DESIGN, MODELLING AND FABRICATION OF POLYCAPROLACTONE ELECTRO-ACTIVE SCAFFOLDS FOR TISSUE ENGINEERING

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<td>1D</td>
<td>One dimensional</td>
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<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>2PP</td>
<td>Two-photon photopolymerisation process</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AM</td>
<td>Additive manufacturing</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARS</td>
<td>Alizarin red-S</td>
</tr>
<tr>
<td>ASC</td>
<td>Adipose stem cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaP</td>
<td>Calcium phosphate</td>
</tr>
<tr>
<td>CAD</td>
<td>Computer aided design</td>
</tr>
<tr>
<td>CC</td>
<td>Cell-to-cell coupling</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
</tr>
<tr>
<td>CCVD</td>
<td>Catalytic chemical vapour deposition</td>
</tr>
<tr>
<td>CD</td>
<td>Cell-to-cell decoupling</td>
</tr>
<tr>
<td>CHA</td>
<td>Carbonated hydroxyapatite</td>
</tr>
<tr>
<td>CNC</td>
<td>Computer numerical control</td>
</tr>
<tr>
<td>CNM</td>
<td>Carbon nanomaterial</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon nanotube</td>
</tr>
<tr>
<td>CPC</td>
<td>Cartilage progenitor cell</td>
</tr>
<tr>
<td>CPD</td>
<td>Critical point dryer</td>
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14
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSMA</td>
<td>Chondroitin sulphate methacryloyl</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CVD</td>
<td>Chemical vapour deposition</td>
</tr>
<tr>
<td>CW</td>
<td>Cell-to-cell wavering</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDOT</td>
<td>Ethylenedioxythiophene</td>
</tr>
<tr>
<td>EMF</td>
<td>External magnetic field</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDM</td>
<td>Fused deposition modelling</td>
</tr>
<tr>
<td>FET</td>
<td>Field effect transistor</td>
</tr>
<tr>
<td>FLG</td>
<td>Few layered graphene</td>
</tr>
<tr>
<td>FW</td>
<td>Filament width</td>
</tr>
<tr>
<td>GelMA</td>
<td>Gelatin methacryloyl</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GNR</td>
<td>Graphene nanoribbon</td>
</tr>
<tr>
<td>GO</td>
<td>Graphene oxide</td>
</tr>
<tr>
<td>GONR</td>
<td>Graphene oxide nanoribbon</td>
</tr>
<tr>
<td>hADSC</td>
<td>Human adipose derived stem cell</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HMDS</td>
<td>Hexamethyldisilazane</td>
</tr>
<tr>
<td>HV</td>
<td>High voltage</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ISO</td>
<td>International Organization of Standardization</td>
</tr>
<tr>
<td>LAB</td>
<td>Laser-assisted bioprinting</td>
</tr>
<tr>
<td>min</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mm</td>
<td>Minute</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium tin oxide</td>
</tr>
<tr>
<td>iPSC</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>ISISA</td>
<td>Ice segregation-induced self-assembly</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>Mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCNT</td>
<td>Multi-walled carbon nanotube</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NC</td>
<td>Negative control</td>
</tr>
<tr>
<td>NCC</td>
<td>Newly formed cell-to-cell coupling</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PA</td>
<td>Polyacrylate</td>
</tr>
<tr>
<td>PABS</td>
<td>Plasma-assisted Bioextrusion System</td>
</tr>
<tr>
<td>PANI</td>
<td>Polyaniline</td>
</tr>
<tr>
<td>PBAT</td>
<td>Poly(butylene adipate-co-terephthalate)</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly(ε-caprolactone)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
</tr>
<tr>
<td>PEDOT</td>
<td>Poly(3,4-ethylenedioxythiophene)</td>
</tr>
<tr>
<td>PET</td>
<td>Poly(ethylene terephthalate)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEU</td>
<td>Polyester Urea</td>
</tr>
<tr>
<td>PGA</td>
<td>Polyglycolic acid</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLCL</td>
<td>Poly(l-lactide-co-ε-caprolactone)</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly(lactide-co-glycolide)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic acid-co-glycolic acid)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PNIPAAm</td>
<td>Poly(n-isopropylacrylamide)</td>
</tr>
<tr>
<td>PPy</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>PS</td>
<td>Pore size</td>
</tr>
<tr>
<td>PSS</td>
<td>Polystyrene sulfonate</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>P3TMA</td>
<td>Poly(3-thiophene methyl acetate)</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor-NF-kB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor-NF-kB ligand</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SBF</td>
<td>Simulated body fluid</td>
</tr>
<tr>
<td>SCC</td>
<td>Strengthened cell-to-cell coupling</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SL</td>
<td>Stereolithography</td>
</tr>
<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>SWCNT</td>
<td>Single-walled carbon nanotube</td>
</tr>
<tr>
<td>TGA</td>
<td>Thermogravimetric analysis</td>
</tr>
<tr>
<td>TIPS</td>
<td>Thermally induced phase separation</td>
</tr>
<tr>
<td>TPU</td>
<td>Thermoplastic Polyurethane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Tuj1</td>
<td>Neuron-specific class III β-tubulin</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VCM</td>
<td>Vancomycin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>wt.%</td>
<td>Weight percent</td>
</tr>
<tr>
<td>WCA</td>
<td>Water Contact Angle</td>
</tr>
<tr>
<td>XCA</td>
<td>Xanthan</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometre</td>
</tr>
</tbody>
</table>
List of Notations

\( \sigma \)  
Stress applied to the surface of the scaffold (MPa)

\( \varepsilon \)  
Strain applied to the surface of the scaffold (mm/mm)

\( \Delta h \)  
Scaffold height variation during compression (mm)

\( h_0 \)  
Initial height of the scaffold (mm)

\( A \)  
Area of the scaffold cross section (mm\(^2\))

\( F \)  
Force applied to the scaffold (load)

\( \delta \)  
Solubility parameter

\( E \)  
Compressive modulus

\( \gamma_{lv} \)  
Surface tension between liquid-vapour

\( \gamma_{sl} \)  
Interfacial tension between the solid and liquid

\( \gamma_{sv} \)  
Surface energy

\( \theta \)  
Angle

\( \rho \)  
Density
Abstract

In tissue engineering, scaffolds are physical substrates for cell attachment, proliferation and differentiation, ultimately leading to the regeneration of tissues. They must be designed according to specific biomechanical requirements such as mechanical properties, surface characteristics, biodegradability, biocompatibility and porosity. The optimal design of a scaffold for a specific tissue application strongly depends on both materials and manufacturing processes. Polymeric scaffolds reinforced with electro-active particles could play a key role in tissue engineering by modulating cell proliferation and differentiation.

This research project investigates different aspects related to the design, fabrication and evaluation of electro-active scaffolds made with poly (ε-caprolactone) (PCL) mixed with graphene nanosheets. A series of physical and biological assessments have been performed to assess the properties of produced scaffolds, demonstrating for the first time that 3D printed PCL/graphene scaffolds are suitable for bone tissue engineering.

An extrusion-based additive manufacturing system was used to produce the scaffolds allowing a good dispersion of the inorganic component. No significant changes on the surface energy were observed. However, biological assessments suggest that the addition of graphene to PCL enhance the biological behaviour of scaffolds in terms of stimulating cell proliferation and differentiation. Surface treatment with NaOH and zonal plasma treatment strategies increase the hydrophilicity of scaffolds, allowing higher cell attachment rate and ultimately lead to higher cell proliferation rates. Protein binding to the scaffold also has a positive impact on both cell proliferation and differentiation. Moreover, the immune response analysis of printed scaffolds suggests high potential for in vivo bone regeneration research. Finally, in vivo tests show that the use of PCL/graphene scaffolds with exogenous microcurrent therapy enhance new tissue formation, stimulating the levels of ALP, RANK and higher ratios between OPG and RANKL. The physiological electrical stimulation modulated the bone remodelling phase, leading to well-organized and mineralized tissue deposition. Generally speaking, both non-biological and biological (including in vitro and in vivo) assessments have presented improved bone regeneration effect compared with conventional PCL scaffolds. All the results present in dissertation suggest a promising future for the in-depth clinical study on this newly developed 3D structure for the speed up of large scale bone tissue regeneration.
Declaration

No portion of the work referred to this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.
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Acknowledgements

This thesis represents not only my work at the keyboard, it is also a milestone in more than three years of work at School of Mechanical, Aerospace and Civil Engineering, the University of Manchester, and specifically within prof. Paulo Bártolo’s Biomedical Engineering Research Centre. I would like to extend thanks to the many people, in many countries, who so generously contributed to the work presented in this thesis.

First profound gratitude goes to my enthusiastic supervisor, Prof. Paulo Jorge Da Silva Bártolo. It has been an honour to be a Ph.D researcher in his research group. My Ph.D has been an amazing experience and I thank Paulo wholeheartedly, not only for his tremendous academic support, but also for giving me so many wonderful opportunities. The joy and enthusiasm he has for his research was contagious and motivational for me, even during tough times in the Ph.D pursuit. I am also thankful for the excellent example he has provided as a successful academic researcher and professor.

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In 2016, I spent nearly half a year in Bone and Joint Research Group, Faculty of Medical and Health Sciences, University of Auckland, New Zealand, for the exchange research on regenerative medicine in skeletal tissues. I would like to thank all the researchers form the group: Jillian Cornish, David Musson, Ally Choi, Young-Eun, Damien Carey, Karen Callon, Maureen Watson, Mei Lin Tay and Jian-ming Lin. Their kindly hospitality and generous technical support made this sic month an amazing experience through my PhD.
I gratefully acknowledge the funding sources that made my Ph.D. work possible, my project was fully funded by School of Mechanical Aerospace and Civil Engineering and the Marwick Bequest for Postgraduate Research in Engineering. Funding also received from Marie Curie Action, SKELGEN International Research Staff Exchange Scheme for the exchange research experience in New Zealand.

Special acknowledgement also goes to my girlfriend Yipeng Wang. Writing up the finial thesis for the whole research project is a challenging process, with her company and support, this busy summer became much more enjoyable.

Finally, but by no means least, thanks go to my father, mother, grandfather and grandmother, for almost unbelievable love, support and encouragement. They are the most important people in my world and I dedicate this thesis to them. For everything I’ve received from them during this Ph.D period is so appreciated. Thank you.

Weiguang Wang

University of Manchester

September 2018
CHAPTER I

INTRODUCTION
1.1 Background

With the increasing life expectancy of the population, osteoporotic fractures will have a serious economic impact on society and patient’s quality of life. It is estimated that in Europe 179,000 men and 611,000 women will suffer a hip fracture each year and the cost of all osteoporotic fractures in EU will increase from the current 31.7 billion euros to 76.7 billion euros by 2050. The problem is worse if we consider also significant bone damage due to accidents and diseases [1].

Bone is able to heal and remodel without leaving any scar in cases of very limited damage or fracture. However, in pathological fractures, traumatic bone loss or primary tumour resection, where the bone defect exceeds a critical size, bone is no longer able to heal itself [2, 3]. To solve large scale bone damage defects, bone grafting is required. Bone grafting is a method by which bone-deficient areas are built up, with the use of different materials, such as autografts, allografts, alloplasts and xenografts. Over recent times, the use of frozen bone matrix formulations and synthetic ceramics has been frequently used. Human bone materials (allografts), animal bone materials (xenografts), synthetic materials (alloplasts) and blood components are successful grafting materials. Their applications have shown an effective amount of bone formation and proliferation in the defective sites [4, 5]. However, limitations still exist. Allografts are associated with rejection, transmission of diseases and infections from donor to recipient and cost. Allografts and xenografts produce poor clinical outcome and the rejection of the graft is more likely and aggressive [6]. To solve these limitations, synthetic grafts (scaffolds) seems to be the alternative solution. These scaffolds can be used to support the self-healing mechanism of the human body and promote the regrowth of the damaged tissue. They must be highly porous allowing tissue in-growth and degrade after successful tissue regeneration minimizing the immune reaction and the need for revision surgery [7]. For bone regeneration, scaffolds must also be osteoconductive and osteoinductive structures [8].

1.2 Bone tissue engineering

Bone is a rigid organ that constitutes part of the vertebral skeleton. It supports and protects the various organs of the body, produce red and white blood cells, store minerals and enable mobility. Bone tissue is a type of dense connective tissue. They are lightweight but strong and hard, serving multiple functions [9, 10]:

- Support and protection: structural support for the entire body and protection to various vital organs;
- Movement: bone function allows muscles to provide movement of the body;
Storage of mineral and energy reserves: supplies calcium phosphate and storage energy reserves such as lipids.

According to porosity and unit microstructure, it is possible to identify two types of bones: cortical bone, also known as compact bone, and trabecular bone, also known as cancellous or spongy bone. Cortical bone is much denser and presents low porosity (5% to 10%) (Figure 1.1).

Figure 1.1 The composition of compact bone [11].

Most of bone properties depend on its composition, which consists of 50-70% mineral, 10-20% collagen and 9-20% water by weight, as shown in Figure 1.2. Bone contains between 50% to 70% of calcium phosphate in the crystalline form of carbonate hydroxyapatite (CHA) [12], which in the collagen matrix of bone becomes a highly stable crystalline structure with great mechanical strength and high flexibility. Hydroxyapatite provides strength and resistance to compression, while collagen fibres provide resistance to tensile forces.

Bone is a type of connective tissue formed by four main types of cells [13-16]:

- Osteoprogenitor stem cells;
- Osteoblasts, responsible for regeneration of new bone matrix (osteogenesis);
- Osteocytes, mature cells, responsible for maintaining and monitoring the protein and mineral content of the bone matrix;
- Osteoclasts responsible to dissolve the bone matrix and release stored minerals (osteolysis).
Figure 1.2 Chemical composition of bone [17].

1.3 Research aims

This research project investigates the use of electro-active active scaffolds for bone regeneration, investigating their potential to stimulate and guide cell attachment, proliferation and differentiation. Organic-inorganic composite material scaffolds (PCL/graphene nanosheets) are investigated and the performances of these scaffolds compared to non-electro-active scaffolds (PCL scaffolds). To reach these aims, this thesis covers a wide range of scientific fields, namely, material science, engineering and biology. Key research objectives can be listed as follows:

- To evaluate current tissue engineering strategies for bone regeneration;
- To develop a new strategy to create scaffolds for bone regeneration with improved mechanical and biological properties;
- To develop a strategy to electrical stimulate bone in-growth;
- To compare the PCL/graphene scaffolds with PCL scaffold reinforced with other carbon nanomaterials in order to define the most suitable inorganic reinforcement to create electro-active scaffolds;
- To develop a new approach in order to improve cell attachment/proliferation and differentiation by coating the scaffolds with natural protein or/and modify its surface wettability properties.

1.4 Thesis structure

This thesis is divided into five Chapters, which progress in accordance with the identified research objectives as presented in Figure 1.3.
Figure 1.3 Thesis structure-Chapters and contents.
CHAPTER II
STATE OF THE ART
This Chapter provides an overview on the current state of the art of materials and fabrication techniques to produce scaffolds for bone tissue engineering. It comprises two main Sections.

The first Section is based on a review paper entitled “Materials for electro-active scaffolds: A review” submitted to Progress in Materials Science. The use of electrical organic and inorganic materials to impart electrical properties to scaffolds, three dimensional substrates for cell attachment proliferation and differentiation, represents an important strategy in the field of tissue engineering. This Section discusses the concept of electro-active scaffolds and their roles in tissue engineering, accelerating cell proliferation and differentiation, and consequently tissue regeneration. The most relevant organic and inorganic materials used to produce electro-active scaffolds are presented and their main advantages and limitations discussed in detail. Particular emphasis is placed on the biocompatibility and biodegradability characteristics of these materials. Different technologies, allowing the fabrication of three dimensional scaffolds in a controlled way are also presented. Finally, challenges for future research in this area are highlighted.

As bone is a vascularised tissue, the second Section focus on different strategies to create the vasculature structure and it is based on a review paper entitled “Engineering the vasculature with additive manufacturing” published in Current Opinion in Biomedical Engineering. Additive manufacturing encompasses a group of 3D printing technologies enabling the generation of complex, biomimetic 3D structures for tissue engineering and regenerative medicine. The ability of 3D printing to pattern multiple materials, cell types and biomolecules provides a unique tool to create tissue constructs closely resembling the composition, architecture and function of biological tissues. Advances in printable biomaterials and 3D printing strategies allow the fabrication of vascularised tissue constructs composed of multiple cells embedded within suitable extracellular matrix components and supplied by functional vasculature. Thick and perfusable vascular tissue constructs can now be designed, printed and in vitro cultured for relevant time periods, offering a promising alternative to traditional vascularisation strategies. In this Section, a concise overview of recent 3D printing strategies explored to create vascular networks and vascularised tissue constructs are presented, and discusses future perspectives regarding the importance of engineering vascularisation for clinical applications.
2.1 Materials for Electro-active Scaffolds: A Review*

Weiguang Wang ¹, Dean Martinez ², Wei-Hung Chiang ², Paulo Bártolo ¹, *

¹ Manchester Biomanufacturing Centre, School of Mechanical, Aerospace and Civil Engineering, University of Manchester, Manchester, M13 9PL, UK

² Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, E2-514, Taiwan

Contribution to the work: As first author, W.W. participated in the planning, writing and editing of the article.

* Progress in Polymer Science, submitted.
2.1.1 Introduction

The use of scaffolds, physical substrates for cell attachment, proliferation and differentiation, is the most common strategy for tissue engineering [2, 11, 18, 19]. These scaffolds must be designed according to specific requirements to create the right environment for cell attachment, proliferation and differentiation. They must be biocompatible and biodegradable (the degradation rate must match the regeneration rate of the new tissue [2, 11, 18, 19]), with proper porosity and pore interconnectivity [2, 11, 18, 19]. Scaffolds must have adequate mechanical properties, which depend on the type of tissue, and surface characteristics and they must be easily sterilised [2, 11, 18, 19]. Its capacity to stimulate cells is also another important requirement. Electrical signals are critical physiological stimuli that strongly affect cell behaviour [20, 21] controlling cell migration, adhesion and differentiation, DNA synthesis and protein secretion [22-25].

The first evidence of electrical effects on tissues was presented by Bassett et al.[26], which investigated the effects of electric currents on bone regeneration in adult dogs. Tissue regeneration induced by electrical stimulation was also observed in rats presenting sciatic nerve injuries [27]. Other researchers also observed that electrical stimulation significantly increased the DNA synthesis of osteoblasts [28], improved the contractile behaviour of engineered cardiac tissue [29] and improved both myogenic differentiation and deposition of type 1 collagen [30].

Electro-active scaffolds have been produced through the incorporation of conductive materials in polymers and polymer composite materials. The incorporation of conductive materials allow the transmission of electrical signals from external sources through the cell seeded scaffolds without compromising its mechanical, biological and degradation behaviour. These electrically conductive polymer composites consist of conductive fillers (e.g. carbon nanomaterials and conductive polymeric materials) blended with nonconductive biocompatible and biodegradable materials, or polymer/ceramic materials. These scaffolds can be easily processed, through relatively low cost fabrication strategies and their mechanical and electrical properties can be easily tailored. The electrically conductive properties of these structures can be empirically described according to the following power law [31]:

\[
\sigma = \sigma_0 (p - p_c)^t
\]

(2.1)

where \(\sigma\) represents the electrical conductivity of the composite material, \(\sigma_0\) represents the scaling factor, a proportionality constant related to the intrinsic conductivity of the filler, \(p\) is the volume fraction of the filler, \(p_c\) is the percolation threshold and \(t\) is the critical
exponent related to the dimensionality of the conductive networks in the composite material. Composites with high $p_c$ present high melt viscosities, and inferior mechanical properties being more difficult to process [32, 33].

Different processing techniques have been explored to produce scaffolds with different dimensionalities and architectures. These processes include coating, hydrogel bending, electrospinning and additive manufacturing. Electrospinning has been widely used to fabricate electroactive structures. In this process polymer and conducting materials are dissolved in a suitable solvent and the polymer solution is dropped via a needle [2, 11]. Additive manufacturing describes a group of processes that create structures by joining material in a layer by layer approach, and includes the following technologies:

- Material extrusion: an additive manufacturing process in which material is selectively dispersed through a nozzle on surface;
- Material jetting: an additive manufacturing process in which droplets of build material are selectively deposited;
- Binder jetting: an additive manufacturing process in which a liquid binding agent is selectively deposited to join powder materials;
- Vat photopolymerisation: an additive manufacturing process in which liquid photopolymer in a vat is selectively cured by light-activated polymerisation;
- Powder bed fusion: an additive manufacturing process in which thermal energy selectively fuses regions of a powder bed.

Material extrusion is the most commonly used additive manufacturing method to create electro-active scaffolds.

### 2.1.2 Conductive elements

#### 2.1.2.1 Carbon nanomaterials

Carbon nanomaterials (CNMs) exhibit vast structural diversity, owing to carbon atom’s capability of covalently bonding at diverse hybridization states (sp, sp$^2$, and sp$^3$) with other carbon atoms and non-metallic elements [34]. The resulting allotropes are classified according to number of dimensions, i.e. 0D, 1D, and 2D with known models such as quantum dots, nanotubes and graphene, respectively [35]. The electronic properties of carbon are highly influenced by nanostructure anisotropy and its degree of replication [36]. All sp$^2$ carbon materials are intrinsically anisotropic as it contains delocalized last non-hybridized valence π-electrons in a plane perpendicular to its basal plane. The mobility
within lattice and the dynamics in one particular configuration creates “electronic layers”, responsible for the high 2D electric conductance [37]. CNMs are of similar size-scale to biological molecules, and thus can be effective platforms for enhancing biological activities within living organisms. Specifically, high surface area-to-mass ratio CNMs such as graphene and carbon nanotubes (CNTs) (Figure 2.1 (a)-(c)), maximize the scaffold potential for cellular development, interacting with biomolecules such as DNA, enzymes, proteins and peptides [38, 39].

2.1.2.1.1 Graphene

Discovered in 2004, a single layer graphene is an atomically thin film of carbon atoms bonded together in a planar 2D structure. As illustrated in Figure 2.1 (d)-(e), each carbon atoms are sp² (planar) hybridized having covalent σ bonds with three nearest carbon atoms, forming a robust honeycomb lattice. This makes graphene currently the strongest known material [16] with Young modulus of ~1.0 TPa [40]. Perpendicular to the graphene plane are π-bonds that form delocalized electron states across the plane. Due to the ease of electron movement in these π-states, a high carrier mobility of ~200000 cm² V⁻¹ s⁻¹ has been attained for suspended graphene and ~500000 cm² V⁻¹ s⁻¹ for graphene based field effect transistor (FET) [41], making graphene a useful material for biosensing and biomedical applications [42]. Moreover, the exceptional light absorption properties make graphene a promising candidate for phototransistors with high responsivity and sensitivity [43]. Recent reports demonstrated that graphene nanoribbon (GNR), a unique from of graphene, is an exceptional material for various applications including energy storage and conversion [44-47], chemical and biological detections [44, 48], catalysis [49, 50], and electronics [51, 52]. GNR represents a strip shape of graphene while the width ranges from sub -10 nanometer to a few hundred nanometers, with thickness around several nanometers [53-57]. Different from graphene nanosheet that possesses a zero bandgap, GNR has an open bandgap which can be controlled by tuning atomic-scale structures. For example, GNR with designed edges can effectively modulate the electronic energy gap, thus enhancing the charge transfer and chemical reactivity when used for chemical and biological detection.
Graphene can be produced by various approaches. Top-down synthesis methods of graphene are generally detachment or exfoliation from existing graphite crystals [60]. Exfoliation can be done mechanically (scotch-tape method) [61], in liquid phase exploiting ultrasounds to graphite [62-64] or graphite oxide sheets [41] using chemicals with matching surface energy, or by electrical arc-discharge between two graphitic electrodes (Figure 2.2) [65]. On the other hand, bottom-up synthesis deal with directly growing graphene layers on substrate surfaces. This method includes epitaxial growth on silicon carbide crystal [66] and chemical vapour deposition (CVD) [67] where graphene from a hydrocarbon source precipitates from the transition metal surface. Synthesis through CVD is the most viable method in terms of operational control, complexity and throughput [60]. The reduction of graphene oxide nanoribbon (GONR) is a promising alternative for the bulk production of GNR-based materials because it can be synthesized in large quantities [56]. Chemical unzipping carbon nanotubes through chemical oxidation with a suitable oxidant should be one of the most effective means of achieving low-cost and scalable GNR synthesis [45, 56]. In addition, this method can be used to generate oxygen-containing groups around the GNRs, thus adding solution-processing ability to the GNR process for industry-oriented device preparation [68].
Figure 2.2 Graphene top-down synthesis methods. Schematic of (a) arc discharge [69] and (b) CVD [70] set-up; (c) Micromechanical exfoliation of graphite [61] and TEM image [71]; (d) Deoxygenation of exfoliated GO under alkaline conditions [72].

After production, graphene can be wrapped up to form a zero dimension nanomaterial, rolled into one dimension nanotube or manipulated into 3D graphite [39]. Dispersed graphene and graphene oxide and its interaction with target cells have been explored [73-75]. For example, doses above 20 μg/mL have induced toxicity to human lung epithelial cells and fibroblasts [73]. Moreover, layered graphene sheets up to 5μm in lateral dimension can be internalized by macrophages by adhering initially, gradually spreading and covering few layered graphene (FLG) surface, without perturbation of their plate like shape (Figure 2.3). Further modifying size, shape and surface chemistry of graphene highly influence its impact as featured by Liao et al. using red blood cells [74]. In addition, common practice to reduce graphene toxicity is to cover its surface with a biocompatible polymer which also improves solubility, stability and retention time in the blood stream [75]. Although conflicting reports have been published on low cell toxicity of these materials [76, 77] it is safe to indicate that lessening graphene toxicity is associated with the biocompatibility of its surface functionalization especially its non-functionalized were found to be more toxic. However, long-term studies should be conducted such as preclinical studies and the consideration that animal models could provide different results from humans [73].
Multiple reports have indicated graphene to be outstanding platforms in promoting cell adhesion, proliferation and differentiation on various cells, e.g., embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) [79-82]. In the case of neural cells, graphene was found to be capable of forming a functional neural network [79] as demonstrated by Serrano et al. where 3D graphene oxide scaffolds were fabricated through a biocompatible freeze-casting procedure named ice segregation-induced self-assembly (ISISA), provided neural network interconnection rich in dendrites, axons and synaptic connections [83]. Graphene has also great potential for neural interfacing, promoting neurite sprouting and outgrowth in primary culture of hippocampal neurons [80]. Heo et al. investigated neural cell-to-cell interactive reactions on graphene/poly (ethylene terephthalate) films, followed by electrical stimulation at low and high magnitude [81]. As shown in Figure 2.4, cell-to-cell interactions were classified into cell-to-cell decoupling (CD) and cell-to-cell coupling (CC). The CC group was further separated to newly formed cell-to-cell coupling (NCC) and strengthened cell-to-cell coupling (SCC). Cell-to-cell wavering (CW) was also covered. Low stimulation was at 4.5 mV/mm and resulted to the highest percentage of cells categorized as CC. The rates of NCC and SCC was at highest and CD categorized cells was at the lowest also at this
setting. With high electrical field simulation (450 mV/mm), the majority of cells that reacted were categorized into the CD and CW groups. These results show that non-contact weak electric field stimulation enables cell-to-cell coupling without cellular death whereas high stimulation enhances cell-to-cell decoupling [81].

**Figure 2.4** Representative images and analysis of cellular response to electrical stimulation. “f1” to “f40” indicated the 1st to 40th imaged taken from optical microscope during stimulation. “f1” cell shapes are outlined in black, as shown in initial. The final shapes are then represented by colour (CC-red, CD-green, and CW-blue). (Top, low stimulation) Stimulation at 4.5 mV/mm where CC categorization was observed in majority of the cells, also with clear NCC and SCC. (Bottom, high stimulation) Stimulation at 450 mV/mm, where CD and CW categorized cells are more evident. Scale bar represents 30mm [81].

Tang _et al._ examined the development of neural network from human neural stem cells (hNSCs) differentiation at graphene by comparing fluorescence images from day 1 to day 14 [84]. After several hours of seeding, the cells had already tightly adhered to the substrates. As indicated in Figure 2.5 (a)-(d), cells showed normal migration ability, by migrating to various directions from the neurospheres after 1 day of seeding, which is typical of healthy neurospheres. At late periods, high portions of the neurites contacted each other resulted in subsequent synapse formation. A study comparing cell differentiation effects on glass and graphene was conducted by Feng _et al._ [82]. As shown in Figure 2.5 (e)-(h), after one-month, differentiated hNSCs were more apparent in graphene, showing also high cell adhesion. In terms of hNSC growth, it was possible to observe that when growth factors were added,
after 5 days the entire surface area was fully covered by cells in both glass and graphene materials. The results shown that graphene worked as an excellent cell-adhesion layer during the long term differentiation process and induced differentiation of hNSCs more towards neuron than glial cells [85].

**Figure 2.5** (a)-(d) NSC differentiation developing neural networks on graphene substrates. Samples of different culturing times are immunostained by antibody against B-tubulin [84]. (e)-(f) bright-field and (g)-(h) fluorescence images of immunostained differentiated hNSCs on glass and graphene. After one-month differentiation, the sample was immunostained with GFAP (red) for astroglial cells, TUJ1 (green) for neural cells, and DAPI (blue) for nuclei [85].

Jakus *et al.* mixed graphene and poly (lactide-co-glycolide) (PLG) as a bioink to produce custom-sized nerve conduit using an extrusion-based additive manufacturing (3D printing) technology [86]. Results show that with the increase of graphene concentration from 20 vol % (or \( \sim 32 \) wt.%) to 60 vol % (or \( \sim 75 \) wt.%), strain decreased from 210% to 81%, conductivity increased from 200 to 600 S/m and hMSC proliferation increased. Moreover, it was also observed that the expressions of neuronal specificmarkers (e.g. Glial and neurogenic relevant genes, glial fibrillary acidic protein (GFAP), neuron-specific class III \( \beta \)-tubulin (Tuj1), nestin (Nes), and microtubule-associated protein 2 (MAP2)) significantly increased after 14 days of cell differentiation [86].

Wang *et al.* [1, 87, 88] explored the use of an extruson-based additive manufacturing system to produce poly(\( \varepsilon \)-caprolactone) (PCL)/graphene scaffolds. The effects of adding graphene to the polymeric scaffolds were studied form morphologiccal, physico-chemical and biological point (Figure 2.6). Results showed that the addition of small quantities of graphene have a positive impact in terms of mechanical properties, cytocompatiability and stimulating cell proliferation. PCL/graphene scaffolds with squared pore size of 350 μm were produced using a screw-assissted extrusion additive manufacturing system. The
results show that by increasing the graphene content from 0 wt.% to 0.78 wt.% the compression modulus increased from 82.2±6.8 MPa to 128.7±6.9 MPa. Cell proliferation of adipose-derived hMSCs was also significantly increased due to the presence of graphene. Other studies also show that graphene can be used to accelerate the osteogenic differentiation of hMSCs [89, 90].

Liao et al. fabricated composite material scaffolds composed of chondroitin sulphate methacryloyl, poly(ethylene glycol) methyl ether-ε-caprolactone-acryloyl chloride, and graphene oxide (CSMA/PECA/GO) using a thermal-initiated free radical polymerization method for cartilage regeneration. In vitro biological assessments suggested chondrocytes grown on these scaffolds were able to proliferate with high viability. Moreover, for the in vivo biological assessment of implanting these scaffolds in the osteochondral defect of a rabbit model, these samples with cell injection induced higher volume of newly formed cartilage/bone tissues, compared to the scaffold only group [91]. Additional biological studies using graphene are presented in Table 2.1.

Figure 2.6 (a) Extrusion-based additive manufacturing system; (b) Design of the PCL and PCL/graphene scaffolds; (c) PCL and PCL/graphene scaffolds after fabrication; (d) mechanical characterisation; (e) Biological characterisation (Alamar Blue assay); (f) Scanning electron microscope and confocal microscope images of cell-seeded scaffolds [1, 87, 88].
2.1.2.1.2 Carbon nanotubes

Carbon nanotubes (CNTs) are cylindrical tubes of sp² bonded carbon atoms, conceptually regarded as rolled-up sheets of graphene [92]. CNTs are considered 1D and highly anisotropic materials as their aspect ratio (length/diameter) frequently exceeds 10,000 [34] with ends uncapped by semi-fullerene molecules (pentagonal ring defect) [68]. CNTs can be classified as single-walled (SWCNT) or multi-walled (MWCNT) depending on the number of concentrically arranged graphene layers. SWCNT diameter typically range from 0.4 to 2 nm [34], while MWCNTs outer diameter varies from 2 to 30 nm [68] with interlayer spacing of 0.34 to 0.39 nm [93] creating a coaxial nanotube assembly resembling a Russian-doll. According to the chirality, SWCNTs are classified as armchair, zigzag and chiral (Figure 2.7 (a)). Chirality and size dictate the electronic properties of CNTs. Armchair SWCNTs exhibit metallic behaviour as they have finite density of states at Fermi level, while chiral SWCNTs are semiconductors given a zero density of state of the small band gap featured in their band structure [34]. Due to the larger outer diameter and lower curvature, MWCNTs are less reactive than SWCNTs [92]. With the distortion of hybrid orbitals due to curvature-induced strain and the presence of sp³ defects sites in the walls, graphene outweighs the electronic properties of SWCNTs with carrier mobility up to $10^4$ cm² V⁻¹ s⁻¹, although superior than silicon, copper and aluminium [94]. Doping CNT with heteroatoms such as nitrogen or boron is an effective way to control its electrical properties [95], thermal properties [96] and chemical properties [97].

Three different methods can be used to synthesize CNTs (MWCNTs and SWCNTs) (Figure 2.7 (b)-(d)): chemical vapour deposition (CVD) [98, 99], carbon arc-discharge [100] and laser ablation [101]. In laser ablation and arc discharge methods, MWCNTs are synthesized in the absence of a catalyst, and SWCNTs are synthesized in the presence of carbon electrodes containing catalytic metal particles [102]. CVD is the most widely used method, allowing large scale production of CNTs and to control the SWCNT chirality [98, 103]. Its variations include plasma enhanced (PE) oxygen assisted CVD [104], microwave plasma (MPECVD) [105], and radio frequency (RF-CVD) [106]. The most powerful and standardized method is catalytic chemical vapour deposition (CCVD) [34].
Figure 2.7 (a) SWCNTs configuration [107, 108]. Three common systems for CNT synthesis: (b) Arc-discharge; (c) Laser ablation and (d) thermal CVD [92].

Although the CNTs with exceptional properties have been shown useful for biomedical applications, the biocompatibility and biosafety are still under investigation [102]. It has been reported that the CNTs with high aspect ratios show similar toxicity with asbestos fibres [109], making it possible to induce inflammation [110] and potentially cause fibrosis [111]. In addition, the surface functional groups attached on the CNTs can change the interaction on the cell membranes and further control the penetration of CNTs into the cells [112, 113]. Moreover, the catalyst particles may affect the biosafety because they can introduce oxidative stress, cross cell membranes, and generate free radicals [114]. Therefore, changing critical parameters such as size, impurities surface chemistry, surface charge, reactivity, morphology and crystal structure can significantly influence toxicity of CNTs towards cells [73]. In addition, there is a huge difference in toxicity patterns of carbon nanotubes in comparison to graphene and graphene oxide, due to the difference in their synthesis route and structural morphology [77]. Reports show that carbon nanotubes have shown superior performance in conductive tissue regeneration. They have been applied to cardiomyocytes for heart engineering [115], nerve tissue replacement [116], bone cell proliferation [117], and stem cell growth [118]. When in contact with CNTs, cells have been found to become electrically more active, mature and better interconnected. Moreover, their high aspect ratio can structurally simulate certain elongated biomolecules (e.g. in the field of nerve tissue engineering and artificial neural networks, CNT has the capability of boosting effect on neuron activity modulate the immune response) useful to mimic morphology of heart and nerve tissues [116].

Electro-active scaffolds of CNT have been produced using different polymers and hydrogels. Sang et al. produced single-walled carbon nanotube-poly(n-isopropylacrylamide) (SWCNT-PNIPAAm) structures using copolymerization of n-isopropylacrylamide, the
oligomeric amphiphilic crosslinker of polyethylene glycol diacrylate-dodecylamine-1-(2-aminoethyl)piperazine (PEGDA-DD-AEP), and single-walled carbon nanotubes [119]. The effects of electrical stimulation on the morphologies of 3D encapsulated SH-SY5Y cells compared with 2D SH-SY5Y cells (2D), PNIPAAm hydrogel (3D), and SWCNT-PNIPAAm hydrogel (3D) was investigated. From Figure 2.8, it is possible to observe that neurite outgrowth is more apparent using 3D SWNT-PNIPAAm hydrogel subjected to electrical stimulation. It was also found that electrical stimulation application not only enhanced the neurite sprouting, but also made the dividing cells gather and form multinucleate cells. The effect of the electrical conductivity of the SWNT-PNIPAAm hydrogel on SH-SY5Y cells was confirmed through significant increase in neurite number and largely enhanced neurite outgrowth.

Gelatin methacryloyl (GelMA) hydrogel structures containing CNTs were investigated by Shin et al. [115] and Ahadian et al. [120]. Ahadian et al. aligned the CNTs (GelMA pre-polymer solution was mixed with the CNT aqueous solution at a ratio of 1:1 to obtain a final GelMA concentration of 5% (w/v)) using dielectrophoresis whereas Shin et al. produced GelMA containing random CNTs (0, 1, 3, 5 mg/ml). Although both systems produced positive results in promoting cell differentiation, the effect of applying electrical stimulation made the difference between them more pronounced. As shown in Figure 2.9, the mouse embryoid bodies (EBs) cultured on the GelMA-aligned CNTs differentiated more towards cardiomyocytes in contrast with the EBs on the pure GelMA and GelMA containing the random CNTs. The different impact of electrical stimulation on the GelMA hydrogels containing aligned CNTs to the pure GelMA and GelMA randomly dispersed CNT hydrogels is due to the higher electrical conductance of CNT containing GelMA in the direction of applied electrical stimulation.
Figure 2.8 (I) The effects of electrical stimulation on SH-SY5Y cell morphology compared to a group with no applied electrical stimulation (II), (scale bar ¼ 40 mm). There was no significant difference in cell morphology in 2D groups (a-f) and 3D PNIPAAm hydrogel groups (g-l). However, neurite outgrowth was enhanced on SWCNT-PNIPAAm with electrical stimulation (m-o) compared with no electrical stimulation (p-r) (scale bar ¼ 40 mm) [119].

Figure 2.9 (a) Cardiac differentiation analysis by applied immunostaining of nuclei (DAPI) and troponin (T) on pure GelMA and CNT-GelMA with embryoid bodies, by Ahadian et al. Low cardiac differentiation is indicated by low expression of Troponin T [120]. (Right) Immunostaining of Troponin I (green) and nuclei (blue) at CNT-GelMA hydrogels by Shin et al. Cardiac cell phenotype examination on the hydrogels showed more aggregated Troponin I presence on (b) pristine GelMA than on (c) CNT-GelMA. (d) Confocal images of pristine GelMA and 1mg/ml CNT-GelMA with cardiomyocytes cultured for 5 days [115].
Ho et al. used an extrusion-based additive manufacturing system to produce 3D porous PCL/MWCNT scaffolds with filament distance ranging from 300 to 450 µm [121]. The effect of adding MWCNT to PCL (1%, 3% and 5% w/w%) on both mechanical and biological properties of the scaffolds were investigated. Nanoindentation studies showed a gradual enhancement in the elastic modulus (increased from 0.51 ± 0.18 GPa for PCL scaffold to 0.87 ± 0.10 GPa for PCL scaffolds containing 5% of MWCNT), hardness (increased from 0.057 ± 0.010 GPa to 0.072 ± 0.003 GPa), and maximum peak load (increased from 1.16 mN to 1.34 mN). The addition of MWCNTs also contribute to increase the crystallization level, broadening the crystallization peak, due to the restricted mobility of polymer chains in the nanocomposite matrix. MTT assay with H9C2 rat myocardial cells showed no cytotoxic due to the presence of MWCNTs [121]. He et al. also used H9C2 cells to assess PCL-polyethylene oxide (PEO)/MWCNT scaffolds [122]. In this case, scaffolds were fabricated with fibre diameter of 10 µm, vertical pores of around 800 µm but almost no porosity in the lateral sides, and different concentrations of MWCNT (0, 0.5, 1.5 w/v%) were produced using a electrohydrodynamic 3D printing system. The PCL solution was prepared using acetic acid and PEO was added to modulate the viscosity. Biological results showed that the addition of MWCNT facilitate cell alignment but had a negative effect on cell attachment, due to its agglomeration in the printed fibres, compared to PCL-PEO scaffolds.

For bone regeneration, Goncalves et al. [123] produced three-phase interconnected porous scaffolds (hydroxyapatite and CNTs mixed with PCL) with different compositions (50 wt.% PCL, CNTs varying between 0 to 10 wt.%, and hydroxyapatite being the balance) and pore size ranging between 450 and 700 µm using a pressure-assisted additive manufacturing system. Biological tests were performed using MG63 osteoblast-like cells. For all compositions, it was possible to observe cell high attachment and proliferation values in scaffolds containing high content of CNTs. Compression tests showed that scaffolds with low CNT content presented larger compressive resistance, while scaffolds with 10% of CNTs were more easily deformed [123]. In another work, a PCL/HA slurry containing ionically modified CNTs (CNT with positive charged surface) was robotic-dispensed producing scaffolds with pore sizes of around 226 µm. The concentration of HA was set to be 40 w/v % and the concentration of CNTs was 0.2 wt.%. Results show that the incorporation of the ionically modified CNTs improved the compressive strength (from 1.5 MPa for PCL scaffolds and 2.0 MPa for PCL/HA scaffolds to 5.5 MPa for PCL/HA/CNT scaffolds) and MC3T3-E1 cell attachment and proliferation. In vivo tests were conducted by implanting the PCL/HA/CNT scaffold into a rat subcutaneous tissue. After four weeks,
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the results show signs of inflammatory effects due to the presence of the scaffold and the formation of soft fibrous tissue and neo-blood vessels [124].

Non-additive techniques like freeze casting and electrospinning were also used to produce different types of polymer/CNT polymer/ceramic/CNT and bioglass/CNT scaffolds [125-131]. In all cases, results show that the presence of CNTs improved compression strength and elastic modulus, and had a positive effect on the biological performance (cell attachment, proliferation and differentiation) of the scaffolds. Additional studies using carbon nanotubes as electro-active scaffolds for cell development are tabulated in Table 2.1.

**Table 2.1** Studies using CNM composites as electro-active scaffolds.

<table>
<thead>
<tr>
<th>Electro-active scaffold</th>
<th>Electrical Stimulation Settings</th>
<th>Cell/Cell Line</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Graphene Nanofibres</td>
<td>Pulse potential of 100 mV/cm (40 s)</td>
<td>Neurons</td>
<td>Neurite growth speed was twice than motor neurons</td>
<td>Feng et al., 2015 [82]</td>
</tr>
<tr>
<td>Graphene foam</td>
<td>-0.2 to 0.8 V, 1–100 ms monophasic cathodic pulses at 10 s intervals, 20 – 30 μA threshold</td>
<td>Neural stem cell</td>
<td>Supported cell growth and enhanced differentiation to neurons than astrocytes</td>
<td>Li et al., 2013 [132]</td>
</tr>
<tr>
<td>Graphene films</td>
<td>10–100 ms monophasic cathodic pulses at 5 s intervals, 0.5 – 1.0 μA threshold</td>
<td>Neural stem cell</td>
<td>Differentiation to neural networks, Neural signal transmission efficacy increased</td>
<td>Tang et al., 2013 [84]</td>
</tr>
<tr>
<td>Graphene/PET</td>
<td>±4.5 mV/mm to ±450 mV/mm (pulse duration 10 s with an interval of 100 s for 32 minutes)</td>
<td>SHSY5Y human neuroblastoma cells</td>
<td>Significant increase in cell number and strengthened new and old cell-to-cell couplings</td>
<td>Heo et al., 2011 [81]</td>
</tr>
<tr>
<td>Material</td>
<td>Current Parameters</td>
<td>Cell Type</td>
<td>Result Description</td>
<td>Reference</td>
</tr>
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<td>----------------</td>
<td>----------------------------------------------------------</td>
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<tr>
<td>mPEG-rGO</td>
<td>1–100 ms monophasic anodic pulses, 10 sec duration, 0.6V pulse potential</td>
<td>PC12 neural cells</td>
<td>Predominant increase in cell percentage with higher action potentials</td>
<td>Zhang et al., 2014 [133]</td>
</tr>
<tr>
<td>Graphene</td>
<td>Cathodic/monophasic voltage-pulses, 500 mV, 100 ms, 10 pulse trains per second</td>
<td>Human neural stem cells</td>
<td>Induced differentiation more towards neurons than glial cells</td>
<td>Park et al., 2011 [85]</td>
</tr>
<tr>
<td>PLLA/CNT</td>
<td>AC: 10 mA (10 Hz, 6 hour/day)</td>
<td>Osteoblasts</td>
<td>46% increase in cell proliferation after 2 days</td>
<td>Supronowicz et al., 2002 [134]</td>
</tr>
<tr>
<td>MWCNT</td>
<td>200 μs pulses of 1–50 V at 40 s intervals</td>
<td>Neurons</td>
<td>Neurite regrowth in spinal explants is favored</td>
<td>Alessandra et al., 2012 [135]</td>
</tr>
<tr>
<td>CNT - PEDOT</td>
<td>-0.9 to 0.5 V at scan rate of 100 mV s⁻¹ followed by 0.30 mC cm⁻² at 50 Hz</td>
<td>NB-39-Nu human Neuroblastoma</td>
<td>Higher cell proliferation and longer neurite lengths</td>
<td>Depan and Misra, 2014 [136]</td>
</tr>
<tr>
<td>PLA/MWCNT</td>
<td>DC: 100 μA (4 hours/day, 6 days)</td>
<td>Osteoblasts</td>
<td>Proliferation and elongation along current direction</td>
<td>Shao et al., 2011 [117]</td>
</tr>
<tr>
<td>CNT-GelMA</td>
<td>50 ms pulses of 0–7 V/cm at 0.5, 1, 2 and 3 Hz</td>
<td>Cardiomyocytes</td>
<td>Improved cell adhesion, organization and cell-to-cell coupling</td>
<td>Shin et al., 2013 [115]</td>
</tr>
<tr>
<td>PCL/CNT</td>
<td>5 V cm⁻¹ for 5 ms duration at 1 Hz every 4 days</td>
<td>Human Mesenchymal Stem Cells</td>
<td>Rapid morphological changes and expressed cardiac genes</td>
<td>Crowder et al., 2013 [118]</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>CNTs aligned in GelMA</th>
<th>1 Hz, 3V, 10 ms duration for two continuous days</th>
<th>129/SVE mouse stem cells</th>
<th>Differentiated more towards cardiomyocytes</th>
<th>Ahadian et al., 2016 [120]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT-PNIPAAm hydrogel</td>
<td>(rectangular wave) 20 Hz, 100 mV potential, for 2 hours</td>
<td>Human SH-SY5Y neuroblastoma cells</td>
<td>Enhanced neurite outgrowth, sprouting and formation of multinucleate cells</td>
<td>Sang et al., 2016 [119]</td>
</tr>
<tr>
<td>PCL nanofibre with CNT</td>
<td>750 mV, 100 Hz AC for 30 minutes daily for 3 to 6 days</td>
<td>PC12 cells</td>
<td>Induced neural differentiation</td>
<td>Su and Shih, 2015 [137]</td>
</tr>
<tr>
<td>NGF-Col-CNT</td>
<td>500 mV using Ag/AgCl electrodes</td>
<td>PC 12 cells</td>
<td>Massive release of NGF consequently supporting neurite sprouting and growth</td>
<td>Cho and Borgens, 2013 [138]</td>
</tr>
</tbody>
</table>

PLA, poly-DL-lactide; GelMA, gelatin methacrylate; PLA, polylactic acid; PEDOT, poly(3,4-ethylenedioxythiophene); PET, polyethylene terephthalate; mPEG, methoxy poly(ethylene glycol); PNIPAAm, carbon nanotube-poly(n-isopropylacrylamide); PCL, polycaprolactone; NGF-Col, nerve growth factor-loaded collagen.

2.1.2.2 Conductive polymers

Electrically conducting polymers have a conjugated backbone with a high degree of π-orbital. These conjugated polymers are initially insulators and it is through a doping process, by chemical or electro-chemical means, that the neutral polymer chain can be oxidized or reduced to become either positively charged (oxidative or p-doping) or negatively charged (reductive or n-doping), introducing the necessary mobile charge carriers for electrical conduction [139-141].

Conductive polymers can be easily blended with other polymers before being processed using for example additive manufacturing technologies or electrospinning techniques to produce porous scaffolds [2, 11, 18, 19]. Blending ensures homogenous distribution of the conducting polymeric chains through the blend, which translate into electrical signals being effectively transmitted through the entire composite structure. As described by Skotheim, there are over 25 conducting polymeric systems [142]. However, the main conductive polymers being used for tissue engineering include polyaniline, polypyrrole, and polythiophene [21, 143]. Table 2.2 summarizes the use of conductive polymers in the
medical field and Table 2.3 presents their conductivity values. Hydrophilic polymers including polysaccharides (hyaluronic acid, alginate, chitosan and cellulose) and proteins (collagen and gelatin) have been also explored as electroconductive hydrogels through blending, doping or chemical modification with electroactive materials [144]. These hydrogels, usually presenting unsatisfactory mechanical properties, potential immunogenicity and difficult to control degradation process, are not considered in this review.

Table 2.2 Medical usage of conductive polymers.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Medical usage</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyaniline</td>
<td>Tissue engineering scaffolds</td>
<td>Gizdavic-Nikolaidis et al., 2010 [145]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Li et al., 2006 [146]</td>
</tr>
<tr>
<td></td>
<td>Bio-actuators</td>
<td>Spinks et al., 2005, 2006 [147, 148]</td>
</tr>
<tr>
<td>Polypyrrole</td>
<td>Implantable electrodes</td>
<td>Cui et al., 2003 [149]; Kim et al., 2004 [150]</td>
</tr>
<tr>
<td></td>
<td>Electrical stimulation and modulation of survival and maintenance of human</td>
<td>Stewart et al., 2014 [151]; Thompson et al., 2010 [152]</td>
</tr>
<tr>
<td>Polythiophene</td>
<td>Substrate to electrically stimulate/regulate adhesion, proliferation and</td>
<td>Ostrakhovitch et al., 2012 [153]; Richardson-Burns et al., 2007 [154]</td>
</tr>
<tr>
<td></td>
<td>signalling of neuronal cells</td>
<td>Furukawa et al., 2013 [155]; Wilks et al., 2009 [156]</td>
</tr>
<tr>
<td></td>
<td>Coating electrodes</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3 Conductive values of the most commonly used conductive polymers [157].

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Conductivity (S cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyaniline</td>
<td>30-200</td>
</tr>
<tr>
<td>Polypyrrole</td>
<td>10⁻³-7.5 x 10⁻¹</td>
</tr>
<tr>
<td>polythiophene</td>
<td>10⁻¹</td>
</tr>
</tbody>
</table>


2.1.2.2.1 Polyaniline

Polyaniline (PANI) is a hydrophilic polymer soluble in water. It is prepared by the chemical oxidation of aniline or anilinium salts in the presence of an oxidizing agent as shown in Figure 2.10 (a) [21, 158, 159]. Other methods to synthesize polyaniline include emulsion, dispersion, solution, interfacial, metathesis, and self-assembling polymerization [160]. Depends on the level of oxidation, polyaniline can be synthesized in a series of insulating forms such as the fully reduced leucoemeraldine base, half-oxidized emeraldine base and the fully oxidized pernigraniline base. The emeraldine base polyaniline (Figure 2.10 (b)-(c)) is the most stable and widely investigated one.

As reported by several groups based on in vivo and in vitro tests, polyaniline can be considered as a biocompatible material [21, 161, 162]. However, because of the non-biodegradability, the long term presence of polyaniline inside the patient could lead to some side effects, such as inflammation caused by wear and debris formation. Additionally, aniline and other reaction by-products formed during the polyaniline synthesis are aromatic amines which have been known to be carcinogenic [163]. Humpolicek et al. investigated the effect of purifying polyaniline through repeated de-protonation and re-protonation on its cytotoxicity behaviour. The results show that the cytotoxicity of polyaniline mainly caused by the reaction of by-products during the synthesis process, and consequently the biocompatibility of polyaniline can be considerably improved by performing additional purification steps during synthesis or by using commercial available polyaniline of high purity [21, 161, 163]. Moreover, the production of electrically conducting polyaniline requires the addition of a dopant, usually strong acids to protonate the polyaniline.
backbone, which leach out from the polyaniline matrix causing a local acidic environment, originating some level of toxicity [164]. Despite some initial toxicity to cells exposed to polyaniline, there are several strategies that can be used to increase the polyaniline biocompatibility showing no adverse reactions. To minimise these problems avoiding adverse biological reactions, several strategies can be considered including pre-soaking the polymer in a proper medium before its contact with cells, purification after synthesis, immobilization of peptide sequences and surface modification (e.g. changing the wettability) [21, 161, 165]. Bidez et al. investigated the adhesion and proliferation of H9c2 cardiac myoblasts on conductive and non-conductive polyaniline films (Figure 2.11) and found that both non-conductive emeraldine base and conductive emeraldine salt allow cell attachment and proliferation [166]. Similarly, Kamalesh et al. investigated the in vivo biocompatibility of polyaniline films subcutaneously implanted in rats during 90 weeks, observing no toxicity, abnormality and inflammatory effects [162].

Figure 2.11 Microfilament arrangement of H9c2 cells growing on E-PANI, PANI and Thermax™. Myoblast cells were seeded at low density (clonal) and cultured for up to 144 h. Cells were stained with the F-actin-specific stain rhodamine-phalloidin and visualized on a Leica DMRx fluorescence microscope. All images were captured with Leica 300FX camera using a 20× fluorescence objective [166].

As a key material for electro-active scaffolds, polyaniline has been used for nerve, bone, and cardiac regeneration [167-169]. Research done by Schmidt et al [167] showed that
relatively short stimulation time (2 hours) during the first 24 hours of cell seeding was an effective mechanism to increase nerve growth factor (NGF)-induced PC12 cell neuritogenesis, compared to the control group that treated with only NGF without electrical stimulation. Therefore scaffolds with different electrical-conductivity properties have been investigated (Table 2.4). Borriello et al. produced polyaniline/PCL short fibres as patches with electrospinning for cardiac muscle regeneration [170]. Human mesenchymal stem cells were successful differentiated into cardiomyocytes. Results also show high survival rates of cardiomyocytes on polyaniline-PCL patches than on PCL ones. Similarly, Li et al. produced electrospun meshes of polyaniline and gelatin, and correlated the effect of adding different contents of polyaniline in terms of fibre diameter and mechanical properties [146]. Results show that an increase in polyaniline concentration reduced the average fibre diameter and increased the tensile modulus. Moreover, cardiac myoblast cells were successfully cultured on the produced electrospun meshes. Initially, cells showed different morphologies on the meshes depending on the concentrations of polyaniline, after one week, all groups obtained similar confluency and morphology (Figure 2.12) [146].

Figure 2.12 Morphology of H9c2 myoblast cells at 20 h of post-seeding on: (a) gelatin fibre; (b) 15:85 PANI-gelatin blend fibre; (c) 30:70 PANI-gelatin blend fibre; (d) 45:55 PANI-gelatin blend fibres; and (e) glass matrices. Staining for nuclei-bisbenzimide and actin cytoskeleton-phalloidin, fibersauto fluorescence, original magnification 400 [146].
Table 2.4 Example of electro-active scaffolds with different electrical-conductivity properties.

<table>
<thead>
<tr>
<th>Electro-active scaffold</th>
<th>Maximum conductivity (S/cm)</th>
<th>Cell line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyaniline/PLACL</td>
<td>0.0138</td>
<td>NIH-3T3 fibroblasts</td>
<td>Jeong et al., 2008 [171]</td>
</tr>
<tr>
<td>Polyaniline/PLCL</td>
<td>0.296</td>
<td>C2C12 myoblasts</td>
<td>Jun et al., 2009 [172]</td>
</tr>
<tr>
<td>Polyaniline/gelatin</td>
<td>0.021</td>
<td>H9c2 cardiac myoblasts</td>
<td>Li et al., 2006 [146]</td>
</tr>
<tr>
<td>Polyaniline/collagen</td>
<td>0.27</td>
<td>Porcine skeletal muscle cells</td>
<td>Kim et al., 2009 [173]</td>
</tr>
<tr>
<td>Polyaniline/PCL</td>
<td>0.00008</td>
<td>HMSCs cardiomyocytes</td>
<td>Chen et al., 2013 [174]</td>
</tr>
<tr>
<td>Polyaniline/PDLA</td>
<td>0.0437</td>
<td>Primary rat muscle cells</td>
<td>McKeon et al., 2010 [175]</td>
</tr>
<tr>
<td>Polyaniline/PLGA</td>
<td>0.0031</td>
<td>Neonatal cardiomyocytes</td>
<td>Hsiao et al., 2013 [176]</td>
</tr>
<tr>
<td>Polyaniline/PGS</td>
<td>0.018</td>
<td>C2C12 myoblasts</td>
<td>Qazi et al., 2014 [161]</td>
</tr>
<tr>
<td>Polyaniline/silk fibroin</td>
<td>0.48</td>
<td>L929 fibroblasts</td>
<td>Xia et al., 2014 [177]</td>
</tr>
</tbody>
</table>

Kim et al. fabricated conductive matrixes of polyaniline nanofibres and dispersed them in collagen to culture porcine skeletal muscle cells [173]. Constructs with a conductivity value of 0.01 S/cm and a high concentration of polyaniline (50 wt.%) were produced using solvent casting. As the conductivity significantly decreased with the decreasing of polyaniline concentration, conductivity was able to be tuned by dedoping the composite in natural water and redoping in acid. It was also able to observe that the contact angle were relatively high (around 80 degrees) for the fabricated samples regardless of the polyaniline concentration in the composite. However, biological tests using porcine skeletal muscle cells showed the amount of cells grown on this composite structure and their morphology were similar to cells grown on collagen samples.

Jun et al. fabricated electrical conducive fibres with composite of polyaniline and poly(l-lactide-co-ε-caprolactone) (PLCL) through electrospinning technology. The
myogenic differentiation of myoblasts on these fibres were investigated [172]. Results indicated produced electrospun fibres show no significant difference in terms of fibre diameter or contact angle with or without the addition of polyaniline material (Figure 2.13 (a)-(b)). The composite fibres were cytocompatible and the results show that with the increase of polyaniline amount, significant higher myotube number, length and area can be observed. Similar results were obtained by Ku et al. using polyaniline-PCL electrospun fibres (Figure 2.13 (c)-(d)) [178]. Moreover, these studies show that the use of an aligned fibre orientation did not have a significant influence on cell attachment and proliferation, also on significant promotion on morphological alignment of cells along the orientation axis of the fibres. However, aligned fibres exhibited significantly higher elastic moduli.
Electrospinning was also used by Bhang et al. to produce polyaniline/PLCL meshes to be used as nerve graphs [179]. Results show that the addition of polyaniline not only increased cell viability, but also promoted the expression of neural differentiation proteins. Ghasemi-Mobarakeh et al. investigated the influence of direct electrical stimulation on nerve stem cells cultured on electrospun composite fibres of polyaniline and PCL/gelatin [139]. A direct current (DC) source was used to stimulate cells at 1.5 V for 15, 30 and 60 minutes, while non/stimulated samples served as control. Electrical stimulation was found
to significantly improve cell proliferation as well as nitrite length and outgrowth, with the best results being achieved after 60 minutes of stimulation.

Whitehead et al. produced conductive composites of polyaniline, PCL and bioactive mesoporous silicon using solvent casting, observing accelerated calcification of the composites in simulated body fluid (SBF) when electrically stimulated [180]. Produced structures were found to be non-cytotoxic to human kidney fibroblasts. Similar results were obtained by Farshi Azhar et al. using freeze drying to produce porous chitosan-gelatin/nanohydroxyapatite-polyaniline scaffolds [181].

Polyaniline is not only relevant to tissue engineering due to its electrical conductivity properties that allows to create electro-active substrates for cell culture and tissue regeneration, but also due to its antibacterial properties. Studies showed that polyaniline presents antibacterial effects against Gram positive and Gram negative bacteria, including *Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus* [182, 183]. Additionally, polyaniline has also been used as drug delivery systems for cancer applications [184-189].

### 2.1.2.2 Polypyrrole

Polypyrrole (PPy) (Figure 2.14) is a rigid and difficult to process conductive synthetic polymer, insoluble in any organic solvent or water [159, 190], synthesized by chemical or electro-chemical processes [191]. Chemical synthesis, usually used to produce large material quantities, requires mixing a strong oxidizing agent with a monomer solution. Electro-chemical synthesis is a simple and versatile technique, allowing high control over material thickness and geometry, facilitating doping during synthesis and the generation of films with a better thickness control. The addition of ester linkages to the pyrrole monomer makes polypyrrole biodegradable, which is a relevant property for tissue engineering applications [22]. Polypyrrole is also an intrinsically porous material. Porosity strongly depends on the preparation method.
The biocompatibility of polypyrrole has been investigated by several authors. Wang et al. demonstrated the biocompatibility of polypyrrole using thin films with endothelial cells [193]. Similar results were observed by Williams and Doherty with L929 mouse fibroblast and neuroblastoma cells [194] and by Jiang et al. using nerve cells [195]. Hodgson et al. investigated the control release of polypyrrole films doped with NGF showing that voltage induced the release of NGF from the films promoting neurite extension from PC12 cells [196]. Similar results were obtained by Gomez and Schmidt [197], which immobilized NGF in polypyrrole through a mechanism illustrated in Figure 2.15. Stauffer and Cui investigated the biological behaviour of polypyrrole films doped with two pipette sequences from laminin (CDPGYIGSR-p31, RNIAEHKDI-p20) using primary neurons cultured in a defined media [198]. Results show that the polypyrrole film with two peptides dopings presented the highest neuron activity. Surfaces doped with pipetted p20 had significantly longer primary neuritis.
Kai and coworkers fabricated PCL/PPy nanofibrous scaffolds with electrospinning and mixed them with gelatin to create a composite structure to mimic the extracellular matrix (ECM) of native cardiac muscle [199]. Results showed with the increase of PPy concentration, the average fibre diameter slightly decreased, and the tensile modulus significantly increased. It was also possible to observe that electroactive meshes acceleratled cardio-myoblasts attachment and proliferation However, better results were obtained with meshes containing lower concentrations of PPy (15\% of PPy) suggesting that high concentrations could have a negative effect on cell growth.

Ezazi et al. used a slurry casting method to create antibiotic drug-loaded composite electro-active scaffolds formed by hydroxyapatite, gelatin and microporous silica, loaded with a model antibiotic, vancomycin (VCM), coupled with or without conductive polypyrrole [200]. The scaffold containing PPy showed good mechanical properties, higher protein adsorption and higher percentage of VCM release over a long duration of time compared to non-conductive scaffolds. In vitro biological assessment showed osteoblasts perfectly immersed in the gelatin matrix and remaining viable for 14 days.

Björninen et al. studied the differentiation of adipose stem cells (ASCs) towards smooth muscle cells (SMCs) on polypyrrole coated poly (trimethylene carbonate) scaffolds through electrical stimulation [201]. Long pulse (1 ms) or short pulse (0.25 ms) biphasic electric current at a frequency of 10 Hz was applied to ASCs to study the effects of electrical stimulation on ASC viability and differentiation towards SMCs on the
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PPy-coated scaffolds. Results suggested PPy-coated scaffolds promoted proliferation and induced stronger calponin, myosin heavy chain (MHC) and smooth muscle actin (SMA) expression in ASCs in comparison with uncoated scaffolds.

Bueno et al. fabricated xanthan/polypyrrole (XCAPPy) scaffolds for tissue engineering by electropolymerising polypyrrole in xanthan hydrogels (XCA) [202]. The obtained 40 ± 10 μm thick scaffolds contained 15 ± 3 wt.% of polypyrrole. Cell adhesion and proliferation of fibroblasts on fabricated scaffolds were evaluated on day 1, 7, 14 and 21, with and without external magnetic field (EMF) of 0.4 T. Based on the result, it can be observed that because of the hydrophobicity and surface roughness, fibroblast proliferation was more pronounced on XCA/PPy scaffolds than on XCA scaffolds. Results also indicated EMF stimulated fibroblast proliferation on both scaffolds.

De Castro et al. electrospun electrical conductive nanoscaffolds using a blend of polypyrrole and a non-conductive biodegradable polymer, poly(butylene adipate-co-terephthalate) (PBAT) [203]. Mechanical and biological assessments showed that the incorporation of PPy to PBAT maintained the ability of the fabricated scaffolds to support cell adhesion with no changes in MG-63 cell viability. Moreover, the PBAT/PPy scaffold presented higher values of alkaline phosphatase, an important indicator of osteoblasts differentiation.

2.1.2.2.3 Polythiophene

Although polyaniline and polypyrrole are the most extensively studied conductive polymer for tissue engineering, there is a growing interest on the use of polythiophene materials such as poly (3,4-ethylenedioxythiophene) (PEDOT) (Figure 2.16 (a)) and poly(3-thiophene methyl acetate) (P3TMA) (Figure 2.16 (b)). Its biocompatibility characteristics have been investigated by different authors.

Del Valle et al. investigated the effect of PEDOT films with epithelial cells confirming its biocompatibility [206]. Luo et al. successfully prepared thin (<100 nm), PEDOT
ultra-smooth electrically conductive nanobiointerfaces using aqueous microemulsion polymerization and different ethylenedioxythiophenes (EDOTs) (e.g., EDOT-OH, C2-EDOT-COOH, C4-EDOT-COOH, C2-EDOT-NHS, EDOT-N3) [207]. After seeding them with NIH3T3 fibroblasts they were subcutaneously implanted. The in vivo results showed that PEDOT films present very low intrinsic cytotoxicity and inflammatory response upon implantation (Figure 2.17).

Figure 2.17 Microscopy images from MTT assay of (A) NIH3T3 cells on poly(EDOT-OH) film and (B) Hep G2 cells on poly(C2-EDOT-COOH) film after 24 h of incubation. (C) Viability of (gray) NIH3T3 and (black) HepG2 cells after 72 h of incubation in the presence of ITO glass substrate with and without films deposited by acid-catalyzed microemulsion electropolymerization of the monomers specified. The cell viability is with respect to the control experiment in the absence of any substrate or film [207].

Bolin et al. fabricated poly(ethylene terephthalate) (PET) nanofibres with electrospinning and coated them with tosylate doped PEDOT with vapour phase polymerization method (Figure 2.18 (a)-(c)) [208]. Results showed a significant impact on the wettability of the produced meshes, transforming hydrophobic PET fibres into super-hydrophilic ones. Produced scaffolds showed excellent adhesion and proliferation of SH-SY5Y neuroblastoma cells, being able to regulate cell signalling. Similarly, Asplund et al. investigated the biocompatibility of PEDOT using coated platinum electrodes with PEDOT
with L929 fibroblasts and human neuroblastoma SH-SY5Y cell lines [209]. Results indicated that platinum electrodes coated with PEDOT were non-cytotoxic and showed no marked immunological differences compared to pure platinum controls (Figure 2.18 (d)-(g)).

![Figure 2.18](image)

**Figure 2.18** Scanning electron microscopy images of the PET fibres, (a) uncoated electrospun PET fibres, (b) with spin coated chemical polymerized PEDOT, and (c) with vapour phase polymerized PEDOT [208]. Phase contrast images of SH-SY5Y cells growing on (d) bare indium tin oxide (ITO), (e) PEDOT: polystyrene sulfonate (PSS), (f) PEDOT: heparin and (g) PEDOT: hyaluronic acid (HA) surfaces on the transparent ITO substrate [209].

Green *et al.* used electrodeposition method to fabricate doped platinum electrodes. The PEDOT was anionically modified laminin peptides, such as DEDEDYFQRYLI and DCDPGYIGSR, and bioactivity of fabricated electrodes were assessed using PC12 nerve cells [210]. Results showed that compared to conventional dopants large peptide dopants produced softer PEDOT films with a minimal decrease in electrochemical stability. It can be observed that PEDOT films doped with synthetic anionic laminin peptides obtained
longer neurite outgrowth compared to PEDOT films doped with conventional paratoluene sulphonate.

Peramo et al. chemically deposit PEDOT on acellularized muscle tissue constructs with a novel method [211]. The chemical polymerization was performed using iron chloride, which remains in the tissue constructs at acceptable metabolic levels. Results revealed that \textit{in situ} polymerization occurs throughout the tissue, converting it into an extensive acellular and non-antigenic substrate crucial for \textit{in vivo} experiments related to nerve repair. A similar strategy was used by Richardson-Burns et al. [154], which describes the use of PEDOT as electrically conductive biomaterials for direct and functional contact with electrically active tissues, such as the nervous system, heart and skeletal muscle (Figure 2.19).

\textbf{Figure 2.19} (A) Optical image of neural cell-templated PEDOT film on gold/palladium electrode shows numerous cell-shaped holes and neurite-templated channels left behind following removal of cells from the PEDOT matrix (dark substance). (B) A new monolayer of SY5Y cells is cultured on the neuron-templated PEDOT film. These cells are stained with Phalloidin-Oregon Green (green fluorescence; F-actin) to visualize cell morphology and position of cellular processes. (C) Merged image suggests that the new cells show preferential adhesion to neuron-templated PEDOT and some cells re-populate the cellshaped holes in the polymer film (see red arrowheads) [154].

Pérez-Madrigal et al. investigated membranes prepared by a soluble polythiophene derivative (poly(3-thiophene methyl acetate)) and a biodegradable polyester (poly(tetramethylene succinate)) [212]. Results show that the blend retains electrochemical properties of the conductive polymer. Degradation studies using hydrolysis and enzymatic mechanisms indicated that the degradation of the polyester domains produces the detachment of the conducting polymer domains. Cellular viability studies using four different cellular lines (MDCK, Du-145, HEp-2 and Cos-7) showed significantly higher results for the blend than for the polyester.

Planellas et al. used electrospinning to produce hybrid scaffolds by mixing a conductive biodegradable polymer (poly(3-thiophene methyl acetate) and a co-polymer derived from
L-leucine [213]. Morphological studies showed a continuous and regular microfiber structure, despite the high conducting polymer content. Produced meshes are thermal stable with good electrochemical behaviour and low crystallinity. A similar work was conducted by Llorens et al. using polylactide acid and poly(3-thiophene methyl acetate) [205]. Operation conditions were optimized to obtain continuous fibres with diameters ranging from 600 to 900 nanometers depending on the polylactide acid/poly(3-thiophene methyl acetate) ratio. Results showed that poly(3-thiophene methyl acetate) does not influence thermal degradation but slightly decreases the fibre diameter and smoother fibre surfaces. Produced meshes presented good electrochemical properties. Other blends were investigated for electro-active scaffolds like polythiophene-g-poly(ethylene glycol) [204].

2.1.3 Summary and conclusion

The field of tissue engineering is experienced exciting advances towards the fabrication of smart and biomimetic constructs as alternatives to clinical therapies. These advances strongly rely on the use of new fabrication techniques and advanced materials. Electrospinning and additive manufacturing have been successfully explored to produce scaffolds for skin, bone, nerve and muscle regeneration. Usually these techniques use single materials (polymers, hydrogels, ceramics and composites) to produce cell substrates designed according to specific requirements like mechanical properties, degradation characteristics, porosity and surface properties. Recent studies showed the relevance of using materials to stimulate cells increasing cell attachment, proliferation and differentiation. Electrical conductive materials, as discussed in this paper, could have a significant impact in tissue engineering, as there is evidence that electrical stimulation is useful for stimulate-guided growth of cells.

Scaffolds made with different polymers reinforced with a wide range of electrical conductive materials have been proposed and assessed from a biological point of view. Most of the electrical conductive materials are toxic in high concentrations. However, the critical concentration of these materials for different cell types is not yet clear. A critical issue is also related to the availability of manufacturing systems capable of producing constructs with different materials allowing to obtain regions within a scaffold containing electrical conductive particles and regions without. Gradient scaffolds like these can be relevant to create tissue interfaces using hMSCs.

As presented in this paper, electro-active scaffolds containing small amounts of electro conductive fillers were successfully used for tissue regeneration. However, a possible alternative route, not explored in this paper, is the fabrication of constructs containing a
very high concentration of electro conductive materials. In this case, the toxicity of these materials can be explored not to stimulate cell proliferation and differentiation, but cell apoptosis, which can be particularly relevant for cancer applications.

As discussed in this paper, the exceptional electrical and surface properties of CNMs, especially graphene and CNTs, together with their controllable morphologies make them key components for the development of novel electro-active scaffolds. A wide range of 2D membranes were produced and assessed with different cell types. Recently the development of additive manufacturing allows the use of CNMs blended with polymers to create 3D porous scaffolds. In this case, the elastic and flexible nature of CNMs not only improves the mechanical properties of the scaffolds, synergizing also the effects of electrical stimulation on both cell proliferation and differentiation. Current major challenges are related to the lack of comprehensive in vivo studies for adequate assessment of long term effects in terms of biocompatibility and cytotoxicity. This requires further investigations, conducting systematic comparative studies which will differentiate results of humans from animals, and further understanding of their cellular and biological interactions, especially relating cellular uptake mechanism and biocorona formation. Current production challenges of synthesis of CNMs with controlled size, shapes and functionalities should also be addressed as it affects the overall biocompatibility and performance of CNM-based electro-active scaffolds.

A key parameter to design optimised scaffolds is the control of its degradability, which must match the regeneration rate of the new tissue. However, some of conductive polymers exhibit non-degradability and the scale of degradation of others are not well optimised for particular tissue engineering applications. Hence, it is necessary to design non-toxic conductive polymers with an optimised degradation rate.

Even though the polyaniline, polypyrrole, polythiophene, graphene and carbon nanotube based conductive polymer materials were proved to be biocompatible in vitro, their biocompatibility and biodegradability in vivo are still not fully investigated, representing an important research challenge. In the future, the development of 3D scaffold structures should extensively be applied for in vivo experiments for better understanding of their interaction with the surrounding tissues and their performance towards tissue regeneration.
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2.2 Engineering the Vasculature with Additive Manufacturing*

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**Contribution to the work:** All authors participated in the planning, writing and editing of the article. As co-author, W.W. mainly focus on the writing of conventional and extrusion-based additive manufacturing processes manuscript text, and manuscript reviewing.

2.2.1 Introduction

Mammalian bodies are highly vascularised which enables healthy functioning and large multicellular organisms to exist. The maintenance of healthy tissues and organs is provided by an extensive and highly organised vascular network which enables the removal of waste products and the diffusion of oxygen and nutrients. Subsequently, cells are located within 100-200 µm of a capillary, regarded as the diffusion limit of oxygen and nutrients within a tissue, with only a few avascular exceptions such as cartilage tissue, the cornea and lens within the eye, and the epithelial layer of the skin [214]. The natural vasculature is organised in vivo as a branched network of larger vessels, small vessels and capillaries. The formation of an organised vascular network is regulated by two fundamental processes of vasculogenesis and angiogenesis [215]. Vasculogenesis is triggered by physical forces and mainly occurs during early embryonic development to form a primitive capillary network, but can also takes place in adults towards tissue revascularisation upon injury. New blood vessels are formed from endothelial progenitor cells, while the recruitment of smooth muscle cells (SMCs), pericytes and fibroblast layers around the endothelial tubes is fundamental for maturation. Angiogenesis is induced by hypoxia in the surrounding tissue and involves the formation of new vessels from an existing vascular network through the sprouting of endothelial cells (ECs) from existing vessels. Angiogenesis firstly leads to the formation of capillary networks consisting of endothelial cell tubes lacking of additional wall structures (e.g., stabilising cells, SMCs), which can subsequently mature through arteriogenesis [216]. Clinical issues arise when the vasculature is extensively disturbed through trauma or disease, leading to a critical reduction on tissue supply with nutrients and oxygen.

The emergence of tissue engineering and regenerative medicine has allowed the development of strategies for tissue and organ repair, replacement, and in vitro tissue models, but has raised the need for vascularised tissue constructs. The size and long-term viability of these constructs are limited by the composition, complexity, and functionality of vascularisation present. Tissue engineered constructs in vitro are dependent on passive diffusion which restricts construct size, however, bioreactors can provide mass transport of nutrients and gases through dynamic culture enabling larger and long-term viability of tissue constructs [217, 218]. Nevertheless, in vivo the rate and quality of vascular ingrowth will be limited which may cause hypoxia, nutrient deficiency, and build-up of waste products with subsequent impact on cell fate and new tissue formation. Subsequently, a variety of strategies to engineer or improve vascularisation have been proposed such as the use of...
pro-angiogenic factors, progenitor or stem cells, decellularised matrices, modular assembly, \textit{in vitro} and \textit{in vivo} pre-vascularisation, synthetic architectures (microfluidic devices and porous scaffolds), novel biomaterials, and combinations of all these approaches [219-223]. Furthermore, the limited control over the spatiotemporal presentation of biological factors (e.g., growth factors) and the requirement of a complex vascular network has prevented successful development of vascularised constructs. Ideally, an optimal engineered vascular network should (i) provide cells with surrounding vessels in close proximity ($100$-$200$ µm), (ii) exhibit hierarchical structural and functional organisation, where a vascular tree with larger vessels branches into small vessels and subsequently into capillaries well-distributed throughout the tissue construct, (iii) present selective permeability to control the delivery and removal of nutrients and metabolites from the surrounding cells, and (iv) allow integration with the host vasculature in the patient to provide immediate perfusion of implanted construct [224]. Thus, it is critical that fabricated vascular constructs possess clinically relevant dimensions and structural integrity to allow easy handling and surgical procedures.

The introduction of additive manufacturing (AM) in the biomedical field has allowed the fabrication of tissue constructs for tissue engineering and regenerative medicine with unique spatial control over the deposition of biomaterials, cells and biomolecules [225-228]. AM includes a range of technologies that automatically fabricate complex three-dimensional (3D) structures through a layer-by-layer process, providing unique control over the architecture and composition of engineered tissues. Through these techniques, the biofabrication of tissue engineered constructs with the inclusion of an existing vasculature is possible, but also the development of tissue-engineered blood vessels [228, 229]. Despite its unique characteristics of automation, reproducibility, scale-up and standardisation, AM technologies have also been combined with conventional biomanufacturing techniques to generate complex, vascularised tissue constructs with potential for clinical translation.

This current opinion classifies the biofabrication strategies currently used to create vasculature and vascularised constructs as using either conventional or additive biomanufacturing techniques, with a focus on the latter. A brief overview of the latest research using both fabrication strategies is provided. Finally, the challenges and future directions in vascular tissue engineering is discussed and how AM can provide new tools to enable researchers within the field.
2.2.2 Conventional techniques

Conventional biofabrication methods have been widely explored for creating vascular grafts, blood vessels and vasculature for tissue constructs. A range of techniques such as cell sheet engineering, micropatterning, phase separation, electrospinning, solvent casting, and combinatorial approaches have been utilised [230-237]. Although these techniques have important limitations regarding the complexity and architecture of engineered constructs as well as over the precise positioning of biomaterials, cells and biological factors in desired 3D locations, their simplicity, low cost and broad range of processable biomaterials make them very applied and useful to develop combinatorial biofabrication strategies [238, 239].

2.2.2.1 Cell sheet engineering

Cell sheet technology is a technique which allows the detachment of a confluent cell layer through the use of a temperature responsive polymer (e.g., poly(N-isopropylacrylamide)) grafted onto cell culture plates [240]. Cell sheets are detached without the need for enzymatic treatments, preserving native extracellular matrix (ECM), cell-cell and cell-ECM anchorage. Cell sheets can be directly transplanted to the host tissue as single sheets or further assembled to create more complex 3D constructs. This technology has been exploited by Sakaguchi et al. [241] to fabricate vascularised 3D cardiac tissue using multiple cell sheet layers. A vascular bed was developed to allow perfusion of the cell sheet construct which allowed 12-layer thick constructs, including both ECs and cardiomyocytes, to survive and develop blood vessel networks within and between layers. Alternatively, Jung et al. [242] have used cell sheets to develop blood vessels by using multiple aligned mesenchymal stem cell (MSCs) sheets which are wrapped around a temporary supporting mandrel (Figure 2.20). This construct is allowed to mature before being perfused with endothelial progenitor cells which attach to the lumen. The organisation of the mature construct resembled the architecture of native blood vessels and exhibited vasodilation, vasoconstriction, and nitric oxide release when exposed to fluid flow and phenylephrine.

2.2.2.2 Solvent casting

Solvent casting is a technology in which porous structures are generated through the polymer dissolution in solvent containing homogeneously distributed salt particles of a specific size. After solvent evaporation, the structure is immersed in water to leach out the entrapped salt particles, resulting in the formation of a porous structure that can be further seeded with the cells of interest [239]. Badhe et al. [243] used a solvent casting-co-particulate leaching technique to produce a biomimetic composite chitosan-gelatine hydrogel scaffold with a bi-layered tubular architecture. The inner
macroporous layer provides a large surface area which facilitates fibroblast attachment and proliferation, while the non-porous outer layer provides mechanical support and protection. The scaffold exhibited desirable mechanical properties and degraded 50% in vitro at day 16. However, the angio- or vasculogenic capability has not been evaluated but the methodology remains a promising approach to fabricate vascular structures. The size of implantable tissue constructs determine the vascularisation strategy pursued as small constructs can utilise diffusion and infiltration from the host tissue. However, larger constructs cannot rely on vessel ingrowth as necrotic zones form before a vascular network is formed. To address this concern, Huling et al. [244] developed an alternative casting strategy, known as vascular corrosion casting technique, to completely replicate the vascular network within a kidney (Figure 2.20). A polycaprolactone (PCL) solution was perfused into a native kidney generating a cast mimicking the vascular network. This serves as a simple, effective and inexpensive method to create a sacrificial template for the formation of a scaffold for the entire volume of a large tissue construct.

2.2.2.3 Micropatterning techniques

Micropatterning techniques such as photolithography, micromolding and microcontact printing have been widely used to regulate cell behaviour on biomaterials through the controlled display of biological factors or by influencing cell behaviour via topographical cues [245, 246]. In photolithographic patterning, photo-reactive species are irradiated with suitable light sources emitting light at certain wavelength through masks to create patterns on substrates. This technology was used to create 3D interconnected vascular networks of varied dimensions within micropatterned gelatine methacryloyl (GelMA) hydrogels of different degrees of methacrylation [247]. Micromolding allows creating complex architectures in biomaterials through the use of master moulds with desired geometry and topography on silicon wafers. After casting and curing a polymer solution on the master mould, a biomaterial substrate is obtained with desired micropatterns. Baranski et al. [248] used a poly(dimethylsiloxane) (PDMS) template fabricated through a micropatterning process to produce aligned ‘cords’ of encapsulated ECs within a collagen gel (Figure 2.20). Capillaries formed along the length of the construct and became perfused with host blood once implanted. The geometric control enabled enhanced hepatic survival and function compared to randomly organised endothelial constructs.

2.2.2.4 Electrospinning

Electrospinning is a biomanufacturing technique that produces fibres at the micro- and nanoscale from polymer solutions or melts through the application of an electric field
between the syringe needle and the collector [249, 250]. By changing the nozzle type and collector design, electrospinning allows the fabrication of electrospun meshes with aligned or random fibres as well as core/shell fibres. Additionally, tailoring operating parameters such as the needle diameter, flow rate or solution viscosity enables to control the fibre diameter and morphology [249, 250].

Considerable interest has been shown in the development of electrospinning techniques for vascular applications [251-253]. Early work by Xu et al. [254] showed that aligned poly(l-lactid-co-ε-caprolactone) copolymer fibres with a diameter of 500 nm mimicked the circumferential orientation of a native artery. This enabled preferential attachment and migration of human artery SMCs along the axis of alignment. This study established the potential of topographical alignment of electrospun nanoscale fibres in modulating cell behaviour and providing a ECM-like structure. Vaz et al. [255] demonstrated the fabrication of multi-layered electrospun tubular structures using a rotating mandrel collector, now a common methodology, to create vascular-like structure that could support cell viability. Currently, optimisation of material properties, fibre morphology, and electrospinning parameters are being investigated towards better modulating cell response and inducing specific cell behaviour. Wang et al. [256] investigated the role of fibre size on cell infiltration and scaffold remodelling in a vascular graft. Fibre and pore size influenced infiltration and regulated macrophages phenotype with larger sizes exhibiting increased cell infiltration and an immunomodulatory and tissue remodelling macrophage phenotype. On the contrary, smaller sizes reduced cell infiltration and promoted a pro-inflammatory macrophage phenotype. Vatankhah et al. [257] electrospun tubular scaffolds using a novel hydrophilic polyurethane, Tecophilic® (TP), blended with gelatine. The scaffolds had similar mechanical properties to native blood vessels and the hydrophilic properties of the composite scaffold enabled non-thrombogenicity. Whilst the gelatine provided binding sites for vascular SMC attachment and maintained the contractile phenotype. The use of coaxial electrospinning was explored by Duan et al. [258] to fabricate core-shell fibres with a PCL core and collagen shell into a tubular scaffold for vascular graft applications. The scaffold exhibited EC attachment and proliferation whilst allowing SMC infiltration. Coaxial electrospinning allows multi-layered tubes to be constructed which more closely mimics the vascular structure and also allows tuning of the mechanical and biological properties of the scaffold. Tan et al. [259] demonstrated the use of a co-electrospinning method to fabricate composite vascular grafts using PCL, polyvinyl alcohol (PVA), and gelatine with subsequent functionalisation with immobilised heparin. PVA rapidly degraded in vivo which increased porosity allowing enhanced cell proliferation and infiltration. Functionalisation
with heparin had an anti-thrombogenic effect and enhanced proliferation of ECs. This study demonstrates the important role of material and biomolecule selection in enhancing scaffold properties. Long-term in vivo studies of electropsun vascular scaffolds by Gao et al. [260] have shown that further research is required to improve scaffold design. Two electrosyn acellular scaffold designs with different wall thicknesses (250 µm and 350 µm) were fabricated using a biodegradable amino acid based polyester urea (PEU) and implanted in a mouse model. The smaller thickness scaffold remained unobstructed and exhibited tissue remodelling mimicking native arteries, over the one year study. However, the larger scaffold becomes occluded due to intimal hyperplasia. Both scaffolds demonstrate slow degradation and remain present after one year. This study shows that PEU is promising material for vascular constructs but also the importance of in vivo studies to prove vascular design and efficacy. Pu et al. [261] fabricated a poly(L-lactic acid) fibrous bilayer scaffold with aligned and random layers which was validated in vivo. The scaffolds had a gradual variation in porosity and fibre alignment. The scaffolds were implanted in Sprague-Dawley rats which confirmed biocompatibility and significantly enhanced cell infiltration, compared to the control, with homogenous cell distribution after 14 days. Collagen matrix deposition and indicators of neovascularisation, α-actin and CD31 which are markers of SMCs and ECs respectively, were increased in comparison to the controls. However, long-term studies are required to assess the potential of fibrous encapsulation and if the scaffolds have functional vascularisation.

2.2.2.5 Combinatorial techniques

Since electrosyn meshes can be designed with required fibre alignment and sufficient mechanical properties to support hemodynamic forces and maintain structural integrity during tissue maturation, the capabilities of electrospinning have been explored in combination with other processes to engineer vascular grafts. Mi et al. [262] fabricated a biomimetic triple-layered vascular scaffold combining electrospinning and thermally induced phase separation (TIPS) (Figure 2.20). The inner layer consisted of electrosyn thermoplastic polyurethane (TPU) nanofibers, the middle layer was composed of a porous TPU produced by TIPS, and the outer layer was either electrosyn TPU or poly(propylene carbonate). The electrosyn layers, due to its compact fibre arrangement, had a high Young’s modulus providing suture retention and preventing bursting. The TIPS layer aided EC penetration due to the high porosity and pore interconnectivity whilst the inner layer showed cell attachment and viability. The approach outlined is potentially suitable for use in vascular grafts especially as the material degradation rates have been considered to match the required in vivo rates. An alternative approach by Ahn et al. [236] explored the
combination of cell sheet engineering and electrospinning. This novel approach utilised an electrospun blend of PCL and collagen type I to create a tubular scaffold which was wrapped in a SMC sheet. The SMC sheet provided a mature cell layer with pre-existing cell-cell interactions and enabled high cell seeding efficiency of the electrospun scaffold. Culturing within a perfusion bioreactor improved cell viability, infiltration, and mechanical properties. This combined fabrication process enables biomimetic architectures and relevant cells types to be combined into a mature vascular construct which has the potential for immediate implantation after bioreactor culturing.

![Diagram of biofabrication techniques](image)

**Figure 2.20** Examples of conventional biofabrication techniques used to produce vascular grafts and perfusible channels. Cell sheet technology using a nanoimprinted substrate to fabricate aligned MSC sheets that are wrapped around a mandrel to create a tubular structure [242]. Micropatterning has been used to create aligned EC-collagen cords using a patterned PDMS substrate [248]. The PDMS is removed and cords are encased in a fibrin gel. Images show cord formation at different stages of fabrication. Casting technique used to replicate the vascular structure of an organ by the injection of a PCL solution which is coated in collagen [244]. The PCL is removed with acetone leaving a hollow collagen scaffold. Combinatorial approach using electrospinning and thermally induced phase separation to fabricate a triple-layered vascular scaffold [262].

### 2.2.3 Additive manufacturing techniques

AM, commonly termed 3D printing, has allowed tissue construct fabrication with multiple materials and complex architectures that are not possible or difficult to achieve with conventional techniques [263, 264]. AM provides superior control, compared with
conventional methods, over the design and fabrication process resulting in precise spatial deposition of biomaterials, cells and biomolecules, which closely resemble the designed structure. This enables the continuous deposition of complex channels and tubular structures, patterning of angiogenic factors in 3D, and the inclusion of complex vasculatures within larger printed tissue constructs, allowing the development of thick constructs with embedded vasculature. A variety of AM techniques are currently used, either alone or in combination with other AM and conventional processes, to engineer vascular networks for tissue engineering, including extrusion, inkjet, vat photopolymerisation, and laser-assisted processes. Two key approaches in vascular printing have been explored, namely direct printing which fabricates vascular constructs immediately through the use of cell-laden bioinks and coaxial nozzles, and indirect printing which utilises a sacrificial or fugitive component that is printed and subsequently removed to create the vascular channel or network [265-268]. Combination of both approaches is also feasible and has been explored to scale up the complexity and dimension of engineered constructs.

2.2.3.1 Extrusion-based processes

Extrusion-based processes include a range of techniques that extrudes a polymer solution or melt through a nozzle onto a platform, using a pneumatic, mechanical (piston and screw-assisted), or solenoid deposition process [269]. Depending on the strategy, extrusion processes can be applied to create 3D structures made of thermoplastic polymers, known as Fused Deposition Modelling (FDM), or to print either acellular or cell-laden hydrogel bioinks, often termed extrusion bioprinting [270, 271]. Extrusion processes are the most popular and affordable due to their versatility, broad range of processable biomaterials and ability to generate 3D tissue constructs at clinically relevant scales.

The most common strategy to directly print perfusable 3D vessel-like channels makes use of a coaxial nozzle to generate either acellular or cellularised hollow fibres, which can be subsequently endothelialised, perfused and even embedded within hydrogels. This strategy was explored by Zhang et al. [272] to fabricate vessel-like microfluidic tubular channels. Alginate and chitosan hydrogels, with encapsulated cartilage progenitor cells (CPCs), were simultaneously crosslinked with a calcium chloride solution during extrusion through the coaxial nozzle. The technique generated channels capable of supporting cell viability, media transportation, and perfusion. This was followed by an investigation of human umbilical vein smooth muscle cells encapsulated within printed alginate conduits [273]. Cell viability and proliferation was observed as well as deposition of smooth muscle matrix and collagen. The printed conduits also allowed for long term media perfusion which is particular relevant
in the development of thick tissue constructs. Similarly, Gao et al. [274] reported the use of a coaxial nozzle to fabricate a hollow 3D cell-laden hydrogel structure by controlling the crosslinking rate. The microchannels showed higher mechanical strength with increasing concentration of sodium alginate and decreasing the distance between adjacent hollow filaments. Higher fibroblast viability was observed in alginate hydrogels with microchannels in comparison with solid hydrogels. This technique is a promising methodology and requires validation with vascular relevant cell types.

Although additive manufacturing technologies provide the ability to generate perfusable and interconnected vascular channels, the fabrication of functional vascular networks strongly depends on the cell-instructive capabilities of developed biomaterials. There is wide recognition that in vivo cells can dynamically interact and remodel the surrounding ECM, which in turn dictates and influences cell fate [275-277]. Rather than printing cells within inert hydrogel materials, efforts have been made to develop cell-responsive hydrogels able to modulate cell behaviour. Jia et al. [278] used a cell responsive bioink based on GelMA, sodium alginate, and 4-arm poly(ethylene glycol)-tetra-acrylate and a multi-layered coaxial nozzle extrusion system to directly print perfusable 3D structures. During the extrusion the bioink is exposed to calcium chloride allowing fast crosslinking with the alginate component to enable shape maintenance. The printed structure is then exposed to ultraviolet light to induce covalent photocrosslinking. The cell-laden (EC and MSC co-culture) perfusable tubular structures supported cell viability, proliferation, and spreading. This approach shows promise in directly creating tubular structures with tuneable mechanical properties and variable diameters through the novel coaxial extrusion and crosslinking process. Exploring a similar approach, Zhang et al. [279] have demonstrated the fabrication of endothelialised myocardial tissue constructs using a coaxial nozzle to print a microfibrous scaffold with ECs embedded within a blend bioink of alginate and gelatine methacryloyl (Figure 2.21). ECs migrate to the periphery of the fibres and form a confluent endothelium. The construct was then seeded with cardiomyocytes and cultured within a perfusion bioreactor which allowed development of an endothelialised myocardium. The strategy utilised enables the vascularisation of large constructs and may be used initially for drug screening and disease modelling but with further development could be useful within regenerative medicine.

An alternative strategy to generate perfusable channels relies on the printing of sacrificial microfibers within hydrogel matrices. After deposition, sacrificial template material is removed, leaving hollow channels which can be endothelialised and perfused. This strategy was explored by Lee et al. [280] to print functional vascular channel exhibiting a perfused open lumen (Figure 2.21). Collagen layers were deposited and a gelatine/EC mixture printed
in a straight line which was then concealed by further layers of collagen. The chamber was incubated at 37°C to allow gelation and liquefaction of collagen and gelatine, respectively, which created a channel within the collagen matrix. The channels allowed cell viability up to 5 mm in distance from the channel under physiological flow conditions whilst also exhibiting a barrier function by preventing transfer of both plasma protein and high-molecular weight dextran. The unique ability of extrusion bioprinting to generate 3D constructs at clinically relevant scales was demonstrated through the fabrication of thick, perfusable and vascularised cell-laden tissue constructs. Kolesky et al. [281], reported the fabrication of tissue constructs with embedded vasculature by co-printing multiple inks containing different cell types, ECM components, and a fugitive ink. Pluronic F127 was selected as fugitive ink, which can be removed under mild conditions to create channels within the construct for subsequent endothelialisation. Pluronic compares favourably to other sacrificial materials which typically require high temperatures or harsh solvents for removal [235, 282]. Lately, this group improved on this approach to generate large tissue constructs (>1 cm) with integrated vasculature, allowing long-term (>6 weeks) perfusion with growth factors (Figure 2.21) [283]. This study is a key step forward in demonstrating the fabrication of large vascularised constructs which is a fundamental advance within vascular tissue engineering itself but will also enable clinical translation of tissue engineered constructs.

Bioprinting systems have also been developed combining polymer melt extrusion, direct, and indirect printing of hydrogel inks towards the generation of human-scale tissue constructs with suitable mechanical stability, biophysical and biological response. Kang et al. [263] developed a bioprinting system capable of fabricating complex tissue constructs with incorporation of microchannels to enhance mass transfer, vascularisation and new tissue formation (Figure 2.21). Constructs were created by combining the extrusion of polymer melt, sacrificial ink and cell-laden bioinks and tested for the regeneration of bone, cartilage and skeletal muscle.
Figure 2.21 Examples of extrusion-based processes applied to create vascular networks in 3D tissue constructs through three major approaches. Direct printing can be achieved through use of a coaxial nozzle enabling co-deposition of a solution and crosslinking agent which allows immediate crosslinking to generate stable structures [279]. Indirect printing (left) of vascular networks can be achieved through the use of sacrificial materials [280]; (right) and fugitive inks [283]. Combinatorial approaches employ both direct and indirect methods to fabricate tissue constructs which exhibit vascular networks [263].
2.2.3.2 Inkjet printing

Inkjet printing is a non-contact technology that deposits small droplets of a bioink onto a build platform through thermal or piezoelectric effects. In thermal inkjet, a heating element is used to induce the vaporisation and consequent ejection of a small volume of bioink, while piezoelectric inkjet makes use of piezoelectric transducers to promote droplet formation [227, 284]. Inkjet printing has been widely used mainly due to its unique characteristics of high-throughput efficiency, resolution and possibility of parallel printing. However, the need for low viscous bioinks to prevent nozzle clogging has limited its application on the fabrication of 3D tissue constructs. Alternative strategies involving the printing of either endothelial cells with crosslinkers or cell-laden bioinks onto a biomaterial substrate have been successfully implemented [285, 286].

Christensen et al. [287] have demonstrated the ability to fabricate complex vascular-like structures that have bifurcations and present both overhanging and spanning features (Figure 2.22). This is achieved by printing alginate droplets directly into a calcium chloride solution which acts as both crosslinker for alginate and supporting buoyant force on the printed structure. High fibroblast cell viability was observed post-printing. This technique is promising as it potentially allows complex networks to be fabricated which more accurately reflect the in vivo vascular structure, though further work using ECs and fabrication of complex networks is necessary. An early example by Cui et al. [288] used a bioink composed of human microvascular ECs, thrombin, and Ca^{2+} that when deposited by a thermal inkjet printer onto a fibrinogen substrate forms fibrin channels, due to the polymerisation of fibrinogen and thrombin into fibrin. This demonstrated the potential of inkjet printing to fabricate precise channels with encapsulated cells which proliferate and form tubular structures within the fibrin channels. This strategy has been continued by Xu et al. [286] through the fabrication of multi-cell heterogeneous constructs including ECs to induce vascularisation of the construct (Figure 2.22). Alginate and collagen substrates were crosslinked by the deposition of a bioink containing cells and calcium chloride which resulted in rapid formation of a gel. Implantation in vivo demonstrated construct viability and maturation but also that adequate vascularisation occurred.

2.2.3.3 Vat photopolymerisation

Vat photopolymerisation or stereolithography (SL) is a light-based technology that fabricates 3D structures through the selective photo-initiated curing reaction of a liquid photosensitive material using either laser writing or mask-based writing approach [289-291]. In the first approach, a focused laser beam is applied to selectively induce the polymerisation
of a liquid photopolymer, while in the second a mask is used to transfer an entire image to a liquid photopolymer reducing the printing time. These techniques have the highest print resolution due to the optical based process which allows resolutions of up to 100 nm using two-photon-photopolymerisation processes (2PP) [292, 293]. SL uses a single-photon to initiate photopolymerisation whilst 2PP requires a material that allows the absorption of two photons, simultaneously, which results in a localised reaction thus allowing high resolution features [293-295].

Meyer et al. [296] successfully fabricated vessel-like structures using SL and 2PP with the aim of creating tubes that mimic the diameter of capillaries (Figure 2.22). Branched tubular structures with inner diameter of 18 µm and wall thickness of 4 µm were created. The polytetrahydrofuranether-diacylate mechanical properties and shape remained relatively unchanged within the aqueous environment and showed good biocompatibility. Huber et al. [297] demonstrated the use of SL to fabricate 3D tubular structures with a photosensitive cytocompatible polyacrylate (PA) (Figure 2.22). Tubular structures, including branching vessels, with wall thickness of 300 µm, inner diameters of 1-2 mm, and defined pores throughout the tubes of 100 or 200 µm were fabricated. The PA was functionalised with thio-modified heparin and cysteine-coupled arginine-glycine-aspartic acid peptides to enhance EC attachment which allowed a confluent endothelial lining of the inner surface. The combination of using AM, optimised rinsing and sterilisation protocols, material biofunctionalisation, and use of bioreactors in this study has allowed a platform to be developed which can be further advanced for vasculature engineering.

An alternative approached explored by Raman et al. [298] is the use of projection µSL for the patterning of angiogenic patches for tissue ischemia applications (Figure 2.22). Hydrogel patches with patterned microchannels exhibited increased secretion of vascular endothelial growth factor (VEGF) by encapsulated cells. The presence of channels increased the surface area to volume ratio which enabled enhanced diffusion of both VEGF and nutrients from the media. The angiogenic properties were confirmed through a chick chorioallantoic membrane model which demonstrated the formation of neovascularature.

2.2.3.4 Laser-assisted bioprinting

Laser-assisted bioprinting (LAB) is an AM technique that uses the principle of laser-induced transfer, originally developed for metal transfer, to deposit biological materials onto a substrate (Figure 2.22) [299, 300]. Although less commonly used than other methods, a range of biological materials have been printed using this method [301-303]. LAB employs
a high-energetic pulsed laser to promote the ejection of small droplets of a bioink from a donor ribbon towards a receiving substrate.

Wu and Ringeisen [304] used LAB to create vascular structures by guiding the development of lumen formation through direct positioning of individual ECs and SMCs (Figure 2.22). Through the co-deposition of both cell types the lumen structure was maintained for longer periods than ECs alone. This co-culture allowed the beginning of a network structure due to cell-cell interactions playing a vital role in maintaining cell phenotype and controlling tissue development. Similar work by Guillotin et al. [305] using ECs indicated that it is possible to direct the formation and growth of lumen and lumen network using LAB (Figure 2.22). Bourget et al. [306] used LAB to investigate EC and MSC cell migration in both single cell islets and in co-culture showing the presence of MSCs maintained EC presence within the printed area. This may allow development of vascular constructs which maintain a specific shape over long periods.

**Figure 2.22** Examples of inkjet, stereolithography, and laser-assisted bioprinting based processes. Inkjet (left) schematic of a bioprinting technique to fabricate multi-cell tissue constructs with light and fluorescent microscopy images showing multiple cell types precisely arranged. Gross images indicate considerable vascularisation compared with...
control after implantation [286]; (right) schematic of a printing technique which enables the fabrication of spanning and overhanging sections, in both horizontal and vertical directions, using the surrounding solution as a buoyant support [287]. Images show fabricated bifurcated structures. Stereolithography (top-left) scanning electron microscopy images of branched tubular structures (height \(\sim 160\) \(\mu\)m, inner diameter \(\sim 18\) \(\mu\)m, wall thickness \(\sim 1\)\(\mu\)m) fabricated using 2PP [296]; (bottom-left) design and CAM assay schematic of a hydrogel patch with integrated channels enabling enhanced diffusion of nutrients and biomolecules [298]; (right) biomimetic microporous PA vascular network [297]. Laser-assisted bioprinting (left) diagram of technique [227]; (middle) ECs deposited sequentially, stained with two separate fluorescent probes, in concentric circles showing precise spatial positioning [305]; (right) double branch structure of printed ECs demonstrating the feasibility of printing complex vascular-like networks [304].

### 2.2.4 Conclusions and future outlook

Several strategies have been tested to tackle the problem of vascularisation in tissue engineering and regenerative medicine, including the design of angiogenic biomaterials and the delivery of endothelial cells and growth factors. These strategies are mostly based on vascular induction rather on the development of perfusable vascular networks, which limits their efficacy in the vascularisation of 3D constructs of clinically relevant dimensions. AM technologies represent a promising and effective alternative to directly incorporate functional vascular networks with complex organisation throughout the 3D construct by the layered deposition of biomaterials, cells and biomolecules. While inkjet and LAB have been mainly explored for cell printing and growth factor patterning, extrusion technologies are currently applied to create complex hollow channels that can be endothelialised and perfused, providing nutrition to the surrounding cells. More importantly, AM technologies permit the precise co-printing of stromal and endothelial cells into predesigned spatial locations to support and maintain vascular-like structures, which is extremely difficult or even impossible when using conventional biofabrication technologies. The versatility of AM technologies has also been explored for the development of combinatorial approaches with potential to improve the biomimicry and the performance of engineered constructs. Future directions of functional vascularised tissue-engineered constructs need to combine the scalability and automation of AM technologies with the design of cell-instructive biomaterials capable of selectively modulating the fate of embedded cells to take the field to the next level. In particular, the design of advanced materials capable of regulating the spatiotemporal release and the sequestration of multiple pro-angiogenic growth factors (e.g., VEGF and basic fibroblast growth factor) and simultaneously exhibiting suitable printability will be required to create...
functional constructs with potential for clinical translation. These developments will be important to advance the biofabrication of engineered constructs with embedded stable, hierarchical vascular trees composed of interconnected channels of variable dimension in order to better recapitulate the *in vivo* organisation of the vascular system and to promote the integration with the host vasculature. In parallel, key aspects related to the safety, efficacy and clinical translation of tissue engineered vascularised products must be taken in consideration during the development and testing phases. A clear understanding of the regulatory hurdles and pathways is required during the product design and material selection phase to ensure that the materials and processes chosen are compatible with the necessary regulatory standards and can be scaled up to good manufacturing practice (GMP) levels [307-309]. Specific properties must be considered during the formulation of a regulatory strategy for product commercialisation, including shelf-life, sterility, and biological interaction. The safety and long-term efficacy of any implantable product is of the highest priority. This is especially important in strategies which involve allogenic, xenogeneic, and genetically modified materials to ensure that no immunological response is triggered. However, the development of personalised medicine strategies using patient stem cells or gene therapies combined with biocompatible materials may avoid an immune response entirely. Subsequently, improved *in vitro* models are required to assess safety and efficacy at an earlier stage to inform product development and to reduce the ethical issues and costs associated with the animal studies. Furthermore, once animal trials are required the correct animal models are needed which provide the closest model to human vascular regenerative capabilities. If these factors are considered the success of clinical translation and commercialisation is more assured. However, an over-engineered solution which requires costly materials or manufacturing processes may not have a high enough cost-benefit ratio to be accepted by healthcare purchasers or authorisation bodies such as the National Institute for Health and Care Excellence (NICE) in the United Kingdom.
2.3 Summary of the Chapter

This Chapter provides a review of the state of the art of materials and fabrication techniques to produce scaffolds for bone tissue engineering. Firstly, the concept of electro-active scaffolds and the materials commonly used to produce them are presented. Followed by the introduction of the concepts of tissue engineering, and the role of scaffolds. Different conventional and non-conventional (additive biomanufacturing) techniques to produce scaffolds are presented. Strategies to create vascularized tissues are presented and discussed.
CHAPTER III

PHYSICAL AND IN VITRO BIOLOGICAL CHARACTERISATION OF ELECTRO-ACTIVE SCAFFOLDS
Chapter III: Physical and in vitro biological characterisation of electro-active scaffolds

This Chapter focuses on the fabrication and characterization of electro-active scaffolds. Scaffolds, produced using a screw-assisted extrusion-based additive manufacturing system, were extensively characterized from a morphological, thermal, mechanical and biological point of view. To identify the optimal process parameters, PCL scaffolds were produced considering different temperatures, screw rotational and deposition velocities and slice thickness values. The effect of these parameters on both pore size and filament diameter was experimentally assessed and reported in Appendix A, which corresponds a conference paper entitled “Process optimisation of extrusion-based additive manufacturing to produce PCL scaffolds for bone regeneration”, published in Proceedings of 39th MATADOR Conference.

The Chapter consists of six Sections detailing the work carried out to characterize the scaffolds, considering different ratios between PCL and graphene nanosheets. The first Section, entitled “Morphological, mechanical and biological assessment of PCL/pristine graphene scaffolds for bone regeneration”, is based on a paper published in the International Journal of Bioprinting. It focuses on the scaffold fabrication process and investigates the effect of graphene on the morphological, mechanical and biological behaviour of the produced scaffolds using human adipose-derived stem cells (hADSC). Additional information is also provided by two conference papers in Appendix B and Appendix C.

The second Section, investigates the effect of a simple chemical modification (NaOH treatment) to change the surface wettability of produced electro-active scaffolds. This Section, entitled “Enhancing the hydrophilicity and cell attachment of 3D printed PCL/graphene scaffolds for bone tissue engineering”, is based on a paper published in Materials. As PCL is a highly hydrophobic material, methods to increase hydrophilicity are critical to improve cell attachment. Thus, the influence of chemical surface modification on their biological behaviour is investigated, and the improved cell response induced by surface modification is reported.

In order to further improve the biological performance, the electro-active scaffolds were bioactivated using a natural protein (P1 protein) extracted from a South America tree (Hevea brasiliensis rubber tree). This protein was transferred to the scaffolds through an adsorption method and its effect investigated in terms of cell proliferation and cell differentiation (osteogenesis). The results are presented and discussed in Section three, entitled “3D printed PCL/graphene scaffolds activated with P1-latex protein for bone regeneration”, based on a paper published in 3D Printing and Additive Manufacturing.
Electro-active scaffolds can be produced by combining non-conductive organic materials with conductive organic materials or by combining non-conductive organic materials with conductive inorganic ones. The forth Section of this Chapter, compares the physical and biological performance of two types of electro-active scaffolds produced using a non-conductive organic material (PCL) and two types of inorganic conductive materials (graphene and CNTs). Both PCL/graphene and PCL/CNT scaffolds loaded with the same amount of inorganic materials. This Section is based on a paper entitled “Assessment of PCL/carbon material scaffolds for bone regeneration” submitted to Scientific Reports. Additional information is also provided in Appendix D, which corresponds to a conference paper published in “Proceedings of the 3rd International Conference on Progress in Additive Manufacturing”.

Producing synthetic scaffolds with adequate physical, chemical and biological properties remains a challenge for tissue engineering. Internal architecture, surface chemistry and material properties have strong impact on the cell biological behaviour. This requires sophisticated systems not only able to process multiple materials with different characteristics, creating fully interconnected 3D porous structures with high reproducibility and accuracy, but also able to modify their properties during the fabrication process. Section five and six introduce a novel additive manufacturing system comprising a multi-printing unit (screw assisted and pressure assisted printing heads) together with a plasma unit that enables the surface modification of printed scaffolds. PCL scaffolds with a laydown pattern of 0/90° were fabricated using the screw assisted printing head and the plasma jet unit was used to uniformly modify each layer, a specific region of each layer during the printing process or the external surface of the printed scaffolds. Scaffolds were produced using different plasma exposure times and different distance between the plasma head and the printed layer, and fixed printing conditions. Produced scaffolds were morphologically, mechanically, chemically, and biologically characterised. As expected, plasma treatment increases hydrophilicity and consequently the biological performance of the scaffold, suggests a potential of using the proposed fabrication system to create functional gradient or scaffolds with tailored properties. Section five is based on the paper entitled “A plasma-assisted bioextrusion system for tissue engineering” published in CIRP Annals-Manufacturing Technology, while Section six is based on the paper entitled “Hybrid additive manufacturing system for zonal plasma treated scaffolds” published in 3D Printing and Additive Manufacturing.
3.1 Morphological, Mechanical and Biological Assessment of PCL/Pristine Graphene Scaffolds for Bone Regeneration*

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Contribution to the work: As first author, W.W. conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

3.1.1 Introduction

Bone is a highly anisotropic tissue, able to heal and remodel without leaving any scar in cases of very limited damage or fracture. However, in pathological fractures, traumatic bone loss or primary tumour resection, where the bone defect exceeds a critical size, bone is no longer able to heal itself [310, 311]. Additionally, this regenerative ability reduces with age [312]. With the increasing life expectancy of the population, osteoporotic fractures will have a serious economic impact on society and patient’s quality of life. It is estimated that in Europe 179,000 men and 611,000 women will suffer hip fracture each year and the cost of all osteoporotic fractures in the EU will increase from the current 31.7 billion euros to 76.7 billion euros by 2050 [313].

In these cases, the clinical approach is the use of bone grafts, defined as an implanted material that promotes bone healing alone or in combination with other materials, through osteogenesis, osteoinduction and osteoconduction [6]. Bone grafts can be divided into autografts, allografts and xenografts [310, 311, 314]. However, there are many inherent limitations with this procedure. Autografts are considered to be the most effective approach, however, they present some drawbacks such as site morbidity, pain and prolonged hospitalization [311, 312]. Allografts are associated with rejection problems, transmission of diseases and infections from donor to recipient and cost [310, 311, 314]. Xenografts major limitations are related to their lack of osteogenic properties, risk of immunogenicity and transmission of infections and zoonotic diseases and poor clinical outcome [6, 314]. Therefore, biofabrication, the combined use of additive manufacturing techniques, biocompatible and biodegradable materials, cells, growth factors, etc. for the fabrication of bioactive scaffolds (synthetic grafts), is becoming a promising alternative for grafting [2, 7, 19, 315-319]. In this approach, scaffolds provide an initial biochemical substrate for the novel tissue until cells can produce their own extra-cellular matrix. An ideal scaffold for bone tissue engineering must be designed according to the following requirements [2, 19, 312, 320, 321]:

- The scaffold material must be non-toxic and allow cell attachment, proliferation and differentiation;
- The scaffold material must degrade into non-toxic products with a controlled degradation rate;
- The scaffolds should promote osteointegration, which corresponds to the formation of a chemical bond between bone and the surface of the implanted scaffold without
the formation of fibrous tissue. They must also promote osteocunduction and osteogenesis, inducing chemical stimulation of human mesenchymal stem cells into bone-forming osteoblasts;

- Scaffolds must be able to delivery growth factors, cytokines and antibacterial materials;
- They must present sufficient strength and stiffness to withstand stresses in the host tissue environment and adequate surface properties like wettability and surface roughness guaranteeing that a good biomechanical coupling is achieved between the scaffold and the tissue.

Its capacity to stimulate cells is also another important requirement. As electrical signals are critical physiological stimuli that strongly affect cell behavior, electro-active scaffolds could have a great potential as substrates for tissue engineering, enabling cell stimulation, increasing their proliferation and differentiation [322-325]. To produce these scaffolds, different routes are explored, including the use of conductive polymers mixed with non-conductive polymers, and the use of inorganic conductive materials with non-conductive polymers. Pristine graphene (highly pure graphene material) is a two-dimensional carbon nano-filler that can be used to create electro-active scaffolds, with potential to improve mechanical and conductivity properties. Different manufacturing techniques, like solvent precipitation/casting and electrospinning, have been used to produce graphene composites substrates (2D), foams and scaffolds (3D) [146, 326, 327]. However, these techniques are not fully reproducible and do not allow a good control over pore shape, pore size and pore interconnectivity, which are critical parameters to design optimised 3D scaffolds. Additionally, a number of studies reported on the cytotoxicity of graphene-based composite materials and their potential risks [74, 328, 329], while others reported that graphene-coated surfaces presented good cytocompatibility, stimulating cell proliferation [80, 330].

This paper, investigates the potential usage of PCL/pristine graphene scaffolds, containing very small concentrations of pristine graphene (to avoid potential cytotoxicity effects), for tissue engineering applications. Two major effects were considered: how effective is pristine graphene to improve the mechanical properties even in small concentrations; the effect of small concentrations of pristine graphene on both cell viability and proliferation. Scaffolds with different material compositions were produced using an extrusion-based
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additive manufacturing technique, which allows high reproducibility and the fabrication of scaffolds with good control over its topology (pore size, pore shape, pore distribution, etc.).

### 3.1.2 Materials and methods

#### 3.1.2.1 Materials

1. **Poly (ε-caprolactone)**

Poly (ε-caprolactone) (PCL) used in the research was Capa 6500 (Perstorp, UK). PCL is a semi-crystalline biocompatible and biodegradable linear aliphatic polyester with a low melting point and glass transition temperature [331, 332]. Relevant properties are indicated in Table 3.1.

<table>
<thead>
<tr>
<th>Table 3.1</th>
<th>Material properties of PCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>1.146 g/mL at 25 °C</td>
</tr>
<tr>
<td>Melting point</td>
<td>58 – 60 °C</td>
</tr>
<tr>
<td>Glass transition temperature</td>
<td>≈ -60 °C</td>
</tr>
<tr>
<td>Molecular weight (Mw)</td>
<td>50000 g/mol</td>
</tr>
<tr>
<td>Specific heat of vaporization</td>
<td>1 kJ/g</td>
</tr>
<tr>
<td>Solubility parameter (δ)</td>
<td>9.34 – 9.43 (cal/cm³)</td>
</tr>
</tbody>
</table>

2. **Pristine graphene**

Pristine graphene was prepared via water-assisted liquid phase exfoliation of graphite. Briefly, 50 mg microcrystalline graphite powder (325 mesh, 99.995% pure, purchased from Alfa Aesar) was immersed in *N*-methyl-2-pyrrolidone (NMP) mixture with a 0.2 mass fraction of water. The initial concentration of graphite was fixed at 5 mg mL⁻¹ for exfoliation. NMP, 99% extra pure was purchased from ACROS ORGANICS. The materials were batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H, Switzerland) at a fixed nominal power and frequency of 100 W and 37 kHz respectively. Sample dispersions were hanged on for overnight in between sonication and centrifugation and were centrifuged at 3000 rpm for 30 minutes using a Hettich, EBA20. The upper 75% of the colloidal supernatant were collected and dried in an oven to yield the graphene nano-sheets.

3. **Melt-blending process**

PCL/pristine graphene blends were prepared according to the following steps:

- PCL pellets were melted up 70°C;
- Pristine graphene flakes were added to the polymer melt at desired concentration;
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- The blend was physically mixed for 15 min to ensure a good pristine graphene dispersion;
- After mixing, the blended material was cool down and cut into small pellets.

### 3.1.2.2 Scaffold fabrication

A three-dimensional block model was initially designed in a CAD software (SolidWorks, Dassault Systems). A 0°/90° lay-down pattern was adopted to obtain pores with a regular square geometry while maintaining a constant filament distance of 680μm. Scaffolds were produced using the following process parameters: melting temperature (90 °C); slice thickness (220 μm); screw rotation velocity (22 rpm) and deposition velocity (20 mm/s). PCL and PCL/pristine graphene scaffolds containing different concentrations of graphene (0.25 wt.%; 0.50 wt.% and 0.75 wt.%) were produced using a screw-assisted additive manufacturing system from RegenHU (3DDiscovery, Switzerland). In this process, the material is molten in the liquefier tank, pressed to the barrier screw tank by compressed air, and extruded out through a 330 μm nozzle. PCL/pristine graphene pellets were initially prepared by melt blending. Produced scaffolds presented well dispersed pristine graphene, as previously reported using Raman spectroscopy and micro Raman mapping [313].

### 3.1.2.3 Thermogravimetric analysis

The onset of thermal degradation and pristine graphene content in the scaffolds was assessed using a TA Instruments Q500 TGA equipped with an evolved gas analysis furnace. Thermogravimetric analysis (TGA) was performed on neat PCL scaffolds as controls, and pristine graphene loaded PCL scaffolds. Scans were performed in an air atmosphere (flow at 60 mL/min) with a temperature range from room temperature to 560°C at a rate of 10°C/min. Measurements were taken using sample mass of 6 ± 1 mg in platinum pans. The weight losses of the PCL/pristine graphene composite structures were monitored and used to calculate the final pristine graphene contents.

### 3.1.2.4 Morphological characterization

Scanning electron microscopy (SEM) was used to investigate the morphology of produced scaffolds and to measure pore size (PS) and filament width (FW), comparing obtained values with the initial design parameters (Figure 3.1). SEM was conducted with a Quanta 200 SEM system, using an accelerating voltage of 10 kV. All relevant dimensions were measured using the software Image J. The average and standard deviation obtained from 6 measurements are reported for each scaffold.
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Figure 3.1 Design parameters of the PCL/pristine graphene scaffolds

3.1.2.5 Apparent water-in-air contact angle

The contact angle indicates the wettability of the material surface, being an important parameter in order to understand the biological interaction between the scaffolds and cells. The balance of forces regarding the surface tension between liquid-vapour ($\gamma_{lv}$) for a liquid drop and the interfacial tension between the solid substrate and the liquid drop ($\gamma_{sl}$) depends on the angle ($\theta$) between the drop and the surface. Thus, the surface energy ($\gamma_{sv}$) can be evaluated using the following equation:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta$$  (3.1)

The contact angle enables to understand the hydrophilic/hydrophobic characteristics of the structure. A contact angle below 90º means a hydrophilic surface while a contact angle values above 90º corresponded to hydrophobic surfaces.

Static contact angle measurements were performed using the equipment OCA 15 (Data Physics) and deionized water (4µl of volume drop, 1µl/s of velocity). For each condition, five measurements were performed using the sessile drop method. The drop shape was recorded with a high speed framing camera. Measurements were performed after a static time of 20 s.
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### 3.1.2.6 Mechanical characterization

Compression tests were performed on both PCL and PCL/pristine graphene scaffolds to assess the effect the addition of pristine graphene on the mechanical properties of scaffolds. All tests were carried out using scaffolds (5 x 5 x 6 mm) in the dry state at a rate of 1 mm/min, to a strain limit of 0.3 mm/mm (30%), using the INSTRON 4507 system equipped with a 1 kN load cell. During uniaxial compression tests, the software captured force, $F$ and corresponding displacement values, which were converted into engineering stress ($\sigma$) and strain ($\varepsilon$) as follows:

$$\sigma = \frac{F}{A}$$  \hspace{1cm} (3.2)

$$\varepsilon = \frac{\Delta h}{h_0}$$  \hspace{1cm} (3.3)

where $A$ is the initial sample cross section area and $\Delta h$ is the scaffold height variation. The obtained stress-strain data was further processed to determine the compression modulus, $E_c$, according to the procedure previously reported by Fiedler [7].

### 3.1.2.7 Biological test (*in vitro*)

1. **Scaffold preparation**

For biological tests, PCL and PCL/pristine graphene scaffolds were cut into small blocks (11 mm x 11 mm x 6 mm) and placed into 24-well plates for further *in vitro* measurement. All scaffolds were sterilized by immersion in 70% ethanol for at least 4 hours, then rinsed twice with phosphate buffer solution (PBS) and dried 12 hours in a 37 ºC incubator. Prior to cell seeding, scaffolds were wet using cell culture media (MesenPRO RS™ Basal Medium) for 4 hours to enhance cell attachment and to prevent drying.

2. **Cell seeding**

*In vitro* tests were performed by seeding human adipose-derived stem cells (hADSCs) (STEMPRO®, Invitrogen, USA) on the scaffolds, using passages 3 to 5 suitable for seeding. Cells were cultured in T75 tissue culture flasks (Sigma-Aldrich, UK) with MesenPRO RS™ Basal Medium (Invitrogen, USA) until 80% confluence and harvested by the use of 0.05% trypsin-EDTA solution (Invitrogen, USA), and finally seeded on the scaffolds (100 µL of medium containing around 5 x 10^4 cells per sample). The cell-seeded scaffolds were incubated at standard conditions (37 ºC under 5% CO₂ and 95% humidity) for 2 hours to allow cell attachment, before the addition of 1 mL fresh basal medium [321, 333].

3. **Cell viability/proliferation**
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Cell viability/proliferation was assessed using the Resazurin assay, commercially known as Alamar Blue assay (Sigma-Aldrich, UK). Resazurin (7-hydroxy-10-oxido-phenoxazin-10-iium-3-one) dye is used to measure cytotoxicity and proliferation [334, 335]. Cells are able to reduce resazurin to resorufin intracellularly by mitochondrial enzyme activity based on their cellular metabolic activity [335, 336]. Briefly, cell viability/proliferation was measured at 3, 7 and 14 days after cell seeding. The medium was changed every 3 days. At each time point, the cell-seeded scaffolds were placed in a new 24 well plate and 1 mL Alamar Blue solution (0.001% in culture medium) was added to each well. The plates were incubated for 4 hours under standard conditions. After incubation, 100 µL of each sample was transferred to a 96-well plate and the fluorescence intensity was measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise; Tecan, Männedorf, Zurich, Switzerland). Experiments were performed at least three times in duplicate.

(4) Cell morphology

After incubation for 14 days, cell-seeded scaffolds were observed with SEM to assess the cell attachment and morphology. Scaffold samples were fixed with 2% glutaraldehyde solution (Sigma-Aldrich, UK) for 2 hours at room temperature. Then scaffolds were rinsed twice with PBS, dehydrated with graded ethanol series, following 50%, 70%, 80%, 90% and 100%, then 50:50 ethanol:hexamethyldisilazane (HMDS), and 100% HMDS (with 10 minute exposure steps) and then air dried for removal of HMDS [337]. Samples were coated with platinum and imaged using SEM, as described above.

3.1.2.8 Data Analysis

All data were represented as mean ± standard deviation. Biological results were subjected to one-way analysis of variance (one-way ANOVA) and Tukey’s post-hoc test using GraphPad Prism software. Significance levels were set at \( p < 0.05 \).

3.1.3 Results and discussion

3.1.3.1 Thermogravimetric analysis

The final content of pristine graphene in the PCL/pristine graphene composite scaffolds was calculated by TGA. The results (Table 3.2) suggest that the pristine graphene was effectively incorporated into the composite scaffolds, without significant losses during the melt blending process. It is also possible to observe from Table 3.2 that there is no significant difference on the onset of thermal degradation due to the presence of pristine graphene.
Results also indicate that no degradation events occur during the scaffold fabrication process, since the extrusion temperature was 90 °C.

**Table 3.2** Pristine graphene loading verification, assessed by TGA (reported at 555°C).

<table>
<thead>
<tr>
<th>Target graphene loading (wt.%)</th>
<th>Determined pristine graphene concentration (%)</th>
<th>$T_d$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>297.7 ± 0.6</td>
</tr>
<tr>
<td>0.25</td>
<td>0.131 ± 0.065</td>
<td>294.7 ± 3.8</td>
</tr>
<tr>
<td>0.50</td>
<td>0.503 ± 0.004</td>
<td>296.3 ± 1.5</td>
</tr>
<tr>
<td>0.75</td>
<td>0.78 ± 0.001</td>
<td>297.3 ± 0.6</td>
</tr>
</tbody>
</table>

### 3.1.3.2 Morphological analysis

Figures 3.2 and 3.3 show the SEM micrographs (top and cross-section) of PCL and PCL/pristine graphene scaffolds. From the top view of these micrographs (Figure 3.2) it is possible to observe that scaffolds present a well-defined internal geometry taken of the scaffold structure show a well-defined internal geometry and uniform pore distribution. Cross-section micrographs (Figure 3.3) show a good adhesion between adjacent layers. The values of pore size and filament width are summarised in Table 3.3. Results show an increase in the filament width due to the increase in pristine graphene, resulting in a slight decrease in both pore size and porosity. Figure 3.4 shows the corresponding variation trends.
Figure 3.2 SEM images of scaffolds (top view) produced using different pristine graphene concentration (a) 0 wt.%; (b) 0.13 wt.%; (c) 0.50 wt.%; (d) 0.78 wt%.
Figure 3.3 SEM images of scaffolds (cross section) produced using different pristine graphene concentration (a) 0 wt.%; (b) 0.13 wt.%; (c) 0.50 wt.%; (d) 0.78 wt.%.

Table 3.3 Morphological characteristics of scaffold structures for different pristine graphene concentration.

<table>
<thead>
<tr>
<th>Pristine graphene concentration (wt.%)</th>
<th>Filament width (μm)</th>
<th>Pore size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>343 ± 5</td>
<td>368 ± 24</td>
</tr>
<tr>
<td>0.13</td>
<td>361 ± 5</td>
<td>366 ± 11</td>
</tr>
<tr>
<td>0.50</td>
<td>367 ± 4</td>
<td>347 ± 13</td>
</tr>
<tr>
<td>0.78</td>
<td>379 ± 14</td>
<td>343 ± 6</td>
</tr>
</tbody>
</table>
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**Figure 3.4** Variation of filament diameter and pore size as a function of pristine graphene concentration.

### 3.1.3.3 Mechanical compression test

The mechanical behaviour of the scaffolds is strongly correlated to the amount of pristine graphene in PCL. Results show that by increasing the concentration of pristine graphene from 0.13% to 0.78% the compression modulus increased from 80 MPa to circa 130 MPa (Table 3.4 and Figure 3.5). This effect is due not only to the reinforcement effect of the pristine graphene but also to the differences in terms of pore size (Table 3.3). In comparison to PCL scaffolds, a slight decrease in mechanical properties was observed for scaffolds containing 0.13% pristine graphene, which the authors hypothesize, could be due to the stress concentration in the interface between the polymer and the pristine graphene, which superpose the reinforcement effect of pristine graphene and the reduction on the pore size. Maximum values of compression modulus observed for scaffolds containing higher concentrations of pristine graphene are in the mid range of properties reported for human cortical bone [3].

**Table 3.4** Mechanical properties of scaffolds for pristine graphene concentration values.

<table>
<thead>
<tr>
<th>Pristine graphene concentration (wt.%)</th>
<th>Compression modulus, $E_c$ [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82.2 ± 6.8</td>
</tr>
<tr>
<td>0.13</td>
<td>80.6 ± 3.1</td>
</tr>
<tr>
<td>0.50</td>
<td>109.3 ± 5.3</td>
</tr>
<tr>
<td>0.78</td>
<td>128.7 ± 6.9</td>
</tr>
</tbody>
</table>
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**Figure 3.5** Compression test results for scaffolds containing different pristine graphene concentrations

### 3.1.3.4 Apparent water-in-air contact angle analysis

To access the hydrophilicity, the static contact angle was determined and the results presented in Table 3.5 and Figure 3.6. The results show a decrease in the static contact angle by increasing the pristine graphene concentration. It is also possible to observe that pristine graphene, even in very small concentrations, can be used to shift from hydrophobic PCL scaffolds to hydrophilic composite ones. Statically, 0.50% and 0.78% PCL/pristine graphene scaffold exhibited lower contact angle than PCL scaffolds.

**Table 3.5** Apparent water-in-air contact angle after 20 seconds.

<table>
<thead>
<tr>
<th>Pristine graphene concentration (wt.%)</th>
<th>Contact Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96.10 ± 1.51</td>
</tr>
<tr>
<td>0.13</td>
<td>91.78 ± 5.32</td>
</tr>
<tr>
<td>0.50</td>
<td>84.68 ± 2.98</td>
</tr>
<tr>
<td>0.78</td>
<td>88.25 ± 3.95</td>
</tr>
</tbody>
</table>
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![Contact Angle Figure](image)

**Figure 3.6** Contact angle test results for scaffolds containing different pristine graphene concentrations. * Statistical evidence (p<0.05) analysed by one-way ANOVA, Tukey post test.

### 3.1.3.5 *In vitro* evaluation

The Alamar Blue assay was performed to evaluate cell viability and cell proliferation on PCL/pristine graphene scaffolds compared to PCL scaffolds. The fluorescence intensity after measurement is given in Figure 3.7. Higher fluorescence intensity corresponds to more metabolically active cells. It can be observed that at day 3, all scaffolds exhibited similar biological performance. At day 7, 0.78% PCL/pristine graphene scaffolds exhibited greater fluorescence intensity, statistically different from 0%, corresponding to a high cellular activity. This observation can indirectly be correlated to higher cell proliferation rate. On the other hand, based on the statistical analysis, it is possible to notice that on day 14, 0.50% and 0.78% PCL/pristine graphene scaffolds positively deviated from PCL scaffolds and 0.13% PCL/pristine graphene scaffolds, showing higher cell viability/proliferation rates. It is also possible to observe that through all the time points, the fluorescence activity increased, which indicates an increase in the cell proliferation rate. The negative control (NC) shows no metabolically active cells.

According to SEM observations of the cells on the scaffolds (Figure 3.8), extensive cell attachment and cell spreading (pseudopodia) are evident. Cell sheets are seen to bridge orthogonal scaffold filaments, indicating that scaffolds are able to support the growth and proliferation of cells. The isolation and characterisation tests of ADSCs were performed by the supplier (ThermoFisher Scientific, UK). The focus of this paper was to access the effect
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of low concentration of pristine graphene on both cell viability and proliferation. Differentiations studies, not reported here, are being conducted.

![Graph showing cell viability/proliferation](image)

**Figure 3.7** Cell viability/proliferation (Alamar blue assay). NC (negative control); * Statistical evidence (p<0.05) analysed by one-way ANOVA, Tukey post test.
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3.1.4 Conclusions

This paper presents the morphological, mechanical and biological properties of PCL/pristine graphene scaffolds containing different concentrations of pristine graphene. Results indicate that the screw assisted additive manufacturing system considered in this research work is a viable technique to produce these composite scaffolds. The results also show that the addition of pristine graphene has an impact on both the scaffold topology and mechanical properties. For the same process conditions, increasing the content of pristine graphene increases the filament width, decreases pore size and increases the mechanical performance of the scaffold.

It was also observed that pristine graphene slightly reduces the contact angle, increasing the cell-scaffold affinity, cell viability/proliferation. These results suggest that PCL/pristine graphene scaffolds are promising biomaterial for bone tissue engineering applications.
3.2 Enhancing the Hydrophilicity and Cell Attachment of 3D Printed PCL/Graphene Scaffolds for Bone Tissue Engineering*

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† Co-first authors, contributed equally to this work.

Contribution to the work: As first author, W.W. conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

3.2.1 Introduction

Three-dimensional (3D) scaffolds fabricated by additive manufacturing are a promising strategy in tissue engineering for the replacement and regeneration of damaged tissue. Such scaffolds should ideally be stimulatory, as well as biocompatible, degradable, and designed according to specific requirements to create a highly porous structure with interconnected pores [2, 19, 338]. These characteristics can provide an appropriate environment for cells and play an important role as a physical substrate for cell attachment, proliferation, and differentiation, as well as integration to the host tissue in order to regenerate the defect [11, 18, 319]. Although some new methods using shape memory materials, such as bioprinting and 4D printing, are under development [318, 339-341], they are very much at infancy and less mature than scaffold technology.

An ideal approach is to combine porous scaffolds with living progenitor cells, especially for elderly people whose cell growth and cell differentiation are age-compromised. Cellularised scaffolds, as previously reported using human adipose-derived mesenchymal stem cells (hADSCs) in a bone regeneration animal model [321], might stimulate the tissue around the damage area towards regeneration whilst playing an important role to support cell migration, cell attachment, proliferation, and differentiation.

Achieving suitable cell attachment to the scaffold is key to success; however, it is challenging with highly hydrophobic scaffold matrices, which may result in inefficient cell colonisation. Material surface modification such as plasma, laser and chemical treatment, and protein coating is commonly used in order to improve cell attachment, leading to more efficient scaffold colonisation [315, 325, 337, 342-344]. However, some of these techniques are expensive, require long times, and in some cases are non-reproducible [315, 337, 342].

Pristine graphene, a two-dimensional carbon nanofiller, could play an important role in enhancing polymer material properties because it can improve solubility, processability, and mechanical and conductivity properties. Furthermore, it is suggested that graphene composites are able to provide a dramatic improvement in these properties with very low filler content [345-349]. Controversial discussion about graphene usage to develop and produce biomaterials has been reported in terms of cytotoxicity. Some research works have presented graphene-based composites as materials that might have potential cytotoxicity risks [74, 328, 329], while other studies report good cytocompatibility and the ability to stimulate cell proliferation, for example, with graphene coatings [80, 330].
In order to enhance cell attachment and biological performance of poly(ε-caprolactone) (PCL) scaffolds, this paper investigates the addition of three different small concentrations of pristine graphene, as well as a simple sodium hydroxide (NaOH) surface treatment, to render the scaffolds more hydrophilic. Two major effects were considered: the effect of pristine graphene in small concentrations on the cell viability/proliferation rate and the effect of NaOH chemical treatment on the surface properties such that the hydrophobic feature of PCL scaffolds is changed.

3.2.2 Results and discussion

3.2.2.1 Surface modification evaluated by contact angle

The apparent water-in-air contact angle on scaffolds untreated and treated with NaOH are given in Figure 3.9. As previously reported [1, 350], the contact angle indicates the wettability of the material surface, indicating hydrophilic/hydrophobic characteristics of the material. In general, a contact angle above 90° corresponds to a hydrophobic surface, while a contact angle value below 90° represents a hydrophilic surface. In the untreated case, the values slightly decreased with the addition of pristine graphene, ranging from 96° ± 1.50° (neat PCL) to 84° ± 2.90° (0.50 wt.% pristine graphene). Contrary to the common assumption that graphene, as other carbon-based materials, is hydrophobic, these results are in line with recent observations from Munz and co-workers [351]. Those researchers investigated the adhesion and friction properties of single-layer and double-layer graphene using chemical force microscopy with a hydrophobic probe. Results showed a large adhesion force between the probe and double-layer graphene compared to single-layer, showing that double-layer graphene is ideal for hydrophobic applications and single-layer graphene for applications where a hydrophilic surface is required. Figure 3.9 shows a bar chart with the contact angle values. A statistical difference was observed between pure PCL scaffolds and both 0.50 wt.% and 0.78 wt.% pristine graphene scaffolds before NaOH treatment, indicating that the hydrophilicity of the surface increased with a small concentration of pristine graphene. After NaOH treatment, neat PCL (61° ± 6.50°), 0.13 wt.% (69° ± 6.72°), and 0.50 wt.% (67° ± 6.09°) pristine graphene scaffolds had a significant reduction in contact angle, compared to untreated scaffolds, and were statistically different from 0.78 wt.% (83° ± 7.06°).
Figure 3.9 Summary of the apparent water-in-air contact angle for scaffolds containing different pristine graphene concentrations untreated and treated with NaOH 5M. * Statistical evidence (p < 0.05) analysed with a one-way ANOVA and Tukey's post-hoc test.

3.2.2.2 Morphological evaluation of scaffolds

Figure 3.10 represents the fibre surface of 0.78 wt.% pristine graphene and neat PCL scaffolds treated and untreated with 5M NaOH for 3 h. Results show that, for the NaOH chemical treatment time considered, there is no negative impact on the fibre structure in the produced scaffolds. Similar results were obtained for the other compositions. It is also evident that the produced scaffolds have regular, well-defined, and uniform pore distribution.
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![NaOH - untreated](image1)

![NaOH - treated](image2)

**Figure 3.10** Top surface and cross-section scanning electron microscope images of neat PCL and 0.78 wt.% pristine graphene scaffolds treated and untreated with NaOH.

### 3.2.2.3 Biological evaluation

Cell viability and proliferation on scaffold samples were assessed using an Alamar Blue assay. Fluorescence intensity is reported in Figure 3.11. Higher fluorescence intensity corresponds to more metabolically active cells. Comparing the three different time points in Figure 3.11a or Figure 3.11b, it can be observed that, for scaffolds both treated and untreated with NaOH, fluorescence intensity increases from one point in time to another, suggesting that scaffolds fabricated with the additive manufacturing system are suitable substrates for cell proliferation. Compared with values at the same point in time in Figure 3.11a or Figure 3.11b for untreated scaffolds, the addition of pristine graphene has a positive impact on the biological behaviour of polymer scaffold, but not a significant one. At Day 14, the fluorescence intensity of the 0.78 wt.% PCL/pristine graphene scaffold was statistically higher than the neat PCL scaffold, representing a higher cell viability/proliferation rate. For NaOH-treated scaffolds, it can be observed that the addition of pristine graphene had a significant positive impact on cell viability/proliferation. According to statistical analysis, at Day 3, 0.50 wt.% and 0.78 wt.% scaffolds exhibited greater fluorescence intensity, statistically different from the neat and 0.13 wt.% scaffolds, corresponding to higher cell viability/proliferation rates. At Days 7 and 14, all PCL/pristine graphene scaffolds exhibited better biological performance over the neat PCL scaffolds.
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Comparing Figures 3.11a, b, it is evident that, at all three time points, scaffolds treated with NaOH had higher fluorescence intensity than non-treated scaffolds, presenting an improved biological performance. After 14 days, results indicated that, after chemical treatment with NaOH, the improvements on biological behaviour caused by the addition of pristine graphene still exist. For each particular time point in Figure 3.11b, PCL/pristine graphene scaffolds showed higher fluorescence intensity than neat PCL scaffolds, with cell proliferation rate increasing concomitant to increase graphene addition.

Figure 3.12 represents the assessment of cells attached on the scaffolds and left on the surface of the well plate. The measurement was performed at the first time point (3 days) after cell seeding, which is a representation of the cell attachment rate. It is evident that scaffold samples treated with NaOH had a higher cell attachment rate than the untreated scaffolds. Scaffolds untreated had around 30% cell attachment, while scaffolds treated with NaOH varied widely among samples. Moreover, 0.50 wt.% and 0.78 wt.% pristine graphene scaffolds had statistically higher cell attachment rate after 3 days compared to the neat PCL scaffold. Cell attachment is closely related to the surface properties of the scaffolds. The chemical treatment with NaOH leads to enhanced hydrophilicity and more cell attachment when compared to the untreated scaffolds. Furthermore, the addition of pristine graphene (0.50 wt.% and 0.78 wt.%) improved the cell attachment and proliferation rate even more after three days.
As is evident from Figures 3.11 and 3.12, pristine graphene has a significant impact on the biological performance of produced scaffolds, increasing cell attachment and proliferation. This can be attributed to the high surface area, the elastic modulus, and the stiffness of graphene. It is also related to the presence of wrinkles and ripples on graphene, created during the production of graphene [89]. Graphene was also found to be useful as a cellular adhesive, preventing implanted cells from reactive oxygen species (ROS)-mediated cell death, enhancing cell proliferation [318].
Figure 3.12 Percentage of cells attached on the well plate surface (cells not attached to the scaffold) and percentage of cells attached on the scaffold after 3 days of cell seeding. * Statistical evidence (p < 0.05) analysed with a one-way ANOVA and Tukey's post-hoc test.

3.2.2.4 Cell attachment and cell morphology

Cell attachment and morphology on the scaffolds was assessed via scanning electron microscopy (SEM) and laser confocal microscopy. Extensive cell attachment and cell spreading was observed, as shown in Figure 3.13. Confluent cell sheets were also observed, with many cells bridging orthogonal scaffold filaments. This indicates that the scaffolds are able to provide a suitable environment and support the growth and proliferation of cells. The confocal images show that cell morphology is maintained (nuclei stained blue, cell membrane red). Qualitatively, for all types of scaffolds, both SEM and confocal images show a greater number of cells present for scaffolds containing pristine graphene. NaOH-treated scaffolds presented higher cell confluency than untreated scaffolds.
Figure 3.13 Cells attached on the scaffold. (a) SEM images after 21 days culture; (b) confocal images after 28 days culture.

3.2.3 Materials and methods

3.2.3.1 Scaffold fabrication

Poly (ε-caprolactone) ($M_w$ 50,000, Capa 6500, Perstorp, Warrington, UK) and pristine graphene were used to produce scaffolds through a screw-assisted additive biomanufacturing system, the 3D Discovery (RegenHU, Villaz-St-Pierre, Switzerland), as previously reported [1]. PCL/pristine graphene pellets were initially prepared by melt blending in three different pristine graphene concentrations (0.25, 0.50, and 0.75 wt.%) [1]. Briefly, pure PCL pellets were heated above 70 °C in a bowl to ensure all material is in a molten state prior to pristine graphene flake addition, at the desired concentrations. The materials were mixed for 15 min to guarantee a homogenously dispersion. After cooling down for 2 h, the blended material was cut into small pellets to be loaded to the
screw-assisted additive biomanufacturing system. As previously reported [1], the obtained pristine graphene concentrations are 0.13, 0.50, and 0.78 wt.

The extrusion-based additive manufacturing technique used in this work allows high reproducibility and good control over scaffold topology (including pore size, pore shape, and pore distribution), which are critical parameters when designing optimised 3D interconnected porous scaffolds [1, 313]. The 0°/90° lay-down pattern was adopted to obtain pores with a regular square geometry and a constant filament distance of 680 μm. The optimal combination of processing parameters—melting temperature (90 °C), slice thickness (220 μm), screw rotation velocity (22 rpm), and deposition velocity (20 mm/s)—allows for the filament diameter after extrusion to be close to the desired diameter of 330 μm [1, 313]. As previously reported, the presence of pristine graphene at the surface of the scaffold filaments was observed using Raman spectroscopy, and micro Raman mapping showed a uniform distribution of pristine graphene [313].

After fabrication, scaffold samples were cut with fine, double-edged razor blades into small blocks (~11 mm × 11 mm × 6 mm) to fit in a 24-well culture plate.

3.2.3.2 Surface modification

Scaffold samples for the NaOH-treated group were processed by soaking in 5 M NaOH for 3 hours using 50 mL conical tubes, at room temperature [321]. The scaffolds were washed exhaustively with distilled water and air-dried for 24 h in an incubator at 37 °C.

3.2.3.3 Apparent water-in-air contact angle

Static contact angle measurements were performed on produced scaffolds using an OCA 15 (Data Physics) machine using the sessile drop method. Deionised water droplets of ~4 μL were deposited via a motorised syringe at a velocity of 1 μL/s. Five measurements per sample type were performed. The drop shape was recorded with a high speed framing camera. Measurements were performed 20 s after droplet addition.

3.2.3.4 Morphological characterisation

Scanning electron microscopy (SEM) was used to investigate the morphology of produced scaffolds treated and untreated with 5 M NaOH. SEM was conducted with a Quanta 200 SEM system, using an accelerating voltage of 3.0 kV.

3.2.3.5 Cell culture studies

In vitro cell culture studies were conducted using human adipose-derived stem cells (hADSCs) (STEMPRO®, Invitrogen, Waltham, MA, USA) ranging from passage 3 to 5.
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Cells were cultured in T75 tissue culture flasks (Sigma-Aldrich, Dorset, UK) with MesenPRO RS™ Basal Medium (Invitrogen, Waltham, MA, USA) until 80\% confluence and harvested by the use of a 0.05\% trypsin solution (Invitrogen, Waltham, MA, USA).

(1) Cell Seeding

Scaffolds were sterilised in 70\% ethanol for 4 h, rinsed in phosphate buffered saline (PBS) three times, placed in 24-well plates, and air-dried for 24 h in an incubator at 37 °C. The scaffolds were wet with 200 µL of media containing 10\% fetal bovine serum (FBS) and kept in standard conditions (37 °C under 5\% CO$_2$ and 95\% humidity) for 2 h prior to cell seeding [321]. Cells were seeded on each scaffold (150 µL of medium containing around $5 \times 10^5$ cells). A tissue culture plastic control containing the same amount of cells was included for consideration as 100\% of cells seeded to evaluate the seeding efficiency after 3 days. The cell-seeded scaffolds and control were incubated at standard conditions (37 °C under 5\% CO$_2$ and 95\% humidity) for 2 h to allow cell attachment, before the addition of 1 mL of fresh basal media [321, 333].

(2) Cell Viability/Proliferation

In order to study the cell viability/proliferation and the percentage of cells attached in the scaffolds (cell-seeding efficiency), the Alamar Blue assay (also termed the Resazurin assay) was used (reagents from Sigma-Aldrich, Dorset, UK) [334, 336]. Briefly, cell viability/proliferation was measured at 3, 7, and 14 days after cell seeding to PCL and PCL/pristine graphene scaffolds treated and untreated with NaOH. After 3 days of cell seeding, but before the Alamar Blue test, the cell-seeded scaffolds were moved to a new 24-well plate and 1 mL of the Alamar Blue solution was added to each well and the control. Cells attached on the surface of the wells were also quantified on the 3rd time point. The plates were incubated for 4 h under standard conditions. After incubation, 150 µL of each sample was transferred to a 96-well plate, and the fluorescence intensity was measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland). Experiments were performed three times in duplicate.

(3) Cell Morphology and Attachment

Samples of scaffolds used in the cell viability/proliferation study were kept in culture up to 21 days to assess cell morphology and qualitative attachment to the scaffolds via scanning electron microscopy (SEM). For SEM preparation, scaffolds were fixed with a 3\% glutaraldehyde solution (Sigma-Aldrich, UK) for 30 min at room temperature, rinsed twice
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with PBS, dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, and 100% (twice)), in 50:50 ethanol/hexamethyldisilazane (HMDS, Sigma-Aldrich, Dorset, UK) and then in 100% HMDS (with 10 min exposure at each step), and then air dried for HMDS removal [321]. Thin cross-section layers of each sample (around 1 mm) was cut and platinum-coated for imaging using a Gatan Model 682 Precision Etching Coating System, to an approximate thickness of 7 nm. SEM images were obtained using a Hitachi S300N microscope (Hitachi, Maidenhead, UK).

Cell morphology was further assessed using laser confocal microscopy with scaffolds cultured up to 28 days, with cell membranes and nuclei stained. Samples were removed from the cell culture plate, rinsed twice in PBS, fixed using 4% paraformaldehyde for 40 min, and then washed twice with PBS prior to immersion for 30 min in an immunocytochemistry blocking buffer comprised of 2% goat serum and 1% bovine serum albumin in PBS. Samples were again rinsed twice in PBS. Cell nuclei were stained blue by soaking them in a PBS solution containing Hoescht 33342 (C62249, ThermoFisher, Waltham, MA, USA) at a 2 µM concentration; the cell membranes stained using CellMask™ Orange plasma membrane stain (C10045, ThermoFisher, Waltham, MA, USA) were diluted to the manufacturer recommended level [352, 353]. Samples were left in the staining solution for 10 min prior to removal, rinsed twice thoroughly with PBS, and mounted using ProLong® Diamond Antifade (P36962, ThermoFisher, Waltham, MA, USA). Confocal images were obtained on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

### 3.2.3.6 Data analysis

All data were represented as mean ± standard deviation. A one-way analysis of variance (one-way ANOVA) and Tukey’s post-hoc test using GraphPad Prism software was applied. Significance levels were set at *p* < 0.05.

### 3.2.4 Conclusions

Scaffolds with filaments containing well dispersed pristine graphene produced by an additive manufacturing system presented good biological behaviour in terms of cell viability and proliferation, making them a good substrate for bone tissue regeneration. The addition of low concentrations of pristine graphene exhibited no cytotoxicity, and enhanced cell viability/proliferation.

The addition of pristine graphene, served to moderately reduce the apparent water-in-air contact angle compared to the neat PCL filaments. Chemical treatment with 5 M NaOH
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further increased the hydrophilicity, leading to better cell attachment and enhanced biological behaviour. Test results also proved that the NaOH treating process did not change the enhancement in biological performance due to the addition of pristine graphene.

It can be concluded that PCL/pristine graphene scaffolds fabricated by an extrusion-based additive manufacturing system could be a promising substrate for bone tissue engineering, and that NaOH chemical treatment could effectively improve the biological behaviour of these composite scaffolds.
3.3 3D Printed Poly(ε-caprolactone)/Graphene Scaffolds Activated With P1-Latex Protein for Bone Regeneration*

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Contribution to the work: As co-first author, together with first author G.C., W.W. conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

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### 3.3.1 Introduction

Tissue engineering is recognized as a promising field to overcome some of the limitations of existing clinical treatments for the repair of damaged and dysfunctional tissues or organs [354-356]. A key strategy involves the use of biocompatible and biodegradable materials, cells and growth factors combined with additive manufacturing techniques to produce tissue constructs [19, 291, 354, 357]. Two main approaches are usually considered [358-360]. The first approach is based on the use of biodegradable and biocompatible porous scaffolds that are implanted into the lesion site without cells, seeded with autologous or allogeneic cells and then implanted, or seeded with cells and then cultured *in vitro* to produce tissue-engineered constructs prior implantation [357-361]. In this approach, scaffolds, from either natural or synthetic materials, provide the appropriated biomechanical environment to allow cells to produce their own extra-cellular matrix. In the second approach, specific additive manufacturing techniques (e.g. extrusion-based processes, laser bioprinting or material jetting processes) are used to print cells immobilized within polymeric hydrogels producing cell-laden 3D constructs [357-359, 362, 363].

Synthetic polymers (e.g. poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(caprolactone) (PCL), poly(lactide-co-glycolide) (PLGA)) are the most commonly used materials for certain applications such as bone and osteochondral tissue engineering [364-366]. Among these polymers, PCL is widely used due to its biocompatibility and favourable rheological properties including low glass transition temperature (~ -60 ºC), low melting point (~60 ºC) and decomposition temperature of around 350ºC, which facilitates its printability [315, 332, 367]. However, PCL does not provide specific motifs for cell attachment and is highly hydrophobic, which compromises its bioactivity and does not possesses the required strength to match the mechanical properties of bone.

In order to improve the performance of PCL scaffolds, this paper investigates the use of a screw-assisted extrusion-based additive manufacturing system to produce totally novel 3D PCL/graphene/P1-latex protein-coated scaffolds for bone regeneration. Graphene is used to improve the mechanical properties of the scaffolds and to impact the biological properties due to its high surface area, stiffness and the presence of wrinkles and ripples created during the production of graphene. Composite scaffolds with low graphene contents are considered to minimise potential cytotoxicity risks. The P1-latex protein extracted from the *Heavea brasiliensis* rubber tree is used to improve the biological performance of produced scaffolds. Preliminary results showed that latex bio-membranes can be successfully used for
wound healing applications, enabling cell adhesion and stimulating various cell types involved in the healing process [368-372]. These bio-membranes were also used to treat critical size bone defects created in rabbit’s calvaria [370].

PCL/graphene scaffolds containing different quantities of graphene nanosheets were printed and coated by soaking them in the P1-latex protein solution. The biological performance of these scaffolds was assessed using human adipose-derived stem cells (hADSCs) for both cell viability/proliferation and cell differentiation tests.

### 3.3.2 Materials and Methods

#### 3.3.2.1 Materials and scaffold fabrication

PCL, Capa 6500 (Mw=50,000), was purchased from Perstorp (UK). Graphene nanosheets were prepared by a water-assisted liquid phase exfoliation of graphite as reported previously [63, 64]. Lyophilized P1 latex-protein from the *Hevea brasiliensis* rubber tree was purchased from PeleNova Biotecnologia (Brazil). PCL/graphene composite blends were prepared by melt blending. In this case PCL pellets were heated above 70 °C in a bowl to ensure that all material is in a molten state prior to graphene addition. PCL and graphene were mixed for 15 minutes to guarantee a homogeneously dispersion. After cooling down for 2 hours, the blended material was cut into small pellets. The exact amount of graphene present in the mixtures was determined using a TA Instruments Q500 TGA. Thermogravimetric analysis was performed using PCL as control and PCL/graphene blends. Scans were performed in an air atmosphere (flow at 60 mL/min) with a temperature ranging from room temperature to 560 °C at a rate of 10 °C/min.

Scaffolds containing different concentrations of graphene (0.13, 0.50 and 0.78 wt.%) were produced using a screw-assisted additive manufacturing system from RegenHU (3DDiscovery, Switzerland) (Figure 3.14a). Scaffolds were produced using a 0°/90° lay-down pattern and the following process parameters: 90°C of melting temperature, 220 μm of slice thickness, 22 rpm of screw rotation velocity and 20 mm/s of deposition velocity. A nozzle of 330 μm of diameter and a constant filament distance of 680 μm were also considered.

After fabrication, the scaffolds were cut into small blocks to fit into the wells of a 24-well plate (11mm x 11mm x 6mm), sterilised in 70% ethanol, prepared with sterile water, and then P1-latex protein adsorption, before soaking them in the cell culture media, basal media (MesenPRO RSTM Basal Media, Thermo Fisher Scientific, Waltham, MA, USA). P1-latex
protein was analysed by Limulus Amebocyte Lysate (LAL, from LONZA), a standard endpoint assay for detection of Gram-negative bacterial endotoxin (LPS), presenting LPS levels below the limited point.

3.3.2.2 Scanning Electron Microscopy (SEM)

To assess the morphology of fabricated scaffolds, thin top and cross-section layers of each scaffold sample (around 3 mm) was cut and gold/palladium coated for imaging using a Q150T turbo-pumped sputter coater (Quorum technologies, UK), to an approximate thickness of 10 nm. SEM images were obtained using a Hitachi S3000N scanning electron microscope (Hitachi, Maidenhead, UK). Corresponding parameters were measured through ImageJ software (NIH, Bethesda, USA) on the obtained SEM images.

3.3.2.3 Mechanical Characterisation

Compression tests were performed to assess the effect of different graphene concentrations on the mechanical properties of the scaffolds. Blocks of 5.0 mm in length, 5.0 mm in width and 5.72 mm in height \((h_0)\) were considered. Tests were carried out on scaffolds in dry state at a rate of 1 mm/min up to a strain limit of 0.3 mm/mm (30%), using the INSTRON 4507 testing system equipped with a 1 kN load cell. During the uni-axial compression tests, the software determined force \(F\) and corresponding displacement values, which were converted into engineering stress \(\sigma\) and strain \(\varepsilon\) as follows:

\[
\sigma = \frac{F}{A} \quad \varepsilon = \frac{\Delta h}{h_0}
\]

Where \(A\) is the initial sample cross section area and \(\Delta h\) is the scaffold height variation. The obtained stress-strain data was further processed to determine the compressive modulus, \(E\), according to the procedure reported by Fiedler et al [7].
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**Figure 3.14** (a) Screw-assisted additive manufacturing system from RegenHU (3DDiscovery, Switzerland); (b) SEM images of top and cross-section view of PCL scaffolds; (c) SEM images of top and cross-section view of 0.78 wt.% PCL/graphene scaffolds; (d) Morphological data (filament diameter and pore size) of PCL and PCL/graphene scaffolds; (e) Mechanical characterisation (compressive modulus) of PCL and PCL/graphene scaffolds.

### 3.3.2.4 Graphene dispersion analysis

*Ex situ* characterization of the graphene dispersion was performed by transmission electron microscopy (TEM) and micro Raman spectroscopy. Spherical-aberration corrected field emission TEM (ARM-200F, JEOL, Tokyo, Japan) with 200 kV accelerating voltage was used. The TEM samples were prepared by dispersing few drops of graphene dispersion onto TEM copper micro-grids (400 mesh, Ted Pella, CA, USA) and then drying in ambient conditions. Raman scattering studies were performed at room temperature using a JASCO 5100 (JASCO, Tokyo, Japan) spectrometer (λ= 533 nm). Spectra were averaged from 10 random positions on each sample.

### 3.3.2.5 P1-latex protein adsorption

After sterilization, scaffolds were rinsed twice with phosphate buffer solution (PBS) and dried for 24 hours in a sterile 37°C incubator environment to remove any remaining ethanol and PBS. The scaffolds were then adsorbed with P1-latex protein using a procedure described by Patel *et al.* to coat PCL substrates [373]. Briefly, lyophilized P1-latex protein was prepared in 1 ml Dulbecco’s phosphate-buffered saline (DPBS) to obtain 1% (w/v) stock, with a concentration of 0.01 g/ml. The stock solution was further diluted to the desired concentration of 0.05% (w/v) in DPBS and filter-sterilized using a 0.22 µm filter. Scaffolds were then exposed to 1ml of 0.05% (w/v) P1 solution at 4°C for 24 hours (n=3). The amount of protein adsorbed after 24 hours was determined by quantifying the
remaining P1-latex protein present in the solution using the Micro BCA™ Protein Assay kit (Thermo Scientific, Waltham, MA, USA).

3.3.2.6 Scratch assay

For the preliminary assessment of the effect of P1-latex protein on cell spreading and cell migration a scratch assay was conducted following a procedure previously reported [374]. The cells were seeded into 24-well tissue culture plates at a concentration of $4 \times 10^4$ cells/well and incubated overnight to allow cell attachment and nearly confluent cell monolayers. A linear wound was created on the monolayer using a sterile 200µl plastic pipette tip. Any cellular debris was removed by washing the wells with phosphate buffer saline. A 1ml cell culture media was added according to each group: media with dimethyl sulfoxide (50%), as negative control group; basal media as basal control group; and three concentrations of P1 (0.01µg, 1µg, 100µg) diluted in media.

After 24 hours of incubation, cells were fixed with 3.7% paraformaldehyde for 15 min and stained with 4’,6-diamino-2-phenylindole (DAPI from Invitrogen, USA) for 20 min. Twelve representative images from each group were photographed to estimate the relative cell migration. The data was analysed using ImageJ software, and the percentage of cell migration calculated as $\left[ \frac{\text{cells (basal media with P1)} - \text{cells (basal control)}}{\text{cells (basal control)}} \right] \times 100\%$.

3.3.2.7 Cell seeding

*In vitro* tests were performed using human adipose-derived stem cells (hADSC) (STEMPRO®, Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured in T75 tissue culture flasks (Sigma-Aldrich, Dorset, UK) with MesenPRO RS™ Basal Media until 80% confluence and harvested using 0.05% trypsin-EDTA solution (Thermo Fisher Scientific, Waltham, MA, USA). After cell counting, cells were seeded on the scaffolds (100 µL of media containing around $5 \times 10^4$ cells per sample), and the cell-seeded scaffolds were incubated at standard conditions (37°C under 5% CO₂ and 95% humidity) for 4 hours to allow cell attachment, before the addition of 1 ml fresh basal media.

3.3.2.8 Cell viability/proliferation

Cell viability/proliferation was assessed at 7, 14 and 21 days after cell seeding, using the Resazurin assay (reagents from Sigma-Aldrich, Dorset, UK) [335, 337]. The media was changed every 3 days. At each time point, the cell-seeded scaffolds were placed in a new 24-well plate and 1 ml Alamar Blue solution 0.001% (v/v) in culture media was added to
each well. The plates were incubated for 4 hours under standard conditions. After incubation, 150 µL of each sample was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland). Cell proliferation experiments were performed three times in triplicate.

3.3.2.9 Cell differentiation

In order to determine the osteogenic differentiation of hADSCs cultured in the scaffolds, in basal media and also in osteogenic media (StemPro Osteocyte/Chondrocyte Differentiation Basal Media, Thermo Fisher Scientific, Waltham, MA, USA), alkaline phosphatase (ALP) enzyme activity was quantified using a colorimetric assay (SensoLYTE® pNPP Alkaline Phosphatase Assay Kit), according to the manufacturer. Cell/scaffold constructs were washed with buffer solution, incubated in 0.2% (v/v) Triton-X 100 in buffer solution for 10 minutes, sonicated twice at 2 minutes and incubated at -20ºC for 10 minutes. Cell lysates were collected, centrifuged at 3000 rpm for 5 min at 4ºC and 50 µl of the cell lysate supernatant was transferred to a 96-well plate and incubated with 50 µl of pNPP alkaline phosphatase substrate (p-nitrophenyl phosphate). After 40 min, the absorbance was measured using a plate reader (Sunrise, Tecan, Männedorf, Zurich, Switzerland) at 405 nm.

Osteogenic differentiation tests were performed three times in triplicate. ALP is reported as mean value ± standard deviation.

3.3.2.10 Cell morphology

Cell-seeded scaffolds were observed with scanning electron microscopy (SEM). Sample preparation was performed in accordance with the standards for cell viability/proliferation (21 days) and differentiation tests (28 days). Briefly, scaffolds were fixed with 2.5% (v/v) glutaraldehyde solution (Sigma-Aldrich, Dorset, UK) for 30 min at room temperature, rinsed twice in PBS, then dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, and 100%, then 50:50 ethanol/hexamethyldisilazane (HMDS) (Sigma-Aldrich, Dorset, UK), and 100% HMDS with 10 min exposure at each step), and then air dried to remove residual HMDS. Thin cross-section layers of each sample (around 2 mm) were cut to size and then platinum-coated for imaging (Gatan Model 682 Precision Etching Coating System, approximate coating thickness 10 nm). SEM images were obtained using a Hitachi S3000N microscope (Hitachi, Maidenhead, UK).

Cell morphology and cell spreading were further assessed using laser confocal microscopy. Samples were prepared in accordance with the standards for cell viability/proliferation (21
days) and differentiation tests (28 days). Cell membranes and nuclei were stained by removing samples from the cell culture plate, rinsed twice in PBS, fixed using 4% (v/v) paraformaldehyde for 40 minutes. Samples were washed twice with PBS prior to immersion for 30 min in an immunocytochemistry blocking buffer comprised of 2% (w/v) goat serum (31872, ThermoFisher, Waltham, MA, USA) and 1% (w/v) bovine serum albumin (37525, ThermoFisher, Waltham, MA, USA) in PBS. Samples were again rinsed twice with PBS. Cell nuclei were stained blue by soaking in a PBS solution containing Hoescht 33342 (C62249, ThermoFisher, Waltham, MA, USA) at a 2 µM concentration while cell membranes were stained using CellMask™ Orange plasma membrane stain (C10045, ThermoFisher, Waltham, MA, USA). Samples were left in the staining solution for 10 minutes prior to removal, rinsed twice with PBS, and mounted using ProLong® Diamond Antifade (P36962, ThermoFisher, Waltham, MA, USA) on glass cover slips. Confocal images were obtained using a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

### 3.3.2.11 Data Analysis

Data is represented as mean ± standard deviation. Data were subjected to analysis of variance, one-way ANOVA, followed by post hoc Tukey’s test and two-way ANOVA, followed by post hoc Bonferroni test, depending on the number of variables to analyse. The first statistical test is applied when only one variable is considered, in this case concentration, while the second method is applied in cases where more than one variable are considered, in this case concentration and time. Significance levels were set at $p < 0.05$. GraphPad Prism software was used for statistical analysis and graphing.

### 3.3.3 Results

#### 3.3.3.1 Scaffold characterisation

Printed scaffolds present uniformly distributed regular pores (Figures 3.14b and 3.14c) with key geometric characteristics indicated in Figure 3.14d. The addition of graphene nanosheets to PCL showed no significant influence on the filament diameter and pore sizes compared with original designed geometric characteristics (330µm filament diameter and 350µm pore size).

Mechanical compression results are presented in Figure 3.14e. Results show that by increasing the concentration of graphene from 0.13 wt.% to 0.78 wt.%, the compressive modulus increased from 80 MPa to around 130 MPa. In comparison to PCL scaffolds, a
slight decrease in mechanical properties was observed for scaffolds containing 0.13% pristine graphene, which the authors hypothesise, could be due to the stress concentration in the interface between the polymer and the pristine graphene, which superpose the reinforcement effect of pristine graphene and the reduction on the pore size. As expected, the mechanical behaviour of the scaffolds is strongly correlated to the amount of graphene nanosheets incorporated in PCL matrix. Moreover, maximum values of compressive modulus observed for scaffolds containing higher concentrations of graphene are in the mid-range of properties reported for human cortical bone [375].

3.3.3.2 Graphene distribution

TEM was performed to determine the morphology of the prepared graphene nanosheets (Figure 3.15a). Results show exfoliated graphene with lateral size ranging between 1500 and 2000 nm and a structure consisting of few layers. The prepared graphene nanosheets showed three typical peaks assigned as D, G, and 2D peaks at 1340, 1577, and 2692 cm\(^{-1}\), respectively (middle in Figure 3.15b).

Moreover, the Raman spectrum of PCL shows several characteristic Raman peaks of PCL at 1438 and 2927 cm\(^{-1}\). The Raman spectrum of PCL/graphene composite also exhibits the D, G and 2D bands of graphene, indicating the presence of graphene in the PCL/graphene scaffold.

Micro Raman spectroscopy was used to assess the graphene dispersion in the composite scaffolds. Figure 3.15c shows the region of PCL/graphene scaffold with 0.50 wt.% graphene considered for the Raman mapping study. Figure 3.15d is a high magnification image of the PCL/graphene scaffold highlighted in red in the SEM image (Figure 3.15c). The image shows the smooth surface of the PCL/graphene scaffold. Figures 3.15e-h present the corresponding two-dimensional micro Raman mapping of the PCL/graphene scaffolds with different graphene concentrations from 0, 0.13, 0.50, and 0.78 wt.%.

The false colour maps were generated by scanning a 532 nm laser beam over an area of 250 × 450 μm\(^2\) in 5 μm steps. The mapping intensity indicates the ratio of integrating the intensity of the G band from 1574 to 1580 cm\(^{-1}\) for graphene to the intensity of 1435 to 1441 cm\(^{-1}\) peak for PCL. Results show uniform dispersion of graphene. Figure 3.15i represents a small region of PCL/graphene scaffold with 0.50 wt.% graphene considered in order to obtain more detailed information. The corresponding mapping intensities presented in Figures 3.15j and 3.15k indicate the ratios of integrating the intensities of the G band from 1574 to 1580 cm\(^{-1}\) for graphene to the intensities of 1435 to 1441 cm\(^{-1}\) peaks.
and 2924 to 2931 cm\(^{-1}\) peaks for PCL, respectively. Overall, the micro Raman mappings show uniform distribution of graphene nanosheets in the printed scaffold.

Figure 3.15 (a) TEM image of graphene nanosheet; (b) Raman spectra of PCL, graphene, and PCL/graphene scaffold containing 0.50 wt.% of graphene; (c) SEM image of PCL/graphene scaffold (0.50 wt.%). Scale bar = 400 μm. (d) Magnified SEM image of PCL/graphene scaffold (0.50 wt.%). Scale bar = 100 μm. Raman mapping of PCL/graphene scaffold with varying concentration of graphene (e) 0 wt.%, (f) 0.13 wt.%, (g) 0.50 wt.% and (h) 0.78 wt.%. Scale bar = 100 μm. (i) SEM image of PCL/graphene scaffold with 0.50 wt.% graphene. Scale bar = 100 μm. Raman mappings of PCL/graphene scaffold (0.50 wt.%) with ratios of integrating the intensities of the G band from 1574 to 1580 cm\(^{-1}\) for graphene to the intensities of (j) 1435 to 1441 cm\(^{-1}\) peak and (k) 2924 to 2931 cm\(^{-1}\) peak for PCL, respectively. Scale bar = 100 μm.
3.3.3.3 Cell viability/proliferation and P1-latex protein

Figure 3.16a presents representative images after 24h of scratching. Figure 3.16b shows the results obtained from the scratch assay on a cell monolayer experiment with the addition of P1-latex protein to the cell culture media. The results suggest that the P1-latex protein has a positive impact on the cell proliferation from an early stage. A significant increase on cell migration/proliferation was observed in the media supplement with 1µg P1-latex protein. In the case of the other two concentrations (0.01µg and 100µg) it was also possible to observe higher percentage of cell migration/proliferation than in the basal control (media without P1), but the results are not statistically significant within 24 hours.

Figure 3.16d shows both the protein adsorbed by the scaffolds and the protein remaining in the solution. It also shows the level of P1 measured before and after filtration (Figure 16c). As observed, there are no significant differences between the P1 level measured before and after filtration, and low amount of P1-latex protein was adsorbed by scaffolds.

Figure 3.16e shows cell viability/proliferation results, based on the fluorescence intensity, measured at different time points for all scaffolds. Similar fluorescence intensity values are observed for all scaffolds at day 7. At day 14, PCL/graphene scaffolds (0.78 wt.%) show statistically higher cell viability/proliferation rate than PCL scaffold. Results also show that P1-latex protein coated scaffolds allow higher cell proliferation, as indicated by higher fluorescence intensity. At day 21, P1-coated scaffolds show a better biological performance enhancing cell proliferation. Additionally, results show that P1-coated scaffolds containing graphene present a better biological performance compared to P1-coated PCL scaffolds.
Figure 3.16 Human ADSC monolayer migration and proliferation with P1 assay. (a) Images presenting the negative control group (basal media + DMSO), basal media as control group and basal media with 1µg P1. (b) Percentage of cell migration/proliferation related to the basal media control group. (c) P1 protein concentration before and after filtration previously to scaffold adsorption. (d) Amount of P1 protein adsorbed by the scaffolds and remaining in the solution. (e) Alamar Blue assay. Results are expressed according to fluorescence intensity performed after 7, 14 and 21 days of cell seeding. Scaffolds uncoated with P1 protein and scaffolds coated with P1 protein were considered. Data are graphically reported as mean value and the standard deviation. Statistical analysis was performed by two-way ANOVA followed by post hoc Bonferroni test (p <0.05).
3.3.3.4 Osteogenic differentiation

Figure 3.17 shows the ALP activity after 7, 14 and 21 days (Figure 3.17a, 3.17b, 3.17c, respectively). After 7 days, P1-coated scaffolds cultivated in osteogenic media show high ALP activity, statistically different from the other 3 groups (scaffold in osteogenic media; Scaffold + P1 in basal media; scaffold in basal media). After 14 days, P1-coated scaffolds cultivated in osteogenic media still presented statistically higher ALP than the other groups. Uncoated scaffolds cultivated in osteogenic media presented higher ALP than the other 2 groups, both of them cultivated in basal media. Similar results are observed after 21 days in all groups, with P1-coated scaffolds presenting increased values when compared to the others. Contrary to proliferation, the addition of small graphene contents seems to have no effect on cell differentiation.
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Figure 3.17 Alkaline phosphatase activity measurement. (a) after 7 days, (b) after 14 days, (c) after 21 days. Statistical analysis was performed by one-way ANOVA followed by *post hoc* Tukey test. * Statistical analysis difference (p<0.05) among Scaffold + P1 in osteogenic media group and all other groups; # statistical analysis difference (p<0.05) between scaffold in osteogenic media group and basal media groups.
3.3.3.5 Cell morphology and cell spreading

Figures 3.18a and 3.18b show cell seeded on the scaffolds at different regions. As observed, cells were able to grow forming colonies and spreading around. Cells are well-spread and long extensions are observed, with cells bridging from one filament to the other. Confocal images (Figure 3.18c) show that spindle-shaped cell morphology is maintained (nuclei stained blue, cell membrane red).

SEM images (Figure 3.18d) and confocal microscopy images (Figure 3.18e) were also acquired from scaffolds coated with P1-latex protein cultivated in osteogenic media after 28 days. As observed, hADSCs were able to undergo the differentiation process induced by the osteogenic media. It is also possible to identify a fibrillary extracellular matrix network formed by calcium deposition. The confocal images also showed a significant spread of cells on scaffolds after osteogenic differentiation, with cells showing a rounded-shape rather than spindle-shaped with long extension.

Figure 3.18  Cell morphology/attachment and cell spreading on neat PCL and PCL/graphene scaffolds. (a)-(b) Scanning electron microscopy images at different magnifications. (c) Laser confocal microscopy images. (d) SEM and (e) laser confocal microscopy images of cell osteogenic differentiation on PCL/graphene scaffolds after 28 days. Scaffolds coated with P1 protein.
3.3.3 Discussion

As observed, the screw-assisted extrusion-based system operating with optimised process parameters was able to produce scaffolds with uniform distribution of graphene nanosheets, and geometric characteristics similar to the initial designed ones. Moreover, the low intensity ratio of D to G band (ID/IG) and strong 2D intensity peaks suggest that the prepared graphene has high crystallinity and low defects [376].

As a strong material, the addition of even small graphene levels to PCL scaffolds improved mechanical properties, with scaffolds containing 0.78 wt.% presenting a compressive modulus close to the cortical bone. Besides improving the mechanical properties of printed scaffolds the multifunctional nature of graphene substrates influence the behaviour of different cell types, mainly due to its nano-topographic features, which provides an appropriate surface for cells attaching. As the surface properties of graphene can be controlled, this 2D material offers opportunities for cellular stimulation to maximize the desired biological response, as proposed by Dubey et al. and Goenka et al [377, 378]. Preliminary results suggest that the addition of graphene increases protein adsorption [89, 90, 379].

The results presented in this paper show low adsorption profiles of P1-latex protein for both PCL and PCL/graphene scaffolds. This can be explained by the hydrophobic nature of PCL, the relatively low concentration of graphene and the high concentration of P1-latex protein in the initial solution. Nevertheless, the results show that the use of P1-latex protein has a positive impact on both cell proliferation and cell differentiation. P1-latex coated scaffolds containing graphene presented higher cell proliferation, which is in accordance with previous studies on the biological impact of graphene [380]. The results suggest that P1-latex protein released from the scaffolds was able to directly stimulate cell proliferation along the 3D fibres of the scaffolds, displaying growth factor-like behaviour. Moreover, this protein also enhances the osteogenic differentiation, as observed by measuring the ALP activity.

3.3.4 Conclusions

This study demonstrates for the first time the successful combination of additive manufacturing and protein-coating processes to produce PCL/graphene/P1-latex protein for bone regeneration with adequate mechanical properties and improved biological performance, promoting hADSCs adhesion, proliferation and differentiation. Graphene nanosheets are well distributed in the PCL scaffold fibres, confirming that screw-assisted
extrusion additive manufacturing is a viable technique to produce scaffolds with geometric characteristics (filament diameter and pore size) close to the designed ones. Results show that the presence of low levels of graphene (maximum 0.78 wt.%) improves cell attachment and proliferation but has no significant impact on cell differentiation. Moreover, results from coated scaffolds compared to uncoated ones show that the P1-latex protein has a positive impact on the cell biological behaviour, enhancing both cell proliferation and osteogenic differentiation. The results suggest that the scaffolds investigated in this paper can be used for bone regeneration.
3.4 Assessment of PCL/Carbon Material Scaffolds for Bone Regeneration*

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**Contribution to the work:** As first author, W.W. conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

* Scientific reports, submitted.
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### 3.4.1 Introduction

Bone defects due to chronic health conditions and fractures are significantly growing associated to population age. This problem is worse if we consider also significant bone defects due to accidents and diseases. To solve large-scale bone problems, bone grafting is usually required [311, 312]. However, natural bone grafts (allografts, autografts and xenografts), the most common clinical approach, present several problems related to site morbidity, disease transmission, accessibility and costs. To solve these limitations, synthetic grafts (scaffolds) represent a promising approach for tissue engineering [6, 314, 357]. These scaffolds are made with biocompatible and biodegradable polymers, ceramics and composites and they must provide an adequate 3D environment for cell attachment, proliferation and differentiation [1, 88, 380].

As cell proliferation and differentiation is a regulated process, which depends on scaffold topography and material cues and also on specific stimulation mechanisms (load or electromagnetic effects), our group is exploring the use of electro-active scaffolds for bone regeneration. To produce these scaffolds, different routes are being investigated including the use of conductive polymers mixed with non-conductive ones, or the use of carbon materials mixed with non-conductive polymers. Developed in 1991 by Lijima [381], carbon nanotubes (CNTs), have been widely used in electronics, reinforcement fillers for composites materials, photovoltaics and energy storage applications due to their extraordinary electrical and mechanical properties [382-385]. In the biomedical field, the use of CNTs is becoming also highly relevant mainly for the development of biosensors, bioimaging applications or as nanocarrieres [386-388]. Its mechanism of cellular internalization comprises active and passive diffusion mechanisms. The active pathways of CNTs through cellular membranes are mainly by endocytosis, through which the CNTs are internalized inside vesicles (endosomes) prior being directly transported to the lysosomes. Passive diffusion is an energy-independent process that starts through the contact between CNTs and cell membranes and the process follows by a sequence of landing on the membrane surface, penetrating into the lipid head group of phospholipid bilayer membrane and eventually sliding through the lipid tails. CNTs are expelled from cells through exocytosis after several hours or days of internalization or through enzymatic degradation[389, 390]. In general, CNTs-induced cytotoxicity depends on the physical-chemical characteristics of CNTs including the length, diameter, surface area and tendency of agglomeration [391].
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Graphene nanosheets share a similar hexagonal structure with CNTs, but present better conductive properties [392-394]. Moreover, graphene has lower aspect ratio, larger surface area and better dispersibility in most solvents compared to CNTs. Due to its 2D nature, graphene nanosheets present very complex biological characteristics, including beneficial ones such as facilitating intracellular transport of therapeutic agents and providing antimicrobial protection; and also negative effects such as membrane damage, fibrosis and accumulation [395, 396]. For tissue engineering applications, graphene and CNTs are usually with biocompatible and biodegradable polymers. Vicentini *et al.* used functionalized graphene, CNTs and nanohorns as fillers to produce poly(L-lactic acid) composite films through solvent evaporation [397]. The levels of inorganic materials varied among 0.1, 0.25, 0.5, 1 and 5 wt.% with respect to the polymer content. Results showed that all composite films were biocompatible and able to support neuron cell proliferation. However, the results cannot be considered conclusive as cell death and proliferation were assessed only 24 hours and 72 hours after cell seeding. Liu *et al.* incorporated graphene oxide and CNTs into oligo(polyethylene glycol fumarate) hydrogel through a crosslinking process, creating membranes for nerve applications [398]. Previously, graphene oxide and CNTs were covalently functionalised with double bonds to obtain crosslinkable graphene oxide acrylate and CNT poly(ethylene glycol) acrylate. Results showed that all membranes were biocompatible and able to support PC12 cell attachment and proliferation. Oyefusi *et al.* investigated the biological nature of carboxylated CNTs and graphene nanosheets grafted with hydroxyapatite (HA) [399]. Results showed that human fetal osteoblastic cells (hFOB 1.19) were able to proliferate and differentiate in culture media containing graphene-HA and CNT-HA. Although numerous studies have been reported on the use of graphene and CNT combined with other biocompatible materials as substrates for cell attachment, proliferation and differentiation, these studies focused on the use of films and membranes which are not able to emulate the complex *in vivo* environment. Moreover, a comparison of the effects of both graphene and CNTs is still limited [400]. This paper reports for the first time the use of additive manufacturing (3D Printing) to produce 3D polymeric scaffolds with controlled architecture loaded with different levels of graphene and CNTs. Scaffolds are extensively studied based on material characteristics and the interactions with biological components (cells and proteins) discussed.
3.4.2 Results and Discussion

3.4.2.1 Thermogravimetric analysis

The final content of carbon material in the PCL/carbon material composite scaffolds was obtained by thermogravimetric analysis (TGA). The results, presented in Table 3.6, suggest that the inorganic materials were effectively incorporated into the polymer scaffolds, without significant losses during the material preparation and scaffold fabrication process. Results also indicate that no degradation events occur during the scaffold fabrication as the extrusion temperature was 90 °C.

Table 3.6 Carbon material loading verification, assessed by TGA.

<table>
<thead>
<tr>
<th>Target carbon material loading</th>
<th>Determined carbon material concentration (wt.%)</th>
<th>$T_d$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 wt.%</td>
<td>-</td>
<td>559.6</td>
</tr>
<tr>
<td>1 wt.% (graphene)</td>
<td>1.05 ± 0.12</td>
<td>559.7</td>
</tr>
<tr>
<td>1 wt.% (CNT)</td>
<td>1.03 ± 0.54</td>
<td>559.7</td>
</tr>
<tr>
<td>3 wt.% (graphene)</td>
<td>3.08 ± 0.12</td>
<td>559.7</td>
</tr>
<tr>
<td>3 wt.% (CNT)</td>
<td>3.04% ± 0.62</td>
<td>559.8</td>
</tr>
</tbody>
</table>

3.4.2.2 Mechanical characterisation

Results of the mechanical compression tests (Figure 3.19) show that the incorporation of both graphene and CNT significantly enhanced the mechanical properties of PCL scaffolds. By increasing the concentration of graphene, the compressive modulus increased from 37.88±1.24 MPa (PCL scaffolds) to 44.81±1.42 MPa (1 wt.% PCL/graphene scaffolds) and 48.81±1.03 MPa (3 wt.% PCL/graphene scaffolds), while the increase of CNT concentration increases the compressive modulus to 42.73±1.07 MPa (1 wt.% PCL/CNT scaffolds) and 45.47±1.12 MPa (3 wt.% PCL/CNT scaffolds). Results show that for the same concentration of inorganic material PCL/graphene scaffolds present around 10% higher compressive modulus. Similar results were recently observed by Li and co-workers through an extensive study on the mechanical properties of PMMA composites reinforced by CNTs and graphene nanosheets [401]. This can be attributed to the larger interaction area between graphene and PCL compared to CNTs and PCL and consequently higher surface adsorption forces provided by the graphene reinforcement to resist the external loads. During the scaffold deformation, the relative movements between the inorganic
reinforcements and the polymer matrix generates internal shear stresses, higher in the case of graphene due to its 2D nature, that increases the resistibility to the external deformation [401]. Results of mechanical analysis indicate that the produced scaffolds can provide sufficient mechanical strength compared to native cancellous bone (2-12 MPa) [375, 402].

Figure 3.19 Mechanical characterisation (compressive modulus) of PCL, PCL/CNTs and PCL/graphene scaffolds. The values were compared using one-way ANOVA, followed by post hoc Tukey’s test (significance levels were set at * p<0.05).

3.4.2.3 Apparent water-in-air contact angle

The apparent water-in-air contact angle of printed scaffolds is given in Figure 3.20. Contact angle represents the wettability of the material surface, indicating hydrophilic/hydrophobic characteristics of the material. In general, a contact angle above 90° corresponds to a hydrophobic surface, while a contact angle value below 90° represents a hydrophilic surface. Results show that by adding graphene nanosheets, the contact angle values slightly decreased from 92.62±0.24° (PCL) to 88.93±0.76° (1 wt.% PCL/graphene scaffolds) and 85.10±0.89° (3 wt.% PCL/graphene scaffolds). In the case of adding CNT, the contact angle values decreased to 89.99±0.44° (1 wt.% PCL/CNT scaffolds) and 86.18±1.25° (3 wt.% PCL/CNT scaffolds) respectively. As observed PCL/graphene presents slightly lower contact angle values compared with PCL/CNT but the results are not significant. In both cases the addition of the inorganic material decreased the contact angle of PCL scaffolds. Contrary to the common assumption that graphene, as other carbon-based materials, is hydrophobic, these results are in line with recent
observations from Munz and co-workers [30]. Those researchers investigated the adhesion and friction properties of single-layer and double-layer graphene using chemical force microscopy with a hydrophobic probe. Results showed a large adhesion force between the probe and double-layer graphene compared to single-layer, showing that double-layer graphene is ideal for hydrophobic applications and single-layer graphene for applications where a hydrophilic surface is required. CNTs, sharing the same graphitic structure of graphene, present a similar effect. However, the 2D nature of graphene and increased surface area on the scaffold fibre can partially explain the differences between PCL/CNT and PCL/graphene.

![Figure 3.20](image_url) Apparent water-in-air contact angle values for scaffolds containing different concentrations of inorganic materials. The values were compared using one-way ANOVA, followed by post hoc Tukey’s test (significance levels were set at * p<0.05).

### 3.4.2.4 Protein adsorption

Protein adsorption on PCL and PCL/graphene scaffolds was studied through BCA assay, and the results are presented in Figure 3.21. All scaffolds were able to adsorb protein as the adsorption time increases. Among all types of scaffolds, PCL/graphene (1 and 3 wt.%) and PCL/CNT (3 wt.%) scaffolds show higher protein adsorption compared to the other scaffolds after 6 hours of incubation. After 12 hours of incubation, PCL/graphene (3 wt.%) scaffolds showed higher protein adsorption compared to all other scaffolds. This might be attributed to the non-covalent interactions between the basic residuals of proteins and the graphitic structure presented on the graphene and CNT surfaces, where aromatic residuals significantly contribute to protein adsorption due to the strong π-π stacking interactions.
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[339]. However, due to its unique 2D structure graphene present larger interaction area than CNT, thus leading to higher protein binding to scaffolds containing graphene nanosheets.

Figure 3.21 Protein adsorption values on all scaffolds after 6 and 12 hours. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05).

3.4.2.5 Biological assessment

Cell viability/proliferation results are presented in Figure 3.22. Results show that for all types of scaffolds, the fluorescence intensity increases from one time point to another, suggesting that the produced scaffolds are suitable substrates for cell proliferation. At both day 1 and day 3, only PCL/graphene scaffolds showed statistically higher fluorescence intensity compared to PCL scaffolds, indicating higher cell affinity, while no statistically significant differences were observed between PCL/CNT scaffolds and PCL scaffold. However, from day 7, all scaffolds containing both graphene and CNTs present statistically higher cell affinity and cell proliferation values compared to PCL scaffolds. These results indicate that the addition of carbon materials into the polymer matrix promotes hADSCs proliferation and for the same content on carbon material, PCL/graphene scaffolds present faster cell proliferation rates compared to PCL/CNT scaffolds. This might be correlated to both higher protein adsorption and the stiff nature of graphene scaffolds. Additionally, the presence of conductive materials at the surface of the scaffolds facilitates cell-cell signalling.
Figure 3.22 Cell viability/proliferation results (fluorescence intensity) of printed scaffolds at day 1, 3, 7 and 14 of cell culture. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05).

Scanning electron microscopy (SEM) images (Figure 3.23a and 3.23b) and confocal microscopy images (Figure 3.23c and 3.23d) present cell attachment and distribution after 14 days of proliferation. It is possible to observed that all scaffolds are able to support cell proliferation along the fibre direction. Extensive cell attachment and spreading can also be observed, with confluent cell sheets bridging through scaffold fibres. These images also show that PCL/graphene and PCL/CNT scaffolds present better confluence and cell distribution than PCL scaffolds.
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Figure 3.23 (a)-(b) SEM images at 65x and 230x magnification, (c)-(d) confocal microscopy images at 100x and 200x magnification of PCL, PCL/graphene (3 wt.%) and PCL/CNT (3 wt.%) cell seeded scaffolds, 14 days after cell proliferation.

Figure 3.24 shows the ALP activity after 14 and 21 days. After 14 days, PCL/graphene (3 wt.%) scaffolds cultivated in osteogenic media show high ALP activity, statistically different from the other groups. After 21 days, all PCL/graphene scaffolds show statistically higher ALP activity than the other groups (PCL and PCL/CNT scaffolds), with better results being observed for PCL scaffolds loaded with 3 wt.% of graphene.
Figure 3.25 shows the calcium deposition on hADSC seeded scaffolds cultured in osteogenic media for 21 days, which is a typical consequence of the osteogenesis process. Results show statistically significantly higher values for PCL/graphene scaffolds and particularly for PCL scaffolds loaded with 3 wt.% of graphene. As observed, graphene strongly stimulates cell proliferation and differentiation, by enhancing cell adhesion and preventing implanted cells from reactive oxygen species (ROS)-mediated cell death [318, 379]. The extraordinary electrical properties of graphene nanosheets also enhance the transmission of cell signals, promoting cell-cell communication and cellular activity. The 2D nature of graphene presenting large surface area contributes to this effect. SEM images (Figure 3.26a, 3.26b and 3.26c) and confocal microscopy images (Figure 3.26d and 3.26e) were also acquired from hADSC seeded scaffolds. It is also possible to observe a fibrillary extracellular matrix network formed by calcium deposition. Moreover, confocal images also showed a significant spread of cells on scaffolds after osteogenic differentiation, with cells showing a rounded-shape rather than spindle-shaped with long extensions.

![Graph showing Alkaline phosphatase activity](image)

**Figure 3.24** Alkaline phosphatase activity measurement after 14 and 21 days. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05).
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Figure 3.25 Calcium deposition measurement after 21 days of cell differentiation. The values were compared using one-way ANOVA, followed by *post hoc* Tukey’s test (significance levels were set at * p<0.05).
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Figure 3.26 (a)-(c) SEM images at 65x, 230x and 1500x magnification, (d)-(e) confocal microscopy images at 100x and 200x magnification of PCL, PCL/graphene (3 wt.%) and PCL/CNT (3 wt.%) cell seeded scaffolds, after 21 days of cell differentiation.
3.4.3 Materials and Methods

3.4.3.1 Materials

3.4.3.1.1 PCL

PCL, CapaTM 6500, a high molecular weight (≈50 KDa) polyester derived from caprolactone monomer, was purchased from Perstorp (Warrington, UK), in the form of 3 mm pellets. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm³, a melting temperature between 58-60 °C, and a glass transition temperature of -60 °C.

3.4.3.1.2 Graphene

Graphene nanosheets (3-4 layers, ~500 µm surface diameter) were prepared via water-assisted liquid phase exfoliation of graphite. Briefly, 50 mg microcrystalline graphite powder (325 mesh, 99.995% pure, purchased from Alfa Aesar, UK) was immersed in N-methyl-2-pyrrolidone (NMP) mixture with a 0.2 mass fraction of water. The initial concentration of graphite was fixed at 5 mg mL⁻¹ for exfoliation. NMP, 99% extra pure was purchased from Acros Organics (USA). The materials were batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H, Switzerland) at a fixed nominal power and frequency of 100 W and 37 kHz respectively. Sample dispersions were hanged on for overnight in between sonication and centrifugation and were centrifuged at 3000 rpm for 30 minutes using a Hettich EBA20 centrifuge (Germany). The upper 75% of the colloidal supernatant were collected and dried in an oven to yield the graphene nanosheets.

3.4.3.1.3 Carbon nanotube

Multiwall carbon nanotubes (MWCNTs) (approximate dimensions of 20 nm in diameter and 1-8 µm in length) were synthesized using a catalytic chemical vapour deposition (CVD). In brief, Fe films (3.0 nm thickness) and an alumina (Al₂O₃) support layer (40 nm thickness) sputtered onto 1 cm × 1 cm polished silicon (Si) substrates with a silicon dioxide (SiO₂) layer of 600 nm were used as the catalyst films for CNT growth. The CNT were synthesized at one atmospheric pressure in a 3 inch quartz tube furnace with two process steps, including catalyst particle formation and CNT growth. For catalyst particle formation, 200 sccm (standard cubic centimetre per minute at 1 atm) helium (He) and 1800 sccm hydrogen (H₂) were flowed for 15 minutes while ramping the temperature from room temperature to 810 °C, then keep the same gas flow rates for 15 minutes to anneal the catalyst particles. Then the CNT growth began for 10 minutes using a water-assisted CVD process at 810 °C with the gas mixture of 100 sccm ethylene (C₂H₄) and 900 sccm H₂, and
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100 ppm water vapour as the carbon precursor and the catalyst preserver and enhancer, respectively. Water vapour of 100 ppm was supplied by passing 1000 sccm He carrier gas through a water bubbler with deionized (DI) water at standard conditions for temperature and pressure (temperature of 0 °C and absolute pressure of 100 kPa) condition. Water vapour concentration was monitored by a single-channel moisture meter (MMS 35-211-1-100, General Electric, USA) coupled with a moisture probe (M2LR, General Electric, USA) installed before the CVD reactor. All gas flows were controlled by mass flow controllers that were carefully calibrated before experiments to precisely control the gas concentrations in the CVD reactor.

3.4.3.1.4 Scaffold Fabrication

PCL/carbon pellets were prepared by melt blending in two different concentrations (1 and 3 wt. %). Briefly, pure PCL pellets were heated above 70 °C in a bowl to ensure all material is in a molten state prior to carbon nanomaterial addition, at the desired concentrations. The materials were mixed for 15 minutes to guarantee a homogenously dispersion. After cooling down for 2 hours, the blended material was cut into small pellets before scaffold fabrication.

A screw-assisted additive biomanufacturing system, 3D Discovery (RegenHU, Villaz-St-Pierre, Switzerland), was used for scaffold fabrication. This system allows high reproducibility and good control over scaffold topology (including pore size, pore shape, and pore distribution), which are critical parameters when designing optimised 3D interconnected porous scaffolds. The 0°/90° lay-down pattern was adopted to obtain pores with a regular square geometry and a constant filament distance of 680 μm was considered. The optimal combination of processing parameters—melting temperature (90 °C), slice thickness (220 μm), screw rotation velocity (12 rpm), and deposition velocity (20 mm/s)—allows the filament diameter after extrusion to be close to the desired diameter of 330 μm After fabrication, scaffold samples were cut with fine, double-edged razor blades into small blocks (~11 mm × 11 mm × 2.8 mm) to fit in a 24-well culture plate.

3.4.3.2 Thermogravimetric analysis

The thermal degradation and carbon nanomaterial content in the scaffolds was assessed using a TA Instruments Q500 thermogravimetric analyzer (New Castle, DE, USA) equipped with an evolved gas analysis furnace. Thermogravimetric analysis was performed on PCL scaffolds as controls, and carbon nanomaterial loaded PCL scaffolds. Scans were performed in an air atmosphere (flow at 60 mL/min) with a temperature range from room
temperature to 560°C at a rate of 10°C/min. Measurements were taken using sample mass of 6 ± 1 mg in platinum pans. The weight losses of the PCL/carbon material composite structures were monitored and used to calculate the final carbon material contents.

3.4.3.3 Mechanical characterisation

Mechanical compression tests were performed to assess the effect of different graphene concentrations on the mechanical properties of the scaffolds. Blocks of 5.0 mm in length, 5.0 mm in width and 5 mm in height (h₀) were considered. Tests were carried out on scaffolds in dry state at a rate of 1 mm/min up to a strain limit of 0.3 mm/mm (30%), using the INSTRON 3344 testing system (Norwood, MA, USA) equipped with a 500N load cell. During the uni-axial compression tests, the software determined force F and corresponding displacement values, which were converted into engineering stress σ and strain ε as follows:

\[
\sigma = \frac{F}{A}
\]

\[
\varepsilon = \frac{\Delta h}{h_0}
\]

where A is the initial sample cross section area and Δh is the scaffold height variation. The obtained stress-strain data was further processed to determine the compressive modulus, E, according to the procedure reported by Fiedler et al [7].

3.4.3.4 Apparent water-in-air contact angle

Static contact angle measurements were performed on produced scaffolds using an OCA 15 contact angle measuring machine (Data Physics, San Jose, CA, USA) using the sessile drop method. Deionised water droplets of ~4 µL were deposited via a motorised syringe at a velocity of 1 µL/s. Five measurements per sample type were performed. The drop shape was recorded with a high speed framing camera. Measurements were performed immediately after droplet addition.

3.4.3.5 Protein adsorption

Protein adsorption was performed using fetal bovine serum (FBS) as a model protein system and a bicinchoninic acid assay (Micro BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA). Briefly, scaffolds were cut into 5mm x 5mm x 5mm cubic blocks (n=3 for each group) and placed into a 48 well plate. 500 µl of 10% FBS solution was added into each well and kept in the incubator at 37 °C. After 6 and 12 hours, the samples were gently washed with PBS to remove excess and unattached protein and transferred to new wells to ensure that proteins attached to the walls and to the bottom of the plate were
not measured. The amount of protein present was quantified using the BCA kit following the manufacturer’s protocol. 500 μl working reagent was added to the samples as well as into a series of bovine serum albumin (BSA) standards, and incubated at 37 °C for 2 hours. Before measuring absorbance, the supernatant was pipetted up and down to ensure complete mixing of the reagents and transferred to a 96 well plate in order to remove any interference from the scaffold. The analysis was performed using a TECAN Infinite 200 plate reader (Tecan, Männedorf, Zurich, Switzerland) at an absorbance of 562 nm. The amount of protein adsorbed was calculated based on a standard curve generated by a dilution series of BSA standards [403, 404].

3.4.3.6 In vitro biological assessment

In vitro cell culture studies were conducted using human adipose-derived stem cells (STEMPRO®, Invitrogen, Waltham, MA, USA) ranging from passage 3 to 5. Cells were cultured in T75 tissue culture flasks (Sigma-Aldrich, Dorset, UK) with MesenPRO RS™ Basal Media (Invitrogen, Waltham, MA, USA) until 80% confluence and harvested by the use of a 0.05% trypsin solution (Invitrogen, Waltham, MA, USA).

3.4.3.6.1 Cell Seeding

Scaffolds were sterilised in 70% ethanol for 1 hour, rinsed in phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK) three times, placed in 24-well plates, and air-dried for 24 hours in an incubator at 37 °C. The scaffolds were wet with 200 μL of media containing 10% foetal bovine serum (FBS) and kept in standard conditions (37 °C under 5% CO₂ and 95% humidity) for 2 hours prior to cell seeding. Cells were seeded on each scaffold (150 μL of medium containing around 5 × 10⁴ cells). The cell-seeded scaffolds were incubated at standard conditions (37 °C under 5% CO₂ and 95% humidity) for 2 hours to allow cell attachment, before the addition of 550 μL of fresh basal media. For cell viability/proliferation test, basal media is changed every 3 days during the cell culture. For cell differentiation test, after the first three days allowing cells proliferation with basal media, media is changed to osteogenesis media (StemPro Osteocyte/Chondrocyte Differentiation Basal Media, Thermo Fisher Scientific, Waltham, MA, USA) to allow cell differentiation.

3.4.3.6.2 Cell viability/proliferation

In order to study the cell viability/proliferation and the percentage of cells attached in the scaffolds (cell-seeding efficiency), the Alamar Blue assay (also termed the Resazurin assay) was used (reagents from Sigma-Aldrich, Dorset, UK) [35,36]. Briefly, cell
viability/proliferation was measured at 1, 3, 7, and 14 days after cell seeding to scaffolds. At each testing time point, before the Alamar Blue test, the cell-seeded scaffolds were moved to a new 24-well plate and 0.7 mL of the Alamar Blue solution (0.001 wt.%) was added to each well and the control. The plates were incubated for 4 hours under standard conditions. After incubation, 150 µL of each sample was transferred to a 96-well plate, and the fluorescence intensity was measured at 540 nm excitation wavelength and 590 nm emission wavelength with TECAN Infinite 200 plate reader.

3.4.3.6.3 Cell differentiation

In order to determine the osteogenic differentiation of hADSCs cultured in the scaffolds, alkaline phosphatase (ALP) enzyme activity was measured at day 14 and 21 using a colorimetric assay (SensoLYTE® pNPP Alkaline Phosphatase Assay Kit, AnaSpec, Fremont, CA, USA), according to the manufacturer’s guide. At each time point, scaffolds with hADSCs were rinsed twice with PBS to remove osteogenesis media, followed by washing twice with 1x alkaline phosphatase dilution assay buffer. The scaffolds were then transferred from 24-well plates into centrifuge 50 mL tubes, adding 1mL 1x assay buffer containing 0.2% (v/v%) Triton X-100 to each tube. To collect the cell lysates, each sample were vortex for 30 seconds and sonicated for 1 minute twice. Samples were then stored under -80°C for 20 minutes using ice crystals to destroy the cell membrane. This process was also operated twice. Afterwards, all samples were centrifuged using 2500xg for 10 minutes at 4°C. After dephosphorylation of p-nitrophenyl phosphate by ALP enzyme for 1 hour at room temperature, the supernatants were collected and detected at absorbance of 405 nm using a microplate reader. The concentration of ALP was calculated based on a standard curve and normalised to the total protein concentration which was determined by using the bicinchoninic acid assay (BCA) (Micro BCA Protein Assay Kit).

In order to determine the mineralization of hADSCs cultured in the scaffolds after 21 days of differentiation, samples were analysed by Alizarin red-S (ARS) (Sigma-Aldrich, Dorset, UK). All samples were rinsed with PBS twice to remove the osteogenic media, followed by immersing in 10% neutral formaldehyde solution (Sigma-Aldrich, Dorset, UK) for 15 minutes. The scaffolds were then washed with deionised water three times and added in 0.2 % ARS staining dye for 40 minutes to bind the calcium salts. After wash with deionised water 5 times to remove residual ARS staining, the scaffolds were transferred to 15 mL centrifuge tubes, added into 800 µL of 10 % acetic acid solution for 30 minutes with gently shaking, and then heated up to 60 °C for 10 minutes. All samples were centrifuged for 15 minutes after stored in ice for 5 minutes. The supernatants were
eventually detected by microplate reader at absorbance of 405nm. The final absorbance was obtained by subtracting absorbance of scaffolds without cell culture.

Osteogenic differentiation tests were performed three times in triplicate. ALP and ARS are reported as mean value ± standard deviation.

3.4.3.6.4 Bioimaging

Scaffolds used in the cell viability/proliferation study were kept in culture up to 21 days to assess cell morphology and qualitative attachment to the scaffolds via scanning electron microscopy (SEM). For SEM preparation, scaffolds were fixed with a 3% glutaraldehyde solution (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature, rinsed twice with PBS, dehydrated with a graded ethanol series (50%, 70%, 80%, 90%, and 100% (twice)), in 50:50 ethanol/hexamethyldisilazane (HMDS, Sigma-Aldrich, Dorset, UK) and then in 100% HMDS (with 10 minutes exposure at each step), and then air dried for HMDS removal [11]. Thin cross-section layers of each sample (around 1 mm) were cut and platinum-coated for imaging using a Gatan Model 682 Precision Etching Coating System, to an approximate thickness of 7 nm. SEM images were obtained using a Hitachi S300N microscope (Hitachi, Maidenhead, UK).

Cell morphology was further assessed using laser confocal microscopy with scaffolds cultured up to 28 days, with cell membranes and nuclei stained. Samples were removed from the cell culture plate, rinsed twice in PBS, fixed using 4% paraformaldehyde for 40 minutes, and then washed twice with PBS prior to immersion for 30 minutes in an immunocytochemistry blocking buffer comprised of 2% goat serum and 1% bovine serum albumin in PBS. Samples were again rinsed twice in PBS. Cell nuclei were stained blue by soaking them in a PBS solution containing 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) at the manufacturer recommended concentration; cell actin stained using Alexa Fluor™ 488 Phalloidin (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) diluted to the manufacturer recommended level. Samples were left in the staining solution for 10 minutes prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

3.4.3.7 Statistical Analysis

All experimental data are represented as mean ± standard deviation (SD). Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (one-way ANOVA) with post hoc Tukey’s test and
Two-way analysis of variance (two-way ANOVA) with post hoc Bonferroni’s test were applied. Significance levels were set at $p < 0.05$.

3.4.4 Conclusions

This is the first paper investigating the use of additive manufacturing technique to produce polymeric scaffolds incorporating carbon nanomaterials, and the assessment on their chemical, physical and in vitro biological properties. PCL/graphene and PCL/CNT blends were efficiently mixed with a melt blending process and processed using a screw-assisted extrusion-based additive manufacturing system. Both graphene and CNTs enhance mechanical properties, reduce surface contact angle reducing the hydrophobic nature of printed scaffolds, and increase protein adsorption. For the same amount of carbon nanomaterials, graphene scaffolds present better chemical and physical properties than CNT scaffolds. Results also suggest that graphene scaffolds present high cell affinity, improving cell attachment, proliferation and differentiation.
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3.5 A Plasma-assisted Bioextrusion System for Tissue Engineering*

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**Contribution to the work:** As co-author, W.W. conceived and designed the experiments; performed wettability, mechanical and biological assessments, analysed corresponding data, prepared figures 3.31, wrote the main manuscript, and responsible for manuscript reviewing.


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3.5.1 Introduction

Tissue engineering is promising for organ replacement as it minimizes the side effects of organ transplantation. Biomanufacturing is one of the key stages in tissue engineering as it is concerned with the development of biological substitutes that restore, maintain, or improve tissue function. Biomanufacturing requires the combined use of additive manufacturing (AM), biocompatible and biodegradable materials, cells and biomolecular signals [361]. AM techniques are commonly applied in scaffold fabrication due to their superior ability in controlling pore size, shape and distribution, thus creating interconnected porous structures [312, 357]. However, cell-seeding and proliferation efficiency are currently big challenges due to the following limitations [405-407].

(i) Most AM techniques are limited to single-material fabrication, which makes it difficult to provide an appropriate environment for cells due to inadequate chemical, physical and biological cues provided during the AM process.

(ii) Non-uniform cell distribution, with rare cell adhesion in the core region of scaffolds, often caused by tortuosity of the constructs.

(iii) Limited cell colonization due to the hydrophobicity of the most commonly used synthetic biopolymers.

Different strategies have been explored to overcome the above limitations. Different materials have been developed and utilized to produce multiple-material scaffolds [359, 408, 409]. However, most of these systems can only fabricate the scaffolds from one type of biomaterial, either soft hydrogels containing cells or bio-signals (the average Young’s modulus is a few kPa), or rigid biopolymers and composites (the average Young’s modulus is a few hundred MPa); the disadvantage with the latter is that it fails to mimic natural tissues. Increasing the hydrophilicity of polymer scaffold to achieve a better cell attachment rate can be achieved by different chemical surface treatment methods. One of them uses a simple sodium hydroxide treatment, rendering the scaffolds more hydrophilic [380]. A low-temperature plasma modification is also capable of improving the hydrophilicity of biopolymers by inducing certain functional groups on the surface to change the chemical signalling, wettability and energy without altering the bulk properties [410-413]. These researchers conducted the plasma treatment after the scaffolds were printed, thus limiting the penetration depth, which subsequently resulted in non-uniform cell distribution along the scaffold.
This paper introduces a novel plasma-assisted bioextrusion system (PABS), which represents an advancement in the manufacture of functional-gradient scaffolds, as it not only permits allowing the processing of different biomaterials but also makes in-process plasma surface modification possible. The system can print a structure and deposit amine groups layer by layer within the scaffold with Nitrogen-based plasma modification.

Water contact angle (WCA) is used to evaluate the hydrophilicity changes in PCL scaffolds after a N$_2$ plasma treatment. In vitro adhesion and proliferation of Human Adipose Derived Stem Cells (hADSC) on treated and untreated PCL scaffolds are also assessed by means of fluorescence microscopy.

### 3.5.2 Materials and Methods

#### 3.5.2.1 Materials

PCL (CAPA™6500, Mw = 50,000 g/mol) is a semi-crystalline polymer with a density of 1.1 g/cm$^3$, a melting temperature between 58-60 °C and a glass transition temperature of -60 °C. This polymer was purchased, in the form of 3 mm pellets, from Perstorp (Cheshire, United Kingdom) and used in this project.

#### 3.5.2.2 Experimental rig

The experimental rig (Figure 3.27(a)) described in Refs. [414, 415] has been further developed to include the plasma modification unit which is described in detail below. For the sake of completeness, some of the more important parts of the rig are also briefly mentioned below.

(i) The rig consists of a multi-extrusion unit consisting of three heads, two pressure-assisted and one screw-assisted. The extrusion unit has four movements, one rotational movement (C1) for indexing the extrusion heads, a second rotational movement for controlling the screw rotational speed of the screw-assisted extruder (C2) and two movements (X and Y axes) in the X-Y plane; all four movements are controlled by CNC drives.
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Figure 3.27 (a) PABS consisting of a control system, multi-extrusion heads, and plasma modification unit; (b) plasma modification unit working at atmospheric conditions: a glass tube reaction jet with three gas inlets for gas mixture and different chemical groups deposition; (c) control system diagram (d) Sequence of operations to fabricate full-layer plasma treated scaffolds.

(ii) The plasma unit is an atmospheric plasma jet and is mounted on an X-Y platform, which is co-planar with the extrusion platform. Since both the platforms share common cylindrical guide rails in the X-direction, the movement of this platform in the X-Y plane gives rise to only one additional axis (V axis), the fifth axis of the rig, with the multi-extrusion platform. A quartz capillary (outside diameter = 7 mm; inner diameter = 5 mm; length = 70 mm) with three gas inlets serves as the reaction jet. A tungsten rod (D=2 mm) and one copper film (width=10 mm) wrapped around the quartz tube serve as the high-voltage and ground electrodes, respectively. The electrode is connected to a high-voltage DC power supply (applied voltage = 10 kV; frequency = 50 kHz). The plasma is generated from the top central electrode, expanding to the surrounding air inside and outside the nozzle (Figure 3.27(b)).

(iii) The build platform moves in the Z-direction and constitutes the sixth controllable axis. Two different types of extrusion heads have been incorporated in the rig, so that scaffolds can be fabricated from materials with both low and high viscous materials such as hydrogels, biomaterials and polymer composites. As shown in the diagram in Figure 3.27(c), stepper motors rotate the extrusion unit, control the rotational speed of the extruder screw, and drive the two platforms in the X-Y plane. Because they are CNC controlled, it means that the region to be plasma treated on a layer of the scaffold, can be of any arbitrary shape; this region is defined using a user-friendly interface, and then the in-house software automatically generates a word-address program, containing G, M, etc. codes, to move the plasma modification unit over the user-defined region. In the work reported herein, for the sake of simplicity, the entire area of each layer is plasma modified. Figure 3.27(d) shows the steps to fabricate full-layer modified scaffolds; the plasma modification is carried out after
each layer of the scaffold has been printed, thereby sandwiching plasma-induced chemical membranes between printed layers of the scaffold.

Scaffolds with a cross-section of 10x10 mm and a height of 3 mm were fabricated using the single lay pattern of 0/90° and a filament distance of one mm for fabricating inter-connected structures. The plasma treatment was conducted at a pressure of 0.689 bar and a flow rate of 5 L/mm. The deposition speed of the plasma jet was 3 mm/s and each layer was subjected to the plasma treatment for one minute. The distance from the bottom of the jet to the surface of the PCL filaments was 10 mm. The other processing conditions for the scaffolds were: slice thickness = 0.5 mm; liquefier temperature = 90°C; extrusion screw rotational speed = 15 rpm; and nozzle tip size = 0.5 mm.

3.5.2.3 Morphological characterization

Scanning electron microscopy (SEM) was used to image the morphology of the scaffolds for visualization and evaluation of the surface characteristics for both plasma-treated and untreated scaffolds. The scaffolds were coated with platinum (~ 40 s sputtering) and imaged at 10 kV (Hitachi S3000N). These images were then analysed using ImageJ [416] to measure the fibre diameter and pore size, and to ascertain whether the plasma surface modification process has a physical impact on these geometrical parameters. At least 40 measurements were made each time.

3.5.2.4 Wettability measurement

WCA tests on the surfaces of untreated and plasma-treated PCL scaffolds were carried out with a commercial KSV CAM 200 system (KSV Instruments, Finland). The system is equipped with a CCD video camera and a micrometric liquid dispenser to drop 2 µL of distilled water on the surface of the scaffold. The measurements of the contact angles are automatically calculated with the instrument software. The temporal effect of plasma treated scaffolds was also assessed with WCA tests on days 1, 3, 7 and 14.

3.5.2.5 Mechanical compression test

Compression tests were conducted to determine the effect of full-layer plasma modification on the mechanical behaviour of the scaffolds. The tests were carried out in a dry state at a speed of 0.5 mm/min and a maximum strain value of 0.45, using an INSTRON 3344 testing device with a 2000N load cell.
3.5.2.6 Biological test

*In vitro* biological assessments were conducted with human adipose-derived stem cells (STEMPRO, Invitrogen, Waltham, MA, USA). Before cell seeding, the scaffolds were sterilized by soaking in 70% ethanol for 2 hours. After sterilisation, samples were rinsed twice in phosphate buffered saline (Gibco, ThermoFisher Scientific, Waltham, MA, USA), transferred to 24-well plates and air-dried for 24 hours at room temperature. 50,000 cells were seeded on each sample, including plasma-treated and untreated scaffolds.

Cell viability/proliferation behaviour and the percentage of cells attached to the scaffolds (cell-seeding efficiency) were assessed through Alamar Blue assay (also referred to as Resazurin assay, reagents from Sigma-Aldrich, UK) [334]. Cell viability/proliferation was measured on the first, third, seventh and fourteenth day after cell seeding [1, 380]. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate and 0.7 mL of Alamar Blue solution was added to each well, the plate was incubated for four hours under standard conditions (37 °C, 5% CO₂ and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland) [380, 417].

3.5.2.7 Data analysis

All data are represented herein as mean ± standard deviation. Biological results were subjected to one-way analysis of variance (one-way ANOVA) and post hoc Tukey’s test using GraphPad Prism software. Significance levels were set at p < 0.05.

3.5.3 Results and Discussion

3.5.3.1 Morphology properties

SEM images of the 3D structures are shown in Figure 3.28(a). SEM images of the top and side views clearly show a well-bonded structure; although the structure was programmed to have a uniform pore size of 500x500 µm, this was not the case in practice. For instance, in the left image in Figure 3.28(a), the four pores are not of the same size, and their dimensions vary between 386±15µm to 567±7µm; this variation in the pore size can probably be attributed mostly to the positioning errors in the open-loop stepper motor drives.

Figure 3.28(b) shows SEM images of filament surfaces of top layers from untreated and plasma-treated scaffolds. The results confirm that the plasma surface modification has an impact on the surface roughness, which is an important factor in biomedical materials, as it
may affect cell adhesion [10]. The surface of an untreated filament has small pores (~5-20 µm diameters). After treatment, these pores disappear; instead lines in the direction of the plasma jet movement are generated, which result in a rougher surface topography. Their occurrence is due to the gas flow generated by the plasma jet during the modification process. Because the plasma modification is done during the fabrication process, it means that the material is still in the molten state when the modification is performed, resulting in the gas flow having a more pronounced effect on the surface topography.

Figure 3.28 SEM images of (a) top and cross-section views of 3D printed PCL scaffolds after N₂ plasma treatment; (b) top view of filament surfaces of untreated and plasma-treated scaffolds.

3.5.3.2 Surface wettability characterization

Since synthetic polymers, such as PCL, are hydrophobic, it is important to know the surface wettability before and after the plasma treatment. WCA measurements were performed on untreated and fully treated plasma PCL scaffold surfaces to determine the effect of plasma modification on the surface wettability. Table 3.7 highlights the WCA results at different instances after the droplet was dropped on the surface of the scaffolds. The results show that in the case of an untreated PCL scaffold, there is little or no change in the WCA values with time. These values vary between 83.2±2.0° and 80.9±2.7°. For the treated scaffolds, the WCA value, at 0 s, was lower at 63.0±3.1°, leading to a fully wetting value of 26.7±0.9° at 0.5 s. The droplet was quickly absorbed into the structure and no value was obtained at 3 s. For the treated scaffolds, the WCA value, at 0 s, was lower at 63.0±3.1°, leading to a fully wetting value of 26.7±0.9° at 0.5 s. The droplet was quickly absorbed into the structure and no value was obtained at 3 s. The results reveal that the hydrophilicity of the surface is dramatically improved due to the ionisable groups introduced on the surface by the N₂
plasma, which enhances the hydrogen bonding with water. When these results are compared with those published in [11, 14], the absorption speed is faster, probably because, with each layer being plasma modified, chemical heterogeneity is generated on the surface of every layer.

**Table 3.7** Temporal variation of water contact angles for treated and untreated scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>PCL scaffolds (°)</th>
<th>N\textsubscript{2} plasma fully treated(°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0s</td>
<td>83.2±2.0</td>
<td>63.0±3.1</td>
</tr>
<tr>
<td>0.5s</td>
<td>82.9±1.2</td>
<td>26.7±0.9</td>
</tr>
<tr>
<td>3s</td>
<td>80.9±2.7</td>
<td>Fully absorbed</td>
</tr>
</tbody>
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Figure 3.29 shows the temporal variation of WCA but on a much longer scale; the WCA values increase linearly with time, which means the plasma modification effect decreases with time. These results confirm that PABS has the capability of creating highly hydrophilic structures but the effect decreases with time.

**Figure 3.29** Temporal variation of the water contact angle for treated scaffolds.

### 3.5.3.3 Mechanical properties characterization

The stress-strain curves (Figure 3.30) for both untreated and fully-treated PCL scaffolds reveal that the mechanical behaviour of both scaffolds is almost identical. Except for some non-linearity between strains of 0.1 and 0.25, the stress-strain curves exhibit a linear variation. These observations indicate that the in-process plasma modification process does not affect the mechanical properties of scaffolds.
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Figure 3.30 Typical stress-strain curve for untreated and full-layer N\textsubscript{2} plasma treated PCL scaffolds.

3.5.3.4 Biological characterization

The adhesion and proliferation of hADSCs cells on plasma full-layer modified PCL scaffolds was studied and compared with untreated ones. The biological characterization was assessed using Alamar Blue Assay. The fluorescence intensity of cells seeded scaffolds was measured at four different culture time points (1, 3, 7 and 14 days), as shown in Figure 3.31. Higher fluorescence intensity corresponds to more metabolically active cells. Comparing the results at the four different time points, it can be observed that for both plasma treated and untreated scaffolds, the fluorescence intensity, which represents the growth in cell viability/proliferation, increases with time, suggesting that scaffolds fabricated with this particular additive manufacturing system are suitable substrates for cell proliferation. Comparing the fluorescence intensities on day one (see Figure 3.31), there is no significant difference between the plasma treated and untreated scaffolds. But on day three, the plasma treated scaffolds present a statistically higher fluorescence intensity than the non-treated scaffolds, suggesting a higher cell attachment/proliferation rate caused by the plasma surface modification through increased hydrophilicity. The phenomenon also exists on the seventh and fourteenth day.
3.5.4 Conclusions

This paper describes the development of a multi-extrusion system, which can print a PCL scaffold, each layer of which has been modified using a plasma jet to create a fully modified structure. When compared to an untreated scaffold, a scaffold subjected to N$_2$ plasma jetting exhibits the following advantages.

(i) Its filament surface morphology contains no pores.
(ii) Its tensile properties are not affected.
(iii) Its surface wettability characteristics are far superior.
(iv) It exhibits better biological performance in terms of cell attachment/proliferation rate.

Applications that may benefit from this technology include hybrid tissue, which has compositional variations depending on the region or organ-like structure which require continuous vascular network to facilitate nutrient diffusion.
3.6 Hybrid Additive Manufacturing System for Zonal Plasma Treated Scaffolds*

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† Co-first authors, contributed equally to this work.

Contribution to the work: As co-first author, together with first author F.L., W.W. conceived and designed the experiments, performed the experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

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3.6.1 Introduction

Tissue engineering is a multidisciplinary field for the development of biological substitutes that restore, maintain or improve tissue function by combining cells with three dimensional (3D) porous scaffolds [357, 418]. In this field, the fabrication of 3D highly porous, biocompatible and biodegradable scaffolds, with proper porosity, pore size, shape and interconnectivity, degradation rate, chemical composition and mechanical properties, is critical.

Recently, additive manufacturing technologies have emerged as a promising approach to produce scaffolds [1, 320, 357, 419]. The main advantage they provide are the capacity to rapidly produce very complex 3D constructs in a layer by layer fashion, and the ability to actively design the porosity and interconnectivity of scaffolds enabling repeatable macro-architectures. However, low resolution limits the fabrication of scaffolds to those with large pores compare with the size of cells. This leads to low cell-seeding efficiency and a non-uniform distribution of cells, and consequently to low rates of tissue formation, less uniform tissue and different cell differentiation behaviour [406, 407]. Moreover, most additive fabrication techniques are usually limited to the fabrication of single material scaffolds, which may not be fully chemically compatible with cells [420]. Furthermore, cell adhesion in the core regions of these scaffolds is often obstructed by the tortuosity of the scaffold, leading to limited cell colonisation [421]. Additionally, as synthetic biopolymers, the most commonly used materials, are hydrophobic, limited cell adhesion and proliferation is observed [407].

As cells are sensitive to the surface characteristics and topography of surfaces, surface-modification techniques such as cold plasma deposition (PE-CVD), etching and grafting processes, can be effective in tailoring the morphology and properties of surfaces to improve and orient their interaction with cells [419, 422]. In particular, PE-CVD of stable functionalised coatings (i.e., characterised by surface functional groups such as -NH\textsubscript{2}, -COOH and others) and plasma-grafting of polar groups allows the hydrophilic character of surfaces to be enhanced [423-425]. This provides anchor groups for biomolecule immobilisation which ultimately improve cell adhesion, colonisation and growth.

Despite its efficiency on the treatment of 2D substrates, the application of plasmas for the homogeneous modification of 3D scaffolds remains unviable due to limited penetration depth. Cold plasmas at low pressure (10-1000 mTorr), the most versatile plasma processes, are not ignited in 10-400 μm pores [426]. In these conditions, plasma active species can
reach the walls of the pores only by diffusion [413]. At atmospheric pressure, it may be possible to make some progress with dielectric barrier discharges (DBD), or plasma jets, but the problem of limited penetration in a 3D scaffold remains.

This paper presents a novel additive manufacturing system, named as Plasma-assisted Bio-extrusion System (PABS), aiming to radically move behind the current state of the art to circumvent the previously mentioned problems by developing a plasma integrated hybrid additive manufacturing system [427]. The system operates both the fabrication and the plasma treatment in a layer by layer manner, allowing highly efficient use of plasma 2D surface treatments, overcoming challenges related to full cell penetration and colonisation. Moreover, this system opens up the possibility of tailoring the plasma treatment for particular areas of the scaffolds. To assess the system, PCL scaffolds were produced and plasma treated considering three strategies: surface treated, centre treated and full-layer treated. The effect of plasma exposure time (i.e., plasma deposition velocity) and intensity (i.e., distance between plasma head and scaffold) were investigated. Hydrophilicity changes under different plasma conditions were determined with water contact angle measurement and compression tests were carried out to evaluate the effect of plasma treatment on the mechanical properties of the scaffolds. In vitro biological assessments were conducted to assess the adhesion and proliferation behaviour of human adipose-derived stem cells (hADSCs) on fabricated scaffold samples (untreated, surface treated, centre treated, full-layer treated PCL scaffolds).

3.6.2 Materials and Methods

3.6.2.1 PCL

PCL (CAPA™6500, Mw = 50,000 g/mol), purchased from Perstorp (Cheshire, UK) in the form of 3 mm pellets, was used to produce the scaffolds. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm³, a melting temperature between 58-60 °C, and a glass transition temperature of -60 °C.

3.6.2.2 Experiment setup

The PABS setup, shown in Figure 3.32(a), allows the fabrication of multi-material scaffolds with either uniform pore size or gradient pore size, specially controlled mechanical properties and chemical homogeneous and heterogeneous surface to modulate cell adhesion, proliferation and differentiation. The system comprises two main units, a multi-extrusion printing system (Figure 3.32(b)), and a plasma jet system (Figure 3.32(c)). The multi-extrusion system comprises two pressure assisted printing heads and one screw
assisted printing head, allowing to operate with a range of thermoplastic biomaterials, hydrogels, and thermoplastic/ceramic materials. All printing heads are equipped with a heated material chamber operating with a set of nozzle diameters ranging from 0.1 to 1 mm. The plasma jet head consists of a quartz capillary with a pin-type electrode mounted in co-axial geometry at the centre of the capillary, and a ground electrode outside. Different types of gas can be applied. The control software was developed in MATLAB as previously reported and generated G code files contains all the instructions for the fabrication process [415].

3.6.2.3 Scaffold design and fabrication

Rectangular scaffolds of 12 x 12 x 3 mm$^3$ (length x width x height) were designed with a lay-down pattern of 0/90° and filament distance of 1 mm. The printing conditions were: deposition rate of 3 mm/s; slice thickness of 0.5 mm; heating temperature of 90°C; extrusion rate of 15 rpm; and nozzle tip size of 0.5 mm.

3.6.2.4 Plasma modification strategies and processing parameters

The plasma treatment was performed using nitrogen (N$_2$) at a pressure of 10 psi with a flow rate of 5 litre/mm directly exposed to selected areas of the scaffolds in atmospheric environment. Tests were performed at different deposition velocities (3 mm/s, 6 mm/s and 12 mm/s), and different distances between the plasma unit and the surface of the deposited material (5mm, 10mm and 15 mm). High voltage (10 kV) and radiofrequency (repetition rate =50 KHz) were ignited between the tungsten electrode and copper film. Three different strategies were considered (Figure 3.32(d)):

i. Surface treated scaffolds: PCL scaffolds were printed using the screw-assisted printing head and then uniformly treated with the plasma system.

ii. Centre treated scaffolds: PCL scaffolds were printed, and during the printing process, the centre of each layer was treated with the plasma system. The centre treated area is determined by the programmed tool path of the plasma jet, which was set as a circular area with 5mm diameter. For short treatment distances (e.g. 5mm), the treatment area is approximately the same as the programmed one. Currently, the plasma exposure area is controlled by adjusting the distance between the plasma system and the material surface, with the other parameters (e.g. power and deposition velocity) enabling to control the degree of surface modification. An improvement on the plasma jet is being considered by creating a well-defined gas curtain around the reaction jet to shield the effluent and get it focused [428].
iii. Full-layer treated scaffolds: The screw-assisted printing head was used to print a layer of PCL and then the plasma system was used to treat the layer.

![Figure 3.32](image-url) (a) Set-up of plasma-assisted bio-extrusion system; (b) Multi-extrusion system; (c) Plasma modification system; (d) schematic images of untreated PCL scaffolds, surface-treated scaffolds, centre treated scaffolds and full-layer treated scaffolds.

3.6.2.5 Morphological characterisation

Scanning electron microscopy (SEM) was used to assess the morphology and surface characteristic of both plasma-treated and untreated scaffolds. The scaffolds were gold/palladium coated using a Q150T turbo-pumped sputter coater (Quorum technologies, UK) and imaged at 10 kV (Hitachi S3000N, Japan). These images were then analysed using ImageJ software.

3.6.2.6 Chemical characterisation

The X-ray photoelectron spectroscopy (XPS) analysis was performed using a Kratos Axis Ultra (Kratos Analytical Ltd, Manchester, UK) with CasaXPS software for data acquisition and analysis. The monochromatic Al (Kα) X-ray source (hv = 15 kV; 300 W set power) was used. The spectrometer was operated in constant analyser energy (CAE) mode with a pass energy of 100eV for survey scan, while for the carbon (C) 1s, oxygen (O) 1s and nitrogen (N) 1s individual high-resolution spectra were taken at a pass energy of 50 and a 0.1 eV energy step. The binding energy (eV) scales were referenced to the hydrocarbon component (C-C) in the C 1s spectra at 285 eV.
3.6.2.7 Surface wettability characterisation

Water contact angle measurements on the surfaces of untreated and plasma-treated PCL scaffolds were carried out with a KSV CAM 200 system (KSV Instruments, Finland). The system is equipped with a CCD video camera and a micrometric liquid dispenser to drop 2µl of distilled water on the surface of the scaffold. The contact angle measurements were automatically calculated with the instrument software. The water droplet was deposited at the centre of the scaffold surface for each sample.

3.6.2.8 Mechanical characterisation

Compression tests were performed to analyse the effect of plasma processing parameters on the mechanical properties of fully plasma treated PCL scaffolds. Before the compression test, the edges of all scaffolds were vertically cut with a razor blade creating a cross-section of ~10 x 10 mm. All tests were carried out using an unconfined Instron 3344 single column universal testing systems (Instron Corporation, Buckinghamshire, UK) equipped with a 2000 N load cell. Specimens were compressed between two fixed stainless steel plates at a rate of 0.5 mm/min up to a strain value of 0.45 mm/mm. For each type of specimen the tests were repeated four times in quadruplicate. Data were collected and analysed using the OriginPro 8.5.1 software (OriginLab Corporation). The stress (σ) was evaluated as the ratio between the load (F) and the total area (A) of the apparent cross section of the scaffold according to the following equation:

\[ \sigma = \frac{F}{A} \]

while strain, ε, was defined as the ratio between the scaffold height variation Δh (the difference between initial height and the height after compression) and the scaffold initial height \( h_0 \):

\[ \varepsilon = \frac{\Delta h}{h_0} \]

The compressive modulus was defined as the slope of the tangent line to the stress-strain curve at 12.5% strain.

3.6.2.9 Biological assessment

Cell proliferation tests were performed using human adipose-derived stem cells (hADSCs) (STEMPRO, Invitrogen, Waltham, MA, USA). Before cell seeding, scaffolds were sterilized by soaking in 70% ethanol for 2 hours. After sterilisation, samples were rinsed twice with phosphate buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA,
USA), transferred to 24-well plates and air-dried for 24 hours at room temperature. 50,000 cells were seeded on each sample, including untreated scaffolds, plasma surface treated, centre treated and full-layer treated scaffolds.

Cell viability/proliferation behaviour and the percentage of cells attached on the scaffolds (cell-seeding efficiency) were assessed through Alamar Blue assay (also termed the Resazurin assay, reagents from Sigma-Aldrich, Dorset, UK). Cell viability/proliferation rate was measured at 1, 3, 7 and 14 days after cell seeding. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate and 0.7 ml of Alamar Blue solution was added to each well, the plate was incubated for 4 hours under standard condition (37 °C, 5% CO₂ and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland).

Cell attachment and distribution are assessed using laser confocal microscopy, with cell nuclei stained. At day one of cell culture and after cell culture for 14 days, scaffolds were removed from 24-well cell culture plate, rinsed twice in phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature. After fixation, samples were rinsed twice with PBS for the removal of formalin, then permeabilised with 0.1 % Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at room temperature for 10 minutes, rinse twice for the removal of Triton-X100. Cell nuclei were stained blue by soaking scaffolds in a PBS solution containing 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Dorset, UK) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 minutes prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained using a 3D rendering mode on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope, and cell colonization was quantified using a standard z-stack method. All images were taken at the centre area of the scaffolds and the experiments were performed three times in triplicate.

3.6.2.10 Data analysis

All data are represented as mean ± standard deviation. Biological results were subjected to one-way analysis of variance (one-way ANOVA) and post hoc Tukey’s test using GraphPad Prism software. Significance levels were set at p < 0.05.
3.6.3 Results

3.6.3.1 Morphological characterisation

SEM images of full-layer plasma treated PCL scaffolds are shown in Figure 3.33(a). As seen from the top view and side view of the SEM images, the structure presented an interconnected structure with pores of ~500 µm uniformly distributed. Figure 3.33(b) represents the top view of a filament surface from untreated and plasma-treated scaffolds. The surface of untreated filaments (Figure 3.33(b) (left)) exhibited a non-smooth morphology with small micropores (~5-20 µm diameters), while the surface of N₂ plasma treated filaments show a denser and smaller nano-size roughness with lines in the direction of the plasma jet movement (Figure 3.33(b) (right)).

3.6.3.2 Chemical characterisation

The chemical composition of untreated and plasma-treated scaffolds, evaluated by XPS, are presented in Table 3.8 and Figure 3.33(c)-(d). Results show that after plasma modification, the shape of the C1s peak dramatically changed with the increase in both C2 and C3, and the appearance of amide groups (N-C(=O), C5), confirming that the PCL chemical composition was modified with a N-rich coating.

Table 3.8 The concentration of Carbon (C), Oxygen (O) and Nitrogen (N) on untreated scaffold and N₂ plasma treated scaffolds (10 mm treatment distance and 3 mm/s plasma deposition velocity) as determined by XPS analysis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Untreated</th>
<th>N₂ Plasma treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>C %</td>
<td>76.7 ± 1</td>
<td>72.4 ± 1</td>
</tr>
<tr>
<td>O %</td>
<td>23.3 ± 1</td>
<td>22.6 ± 1</td>
</tr>
<tr>
<td>N %</td>
<td>0</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>C-C %</td>
<td>52.4 ± 1</td>
<td>53.4 ± 1</td>
</tr>
<tr>
<td>-C-N- %</td>
<td>0</td>
<td>4.4 ± 1</td>
</tr>
<tr>
<td>-C-O- %</td>
<td>19.2 ± 1</td>
<td>18.3 ± 1</td>
</tr>
</tbody>
</table>
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Figure 3.33 SEM images of (a) top view and cross-section view of 3D printed PCL scaffolds after N\textsubscript{2} plasma treatment, scale bar 500 \(\mu\)m; (b) filament surfaces of untreated and plasma-treated scaffolds, scale bar 100 \(\mu\)m. The C1s spectra of (c) untreated and (d) N\textsubscript{2} plasma treated PCL scaffolds.

3.6.3.3 Surface wettability characterisation

Figure 3.34(a) shows that the untreated PCL scaffolds present higher water contact angle (85.48±1.5°), typical of hydrophobic surfaces. The figure also revealed a slightly lower water contact angle of full-layer treated PCL scaffolds (59.07±4.3°) compared with surface treated scaffolds (62.56±2.7°). Moreover, the centre treated scaffold presented lowest water contact angle (52.04±2.4°) in comparison with the full-layer and surface treated scaffolds.

The effect of plasma processing parameters, such as the distance between the plasma head and deposited material and plasma deposition velocity on the surface wettability, was also
investigated considering full-layer treated scaffolds. Figure 3.34(b) shows the water contact angle measurements after water dropped on the surface for 0s, 3s and 5s for samples treated with the same deposition velocity of 3 mm/s, and different distances between the plasma jet and the deposited material. The results show that by increasing the distance the water contact angle decreases which is associated to a decrease on the plasma effect. Figure 3.34(c) shows the effect of plasma deposition velocity considering a 10 mm distance between the plasma head and the printed material. The results show that the plasma modification effect decreases with increasing plasma deposition velocity.

![Graph showing water contact angles](image)

**Figure 3.34** (a) Water contact angles of untreated, surface-treated, center–treated, and full-layer-treated PCL scaffolds after the water drop on the surface at 0 s; effect of (b) the distance from plasma jet on the deposited material (at deposition velocity of 3 mm/s), and (c) the plasma deposition velocity (with 10mm distance) on the surface wettability detected by water contact angle results at time points 0, 3, and 5 s.

### 3.6.3.4 Mechanical characterisation

Mechanical compression tests were performed to assess the effect of the plasma treatment processing conditions on the mechanical performance of the scaffolds. Obtained results are presented in Figure 3.35 and Tables 3.9 and 3.10. As observed, for a fixed distance between the plasma head and the deposited material, the plasma deposition velocity has a significant
effect on the mechanical properties with the compression modulus varying between 21.41 MPa at 3 mm/s and 34.30 MPa at 12 mm/s. For a fixed plasma deposition velocity, the effect of changing the distance between the plasma head and the deposited material on the compressive modulus is less significant.

**Table 3.9** Compressive modulus for plasma treated scaffolds with different treatment distances and a constant plasma deposition velocity of 3 mm/s.

<table>
<thead>
<tr>
<th>Plasma treatment distance between plasma head and deposited material (mm)</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compressive Modulus (MPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.55 ± 1.2</td>
<td>21.41 ± 0.8</td>
<td>23.23 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.10** Compressive modulus for plasma treated scaffolds with different deposition velocities and a constant plasma treatment distance of 10 mm.

<table>
<thead>
<tr>
<th>Plasma deposition velocity (mm/s)</th>
<th>3</th>
<th>6</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compressive Modulus (MPa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.41 ± 0.8</td>
<td>26.14 ± 1.2</td>
<td>34.30 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.35** Effect of full-layer plasma modification on the compressive mechanical properties with (a) different distance between plasma jet and deposited material (at velocity of 3 mm/s) and (b) plasma deposition velocity (with 10 mm distance). Typical stress-strain curves for PCL scaffolds characterised by a 0/90° lay-down pattern and filament distance of 500 µm, compressed at a rate of 0.5 mm/min up to a strain value of 0.4 mm/min.
3.6.3.5 Biological assessment

Cell adhesion and proliferation of four types of 3D scaffolds (N₂ plasma surface treated, centre treated, full-layer treated and untreated) are assessed using the Alamar Blue Assay. Fluorescence intensity of cell seeded scaffolds were measured at four different culture time points (1, 3, 7, 14 days), as presented in Figure 3.36(a). The results show that for both plasma treated and untreated scaffolds, the fluorescence intensity increases from one time point to another, suggesting that the scaffolds fabricated with the developed additive manufacturing system are suitable substrates for cell proliferation. At both day 1 and day 3, no significant differences were observed between the different types of scaffolds. However at day 7, plasma surface treated and full layer treated scaffolds present statistically higher fluorescence intensity than untreated scaffolds, suggesting higher cell attachment/proliferation rate due to the plasma surface modification. At day 14, plasma surface treated scaffolds show statistically higher cell proliferation than both untreated scaffolds and centre treated scaffolds. No statistical differences were observed between plasma surface treated and full-layer treated scaffolds.

Confocal microscopy images (Figure 3.36(b)) present the cell attachment and distribution after cell seeding (day 1) and proliferation (day 14). It can be observed that plasma treated scaffolds presented higher numbers of cells than untreated scaffolds. Additionally, it is also possible to observe that plasma surface treated scaffolds presented best cell attachment and dispersion.
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**Figure 3.36** (a) Cell viability/proliferation test result (fluorescence intensity) of cell seeded PCL scaffolds (untreated and N\textsubscript{2} plasma centre, surface and full layer treated) at day 1, 3, 7 and 14 of cell culturing; (b) Confocal microscope images of all types of cell seeded scaffolds, 1 day and 14 days after cell culture. Scale bar 250 µm.

### 3.6.4 Discussion

The SEM results indicate that the plasma surface modification has an impact on the surface roughness, with the results focusing on the extruded filament confirming this impact. The micropores observed on the surface of untreated filament are due to the structure evolution during the screw-assisted melt extrusion process [429]. After plasma treatment, the denser surface appearance indicates an increase in the surface roughness due to the etching process,
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during which the melting and etching consecutively occurred on the PCL surface [430, 431]. This etching results in the stripping off the topmost layer of the polymer due to the weight loss during the plasma exposure [413]. Moreover, the textures on the treated filament surface are attributed to the gas flow generated by the plasma jet during the modification process. In this case, as the printed material is not totally cooled down, when the plasma modification occurs, the gas flow effect is stronger, significantly influencing the surface topography.

The WCA measurement results indicate that N₂ plasma modification dramatically increased the wettability of PCL scaffolds. The effect of the chemical groups deposited in the bottom layers of full layer-treated scaffolds enhanced the better hydrophilicity behaviour than surface treated scaffolds. Since the centre treatment guided the direction of water absorption in the vertical direction, limiting the spreading in the horizontal direction, the centre treated scaffolds present time-proved wettability. The effect of plasma modification can be tailored by changing the plasma processing parameters, including the distance between the plasma head and deposited material, and plasma deposition velocity. The plasma modification effect is related to the spatial distribution of active species and temperature and also the pressure associated with the plasma treatment [432, 433]. For smaller distances, the higher temperature, higher pressure and more concentrated active species induces lower WCA on the treated surface. Higher plasma deposition velocity results in the shorter plasma exposure time, which leads to less active species deposited on the surface.

The effect of plasma modification process on the mechanical properties is further investigated in this study by changing the plasma processing parameters. The mechanical analyses revealed that with a constant plasma modification velocity, the distance does not affect the bulk properties, which is consistent with previous studies [410, 413], while the plasma modification velocity has an effect on the compressive behaviour. Results show that slower plasma deposition velocities resulted in reduced compressive modulus. Since the deposited material is not fully solidified when conducting the plasma modification, lower plasma velocities may influence the microstructure development contributing to reduced mechanical properties [429].

According to other studies [410, 413], a significant increase in the biological behaviour is observed after plasma modification. Due to the zonal plasma modification capability of PABS, the biological behaviour was further assessed through comparison of surface treated, centre treated and full-layer treated scaffolds. The centre treated scaffolds presented insignificantly improved results as the space for cell proliferation is limited in the core region of scaffolds. This also explained the higher fluorescence intensity observed for
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surface treated and full-layer treated scaffolds. Furthermore, the full-layer treated scaffolds resulted in the superhydrophilic properties, which may lead to decreased cell attachment and proliferation [434], and this explains the observed lower cell intensity compared with surface-treated scaffolds.

### 3.6.5 Conclusions

This paper presented a novel additive manufacturing system comprising a multi-material printing unit and a plasma jet unit. To assess the system, non-plasma-treated and plasma treated PCL scaffolds were produced. The effect of plasma deposition velocity and the distance between the plasma head and the surface of the deposited material was investigated. Results from SEM analysis confirm the capability of the proposed system to produce full-layer or zonal plasma modified scaffold with fully interconnected channels and regular pore size. As observed, the filament morphology changed from a non-smooth morphology with small micropores in the case of untreated scaffolds to a morphology with textures aligned with the direction of the plasma jet movement in the case of treated scaffolds. XPS results indicate the deposition of nitrogen-rich chemical groups on the top surface and throughout the entire 3D construct based on the treatment strategy. The water contact angle measurement results reveal that the scaffolds become more hydrophilic after the plasma treatment, and additionally, centre-treated scaffolds appear to show the best hydrophilicity. Moreover, the effect of plasma treatment seems to be more significant with reduced deposition velocities and distances between the plasma head and the printed material. Mechanical compression tests show that for a fixed plasma deposition velocity, the effect of changing the distance between the plasma head and the deposited material is not significant. However, for a fixed distance, the compressive modulus decreases with the decrease in the plasma deposition velocity. *In vitro* biological assessment reveals better cell adhesion and proliferation behaviour of hADSCs on the plasma-treated scaffolds, and the plasma surface treated and plasma full-layer treated scaffolds presented high biological performance. Bioimaging showing cell attachment and distribution also confirmed this observation.

Applications that may benefit from this technology include functional graded scaffolds, which have compositional variations tailored by plasma treatment with different gases, various plasma deposition velocities and distances.
Chapter III: Physical and *in vitro* biological characterisation of electro-active scaffolds

3.7 Summary of the Chapter

This Chapter reports the fabrication and main results obtained with PCL and PCL/graphene (different contents of graphene) scaffolds. Scaffolds were mechanically tested using static compression method, and morphologically assessed through optical microscopy and scanning electron microscopy. To obtain electro-active scaffolds using graphene, it is important to guarantee a good dispersion of this inorganic material in the polymer fibre. Dispersion analysis was investigated using Raman and micro Raman spectroscopy. Transmission electron microscope was used to investigate the size of the graphene nanosheet in the scaffold fibre. This Chapter also reports the strategies to modify the surface properties of produced scaffolds. Firstly, a NaOH chemical treatment was used to improve hydrophilicity. Secondly, scaffolds were coated with a natural protein to improve bioactivity. Plasma zonal surface modification is also considered to increase the hydrophilicity and consequently the biological performance of PCL scaffolds, using a novel additive manufacturing system developed by our group. Biological studies were conducted using human adipose-derived stem cells, cytotoxicity/biocompatibility and cell proliferation tests were performed using Alamar Blue assay, while cell differentiation was performed using Alkaline Phosphatase method. Cell morphology was investigated using both laser confocal and scanning electron microscopy. A comparison between scaffolds reinforced with two types of carbon nanomaterials (graphene nanosheet and multi-walled carbon nanotube) is also presented, investigating their physical and biological properties.
CHAPTER IV

IN VIVO BIOLOGICAL CHARACTERISATION OF ELECTRO-ACTIVE SCAFFOLDS
Chapter IV: *In vivo* biological characterisation of electro-active scaffolds

This Chapter mainly focuses on the pre-*in-vivo* and *in vivo* biological characterization of electro-active scaffolds, based on a research article entitled “Pre-*in-vivo* and *in vivo* investigations of engineered 3D printed scaffolds for bone applications” submitted to Biofabrication.

Scaffolds are important physical substrates for cell attachment, proliferation and differentiation. Multiple factors could influence the optimal design of scaffolds for a specific tissue, such as the geometry, the materials used to modulate cell proliferation and differentiation, its biodegradability and biocompatibility. The optimal design of a scaffold for a specific tissue strongly depends on both materials and manufacturing processes. Previous studies of human adipose-derived stem cells (hADSCs) seeded on PCL/graphene scaffolds have proved that the addition of small concentrations of graphene to PCL scaffolds improves cell proliferation. Based on such results, this Section further investigates, for the first time, the pre-*in-vivo* characteristics of 3D printed PCL/graphene scaffolds, using an *in vitro* approach. Scaffolds were evaluated from morphological, biological and short term immune response points of view. Results show that the produced scaffolds induce an acceptable level of immune response, suggesting high potential for *in vivo* applications. Finally, the scaffolds were used to treat a rat calvaria critical size defect with or without applying micro electrical stimulation (10 µA). Quantification of connective and new bone tissue formation and the levels of ALP, RANK, RANKL, OPG were considered. Results show that the use of scaffolds containing graphene and electrical stimulation seems to increase cell migration and cell influx, leading to new tissue formation, well-organised tissue deposition and bone remodelling.
Pre-in-vivo and In vivo Investigations of Engineered 3D Printed Scaffolds for Bone Applications*

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³ Bone and Joint Research Group, Faculty of Medical and Health Sciences, University of Auckland, Auckland, 1023, New Zealand

Contribution to the work: As first author, W.W. conceived and designed the experiments, performed non-biological and in vitro biological experiments, analysed the data, prepared figures and tables, wrote the main manuscript text, and responsible for manuscript reviewing.

* Biofabrication, submitted.
4.1 Introduction

Bone defect caused by trauma, infections or tumour resection is a relevant clinical problem. It is estimated that in Europe 179,000 men and 611,000 women will suffer a bone fracture each year and the cost of all osteoporotic fractures in EU will increase from the current 31.7 billion euros to 76.7 billion euros by 2050 [1]. Bone tissue engineering, combining biodegradable scaffolds, cells and growth factors represents a promising approach for bone regeneration. The in vitro and in vivo relevance of 3D porous scaffolds has been widely demonstrated by several studies [435-437], being evident from those studies the importance of porosity, pore size, pore shape, pore distribution and pore interconnectivity on the osteogenesis process. A wide range of techniques have been explored to produce these biodegradable scaffolds including electrospinning, solvent casting, particulate leaching, laser ablation and melt moulding techniques [2, 11, 19]. However, these techniques are in most cases laborious process, presenting poor reproducibility and repeatability characteristics and limited capabilities to control pore shape, pore size, pore distribution and interconnectivity. Recently, additive manufacturing also known as 3D Printing emerged as a group of fabrication techniques that allow to create 3D interconnected porous scaffolds in a reproducible and repeatable way with minimal human intervention. A wide range of techniques such as extrusion-based (an additive manufacturing process in which a melted material is selectively dispensed through a nozzle), powder-bed fusion (an additive manufacturing process in which thermal energy selectively fuses regions of a powder material), vat-photopolymerisation (an additive manufacturing process in which a liquid photopolymer in a vat is selectively polymerized by light-activated polymerisation) and binder jetting (an additive manufacturing process in which a liquid bonding agent is selectively deposited to join powder materials) processes have been successfully used to produce 3D bone scaffolds in a wide range of polymers, ceramics and polymer/ceramic materials [7, 314, 438, 439].

As bone is an electrically active tissue, scaffolds must be designed not only based on key mechanical and biological requirements but also in terms of electrical compatibility. However, these characteristics have been largely unexplored in terms of the design of 3D porous scaffolds. The use of electrical stimulation to induce osteogenesis was initially described by Yasuda [440]. Since then, its use has been investigated for bone consolidation, incorporation of grafts and treatment of osteoporosis [441, 442]. Results demonstrated that the exogenous application of electric currents at physiological levels plays a role in cellular and molecular signalling pathways. The application of low-intensity electric current, also
known as microcurrent, ranging from 10 to 20 μA was also reported to stimulate bone repair. However, it was also observed that electric current above 50μA promote cell death and bone necrosis [443-445].

Even though the predominant mechanism of electric current interactions with biological systems remains unknown, it is accepted that it can trigger the cell surface, affecting membrane protein functions like enzyme activity, membrane-receptor complexes and ion-transporting channels [446-448]. Furthermore, electrical current seems to stimulate the synthesis and release of growth factors for cell proliferation and osteogenic differentiation, like alkaline phosphatase (ALP) and bone morphogenetic proteins, proteoglycans and cytokines involved in angiogenesis and collagen maturation and reorganization such as RANK (receptor activator of nuclear factor-NF-kB), RANKL (receptor activator of NF-kB ligand) and OPG (osteoprotegerin) [447-450].

For bone tissue engineering, we have fabricated 3D porous electro-active scaffolds made of poly(ε-caprolactone) (PCL) and graphene nanosheets using an extrusion-based additive manufacturing system. PCL, a semi-crystalline biodegradable polymer, was selected due to its low melting point, making it easy to process. However, it is a non-conductive hydrophobic polymer presenting limited mechanical properties. Contrary, graphene is a 2D carbon material with extremely high mechanical and electrical properties [451, 452]. However, its use for biomedical application has been investigated without a conclusive result. Some authors reported good cytocompatibility and the ability to stimulate cell proliferation [80], while others reported some cytotoxicity risks [329]. Previously we showed that small concentrations of graphene slightly improve the hydrophilicity of PCL scaffolds and mechanical properties [1, 453]. In vitro tests with hADSCs showed that the presence of graphene stimulates cell proliferation, but no significant differences between PCL scaffolds and PCL/graphene scaffolds were observed regarding cell differentiation [454]. All tests were performed without electrical stimulation. So far, no previous work studied graphene-based scaffolds and electrical stimulation for bone regeneration considering an animal model. Therefore, this paper investigates the use of electro-active scaffolds (PCL/graphene) associated with exogenous microcurrent therapy (non-invasive therapy) to treat a critical size bone defect created in the calvaria of rats. In this study, PCL and PCL/graphene (loaded with 0.13 wt.%, 0.50 wt.% and 0.78 wt.% of graphene) were initially screened using MC3T3 pre-osteoblastic cells and THP-1 human monocytic cells to assess their in vitro characterisation and immune response, and to select the best composition for the in vivo tests. After, PCL and PCL/graphene (loaded with 0.78 wt.% of
graphene) scaffolds were assessed in vivo, critical size calvaria defects were induced in male Wistar rats and bone regeneration investigated with and without electrical stimulation.

4.2 Materials and methods

4.2.1 Scaffold design and fabrication

PCL, Capa™ 6500, a high molecular weight (≈50 KDa) polyester derived from caprolactone monomer, purchased from Perstorp (Warrington, UK) in the form of 3mm pallets, and graphene nanosheets (3-4 layers, ~500 um surface diameter) synthesized by a water-assisted liquid phase exfoliation of graphite, were used to produce scaffolds through a screw-assisted additive biomanufacturing system (3D Discovery from RegenHU, Villaz-St-Pierre, Switzerland).

For the synthesis of graphene nanosheets, 50 mg microcrystalline graphite powder (325 mesh, 99.995% pure, purchased from Alfa Aesar, UK) was immersed in N-methyl-2-pyrrolidone (NMP) mixture with a 0.2 mass fraction of water and batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H, Switzerland) at a fixed nominal power and frequency of 100 W and 37 kHz. Sample dispersions were centrifuged at 3000 rpm for 30 minutes using a Hettich EBA20 centrifuge (Hettich Instruments, Germany), collecting the upper 75% of the colloidal supernatant and dried in an oven to yield the graphene nanosheets.

PCL/graphene pellets were initially prepared by melt blending considering three different graphene concentrations (0.25, 0.50, and 0.75 wt.%). Briefly, pure PCL pellets were heated above 70°C in a bowl to ensure all material is in a molten state prior to graphene flake addition, at the desired concentrations. The materials were manually mixed for 15 minutes to guarantee a homogeneously dispersion. After cooling down for 2 hours, the blended material was cut into small pellets for processing. The obtained graphene concentrations are 0.13, 0.50, and 0.78 wt.%, as determined by thermogravimetric analysis (TGA).

The extrusion-based additive manufacturing technique used to produce the scaffolds allows high reproducibility and good control over scaffold topology (including pore size, pore shape and pore distribution), which are critical parameters to design optimised 3D interconnected pores scaffolds. The 0°/90° lay-down pattern was adopted to obtain pores with a regular geometry and a constant filament diameter of 330 μm, and a filament distance of 680 μm. The optimal combination of processing parameters allowing the
fabrication of scaffolds with similar geometric characteristics to the designed ones, were 90 °C of melting temperature, 220 μm of slice thickness, 22 rpm of screw rotational velocity, and 20 mm/s of deposition velocity, the fabrication process was performed at room temperature.

After fabrication, scaffolds were cut with fine, double edged razor blades into small blocks (~11 mm x 11 mm x 3 mm) to fit in a 24-well culture plate.

4.2.2 Morphological analysis

The morphology of produced scaffolds was assessed using both scanning electron microscopy (SEM) and microCT. A Zeiss EVO60 SEM (Oberkochen, Germany) was used to assess pore and filament morphology. After scaffold fabrication, both top and cross-section layers of each sample were platinum-coated (Gatan Model 682 Precision Etching Coating System, Pleasanton, CA, USA), approximate coating thickness of 10 nm) and imaged through SEM. SEM images were analysed through ImageJ software (NIH, Bethesda, USA).

SkyScan1172 microCT from Bruker (Kontich, Belgium) was used to assess pore distribution and interconnectivity. Edges of scaffold samples were removed and then samples were cut into small pieces (3mm x 3mm x 6mm) to fit into the microCT chamber. The 3D scaffold structure was built after post processing, applying smoothing filter to improve image quality. Scaffold pore image was also processed with the same procedure.

4.2.3 Protein adsorption

Protein adsorption was performed using fetal bovine serum (FBS) as a model protein system and a bicinchoninic acid assay (Micro BCA Protein Assay Kit, Thermo Scientific, Waltham, MA, USA). Briefly, scaffolds were cut into 5mm x 5mm x 5mm cubic blocks (n=3 for each group) and placed into a 48 well plate. 500 μl of 10% FBS solution (diluted by phosphate-buffered saline (PBS) solution (Sigma-Aldrich, Dorset, UK)) was added into each well and kept in the incubator at 37 °C. After 6 and 12 hours, the samples were gently washed with PBS to remove excess and unattached protein and transferred to new wells to ensure that proteins attached to the walls and to the bottom of the plate were not measured. The amount of protein present was quantified using the BCA kit following the manufacturer’s protocol. 500 μl working reagent was added to the samples as well as into a series of bovine serum albumin (BSA) standards, and incubated at 37 °C for 2 hours. Before measuring absorbance, the supernatant was pipetted up and down to ensure complete mixing of the reagents and transferred to a 96 well plate in order to remove any interference from the
scaffold. The analysis was performed using a TECAN Infinite 200 plate reader (Tecan, Männedorf, Zurich, Switzerland) at an absorbance of 562 nm. The amount of protein adsorbed was calculated based on a standard curve generated by a dilution series of BSA standards.

4.2.4 In vitro biological assessment

4.2.4.1 Cell viability/proliferation

In order to study cell viability/proliferation and cell attachment to the scaffolds (cell-seeding efficiency), the Alamar Blue assay (also termed Resazurin assay) was used (reagents from Sigma-Aldrich, Dorset, UK) with mouse pre-osteoblastic cells, MC3T3 (purchased from ATCC, Cryosite Distribution, Lane Cove, NSW, Australia, method of authentication STRS analysis). Briefly, cell viability/proliferation was measured at 1, 3, 7 and 14 days after cell seeding on PCL and PCL/graphene scaffolds. The upper surface of each scaffold was seeded with 700 μl of cell suspension containing 5 x 10⁴ cells. For each time point, cell-seeded scaffolds were moved to a new 24-well plate, 1 ml of Alamar Blue solution (0.001 wt.%) was added to each well and the plate was incubated for 4 hours under standard condition (37 ºC, 5% CO₂ and 95% humidity). After incubation, 150 μL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a TECAN Infinite 200 spectrophotometer (Tecan, Männedorf, Zurich, Switzerland).

Scaffold samples used in the cell viability/proliferation study were kept in culture up to 14 days to assess cell morphology and qualitative attachment to the scaffolds using scanning electron microscopy (TM3030Plus, Hitachi, Japan).

4.2.4.2 Immune response

An immune response test based on gene expression assay was conducted to assess the immune response of fabricated scaffolds. To ensure maximum contact surface between scaffold material and cells, different scaffold samples were cut into small pieces, sterilised with 70% ethanol for two hours, then transferred to 48-well plate enough to cover the bottom of the well. Around 1,000,000 THP-1 human monocytic cells (TIB-202™, ATCC®, American Tissue Culture Collection) were seeded to each well. Cells were cultured in Roswell Park Memorial Institute (RPMI) media (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Utah, USA), 10,000U/ml Penicillin/Streptomycin mixture (Gibco, ThermoFisher Scientific, Waltham, MA, USA) and 1mM Sodium Pyruvate. At day 1 and
day 3, cells attached to the scaffolds and culture media were collected separately for further analysis. Clinical suture material (Coated VICRYL® (polyglactin 910) Suture, ETHICON, Somerville, NJ, USA) was used as positive control group, while the negative control group consisted in 1,000,000 THP-1 cells cultured alone using the above protocol. Main pro-inflammatory cytokines, tumour necrosis factor alpha (TNFα) and interleukin-1 beta (IL-1β) are considered as the gene probe for immune response test. For the gene expression, RNA was prepared with the RNeasy kit (QIAGEN Pty Ltd, Melbourne, VIC, Australia), following the manufacturer’s instructions after cells were lysed using β-mercaptoethanol in RLT buffer (Qiagen Pty Ltd, Melbourne, VIC, Australia). RNase-free DNase set was used to remove genomic DNA (Qiagen Pty Ltd, Melbourne, VIC, Australia). The quantity and purity of RNA were measured using a Nanodrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesised with Superscript III (Life Technologies, Carlsbad, CA, USA) using a PTC-100TM programmable thermal controller (MJ Research, Inc, Waltham, MA, USA). Real-time polymerase chain reaction (PCR) was performed with a QuantStudio™ 12K Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). The predesigned FAM-labelled primer-probe set was purchased as TaqMan® probe assay (Cat.#4331182; Life Technologies, Carlsbad, CA, USA). The VIC-labelled 18s ribosomal RNA was used as endogenous control gene (Life Technologies, Carlsbad, CA, USA). For the analysis, the ΔΔCt method was used to calculate the mRNA gene expression by normalise the expression to endogenous control.

4.2.4.3 Imaging

For SEM preparation, scaffolds were fixed with 3% glutaraldehyde solution (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature, rinsed twice with PBS, dehydrated with graded ethanol series (50%, 70%, 80%, 90% and 100%), post fix with 1% osmium tetroxide in 0.1M phosphate buffer, rinse twice with 0.1M phosphate buffer for removal, then dried with critical point dryer (CPD). Thin cross-section layers of each sample (around 3mm) were cut and gold coated for imaging, using a Gatan Model 682 Precision Etching Coating System, to an approximate thickness of 10nm.

Cell attachment and distribution were assessed using laser confocal microscopy, with cell nuclei and actin stained. At day one of cell culture and after cell culture for 14 days, scaffolds were removed from 24-well cell culture plate, rinsed twice in PBS, and fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature.
Then, samples were rinsed twice with PBS for the removal of formalin, permeabilised with 0.1 % Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at room temperature for 10 minutes, rinse twice for the removal of Triton-X100. Cell nuclei were stained blue by soaking scaffolds in a PBS solution containing 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) at the manufacturer recommended concentration. Cell actin was stained red by soaking scaffolds in a PBS solution containing Alexa Fluor™ 594 Phalloidin (Invitrogen™, ThermoFisher Scientific, Waltham, MA, USA) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 minutes prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained using a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

4.2.5 *In vivo* biological assessment

4.2.5.1 Calvaria defects

Defects were induced in male Wistar rats (weight of 300 – 350 g), according to the experimental standards and biodiversity rights (NIH Publication 80-23, revised 1996 and Arouca Law-11, 794, 2008), approved by CEUA/UNIARARAS (026/2017). All animals were healthy and the experimental procedure did not promote stress.

The animals were anesthetized by intraperitoneal administration of a mixture of 10% of ketamine hydrochloride (30mg/Kg) and 2% of xylazine hydrochloride (10mg/Kg). Tricotomy was performed in the occipital region of the animals followed by asepsis with 4% of chlorhexidine digluconate. A 5 mm x 5 mm square defect was created using a sharp tip Osteo I (PiezoHelse, Hense Dental Technology, Santos Rosa do Viterbo, SP, Brazil) coupled on an ultrasound device for cutting the bone (Olsen, O Piezo Light D5 LED, Palhoça, SC, Brazil) under constant irrigation of a sterile physiological solution (NaCl 0.9 %), creating a critical defect of 25 mm$^2$ in preserving the dura mater.

Sixty animals were considered, divided into six testing groups (n=10), as follows: NBR (natural bone regeneration) or control group; NBR+ES (natural bone regeneration with electrical stimulation); PCL (PCL scaffolds); PCL+ES (PCL scaffolds with electrical stimulation); PCL/G (PCL composite scaffolds containing 0.78 wt.% of graphene); PCL/G+ES group (PCL composite scaffolds containing 0.78 wt.% of graphene with electrical stimulation). After the scaffold implantation, wounds were sutured with nylon 5-0 sutures (Johnson&Johnson, Brazil).
4.2.5.2 Electrical stimulation

The microcurrent (microgalvanic) application was performed using a low-intensity transcutaneous electrical stimulator (Physeotonus microcurrent, BIOSET, Indústria de Tecnologia Electrônica Lda, Rio Claro, SP, Brazil). Two metal electrodes were placed around the bone defect (10 μA for 5 minutes). The applications started immediately after surgical procedure and were performed twice a week. The animals were euthanized after 60 and 120 days with excessive anesthetic dose, and bone defect samples were collected for further analysis.

4.2.5.3 Histology

Bone biopsies were fixed in 3.7% buffered formaldehyde solution (pH 7.4) for 48 hours, and demineralized with 10% ethylenediaminetetraacetic acid for 30 days. After this period, biopsies were dehydrated and embedded in paraffin, and cross-sections of 4.0μm thick were cut with a micrometer, mounted on glass slides, dewaxed, rehydrated and stained with hematoxylin-eosin and Masson’s Trichrome. The histological sections were analysed by light field microscopy using a LEICA DM 4000BVR microscope equipped with a LEICA camera (Leica Microsystems, Germany) and images were captured at 50x and 100x magnification. Colour Deconvolution ImageJ software was used to evaluate the percentage of new bone and connective tissue formation by separating blue and red colours. The presence of immature to organized mature collagen fibres is indicated as blue colour, depending on its intensity, while red colour indicates greater mineralization. The morphometric analysis corresponding to both colours was measured as the percentage of the total number of pixels in each original image, as described elsewhere.

4.2.5.4 Immunohistochemistry

Cross-sections of 4.0μm thick were used for immunohistochemistry. In order to evaluate osteoblasts and osteoprogenitors cells, alkaline phosphatase (ALP) was measured by using anti-ALP antibodies (1:400, Santa Cruz Biotechnology, Dallas, USA). Besides, anti-RANK, anti-RANKL and anti-OPG (1:200, Santa Cruz Biotechnology, Dallas, USA) were used to evaluate osteoclastic activation during bone resorption and bone remodelling, following the manufacturer's recommendation, and then incubated with secondary antibodies for 30 minutes. Liquid DAB (Springer-DAB-125) was used as substrate-chromogen for 5 minutes at room temperature to reveal markings. Biopsies were analysed using a LEICA DM 4000BVR microscope (Leica Microsystems, Germany) and images were captured at 400x magnification.
4.2.6 Statistical analysis

All experimental data are represented as mean ± standard deviation (SD). Data were analysed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Two-way analysis of variance (two-way ANOVA) with post hoc Bonferroni’s test were applied. Significance levels were set at * p<0.05; ** p<0.01; *** p<0.001.

4.3 Results

4.3.1 Morphological analysis

Figure 4.1 shows the screw-assisted additive manufacturing system used to print the scaffolds, microCT micrographs and SEM images of both PCL and PCL/graphene scaffolds. As seen from the top view and side view of the SEM images, the structure presented well-defined, regular shape and uniformly distributed pores. Printed scaffolds have an average pore size of 356μm (the designed value was 350μm), filament diameter of 362.5μm (the designed value was 350μm) and an average porosity of 45%. MicroCT scanning results show that the scaffolds present well-defined pores and pore interconnectivity. Results are also repeatable.

Figure 4.1 (a) 3D Discovery from RegenHU used to print the scaffolds; (b) PCL and graphene scaffolds; (c) and (d) MicroCT scan images of PCL scaffold (scaffold structure and pores); (e) and (f) SEM image top and cross section views of PCL/graphene scaffold.

4.3.2 Protein adsorption

The adsorption of protein is one of the first events occurring on the surface of a scaffold once implanted in a biological environment, with significant impact on further cellular interaction. Results of protein adsorption on PCL and PCL/graphene scaffolds are presented in Figure 4.2. It is possible to observe that all scaffolds were able to absorb
protein and that the amount of protein adsorption increase with time. After 6 hours of incubation, PCL/graphene scaffolds show statistically higher protein adsorption compared to PCL scaffolds, with the scaffolds loaded with the higher amount of graphene (0.78 wt.%) showing also higher protein adsorption. After 12 hours, PCL/graphene (0.50 and 0.78 wt.%) scaffolds show statistically higher protein adsorption compared to PCL scaffolds, but no significant difference between them.

Figure 4.2 Adsorption of protein on PCL and PCL/graphene scaffolds. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05; ** p<0.01; *** p<0.001).

4.3.3 In vitro biological assessment

4.3.3.1 Cell viability/proliferation

Cell viability and proliferation rates on PCL and PCL/graphene scaffolds were assessed by Alamar Blue assay. Figure 4.3 shows the fluorescence intensity values at different time points for scaffolds produced with different concentrations of graphene nanosheets. As higher fluorescence intensity corresponds to more metabolically active cells, results show that cells are growing in all scaffolds at different rates. At day 1, all scaffolds exhibited similar cell attachment and biological performance. At day 3 and afterwards, the presence of graphene became relevant, as 0.50 and 0.78 wt.% PCL/graphene scaffolds exhibited higher cell viability/proliferation rate, statistically higher than PCL scaffolds. A significant difference between 0.78 wt.% and PCL scaffolds is observed at days 3, 7 and 14. Results suggest that with the increase of graphene concentration, cell viability/proliferation rate increases. Similar trend was also previously observed using hADSCs and scaffolds with the
same architecture and material composition, which verifies the assumption that graphene improves the biological properties of PCL scaffolds [1, 453].

Figure 4.3 Cell viability/proliferation behaviour (fluorescence intensity) at day 1, 3, 7 and 14 after cell seeding. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05; ** p<0.01; *** p<0.001).

After 14 days, extensive cell attachment and cell spreading was observed, as shown in Figure 4.4. Confluent cell sheets are observed, with many cells bridging orthogonal scaffold filaments. These results indicate that cells are able to grow forming colonies and spreading around in the 3D printed scaffolds, and scaffold samples are able to provide a suitable environment to support cell growth and proliferation.
Figure 4.4 SEM images of cell seeded scaffolds cultured up to 14 days. (a) PCL; (b) PCL/graphene (0.13 wt.%); (c) PCL/graphene (0.50 wt.%); (d) PCL/graphene (0.78 wt.%).

Confocal microscopy images (Figure 4.5) present the cell attachment and distribution after proliferation for 14 days. It can be observed that PCL/graphene scaffolds presented higher numbers of cells than PCL scaffolds. Additionally, it is also possible to observe that PCL/graphene scaffolds presented higher cell attachment and dispersion.
Figure 4.5 Confocal microscope images of cell seeded scaffolds, 14 days after cell culture. Scale bar = 250 µm (10 x), 100 µm (20 x).

4.3.3.2 Immune response

Figure 4.6 presents the scaffolds immune response results, in terms of relative expression values. The results show no significant differences at day 1. At day 3, the expression of both TNFα and IL-1β for all scaffolds present significantly lower relative expression values than
the clinical suture material used as positive control. As previously reported, clinical suture material, coated VICRYL® won’t cause any immune/inflammatory response [455]. Therefore, the results suggest that the different PCL/graphene scaffolds have high potential for in vivo applications as they induce lower inflammatory response than clinical suture material.

**Figure 4.6** Immune response result (relative expression) at day 1 and day 3 after cell seeding. * Statistical analysis difference (p<0.05) between scaffold group and positive control group (coated VICRYL® clinical suture material).

**4.3.4 In vivo biological assessment**

**4.3.4.1 Histology**

A rat calvaria defect model was used to study the in vivo osteogenesis and the stimulatory effect of electrical stimulation. Figure 4.7a presents bone defects immediately after surgery (osteotomy), while Figures 4.7b, 4.7c and 4.7d present animals treated with no scaffold (NBR group, as natural bone regeneration), PCL scaffold and PCL/graphene scaffolds,
respectively, after 4 post-operative months. The bone defect area was collected after euthanasia.

Figure 4.7 (a) Initial calvaria defect created; Bone defect area after 120 days to (b) no scaffold; (c) PCL scaffold; (d) PCL/graphene scaffold.

Figure 4.8 shows images at 50x magnification considering new tissue formation after 2 and 4 post-operative months for all six groups. No inflammatory effects were observed in all groups neither before the euthanasia of the animals (macroscopic analysis) nor in the histological images (microscopic analysis). In the control group (NBR group), it is possible to observe that the bone defect presents a thin connective tissue layer, even after 120 days of follow-up. In this case, only a small portion of new bone is observed, mostly close to the edges of the defect. A similar layer of connective tissue is also observed in the NBR + ES group. However, in this group, small portions of new bone tissue can be seen near the bone edges 60 days after implantation, and along the bone defect 120 days after implantation. For both PCL and PCL + ES groups, it is clear the presence of connective tissue on the fibres of scaffolds and also “islands” of bone tissue spreading along the defect embedded in a connective tissue at both 60 and 120 days. Similarly, it is also possible to observe more evident organized bone formation and greater portions of new bone in the PCL/G and PCL/G + ES groups at 120 days.
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Figure 4.8 Photomicrography of the defect area for all groups after 60 and 120 days, stained with hematoxylin and eosin at 50x magnification showing the entire bone defect. From these images it is possible to observe the bone edge (B.E), scaffold (S), connective tissue (C.T) and matured / organized tissue (O.T).

Figure 4.9 shows histological images stained with Masson’s Trichrome staining at 100x magnification. The blue dye indicates connective tissue (collagen) and primary mineralized bone (depending on the tissue organization and colour intensity), while the red dye indicates mineralized tissue after 2 and 4 post-operative months for all six groups. At day 60, in the NBR and NBR + ES groups, relatively small collagen deposition can be observed (blue dye). After 120 days, the tissue seems to be more organized with the presence of some new bone tissue (blue-red dye) embedded in the collagen. For the groups where scaffolds were used to fill the bone defect, it is clear the presence of the scaffold filaments in white round structures (S). It is also possible to observe the presence of both connective and mineralized tissue formed in the interconnected pores of the scaffolds. No differences were observed for PCL and PCL+ES groups at day 60. However, at day 120 it can be seen the formation of organized tissue (blue dye) and mineralized tissue (red dye) around the scaffold filaments. For scaffolds containing graphene nanosheets, it is also possible to observe the presence of the nanomaterial represented by small black tiny dots. Greater tissue formation
and well-organized islands are observed in the case of scaffolds containing graphene nanosheets, especially at day 120. The presence of mixed blue-red dye show the tissue maturation observed in PCL/graphene groups. Histological images stained with Masson’s Trichrome staining at 100x magnification were used for tissue quantification, as shown in Figure 4.10.

![Figure 4.9 Photomicrography of the defect area for all groups after 60 and 120 days, stained with Masson’s Trichrome at 100x magnification showing areas of the bone defect; In these images, it is possible to observe the bone edge (B.E), scaffold (S), connective tissue (C.T), bone tissue (B.T) and the presence of graphene nanosheets (*).](image)

4.3.4.2 Quantification of new tissue formation

Figure 4.10 shows the percentage of connective tissue and new bone formation in all groups. After 60 days of implantation, all scaffold-based groups, except the PCL group, are associated to higher connective tissue formation (p<0.05) compared to the NBR group, showing that scaffolds enable cell influx and tissue formation (Figure 4.10a). After 120 days, the PCL group seems to be associated to the formation of less connective tissue in
comparison to both control and PCL/G group and that the application of electrical stimulation also increases the formation of connective tissue. However, the results are not statistically significant. Regarding bone (mineralized tissue) formation (Figure 4.10b), the PCL/G+ES group is associated to higher percentage of new bone formation (7.1%) compared to the NBR group (2%). Figure 4.10c presents the cumulative tissue formation (connective and mineralized bone tissue) for all investigated cases. Results show that at day 60, PCL+ES (31% of cumulative tissue formation), PCL/G (38.2%) and PCL/G+ES (41.2%) present higher percentage of new tissue formation compared to the NBR group (17.6%). These results are statistically significant. Additionally, the PCL/G+ES group (41.2% of cumulative tissue formation) presents higher volumes of cumulative tissue formation compared to both NBR+ES (26%) and PCL (26%) groups. At day 120, the differences tend to be less significant between groups, mostly due to the bone remodelling phase, instead of tissue formation. However, the PCL/G+ES group presents significantly higher values of cumulative tissue formation compared to PCL group.
Figure 4.10 The percentage of tissue formation in all groups. (a) Connective tissue; (b) Mineralized bone tissue; (c) Cumulative tissue formation (connective + bone tissues) quantified by ImageJ software from 100x magnification images (Masson’s Trichrome staining). The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05; ** p<0.01; *** p<0.001).

4.3.4.3 Quantification of ALP, RANK, RANKL and OPG

The percentage of ALP, RANK, RANKL and OPG after 60 and 120 days is presented in Figure 4.11. At day 60, all groups show higher percentage of ALP than RANK, RANKL and OPG. All groups present also higher percentage of ALP compare to the NBR group. The percentage of ALP increases with both electrical stimulation and presence of graphene, with the exception of the PCL group where the higher values were observed without electrical stimulation (Figure 4.11a). At day 120, NBR+ES group show significantly higher percentage of ALP compared to NBR group, and no significant difference were observed between the NBR group and the PCL group. Similarly, PCL+ES and PCL/G groups show significantly higher ALP values in comparison to the other groups. Results also show a
decrease of ALP from day 60 to day 120 in the case of both PCL/G and PCL/G+ES groups.

At day 60, higher percentages of RANK were observed in the PCL and PCL/G+ES groups (Figure 4.11b). It is also possible to observe that the NBR+ES group presents significantly higher values of RANK compared to the NBR group. From day 60 to day 120, RANK values tend to decrease, with the exception of the NBR and PCL/G groups. Similarly, at day 60, the NBR+ES group presents higher values of RANKL compared to the NBR group (Figure 4.11c). The groups containing graphene present also high values of RANKL compared to both PCL and PCL+ES group. The RANKL levels are also higher in the case of the NBR+ES group compared to the PCL groups (PCL and PCL+ES). From day 60 to day 120, the RANKL levels tend to decrease except in the PCL and PCL+ES groups.

The level of OPG at day 60 and 120 for all groups is presented in Figure 4.11d. The results show significantly high values of OPG compared to RANKL for all considered groups. At day 60, higher levels of OPG were observed for both the NBR+ES and PCL/G groups compared to the other groups. OPG levels are also higher in the case of the NBR group compared to both PCL and PCL+ES groups. From day 60 to day 120, the OPG levels decreased in the NBR, NBR+ES and PCL/G groups, increasing in the other cases. Contrary to day 60, results show that the OPG level at day 120 is significantly higher in the case of PCL/G+ES group compared to the PCL/G group and also higher in comparison to the other groups. At day 60, the highest value of OPG/RANKL was observed in the PCL/ES group while at day 120 it was observed in the PCL/G+ES group.
Figure 4.11 The percentage of a) ALP, b) RANK, c) RANKL and d) OPG in new bone tissue for all groups after 60 and 120 days. The values were compared using two-way ANOVA, followed by post hoc Bonferroni’s test (significance levels were set at * p<0.05; ** p<0.01; *** p<0.001).

4.4 Discussion

Any biomaterial to be used in clinical applications must comply with a set of assays of cytotoxicity and regulatory rules such as the International Organization of Standardization (ISO) protocols for the biological evaluation of medical devices. These assays provide an indication of the toxicity to tissues in direct contact with a biomaterial. Although this may be sufficient for some long-term medical implants, it is not for novel biomaterial scaffolds that are designed to interact with the host tissue to facilitate appropriate cellular responses, such as new tissue formation. In this way, one of the main failings of degradable scaffolds results from immune reactions rather than cellular toxicity. In this research work, PCL/graphene scaffolds containing different concentrations of graphene were morphologically and biologically characterised. In vitro assays of cytocompatibility, cell proliferation and
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scaffold immunogenicity were performed to assess the scaffolds providing a preliminary indication of its potential use in bone tissue regeneration.

Scanning electron microscope images and microCT scanning (Figure 4.1) confirmed that the screw-assisted extrusion additive manufacturing is a viable technique to produce scaffolds with geometric characteristics close to the designed ones and able to produce interconnected porous scaffolds. This fabrication technology is highly reproducible allowing the fabrication of repeatable scaffolds with controlled geometric characteristics.

As previously reported, protein adsorption strongly depends on the characteristics of a structure, such as surface area, wettability, surface roughness, electrostatic interactions, surface chemistry, and presence of functional groups [456-458]. The results presented in Figure 4.2 show that the protein adsorption increases with the presence of graphene. Since the graphene nanosheets used in this research were not functionalised, there are no specific binding motifs or chemistries present to specifically enhance protein attachment to the graphene nanosheets. As previously reported by the authors, the printing technology considered here to fabricate the scaffolds allow the fabrication of filaments with uniformly dispersed graphene [1]. Therefore, the inherent structural characterisation of the graphene nanosheets can partially explain the higher protein adsorption of PCL/graphene scaffolds compared to PCL scaffolds. The large surface area of graphene nanosheets and the impact of high levels of graphene on the surface texture of the filaments of the scaffolds together with the electrical properties of graphene lead to higher protein bonding. Additionally, authors also reported that these relatively small loading levels of graphene contribute to slightly reduce the hydrophobic nature of PCL scaffolds [87], which can also partially explain the higher protein adsorption of PCL/graphene scaffolds compared to PCL scaffolds.

Cell viability/proliferation tests with MC3T3 osteoblasts (Figure 4.3) confirmed that the PCL/graphene scaffolds are able to provide a proper environment for cells to attach and proliferate. Cell proliferation is stimulated with the increase of graphene concentration and no significant cytotoxicity was observed. This can be explained by the high surface area, the elastic modulus, the stiffness and the presence of wrinkles and ripples created during the production of graphene [89, 377]. Graphene has also been reported to enhance cell adhesion for anchorage dependant cells, preventing implanted cells from reactive oxygen species (ROS)-mediated cell death, and enhancing cell proliferation [315, 379]. The
electrical properties of graphene can also enhance the transmission of cell signals, promoting cell-cell communication.

Besides potential cytotoxicity effects not observed in all scaffolds, it is also important to evaluate potential inflammatory response. During the healing process of different tissue, inflammatory events are expected. To prevent the organism against microorganisms such as bacteria, which are able to provide contamination and impair the healing process. However, if the inflammatory response persists, the defect area is barely able to heal, due to metalloproteinase types released by inflammatory cells [456-461]. To assess the immune response of fabricated scaffolds, an immune response test was conducted based on gene expression assay. TNFα and IL-1β are the main pro-inflammatory cytokines, and therefore the most likely to be upregulated in a foreign body response, and were considered as the probe of immune response test [455, 462]. Experimental results show that both PCL and PCL/graphene scaffolds induce less inflammatory response than clinical suture material, as revealed by the low levels of TNFα and IL-1β, suggesting that these scaffolds do not stimulate inflammatory response, being appropriate for in vivo applications.

As bone is a piezoelectric tissue, the application of electrical stimulation (ES) offers substantial therapeutical potential and several authors reported the in vitro of using ES in terms of cell proliferation and cell osteogenic differentiation [446, 463-468]. It was also reported that ES is capable of inducing cell elongation, modulating cell alignment and facilitating cell migration [446].

Results presented in this paper show that the combined use of scaffolds containing graphene and electrical stimulation is the most effective approach in terms of new tissue formation. The corresponding high levels of ALP suggest more pre-osteoblasts and osteoblast cells in the tissue, which might contribute to bone tissue formation. The NBR group presented the lowest level of ALP at both day 60 and day 120, but through the application of electrical stimulation these levels increased at both time points. Moreover, the ALP values significantly decreased from day 60 to day 120 in the PCL group, but again this trend was not observed by applying electrical stimulation. Therefore, the result seems to indicate a positive impact of electrical stimulation on the physiological stimulation on osteoprogenitor cells and osteoblast. However, at day 120, the PCL/G+ES group presents lower values of ALP than the PCL/G group. This might be explained by the bone remodelling phase, once the PCL/G and PCL/G+ES presented higher and well-organised tissue formation than other groups, at both day 60 and 120. Moreover, this is also a
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consequence of a more evident cell influx due to the presence of graphene on scaffolds, in agreement with the *in vitro* cell proliferation tests. In this scenario, the electrical stimulation modulated the tissue formation in graphene nanosheets groups.

The higher connective and mineralized tissues produced in the case of PCL/G and PCL/G+ES groups is a consequence of the extracellular matrix production by osteoblasts, and bone remodelling by osteoclasts. The balance between bone formation and bone resorption stimulated by graphene and electrical stimulation was assessed by immunohistochemistry for anti-RANK/RANKL/OPG, as this molecular triad plays a crucial role in the osteoclastic activation and bone remodelling [469]. RANK, RANKL and OPG have been identified as regulators of osteoclastogenesis modulating the tissue formation towards well-organized deposition of mineralized tissue [469, 470]. Several cell types produce osteoprotegerin (OPG), a cytokine with affinity for RANKL in order to perform an inhibitory effect (feedback system), preventing RANKL to bind to RANK membrane receptor. RANKL is produced by osteoblasts and endothelial cells and activate osteoclasts when linked to their RANK membrane receptors, a marker of bone resorption [470].

The higher OPG and low RANK levels in the PCL/G group at day 60 suggest an inhibitory effect of bone adsorption. Although the PCL/G+ES group present an opposite trend to that observed in the PCL/G group, two graphene-composite groups show higher tissue formation, especially PCL/G+ES regarding mineralized tissue. Authors believe that graphene was able to improve tissue formation by stimulating the osteoblasts activity. Moreover, microcurrent therapy modulated the bone remodelling, leading to the deposition of well-organized mineralized tissue, a balanced event to produce bone tissue. Similar levels of tissue formation were observed for the PCL and NBR+ES groups. A possible explanation can be attributed to the hydrophobic nature of PCL limited the cell migration and extracellular matrix production compared to graphene. However, electrical stimulation modulated this effect, preventing over-bone adsorption, as demonstrated by low levels of RANK and RANKL.

At day 120, the RANK levels are lower in the groups treated with microcurrent, while the OPG levels increase in the groups treated with microcurrent, suggesting an important role of this therapy during bone formation and bone remodelling phases. Moreover, it seems that the PCL group is associated to higher osteoclastic activity, presenting high percentage of RANK and low percentage of OPG.
4.5 Conclusions

In this study, morphological and different types of biological assessments have been performed to investigate the properties of PCL/graphene scaffolds containing different concentrations of graphene fabricated through additive manufacturing. Morphological evaluations showed that fabricated scaffolds are close to the original designs in terms of geometry and inner structure. *In vitro* biological assessment using two different cell lines, MC3T3 and THP-1, showed that all scaffolds are suitable for cell growth, and that the addition of graphene nanosheets stimulates cell proliferation. Moreover, the immune response analysis suggested high potential for *in vivo* bone regeneration research. *In vivo* tests suggest that the use of PCL/graphene scaffolds with exogenous microcurrent therapy enhance new tissue formation, stimulating the levels of ALP, RANK and higher ratios between OPG and RANKL. The physiological electrical stimulation modulated the bone remodelling phase, leading to well-organized and mineralized tissue deposition.
4.6 Summary of the Chapter

This Chapter investigates, for the first time, the pre-in-vivo characteristics of 3D printed PCL/graphene scaffolds, using an in vitro approach. An immune response test based on gene expression assay was performed to compare the inflammatory response between clinical suture material and fabricated scaffolds, verifying the potential of printed scaffolds for in vivo applications. Finally scaffolds were implanted into the calvaria defects of rats, and the bone regeneration process was investigated with and without electrical stimulation.
CHAPTER V

CONCLUSIONS AND FUTURE WORK
5.1 Discussion and conclusions

As discussed through the whole dissertation, scaffolds play a very important role as physical substrates for cell attachment, proliferation and differentiation, ultimately leading to the regeneration of corresponding tissues. Thus, this research project focuses on the optimal design of scaffolds for bone tissue regeneration in terms of both materials and manufacturing processes. As bone is piezoelectric tissue, the application of electrical stimulation offers substantial therapeutical potential, this lead to the use of extrusion-based additive manufacturing technology to fabricate poly (ε-caprolactone) scaffolds, with the incorporation of carbon nanomaterials in order to enhance the electro-conductivity.

Scaffolds investigated in this dissertation were produced considering mixtures of an organic non-conductive material (PCL) and a conductive inorganic material (graphene nanosheets/carbon nanotubes). Material pellets were prepared by melt blending considering different PCL/carbon nanomaterial ratios and the results obtained by Thermogravimetric Analysis (TGA) showed that this is an effective method to incorporate graphene nanosheets in the PCL matrix. Scaffolds were successfully produced using a screw-assisted extrusion-based additive manufacturing using optimized processing parameters obtained after an extensive experimental work. The scaffolds were extensively characterized from a chemical, physical, morphological and biological (both in vitro and in vivo) points of view using a myriad of techniques (Scanning Electron Microscopy; optical microscopy; microComputer Tomography, confocal microscopy, static mechanical compression tests, contact angle measurement, Raman and micro Raman Spectroscopy, contact angle measurement, Alamar Blue method, Alkaline Phosphatase method, Alizarin red-S method, gene expression, histology and immunohistochemistry). Therefore, this dissertation was broader not only on the range of techniques used but also on the fields covered, requiring knowledge from manufacturing and mechanical engineering, materials science and cell biology.

The following list of outputs (publications) explicitly stated the contributions of this research work, which aligned with the research aims and objectives.
## Table 5.1 List of outputs

<table>
<thead>
<tr>
<th>Objectives</th>
<th>Outputs</th>
</tr>
</thead>
<tbody>
<tr>
<td>To evaluate current tissue engineering strategies for bone regeneration;</td>
<td>Published and submitted papers:</td>
</tr>
<tr>
<td></td>
<td>“Materials for Electro-active Scaffolds: A Review” (Section 2.1)</td>
</tr>
<tr>
<td></td>
<td>“Engineering the Vasculature with Additive Manufacturing” (Section 2.2)</td>
</tr>
<tr>
<td>To develop a new strategy to create scaffolds for bone regeneration with</td>
<td>Published paper:</td>
</tr>
<tr>
<td>improved mechanical and biological properties;</td>
<td>“Morphological, Mechanical and Biological Assessment of PCL/Pristine</td>
</tr>
<tr>
<td></td>
<td>Graphene Scaffolds for Bone Regeneration” (Section 3.1)</td>
</tr>
<tr>
<td>To develop a strategy to electrical stimulate bone in-growth;</td>
<td>Submitted paper:</td>
</tr>
<tr>
<td></td>
<td>“Pre-in-vivo and In vivo Investigations of Engineered 3D Printed</td>
</tr>
<tr>
<td></td>
<td>Scaffolds for Bone Applications” (Chapter 4)</td>
</tr>
<tr>
<td>To compare the PCL/graphene scaffolds with PCL scaffold reinforced with</td>
<td>Submitted paper:</td>
</tr>
<tr>
<td>other carbon nanomaterials in order to define the most suitable inorganic</td>
<td>“Assessment of PCL/Carbon Material Scaffolds for Bone Regeneration”</td>
</tr>
<tr>
<td>reinforcement to create electro-active scaffolds;</td>
<td>(Section 3.4)</td>
</tr>
<tr>
<td>To develop a new approach in order to improve cell attachment/proliferation</td>
<td>Published papers:</td>
</tr>
<tr>
<td>and differentiation by coating the scaffolds with natural protein or/and</td>
<td>“Enhancing the Hydrophilicity and Cell Attachment of 3D Printed PCL/</td>
</tr>
<tr>
<td>modify its surface wettability properties.</td>
<td>Graphene Scaffolds for Bone Tissue Engineering” (Section 3.2)</td>
</tr>
<tr>
<td></td>
<td>“3D Printed Poly(ε-caprolactone)/Graphene Scaffolds Activated With P1-</td>
</tr>
<tr>
<td></td>
<td>Latex Protein for Bone Regeneration” (Section 3.3)</td>
</tr>
<tr>
<td></td>
<td>“A Plasma-assisted Bioextrusion System for Tissue Engineering” (Section</td>
</tr>
<tr>
<td></td>
<td>3.5)</td>
</tr>
<tr>
<td></td>
<td>“Hybrid Additive Manufacturing System for Zonal Plasma Treated Scaffolds” (Section 3.6)</td>
</tr>
</tbody>
</table>
In conclusion, the novelty of this work emphasised on the use of a novel fabrication technology, allowing good control over the general geometry structure, inner geometry structure and good repeatability, together with novel composite material, having better mechanical biological and electrical properties, to fabricate 3D scaffold to support and speed up bone regeneration process. With novel surface modifications methods and microcurrent therapy, both in vitro and in vivo biological assessments have presented improved bone regeneration effect compared with conventional PCL scaffolds. All the results present in dissertation suggest a promising future for the clinical trial on this newly developed 3D structure, aiming to solve large scale bone defect caused by diseases or accidents, to reduce the pain from the patients, saving billions for the NHS.

5.2 Future work

This thesis presents major in vitro studies considering cell attachment, proliferation and differentiation. In the future, quantification of the extracellular matrix (ECM) formation and gene expression will be conducted allowing to better understanding the role of graphene on bone tissue regeneration. Additional, it will be important to assess the performance of scaffolds containing high levels of graphene in order to determine any threshold value after which the presence of graphene could be toxic. This will be relevant to design scaffolds for both bone regeneration and bone cancer treatment applications.

Preliminary results reported in this dissertation demonstrate a positive role of P1-protein to create scaffolds with improved bioactivity. This is a group of proteins that must be further investigated to understand not only the mechanism responsible for the improvement of cell attachment, proliferation and differentiation but also to identify the specific protein responsible for this behaviour. Additionally, there are some evidences, not reported here, that P1-protein is also angiogenic. Therefore, further in vitro studies using epithelial cells will be conducted with PCL/graphene scaffolds coated with P1-protein to confirm this angiogenic property.

PCL/graphene scaffolds with plasma treatment will be produced and their properties compared with NaOH treated scaffolds to determine the most effective route to create PCL/graphene scaffolds with improved hydrophilicity.

Degradation studies of PCL/graphene scaffolds, together with the definition of mathematical models describing the degradation process will be conducted. The in vitro degradation behaviour will be conducted using both SBF and PBS. The characterisation of the degradation behaviour of the scaffolds will be performed at specific times by
evaluating changes in the average molecular weight, weight loss, thermal properties and mechanical properties.

Based on the current *in vivo* biological analysis, results suggest that the use of PCL/graphene scaffolds with exogenous microcurrent therapy enhance new tissue formation, stimulating the levels of ALP, RANK and higher ratios between OPG and RANKL. The physiological electrical stimulation modulated the bone remodelling phase, leading to well-organized and mineralized tissue deposition. In the future, besides the current quantification on new tissue formation and immunohistochemistry analysis, gene expression tests will be conducted and the production of specific growth factors (BMP-2, BMP-7, etc.) will be investigated during the *in vivo* bone regeneration process.

It will be also important to further investigate the internal mechanism through which graphene nanosheets are able to stimulate cell proliferation and differentiation. Based on current research, electrical properties of graphene can enhance the transmission of cell signals, promoting cell-cell communication. In the future, N-doped graphene, a type of graphene with chemically-modified surface to further enhance electrical conductivity will be used to fabricate scaffolds. Based on the cell biological behaviour, comparisons between non-doped graphene scaffolds and N-doped graphene scaffolds will allow to obtain more information on the effect of electrical conductivity on biological behaviour.

These planned activities will be published in journal papers and also reported in a book on “Electro-active scaffolds for bone tissue engineering” invited by Pan Stanford Publishing expected to be completed in late 2019 beginning of 2020.
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Appendix A

Process Optimisation of Extrusion-Based Additive Manufacturing to Produce PCL Scaffolds for Bone Regeneration


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1 Manchester Biomanufacturing Centre, School of Mechanical, Aerospace and Civil Engineering, University of Manchester, M13 9PL, UK.

Abstract: This appendix reports the work carried out to evaluate the effect of processing conditions on the morphology, porosity and mechanical properties of scaffolds produced using additive manufacturing, in order to determine optimal processing parameters for scaffold fabrication. The obtained results clearly demonstrate the potential of using screw-assisted additive manufacturing system to produce 3D scaffolds with reproducible well organized architectures and tailored mechanical properties.
A.1 Introduction

The colonization of 3D porous matrices is dependent on many factors including architectural features such as pore size/shape, pore interconnectivity, overall scaffold porosity and topography [1-3]. Previous studies showed that pore size and shape can actually influence cell adhesion, migration and tissue ingrowth both in vitro as in vivo [4-6]. In the specific case of load-bearing applications such as bone, 3D scaffolds are required to withstand mechanical stresses during a specific period of time, providing adequate support for cell adhesion/proliferation, vascularization and tissue ingrowth. Therefore, the optimal design of a scaffold is the key for tissue engineering [7]. This design optimization strongly depends on the knowledge of the process-morphological development interrelationship during the scaffold fabrication process. Different additive manufacturing techniques have been developed to produce scaffolds for bone regeneration, including extrusion based process, powder bed fusion, binder jetting and vat photopolymerization process [8, 9]. Among these techniques, extrusion based process is one on the most relevant due to its versatility (e.g. it is possible to process a wide range of synthetic and natural polymers and composites), low cost and accuracy. This paper explores the use of a screw-assisted additive manufacturing system to produce bone scaffolds, investigate effect of processing conditions on morphological and mechanical properties of the scaffolds.

A.2 Materials and methods

A.2.1 Poly (ε-caprolactone)

Poly (ε-caprolactone) (PCL) used in this research work is Capa 6500 purchased from Perstorp (UK). It is a semicrystalline polymer with low melting temperature. It has good biocompatibility and biodegradability, which means that when the scaffold is implanted in the human body, the patient will not reject it and after the healing process, the scaffold would disappear being replaced by new bone tissues. The degradation mechanism of PCL is characterized by random hydrolysis ester cleavage and weight loss. It has been founded that the degradation of PCL with high molecular weight requires 3 years for complete
removed from the human body [10]. However, the degradation of the scaffolds strongly depends on the molecular weight of PCL, scaffold topology and processing conditions.

A.2.2 Scaffold design and fabrication

A scaffold three-dimensional model (30 x 30 x 6 mm$^3$) was initially designed in a CAD software (SolidWorks, Dassault Systemes) and a $0^\circ/90^\circ$ lay-down pattern was adopted to obtain pores with a regular square geometry while maintaining a constant filament distance of 680μm. Scaffold fabrication process was controlled by four processing parameters: melting temperature; slice thickness; screw rotation velocity and deposition velocity. To ensure the fabrication efficiency, the deposition velocity is kept constant at 20 mm/s. Thus, the optimisation is focused on the following three parameters: temperature, slice thickness and screw rotation velocity. Four different values were selected for each processing parameter during the optimisation. PCL scaffolds with different combinations of processing parameters were produced using the 3DDiscovery, a screw-assisted additive manufacturing system from RegenHU (Switzerland).

A.2.3 Characterisation

A.2.3.1 Morphological characterization

ZEISS EVO 60 scanning electron microscope (SEM) was used for morphological analysis, namely, to access pore shape, pore size and pore distribution. Software ImageJ was used for filament diameter and pore size measurement. Porosity was evaluated using the following methodology [11]:

(1) measuring the weight and volume of each scaffold;

(2) calculate the apparent density of each scaffolds;

(3) applying the following equation:

$$\text{Porosity} = (1-\rho^*/\rho_{\text{sub}}) \times 100$$  \hspace{1cm} (A.1)

where $\rho^*$ is the apparent density of the scaffold and $\rho_{\text{sub}}$ sub is the density of the original substance ($\rho_{\text{sub}} = 1,145$ g/cm$^3$).
A.2.3.2 Mechanical characterization

Compression tests were performed to assess the effect of different processing parameters on the mechanical properties of scaffolds. Blocks of 5.0 mm in length (l), 5.0 mm in width (w) and 5.72 mm in height ($h_0$) were considered. Tests were carried out on scaffolds in dry state at a rate of 1 mm/min up to a strain limit of 0.3 mm/mm (30%), using the INSTRON 4507 testing system equipped with a 1 kN load cell. During the uni-axial compression tests, the software determined force $F$ and corresponding displacement values, which were converted into engineering stress $\sigma$ and strain $\varepsilon$ as follows:

$$\sigma = \frac{F}{A}$$  (A.2)

$$\varepsilon = \frac{\Delta h}{h_0}$$  (A.3)

where $A$ is the initial sample cross section area and $\Delta h$ is the scaffold height variation. The obtained stress-strain data was further processed to determine the compressive modulus, $E$, according to the procedure reported by Fiedler [12].

A.3 Results and discussion

A.3.1 The effect of temperature variation

Values of slice thickness, screw rotation velocity and deposition velocity were maintained constant (slice thickness=220 μm, screw rotation velocity =25 rpm, and deposition velocity =20 mm/s) while temperature varied from 90 ºC to 120 ºC with 10 ºC interval. Results show that an increment in the temperature increases the filament diameter from 373 ± 12 μm to 387 ± 8 μm. Contrarily, pore size decreased from 321 ± 8 μm to 314 ± 6 μm, and porosity from 41% to 37%. Obtained results are presented in Figures A.1 to A.2, and Table A.1.
Figure A.1 SEM images of scaffolds (top view) produced using different temperatures (a): 90 °C; (b): 100 °C; (c): 110 °C; (d): 120 °C

Figure A.2 Variation of filament diameter and pore size as a function of temperature.
Appendix A

Table A.1 Morphological characteristics of scaffold structures for different temperature values.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Filament diameter (μm)</th>
<th>Pore size (μm)</th>
<th>Weight (g)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 °C</td>
<td>373±12</td>
<td>321±8</td>
<td>3.4477</td>
<td>41.16</td>
</tr>
<tr>
<td>100 °C</td>
<td>380±9</td>
<td>318±5</td>
<td>3.5264</td>
<td>39.79</td>
</tr>
<tr>
<td>110 °C</td>
<td>385±6</td>
<td>315±5</td>
<td>3.6252</td>
<td>38.09</td>
</tr>
<tr>
<td>120 °C</td>
<td>387±8</td>
<td>314±6</td>
<td>3.7210</td>
<td>36.56</td>
</tr>
</tbody>
</table>

Typical stress-strain curves obtained from mechanical compression tests are reported in Figure A.3, which graphically highlight the effect of temperature on the mechanical performances of the constructs. As observed, temperature has an effect on the morphology of the scaffolds due to its impact on the filament diameter and porosity. Consequently, mechanical property such as compressive modulus (E) was likely to be influenced. The obtained results show that an increase in temperature resulted in thickening the extruded filaments decreasing porosity from 41% to 37%, which causes an increment in the stiffness and strength values (Table A.2 and Figure A.3). The compressive modulus was calculated as the slope of the initial linear region of the stress-strain curve.

Table A.2 The effect of processing temperature on scaffold mechanical properties.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Compressive modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 °C</td>
<td>132.65</td>
</tr>
<tr>
<td>100 °C</td>
<td>141.27</td>
</tr>
<tr>
<td>110 °C</td>
<td>116.63</td>
</tr>
<tr>
<td>120 °C</td>
<td>129.61</td>
</tr>
</tbody>
</table>
A.3.2 The effect of slice thickness variation

By tuning the slice thickness, it is possible to control the layer gap, and hence the pore size. However, the influence of slice thickness is also extended to the filament diameter, pore width and porosity. Theoretically, by reducing the slice thickness, the extruded filaments of adjacent layers will be compressed against each other, causing a change in the geometry of the filament that becomes more elliptical. Therefore, the filament diameter increases while filament gap and porosity decreases. The lower and upper limits for slice thickness may be established based on the adhesion and interconnectivity criteria.

As expected, the most evident effect of slice thickness was observed in the layer gap values. Increasing slice thickness from 220 μm to 330 μm causes a significant decrease in the filament diameter, increasing pore size and porosity from 41% to 59%. Pore size increases as a consequence of a decrease in the filament diameter as shown in Figure A.4. Higher porosity value and lower filament diameter caused by an increment in slice thickness was also observed as shown in Figure A.5 and Table A.3.
Figure A.4 SEM images of scaffolds (top view) produced using different slice thickness (a): ST=220 μm; (b): ST=220 μm; (c): ST=220 μm; (d): ST=220 μm.

Figure A.5 Variation of filament diameter and pore size as a function of slice thickness (data obtained from SEM analysis). The dotted red line corresponds to the theoretical filament diameter value.
Table A.3 Morphological characteristics of scaffold structures for different slice thickness values.

<table>
<thead>
<tr>
<th>Slice thickness (μm)</th>
<th>Filament diameter (μm)</th>
<th>Pore size (μm)</th>
<th>Weight (g)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>373±12</td>
<td>321±8</td>
<td>3.4477</td>
<td>41.16</td>
</tr>
<tr>
<td>260</td>
<td>363±8</td>
<td>323±16</td>
<td>2.9174</td>
<td>50.20</td>
</tr>
<tr>
<td>300</td>
<td>351±9</td>
<td>366±10</td>
<td>2.6348</td>
<td>55.15</td>
</tr>
<tr>
<td>330</td>
<td>342±11</td>
<td>367±16</td>
<td>2.3889</td>
<td>59.24</td>
</tr>
</tbody>
</table>

Results also show that variations of slice thickness influence the mechanical properties of the scaffolds. In general, an increase in the slice thickness decreases the compressive modulus (Table A.4 and Figure A.6). The increment of solid material present in the scaffold (lower porosity), caused by a decrease in pore height and pore width (due to higher filament diameter) explains this behaviour.

Table A.4 The effect of slice thickness on the scaffolds’ mechanical properties.

<table>
<thead>
<tr>
<th>Slice thickness (μm)</th>
<th>Compressive modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
<td>132.65</td>
</tr>
<tr>
<td>260</td>
<td>113.76</td>
</tr>
<tr>
<td>300</td>
<td>43.21</td>
</tr>
<tr>
<td>330</td>
<td>31.47</td>
</tr>
</tbody>
</table>
Appendix A

The effect of screw rotation velocity variation

The rotational velocity imposed to the screw, determines the amount of material that is extruded through a nozzle. Similar to previous cases, temperature, deposition velocity and slice thickness were fixed (90 °C, 20 mm/s and 220 μm respectively) while the screw rotation velocity changed between 17.5 and 25 rpm with a 2.5 rpm interval.

Results show that an increment in the screw rotation velocity from 17.5 to 25 rpm led to an increment in the filament diameter from 293 ± 8 μm to 412 ± 10 μm as a consequence of the higher amount of extruded material. Inversely, higher rotation velocity reduces pore size and porosity, as shown in Table A.5. Variations of filament diameter and porosity as a function of screw rotation velocity are shown in Figures A.7 to A.8, and Table A.5.

Figure A.6 Compression test result for slice thickness variation.

A.3.3 The effect of screw rotation velocity variation

The rotational velocity imposed to the screw, determines the amount of material that is extruded through a nozzle. Similar to previous cases, temperature, deposition velocity and slice thickness were fixed (90 °C, 20 mm/s and 220 μm respectively) while the screw rotation velocity changed between 17.5 and 25 rpm with a 2.5 rpm interval.

Results show that an increment in the screw rotation velocity from 17.5 to 25 rpm led to an increment in the filament diameter from 293 ± 8 μm to 412 ± 10 μm as a consequence of the higher amount of extruded material. Inversely, higher rotation velocity reduces pore size and porosity, as shown in Table A.5. Variations of filament diameter and porosity as a function of screw rotation velocity are shown in Figures A.7 to A.8, and Table A.5.
Figure A.7 SEM images of scaffolds (top view) produced using different screw rotation velocity (a): SRV=17.5 rpm; (b): SRV=20 rpm; (c): SRV=22.5 rpm; (d): SRV=25 rpm.

Figure A.8 Variation of filament diameter and pore size as a function of screw rotation velocity (data obtained from SEM). The dotted red line corresponds to the theoretical filament diameter value.
Appendix A

Table A.5 Morphological characteristics of scaffold structures for different screw rotation velocity values.

<table>
<thead>
<tr>
<th>Screw rotation velocity (rpm)</th>
<th>Filament diameter (μm)</th>
<th>Pore size (μm)</th>
<th>Weight (g)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.5</td>
<td>293±8</td>
<td>434±10</td>
<td>2.4922</td>
<td>57.53</td>
</tr>
<tr>
<td>20</td>
<td>315±13</td>
<td>399±8</td>
<td>2.9310</td>
<td>50.03</td>
</tr>
<tr>
<td>22.5</td>
<td>367±5</td>
<td>353±15</td>
<td>3.3580</td>
<td>42.87</td>
</tr>
<tr>
<td>25</td>
<td>412±10</td>
<td>312±8</td>
<td>3.7680</td>
<td>35.87</td>
</tr>
</tbody>
</table>

Additionally, results show that by increasing the screw rotation velocity, the amount of extruded material increases, resulting in thickening of the filaments and ultimately in structures with lower void spaces (lower porosity). An increase of stiffness and strength is achieved as by increasing screw rotation velocity from 17.5 to 25 rpm (Table A.6 and Figure A.9).

Table A.6 The effect of screw rotation velocity on the scaffolds’ mechanical properties.

<table>
<thead>
<tr>
<th>Screw rotation velocity (rpm)</th>
<th>Compressive modulus E (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.5</td>
<td>61.67</td>
</tr>
<tr>
<td>20</td>
<td>87.47</td>
</tr>
<tr>
<td>22.5</td>
<td>127.55</td>
</tr>
<tr>
<td>25</td>
<td>182.09</td>
</tr>
</tbody>
</table>
A.4 Summary and conclusions

In order to find the optimal set of process parameters to rapidly produce scaffolds without compromising their structural and repeatability characteristics, the effect of all process parameters was investigated in order to produce scaffolds with a filament diameter equivalent to the internal nozzle diameter (330μm). The results show the following trends:

- With the increase of temperature, the filament diameter increases, pore size and porosity decreases, leading to a slight increase in the mechanical behaviour;
- With the increase of slice thickness, the filament diameter decreases, pore size and porosity increases, leading to a significant decrease in the mechanical behaviour;
- With the increase of screw rotation velocity, the filament diameter increases, pore size and porosity decreases, leading to a significant increase in the mechanical behaviour.

Experimental results also confirmed that the screw-assisted additive manufacturing system (3DDiscovery) is able to produce non-random porous structures with consistent pore sizes, spatial distribution and controllable porosity. From the morphological and mechanical analysis it was possible to observe that the variation of temperature, slice thickness, and screw rotation velocity parameters have a profound impact in terms of filament diameter and consequently on pore size, porosity and compression strength. The results and changing

![Figure A.9 Compression test result for screw rotation velocity variation.](image_url)
trends are also in agreement with previous works performed by other researchers, and confirm the porosity dependence of 3D scaffolds when tested under compression [13, 14].

References


Appendix B

Design, Fabrication and Evaluation of PCL/Graphene Scaffolds for Bone Regeneration


Weiguang Wang 1, Wei-Hung Chiang 2, Paulo Bártolo 1

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2 Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei 106, Taiwan

Abstract: Scaffolds are physical substrates for cell attachment, proliferation and differentiation, ultimately leading to the regeneration of tissues. They must be designed according to specific biomechanical requirements such as mechanical properties, surface characteristics, biodegradability, biocompatibility and porosity. The optimal design of a scaffold for a specific tissue strongly depends on both materials and manufacturing processes. Polymeric scaffolds reinforced with electro-active particles could play a key role in tissue engineering by modulating cell proliferation and differentiation. This appendix investigates the use of an extrusion additive manufacturing system to produce PCL/pristine graphene scaffolds for bone tissue applications. PCL/pristine graphene blends were prepared using a melt blend process. Scaffolds with the same architecture but different contents of pristine graphene were evaluated from a chemical, morphological and mechanical view. Scaffolds with regular and reproducible architecture and a uniform dispersion of pristine graphene flakes were produced. It was also possible to observe that the addition of pristine graphene improves the mechanical performance of the scaffolds.
B.1 Introduction

With the increasing life expectancy of the population, osteoporotic fractures will have a serious economic impact on society and patient’s quality of life. It is estimated that in Europe 179,000 men and 611,000 women will suffer a hip fracture each year and the cost of all osteoporotic fractures in EU will increase from the current 31.7 billion euros to 76.7 billion euros by 2050. The problem is worse if we consider also significant bone damage due to accidents and diseases. To solve large scale bone damage, bone grafting is required. Bone grafting is a method by which bone-deficient areas are built up, with the use of different materials, such as autografts, allografts, alloplasts and xenografts. However, limitation exists. Allografts are associated with rejection, transmission of diseases and infections from donor to recipient and cost. Allografts and xenografts produce poor clinical outcome and the rejection of the graft is more likely and aggressive [1]. To solve these limitations, scaffolds can be the solution. Tissue engineering scaffolds can be used to support the self-healing mechanism of the human body and promote the regrowth of damaged tissue. These scaffolds can degrade after successful tissue regeneration minimizing the immune reaction and the need for revision surgery [2].

Electro-active scaffolds are important substrates for bone tissue engineering as cells need to be stimulated, increasing cell proliferation and differentiation. To produce these scaffolds, we are exploring different routes including the use of conductive polymers mixed with non-conductive ones, or the use of graphene mixed with non-conductive polymers. This paper investigates the use of polycaprolactone/pristine graphene for bone applications. Scaffolds with different material compositions are assessed from morphological, mechanical and graphene dispersion point.

B.2 Materials and methods

B.2.1 Materials

The materials considered in the research are Poly (ε-caprolactone) (PCL) and pristine graphene.
B.2.1.1 PCL

PCL used in the research is Capa 6500 (Perstorp, UK). PCL is a semicrystalline polymer with low melting temperature. It has good biocompatibility and biodegradability, which means when the scaffold is implanted in the human body, the patient will not reject it and after the healing process, the scaffold would disappear being replaced by new bone tissues. The degradation mechanism of PCL is characterized by random hydrolysis ester cleavage and weight loss. It has been founded that the degradation of PCL with high molecular weight requires 3 years for complete removed from the human body [3]. However, the degradation of the scaffolds strongly depends on the molecular weight of PCL, scaffold topology and processing conditions. Relevant properties of PCL are listed in Table B.1.

Table B.1 Material properties of PCL.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>1.146 g/mL at 25 °C</td>
</tr>
<tr>
<td>Melting point</td>
<td>58 – 60°C</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>50000</td>
</tr>
<tr>
<td>Specific heat of vaporization</td>
<td>1KJ/g</td>
</tr>
<tr>
<td>solubility parameter (δ)</td>
<td>9.34 – 9.43 (cal/cm³)</td>
</tr>
</tbody>
</table>

B.2.2.2 Pristine graphene

The graphene used in this work was prepared by a water-assisted liquid phase exfoliation of graphite. Briefly, 50 mg graphite microcrystalline powder (-325 mesh, 99.995 % pure) purchased from Alfa Aesar was immersed with water-N-methylpyrrolidinone (NMP) mixture with 0.2 mass fraction of water. The initial concentration of graphite was fixed at 5 mg mL⁻¹ for exfoliation. N-methylpyrrolidinone (NMP, 99% extra pure) was purchased from ACORS ORGANICS. The materials were batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H) at a fixed nominal power and frequency 100 W and 37 kHz respectively. Sample dispersions were hanged on for overnight in between sonication and centrifugation and were centrifuged at 3000 rpm for 30 minutes with Hettich, EBA20. Upper 3/4 th of the colloidal supernatant were collected and dried in an oven to yield the graphene nanosheets.
Appendix B

B.2.2 Scaffold design and fabrication

A three-dimensional block model (30x30x6 mm$^3$) (Figure B.1a) was initially designed in a CAD software (SolidWorks, Dassault Systemes). A 0°/90° lay-down pattern was adopted to obtain pores with a regular square geometry while maintaining a constant filament distance of 680μm.

Scaffold fabrication process was controlled by four processing parameter: melting temperature (90°C); slice thickness (220 μm); screw rotation velocity (22 rpm) and deposition velocity (20 mm/s).

PCL scaffolds containing different concentrations of graphene were produced using a screw-assisted additive manufacturing system from RegenHU (3DDiscovery, Switzerland) (Figure B.1b and B.1c). In this process, the material is molten in the liquefier tank and pressed to the barrier screw by compressed air. The extruder barrier screw and the material liquefier tank can both be heat up to 250°C and thus enable processing a broad range of polymers and polymer-based composite.

![Figure B.1](image)

**Figure B.1** (a) CAD model; (b) PCL scaffold (30mm x 30mm x 6mm); (c) PCL/pristine graphene scaffold (30mm x 30mm x 6mm).

B.2.3 Characterisation

B.2.3.1 Morphological characterization

Scanning electron microscope (SEM) is used for morphological analysis, namely, to access pore shape, pore size and pore interconnectivity. The equipment used was Quanta 200 SEM, which is able to achieve 3.0 nm resolution at 30 kV. Its accelerating voltage is between 200 V and 30 kV. In this study, all images were taken at 10 KV for high resolution. The software imageJ was used for measurement.
Porosity was evaluated using the following methodology: (1) measuring the weight and volume of each scaffold; (2) calculate the apparent density of each scaffolds; (3) applying the following equation:

\[
\text{Porosity} = (1 - \rho^*/\rho_{\text{sub}}) \times 100
\]

where \( \rho^* \) is the apparent density of the cellular structure (scaffold) and \( \rho_{\text{sub}} \) is the density of the original substance (\( \rho_{\text{sub}} = 1,145 \text{ g/cm}^3 \)).

**B.2.3.2 Mechanical characterization**

Compression tests were performed to assess the effect of graphene concentration on the mechanical properties of scaffolds. Blocks of 5.0mm in length (l), 5.0mm in width (w) and 5.72mm in height (\( h_0 \)) were considered. All tests were carried out on scaffolds in dry state at a rate of 1 mm/min up to a strain limit of 0.3mm/mm (30%), using the INSTRON 4507 testing system equipped with a 1kN load cell. During the uni-axial compression tests, the software captured force \( F \) and corresponding displacement values, which were converted into engineering stress \( \sigma \) and strain \( \varepsilon \) as follows:

\[
\sigma = \frac{F}{A} \quad \text{(B.2)}
\]
\[
\varepsilon = \frac{\Delta h}{h_0} \quad \text{(B.3)}
\]

where A is the initial sample cross section area and \( \Delta h \) is scaffold height variation. The obtained stress–strain data was further processed to determine the compression modulus E according to the procedure reported by Fiedler et al [2].

**B.2.3.3 Dispersion analysis**

*Ex situ* characterization of the graphene dispersion were performed by transmission electron microscopy (TEM) and micro Raman. Cold-field emission Cs-corrected TEM (JEOL ARM-200F, Japan) with 200 kV accelerating voltage was used. Carbon-coated copper grids (400 mesh) were used in the TEM sample preparation. Raman scattering studies were performed at room temperature with a JASCO 5100 spectrometer (\( \lambda = 533 \text{ nm} \)).
**B.3 Results and discussion**

**B.3.1 Morphological characterization**

SEM micrographs (Figure B.2) show a well-defined porous structure. It is also possible to observe that an increment in pristine graphene concentration from 0% to 0.75% caused the increasing of filament diameter from 343±5μm to 379±14μm, resulting in slight decrease in pore size and porosity, as shown in Table B.2. Figure B.3 shows the corresponding variation trends.

![SEM micrographs](image)

**Figure B.2** SEM images of scaffolds (top view) produced using different graphene concentration (a) 0 wt.%; (b) 0.25 wt.%; (c) 0.50 wt.%; (d) 0.75 wt.%.

**Table B.2** Morphological characteristics of scaffold structures for different graphene concentration.

<table>
<thead>
<tr>
<th>Pristine graphene concentration (wt.%)</th>
<th>Filament diameter (μm)</th>
<th>Pore size (μm)</th>
<th>Weight (g)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>343±5</td>
<td>368±24</td>
<td>3.2778</td>
<td>44.06</td>
</tr>
<tr>
<td>0.25</td>
<td>361±5</td>
<td>366±11</td>
<td>3.3113</td>
<td>43.53</td>
</tr>
<tr>
<td>0.50</td>
<td>367±4</td>
<td>347±13</td>
<td>3.3183</td>
<td>43.41</td>
</tr>
<tr>
<td>0.75</td>
<td>379±14</td>
<td>343±6</td>
<td>3.3130</td>
<td>43.38</td>
</tr>
</tbody>
</table>
Appendix B

Figure B.3 Variation of filament diameter and pore size as a function of pristine graphene concentration

B.3.2 Mechanical characterization

According to mechanical compression tests, the mechanical behavior of scaffolds strongly depends on the amount of pristine graphene. Results show that by increasing the concentration of pristine graphene from 0.25\% to 0.75\%, the mechanical properties increased from 80 MPa to around 130 MPa (Table B.3 and Figure B.4). Compared to PCL scaffolds, a slightly decrease on the mechanical properties was observed for scaffolds containing 0.25\% pristine graphene, which can be related to the stress concentration in the interface between the polymer and graphene. For this composition, this effect superposed the reinforcement effect of pristine graphene.

Table B.3 Mechanical properties of scaffolds for different pristine graphene concentration values

<table>
<thead>
<tr>
<th>Pristine graphene concentration (wt.%)</th>
<th>Compression modulus, $E$ [MPa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82.198±6.773</td>
</tr>
<tr>
<td>0.25</td>
<td>80.582±3.081</td>
</tr>
<tr>
<td>0.50</td>
<td>109.330±5.334</td>
</tr>
<tr>
<td>0.75</td>
<td>128.742±6.893</td>
</tr>
</tbody>
</table>
Appendix B

Figure B.4 Compression test results for scaffolds containing different pristine graphene concentrations

B.3.3 Dispersion analysis

TEM images of pristine graphene (Figure B.5a) revealed as-exfoliated graphene with lateral size 1500–2000 nm and few layer structure. Raman spectroscopic analysis was performed to study the exfoliated graphene, PCL and PCL/pristine graphene composite. The exfoliated graphene (Figure B.5b) shows three typical peaks assigned as D, G and 2D bands at 1340, 1577 and 2692 cm\(^{-1}\), respectively, which are consistent with previous reports [4, 5]. Moreover, the Raman spectrum of PCL shows several characteristic Raman peaks of PCL at 1438 and 2927 cm\(^{-1}\). The Raman spectrum of PCL/pristine graphene composite also exhibits the D, G and 2D bands of graphene, indicating the presence of graphene in the PCL/pristine graphene scaffolds.
Figure B.5 (a) TEM image of pristine graphene; (b) Raman spectra of PCL, pristine graphene, and PCL/pristine graphene scaffold; (c) SEM and (d) charge-coupled detector images of graphene/PCL scaffold (0.5 wt.%); (e) Raman mapping of PCL/pristine graphene scaffold (0.5 wt.%).

To further study the dispersion of graphene in PCL matrix, micro Raman mapping was performed. Figures B.5c and B.5d show the SEM and charge-coupled detector images of PCL/pristine graphene scaffold for the Raman mapping study. The mapping area is highlighted in red rectangular in Figures B.5c and B.5d. Figure B.5e presents the representative Raman two-dimensional mapping of the PCL/pristine graphene scaffold sample. The false color maps were generated by scanning a 532 nm laser beam over an area of 250 × 450 μm² in 5 μm steps. The mapping intensity indicates the ratio of integrating intensity of the G band from 1574 to 1580 cm⁻¹ for graphene to the integrating intensity from 1435 to 1441 cm⁻¹ for PCL. Figure B.5e shows uniform distribution of pristine graphene in the scaffold sample.

B.4 Conclusions

This paper presents the morphological and mechanical properties of PCL/pristine graphene scaffolds containing different concentrations of graphene. Results indicate that the
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Graphene nano flakes are well distributed in the PCL fibers confirming that the AM system considered in this research is a viable technique to produce electro-active scaffolds. The results also show that the addition of pristine graphene has an impact on the topology and mechanical properties of the produced scaffolds.

References


Appendix C

3D PCL/Graphene Scaffold for Bone Tissue Engineering


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2 Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo (USP), Ribeirão Preto, SP, Brazil

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Appendix C

C.1 Introduction

Bone defects assume importance in growing prevalence of chronic health conditions since fractures and critical defects should increase as the population ages [1]. This problem is worse if we consider also significant bone damage due to accidents and diseases. To solve large-scale bone damage, bone grafting is required [2]. However, natural bone grafts (allografts, autografts and xenografts) present several problems related to site morbidity, disease transmission, accessibility and costs [3, 4]. To solve these limitations, synthetic grafts (scaffolds) represent a promising approach for tissue engineering. These scaffolds are made with biocompatible and biodegradable polymers, ceramics and composites and they must provide the adequate environment for cell attachment, proliferation and differentiation [5].

As cells proliferation and differentiation is a regulated process, which depends on scaffold topology and material cues and also on specific stimulation mechanisms (load or electromagnetic), we are exploring the use of electro-active scaffolds for bone regeneration. To produce these scaffolds, we are exploring different routes including the use of conductive polymers mixed with non-conductive ones, or the use of graphene mixed with non-conductive polymers. This paper discusses the use of polycaprolactone/pristine graphene for bone applications.

C.2 Materials and methods

C.2.1 Materials

C.2.1.1 PCL

PCL used in the research is Capa 6500 (Perstorp, UK). PCL is a semicrystalline polymer with low melting temperature, good biocompatibility and biodegradability. Relevant properties are listed in Table C.1.
Table C.1 Material properties of PCL.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting point</td>
<td>58 to 60 °C</td>
</tr>
<tr>
<td>Glass transition temperature</td>
<td>≈ -60 °C</td>
</tr>
<tr>
<td>Density</td>
<td>1.146 g/mL at 25 °C</td>
</tr>
<tr>
<td>Molecular weight (Mw)</td>
<td>50000 g/mol</td>
</tr>
<tr>
<td>Specific heat of vaporization</td>
<td>1 kJ/g</td>
</tr>
</tbody>
</table>

C.2.1.2 Pristine graphene

Pristine graphene was prepared by a water-assisted liquid phase exfoliation of graphite. The preparation procedure can be found in [6, 7].

C.2.1.3 PCL/Pristine graphene blends

Melt blending was used to prepare PCL/pristine graphene pellets containing different concentrations of pristine graphene. The concentration of pristine graphene (0.13%, 0.5% and 0.78%) was assessed using thermogravimetric analysis as reported by Wang et al [7].

C.2.2 Scaffold design and fabrication

Three-dimensional scaffolds (30mm x 30mm x 6 mm) were produced using a 0°/90° lay-down pattern, regular square pores, filament diameter of 330μm and constant filament distance of 680μm.

The 3D Discovery system (RegenHU, Switzerland), which is a screw-assisted extrusion additive manufacturing system (Figure C.1), was used to produce the scaffolds. Processing conditions are presented in Table C.2.
Table C.2 Processing conditions used to produce the PCL and PCL/pristine graphene scaffolds.

<table>
<thead>
<tr>
<th>Melting Temperature</th>
<th>Slice thickness</th>
<th>Screw Rotational Velocity</th>
<th>Deposition Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>90 °C</td>
<td>220 μm</td>
<td>22 rpm</td>
<td>20 mm/sec</td>
</tr>
</tbody>
</table>

Figure C.1 3D Discovery system.

C.2.3 Characterization

C.2.3.1 Morphological characterization

Scanning electron microscope (SEM) (Quanta 200 SEM) and Micro Computer Tomography, μCT (320/225 Kv Nikon XTEC) was used for morphological analysis, namely, to access pore shape, pore size and pore interconnectivity.

C.2.3.2 Graphene dispersion

Ex situ characterization of the graphene dispersion were performed by transmission electron microscopy (TEM) and micro Raman. Cold-field emission Cs-corrected TEM (JEOL ARM-200F, Japan) with 200 kV accelerating voltage was used. Carbon-coated copper grids (400 mesh) were used in the TEM sample preparation. Raman scattering studies were performed at room temperature with a JASCO 5100 spectrometer (λ= 533 nm).
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C.2.3.3 Apparent water-in-air contact angle

The contact angle enables to understand scaffolds’ hydrophilic/hydrophobic characteristics. A contact angle below 90° means a hydrophilic surface while a contact angle values above 90° corresponded to hydrophobic surfaces. Static contact angle measurements were performed using the equipment OCA 15 (Data Physics) and deionized water (4µl of volume drop, 1µl/s of velocity) and the drop shape was recorded with a high speed framing camera after a static time of 20 s.

C.2.4 Biological evaluation

C.2.4.1 Scaffold preparation

Scaffolds were cut into small blocks (11 mm x 11 mm x 3 mm) with the help of specially designed fixture and clamp, and then sterilised by immersing in 70% ethanol for 1 hour. After sterilization and before cell seeding, scaffold samples were placed into 24-well plates, rinsed twice with phosphate buffer solution (PBS), soaked in cell growth media (αMEM / 5% fetal bovine serum) and incubate during 2 hours in a 37 ºC incubator.

C.2.4.2 Cell seeding

MC3T3-E1 osteoblasts were used for in vitro tests. Cells were cultured in T75 tissue culture flasks (Corning, USA) with cell culturing media (MEM/10% FBS+1mM Sodium Pyruvate) until 80% confluence, harvested by the use of 0.05% trypsin-EDTA solution (Invitrogen, USA), and finally seeded on the scaffolds (1 mL of medium containing around 5 x 10^4 cells per sample). The cell-seeded scaffolds were incubated at standard conditions (37 ºC under 5% CO₂ and 95% humidity).

C.2.4.3 Cell viability/proliferation

During the culturing process, cell viability/proliferation was measured at 1, 3 and 7 days, in triplicate. At each time point, cell-seeded scaffolds were transferred to a new 24 well plate. 1 mL Alamar Blue premix solution (1% dilution in culture media) was added to each well and then incubated for 4 hours under standard conditions. After incubation, 200 µL of each sample was transferred to a 96-well plate and the fluorescence intensity was measured at 530
nm excitation wavelength and 620 nm emission wavelength by a microplate reader (Synergy 2, BioTek, USA). Experiments were performed twice.

**C.2.5 Data Analysis**

All data were represented as mean ± standard deviation. Biological results were subjected to one-way analysis of variance (one-way ANOVA) and Tukey’s post-hoc test using GraphPad Prism software. Significance levels were set at $p < 0.05$.

**C.3 Results and discussion**

**C.3.1 Morphological characterization**

SEM images (Figure C.2) and µCT (Figure C.3 and C.4), show that the 3D Discovery system allows the fabrication of scaffolds with well defined pores, uniform pore distribution and pore interconnectivity.

**Figure C.2** SEM image. PCL/Pristine Graphene scaffold (0°/90°).

**Figure C.3** µ-CT image. PCL/Pristine Graphene scaffold (0°/90°).
C.3.2 Dispersion analysis

TEM images of pristine graphene (Figure C.5a) revealed as-exfoliated graphene with lateral size 1500–2000 nm and few layer structure. Raman spectroscopic analysis was performed to study the exfoliated graphene, PCL and PCL/pristine graphene composite. The exfoliated graphene (Figure C.5b) shows three typical peaks assigned as D, G and 2D bands at 1340, 1577 and 2692 cm$^{-1}$, respectively, which are consistent with previous reports [8, 9]. Moreover, the Raman spectrum of PCL shows several characteristic Raman peaks of PCL at 1438 and 2927 cm$^{-1}$. The Raman spectrum of PCL/pristine graphene composite also exhibits the D, G and 2D bands of graphene, indicating the presence of graphene in the PCL/pristine graphene scaffolds.

Figure C.5 Dispersion evaluation. (a) TEM image of Pristine graphene scaffold; (b) Raman spectra of PCL, pristine graphene and PCL/pristine graphene scaffold.

To further study the dispersion of graphene in PCL matrix, micro Raman mapping was performed. Figures C.6 shows the SEM and charge-coupled detector images of PCL/pristine graphene scaffold for the Raman mapping study. The mapping area is highlighted in red rectangular in Figures C.6a. Figure C.6b presents the representative
Raman two-dimensional mapping of the PCL/pristine graphene scaffold sample. The false color maps were generated by scanning a 532 nm laser beam over an area of 250 × 450 μm² in 5 μm steps. The mapping intensity indicates the ratio of integrating intensity of the G band from 1574 to 1580 cm⁻¹ for graphene to the integrating intensity from 1435 to 1441 cm⁻¹ for PCL. Results show a uniform distribution of pristine graphene.

![Raman mapping](image)

**Figure C.6** (a) SEM of 0.50 wt.% graphene scaffold; (b) Raman mapping of 0.50% graphene scaffold.

### C.3.3 Apparent water-in-air contact angle

Figure C.7 shows the contact angle values for the scaffolds considered in this research work. The results show a decrease in the contact angle, especially to 0.50% and 0.78%, which are statically different from only PCL scaffolds, moving towards a less hydrophobic characteristic.

![Contact Angle](image)

**Figure C.7** Contact angle values for scaffolds containing different pristine graphene concentrations. *Statistical evidence (p < 0.05) analyzed by one-way ANOVA, and Tukey post test.
C.3.4 Biological analysis

The Alamar Blue assay was performed to evaluate cell viability and cell proliferation on PCL and PCL/pristine graphene. MC3T3-E1 osteoblasts were used. The fluorescence intensity after measurement is given in Figure C.8. Higher fluorescence intensity corresponds to more cell metabolically active. It can be observed that at day 1, scaffolds containing 0.78% pristine graphene exhibited statistically higher fluorescence intensity compared to the others compositions, which could mean more efficient cell attachment on graphene composites. On day 3, all PCL/pristine graphene scaffolds exhibited greater fluorescence intensity corresponding to a high cellular activity. At day 3, it is also possible to observe that the results obtained for scaffolds containing 0.78% of pristine graphene were statistically higher than scaffolds containing 0.13% of pristine graphene. Similar trend was also observed on day 7, with all PCL/pristine graphene scaffolds presenting higher cell viability/proliferation rates than pure PCL scaffolds. These results suggest that the addition of pristine graphene has a positive impact on cell viability/proliferation. It is also possible to observe that throughout the time points, the fluorescence activity increased, which indicates an increase in the cell proliferation rate, showing that all scaffolds were able to provide a good structure to support cell proliferation.

Figure C.8 Cell viability/proliferation evaluated after 1, 3 and 7 days using Alamar Blue assay.
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C.4 Conclusion

PCL/pristine graphene scaffolds produced using a screw-assisted extrusion-based additive manufacturing system present well defined pore shapes, pore distribution and pore interconnectivity, with well distributed pristine graphene flakes. Cell viability tests, using osteoblast-like cells show that the addition of pristine graphene increases both cell attachment and proliferation.

References


[9] Bracamonte M, Lacconi G, Urreta S, Foa Torres L. On the nature of defects in
Appendix D

Assessment of PCL/Carbon Material Scaffolds for Bone Regeneration


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Abstract: Biomanufacturing is a relatively new research domain focusing on the use of additive manufacturing technologies, biomaterials, cells and biomolecular signals to produce tissue constructs for tissue engineering. For bone regeneration, researchers are focusing on the use of polymeric and polymer/ceramic scaffolds seeded with osteoblasts or mesenchymal stem cells. However, high-performance scaffolds in terms of mechanical, cell-stimulation and biological performance is still required. This paper investigates the use of an extrusion additive manufacturing system to produce poly(ε-caprolactone) (PCL), PCL/graphene nanosheet and PCL carbon nanotube (CNT) scaffolds for bone applications. Scaffolds with regular and reproducible architecture and uniform dispersion of carbon materials were produced and evaluated in terms of carbon material concentration and biological performance. Results suggest that the addition of both graphene and CNT can improve the biological performance of the scaffolds, while graphene presented better enhancement effect than CNT.
D.1 Introduction

Bone defects due to chronic health conditions and fractures are significantly growing associated to population age. This problem is worse if we consider also significant bone defects due to accidents and diseases. To solve large-scale bone damage, bone grafting is usually required [1, 2]. However, natural bone grafts (allografts, autografts and xenografts), the common clinical approach, present several problems related to site morbidity, disease transmission, accessibility and costs. To solve these limitations, synthetic grafts (scaffolds) represent a promising approach for tissue engineering [3-5]. These scaffolds are made with biocompatible and biodegradable polymers, ceramics and composites and they must provide an adequate environment for cell attachment, proliferation and differentiation [6-8].

As cell proliferation and differentiation is a regulated process, which depends on scaffold topology and material cues and also on specific stimulation mechanisms (load or electromagnetic), we are exploring the use of electro-active scaffolds for bone regeneration. To produce these scaffolds, different routes are being investigated including the use of conductive polymers mixed with non-conductive ones, or the use of carbon materials mixed with non-conductive polymers. This research assesses two different types of carbon material electro-active polymeric scaffolds: PCL/graphene and PCL/CNT. The biological behaviour of these two types of scaffolds is investigated using human adipose-derived stem cells (hADSCs) and their performance compared to PCL scaffolds. All scaffolds were produced by an extrusion additive manufacturing system.

D.2 Materials and methods

D.2.1 PCL

PCL (CAPA\textsuperscript{TM} 6500, Mw = 50,000 g/mol), purchased from Perstorp (Cheshire, UK) in the form of 3 mm pellets, was used to produce the scaffolds. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm\textsuperscript{3}, a melting temperature between 58-60 °C, and a glass transition temperature of -60 °C.
D.2.2 Graphene

Graphene nanosheets (3-4 layers, ~500 um surface diameter) were prepared via water-assisted liquid phase exfoliation of graphite. Briefly, 50 mg microcrystalline graphite powder (325 mesh, 99.995% pure, purchased from Alfa Aesar, UK) was immersed in N-methyl-2-pyrrolidone (NMP) mixture with a 0.2 mass fraction of water. The initial concentration of graphite was fixed at 5 mg mL$^{-1}$ for exfoliation. NMP, 99% extra pure was purchased from Acros Organics (USA). The materials were batch sonicated for 6 hours in a bath sonicator (Elma sonic P60H, Switzerland) at a fixed nominal power and frequency of 100 W and 37 kHz respectively. Sample dispersions were hanged on for overnight in between sonication and centrifugation and were centrifuged at 3000 rpm for 30 minutes using a Hettich EBA20 centrifuge (Germany). The upper 75% of the colloidal supernatant were collected and dried in an oven to yield the graphene nanosheets.

D.2.3 Carbon nanotube

Multiwall carbon nanotubes (MWCNTs) (approximate dimension 20 nm in diameter and 1-8µm in length) were synthesized using a catalytic chemical vapour deposition (CVD). In brief, Fe films (3.0 nm thickness) and an alumina (Al$_2$O$_3$) support layer (40 nm thickness) sputtered onto 1 cm × 1 cm polished silicon (Si) substrates with a silicon dioxide (SiO$_2$) layer of 600 nm were used as the catalyst films for CNT growth. The CNT were synthesized at one atmospheric pressure in a 3 inch quartz tube furnace with two process steps, including catalyst particle formation and CNT growth. For a typical catalyst particle formation experiment, we first flowed 200 sccm (sccm denotes standard cubic centimetre per minute at 1 atm) helium (He) and 1800 sccm hydrogen (H$_2$) for 15 minutes while ramping the temperature from room temperature to 810 °C, then keep same gas flow rates for 15 minutes to anneal the catalyst particles. Then CNT growth began for 10 minutes using a water-assisted CVD process at 810 °C with the gas mixture of 100 sccm ethylene (C$_2$H$_4$) and 900 sccm H$_2$, and 100 ppm water vapour as the carbon precursor and the catalyst preserver and enhancer, respectively. Water vapour of 100 ppm was supplied by passing 1000 sccm He carrier gas through a water bubbler with deionized (DI) water at STP (STP denotes standard condition for temperature and pressure, NIST version) condition. Water vapour concentration was monitored by a single-channel moisture meter.
(MMS 35-211-1-100, General Electric, USA) coupled with a moisture probe (M2LR, General Electric, USA) installed before the CVD reactor. All gas flows were controlled by mass flow controllers that were carefully calibrated before experiments to precisely control the gas concentrations in the CVD reactor.

**D.2.4 Scaffold design and fabrication**

Rectangular scaffolds of 10 x 10 x 3 mm³ (length x width x height) were designed with a lay-down pattern of 0/90° and filament distance of 680µm. Scaffolds were fabricated through a screw-assisted additive manufacturing system (3D Discovery from RegenHU, Villaz-St-Pierre, Switzerland). PCL/carbon material pellets were initially prepared by melt blending considering different material concentrations (1 wt.% and 3 wt.%). The printing conditions were: heating temperature of 90°C, deposition velocity of 20 mm/s; slice thickness of 280µm and screw rotational velocity of 22 rpm. Printing nozzle inner diameter was 330µm.

**D.2.5 Thermogravimetric analysis**

The thermal degradation and carbon material content in the scaffolds was assessed using a TA Instruments Q500 TGA (USA) equipped with an evolved gas analysis furnace. Thermogravimetric analysis (TGA) was performed on PCL scaffolds as controls, and carbon material loaded PCL scaffolds. Scans were performed in an air atmosphere (flow at 60 mL/min) with a temperature range from room temperature to 560°C at a rate of 10°C/min. Measurements were taken using sample mass of 6 ± 1 mg in platinum pans. The weight losses of the PCL/carbon material composite structures were monitored and used to calculate the final carbon material contents.

**D.2.6 Biological assessment**

Cell proliferation tests were performed using hADSCs purchased from STEMPRO, Invitrogen (Waltham, MA, USA). Before cell seeding, scaffolds were sterilized by soaking in 70% ethanol for 2 hours. After sterilisation, samples were rinsed twice with phosphate buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), transferred to 24-well plates and air-dried for 24 hours at room temperature. 50,000 cells were seeded
on each sample, including PCL scaffolds, PCL/graphene scaffolds and PCL/CNT scaffolds.

Cell viability/proliferation behaviour was assessed through Alamar Blue assay (reagents from Sigma-Aldrich, Dorset, UK). Cell viability/proliferation rate was measured at 1, 3, 7 and 14 days after cell seeding. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate and 0.7 ml of Alamar Blue solution was added to each well, the plate was incubated for 4 hours under standard condition (37 °C, 5% CO₂ and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland).

Cell attachment and distribution was also assessed using laser confocal microscopy, with cell nuclei stained. After cell culture for 14 days, scaffolds were removed from 24-well cell culture plate, rinsed twice in phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA), fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 minutes at room temperature. After fixation, samples were rinsed twice with PBS for the removal of formalin, then permeabilised with 0.1% Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at room temperature for 10 minutes, rinse twice for the removal of Triton-X100. Cell nuclei were stained blue by soaking scaffolds in a PBS solution containing 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, Dorset, UK) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 min prior to removal, rinsed twice thoroughly with PBS. Confocal images were obtained on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope.

D.3 Results and discussion

D.3.1 Thermogravimetric analysis

The final content of carbon material in the PCL/carbon material composite scaffolds was calculated by TGA. The results (Table D.1) suggest that the carbon material was effectively incorporated into the polymer scaffolds, without significant losses during the
melt blending process. Results also indicate that no degradation events occur during the scaffold fabrication process, since the extrusion temperature was 90 °C.

**Table D.1** Carbon material loading verification, assessed by TGA.

<table>
<thead>
<tr>
<th>Target carbon material loading</th>
<th>Determined carbon material concentration (%)</th>
<th>$T_d$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 wt.%</td>
<td>-</td>
<td>559.6</td>
</tr>
<tr>
<td>1 wt.% (graphene)</td>
<td>$1.05 \pm 0.12$</td>
<td>559.7</td>
</tr>
<tr>
<td>1 wt.% (CNT)</td>
<td>$1.03 \pm 0.54$</td>
<td>559.7</td>
</tr>
<tr>
<td>3 wt.% (graphene)</td>
<td>$3.08 \pm 0.12$</td>
<td>559.7</td>
</tr>
<tr>
<td>3 wt.% (CNT)</td>
<td>$3.04% \pm 0.62$</td>
<td>559.8</td>
</tr>
</tbody>
</table>

**D.3.2 Biological assessment**

Cell viability/proliferation results for all scaffolds considered in this study are presented in Figure D.1. The results show that for all types of scaffolds, the fluorescence intensity increases from one time point to another, suggesting that the scaffolds fabricated with the additive manufacturing system are suitable substrates for cell proliferation. At both day 1 and day 3, only PCL/graphene scaffolds presented better biological behaviour compared with PCL scaffolds, showing significant higher fluorescence intensity. Start with day 7, 3 wt.% PCL/CNT scaffolds presented higher cell proliferation rate than PCL scaffolds, while on day 14, all carbon material scaffolds presented a better biological behaviour compare to PCL scaffolds. Results also show that for the same content on carbon material, cells proliferate faster in PCL/graphene scaffolds compare to PCL/CNT scaffolds.
Figure D.1 Cell viability/proliferation test result (fluorescence intensity) of cell seeded scaffolds (PCL, PCL/graphene and PCL/CNT) at day 1, 3, 7 and 14 of cell culturing.

Confocal microscopy images (Figure D.2) present the cell attachment and distribution after proliferation for 14 days. It can be observed that all scaffolds can support the cell proliferation along the fibre direction. PCL/graphene and PCL/CNT scaffolds presented better confluency and cell distribution.

Figure D.2 Confocal microscope images of all types of cell seeded scaffolds, 14 days after cell culture. (a) PCL scaffold; (b) 1 wt.% PCL/graphene scaffold; (c) 3 wt.%
PCL/graphene scaffold; (d) 1 wt.% PCL/CNT scaffold; (e) 3 wt.% PCL/CNT scaffold.
Scale bar 750 µm.

D.4 Conclusion

This paper investigates the use of additive manufacturing to produce polymeric scaffolds incorporating carbon materials. PCL/graphene and PCL/CNT blends were efficiently prepared with a melt blending process. For the same amount of carbon materials, graphene seems to be more effective than CNT in stimulating cell proliferation.

References


