMSCs, chondrogenic gene expression of type II collagen, SOX-9 and aggrecan	[58]
GO	GO-PLA	Extrusion-based printing	Decrease surface hydrophobic properties, improve thermal stability, mechanical properties, cell	Tissue engineering - bone	Higher MC3T3-E1 cell viability after 7 days Improved MG-63 cell proliferation and mineralization	[59]

			viability, and mineralization			
rGO	rGO- cyclohexanone -terpineol- ethyl cellulose	Ink-jet printing	Improve mechanical, and electrical properties	Tissue regeneration - peripheral nerve	Differentiation of MSCs into Schwann cell like phenotypes through the application of electrical stimuli from a graphene- based electrode.	[60]
GO/rGO	GO-TCP-Gel- CH	Extrusion- based printing	Improve wettability, mechanical properties, calcium deposition, and antimicrobial properties	Tissue regeneration - bone	hOB cells remained viable after 7 days of incubation Cells proliferated and exhibited elongated and flattened morphology	[61]
rGO	rGO-Gel- Mag-Arg- β - TCP	Extrusion- based printing (pneumatic)	Improve mechanical properties, and enhance cell proliferation	Tissue engineering - bone	DPSCs have higher attachment and proliferation	[62]
rGO	rGO- regenerated silk fibroin	Extrusion- based printing	Improve mechanical properties	Tissue engineering - cartilage	Slight decrease in L929 cell viability and in proliferation	[63]

2.4.2 Sensors and biosensors

A sensor can be defined as a device that can convert an input of a physical quantity into an output usually in an electrical or optical signal, that electronic instruments or humans can read.

Using sensors and their interfaces, it is possible to detect or measure different physico-chemical properties of compounds. A biosensor is formed by a biological component, a bioreceptor, a physico-chemical detector and a transducer. The bioreceptor can be an antibody, aptamer, enzyme, nucleic acid, or cell that can detect and identify the target analyte. Biosensors have high selectivity to the target analyte since the bioreceptor is capable of doing a specific interaction with the analyte. Since this interaction is specific for the bioreceptor and analyte, it prevents interference from other signals. A bioreceptor will recognize the event and the transducer will transform that into a measurable signal [64].

V.A.O.P. Silva *et al.* proposed a solution where the sequential chemical treatment of 3D printed graphene and polylactic acid (PLA) electrodes would result in rGO-PLA electrodes. As proof-of concept, the 3D printed electrodes were applied to serotonin determination in a synthetic urine sample. Another application was turning the electrodes into biosensors using tyrosinase, to determine catechol (a contaminant that can harm health and may be found in natural water from industrial effluent discharges) in natural water samples. Through extrusion-based bioprinting, conductive PLA doped with graphene and non-conductive PLA were used to form an electrochemical system. The electrochemical cell had 30 mL, with 46 mm diameter and 35 mm height, inner diameter of 40 mm and inner height of 32 mm and was printed with non-conductive PLA. The top part of the cell had a screw cap form, to fix the cover, having 49 mm diameter, 8 mm height, and 5 holes with 8 mm diameter each to allow the arrangement of three electrodes (working, pseudo-reference, and counter electrodes). The electrodes were printed with conductive graphene/PLA ink, forming three conductive hollow cylinders with 37.747 mm length and 23 mm deep hollow (to connect the cables). The inner diameter (diameter of the hollow) was 4.5 mm, while the external diameter was 7.5 mm. These cylinders could be attached to the cell top. To enhance the performance of the electrodes, they were immersed in dimethylformamide (DMF) for 15 min. The working electrode was also immersed for 15 min in two sequential solutions, one of 1.0 mol/L nitric acid (HNO_3) and other of 0.15 mol/L sodium borohydride (NaBH_4). This chemical treatment will result in rGO-PLA electrodes, confirmed by Raman spectra. It was seen that treated electrode (rGO-PLA) facilitated electron transfer when compared to G-PLA electrode. The production process of these electrodes can be seen in Figure 2.5.A.a). The rGO-PLA electrode was used for serotonin determination and had a better sensing performance in comparison to other works. To produce the biosensor, the first step was to prepare an enzyme layer film on the working electrode (treated with DMF). For that purpose, dihexadecyl phosphate (DHP) was dissolved in phosphate buffer, achieving a final concentration of 1.0 mg/mL. Exactly 90 μL of this solution were mixed with the enzyme tyrosinase. Then, 40 μL of the enzymatic solution were placed on the surface of the G-PLA working electrode. That process is present in Figure 2.5.A.b). The biosensor could determine the catechol in natural water samples with acceptable features [65].

A. Hiratsuka *et al.* proposed a direct-electron-transfer (DET)-type biosensor. The bio-nanoink for DET, was composed of glycan-chain-rich *Aspergillus terreus* var. *aureus* flavin adenine dinucleotide-dependent-glucose dehydrogenase (FAD-GDH) (*AtaGDH*), SWCNT and surfactant sodium cholate (SC). The bioink was prepared by dispersing SWCNT in the SC aqueous solution, resulting in a solution with 0.15% SWCNT and 2.0% SC (w/v). The ink is a mixture (1:1, v/v) of SWCNT dispersion in SC and FAD-GDH. To print the biosensor, inkjet printing was used, with a piezoelectric-controlled procedure, under the following conditions: 20V, 1kHz, and one drop 25 pL. A biosensor was composed by 8000 drops (0.2 μ L). The production process is represented in Figure 2.5.B. Glycan-chain-rich *AtaGDH* was screened, and it proved to be most suitable for bioink due to its chemical stability and activity. *AtaGDH* can keep its enzymatic activity in the SWCNT/SC solution, because of its glycan chain, whilst FAD-GDH with lower amount of glycan chain would be denatures by SWCNT/SC dispersion. The biosensor showed high sensitivity (70 μ A m/M.cm²), selectivity toward interferences, and low detection limit (1.1 μ m). After 24h, the electrochemical response of the device remained above 90% of the initial current. This response is maintained over 7 days, suggesting that the produced bioink does not cause leaching of SWCNT or *AtaGDH*. The produced sensor exhibited a better performance than most other enzyme glucose biosensors in terms of sensitivity and detection limit. The biosensor was used to measure a real-life sample, using a serum as a model of the real-life sample as a control. The values obtained by the sensor for the sample and the control are similar, with a deviation within 5%, so the produced biosensor exhibited precision, accuracy, and reliability [66].

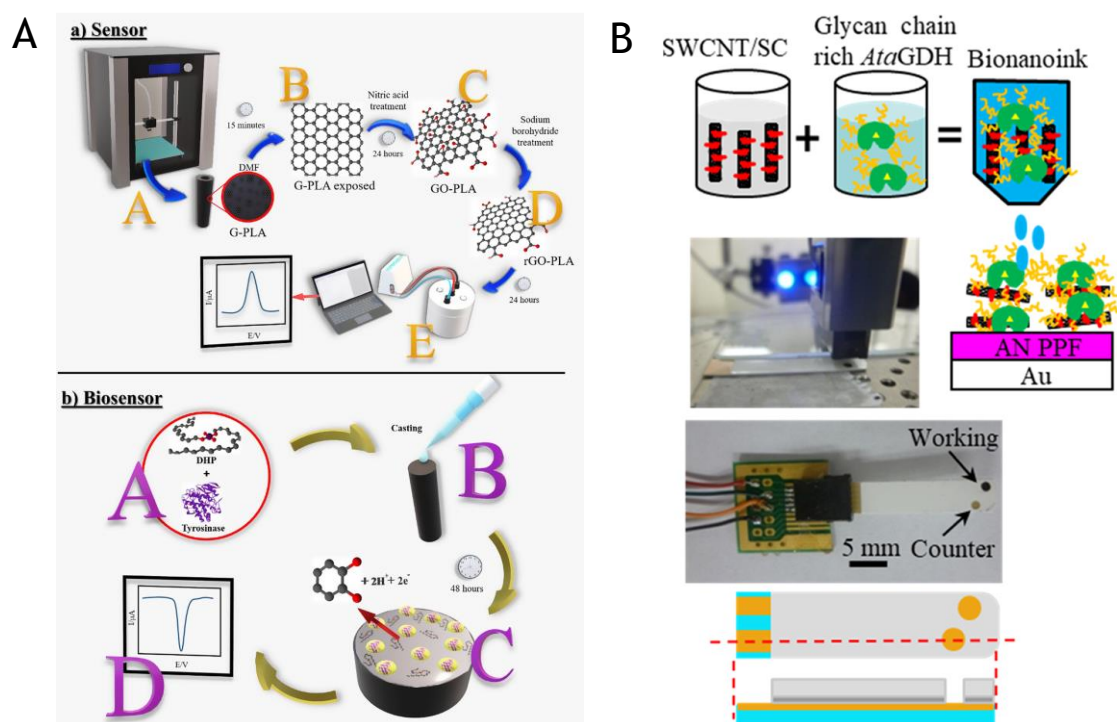


Figure 2.5 – Production of sensors/biosensors from the selected recent studies. A.a) Scheme of the procedure for obtaining the G-PLA 3D-printed working, counter, and reference electrodes; A.b) Scheme of the steps involved in the production of the biosensor, including the mixture of DHP and tyrosinase (from [65]); B) Schematic representation of DET bio-nanoink fabrication processes (from [66]).

2.5 Conclusions and Outlook

In the areas of biofabrication and TE, CNM have drawn more focus toward the creation of biomimetic constructions that capture the distinctive characteristics of tissues and organs. CNM also show their potential in other biomedical areas, such as sensors and biosensors. According to the type of CNMs, bioprinting technology, ways that CNMs affect the bioprinting process, and applications, current state of the design and bioprinting of devices containing CNM was outlined in this review.

The inference was made that the versatility of CNM has generated interest in merging them to produce cutting-edge 3D printed materials with superior properties for biomedical uses. The creation of biomaterial inks that consider elements including printability, biocompatibility, mechanical characteristics, cell guiding, and tissue biomimicry — often necessary for a successful 3D/4D bioprinted tissue implant — has received significant attention from researchers. Studies have been done on a variety of bioprinting techniques, and biomaterial inks, among other aspects.

Due of its accessibility, simplicity, and adaptability, extrusion-based bioprinting has produced the majority of the constructs. The majority of the structures created thus far are based on simple geometries like filaments, films, and bifurcated structures, despite the fact that

technical advancements enable the bioprinting of constructs with intricate and sophisticated geometries. This is mostly due to the fact that this field of study is still in its early stages, and most studies have been concentrated on defining the bioprinting parameters and comprehending the impact of CNM on the bioprinting process. In fact, recent research has concentrated on creating and bioprinting materials that contain CNM but no cells, which are then planted on top of bioprinted constructions.

In addition to extrusion-based bioprinting, light-based bioprinting, in particular stereolithography, has been investigated for the construction of CNM-containing structures, however the number of publications is substantially lower. The extremely limited number of papers in the field using stereolithography may be explained by the lesser availability of the technology, together with the requirement for photocrosslinkable materials and cytocompatible photoinitiators.

There are also very few works using inkjet bioprinting for 3d-printing CNM-containing constructs. This may be connected to the requirement for low viscosity biomaterial inks to facilitate droplet production and reduce nozzle blockage.

Due to the improved interaction between the graphene and the polymer matrix, a lot of investigations have used oxidized graphene forms (GO) in an effort to achieve better dispersions and adjust the viscosity of the biomaterial ink. CNT were also very common, being used mostly for bone TE due to their enhanced mechanical properties or for neural or cardiac TE, because of their electrical conductivity.

It is essential to assess the potential toxicity, biological stability, and interactions of bioprinted CNM composites before using them in the medical industry. This is especially important for GBM and CNT since, in addition to being nanomaterials, they also come in a variety of shapes, sizes, and surface chemistries that unavoidably have diverse toxicological consequences.

Regarding the 3D-production of sensors and biosensors, the field is still lacking, but the availability of the needed materials is an advantage.

Chapter 3: Materials and Methods

3.1 GBM Production

For this work, two graphene-based materials (GBM) were selected and produced, specifically nanographene oxide (GOn) and partially reduced nanographene oxide (p-rGOn).

To produce GOn, graphite powder (size $\leq 20\text{ }\mu\text{m}$, Sigma-Aldrich, Sr. Louis, MO, USA) was oxidised using a modified Hummers method [67]. To summarize the process, a mixture of 160 mL of sulfuric acid (H_2SO_4 , VWR, Darmstadt, Germany) and 40 mL of phosphoric acid (H_3PO_4 , Chem-Lab, Zedelgem, Belgium), was added to 4 g of graphite. In a Lenz reactor, the dispersion was cooled using a cooling bath (Julabo F25), whilst stirred. Afterwards, 24 g of potassium permanganate (KMnO_4 , JMGS, Odivelas, Portugal) were gradually added to the solution, while it was stirred at 420 rpm. Following, 600 mL of H_2O were introduced slowly, under stirring, and the temperate was controlled ($< 40\text{ }^\circ\text{C}$) using a cooling bath. Finally, 26.5 mL of hydrogen peroxide (H_2O_2 , VWR, Darmstadt, Germany) were added and the dispersion was left to rest overnight. The dispersion was then decanted to separate the solid phase from the acidic solution, centrifuged ad 4000 rpm, and redispersed in ultrapure water. This process was repeated until the pH of the supernatant was the same as the pH of the ultrapure water.

To obtain GOn, exfoliation and particle size reduction was done. For this, a home-build system with a peristaltic pump (Matson Marlow 323) and an industrial ultrasonic probe (Hielscher) was used. After diluting GOn to a concentration of 1 mg/mL, it was placed in a Hergeus reactor and photoreduced under magnetic stirring to obtain p-rGOn. For this, the temperature was kept below $40\text{ }^\circ\text{C}$ using a cooling bath (Julabo F25). A schematic representation of the described process can be seen in Figure 3.1.

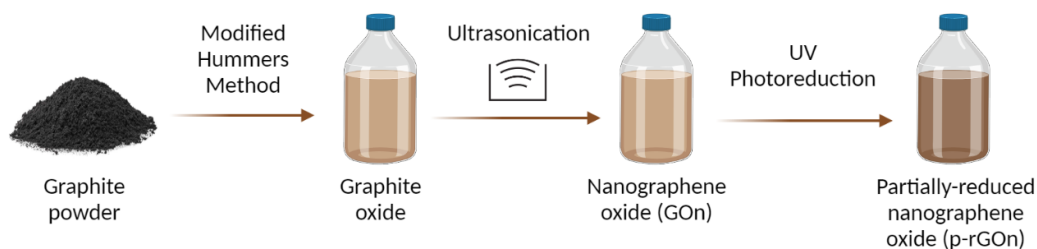


Figure 3.1 – Production method of nanographene oxide (GOn) and partially reduced nanographene oxide (p-rGOn). Created with Biorender.com.

3.2 GBM Characterization

3.2.1 Physical Characterization

3.2.1.1 Transmission electron microscopy (TEM)

Morphology and particle size of GOn and p-rGOn aqueous dispersions were analysed by transmission electron microscopy (TEM) with a JEOL JEM 1400 TEM (Tokyo, Japan) at a concentration of 40 $\mu\text{g/mL}$. The dispersions were sonicated and 10 μL were deposited on a carbon-coated TEM grid and left to sediment for 1 min. Afterwards, the excess material was removed by capillarity with a filter paper. The lateral dimensions of the nanomaterials were measured using the ImageJ software.

3.2.1.2 Particle size measurements by dynamic light scattering

To obtain the particle size distributions of the aqueous dispersions of GOn and p-rGOn, dynamic light scattering (DLS) using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) was performed.

The GOn and p-rGOn dispersions were sonicated and diluted to a concentration of 30 $\mu\text{g/mL}$.

Afterwards, they were placed in a disposable Zetasizer cuvette (Malvern Instruments, Worcestershire, UK). The measurements were made in triplicate at room temperature and results are reported as mean and standard deviation.

3.2.1.3 Zeta potential measurements

To obtain zeta potential measurements, GOn and p-rGOn aqueous dispersions were sonicated and prepared at a concentration of 30 $\mu\text{g/mL}$. Using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK), where the dispersions were placed in disposable Zetasizer cuvette (Malvern Instruments, Worcestershire, UK), measurements were made at room temperature and results are reported as mean and standard deviation.

3.2.2 Chemical Characterization

3.2.2.1 Fourier transform infrared spectroscopy (FTIR)

GOn and p-rGOn samples were heated at 60 °C, during 24h to dehydrate. To obtain the infrared spectra of dehydrated samples of GOn and p-rGOn, Fourier transform infrared (FTIR) spectroscopy was used, resorting to a VERTEX 70 FTIR spectrometer (Bruker, Karlsruhe, Germany) in transmittance mode at room temperature. Samples were measured in attenuated total reflection (ATR) mode with a A225/Q PLATINUM ATR Diamond crystal with a single reflection accessory. Spectra were recorded by 60 scans, at a resolution of 4 cm⁻¹, in a wavenumber range from 4000 and 400 cm⁻¹.

3.2.2.2 Thermogravimetric analysis (TGA)

To evaluate the thermal stability of dehydrated samples of GOn and p-rGOn, Thermogravimetric analysis (TGA) using a Netzsh STA 449 F3 Jupiter (Selb, Germany) was conducted. The dry samples had around 6 mg. The thermograms were recorded between 30 and 1000 °C, at a heating rate of 10 °C/min, under nitrogen flow. Results are presented as percentage (%) of weight loss.

3.2.2.3 Ultraviolet-visible spectroscopy (UV-Vis)

To acquire the absorption spectra of GOn and p-rGOn aqueous dispersion at a concentration of 12 µg/mL, a Lambda 35 UV/vis spectrometer (Perkin-Elmer, Waltham, MA, USA) was used. Samples were placed in a quartz cuvette (Hellma Analytics, Analytica Munich, Germany) with 10 mm light path length and their spectra were acquired between 200-850 nm. The measurements were made in triplicate, at room temperature, using water as a blank control for the baseline correction.

3.2.2.4 X-ray Photoelectron Spectroscopy (XPS)

The data analysis of GOn and p-rGOn was performed with a Kratos Axis Ultra HAS (Kratos Analytical, Manchester, UK) with VISION software for data acquisition and CASAXPS software (Casa Software Ltd, UK) at CEMUP (Centro de Materiais da Universidade do Porto, Porto, Portugal). The analysis was carried out with a monochromatic Al K X-ray source (1486.7 eV), operating at 15 kV (90 W), in fixed analyser transmission (FAT) mode, with a pass energy of 40

eV and a step of 0.1 eV for regions (C, O, and Fe) and 160 eV of pass energy and 1.0 eV of step for the survey. The peaks were fitted using a Gaussian-Lorentzian shape and a Shirley background.

3.2.3 Photothermal Properties

3.2.3.1 NIR Irradiation

To evaluate the photothermal properties, in a 48-well plate, 600 μL of GOn and p-rGOn aqueous dispersions, at the concentrations of 250 and 500 $\mu\text{g/mL}$ were used. Samples were irradiated with a custom LED-system, with a peak-emission around 810 nm (NIR region) and 150 mW/cm^2 irradiance. The samples were irradiated for 30 min, in order to record the light-induced temperature change. For this, a type K thermocouple was placed in the centre and half-height in the dispersion. Three replicates were made per condition, using a control with water only. This assay was performed inside an oven at 37 °C, as to replicate the biological conditions. Results are presented as mean and standard deviation.

Thermal photos of the water control and dispersions at 250 and 500 $\mu\text{g/mL}$ were captured using a FLIR E60 thermal imaging camera (Teledyne FLIR LLC, Wilsonville, Oregon, U.S.) as a way to visually capture the increase of the temperature due to irradiation. In this case, the 48-well plate containing the samples was preheated to 37 °C, and then removed to perform the irradiation. The thermal pictures were analysed using FLIR Research Studio software.

3.3 Hydrogels Production

3.3.1 Pectin purification

Firstly, pectin was purified from LM citrus pectin (Classic CU701) (Herbstreith & Fox, Neuenbürg, Germany) and termed herein as raw pectin (RAWpec). Briefly, the purification consisted in preparing a 1 wt.% solution of RAWpec in ultrapure water (18 MU, Milli-Q UltraPure Water System, Millipore). After the polymer was fully dissolved, the solution was sequentially filtered using 0.80 μm , 0.45 μm , and finally 0.22 μm filter membranes (mixed cellulose esters, MCE, Millipore). Then, activated charcoal (2 wt.%, Norit SX Plus, Norit) was added and the polymer solution was stirred for 1 h at room temperature for subsequent centrifugation at 27 000 $\times$ g for 1 h. Afterwards, the solution was filtered using a 0.22 μm membrane. Finally, the solution was lyophilized, and stored at -20 °C until further use. The result of this protocol is purified pectin, hereafter referred to as PURpec.

3.3.2 Pectin biofunctionalization

The oligopeptidic sequence (Glycine)₄-Arginine-Glycine-Aspartic acid-Serine-Proline (abbreviated as G4RGDSP, Genscript, Piscataway, NJ, USA) was grafted onto PURpec following an adapted method previously described for natural polymers [68, 69]. Firstly, PURpec was dissolved in ultrapure water, at a concentration of 1 wt.%, under agitation. Two separate batches were produced, one for modification and the other, of low volume (around 20 mg) as a control. As soon as the polymer was completely dissolved, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Tokyo Chemical Industry Co.), used as an activator for the carboxyl polysaccharides, was added to both batches and left to react for 30 min, protected from the light. After the 30 min, the peptide was added to the batch of polymer to biofunctionalized and left to react for 24h. When the reaction was finished, the solution was diluted with ultrapure water, purified by dialysis, and lyophilized. The final polymer is hereby referred to as RGDpec.

The coupling efficiency was quantified through colorimetric DC Protein Assay (Bio-Rad). Briefly, the polymer was dissolved in ultrapure water (1 wt.%) and the samples of the modified polymer and control (20 μ L) were transferred to a 96 well-plate. After, alkaline copper tartrate solution (25 μ L) and dilute Folin reagent (200 μ L) were added to the wells, followed by mixing with a multi-channel micropipette. The solutions were left to react for 30 min, being then analysed for absorbance with a micro-plate reader (Synergy MX, BioTek) at 750 nm. In order to determine the protein content, absorbance values were converted to peptide concentrations by the use of a standard curve of free peptide in polymer and normalised against control polymer solutions that were conjugated with the activator but absent of peptide.

3.3.3 Preparation of hydrogels

The crosslinking method used to form pectin hydrogels, is based on the internal gelation strategy [70], [71]. Briefly, it depends on a calcium carbonate (CaCO_3)(Fluka) and glucono-delta-lactone (GDL)(Sigma-Aldrich) system, where GDL hydrolyses throughout time, resulting in a reduction of the solution pH, which will ultimately lead to the release of Ca^{2+} ions from CaCO_3 molecules [72]. The concentrations of polymer, CaCO_3 and GDL were optimized in order to obtain a hydrogel. To reinforce the hydrogels for certain assays, and achieve better shape-retention, hydrogels were also subjected to an external gelation by incubation in calcium dichloride (CaCl_2) solution. In external gelation, the gelation occurs via diffusion of free Ca^{2+} ions. A CaCl_2 bath of 200 mM for 5 minutes was used.

3.3.3.1 Pectin hydrogels

For preparing the hydrogels, the lyophilized PURpec, CaCO_3 and GDL were separately dissolved in ultrapure water. Firstly, the desired amount of CaCO_3 was added to the pectin solution and thoroughly mixed, to ensure the CaCO_3 is dispersed in the pectin. After this, the GDL was added and thoroughly dispersed in the solution. To prepare hydrogel cylinders, the solution was cast in a QGel® 3D disc caster (Lausanne, Switzerland). After some time, the crosslinking is apparent and there is the formation of a hydrogel. For pectin, concentrations between 1.5 and 3 (w/v) % were tested, while for CaCO_3 and GDL, a range of concentrations between 10-100 mM and 20-200 mM, respectively, were tested.

3.3.3.2 Pectin/p-rGOn hydrogels

When preparing pectin hydrogels with GBM, firstly an approach to add the p-rGOn dispersion to the hydrogel precursor solution was tested. The maximum possible concentration of GBM in the hydrogel with this method is around 0.09 wt.%. The production of the hydrogel precursor solution follows the same procedure as described for pectin only solution, except for the addition of the amount of p-rGOn dispersion needed to achieve the desired final concentration of GBM in the hydrogel.

Another approach was to dissolve the pectin, the CaCO_3 and the GDL in the p-rGOn dispersion, using the GBM as buffer as well. With this method, the GBM content is around 0.18 wt.% in the hydrogel.

The concentrations of polymer, CaCO_3 and GDL were chosen according to the previous optimization steps.

3.3.3.3 Pectin/p-rGOn printable hydrogel

To design a printable hydrogel, another step of optimization had to be conducted, in order to modulate viscosity for potential extrusion bioprinting. Polymer concentrations of 2 and 2.5% were tested, along with CaCO_3 and GDL concentrations ranging between 5 and 100 mM, and 1 and 200 mM, respectively. The pre-crosslinked hydrogels were loaded in syringes and extruded through a cylindrical nozzle of 0.41 mm (Nordson EFD).

3.4 Hydrogels Characterization

3.4.1 Physical Characterization

To use the produced hydrogels for in vitro testing and for future applications, they must be stable and have enough mechanical resistance to be handled [73], thus, the mechanical strength must be tested.

The produced hydrogels are meant to be applied to the skin, being constantly subjected to mainly compressive forces [74], so the samples were mechanically characterized by compression tests at room temperature using a software-controlled Texture Analyser (TA.XT plus texture analyser; Stable Micro Systems, Godalming, UK), with a load cell of 5 Kg, force resolution of 0.01 g and distance resolution of 0.001 mm.

Firstly, a test was performed to optimize the parameters to use, like thickness and surface area of the samples to test, probe to be used, and parameters in the software to be chosen. The chosen values were based on this optimization step and on values described on the literature for similar tests, so the prepared samples were produced in cylindrical shapes, with 8 mm diameter and thickness between 2 and 4 mm [73–76].

The mechanical properties were evaluated by compression tests, recording the resistance of the sample to the compression of an aluminium cylinder probe with a diameter of 25 mm (the diameter of the probe should be 2 to 3 times bigger than the sample for an accurate test [74, 75]).

This test was performed by placing each sample in the centre of the plate, where the compression force is applied. Initiating the test, the probe descends until touching the sample, where it starts recording the data. The probe exerts pressure down to 0.8% of the initial thickness of the sample, finishing the test when that point is reached. When finishing the test, the probe returns to the initial position. In table 3.1, the chosen settings for the tests performed are present.

Table 3.1 – Settings used in the software for compression tests.

<i>Test Mode</i>	<i>Pro-test speed</i>	<i>Test speed</i>	<i>Post-test speed</i>	<i>Target Mode</i>	<i>Distance</i>	<i>Trigger type</i>	<i>Trigger force</i>
<i>Compression</i>	1 mm/s	0.1 mm/s	1 mm/s	Distance	0.8% of the initial sample thickness	Auto (Force)	0.01 N

When performing the test, data regarding force (N), time (s) and distance (mm) is collected.

The Young's modulus is calculated as the slope of the stress (MPa) vs. strain (%) curve within the linear region. The linear region corresponds to the elastic region and is usually in a range

between 5 and 15% of strain. Before calculating the Young's modulus, stress and strain must be calculated.

Compressive stress is calculated using equation 3.1:

$$Stress (MPa) = \frac{F(t)}{A} \quad (3.1)$$

where $F(t)$, in N, is the force applied on the sample and A , in mm^2 , is the surface area of the sample. The surface area is calculated based on the diameter of the sample, measured before the test. The stress at each time, in MPa or N/ mm^2 , is calculated by the force in each instant by the surface area.

Compressive strain is calculated following equation 3.2:

$$Strain (\%) = \frac{-\Delta L}{L_o} \quad (3.2)$$

where ΔL is the quantity by which the length of the object changes, and L_o is the initial thickness of the sample. The mechanical strain is a geometric measure of the deformation, representing the relative displacement between particles in a material. The Young's modulus (E) is calculated using equation 3.3:

$$E (MPa) = \frac{Stress}{Strain} = - \frac{F \times L_o}{A \times \Delta L} \quad (3.3)$$

Considering the previous equation, the Young's modulus can be calculated by the slope of the stress-strain curve. As a way to compare the influence of each variable on the mechanical properties of the hydrogels, the hydrogels present in table 3.2 were prepared:

Table 3.2 – Different conditions on hydrogel fabrication to be tested.

Hydrogel		
0 wt.% GBM (pectin)	as prepared	w/ CaCl ₂
		w/o CaCl ₂
	incubated in DMEM	w/ CaCl ₂
		w/o CaCl ₂
0.09 wt.% GBM	as prepared	w/ CaCl ₂
		w/o CaCl ₂
	incubated in DMEM	w/ CaCl ₂
		w/o CaCl ₂
0.18 wt.% GBM	as prepared	w/ CaCl ₂
		w/o CaCl ₂
	incubated in DMEM	w/ CaCl ₂
		w/o CaCl ₂

Hydrogels referred to as “w/ CaCl₂” were reinforced with an external gelation bath and hydrogels “incubated in DMEM” had an overnight incubation. Three samples of each testing group were produced, and the average value of the measurements was recorded for each condition.

3.4.2 Photothermal Properties

To evaluate the photothermal properties of the produced GBM hydrogels, in a 48-well plate, hydrogels with 0, 0.09 and 0.18 wt.% GBM were placed at the bottom of a well and covered by 600 µg of DMEM. Samples were irradiated with a custom LED-system, with a peak-emission around 810 nm (NIR region) and 150 mW/cm² irradiance. The samples were irradiated for 30 min, in order to record the light-induced temperature change. For this, a type K thermocouple was placed in the centre, touching the surface of the hydrogel. Three replicates were made per condition, using a control with water only. Before the irradiation, samples were pre-heated to 37 °C in an oven, as to replicate the biological conditions. Results are presented as mean and standard deviation.

Thermal photos of the DMEM control and hydrogels with 0, 0.09 and 0.18 wt.% GBM were captured using a FLIR E60 thermal imaging camera (Teledyne FLIR LLC, Wilsonville, Oregon, U.S.) as a way to visually capture the increase of the temperature due to irradiation. As before, the 48-well plate containing the samples was preheated to 37 °C, and then removed to perform the irradiation. The thermal pictures were analysed using FLIR Research Studio software.

3.5 *In vitro* biological studies

3.5.1 Cell culture

Biological studies were performed using A-431 human epidermoid carcinoma cells (ATCC, CRL-1555), HFF-1 human foreskin fibroblasts (ATCC, SCRC-1041) and HDFn human dermal neonatal fibroblasts. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, ATCC) supplemented with 10% (v/v) fetal bovine serum (Alfagene, Carcavelos, Portugal) and 1% (v/v) penicillin/streptomycin (Biowest, Pays De La Loire, France). Cells were maintained in a humidified atmosphere with 5% CO₂ and 95% air at 37 °C. The medium was replaced every 2-3 days and cells were detached upon reaching 70% confluence.

For biological experiments, the effect of pectin, and pectin/p-rGOn hydrogels was assessed using both normal (fibroblasts) and cancer cells in the presence or absence of NIR irradiation, as described in detail below.

3.5.2 Cytotoxicity of hydrogels

3.5.2.1 *Preparation of hydrogels for in vitro assays*

For extract exposure assays, hydrogels were made using solely PURpec. For direct contact experiments, PURpec and RGDpec were mixed to achieve a final hydrogel with 25% PURpec and 75% RGDpec, resulting in a final concentration of 450 µM of RGD in the hydrogel, similar to previously reported values found in biological ECMs [72].

Before assays, hydrogels were disinfected to ensure they were as clean as possible before coming into contact with the cells. For this, the hydrogels were washed firstly with ethanol at 95% and left for 15 min. After this time, the materials were washed with 80% ethanol for another 15 min. The last wash was done with ethanol at 70% and this time, the exposure was 30 min. Finally, the hydrogels were rinsed with Hanks' Balanced Salt Solution (HBSS) three times.

3.5.2.2 *Extract exposure assays*

To evaluate the cytotoxic effect of the extracts of pectin and pectin/p-rGOn hydrogels, cells were seeded in 48-well plates at a density of 1×10^4 cells/well for HDFn human dermal neonatal fibroblasts), and at a cell density of 4×10^4 cells/well for A-431 human epidermoid carcinoma cells, incubated at 37 °C and 5% CO₂.

Hydrogels with different concentrations of p-rGOn (0, 0.09, 0.15 wt. %) with 100 µL were produced. After the hydrogels were thoroughly disinfected with ethanol at decreasing

concentrations and rinsed with HBSS, the hydrogels were incubated in complete DMEM for 24h at 37 °C. Upon sub-confluence (24h), cell culture medium was replaced by the culture medium that contained each hydrogel, using a final volume of 250 µL/well.

Then, cell viability was quantified using a resazurin assay. Briefly, the hydrogel culture medium was removed, and cells were incubated in 10% (v/v) resazurin reagent (Sigma-Aldrich, St. Louis, MO, USA) prepared in complete culture medium at 37 °C and 5% CO₂ for 2h. Fluorescence ($\lambda_{ex/em}$ = 530/590 nm) of the supernatant was measured using a microplate reader spectrophotometer (Synergy Mx, Bio-Tek Instruments, Winooski, VT, USA). Controls for cell viability were performed by incubating cells with complete DMEM. Data for each sample were normalized to the control and results are presented as mean % of control and standard deviation. Assays were performed in three replicates for each condition. According to ISO10993-5, the toxicity limit was defined at 70%.

3.5.2.2 Direct contact assays

Pectin only and 0.18 wt.% GBM cylindrical hydrogels with 100 µL were produced. After the hydrogels were disinfected with ethanol at decreasing concentrations and rinsed thrice with HBSS, as previously described, and the hydrogels were incubated in complete DMEM overnight. The next day, the medium was taken and 100 µL of cells (A-431 or HFF-1) were seeded on the surface of the hydrogels. As a control, cells were seeded in the absence of hydrogel. Cells were allowed to adhere for 2h, after which the rest of the complete culture medium was carefully added, to complete a final volume of 600 µL. Cells were incubated at 37 °C and 5% CO₂ for 24h. For A-431 cells, the seeding was done at a final density of 4×10^4 cells/well and for HFF-1 at a density of 1×10^4 cells/well. For A-431, after 24h, cells were irradiated or not with NIR light for 30 minutes. The next day, metabolic activity was measured with resazurin assay, like previously described. In short, the medium was removed, and cells were incubated in 10% (v/v) resazurin reagent (Sigma-Aldrich, St. Louis, MO, USA) prepared in complete culture medium at 37 °C and 5% CO₂ for 2h. Fluorescence ($\lambda_{ex/em}$ = 530/590 nm) of the supernatant was measured using a microplate reader spectrophotometer (Synergy Mx, Bio-Tek Instruments, Winooski, VT, USA). Assays were performed in triplicate, with four replicates for each condition. According to ISO10993-5, the toxicity limit was defined at 70%.

3.5.3 Cell Morphology

Cell morphology was evaluated using fluorescence microscopy 24h after seeding and 24h after treatment (48h after seeding). Cells were stained for f-actin using phalloidin (Alexa Fluor 488 nm, Invitrogen) and nuclei using 4',6'-diamino-2-fenil-indol (DAPI). The cells were washed three times with HBSS and fixed with 4 % (w/v) paraformaldehyde (PFA) in PBS for 15 min at room temperature. After, the cells were washed three times with HBSS and incubated for 10 min

with 0.2 % Triton X-100 (v/v) in HBSS and washed three times with HBSS, at room temperature, to assure cell permeation. The cells were blocked with 1 % Bovine Serum Albumin (BSA) in HBSS (w/v) at room temperature, for 30 min and washed again three times with HBSS, to reduce the non-specific background. Lastly, samples were incubated with phalloidin (1:80) and DAPI (1:1000) solution, diluted in BSA/HBSS solution overnight at 4 °C, covered from light. The following day, samples were washed three times with HBSS. Samples were observed in Motorized inverted epifluorescence microscope (Leica Microsystems, Germany). Images were taken with LAS X Software and processed with ImageJ 1.53t.

3.6 Statistical Analysis

The statistical analysis in this work were performed using GraphPad Prism software (version 9.3.1.471, San Diego, CA, USA). One-way analysis of variance (ANOVA) with Tukey's tests for multiple comparisons were performed. Differences between experimental groups were considered significant with a confidence interval of 95 %, whenever $p < 0.05$.

Chapter 4: Results

4.1 GBM Characterization

4.1.1 Physical Characterization

Transmission electron microscopy (TEM) was used to study the morphology of graphene-based materials (GBM) nanosheets, as presented in Figure 4.1. In the images, well-dispersed sheets can be seen, with no agglomerates in either nanographene oxide (GOn) or partially reduced nanographene oxide (p-rGOn) aqueous dispersions at a concentration of 40 $\mu\text{g/mL}$. The aspect of the sheets can confirm the dispersibility and exfoliation degree of the GBM.

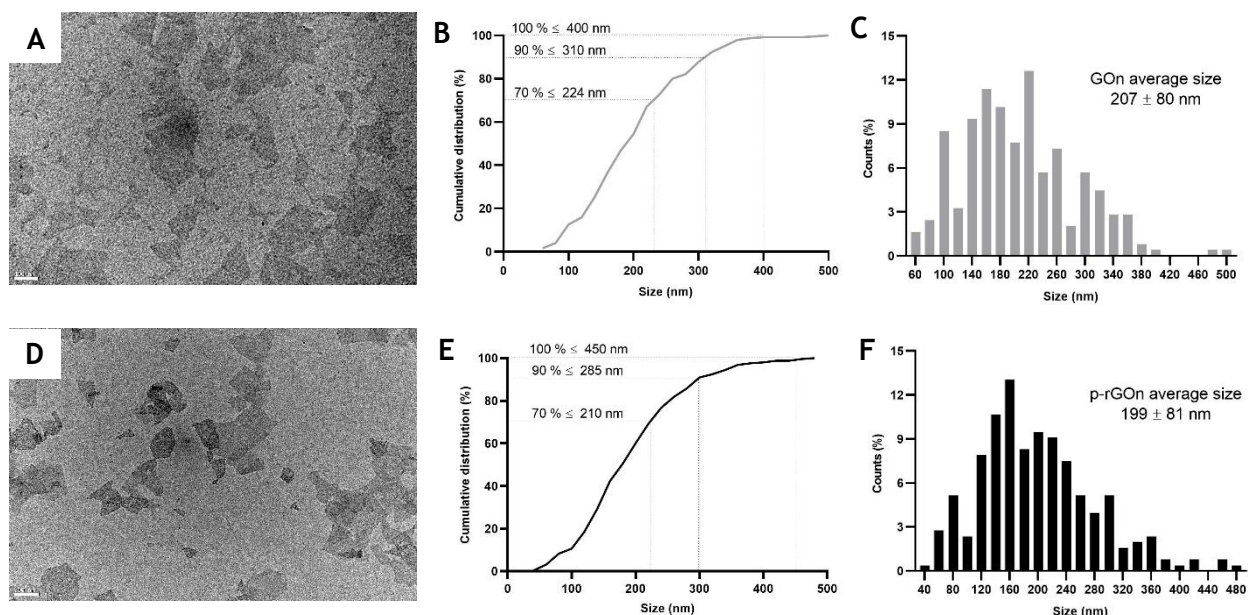


Figure 4.1 – Morphology and lateral dimensions of GOn and p-rGO aqueous dispersions. A, D) Representative TEM images of GOn (A) and p-rGOn (D) at 40 $\mu\text{g mL}^{-1}$. Scale bar represents 200 nm. B, E) Cumulative percentage of size distribution of GOn (B) and p-rGO (E). C, F) Size distribution of GOn (C) and p-rGO (F) in percentage.

Lateral dimensions of the produced GBM were determined by the analysis of TEM images. For GOn and p-rGOn, 70% of particles have sizes below 224 and 210 nm, 90% below 310 and 385 nm and, 100% below 400 and 450 nm, respectively. Histograms with the measurements of the particle size of GOn and p-rGOn is present in Figures 4.1.C and 4.1.F, respectively. On average, GOn has a particle size of 207 ± 80 nm and p-rGOn as an average size of 199 ± 81 nm.

Higher sizes for particle size were expected with the Zetasizer equipment, since with the DLS principle, a spherical model of the particles is assumed, whilst GBM are 2D-sheets. GOn and p-rGOn presented a particle size of 206.7 ± 5.0 nm and 247.0 ± 8.0 nm, respectively. The value of the polydispersity index (PDI) ranges between 0.0 (for samples with perfectly uniform particle size) and 1.0 (for samples that are very polydisperse, having multiple particle size populations) [77]. If the PDI value sits between 0.2 and 0.5, it can indicate that there is a wide particle size distribution [78], which is the case of the produced GBM, since GOn and p-rGOn had PDI values of 0.350 ± 0.017 and 0.361 ± 0.024 , respectively.

Table 4.1 – Particle size and polydispersity index of GOn and p-rGOn aqueous dispersions at a concentration of 10 $\mu\text{g/mL}$, determined by DLS with a Zetasizer equipment ($n = 3$).

GBM	Size (nm)	Polydispersity Index (PDI)
GOn	206.0 ± 5.0	0.350 ± 0.017
p-rGOn	247.0 ± 8.0	0.361 ± 0.024

The surface charges of GOn and p-rGOn were analysed by Zetasizer. In Figure 4.2, zeta potential values are presented. For GOn, the obtained value was -31.9 ± 0.81 mV and, for p-rGOn was -42.8 ± 0.63 mV. For both materials, the surface charge values were under -30 mV, which indicates high water stability [77].

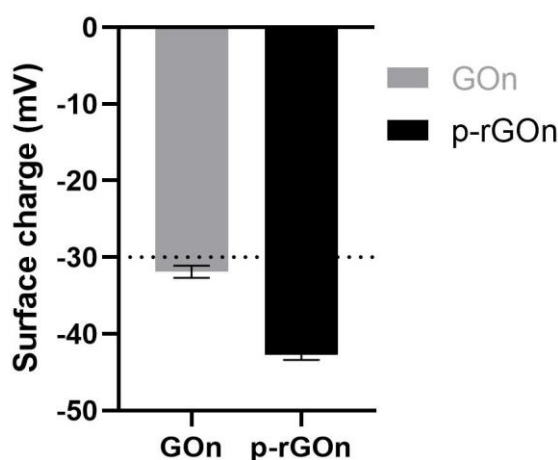


Figure 4.2 – Surface charge of GOn and p-rGOn aqueous dispersions at a concentration of 10 $\mu\text{g/mL}$, determined by Zetasizer equipment ($n = 3$). The dashed line indicates the good water stability limit set at -30 mV.

4.1.2 Chemical characterization

Fourier-transform infrared spectroscopy (FTIR) spectra were obtained to analyse the presence of GOn oxygen functionalities and its partial reduction into p-rGOn (Figure 4.3). In the GOn spectra, a broad band with wavenumbers ranging between 3000 cm^{-1} and 3600 cm^{-1} can be seen, which corresponds to O-H stretching vibrations. These values are usually attributed to adsorbed water molecules, hydroxyl, and carboxyl groups. Around 1728 cm^{-1} , the C=O stretching vibrations are present, which correspond to the carbonyl and carboxyl molecules. Due to the unoxidized graphite, the stretching of cyclic alkaline (C=C) is present at around 1618 cm^{-1} . The presence of ethers can be attested by the absorption bands around 1163 cm^{-1} and 1037 cm^{-1} corresponding to C-O stretching vibrations and through epoxides with C-O bending vibrations around 903 cm^{-1} [24, 78]. In the p-rGOn spectra, a significant difference can be seen, with a reduction of the intensity of the oxygen-containing groups in comparison to GOn, confirming that the reduction was successful. Around 3500 cm^{-1} there is a decrease of intensity, indicating that a large number of O-H groups was lost in the reduction process. There are still some oxygen-containing groups present, confirmed by the C=O stretching and C-O bending at approximately 1728 cm^{-1} and 1037 cm^{-1} , respectively.

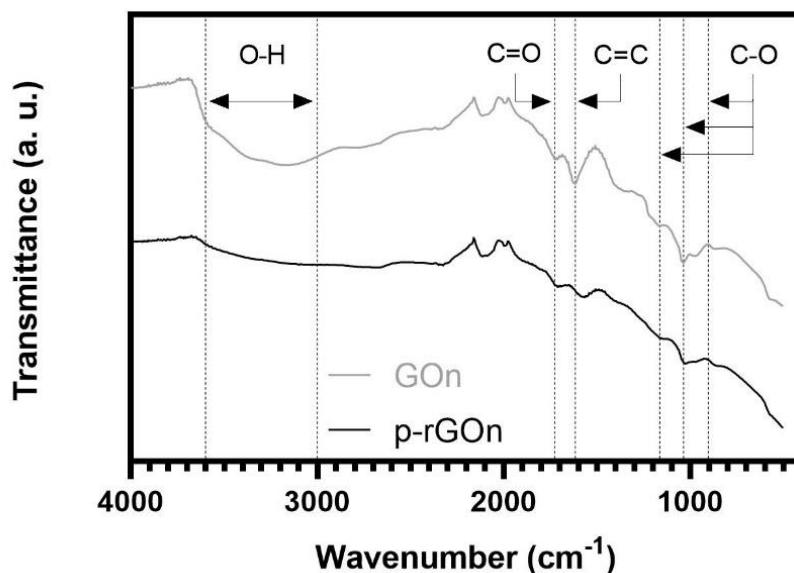


Figure 4.3 – FTIR spectra of GOn and p-rGOn. The lines indicate the contribution of different surface functionalities.

The optical properties of both GBM were analysed by Ultraviolet-visible spectroscopy (UV-Vis) (Figure 4.4). For GOn, there is a maximum absorbance peak around 230 nm, resultant from the $\pi - \pi^*$ electronic transitions in sp^2 clusters and a shoulder peak around 300 nm, characteristic of the $n - \pi^*$ transitions of free electron pairs in the oxygen atoms that exist in the double bonds

between carbon and oxygen, from carbonyl and carboxyl groups [24]. The p-rGOn spectrum shows a 3.34-fold increase in near-infrared radiation (NIR) absorbance (810 nm), compared to GOn. In the p-rGOn spectra the shoulder peak is not present, since the reduction of the GBM removed oxygen-containing functional groups.

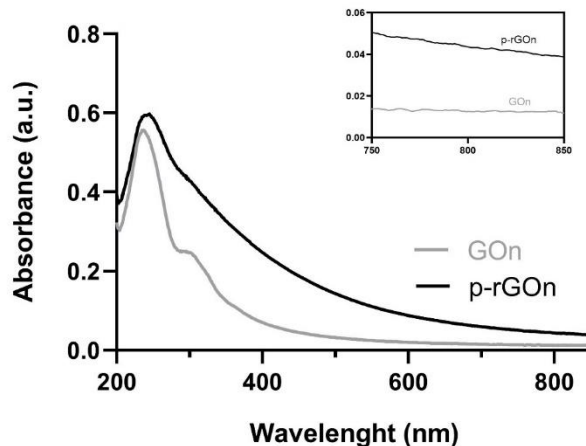


Figure 4.4 – UV-Vis absorption curves of GOn and p-rGOn. The inset shows a zoom-in view of the curves in the NIR range (750-850 nm).

Thermogravimetric analysis (TGA) can be used to study the thermal stability of the produced materials. The obtained thermograms (Figure 4.5) show three main steps regarding the weight loss during the heating of the GBM above 100 °C. Under 100 °C, there is a slight weight loss (approximately 13%), which is attributed to water evaporation. The first weight loss step can be seen between 100 °C and 225 °C. In this step, correspondent to the loss of reactive oxygen-containing functional groups such as carboxyl and epoxy, GOn and p-rGOn presented weight losses of approximately 34.8% and 28.6%, respectively. In the second step, between 225 and 600 °C, GOn had a weight loss of about 15.2% and p-rGOn had a weight loss of 14.5%, which corresponds to the partial combustion of the carbon skeleton and other stable oxygen-containing functional groups, like carbonyls and hydroxyls. A smaller weight loss in p-rGOn in this step suggests the presence of less stable oxygen-containing groups, which further confirms a successful reduction. The third and final step in weight loss, correspondent to the continuous combustion of the carbon skeleton, happened between 600 and 1000 °C. In this last step, GOn and p-rGOn showed weight losses of 23.0% and 8.1%, respectively. In total, GOn presented a weight loss of 86.2% and p-rGOn 66.8%. Since oxygenated groups are thermolabile and the reduction decreased the amount of these functional groups in p-rGOn, p-rGOn allows superior thermal stability when compared with GOn.

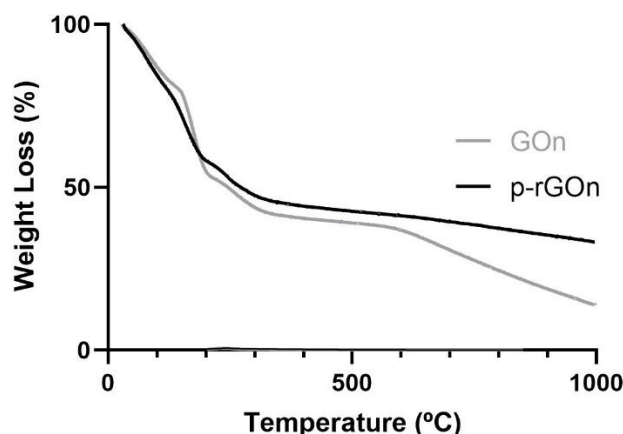


Figure 4.5 – Thermal decomposition of GOn and p-rGOn. TGA curves and weight loss values for GOn (grey line) and p-rGOn (black line), under a nitrogen atmosphere.

X-ray Photoelectron Spectroscopy (XPS) was performed to analyse the oxidation degree and chemical functional groups at the surface of the produced GBM.

As seen in Figure 4.6.A and 4.6.B, GOn exhibited a C at.% of 68.7 and a O at.% of 31.6, which proves that there was a successful oxidation and introduction of oxygen functionalities at the surface. In the C1s spectra of GOn (Figure 4.6.B), there are two large peaks that could be deconstructed into seven peaks. The two large peaks with different binding energies are a result of the formation of sp² and sp³ hybridizations of carbon in the graphitic backbone, which are attributed to C-C (284.80 eV, C1s at.% = 4.19) and C=C (284.20 eV, C1s at.% = 38.79). The third value of binding energy occurs due to single bonds of carbon and oxygen (C-OH) present in hydroxyls. Despite this, the C1s at.% was 0 %, proving that this type of bond was not present in GOn. Double bonds between carbon and oxygen, C=O, attributed to carbonyl groups, were also present (287.50 eV, C1s at.% = 10.5). The existence of carboxyl groups explains the multiple bonds between carbon and oxygen, O-C=O (288.80 eV, C1s at.% = 0.22). The $\pi - \pi^*$ bonds (290.20 eV), that occur due to the presence of delocalized π electrons in the graphene lattice, are not present in GOn since the C1s at.% is 0 [78]. GOn O1s spectra exhibited just one peak, that could be deconvoluted into three peaks (Figure 4.6.C). The first and second binding energy values both have O1s at.% = 0.56 and appear due to C=O bonds in carbonyl groups (531.50 eV) and C-O bonds in hydroxyl groups (533.00 eV), respectively. The third and last peak is caused by the O-C=O bonds in carboxyl groups (533.61 eV, O1s at.% = 98.88). By analysing both C1s and O1s deconvoluted spectra of GOn, it is concluded that the material was well oxidized, since carbon atoms in carbonyl and carboxyl groups are present, with O-C=O bonds dominating the surface chemistry.

In Figure 4.5.D and 4.6.E, it can be seen that p-rGOn presented a C at.% of 70.1 and a O at.% of 29.9, proving a decrease in the oxygen content compared to GOn. Similarly to the C1s spectra of GOn, the C1s spectra of p-rGOn was also deconvoluted in seven peaks (Figure 4.6.E). C-C (284.20 eV) and C=C (284.80 eV) bonds are present as well. Nonetheless, when comparing

to GOn, there was an increase in C at.% of C-C from 4.19% to 27.10%, and a decrease in C at.% of C=C from 38.79% to 20.67%. The most common oxygen functionality in GOn was the C-O-C bond (286.60 eV) and there was a decrease of C at.% from 46.31% to 32.83% after the reduction process to obtain p-rGOn. In p-rGOn spectra, there is also an increase of C at.% in C=O bonds (287.50 eV) to 15.16%, and in the O-C=O bonds (288.80 eV) to 0.76%, showing the effect of the reduction on surface chemistry. The C at.% in $\pi - \pi^*$ bonds (292.00 eV) in p-rGOn show a partial restoration of the aromatic structure, increasing from 0% in GOn to 0.76% [24].

In regard to the p-rGOn O1s spectra, equivalently to GOn O1s spectra, it was also deconvoluted in three peaks (Figure 4.6.F). The spectra show an increase of C=O (531.50 eV, O1s at. % = 4.51) and C-O (532.74 eV, O1s at.% = 4.74) bonds, however a decrease of O-C=O (434.09 eV, O1s at.% = 90.75) bonds could be seen. The C/O ratio of GOn was 2.2, whilst it was 2.3 for p-rGOn, furthermore proving that a successful partial reduction of GOn to p-rGOn occurred.

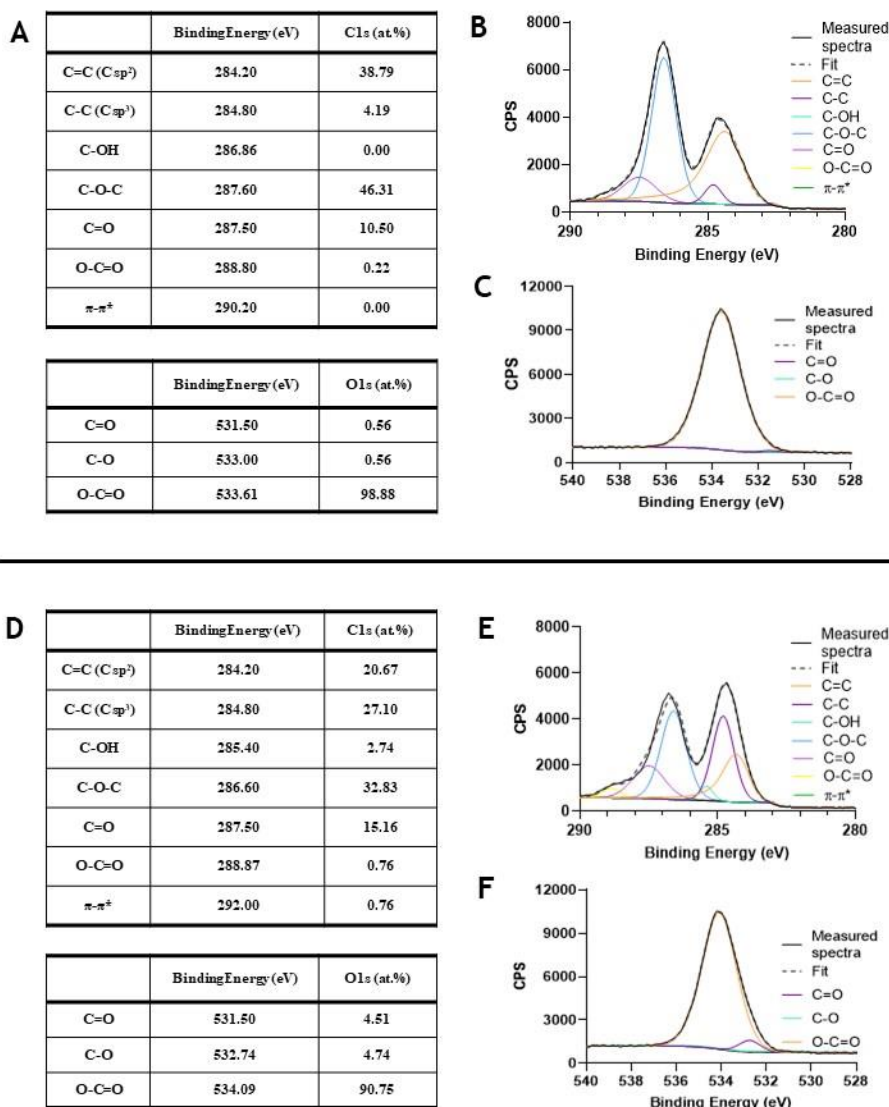


Figure 4.6 – XPS analysis of GOn and p-rGOn. A, D) Atomic composition and content of C 1s and O 1s chemical functional groups resulting from spectra fitting of GOn (A) and p-rGOn (D); B, E) Deconvolution of high-resolution C 1s of GOn (B) and p-rGOn (E); C, F) Deconvolution of high-resolution O 1s XPS spectra for GOn (C) and p-rGOn (F).

4.1.3 Photothermal Properties

With aqueous dispersions of GOn and p-rGOn, at concentrations of 250 and 500 $\mu\text{g/mL}$, the efficiency of these nanomaterials to convert NIR light into heat was evaluated. The samples were placed in an oven, so an atmosphere of 37 $^{\circ}\text{C}$ was maintained throughout the test. With the samples reaching a temperature close to 37 $^{\circ}\text{C}$, the irradiation with a custom-build LED NIR system (810 nm, 150 mW/cm^2) started. The temperature was measured at different time points. In Figures 4.7 and 4.8, the temperature increase during 30 minutes of NIR irradiation with concentrations of 250 and 500 $\mu\text{g/mL}$, respectively, is presented. At 250 $\mu\text{g/mL}$, GOn reached an

average final temperature of 43.1 °C and p-rGOn reached a temperature of 46.7 °C, whilst the control reached 40.5 °C. In total, the water control had a temperature increase of 5.6 °C, GOn and p-rGOn increased 8.9 °C and 12.5 °C, respectively. At 500 µg/mL, the control reached 44.1 °C, having a temperature increase of 7.7 °C, GOn reached a final temperature of 44.6 °C, with a total temperature increase of 8.5 °C whilst p-rGOn reached a temperature of 50.5 °C, with an increase of 14.2 °C in total. At both concentrations of the dispersions, the increase of temperature in GOn is not vastly different from the increase of the temperature in the water control.

With the reduction process, the aromatic structure of p-rGOn was restored, giving the material enhanced NIR absorbance, which allows for higher heat production upon NIR irradiation [79]. It has been proven that mild hyperthermia (43 – 50 °C) can cause damages in cancer cells to a degree that makes them unable to recover [16]. In pre-clinical and clinical settings, tumorous cells have been observed to die at an exponential rate when exposed to temperatures above 42 °C for about 30 minutes [80, 81]. The produced p-rGOn is innovative, since it is stable in water, and has enhanced NIR absorbance, converting the radiation into heat, having enormous potential for the desired phototherapeutic application, and being selected for future studies.

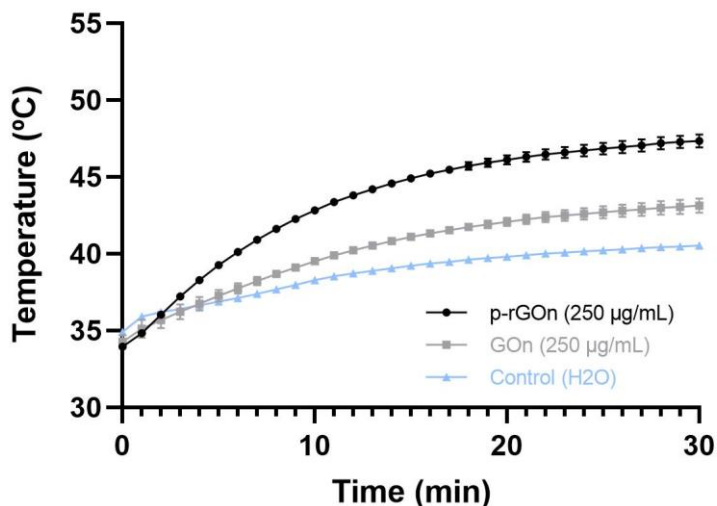


Figure 4.7 – Photothermal heating curves of water, p-rGOn and GOn aqueous dispersions at 250 µg/mL.

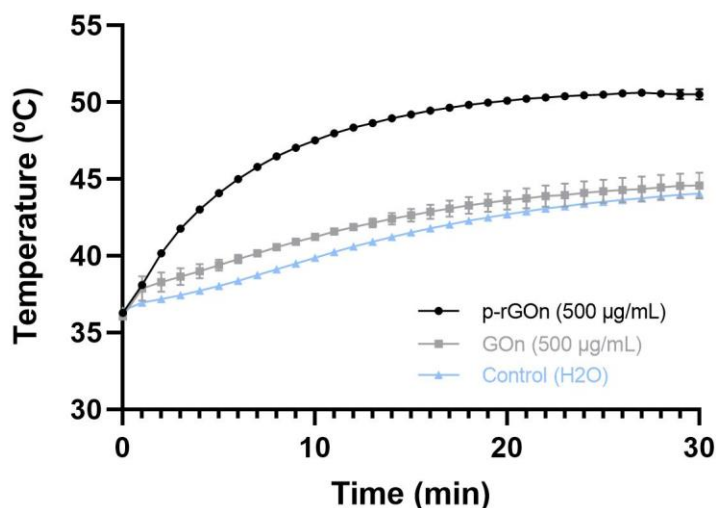


Figure 4.8 – Photothermal heating curves of water, p-rGOn and GOn aqueous dispersions at 500 µg/mL.

In order to take pictures with the thermal camera, the plates were preheated to 37 °C in an oven and taken out to irradiate using the NIR LED system. In Figure 4.9.A and 4.9.C, thermal pictures of the 48-well plate with water as control and aqueous dispersions of the produced GBM at concentrations of 250 and 500 µg/mL, before and after being irradiated with NIR light, are present. Pictures were analysed using the FLIR Research Studio software and use the Rainbow High Contrast filter. Since the atmosphere was at room temperature (RT), the highest temperature reached was not quite as high as the temperature measured when the irradiation was performed in 37 °C atmosphere, because of the equilibrium with RT during the assay.

In Figure 4.9.A, all the chosen samples, marked with a cursor, have temperatures between 36.36 and 36.57 °C. A line that connects the middle points of the wells containing dispersions and control was used to obtain a temperature profile (Figure 4.9.B). In the temperature profile of pre-irradiated samples, it is possible to see that the graph presents 5 major plateaus, corresponding to the space of the actual well. The peaks in between the plateaus correspond to the edges of the wells and the space between wells. All plateaus reach values approximately to 36.5 °C, so all the dispersions and the control are starting the irradiation assay at an accepted biological temperature. After the samples were irradiated for 30 minutes, a picture was taken (Figure 4.9.C). The highest temperature captured is for p-rGOn at 500 µg/mL, showing a temperature increase to 48.7 °C, whilst the lowest temperature happened for the water control, as expected, having reached only 40.86 °C. The second highest temperature was achieved by p-rGOn at 250 µg/mL, with a final temperature of 47.35 °C. The temperature reached by GOn at 500 µg/mL was 41.13 °C, being lower than the temperature reached by GOn at 250 µg/mL (44.05 °C). This could be explained by the placement of the GOn at 500 µg/mL samples, that were placed by the edge of the 48-well plate, reaching equilibrium with RT faster. This same reason can explain why the samples in the first and last columns do not reach higher temperatures. In Figure 4.9.D, there is a temperature

profile of the line connecting the cursors in the middle of each well for each sample. Similarly to the previous temperature profile, there are 5 plateaus corresponding to the different wells and peaks corresponding to the edges of the wells and space in between them. In this profile, the differences in temperature reached are clear, with the highest plateaus happening with p-rGOn samples. As expected, the control showed the lowest final temperature.

In Figure 4.10, a photograph showing the disposition of the samples and control can be seen. From top to bottom the disposition of the samples is: p-rGOn and GOn at a concentration of 250 $\mu\text{g/mL}$, water control, p-rGOn and GOn at a concentration of 500 $\mu\text{g/mL}$.

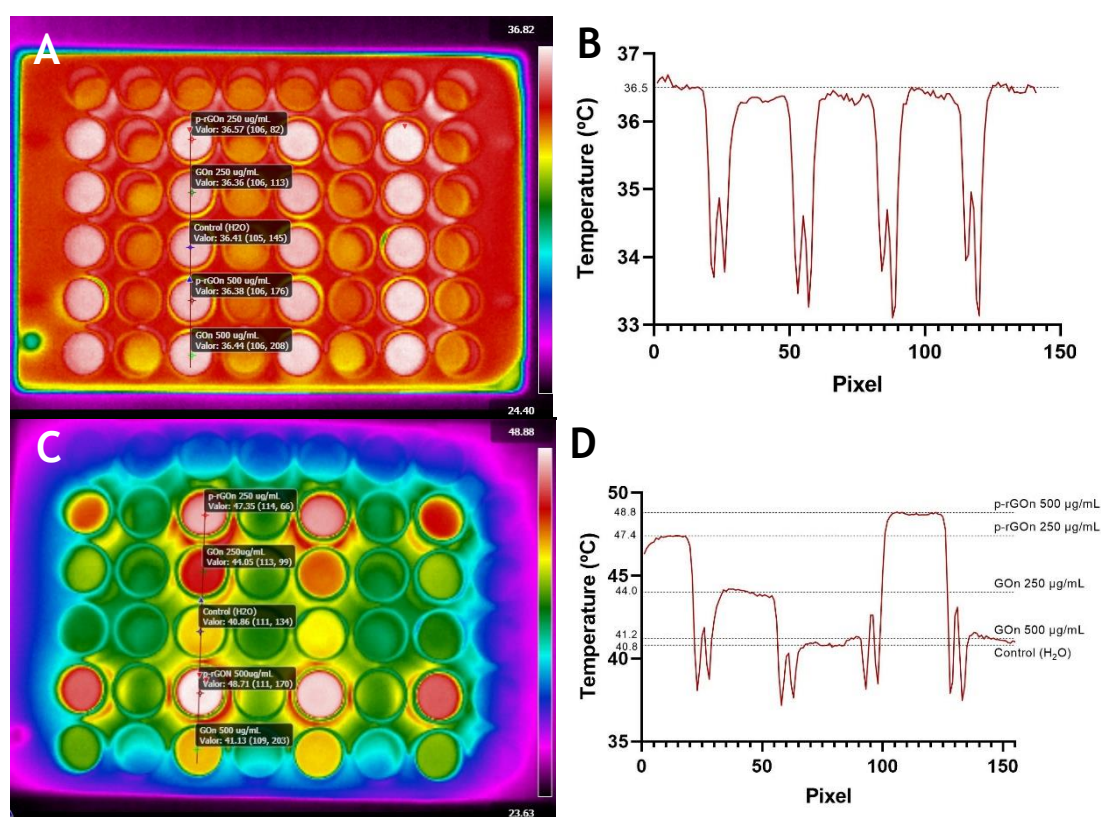


Figure 4.9 – Thermal camera images and temperature profiles of represented lines of the heating of GBM dispersions. A) Thermal image before irradiation; B) Temperature profile of line connecting samples before irradiation; C) Thermal image after 30 minutes of NIR irradiation; D) Temperature profile of line connecting samples after irradiation.



Figure 4.10 – Disposition of GBM dispersions and water control in 48 well-plate to be NIR irradiated. From top to bottom: p-rGOn and GOn at a concentration of 250 µg/mL, water, p-rGOn and GOn at a concentration of 500 µg/mL.

4.2 Hydrogels Characterization

Several concentrations of polymer, calcium carbonate (CaCO_3) and glucono delta-lactone (GDL) were tested in order to evaluate its impact on hydrogel formation. In table 4.2, all the tested combinations of concentrations are presented.

Table 4.2 – Polymer and CaCO_3 :GDL concentrations used in hydrogel production optimization. The highlighted combination was used for further production.

1.5% polymer	2% polymer	2.5% polymer	3% polymer
100:200 mM	100:200 mM	15:30 mM	10:20 mM
		20:40 mM	10:30 mM
		50:100 mM	20:40 mM
		90:180 mM	50:100 mM
			70:140 mM
			85:170 mM
			100:200 mM

After systematic evaluation of all formulations, it was established that the best combination was 2.5% polymer with 15 mM of CaCO_3 and 30 mM of GDL. In Figure 4.11 the fabrication process of pectin hydrogels, is displayed. With this combination, it was possible to handle the pre-crosslinked hydrogel in order to form hydrogel cylinders.



Figure 4.11 – Production of cylindrical pectin hydrogels using QGel® 3D disc caster. A) Deposition of pre-crosslinked hydrogel; B) Internally cross-linked produced hydrogels; C) CaCl_2 bath (external gelation) for hydrogel reinforcement.

4.2.1 Physical Characterization

For mechanical testing, three samples of each condition were produced, in a way to compare the influence of each variable on the mechanical properties of the hydrogels:

- 1) 0, 0.09 and 0.18 wt.% GBM hydrogels as prepared (only internal gelation);
- 2) 0, 0.09 and 0.18 wt.% GBM hydrogels as prepared reinforced with external gelation bath;
- 3) 0, 0.09 and 0.18 wt.% GBM hydrogels (only internal gelation) incubated in DMEM overnight;
- 4) 0, 0.09 and 0.18 wt.% GBM hydrogels reinforced with external gelation bath incubated in DMEM overnight.

The setup for the compression test is showed in Figure 4.12. In order to calculate the Young's modulus, the stress-strain curve was obtained from the collected data, and then the slope in the region between 5 and 15% of strain was calculated. Afterwards, the Young's modulus of each formulation was calculated as the average value of the three samples



Figure 4.12 – Compression test using the texture analyzer for A) GBM hydrogel; B) pectin hydrogel; C) pectin incubated in DMEM hydrogel samples.

In table 4.3, the results of the compression tests for each condition are displayed by average Young's modulus, standard deviation and R^2 value (from the slope in the region between 5 and 15% of strain). In Figure 4.13. the average Young's modulus for each condition is also shown, with the indication of standard variation.

Table 4.3 – Average Young's modulus (E), standard deviation (SD) and average R^2 of each tested condition.

Hydrogel			E(kPa)	SD	R^2
0 wt.% GBM (pectin)	as prepared	w/ CaCl_2	21.85	6.01	0.9968
		w/o CaCl_2	11.50	2.69	0.9992
	incubated in DMEM	w/ CaCl_2	16.50	5.37	0.9902
		w/o CaCl_2	6.60	4.17	0.9746
0.09 wt.% GBM	as prepared	w/ CaCl_2	22.47	3.11	0.9977
		w/o CaCl_2	12.30	1.85	0.9965
	incubated in DMEM	w/ CaCl_2	18.25	4.31	0.9908
		w/o CaCl_2	8.50	1.56	0.9939
0.18 wt.% GBM	as prepared	w/ CaCl_2	26.73	4.93	0.9994
		w/o CaCl_2	12.63	1.82	0.9989
	incubated in DMEM	w/ CaCl_2	21.88	6.13	0.9961
		w/o CaCl_2	9.60	1.27	0.9955

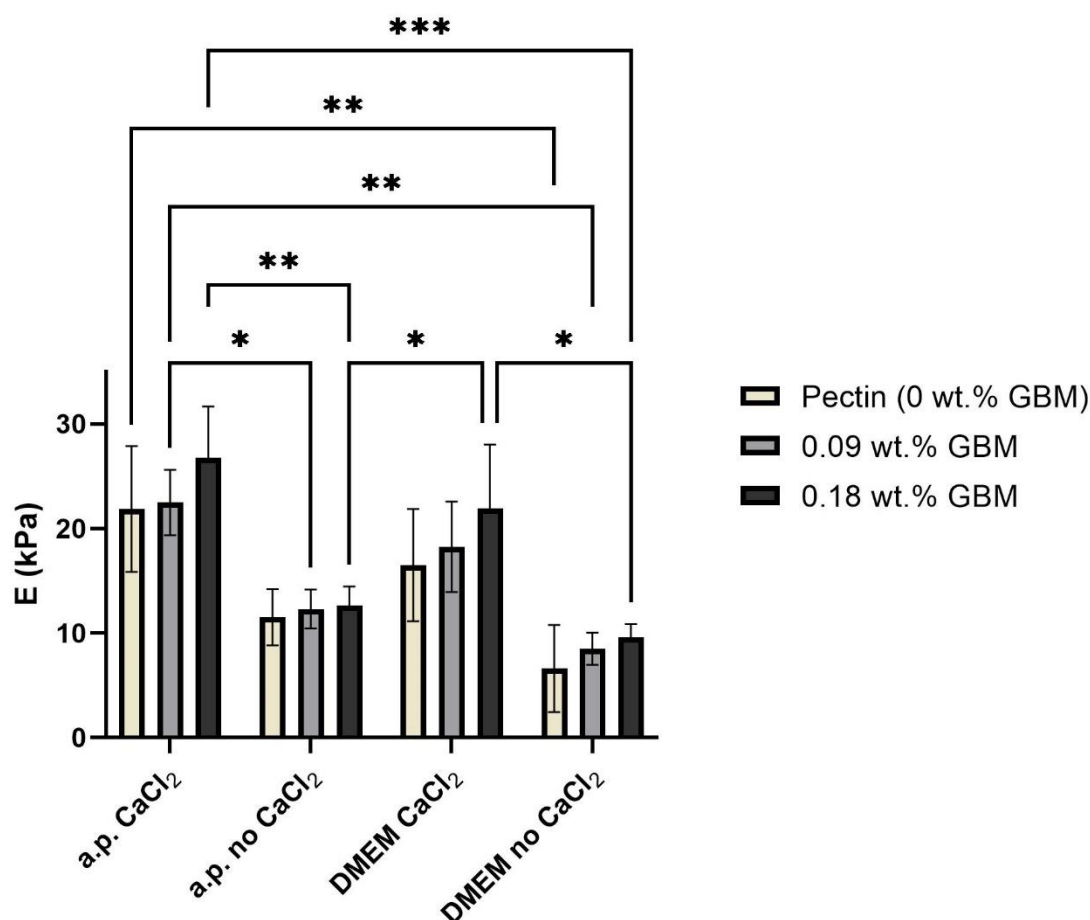


Figure 4.13 – Effects on Young's modulus of all conditions. The conditions are hydrogels as prepared (a.p.) with and without CaCl₂ bath and hydrogels incubated in DMEM overnight with and without CaCl₂ bath. Statistically significant differences are present as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Although the differences are not statistically different, a tendency for an increase in Young's modulus with the increase in GBM content in the hydrogel can be seen. For the conditions where the hydrogels were in a CaCl₂ bath, the Young's modulus is higher. Hydrogels are stiffer when they have an extra gelation step, with the CaCl₂ bath, especially after they have just been prepared, having a higher Young's modulus. After DMEM incubation, the hydrogels tend to become softer, resulting in lower Young's modulus. This could be caused by the monovalent ions in DMEM, that compete with the bivalent ions responsible for reticulating the hydrogel. The obtained results were as expected, since it was possible to notice the difficulty in handling the hydrogels when they were not in a bath of CaCl₂, being almost impossible to handle those hydrogels after incubation without breaking, since they became too soft. Supplementing DMEM is a possibility to avoid the breakage in the future. The results help prove the need for an external gelation step for assays where the hydrogels had to be incubated in DMEM overnight, since without that step the hydrogels became too soft, being too difficult to handle. GBM have long been known for their mechanical properties, with graphene oxide (GO) having an elastic modulus of 220 GPa [82], being used with the purpose of tuning mechanical properties of scaffolds before [83].

The human skin is highly anisotropic and viscoelastic, resulting in a very ample range of Young's modulus. The Young's modulus of the skin can range from 5 kPa to 140 MPa, depending on the region of the skin, the type of mechanical examination, and whether it relates to the dermis or dermis and epidermis. The obtained values are similar to those obtained through indentation techniques in forearm samples (4.75 – 33.4 kPa)[84], which can be important since these are produced with the intent of skin cancer treatment, being applied to the skin.

4.2.2 Photothermal Properties

The efficiency of composite hydrogels to convert NIR light into heat was evaluated. The samples were firstly placed in an oven for pre-heating (37 °C) and were subsequently irradiated with a custom-build LED NIR system (810 nm, 150 mW/cm²). The temperature was measured at different time points. In Figure 4.13 the temperature variation throughout 30 minutes of NIR irradiation of DMEM (as a control), and hydrogels with 0, 0.09 and 0.18 wt.% GBM is presented. The highest temperature achieved was, as expected, the hydrogel with highest GBM content. Hydrogels with 0.18 wt.% GBM reached an average final temperature of 48.6 °C, hydrogels with 0.09 wt.% GBM reached a temperature of 46.9 °C, and pectin only hydrogels (0 wt.% GBM) reached a final temperature of 36.9 °C, whilst the DMEM control reached 32.8 °C.

In total, the DMEM control had a temperature decrease of 3.9 °C, pectin only hydrogels had an average decrease of just 0.2 °C, whilst 0.09 and 0.18 wt.% GBM hydrogels had a temperature increase of 10.0 °C and 11.5 °C, respectively. Since the control and the pectin only hydrogels do not absorb NIR as hydrogels with GBM do, the temperature decreases, since there is an equilibrium of the temperature with RT. Still, it is important to note that pectin only hydrogels can maintain the temperature better than DMEM only.

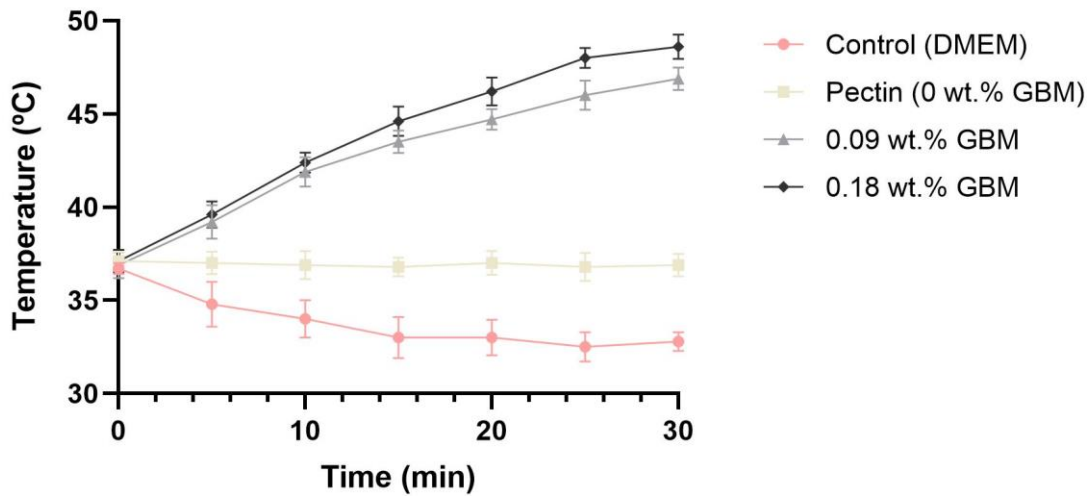


Figure 4.14 – Photothermal heating curves of DMEM and hydrogels with 0, 0.09 and 0.18 wt.% GBM.

In order to take pictures with the thermal camera, the plate was also pre-heated to 37 °C in an oven and taken out to irradiate using the NIR LED system. In Figure 4.15.A, a thermal picture of the 48-well plate with hydrogels and DMEM control, after being irradiated with NIR light for 30 minutes, is present. Pictures were analysed using the FLIR Research Studio software and use the Rainbow High Contrast filter.

After the samples were irradiated for 30 minutes, a picture was taken (Figure 4.15.A). The highest temperature captured is for 0.18 wt.% GBM hydrogels, showing a temperature increase from 37 °C to 46.48 °C, whilst the lowest temperature happened for the DMEM control, as expected, having reached only 32.63 °C. The second highest temperature was achieved by 0.09 wt.% GBM hydrogels, with a final temperature of 45.71 °C. Pectin only hydrogels reached a temperature of 36.35 °C. Similarly to the previous assay, the temperature of the control and the pectin only hydrogel decreases, since there is an equilibrium of the temperature with RT. Still, the pectin only hydrogel can maintain the temperature better than control only. The temperatures in the first and last columns with samples is not as high as in the middle one, which could be explained by the samples reaching equilibrium with RT faster since they do not have other heating samples surrounding them.

In Figure 4.15.B, there is a temperature profile of the line connecting the cursors in the middle of each well for each sample. There are 4 plateaus corresponding to the different wells and peaks corresponding to the edges of the wells and space in between them. In this profile, it is seen that 0.18 wt.% GBM hydrogels reach the highest temperature. As expected, the control showed the lowest final temperature. It is also possible to observe that the point where 0.09 wt.% GBM hydrogel reaches its highest temperature is in the pixels closer to the 0.18 wt.% hydrogel, so the

latter may be influencing the temperature increase of the 0.09 wt.% GBM due to the sample proximity.

In Figure 4.16, a photograph showing the disposition of the samples and control can be seen. From top to bottom the disposition of the samples is: DMEM control, pectin only hydrogel (0 wt.% GBM), 0.09 wt.% GBM hydrogel and 0.18 wt.% GBM hydrogel.

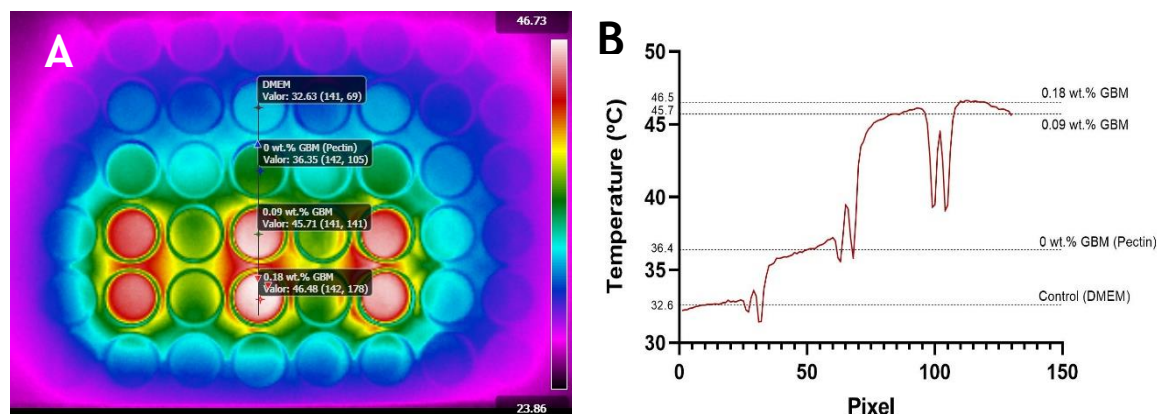


Figure 4.15 – Thermal camera image and temperature profile of represented line after 30 minutes of NIR irradiation of hydrogel samples. A) Thermal image of hydrogels and control after 30 minutes of irradiation; B) Temperature profile of line connecting samples after irradiation.



Figure 4.16 – Disposition of produced hydrogels and DMEM control in 48 well-plate to be NIR irradiated. From top to bottom: DMEM; pectin hydrogel (0 wt.% GBM); 0.09 wt.% GBM hydrogel and 0.18 wt.% GBM hydrogel.

Hydrogels with 0.18 wt.% GBM can reach temperatures higher than 0.09 wt.% GBM hydrogels. Even with only a slight temperature difference, that difference can be decisive in reaching mild hyperthermia temperatures, therefore, for direct cell contact assays, pectin only and 0.18 wt.% p-rGOn were used.

4.2.3 Printability

To ensure that the composite material was suitable for printing, the viscosity of the hydrogel precursor solution had to be modulated. In table 4.4, all the tested combinations of concentrations of polymer, CaCO_3 and GDL are present.

Table 4.4 – Polymer and CaCO_3 :GDL concentrations used in hydrogel printability optimization. The highlighted combinations showed potential for future hydrogel bioprinting.

2% polymer	2.5% polymer
10:20 mM	5:10 mM
15:30 mM	5:15 mM
20:40 mM	7.5:15 mM
25:50 mM	10:20 mM
30:60 mM	10:30 mM
40:80 mM	
50:100 mM	

Several of the tested combinations appeared to have suitable viscosity for bioprinting, with the best performing in filament formation being 2% polymer with 25 mM of CaCO_3 and 50mM of GDL. Hydrogels with 2% polymer and 15:30 mM of CaCO_3 :GDL and 20:40 mM of CaCO_3 :GDL also showed potential as they also had viscosity that allowed them to be poured out of the syringe as stable filaments.

In Figure 4.17, the aspect of the deposition of some of the tested biomaterial ink formulations can be observed. The pre-crosslinked hydrogel was placed on a syringe, with a 0.41 mm nozzle. In Figures 4.17.A and 4.17.B, we can see that the solution is too liquid, whilst in Figures 4.17.F and 4.17.G, there is too high crosslinking, resulting in a filament with aggregates, that is prone to break. Figures 4.17.C, 4.17.D and 4.17.E show solutions that can form stable filaments. In Figure 4.18, a photograph of the deposition of filaments of hydrogel with 2% polymer concentration, and 25:50 mM of CaCO_3 :GDL can be seen.

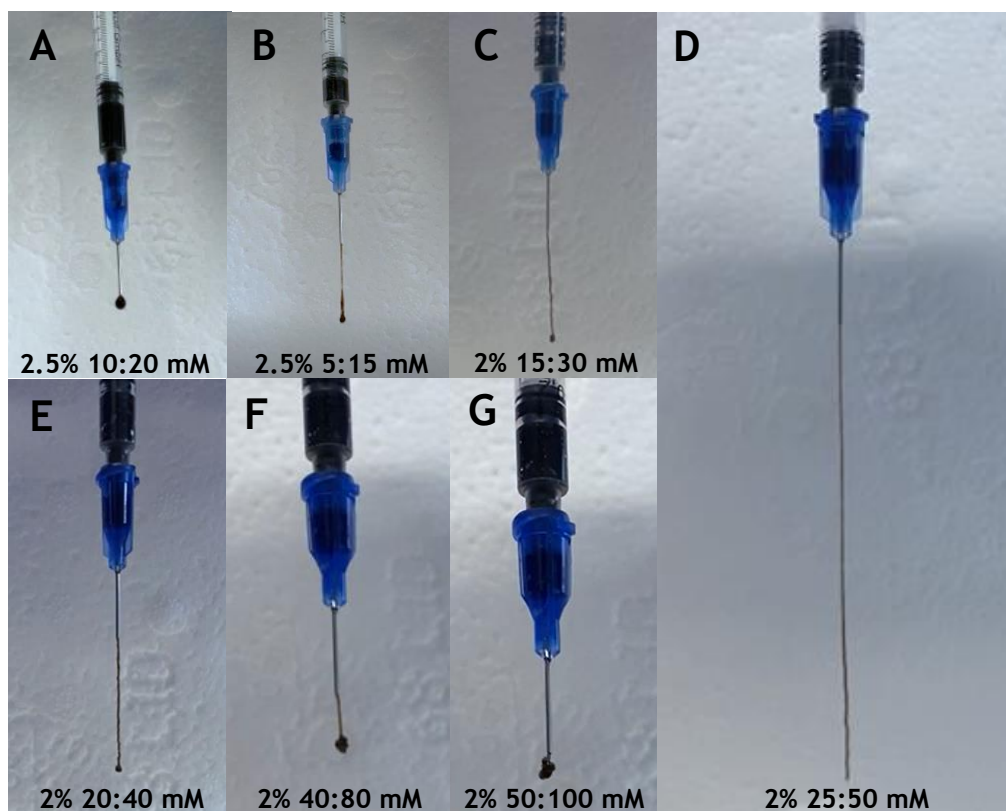


Figure 4.17 – Evaluation of the printability of pectin/GBM composite hydrogels through the modulation of polymer, CaCO_3 and GDL concentrations.

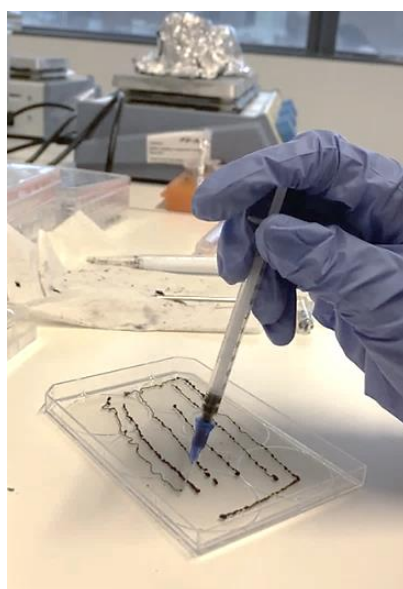


Figure 4.18 – Deposition of hydrogel filaments, with 2% polymer concentration, and 25:50 mM of CaCO_3 :GDL.

4.3 *In vitro* biological studies

In Figure 4.19, the normal appearance and morphology of the used cell lines in culture is displayed. In Figure 4.19.A, A-431 human epidermoid carcinoma cells are present and in Figure 4.19.B, HFF-1 human foreskin fibroblasts can be seen. A-431 cells present their normal epithelial-like morphology, growing in clusters. HFF-1 cells present their usual spindle shape.

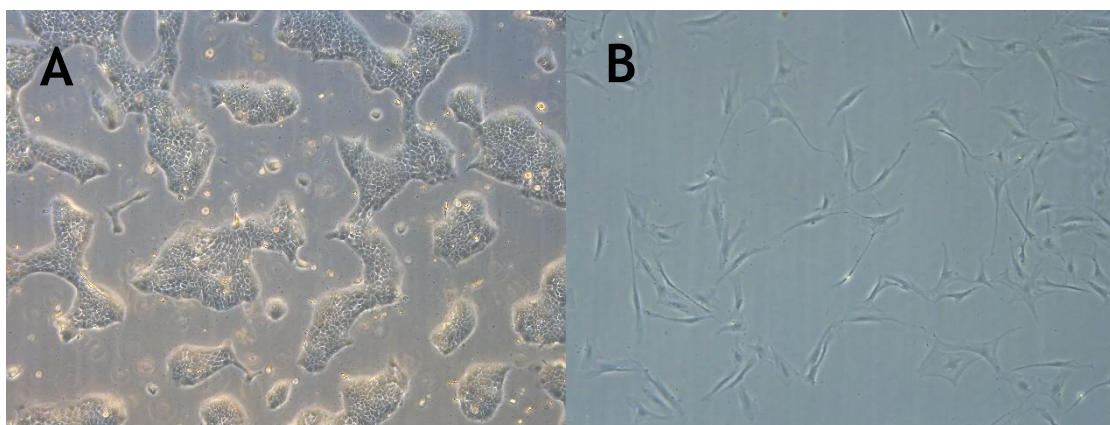


Figure 4.19 – Optical images of tested cell lines in culture. A) A-431 cell line; B) HFF-1 cell line. Magnification used was 5x.

4.3.1 Extract exposure

The cytotoxicity of the produced hydrogels was assessed through an extract exposure assay, analysing if any GBM particles would leach out of the hydrogel samples and eventually cause toxicity. In Figure 4.20.A, the obtained results for HDFn fibroblasts are present. In Figure 4.20.B, results relative to A-431 cells can be seen. Results were normalized concerning values of the cells incubated in complete DMEM, never having been exposed to any hydrogel extract (negative control). Results are presented as average and standard deviation. In HDFn, the lowest value obtained happened for pectin/GBM hydrogels with 0.09 wt.% p-rGOn, with a cell viability of 91.14%. For all other hydrogels (0 and 0.18 wt.% p-rGOn), values were approximately 100%. In A-431, the lowest value obtained happened for pectin/GBM hydrogels with 0.18 wt.% p-rGOn, with a cell viability of 91.63%. For the other hydrogels (0 and 0.09 wt.% p-rGOn), values were approximately 100%. For all conditions, there was no significant difference in cell viability between the cell control and the hydrogels, so if some particle leaching happened, it was not enough to cause any drops in viability either for cancer or healthy (fibroblast) cells. The leaching of any particles was invisible to the naked eye.

It is clear from Figure 7.20 that all extracts of the pectin/GBM hydrogels (0, 0.09 and 0.18 wt.% p-rGOn) have no discernible cytotoxic effects on cells' metabolic activity because it is always higher than 70%, which is the threshold at which a sample is considered to have cytotoxic potential, according to ISO 10993-5:2009(E).

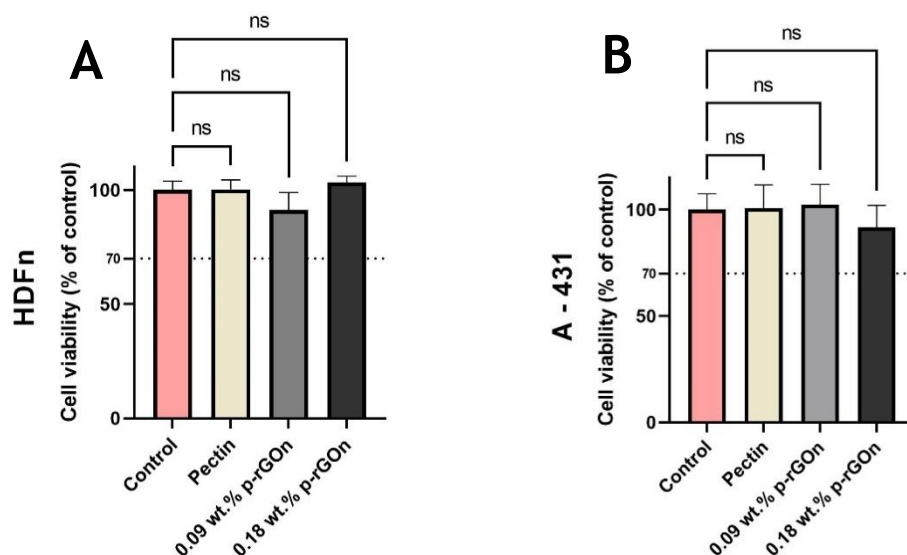


Figure 4.20 – Cell viability after 24 hours of indirect contact as evaluated by resazurin assay for A) HDFn cells; B) A-431 cells. Results are normalized concerning values of the control incubated in complete DMEM. Results are presented as average and standard deviation. Dashed line marks the toxicity limit at 70 % (ISO10993-5).

4.3.2 Direct contact – Preliminary tests

The cytotoxicity and photothermal effect of the hydrogels were assessed through a preliminary direct contact assay of the cells cultured on the surface of the hydrogels. The cell adhesion was also evaluated. Looking at the results of the resazurin assay (Figure 4.21.A and Figure 4.21.B), a decrease in cell viability was seen for both pectin and GBM hydrogels when the results are normalized to cell only control (Figure 4.21.A). For non-irradiated pectin and GBM hydrogels, a decrease in cell viability to 39.07% and 6.03%, respectively, was observed. After 30 min of NIR exposure, the cell viability for pectin hydrogels was 40.47% and for GBM hydrogels was 5.55%. Since the values for both conditions (NIR/no NIR) were similar, it can be suggested that the effect of the irradiation was not visible when the results are compared with cell only control. However, these results could be related to the cells not adhering to the surface of the hydrogel. Figure 4.21.C. shows that the number of adhered cells in pectin and GBM hydrogels is much lower compared to the control. GBM hydrogel have an even lower number of cells than pectin hydrogels. This could indicate that the hydrogels have a cytotoxic effect on the cells, or that the cells cannot adhere to the hydrogels. Since the main components of the hydrogels (pectin and GBM) previously proved they were biocompatible, it can be assumed that the low cell number

is related to the poor cell adhesion of the hydrogels [21, 23, 24, 32]. This could mean that the RGD density needs to be optimized, and/or the mechanical properties were not suited for A-431 cells. GBM hydrogels previously showed higher values of Young's modulus, resulting in a stiffer hydrogel, which may not be ideal for the adhesion of A-431 cells. Figure 4.21.C also shows that NIR irradiation did not change the morphology of A-431 cells.

Considering the poor cell adhesion, resazurin assay results were normalized for the positive control (pectin hydrogels) (Figure 4.21.B). When compared to the positive control, GBM hydrogels showed a cell viability of 15.05% when cells were not irradiated, and 12.53% after 30 min irradiation. Even if not statistically different, the GBM hydrogels caused a decrease in cell viability when irradiated, which can suggest they have a slight photothermal effect. This effect was not visible in the results normalized to cell only control. To further confirm this result, the cell adhesion needs to guarantee ideal cell confluence to avoid other potential effects of the low cell number.

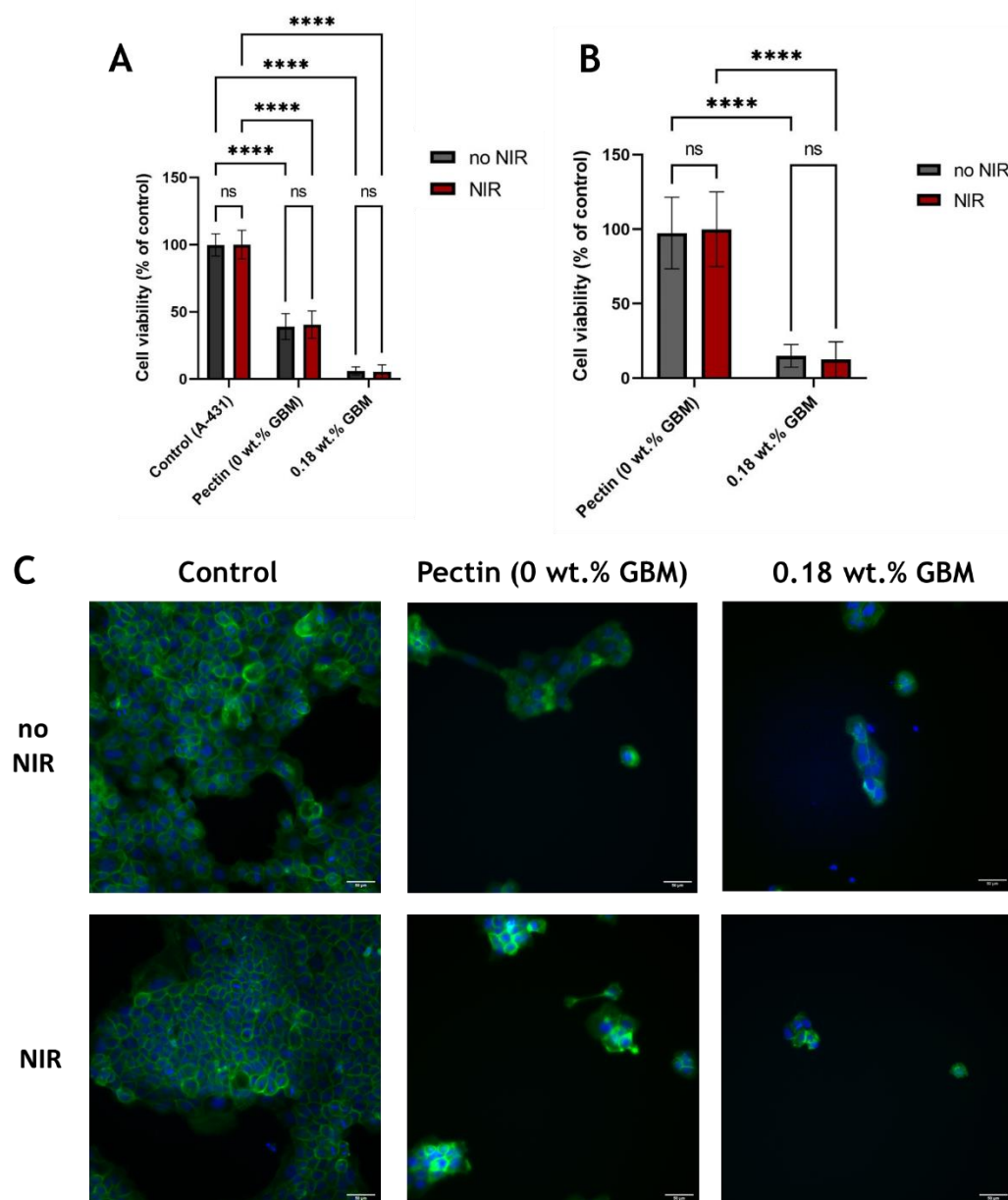


Figure 4.21 – Cytotoxicity of pectin and p-rGOn hydrogels on A-431 cells, with and without 30 min irradiation. A) Cell viability of A-431 cells, normalized to cell only control; B) Cell viability of A-431 cells, normalized to pectin hydrogel. Statistically significant differences are present as ****, $p < 0.0001$; C) Motorized inverted epifluorescence microscope images (10x magnification) of DAPI/Phalloidin stain of A-431 cells for cell only control, pectin and GBM hydrogels. Scale bar represents 50 μm .

The impact of direct contact of hydrogels in HFF-1 cell viability was also evaluated. Since the irradiation can be directed just to cancer cells in the final application, healthy cells (HFF-1) were not tested under NIR irradiation. In Figure 4.22.A, a decrease in cell viability was seen for pectin and GBM hydrogels. For pectin and GBM hydrogels, the cell viability was 15.46% and 0%, respectively. In Figure 4.22.B, the HFF-1 cells in the control presented a morphology characteristic of human skin fibroblasts, with a normal spindle like shape. In contrast with A-431,

cells adhere to the surface of both types of hydrogels, however the morphology was affected, which can explain the resazurin results. In GBM hydrogels, where cell viability was 0%, the cell nuclei appear fragmented, since the DAPI staining resulted in dispersed unrounded shapes. This could be caused by cytotoxicity of the hydrogel components, or to the mechanical properties of the hydrogels. Despite these concerning results, in the desired application, the hydrogels would not be in direct contact with fibroblasts, and as shown before, in extract exposure assays, no cytotoxic effect was observed.

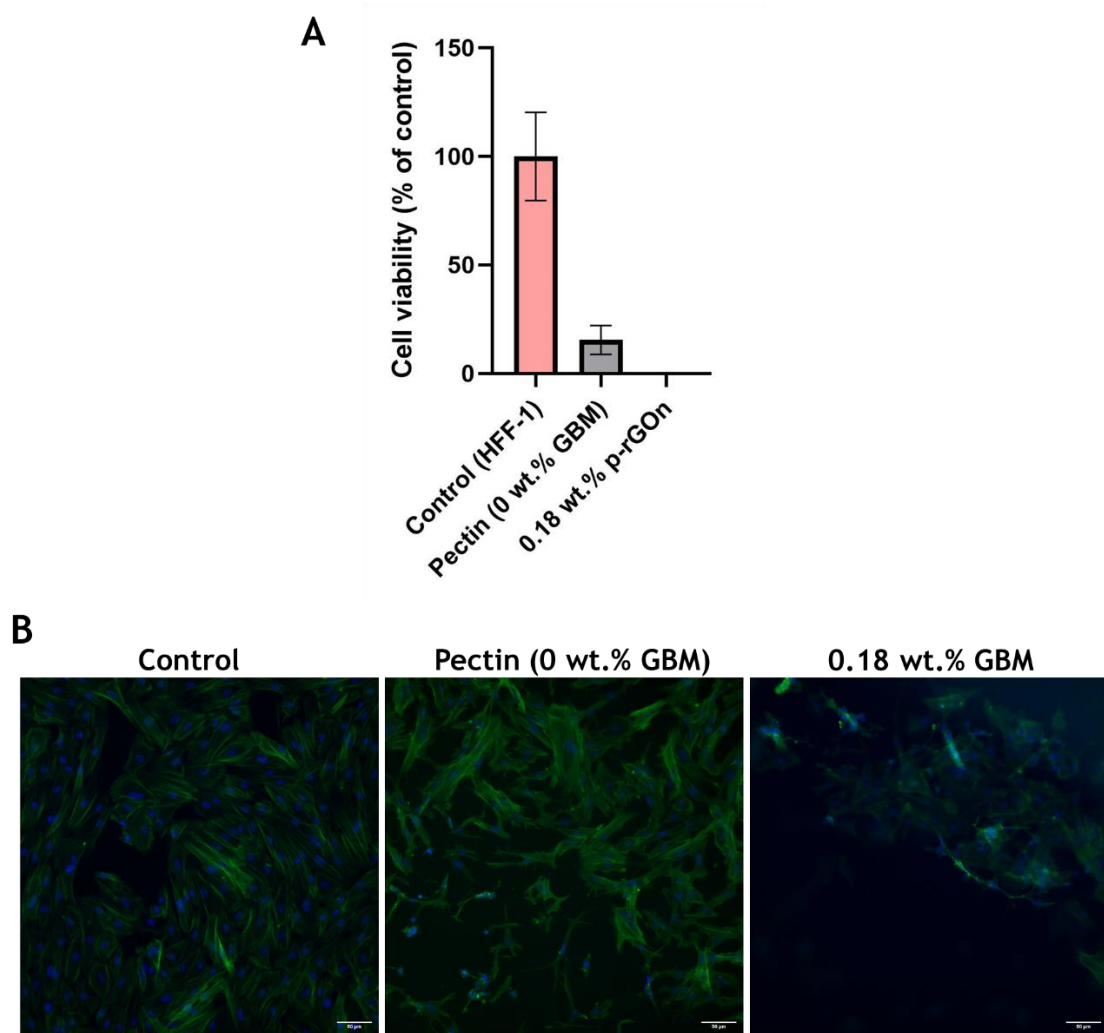


Figure 4.22 – Cytotoxicity of pectin and p-rGOn hydrogels on HFF-1 cells. A) Cell viability of HFF-1 cells, normalized to cell only control; B) Motorized inverted epifluorescence microscope images (10x magnification) of DAPI/Phalloidin stain of HFF-1 cells for cell only control, pectin and GBM hydrogels. Scale bar represents 50 μm .

Chapter 5: Discussion

The main goal of this dissertation was to develop graphene-based materials (GBM), particularly nanographene oxide (GOn) and partially reduced nanographene oxide (p-rGOn) and include them in bioprintable pectin-based hydrogels for skin cancer treatment. The incorporation of GBM into pectin hydrogels had the purpose of conferring photoabsorption properties to those. The ultimate goal was to produce pectin/GBM hydrogels that could be irradiated with near-infrared radiation (NIR) for a non-invasive and localized phototherapeutic effect for the treatment of skin cancer. To achieve this main purpose, several objectives were set: production and characterization of both GBM; production and characterization of pectin/GBM hydrogels; optimization of pectin/GBM hydrogels for potential printability; evaluation of hydrogels' biocompatibility and phototherapeutic effect under NIR.

Typically, graphene oxide (GO) is obtained for biomedical applications following the modified Hummer's method [67], which has routinely been used before in our laboratory [24], and was therefore selected for this study. Some important features GBM should present to be used for desired applications are good water stability, photoabsorption, biocompatibility, and skin penetration, for which particles with sizes below 200 nm are preferable [21]. With this in mind, a recirculation system was implemented to decrease GO particles lateral size using high-power ultrasonication [22]. By ultrasonication GO, GOn was obtained with sizes as low as 60 nm. GOn was subjected to UV photoreduction, which allowed the obtention of an innovative GBM, a NIR absorbant and water stable partially reduced nanographene oxide, with suitable properties for phototherapy. Recently, our group demonstrated that GOn water suspensions (300-1000 $\mu\text{g/mL}$) were able to permeate through human skin, crossing epidermis and dermis layers [21]. In addition, unpublished results indicate that GOn and p-rGOn incorporated in carbopol hydrogels were also capable of permeate deeply into human skin samples.

In this work, both produced GBM were characterized regarding the morphology, size, water and thermal stability, oxygen functionalities, NIR absorption and heat production. Using transmission electron microscopy (TEM), it was possible to observe that most of the GOn and p-rGOn sheets were individualized without forming larger agglomerates, showing sizes around 200 nm, which was the goal for biomedical applications. The particle size of GOn was 207 ± 80 nm and of p-rGOn was 199 ± 81 nm. Zetasizer measurments, the surface charges obtained for GOn

and p-rGOn were -31.9 ± 0.8 mV and -42.1 ± 0.9 mV, respectively, which confirms good water stability of both GBM dispersions [77].

The total reduction of GOn leads to the removal of oxygen-containing functional groups, which causes a decrease in water stability of the dispersion, limiting their potential for biomedical usage. However, the partial reduction of GOn resulted in a GBM with less oxygen-containing groups, whilst maintaining water stability. Furthermore, p-rGOn revealed to be efficient in convert NIR light into heat, demonstrating its potential as phototherapeutic agent. After 30 minutes irradiating using a NIR LED system (810 nm, 150 mW/cm²), 500 µg/mL p-rGOn reached 50.5 °C corresponding to a temperature increase 5.9 °C higher than GOn (44.6 °C) at the same concentration. This temperature (50.5 °C) can be used to achieve mild hyperthermia in photothermal therapy. According to the literature, most studies use high irradiance NIR lasers (< 3 W/cm²), that increase the temperatures of GBM to well above 50 °C, causing necrosis of the irradiated tissues in a unselective way [85, 86]. The used system used lower irradiance LEDs (150mW/cm²), while still reaching the temperatures needed for mild PTT. Our group reported a decrease in A-431 cells viability of 38% caused by a temperature increase to 47 °C using 250 µg/mL rGOn-PEG, irradiated with a NIR LED system (810 nm, 150 mW/cm²) for 30 minutes [24].

The optical properties of GBM were analysed by UV-Vis. The observed temperature increase in the NIR irradiation assays are in agreement with a 3.34-fold increase in NIR region absorbance of p-rGOn (810 nm), in comparison with GOn. It was also observed that GOn and p-rGOn have a maximum absorption peak at 230 nm. However, p-rGOn does not present a shoulder peak at 300 nm, unlike GOn. The shoulder peak present at 300 nm is associated with $n - \pi^*$ transitions of free electron pairs in the oxygen atoms present in the C=O bonds from carbonyl and carboxyl groups. The lack of this peak in the p-rGOn spectra strengthens that there was an efficient partial reduction of GOn. The partially reduction of GOn into p-rGOn was proved by complementary techniques, including the Fourier transform infrared spectroscopy (FTIR), Ultraviolet-visible spectroscopy, thermogravimetric analysis (TGA), and X-ray Photoelectron Spectroscopy (XPS).

In the FTIR analysis, GOn spectrum revealed a band between 3000 and 3600 cm⁻¹, which has been reported to correspond to the O-H stretching vibrations [22] and bands at X and Y assigned to C=O and C-O bonds [22]. In the p-rGOn spectrum, the O-H band shows a significant decrease in intensity, confirming the reduction in the number hydroxyl groups. However, the p-rGOn spectrum still shows some absorption bands at around 1728 and 1037 cm⁻¹, associated to the presence of carbonyl, carboxyl and ethers group [24]. According to the literature, if the GOn reduction had been complete, rGOn would be obtained and these bands would be completely absent [87]. The existence of these peaks, although showing a decrease in intensity when compared to GOn spectrum, confirms the incomplete reduction step and the production of a partially-reduced GOn, since there are still some oxygen-containing groups present [24].

XPS spectra revealed that GOn was well oxidized, since carbon atoms in carbonyl and carboxyl groups were present in the spectra, with O-C=O bonds dominating the surface chemistry. It was also seen that in the C1s spectra of p-rGOn, the most abundant oxygen functionality, C-O-C, decreased from 46.31% to 32.83% when compared to the GOn spectra, proving a successful reduction. There was also an increase in the C at.% in $\pi - \pi^*$ bonds in p-rGOn, showing a partial restoration of the aromatic structure. The C/O ratio was 2.2 for GOn and 2.3 for p-rGOn. All the preceding results suggest that GOn was successfully partially reduced to p-rGOn.

GBM thermal stability was tested by TGA, where GOn and p-rGOn showed three main weight loss steps over 100 °C. In the literature, values below 100 °C have been attributed to water evaporation of the samples [22]. Between 100 and 225 °C was the first weight loss step, where GOn had a weight loss of 34.8% and p-rGOn of 28.6%. This weight loss is associated with the loss of reactive oxygen-containing functional groups, like carboxyl and epoxy [24, 78]. The higher weight loss in GOn than p-rGOn is due to a higher presence of these groups in GOn, a suggestion of a successful partial reduction of GOn into p-rGOn. The second weight loss step happened between 225 and 600 °C, with weight losses of 15.2 and 14.5% for GOn and p-rGOn, respectively. This weight loss step related to the partial combustion of the carbon skeleton and other stable oxygen-containing functional groups, like carbonyl and hydroxyl [24]. Since GOn has more oxygen-containing groups, its weight loss is higher than p-rGOn once more. The final weight loss is between 600 and 1000 °C, with a weight loss of 23.0% for GOn and 8.1% for p-rGOn. This weight loss step is attributed to the combustion of the carbon skeleton. In total, GOn had a weight loss of 86.2% while p-rGOn had a total weight loss of 66.8%, so the removal of some oxygen-containing groups resultant from the partial reduction, resulted in a GBM with superior thermal stability [88].

Since p-rGOn was the produced GBM with higher NIR absorbance, having both good conversion of light to temperature and superior thermal stability, it was the material chosen to produce GBM hydrogels and consequent in vitro studies.

The used method for crosslinking the hydrogels was based on the internal gelation strategy [70, 71], where GDL hydrolyses throughout time, resulting in a reduction of the solution pH, which will ultimately lead to the release of Ca^{2+} ions from CaCO_3 molecules [72]. Hydrogels could also be reinforced and have better shape-retention when subjected to an external gelation by incubation in CaCl_2 solution, where the diffusion of free Ca^{2+} ions will provoke the gelation. The concentrations of polymer, CaCO_3 and GDL were optimized in order to obtain a hydrogel and it was found that the optimal concentrations for disc formation were 2.5% polymer, with 15 mM and 30 mM of CaCO_3 and GDL, respectively.

The produced p-rGOn was added to the hydrogel precursor solution, obtaining a hydrogel with a maximum of 0.09 wt.% p-rGOn content. When all components were dissolved in p-rGOn dispersion, hydrogels with 0.18 wt.% p-rGOn could be achieved.

The mechanical properties of the hydrogels were evaluated using a texture analyzer, in order to obtain the Young's modulus of the hydrogels under certain conditions. For all conditions, higher p-rGOn content in the hydrogels resulted in higher Young's modulus. When the hydrogels were in a CaCl_2 bath, the Young's modulus is higher. The stiffer hydrogels (highest Young's modulus) were the hydrogels with highest GBM content (0.18 wt.%), as prepared, when incubated in CaCl_2 . After hydrogels are incubated in DMEM overnight, they tend to become softer, resulting in lower Young's modulus. The softest hydrogels, incubated in DMEM without CaCl_2 , resulted in the lowest Young's modulus, and were very difficult to handle without breaking. The results help prove the need for an external gelation step for assays where the hydrogels had to be incubated in DMEM overnight, since without that step the hydrogels became too soft, being too difficult to handle. GBM have long been known for their mechanical properties, with graphene oxide (GO) having an elastic modulus of 220 GPa [82], being used with the purpose of tuning mechanical properties of scaffolds before [83].

The efficiency of composite hydrogels to convert NIR light into heat was evaluated, using with a custom-build LED NIR system (810 nm, 150 mW/cm²), with assay starting at 37 °C. The temperature variation throughout 30 minutes of NIR irradiation of DMEM (as a control), and hydrogels with 0, 0.09 and 0.18 wt.% GBM was evaluated. The highest temperature achieved was for the hydrogel with highest GBM content, reaching an average final temperature of 48.6 °C. These results were expected since the temperature increase revealed to be concentration-dependent [21, 24]. Hydrogels with 0.09 wt.% GBM reached a temperature of 46.9 °C, and pectin only hydrogels (0 wt.% GBM) reached a final temperature of 36.9 °C, whilst the DMEM control reached 32.8 °C. In total, the DMEM control had a temperature decrease of 3.9 °C, pectin only hydrogels had an average decrease of just 0.2 °C, whilst 0.09 and 0.18 wt.% GBM hydrogels had a temperature increase of 10.0 °C and 11.5 °C, respectively. The temperature drops because there is an equilibrium of the temperature with RT and the control and pectin only hydrogels do not absorb NIR as do hydrogels containing GBM.

The rheology of hydrogel precursor solutions had to be tuned to allow its processing via bioprinting. For this, various polymer, CaCO_3 , and GDL concentrations were tested. A few of the tested mixtures seemed to have the right viscosity for bioprinting, with 2% polymer with 25 mM CaCO_3 and 50 mM GDL forming the best filaments. Potential was also demonstrated by hydrogels containing 2% polymer, 15:30 mM CaCO_3 :GDL, and 20:40 mM CaCO_3 :GDL, respectively. These hydrogels exhibited a viscosity that permitted them to be dispensed from syringes as stable filaments.

Through extract exposure experiments, the cytotoxicity of hydrogels was assessed, and it was discovered that neither HDFn nor A-431 cells controls significantly differed from hydrogels in terms of cell viability. This would imply that cell viability wouldn't be compromised if some particles leached out of the hydrogels.

Through a direct contact assay in which cells were seeded on the hydrogel surface, the cytotoxicity and photothermal effect of the hydrogels were evaluated. Additionally assessed was cell adhesion. Both pectin and GBM hydrogels had lower cell viability, according to the results. For not irradiated pectin and GBM hydrogels, a decrease in cell viability to 39.07% and 6.03%, respectively, was observed. After 30 min of NIR exposure, the cell viability for pectin hydrogels was 40.47% and for GBM hydrogels was 5.55%. The acquired values were comparable with and without irradiation, indicating that the influence of the irradiation was not discernible when data were normalized to cell only control. These outcomes might be connected to the cells' adherence to the hydrogel's surface. Fluorescence microscopy images revealed that there were much less cells in pectin and GBM hydrogels than in the control, and that there were even fewer cells in GBM hydrogels than in pectin hydrogels. This can mean that the hydrogels are cytotoxic to the cells or that the cells are unable to adhere to the hydrogels. It can be believed that the low cell number is due to the poor cell adherence on the hydrogels, as the primary components of the hydrogels (pectin and GBM) previously demonstrated biocompatibility [21, 23, 24, 32].

F. Silva *et al.* discovered that GOn concentrations greater than those that could penetrate the skin (300–1000 g/mL) had no effect on the morphology or viability of human skin fibroblasts (HFF-1), after 24h of incubation [21].

R. F. Pereira *et al.* developed norbornene-functionalized pectin hydrogels. Biofunctional hydrogels with various levels of crosslinking density were created, eliciting various biological reactions, by choosing appropriate MMP-degradable peptide crosslinkers and pendant cell-adhesive peptides. In more permissive hydrogels, embedded fibroblasts showed an extended shape and reduced compaction, which caused the matrix to compress to a greater extent [32].

GBM hydrogels have in the previously displayed greater Young's modulus values, resulting in a stiffer hydrogel that might not be the best for A-431 cells to adhere to. Fluorescence microscopy images further demonstrated that the morphology of A-431 cells was unaffected by NIR irradiation, pectin hydrogels, or GBM hydrogels. The findings of the resazurin assay were normalized for the positive control because of the weak cell adherence (pectin hydrogels). GBM hydrogels demonstrated a cell viability of 15.05% when cells weren't exposed to radiation for 30 minutes and 12.53% following exposure, as compared to the positive control. The GBM hydrogels reduced cell viability when exposed to radiation, even though the results were not statistically different, which may indicate they have a slight photothermal effect. In order to prevent additional potential impacts of the low cell number, the cell adhesion must ensure optimal cell confluence in order to further corroborate this outcome. Regarding HFF-1 cells, a decrease in cell viability was seen for pectin and GBM hydrogels. For pectin and GBM hydrogels, the cell viability was 15.46% and 0%, respectively. The HFF-1 cells in the control presented a morphology characteristic of human skin fibroblasts, with a normal spindle like shape. In contrast with A-431, cells adhere to the surface of both types of hydrogels, however the morphology was affected, which can explain the resazurin results. The mechanical properties of hydrogels can

cause changes in cell adhesion and proliferation [89]. The cell nuclei in GBM hydrogels, where cell viability was 0%, seemed fractured because the DAPI labeling produced scattered, irregular forms. This can be brought on by the hydrogel components' cytotoxicity or the hydrogels' mechanical characteristics. KH. Liao *et al.* described no toxicity in human skin fibroblasts (BJ) when exposed to rGO concentrations up to 200 $\mu\text{g/mL}$, after 24 h of incubation [90]. Despite these unsettling findings, the hydrogels wouldn't come into direct contact with fibroblasts in the intended application, and as previously demonstrated in extract exposure studies, no cytotoxic effect was seen.

As a result, the proposed method could only be used in combination with focused radiation to avoid adverse effects on the surrounding healthy tissues and selective delivery of hydrogels to the tumour site. To the best of our knowledge, the use of pectin/GBM hydrogels for PTT has not been studied, however several related approaches have been investigated.

W. He *et al.* developed a very effective doxorubicin hydrochloride delivery system using a hydrogel of carboxymethyl chitosan-functionalized reduced graphene oxide/aldehyde functionalized poly (ethylene glycol) (CMC-rGO/CHO-PEG). Modified Hummers method was used to exfoliate and oxidize graphene to create single-layered GO nanosheets, which had high hydrophilicity and productivity. Carboxymethyl chitosan (CMC) was used to obtain CMC-rGO via thermal reduction. Using a NIR laser (808 nm, 1W/cm^2), a small temperature increase was seen in the CMC/CHO-PEG hydrogel control, however a considerable temperature change was seen in the CMC-rGO complex upon NIR light irradiation, reaching temperatures above 50°C in about 8 min. Notably, the CMC-rGO/CHO-PEG hydrogel showed an even stronger photothermal impact, reaching temperatures above 60°C . rGO was drug loaded with antitumor drug, doxorubicin hydrochloride (DOX), and CMC-rGO/CHO-PEG hydrogel exhibited great potential to serve as a smart anticancer drug carrier [91].

X. Zhu *et al.* functionalized GO with chitosan for higher water stability. A docetaxel (DTX) and GO-chitosan hydrogel (DTX-GO/CS) was produced. MCF-7 cells were incubated with various concentrations of DTX and DTX-GO/CS for 24, 48 and 72h. The cells were subjected to NIR laser irradiation (2.5W, 2 min). Due to the combination of DTX's unique chemotherapeutic mechanism and the PTT properties of GO/CS, treatment with DTX-GO/CS with laser demonstrated excellent antitumor activity on MCF-7 cancer cells in vitro. The DTX-GO/CS with NIR Laser group had a higher percentage of apoptotic cells ($18.34 \pm 0.12\%$), indicating that the treatment with DTX-GO/CS with NIR laser promoted the death of tumour cells via the apoptotic route [92].

G. Chang *et al.* mixed 5-FU, rGO, gold nanocages, and spinach extract in a hybrid hydrogel for multimodal cancer therapy. HeLa cell viability dropped to 1.2% during a 48h incubation with the hydrogels and a 10-minute NIR laser irradiation (660 nm , 200 mW/cm^2) while CHO cell viability remained above 50% [40].

Despite the fact that numerous studies have examined the various technical and biomedical applications of GBM, there is still more to investigate using the pectin/GBM hydrogels. The current work shows their PTT potential, but a targeted therapy effect has not yet been seen. The conclusions offer perspectives on potential future work.

Chapter 6: Conclusions and future work

The present dissertation focuses on the development of pectin/p-rGOn hydrogels for photothermal therapy of skin cancer. Currently, available therapies are not effective, and their safety is not ideal, therefore new strategies are required to offer more selective and effective treatments to patients, such as phototherapy.

The state-of-the-art review shows that CNM have great potential to be used in the 3D-printing of biomedical devices. The most common applications of these printed devices are scaffolds for tissue engineering and sensors or biosensors. The spoken applications employed carbon nanofibers, carbon nanotubes, and GBM in bioinks/biomaterial inks. The constructs can be printed through different techniques, like inkjet-, light- and extrusion-based bioprinting. CNM are employed in the inks can modulate several properties in the final constructs, like mechanical properties, thermal and electrical conductivity, and biological response.

Herein, GOn was produced through the modified Hummers' method, followed by recirculation through a custom-built high-power ultrasonication probe to exfoliate the particles. p-rGOn was partially-reduced by UV radiation. Physical characterization by TEM and Zetasizer revealed that the graphene sheets with nanometric sizes were achieved, which is ideal for biomedical applications. Both GOn and p-rGOn had zeta potential values below -30 mV, presenting good water stability. In GO, typical oxygen-containing functional groups were found, and following UV reduction, the strength of their bands decreased. Additionally, TGA results showed weight losses of 86.2% in GOn corresponding to oxygen-functionalities; these losses were smaller in p-rGOn (66.8%), further demonstrating the effectiveness of the partial reduction. UV-Vis spectroscopy showed that p-rGOn displayed a 3.34 times increase in NIR (810 nm) absorption over GOn.

NIR irradiation assays (810 nm, 150 mW/cm², 30 min) revealed that p-rGOn presented a higher light-to-heat conversion, within temperatures of interest for phototherapy (> 50 °C), while GOn only achieved a maximum temperature of $\approx$ 42 °C when irradiated. This material was chosen for additional testing with the goal of developing the intended phototherapeutic application after identification of the p-rGOn enhanced photoproperties and prior confirmation of its water

stability. The development of a material with these qualities is entirely novel. The novel GBM, p-rGOn, was further investigated with the goal of evaluating its photothermal when mixed in pectin-based hydrogels.

Hydrogels were crosslinked by internal gelation using the CaCO_3 :GDL system and eventually subjected to a secondary crosslinking by external gelation using CaCl_2 . The optimal concentration to produced hydrogel disks was found to be 2.5% polymer, with 15 mM and 30 mM of CaCO_3 and GDL, respectively.

Compressive tested showed that increased p-rGOn content in the hydrogels led to higher Young's modulus under all circumstances.

When the effectiveness of composite hydrogels in converting NIR light into heat was tested, it was seen that hydrogels with 0.18 wt.% p-rGOn reached a maximum temperature of 48.6 °C.

Hydrogel printability could be achieved using a hydrogel precursor solution with 2% polymer, 25 mM CaCO_3 and 50 mM GDL.

With *in vitro* assays, it was seen that there was no drop in HDFn and A-431 viability when exposed to hydrogel extracts. Hydrogels showed poor A-431 cell viability and adhesion, after the cells were seeded on the surface of the hydrogels. GBM hydrogels showed even poorer adhesion when compared to pectin ones. When seeded hydrogels were irradiated with NIR light for 30 min, a minor photothermal effect could be seen in GBM hydrogels, but cell adhesion must guarantee optimal cell confluence in order to further support this result and prevent any other potential effects of the low cell number. Pectin and p-rGOn hydrogels do not cause changes in A-431 morphology, nor does NIR irradiation. HFF-1 cells appeared to adhere to the hydrogels after being seeded there, although their morphology underwent significant alterations, causing implications in the cellular metabolic activity.

Overall, the produced p-rGOn showed potential for photothermal therapy, and pectin hydrogels containing it are also capable of converting NIR light into heat. The mechanical, photothermal and cell adhesive properties of these hydrogels should be optimized to correctly evaluate their effect on cancer cell death.

Based on current data, in the future there are several aspects that would require improvement and further investigation.

The protocols for evaluation of hydrogel cytotoxicity and photothermal effect on cells must be optimized, starting first by altering the hydrogel composition to allow A-431 cell adhesion. Increasing the RGD content of the final hydrogels is one technique to potentially improve cell adherence. Trying multiple hydrogel crosslinking techniques might result in diverse mechanical qualities, which might also help with cell attachment. Evaluating cell invasion of the hydrogels could also be conducted, by means of confocal microscopy.

An interesting approach to evaluate the photothermal effect of the produced hydrogels would be to have the cells on top of the hydrogel in a 3D structure, embedding the cells in gelatin,

for instance. This approach is more biomimetic, which can produce very interesting results. Using this strategy, studying the influence of NIR irradiation presents results closer to the reality.

To have a hydrogel that could be used for combined photothermal-chemo-therapy, anticancer drugs could be introduced in the hydrogels. Drugs like 5-FU or imiquimod may be used for this purpose.

Since a printable hydrogel was discovered in this experiment, 3D-printed constructs are possible.

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Appendix A

Supplementary Information for Chapter 4

In this Appendix, the characterization of GOn and p-rGOn batch 027 is presented. For the work presented in Chapter 4, batch 028 was used.

Particle size distribution was evaluated by dynamic light scattering (DLS) using Zetasizer equipment (Table A.1).

Table A.1 – Particle size, polydispersity index and zeta potential of GOn and p-rGOn (from batch 027) aqueous dispersions at a concentration of 30 µg/mL, determined by DLS with a Zetasizer equipment (n = 3).

GBM	Size (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
GOn	304.7 ± 11.34	0.444 ± 0.031	-45.8 ± 1.16
p-rGOn	258.2 ± 8.155	0.432 ± 0.042	-44.4 ± 3.92

Higher sizes for particle size were expected with the Zetasizer equipment, since with the DLS principle, a spherical model of the particles is assumed, whilst GBM are 2D-sheets. GOn and p-rGOn presented a particle size of 304.7 ± 11.34 nm and 258.2 ± 8.155 nm, respectively. The PDI values of GOn and p-rGOn are 0.444 ± 0.031 and 0.432 ± 0.042, respectively. Like previously mentioned, if the PDI value sits between 0.2 and 0.5, it can indicate that there is a wide particle size distribution, which is the case for the produced GBM.

The surface charges of GOn and p-rGOn were analysed by Zetasizer. For GOn, the obtained value was -45.8 ± 1.16 mV and, for p-rGOn was -44.4 ± 3.92 mV. For both materials, the surface charge values were under -30 mV, indicating high water stability.

In Figure A.1, the FTIR spectra of GBM from batch 027 are present. In the GOn spectra, a broad band with wavenumbers ranging between 3000 cm⁻¹ and 3600 cm⁻¹ can be seen, which corresponds to O-H stretching vibrations. These values are usually attributed to adsorbed water molecules, hydroxyl, and carboxyl groups. Around 1725 cm⁻¹, the C=O stretching vibrations are present, which correspond to the carbonyl and carboxyl molecules. Due to the unoxidized graphite, the stretching of cyclic alkaline (C=C) is present at around 1615 cm⁻¹. The presence of

ethers can be attested by the absorption bands around 1222 cm^{-1} and 1042 cm^{-1} corresponding to C-O stretching vibrations and through epoxides with C-O bending vibrations around 971 cm^{-1} . In the p-rGOn spectra, a significant difference can be seen, with an increase of the intensity of the oxygen-containing groups in comparison to GOn, meaning that the reduction was unsuccessful. There is a major peak between 3000 and 3600 cm^{-1} , and peaks at 1725 , 1222 and 1042 cm^{-1} , all peaks indicated are characteristic of oxygen-containing groups. It was possible to conclude that GOn from lot027 was not successfully reduced into p-rGOn.

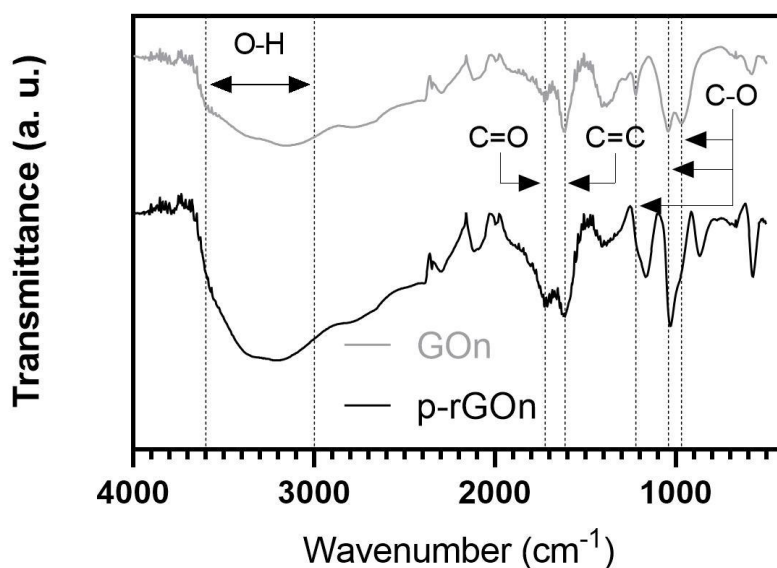


Figure A.1 – FTIR spectra of GOn and p-rGOn (from batch 027). The lines indicate the contribution of different surface functionalities.

The optical properties of both GBM were analysed by UV-Vis spectroscopy (Figure A.2). For GOn, there is a maximum absorbance peak around 230 nm, resultant from the $\pi - \pi^*$ electronic transitions in sp^2 clusters and a shoulder peak around 300 nm, characteristic of the $n - \pi^*$ transitions of free electron pairs in the oxygen atoms that exist in the double bonds between carbon and oxygen, from carbonyl and carboxyl groups. The p-rGOn spectrum also presents a maximum absorbance peak around 230 nm and shows a 1.25-fold increase in NIR absorbance (810 nm), compared to GOn. The existence of a slight shoulder peak shows that p-rGOn was not reduced enough to remove the oxygen-containing functional groups.

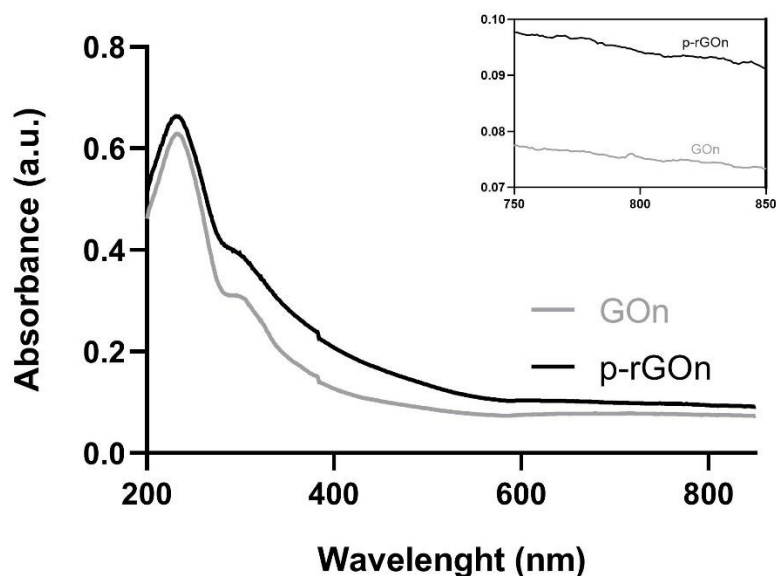


Figure A.2 – UV-Vis absorption curves of GOn and p-rGOn (from batch 027). The inset shows a zoom-in view of the curves in the NIR range (750-850 nm).

The obtained thermograms (Figure A.3) show three main steps regarding the weight loss during the heating of the GBM above 100 °C. For GOn, the first weight loss step can be seen between 160 °C and 220 °C and for p-rGOn between 120 °C and 170 °C. In this step, correspondent to the loss of reactive oxygen-containing functional groups such as carboxyl and epoxy, GOn and p-rGOn presented weight losses of approximately 27 % and 20%, respectively. In the second step, between 225 and 600 °C, GOn had a weight loss of about 14.6% and between 175 °C and 600 °C p-rGOn had a weight loss of 16.5%, corresponding to the partial combustion of the carbon skeleton and other stable oxygen-containing functional groups, like carbonyls and hydroxyls. The third and last step in weight loss, correspondent to the continuous combustion of the carbon skeleton, happened between 600 and 1000 °C. In this last step, GOn and p-rGOn showed weight losses of 22.6% and 5%, respectively. In total, GOn presented a weight loss of 81.8% and p-rGOn 64.2%.

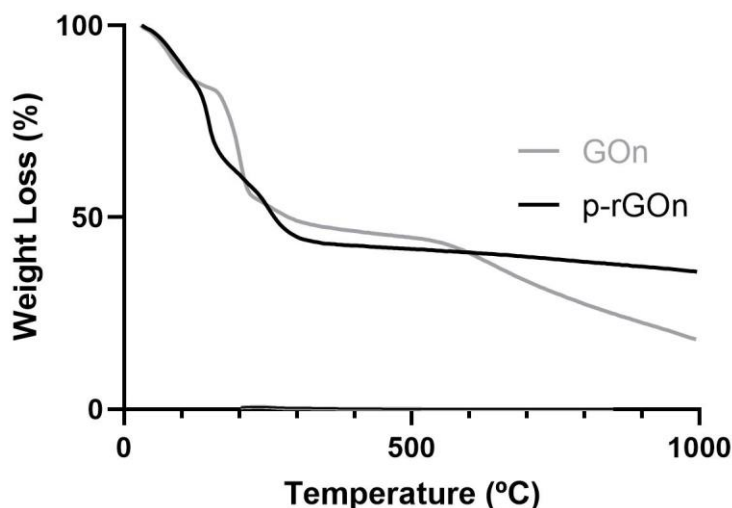


Figure A.3 – Thermogravimetric analysis of GOn and p-rGOn (from batch 027) under a nitrogen atmosphere.

With aqueous dispersions at a concentration of 500 $\mu\text{g/mL}$ of both GOn and p-rGOn, the efficiency of these nanomaterials to convert NIR light into heat was evaluated (Figure A.4). The samples were placed in an oven, so an atmosphere of 37 °C was maintained throughout the test. With the samples reaching a temperature close to 37 °C, the irradiation with a custom-build LED NIR system (810 nm, 150 mW/cm^2) started. The temperature was measured at different time points. In Figure A.4, the temperature increase during 30 minutes of NIR irradiation is presented. At 500 $\mu\text{g/mL}$, the control reached 42.7 °C, having a temperature increase of 7.0 °C, GO reached a final temperature of 42.9 °C, with a total temperature increase of 7.7 °C whilst p-rGOn reached a temperature of 45.9 °C, with an increase of 11.1 °C in total. The increase of temperature in GOn is almost the same as the increase of the temperature in the water control. Even with p-rGOn, there is only an increase of 3.2 when compared to the water control, showing that the material does not have very increased absorption properties in the NIR regions, presenting itself as potentially inadequate for photothermal therapy (PTT).

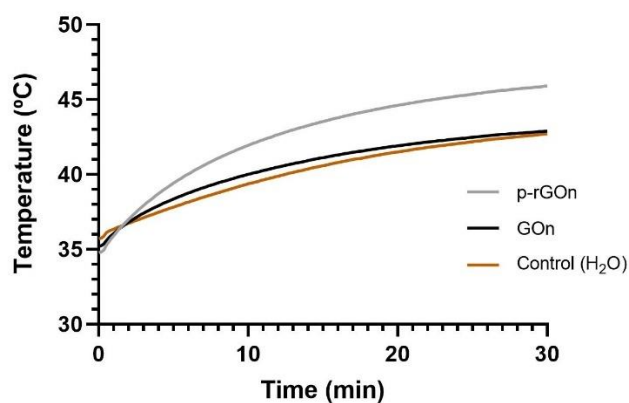


Figure A.4 – Photothermal heating curves of water, p-rGOn and GOn (from batch 027) aqueous dispersions at 500 $\mu\text{g/mL}$.

Hydrogels produced with p-rGOn from batch 027 were irradiated with a NIR laser from room temperature. The irradiance of the laser was measured, obtaining an average value of 1075,1 W/m². In Figure A.5, the results of the light-to-heat conversion of hydrogels during 30 minutes of irradiation is displayed. The hydrogels reach a higher temperature than they would with the custom LED system, but that temperature increase is also due to the laser being non-isolated, producing a lot of heat that will influence the sample. This can be proved by the final temperature of the control and the pectin hydrogel, that cannot absorb NIR irradiation but still have a temperature increase of 13.3 °C and 16.0 °C. When compared to the control, the hydrogel with the highest GBM content has a difference in temperature increase of 10.4 °C.

Another inconvenience of the use of the laser is that it can only irradiate one sample at the time, so its use in cell assays would result in experiments that are too long and generate unreliable results. For this reason mainly, the laser was not used for further assays.

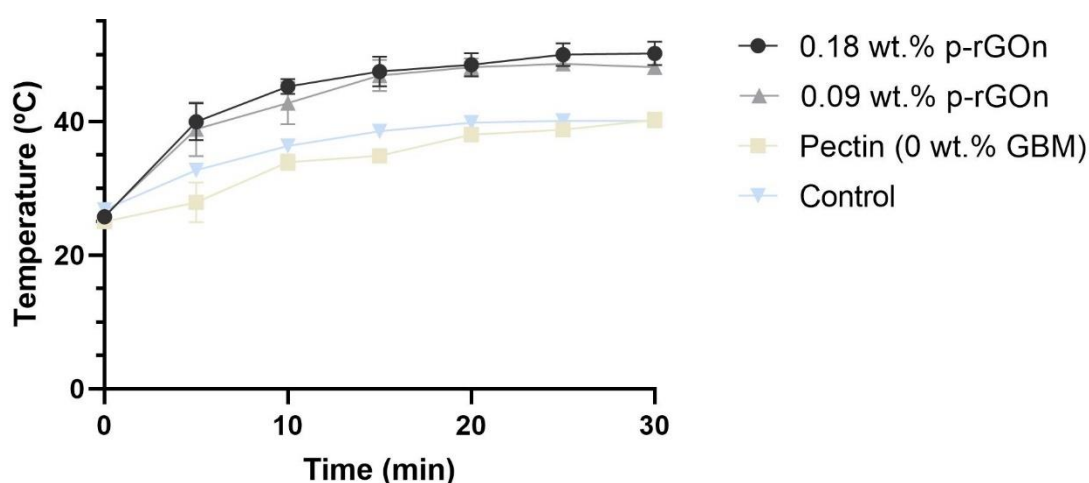


Figure A.5 – Photothermal heating curves of water control and hydrogels with 0, 0.09 and 0.18 wt.% GBM, using a laser as NIR light source.

Appendix B

List of presentations

- I. Pedro, T. van de Kemp, B. Freitas, J. Moreira, L. Pires, F. Silva, R. Pereira, F. Magalhães, M. Castilho, A. Pinto, “Graphene/pectin-based hydrogels for cancer treatment and tissue regeneration”, IJUP, 2022.
- T. van de Kemp, J. P. S. Meneses, I. Pedro, B. Freitas, J. Moreira, L. Pires, F. Silva, R. Pereira, F. Magalhães, M. Castilho, A. Pinto, “3D printing of polycaprolactone/graphene-based materials scaffolds for tissue engineering”, IJUP, 2022.