



Engineering of Osteochondral Grafts by Electrospinning

Pierre-Alexis Mouthuy

Linacre College, University of Oxford

A thesis submitted for the degree of
Doctor of Philosophy
in *Engineering Science*

Michaelmas 2011

Abstract

Articular cartilage (AC) morbidity represents a substantial burden to global and public health, and continues to afflict millions of people in the UK. This costs the economy more than £3 billion annually and requires approximately 80,000 knee replacements every year. To avoid these radical surgery procedures, various strategies have been recently developed to repair or restore AC through implantation of osteochondral grafts. However, their success remains limited, mainly with respect to the quality of the newly formed cartilage.

To contribute to the development of improved treatment options, this thesis explored a tissue engineering approach towards the potential use of the electrospinning technique to build osteochondral grafts. The main objectives of the research were to produce and characterise a range of electrospun membranes with various compositions, to construct three-dimensional (3D) scaffolds seeded with cells, and to study the release of therapeutic agents from electrospun materials.

Poly(lactic-co-glycolic acid) (PLGA) and Poly- ϵ -caprolactone (PCL) were the two FDA-approved polymers used to create the electrospun membranes. Collagen and hydroxyapatite were also added to increase the biocompatibility of the scaffolds. Tissue constructs were created by layering electrospun membranes with seeded sheets of human mesenchymal stem cells. Bovine Serum Albumin (BSA) and recombinant Transforming Growth Factor β 3 (TGF- β 3) were also incorporated into the fibres and used as model proteins to study the release of therapeutic agents from the core of co-axial electrospun fibres.

Results suggest that minor alterations in composition cause gradual but significant changes in morphology, surface properties, mechanical characteristics and biocompatibility of the scaffold. The combination of electrospinning and cell sheet engineering presents a unique and effective strategy that can be used to create 3D tissue constructs with high cell density and viability. Differentiation results also indicate that intrinsic properties of PLGA and PCL affect the chondrocyte phenotype. With regard to drug delivery, BSA and TGF- β 3 were successfully incorporated into electrospun materials and subsequently released into an aqueous environment. The release profiles recorded exhibited a pronounced initial burst release that was significantly reduced by mineral deposition onto the membranes.

To conclude, this thesis presents several contributions that demonstrate the use of multilayered electrospun scaffolds as a successful approach for the generation of tissue engineered osteochondral grafts. Furthermore, this work supports the use of electrospinning as a key technology for future AC repair.

Acknowledgments

This thesis would not have been possible without contribution of many people, to which I would like to express my deepest gratitude.

First of all, I would like to thank Dr. Cathy Hua Ye for her continuous help and support. It was very motivating and enjoyable to carry out my research under her supervision. I am also grateful to Professor Zhanfeng Cui for his highly valuable guidance and his numerous encouragements.

I am very thankful to Professor Andrew Carr and Professor Robert Brown for devoting their time as examiners and for their insightful comments.

It is with sincere appreciation that I thank Dr. Alison Crossley, Dr. Robert Jacobs, Dr. Heiko Shiffter and Dr. Marc Thompson for sharing their equipment and knowledge. I am also very grateful to James Fisk, Roger Lewis, Peter Rockett, and David Salisbury for their great help in designing and fabricating various pieces of equipment without which the realisation of this project would not have been possible. Big thanks also to the IBME staff for making this project progress faster through their daily support. My thanks are also going to Gabriella Chapman and Chris Salter for their assistance in electron microscopy.

I am indebted to many members of the Tissue Engineering group. In particular, I would like to thank Jangho Lee, Yang Liu, George Oommen, Sammy Sambu, Nikolas Weissmueller, Amy Jie Yang, Jinnan Zhang, and Yanxia Zhu. I also thank Dr. Yasser El-Sherbini and Dr. Renchen Liu for their various contributions and Dr. Julian George for his helpful comments during the write-up period.

It is with immense gratitude that I thank Chris Mooney Sensei, and all the members of the Ei Mei Kan dojo. The regular practice of aikido and related disciplines have tremendously help me to keep myself focussed and grounded during the whole project.

Last but not the least, I would like to dedicate this thesis to my family and my friends as a recognition of their constant support and encouragement during these past years. Among them, a special thank goes to Jelena, whose smile and presence have enlightened my days ever since we met.

List of publications

Journal paper

- Mouthuy P.-A., Ye C. H., Triffitt J., Oommen G., Cui Z. (2010). Physico-chemical Characterisation of Functional Electrospun Scaffolds for Bone and Cartilage Tissue Engineering, *Proceedings of the Institution of Mechanical Engineers, Part H, Journal of Engineering in Medicine* 224, 1-14.

Book Chapter

- Mouthuy P.-A., Ye C. H. (2011). Biomaterials: electrospinning. In: M. Moo-Young. *Comprehensive Biotechnology*, 2nd Edition, Vol 5, 25, pp 23-36.

Conferences

- Mouthuy P.-A., Ye C. H. (2009). UKNSCN Scientific Conference, Oxford. "Towards engineering of osteochondral grafts" (Poster).
- Mouthuy P.-A., Ye C. H., Cui Z. (2009). Graduate seminar, Department of Engineering Science, Oxford. "Scaffold design for bone and cartilage tissue engineering" (Oral presentation).
- Mouthuy P.-A., Ye C. H. (2010). Termis EU, Galway, Ireland. "Physico-chemical Characterisation of Functional Electrospun Scaffolds for Bone and Cartilage Tissue Engineering" (Poster).
- Mouthuy P.-A., Ye C. H. (2010). Poster Presentation, Department of Engineering Science, Oxford. "Electrospinning and cell sheet engineering for bone-cartilage interface reconstruction" (Poster).
- Mouthuy P.-A., Ye C. H. (2011). Asian Pacific Termis meeting 2011, Singapore. "Electrospinning and cell sheet engineering for cartilage tissue engineering" (Oral presentation).

Abbreviations

- AC: Articular Cartilage
- AB: Alamar Blue
- ARS: Alizarin Red S
- BMP: Bone Morphogenetic Protein
- BSA: Bovine Serum Albumin
- CaP: Calcium Phosphate
- Col: Collagen Type I
- CNT: Carbon Nanotube
- DAB: 3,3'-diaminobenzidine tetrahydrochloride
- DHA: Deficient Hydroxyapatite
- ECM: Extracellular Matrix
- FAK: Focal Adhesion Kinase
- FITC: Fluorescein Isothiocyanate Conjugate
- FTIR: Fourier Transform Infrared spectroscopy
- HA: Hydroxyapatite
- HEPES: 2-(4-(2-hydroxyethyl)-1-piperazine)ethanesulfonic acid
- hMSC: human Mesenchymal Stem Cell
- HV: High Voltage
- IHC: Immunohistochemistry
- MCGS: Mesenchymal Cell Growth Supplement
- MC: Methylene Chloride (Dichloromethane)
- MSC: Mesenchymal Stem Cell

- MSCGM: Mesenchymal Stem Cell Growth Medium
- Mw: Molecular weight
- nHAp: Hydroxyapatite Nanoparticles
- NHS: National Health Service
- OA: Osteoarthritis
- OD: Osteochondral Defect
- PBS: Phosphate Buffered Saline
- PCL: Poly- ϵ -caprolactone
- PCL-c: blend material containing PCL, Collagen and nHAp
- PE: Polyester
- PHBV: Polyhydroxybutyrate-valerate
- PLGA: Poly(lactic-co-glycolic acid)
- PLGA-c: blend material containing PLGA, Collagen and nHAp
- RGD: Arginine-Glycine-Aspartic acid
- SBF: Simulated Body Fluids
- SBF10: 10x concentrated Simulated Body Fluids
- SEM: Scanning Electron Microscope
- TBS: Tris-Buffered Saline
- TGF- β 3: Transforming Growth Factor β 3
- TRIS: Tris(hydroxymethyl)aminomethane
- TE: Tissue Engineering
- WHO: World Health Organisation
- XPS: X-Ray Photoelectron Spectroscopy
- XRD: X-Ray Diffraction

Contents

1	Introduction	1
1.1	Background	1
1.2	Objectives	4
1.2.1	Design of a range of electrospun membranes	4
1.2.2	Creation of a multilayered tissue construct	5
1.2.3	Electrospun fibres as a drug delivery system	7
1.3	Scope	7
2	Literature Review	9
2.1	The bone-cartilage interface	9
2.1.1	Osteochondral defects	9
2.1.2	Structure and function of articular cartilage	11
2.1.3	Structure and function of bone	14
2.1.4	Bone-cartilage transition and interaction	15
2.2	Regenerative medicine and tissue engineering	16
2.2.1	Regenerative medicine	16
2.2.2	Tissue engineering of osteochondral grafts	17
2.2.3	Pre-clinical and clinical use of osteochondral engineered constructs	18
2.3	Stem cells for tissue engineering	21
2.3.1	Stem cell strategies	21
2.3.2	Adult stem cells for osteochondral TE	22
2.3.3	Stem cell fate	25
2.4	Scaffolds	27
2.4.1	Definition and design considerations	27
2.4.2	Conventional scaffold fabrication techniques	28
2.4.3	Scaffolds for osteochondral TE	28
2.5	Electrospinning	32
2.5.1	Principle of electrospinning	32
2.5.2	Electrospun materials: a wide range of possibilities	39
2.5.3	Biocompatibility of electrospun materials	44

2.5.4	Electrospun fibres for three dimensional tissue regeneration . . .	49
2.5.5	Electrospun materials for bone and cartilage TE	54
2.6	Drug delivery	58
2.6.1	Drug delivery from TE scaffolds	58
2.6.2	Drug delivery from electrospun fibres	59
2.6.3	Calcium phosphate materials for drug delivery	61
2.7	Summary	62
3	Materials and Methods	64
3.1	Materials and equipment	64
3.2	Electrospinning	67
3.2.1	Electrospinning apparatus	67
3.2.2	Preparation of the electrospinning apparatus	69
3.2.3	Preparation of electrospinning solutions	71
3.2.4	Preparation of electrospun membranes for TE applications	72
3.2.5	Preparation of calcium phosphate fibres	73
3.3	Physico-chemical characterisation	73
3.3.1	Scanning electron microscopy (SEM)	73
3.3.2	Fourier transform infrared spectroscopy (FTIR)	74
3.3.3	Tensile testing	74
3.3.4	Contact angle measurements	75
3.3.5	Simulated body fluids (SBF)	75
3.3.6	X-Ray diffraction (XRD)	76
3.3.7	X-Ray photoelectron spectroscopy (XPS)	76
3.3.8	Statistical analysis	76
3.4	Biological characterisation	77
3.4.1	hMSCs culture	77
3.4.2	Cell adhesion study	77
3.4.3	Cell proliferation study	78
3.4.4	Cell sheet engineering and multilayering	80
3.4.5	Assessing the cell coverage after transfer of the cell sheet	82
3.4.6	Differentiation of hMSCs in 3D constructs	82
3.4.7	Viability test	82
3.4.8	hMSCs surface markers	83
3.4.9	RT-PCR	83
3.4.10	Paraffin embedding and sectioning	85
3.4.11	Immunohistochemistry	85
3.5	Drug delivery from electrospun fibres	86
3.5.1	Preparation of drug releasing membranes	86
3.5.2	Preparation of the SBF solution	87

3.5.3	Membrane mineralisation	88
3.5.4	BSA release study	88
3.5.5	TGF release study	89
4	Functional Scaffolds for Bone & Cartilage TE	91
4.1	Introduction	91
4.2	Objective and methodology	92
4.3	Results	94
4.3.1	Membrane morphology	94
4.3.2	nHAp incorporation	94
4.3.3	Fourier transform infrared spectroscopy (FTIR)	97
4.3.4	Tensile testing	103
4.3.5	Biological characterisation	104
4.3.6	Simulated body fluids	110
4.4	Discussions	111
4.4.1	Membrane collection	112
4.4.2	Homogenisation and distribution of nHAp	113
4.4.3	Morphological characteristics	115
4.4.4	Surface properties	116
4.4.5	Mechanical properties	117
4.4.6	Biocompatibility	118
4.5	Conclusions	120
5	Electrospinning for Cartilage TE	122
5.1	Introduction	122
5.2	Objectives and methodology	124
5.3	Results	125
5.3.1	Membrane characteristics	125
5.3.2	Culture of cell sheet on electrospun membranes	127
5.3.3	Monophasic layered constructs	130
5.4	Discussions	137
5.4.1	Membrane characteristics	137
5.4.2	Electrospun membrane maintain the hMSCs phenotype	140
5.4.3	Cell coverage after transfer of the cell sheet	140
5.4.4	Integrity of the multilayered construct	141
5.4.5	Viability in the multilayered construct	141
5.4.6	Cell morphology in the multilayered construct	142
5.4.7	Gene transcription and expression	142
5.5	Conclusions	145

6	Protein Delivery from Electrospun Fibres	147
6.1	Introduction	147
6.2	Objectives and methodology	148
6.3	Results and discussions	150
6.3.1	BSA study	150
6.3.2	TGF- β 3 release study: a preliminary work	162
6.4	Conclusions	169
7	Creation of Calcium Phosphate Fibres	171
7.1	Introduction	171
7.2	Objectives and methodology	173
7.3	Results and discussions	174
7.3.1	Morphology of HA fibres	174
7.3.2	FTIR analysis	176
7.3.3	XPS analysis	178
7.3.4	XRD analysis	182
7.4	Discussions	182
7.5	Conclusions	188
8	Conclusions and Future Work	189
8.1	Conclusions	189
8.2	Future work	194
	Bibliography	199
	Appendix	I

List of Figures

2.1	Osteochondral defect	10
2.2	Zonal arrangement of AC	12
2.3	Calcified cartilage and underlying bone in human articular cartilage	16
2.4	Multi-lineage potential of adult mesenchymal stem cells	23
2.5	Strategies used to build heterogeneous osteochondral scaffolds	29
2.6	Papers published on electrospinning	32
2.7	Effect of the polymer solution concentration on electrospun fibres	34
2.8	Typical electrospinning set-up	35
2.9	Model electrospinning jet	37
2.10	Buckling observed at the collector	38
2.11	Nanostructures on electrospun fibres	41
2.12	Aligned electrospun fibres	42
2.13	Ceramic hollow fibres	43
2.14	Calcium phosphate crystals on electrospun fibres	43
2.15	Laser-machined pattern in electrospun mat	44
3.1	Electrospinning setup	67
3.2	Electrodes within the electrospinning glove box	69
3.3	Single channel and co-axial nozzles	70
3.4	Molecular structure of PLGA and PCL	71
3.5	Preparation of multilayered constructs	81
4.1	Morphologies and fibre diameter distributions of typical PLGA membranes	95
4.2	Morphologies and fibre diameter distributions of typical PLGA-collagen membranes	96
4.3	SEM images of electrospun fibres from Group 1	99
4.4	SEM images of electrospun fibres from Group 2	100
4.5	FTIR spectra obtained for groups 1 and 2	102
4.6	Contact angles for groups 1 and 2	103
4.7	Mechanical properties of group 1	105
4.8	Mechanical properties of group 2	106

4.9	Morphologies of hMSCs on the electrospun membranes shown by actin staining	108
4.10	Adhesion of hMSC on electrospun membranes shown by SEM	109
4.11	AB standard curve	109
4.12	AB reduction by hMSCs growing on electrospun membranes	110
4.15	Summarising diagram	121
5.1	Morphologies of PLGA-c and PCL-c mats	126
5.2	Fluorescence and SEM images of hMSCs on PLGA-c and PCL-c membranes	128
5.3	Flow Cytometry analysis of CD14, CD29, CD34, CD44, CD45, CD105, and CD166 expression on hMSCs	129
5.4	Morphology of the multilayered arrangement observed after 21 days of differentiation	130
5.5	Size and granularity of hMSCs in PLGA-c and PCL-c construct	132
5.6	mRNA expression in PLGA-c constructs	134
5.7	mRNA expression in PCL-c constructs	135
5.8	Multiphoton microscope images of tissue constructs cultured for 21 days .	136
5.9	Immunohistochemistry performed on PLGA-c tissue constructs	137
5.10	Immunohistochemistry performed on PCL-c tissue constructs	138
6.1	Morphology of the PCL-BSA/PLGA-col electrospun fibres	151
6.2	Morphology of the PCL-BSA/PLGA-col-nHAp electrospun fibres.	152
6.3	Fluorescence images of PCL-BSA/PLGA-collagen electrospun fibres . .	154
6.4	SEM images showing the morphology of PCL-nHAp/PLGA-col electrospun fibres	155
6.5	Standard curve for BSA	156
6.6	Accumulative profiles of BSA released from PCL-BSA/PLGA-col fibres .	158
6.7	BSA release profile from PCL-BSA/gelatin fibres and PCL-BSA/PLGA-col fibres	159
6.8	BSA release profile from PCL-BSA/PLGA-col-nHAp fibres	160
6.9	BSA release profile from mineralised PCL-BSA/PLGA-col fibres	161
6.10	BSA release profile from mineralised PCL-BSA/PLGA-col-nHAp fibres .	161
6.11	Fluorescence spectra assessing the BSA tertiary structure	163
6.12	Morphology of PCL-TGF- β 3/PLGA-col fibres	163
6.13	Standard curve for TGF- β 3 in PBS solution	164
6.14	TGF- β 3 release profile from PCL-TGF β 3/PLGA-col fibres	165
6.15	mRNA expression for the drug delivery experiment	168
7.1	Morphology of PLGA-nHAp fibres and sintered samples	175
7.2	FTIR spectra of sintered fibres	177

7.3	XPS spectrum obtained for PLGA-HA fibres	179
7.4	XPS spectrum obtained for HA powder	180
7.5	XPS spectrum obtained for sintered fibres	181
7.6	XRD pattern of sintered fibres	183
7.7	XRD pattern of sintered fibres	184
7.8	XRD pattern of sintered fibres	185

List of Tables

3.1	List of materials	64
3.2	List of equipment	66
3.3	Preparation of SBF solution	75
3.4	Composition of electrospinning solutions and voltage used for the electrospinning process	80
3.5	Primer sequences for GAPDH, Collagen II, Collagen X, Sox9 and Aggrecan	84
3.6	Probe sequences for GAPDH, Collagen II, Collagen X, Sox9 and Aggrecan	84
3.7	Volumes of PCR reagents	85
3.8	Preparation of SBF10 solution	88
4.1	Detailed compositions of Group 1 and Group 2 membranes	93
4.2	Characteristics of Group 1 membranes	97
4.3	Characteristics of Group 2 membranes	98
5.1	Morphological properties of PLGA-c and PCL-c mats	125
5.2	Mechanical properties of PLGA-c and PCL-c membranes	126
5.3	Fraction of the membrane covered by hMSCs three days after the transfer of a cell sheet	127
5.4	Viable, apoptotic and dead hMSCs in the multilayered arrangements . . .	131
6.1	Average fibre diameters observed for different coaxial fibre compositions	151
6.2	Characteristics of TGF- β 3-loaded coaxial fibres	164
7.1	Binding energies and proportion of elements measured in PLGA-HA fibres	179
7.2	Binding energies and proportion of elements measured in HA powder . .	180
7.3	Binding energies and proportions of elements measured in sintered fibres	181

Chapter 1

Introduction

1.1 Background

During normal physiological activity throughout life, articular cartilage (AC) is able to withstand high levels of friction and loading stresses. Mature AC continuously renews its extracellular matrix, but it has very poor self-repair capacity after injury. Cartilage is avascular and the normal mechanisms of tissue repair, via the recruitment or activation of progenitor cells through vascular invasion to the defect site, do not take place in this tissue (Mow and Rosenwasser, 1988). As a result, irreparable damages can be caused if an injury or infection occurs in the articular cartilage. Ultimately, such damage to the joint surface might lead to premature osteoarthritis (OA), a degenerative disease involving both AC and subchondral bone.

For young people, cartilage damage results mostly from sport and occupational in-

juries. However, there might be an important delay between the moment the lesion occurs and the moment the symptoms appear, which makes it difficult to know the history of the problem and its primary cause. In general, joint injuries sustaining haemarthrosis are more likely to result in OA. Disease, such as osteochondritis dissecans, are an other cause of damage leading to OA and it occurs primarily in young patients. However, the incidence of such disease is low, representing 30-70 patients per 500,000 population in the UK, according to the National Health Service (NHS, 2008). Knee instability, abnormal loading, and genetic predisposition are other factors that might lead to early cartilage degeneration. Last but not least, age is an important factor that increases the incidence of OA. The World Health Organisation (WHO) has estimated that 9.6% of men and 18.0% of women aged over 60 years have symptomatic OA (WHO, 2010). With regard to the prediction of demographic trends, these numbers are expected to increase further. In the UK, OA affects in total at least 8 million people, the knee being the most commonly affected joint.

The impact of OA on health and quality of life is considerable. In the UK, it is identified as the most common cause of disability. About 80% of those suffering from OA will have limitations to perform their daily activities due to pain and reduction of movement. For those involved in professional activities that involve physical work, it might result in the loss of employment. OA has also been linked to problems of depression and anxiety (WHO, 2010).

Every year, over 2 millions of british people consult their general practitioner with symptoms related to OA, which represents a significant cost to the NHS (Arthritis Research UK, 2008). OA also has an important impact on the UK economy, since around 36 million working days are lost each year because of osteoarthritis, costing the economy £3.2 billion in lost production. The total economic impact of OA is estimated as equivalent of 1% of gross national product per year (Arthritis and Musculoskeletal Alliance, 2004).

The damage to the joint can be such that it needs partial or total replacement. The first knee arthroplasty was reported in the early 50's of the last century. Since then, millions of arthroplasty procedures have been conducted. In 2010, more than 80,000 total knee replacements were performed in Great Britain (National Joint Registry, 2011). However, a growing tendency is to avoid radical solutions such as joint replacement. Therefore, various strategies have been proposed to repair or restore bone-cartilage (or osteochondral) defects rather than to resurface the joints. Approaches include surgical techniques such as mosaicoplasty, microfracturing, autologous chondrocyte implantation, in addition to tissue engineering. There are variations in diagnostics and surgical approaches and the number of knee repair procedures performed per year remains unknown for these strategies. Moreover, the methods are still restricted in use because of limitations related to size of the defect and site of implantation together with age of the patient. Furthermore, although pain may be relieved, the function of the tissue is rarely completely restored. In fact, clinical evidence has shown that disruption of the interface between bone and

cartilage can significantly impact on the integration of a graft, strongly suggesting that a functional bone-cartilage interface is critical for the repair of osteochondral damage.

1.2 Objectives

The objective of this research is to explore the possibility of using electrospun fibres to engineer osteochondral grafts, with a particular focus on creating a functional interface between bone and cartilage tissues. The project can be broken down into the following stages:

1. Design and fabrication of a range of electrospun membranes with varying compositions, with an effort to replicate the different parts of the bone-cartilage transition.
2. Creation of a three-dimensional (3D) tissue construct by layering the previously made electrospun membranes, seeded with human mesenchymal stem cells.
3. Investigation of the potential to improve the tissue construct by use of electrospun fibres as a delivery system for growth factors.

1.2.1 Design of a range of electrospun membranes

Electrospinning has recently attained broad interest in the field of regenerative medicine because it enables the production of sub-microfibres that can better mimic the features of the extracellular matrix (ECM) surrounding the cells in our body (Ashammakhi et al., 2007; Li et al., 2006). These electrospun fibres can be used as a support (or scaffold) for

tissue engineering, and can be tailored, in terms of architecture and composition, in order to optimise the cellular response.

The concept behind this approach is to enable the surgeon to select a particular combination from a wide range of membranes, and stack them to create a patch or pad with a gradient of properties tailored to fit the patient's damaged tissue. Soft scaffolds are desirable for the upper parts of the cartilage, and our design will involve synthetic biodegradable polymers such as Poly(lactic-co-glycolic acid) (PLGA) and Poly- ϵ -caprolactone (PCL). Natural polymer such as collagen type I will also be added to improve the biocompatibility of the material. For the area of cartilage close to the bone that is naturally calcified, we will create composite fibres by incorporating hydroxyapatite nanoparticles into the polymer matrix. Hydroxyapatite forms the primary mineral constituent of bone and is known to influence the process of osteogenic differentiation (Suominen et al., 1996). Furthermore, a thermal treatment will also be applied to those composite fibres containing hydroxyapatite to investigate possibility of creating ceramic fibres through the simultaneous decomposition of the polymer matrix and sintering of the nanoparticles.

1.2.2 Creation of a multilayered tissue construct

Multilayered tissue constructs will be created by alternating electrospun fibre layers with seeded cell sheets. Cell sheets can be obtained by a recent technology involving a thermoresponsive tissue culture surface, that detaches a whole cell sheet when exposed

to reduced temperatures (Yamato, 2004). The use of stem cells has huge potential, displaying the ability to self-renew and later differentiate into any desirable cell type through exposure to specific signalling molecules and conditions. In the context of engineering an osteochondral graft, this is particularly useful since the same cell source can potentially be used to produce both bone and cartilage tissues. The general concept that underlies the use of stem cells in the field of tissue engineering is to isolate cells directly from the patients or a donor, grow these cells *in vitro*, seed them onto the matrix, differentiate the resulting scaffold-cell assembly and finally implant the tissue graft into the damaged site, in the patient's body.

Further work will consist of culturing the resulting 3D constructs in chondrogenic conditions and characterise the differentiated tissue constructs in order to assess the potential of the scaffold for cartilage tissue engineering. The layer-by-layer approach makes it possible to generate a gradient within the tissue construct. However, this work will only focus on homogeneous constructs to limit the number of parameters involved. Two types of scaffolds, based on PLGA and PCL polymers, will be investigated to determine the influence of these materials on the cellular behaviour. Finally, a preliminary attempt to create a whole osteochondral construct will be carried out by using biphasic scaffolds, made of electrospun membranes stacked on hydroxyapatite disks.

1.2.3 Electrospun fibres as a drug delivery system

The incorporation of drugs into electrospun fibres is of growing interest within the scientific community (Huang et al., 2003; Sill and von Recum, 2008). The high specific surface area of fibrous scaffolds makes them a good candidate to serve as drug delivery system. Delivering growth factors from the fibres would indeed help to avoid the problems of storage and of supply of the molecule during the time of culture. Furthermore, this strategy may potentially lead to the creation of multiphasic tissues, since different signalling molecules can be used in different parts of the scaffold. During this work, we will study the effect of an additional polymer coating through co-axial electrospinning, the impact of the presence of hydroxyapatite nanoparticles in the polymer matrix, and the influence of mineralisation by immersion in simulated body fluids on the release profiles of bovine serum albumin. Preliminary work will also be carried out with the transforming growth factor, TGF- β 3.

1.3 Scope

This thesis is organised into eight chapters. Chapter 2 reviews the literature and provides an overview of the different scientific areas involved in the project. Chapter 3 presents all materials and methods used for the data collection and analysis. Chapter 4 discusses the physico-chemical characterisation of composite scaffolds for cartilage tissue engineering. Chapter 5 describes the application of combining electrospinning and

cell sheet engineering to engineer multilayered tissue constructs. Chapter 6 investigates the use of mineralised electrospun fibres as a direct delivery system for growth factors. Chapter 7 is an extension of Chapter 4, which details the production and characterisation of calcium phosphate fibres through electrospinning and sintering. To conclude, Chapter 8 summarises the scientific contributions made by this project and discusses possible future research directions.

Chapter 2

Literature Review

2.1 The bone-cartilage interface

2.1.1 Osteochondral defects

An "osteochondral defect" (OD) can be described as an area of damaged tissue that permeates the surface of the joints to affect both the cartilage layer and the subchondral bone. Such damage can have many possible origins. It can be created instantly, during a high energy impact to the cartilage (e.g. sport injury). It can also progressively appear through wear and tear following a less damaging trauma, a repetitive injury, a miss-alignment or an instability in the joint. Disease, such as osteochondritis dissecans, may also cause an OD. In this case, poor blood supply within the underlying bone can result in disruption of the bone-cartilage interface and, consequently, in detachment of the cartilage from the joint. Moreover, since the integrity of articular cartilage is partially maintained by the movements of the joint, immobilisation of the joint for long periods of time can also give

rise to an osteochondral lesion. OD can rapidly progress to joint malfunction (such as loss of integrity or stability, or a reduction of motion) and eventually to the development of premature osteoarthritis. Indeed, cartilage is known for its poor self-repair ability due to the absence of a vascular system and the limited capacity of mature chondrocytes to migrate and proliferate to produce new cartilage. Age is a factor that also strongly influences cartilage degeneration. Figure 2.1(b) shows an example of several osteochondral degenerative lesions observed at the articular cartilage of a 53 years old patient's knee. The reddish part seen at the surface of the joint indicates that the damage has reached the underlying bone.

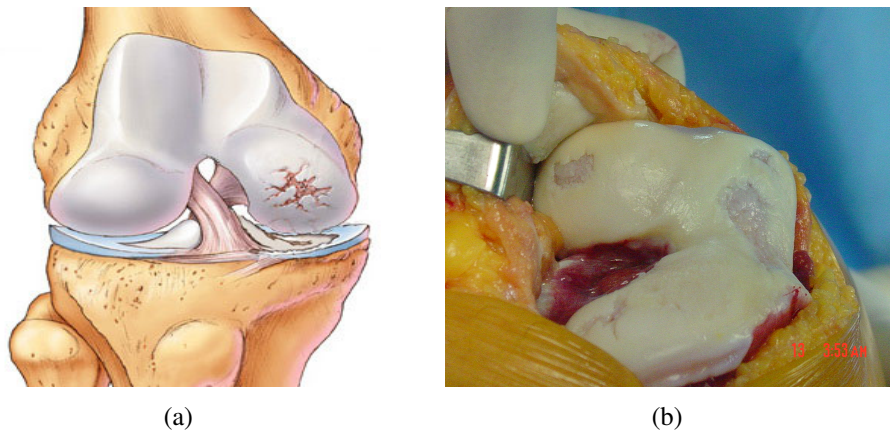


Figure 2.1: Images showing typical osteochondral lesions observed at the knee: (a) sketch (illustration from Zimmer, Inc.) and (b) defects observed in human articular cartilage. The picture was kindly provided by Jangho Lee (Department of Engineering Science, University of Oxford). The reddish areas indicate that the lesions permeate down to the subchondral bone.

Besides the possibility of performing arthroplasty to replace the joint with an implant, a number of strategies are available to surgeons for the repair of osteochondral damage. These include the mobilisation of marrow stem cells by microfracturing or drilling of the subchondral bone, implanting autograft tissue using mosaicoplasty procedures, the re-

placement with a full allograft implant and autologous chondrocyte implantation (Alford and Cole, 2005, a and b). However, the success of these methods is limited with respect to the quality of the cartilage formed, the durability of the repair, the size of the defect that can be treated and whether the site of implantation is treatable. To overcome these challenges, various alternatives are currently being investigated. Among these, regenerative medicine and tissue engineering techniques offer significant advantages, promising to revolutionise the repair of damaged tissue.

2.1.2 Structure and function of articular cartilage

Articular cartilage (AC) is a specialised form of hyaline cartilage situated in a layer, typically 3-4 mm thick, at the surface of articular joints. As for all types of cartilage, it is an aneural, avascular, alymphatic connective tissue with poor self-repair capacity. AC serves as a lubricated, wear resistant, friction-reducing surface that is slightly compressible, evenly distributing the forces onto the underlying bone. Its functional properties arise through complex composition and structure. AC is composed of a liquid phase (65-85% by wet weight), which consists of water with physiological concentrations of ionic and non-ionic solutes, and a solid phase containing chondrocytes (about 1-2%), collagen II (about 10-20%), proteoglycans (3-10%), as well as small amounts of lipids and glycoproteins (Hu and A., 2003). AC can be divided into four distinct zones: the superficial, middle, deep, and calcified zones (Figure 2.2).

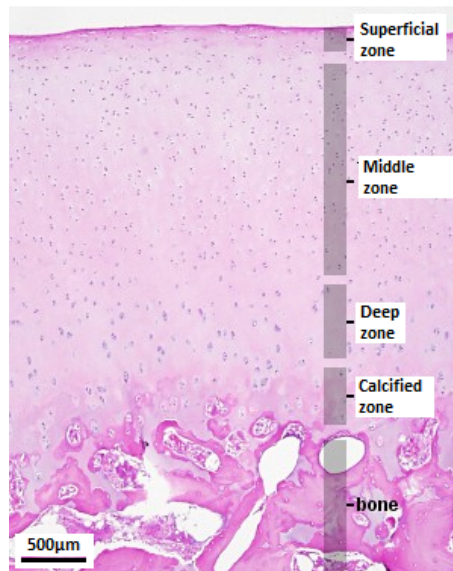
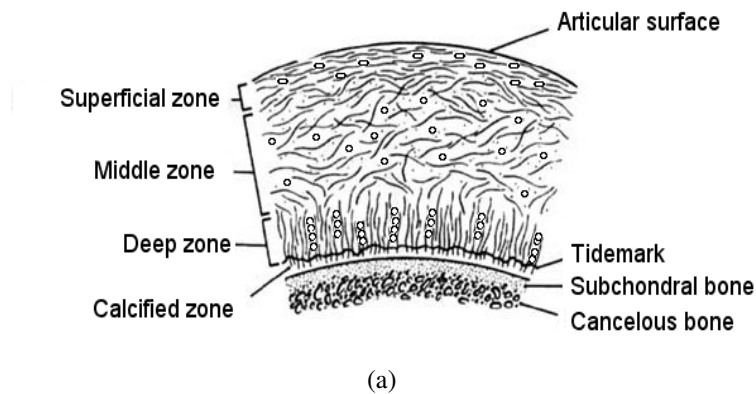


Figure 2.2: The four distinct regions of articular cartilage: superficial, middle, deep, and calcified zones. The associated areas of bone are also represented. (a) chondrocytes (circles) and collagen fibres (filaments) are not drawn to scale (modified from Mow and Hayes, 1997), (b) cross-section of articular cartilage observed by optic microscopy (adjusted from Alford and Cole, 2005a).

The superficial zone (10% to 20% of AC total thickness), lying at the joint surface, is characterized by flattened chondrocytes, relatively low quantities of proteoglycan, and high quantities of collagen II fibrils arranged parallel to the articular surface. Cell density is the highest in this region. In contrast, the middle zone (40% to 60% of AC total

thickness) has rounded chondrocytes, and the highest level of proteoglycan of the four areas. Also, the collagen fibrils are larger in diameter and have a random arrangement. In the deep zone (about 30% of AC total thickness), both chondrocytes and collagen fibres are organized perpendicular to the articular surface, the chondrocytes being arranged in vertical columns (Mow and Hayes, 1997). Collagen fibril diameter is maximal in this region, but collagen content is at its lowest. In the deep region, radial collagen fibres extend into the calcified zone, to reinforce the bond between cartilage and bone. An undulating basophilic layer of about 0.1 mm thick, termed the tidemark, acts as a strong, stable interface between the deep and the calcified regions. The calcified zone is partly mineralized, and acts as the transition between the cartilage and the underlying subchondral bone. In this area, chondrocytes are larger, being found in a hypertrophic state. The presence of collagen X is also specific to the calcified region.

In all areas of the cartilage, collagen fibrils form a dense and highly interconnected matrix filled with the ground substance mainly made of proteoglycans. The proteoglycan aggregates have a highly negative electrostatic charges which strongly attract the water molecules. The swelling resulting from the influx of water is countered by the resistance of the collagen matrix. This generates a viscoelastic gel with large internal pressure and gives the AC its unique mechanical properties (Mow and Rosenwasser, 1988). Since the zones in AC vary both in the distribution and arrangement of components, each zone of tissue is characterised by significantly different mechanical properties. The approximate compressive moduli of the superficial zone, middle zone, deep zone, and calcified carti-

lage are 0.079, 0.14, 2.1, and 320 MPa, respectively (Schinagl et al., 1997; Mente and Lewis, 1994; Huang et al., 2005). This gradient of moduli can be mainly related to the distributions of the components: the cartilage is stiffer in the lower zones where proteoglycan and hydroxyapatite concentrations are the highest. Moreover, as mentioned in the previous paragraph, the diameter of collagen fibrils is increasing when going deeper in the cartilage and this may also contribute to improving the modulus.

2.1.3 Structure and function of bone

Bones are rigid organs that form part of the endoskeleton and fulfil many functions including mechanical and metabolic. The bone tissue is a dense type of connective tissue and can be classified into trabecular and cortical bone. Trabecular (or cancellous) bone is a porous tissue filled with marrow, which is involved in generation of red and white blood cells. Cortical (or compact) bone is a dense tissue that forms a smooth, white, and rigid layer at the exterior surface, and accounts for 80% of the total bone mass of an adult skeleton (Currey, 2002). Three cell types coexist within cortical bone: osteoclasts, responsible for bone resorption, osteoblasts, producing calcified tissue, and osteocytes, originating from osteoblasts that surround themselves with bone matrix. In joints, the cortical bone lies immediately below the cartilage. Its compressive modulus is about 5.7 GPa (Mente and Lewis, 1994).

Bone is mainly composed of water, collagen type I, and calcium-phosphate nanocrystals, with proportions varying according to the bone type. Hydroxyapatite is well-known

as the main form of calcium-phosphate crystal found in human bone. The crystals are formed on and between the collagen fibres. These components are intimately associated into an ordered structure, with hierarchical levels of organisation that gives bone its unique mechanical properties (Weiner and Wagner, 1998).

2.1.4 Bone-cartilage transition and interaction

The transition between unmineralised cartilage and mineralised bone occurs very gradually. Sharma and Elisseeff (2004) have suggested that mimicking this transition is a crucial requirement to obtain a successful osteochondral engineered graft. Calcium-phosphate content progressively increases across the zone of calcified cartilage to reach high levels in the subchondral bone. Similar observations are made for collagen type I. Alternatively, type II collagen content dramatically decrease from the superficial cartilage to disappear in subchondral bone. As mentioned earlier, some components are found only in specific regions, such as collagen type X, which is located in calcified cartilage.

Calcified cartilage is interdigitated with subchondral bone, but collagen fibres do not extend across the interface. This is illustrated in Figure 2.3, where interdigitations can be seen between the calcified cartilage and the underlying bone in human AC. The tight connection between the fibrous calcified matrix and the underlying bone can also be observed. The interdigitations presumably provide for tighter adherence at the interface, eliminating lateral tissue strain. This may reduce the possibility of cartilage cracking.

The vertically oriented fibres at the tidemark and interdigitations present at the interface may act to reduce stress concentrations, as well as providing better integration with the underlying bone (Oegema et al., 1997).

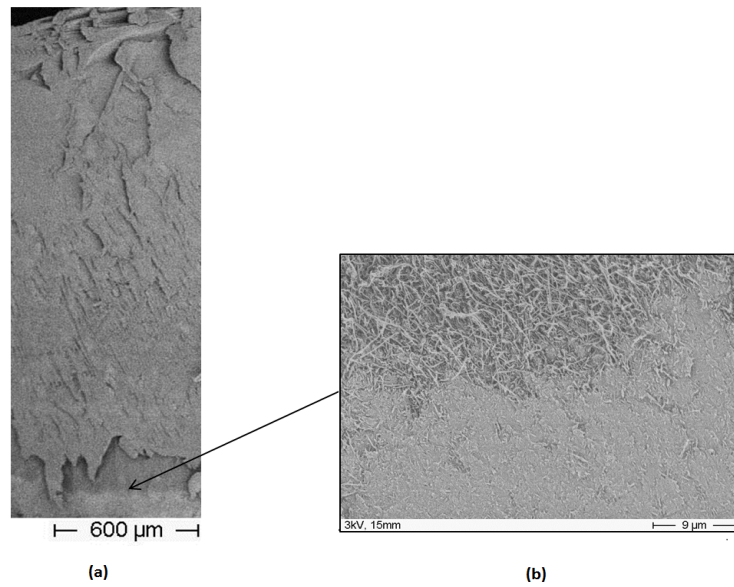


Figure 2.3: Cross-section of human articular cartilage observed by electron microscopy: (a) low magnification image, representing the whole section of the articular cartilage (with subchondral bone), (b) high magnification image, showing the interface between the calcified cartilage and the underlying bone. These images have been kindly provided by Jangho Lee (Department of Engineering Science, University of Oxford).

2.2 Regenerative medicine and tissue engineering

2.2.1 Regenerative medicine

Regenerative medicine is an interdisciplinary field that seeks to heal damaged tissues by replacing or regenerating cells, tissues or whole organs, and restoring or re-establishing

normal tissue function (Mason and Dunnill, 2008). There is a constant need to replace and regenerate body parts, damaged due to congenital defect, disease, trauma and aging. Interest in regenerative medicine began out of a need to find solutions for overcoming issues arising from the shortage of donors organs, high cost, and possible complications of transplant surgery. Today, the field encompasses many different approaches: traditional transplantation, engineered tissues, drugs, gene therapy, and stem cell transplantation.

2.2.2 Tissue engineering of osteochondral grafts

Tissue engineering (TE) is one of the most promising approaches in regenerative medicine for the repair of osteochondral lesions. It is defined by Langer and Vacanti (1993) as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ. A typical strategy in TE is to create *ex vivo* three-dimensional tissue mimetic structures, through the association of cells, induction factors and a porous matrix cultured in a bioreactor. The cell source is crucial, and in a clinical setting, the choice should take into consideration not only the intrinsic characteristics of the cells but also the final application, the age of the patient and the size and location of the defect. The matrix or "scaffold" provides a template for tissue development, and can be tailored to resorb at a defined rate (Guelcher and Hollinger, 2005). Bioreactors provide a controlled environment for tissue formation. Engineered tissues may be used for transplantation purposes, but also as a model for drug screening and for tissue related

studies (Hellman, 1995).

The creation of a functional osteochondral interface is one of the most important challenges in bone and cartilage TE. It is particularly challenging given that the two tissues concerned, bone and cartilage, have very different requirements, which vary considerably according to the site of implantation. A functional interface should allow for a proper transfer of gases and nutrients, and should adequately distribute and transmit the compressive forces that naturally take place at the joint *in vivo*. One way to engineer such an interface is to create scaffolds that mimic the soft transition and binding between bone and cartilage. Moreover, through the use of systems of control, such as drug delivery systems and multi-chambers bioreactors, it should be possible to encourage localised differentiation in multiphasic engineered tissues. The ultimate challenge for osteochondral repair is to fabricate custom-made individual implants that are tailored to a patient's specific requirements (Rodrigues et al., 2011).

2.2.3 Pre-clinical and clinical use of osteochondral engineered constructs

Although TE applications for bone and cartilage repair have been the object of recent investigations (Iwasa et al., 2009), little work has been done regarding the use of osteochondral constructs for pre-clinical and clinical applications. Several examples of osteochondral constructs are presented in Section 2.4.3. Pre-clinical studies using osteochon-

dral tissue constructs have already been carried out with different animal models. Xue et al. (2010) studied the osteochondral repair in a rat model using porous Poly(lactide-co-glycolide)/nano-hydroxyapatite hybrid scaffolds with undifferentiated mesenchymal stem cells. Twelve weeks after the operation, the authors reported that the treated defects were filled with smooth and hyaline-like cartilage, with abundant glycosaminoglycan and collagen type II deposition, suggesting that these scaffolds could have potential for clinical applications. Ito et al. (2008) used a construct consisting of chondrocytes dispersed in a collagen gel covering a porous hydroxyapatite cylinder to treat defects in a rabbit model. They found that, twelve weeks after transplantation, the defects were filled with cartilage-like tissue with some subchondral bone formation. Tanaka et al. (2005) used a β -tricalcium phosphate solid block covered with chondrocytes to treat defects in a rabbit model. After 12 weeks, the authors found that most of the calcium phosphate block was replaced with bone, whilst in the cell-seeded layer, newly formed cartilage was observed, with fibrocartilage being dominant. They also observed hypertrophic cells at the bottom of the cartilaginous layer indicating the possible formation of calcified cartilage near the bone. Holland et al. (2005) studied the repair of osteochondral lesions in a rabbit model through implantation of bilayered hydrogel scaffolds filled with chondrocytes. The authors indicated that after 14 weeks, hyaline cartilage filled the upper region of the defect and newly formed subchondral bone was found in the lower region. They observed that the chondrocyte organisation in the formed cartilage had similarities with the arrangement found in the native tissue and that the bone portion of the scaffold was completely integrated with the surrounding tissue. Schlichting et al. (2008) investigated a sheep model

to demonstrate that scaffolds which integrate with bone should have a comparable stiffness to the healthy subchondral bone, rather than being too flexible. The team observed that soft scaffolds provided less support for bone formation, and consequently resulted in the generation of pathologic bone. Jiang et al. (2007) used porous plugs made of Poly(lactic-co-glycolic)acid, with the lower body impregnated with β -tricalcium phosphate to fill osteochondral defects created in a porcine model. Autologous chondrocytes were seeded into the upper part of the plug. The authors demonstrated that implanted autologous chondrocytes survived and yielded hyaline-like cartilage *in vivo*. They also reported that pre-seeding of osteogenic cells did not appear to be necessary to regenerate the subchondral bone.

With regard to clinical applications, trials involving osteochondral systems are in early stages, although applications that employ TE scaffolds have already begun to be used for cartilage tissue (Iwasa et al., 2009). A pilot clinical trial is currently being carried out by Kon et al. (2010). The authors have treated more than thirty osteochondral lesions with a cell-free trilayered scaffold made of collagen and hydroxyapatite, and early follow-up has revealed promising results, indicating enhanced repair of the defects.

2.3 Stem cells for tissue engineering

2.3.1 Stem cell strategies

Stem cells play a unique and pivotal role in the field of TE. They possess the ability to self-renew and to differentiate into specialised cells (Bianco and Robey, 2001; Liao et al., 2008; Tuan et al., 2002). A challenge when working with stem cells is to provide the appropriate cues to control differentiation. These cues may come from exposure to recombinant biomolecules (soluble or surface-bound molecules), specific culture conditions (*e.g.* cell seeding density, type of bioreactor), or the properties of the scaffold itself (*e.g.* composition, structure) (Ashammakhi et al., 2007). Stem cells have already been used successfully to engineer a number of different tissues. In the human body, most tissues and organs are multiphasic in nature and contain multiple cell types. In this instance, use of stem cells can serve in the creation of multiphasic tissue constructs, such as osteochondral graft.

There are different approaches towards the use of stem cells in TE. One approach is to expand a population *ex vivo* prior to transplantation. A second approach is to create and implant substances and/or devices that enhance *in vivo* activation of stem cells. A third strategy -the ultimate approach for tissue engineers- is to recreate, *ex vivo*, a tissue or an organ prior to transplantation.

Sources of stem cells may be found either in the embryo or in adult tissues. Embryonic

stem cells are pluripotent and may prove to be the ideal choice for the creation of new organs. However, their usage is limited by the tumorigenicity of the cells, and the ethical issues associated with their harvest, as well as the lack of understanding precludes their widespread use (Lanza, 2006). This has increased interest in use of adult stem cells, which have been discovered and characterised in a multitude of tissues. Moreover, reports have provided evidence supporting the concept that adult stem cells are part of the natural system for tissue repair (Tuan et al., 2002; Lanza, 2006). This suggests the potential of adult stem cells for repairing damage in their host tissues.

2.3.2 Adult stem cells for osteochondral TE

Among the best known adult stem cells are the mesenchymal stem cells (MSCs). These are characterised by their capability to differentiate into different lineages such as osteocytes and chondrocytes. Of particular interest, bone marrow mesenchymal stem cells (found in bone marrow) are multipotent and can be purified, expanded and differentiated into multiple lineages as osteocytes, chondrocytes, fibroblasts, adipocytes, astrocytes and myocytes (Caplan, 1991; Pereira et al., 1995). The multi-lineage potential of adult mesenchymal stem cells is shown in Figure 2.4. Even though MSCs are mainly located in the bone marrow they have been localised in tissues such as muscle and adipose tissues (Deans and Moseley, 2000).

To date MSCs have been successfully used in animal models to treat bone defects and cartilage lesions (Hui et al., 2005) spinal cord injuries (Sykova et al., 2006), cardiovas-

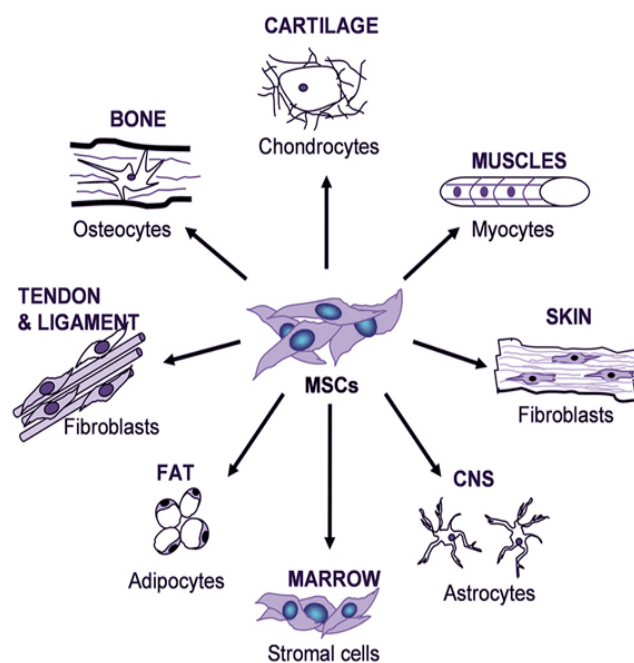


Figure 2.4: Multi-lineage potential of adult mesenchymal stem cells. Mesenchymal stem cells which are present in adult bone marrow are multipotent: they can differentiate into multiple lineages as osteocytes, chondrocytes, fibroblasts, adipocytes, astrocytes and myocytes. (Grassel and Ahmed, 2007).

cular diseases (Perin et al., 2004) and haematological pathologies (Fouillard et al., 2007). Similar success was achieved using MSCs in human clinical applications for orthopaedic diseases. In one clinical study, new dense bone was successfully developed in children suffering from *osteogenesis imperfecta* three months after administration of MSCs. These results were further supported by increased total mineral bone content and decreased fracture rate (Keating et al., 1998). In another clinical study, MSCs were injected in patients with spinal cord injury and the authors showed that motor and sensory functions were significantly improved (Yoon et al., 2005).

MSCs have been used for various bone and cartilage TE applications (Caplan, 2007). However, in the context of engineered osteochondral grafts, little work has been done using MSCs. Jeong et al. (2008) reported the use of MSCs to repair osteochondral defects on the distal femurs of rabbits using Polydioxanone/Poly(vinyl alcohol) scaffolds. MSCs isolated from the rabbits were cultured in the scaffolds, differentiated into cartilage and implanted into osteochondral defects on the distal femurs of the same rabbits. More recently, Guo et al. (2009, a and b) reported osteochondral repair in rabbits using MSCs encapsulated in biodegradable hydrogel composites. In that study, MSCs in the hydrogel were directly implanted within the defects, without initial differentiation into either bone or cartilage.

Adipose-derived stem cells have also been extensively studied and showed their potential for osteogenic and chondrogenic differentiation (Strem et al., 2005). Other sources

of adult stem cells that have also been introduced for cartilage and bone tissue engineering include stem cells from the sinovial tissue, periosteum-derived stem cells, umbilical cord stem cells, and amniotic fluid derived stem cells (Rodrigues et al., 2011).

2.3.3 Stem cell fate

Stem cell adhesion, self-renewal and differentiation are dependent on many interconnected properties and therefore, sophisticated control is necessary to create a successful tissue construct. Cell fate is influenced by many factors that can be classified into chemical, mechanical, and topographical cues.

2.3.3.1 Chemical cues

Providing proper biological and chemical signals to the cells is necessary to induce the formation of a specific tissue. With an appropriate cocktail of biomolecules, MSCs can be successfully differentiated into the cell types of many different tissues such as bone, cartilage, muscle, tendon/ligament, and connective tissue (Grassel and Ahmed, 2007). Of particular interest is that the differentiation of MSCs into chondrocytes requires the addition of transforming growth factors beta (TGF- β family), while their differentiation into bone cells involves bone morphogenic proteins (BMPs). The success of the differentiation of stem cells is often limited by problems of dosages, activity and supply of the signalling molecules (Bianco and Robey, 2001). New methodologies that incorporate and optimise bioactive compounds into biocompatible scaffolds are required. In prac-

tice, bioactive molecules are either provided with the culture medium or incorporated into the scaffold by initial blending with the polymer, or by surface modifications (coating or grafting) of the scaffold (Lee and Shin, 2007). It is important to note that the signals are not only provided by molecules such as growth factors. The composition of the scaffold also influences stem cell fate to some extent. For instance, the use of collagen in a TE scaffold will provide binding sites for cell adhesion, since the Arginine-Glycine-Aspartic acid (RGD) sequences contained in collagen are recognised and bound by cell integrin receptors. Cell fate can also be modified through exposure to the degradation product of the scaffold materials. This often results in unfavourable toxicity, and may impede proper control over stem cell fate.

2.3.3.2 Mechanical cues

Chemical cues are not sufficient to provide an *in vivo*-like differentiation. In the body, cells are subjected to mechanical forces that significantly contribute to direct the stem cell differentiation (Cohen and Chen, 2008). Therefore, appropriate mechanical stimulation *in vitro* should also support desirable differentiation. Mechanical responsiveness, for instance, is very important for articular cartilage formation, particularly since cartilage is partially maintained by the movements of joints (Buckwalter and Mankin, 1998). In TE, mechanical stresses can be provided by the bioreactor. Mechanical loading, shear stresses and other forces applied directly on the tissue construct are known to affect the fate of stem cells (Gooch and J., 1997; Sarraf and Eastwood, 2005; Lee et al., 2006). For instance, Davisson et al. (2002) have shown that compressive loading had a positive effect

on synthesis glycosaminoglycans in tissue engineered cartilage. More recently, it has also been reported that the mechanical stiffness of the substrate itself may influence stem cell differentiation (Even-Ram et al., 2006; Engler et al., 2006).

2.3.3.3 Topographical cues

Topographical features have an important effect on cellular fate, from the early stages of cell adhesion to the final differentiated state. Many studies using monolayer culture have shown that nanostructured surfaces affect the cell differentiation (Lim and Donahue, 2007; Yim et al., 2007; Mendonça et al., 2009; Wang et al., 2011). In three dimensions, architectural properties such as matrix density, presence of nanostructures, porosity and pore size, have also been found to have a potential effect on the stem cell differentiation (Liao et al., 2008). Therefore, today's processing of biomaterials to produce medical devices has become a critical step which requires consideration on both the macro- and nano-scale. The interest in electrospun materials for tissue engineering is mainly due to the ability of the technique to produce fibres that mimic the sub-microstructures found in the extracellular matrix (ECM), surrounding cells in the body (Teo et al., 2006).

2.4 Scaffolds

2.4.1 Definition and design considerations

Scaffolding is one of the key concepts in TE. The role of the scaffold is to provide a temporary support for cell attachment, migration, proliferation and differentiation

(Venugopal et al., 2008). Therefore, the scaffold must satisfy different criteria: biocompatibility, biodegradability, and mechanically properties similar to natural tissue. Porosity and pore size are also important, as the scaffold should allow a uniform cell distribution in order to facilitate the new tissue formation (Yang et al., 2005). Porosity is also important for the supply of gas and nutrient to the cells. Polymers, either natural or synthetic, are frequently used to make scaffolds for TE (Ramakrishna et al., 2005; Ashammakhi et al., 2007). Ceramics are also used, mainly for bone tissue engineering (Porter et al., 2009).

2.4.2 Conventional scaffold fabrication techniques

Numerous techniques have been used to process synthetic or natural scaffolds into porous structures that can serve for tissue engineering. Solvent-casting and particulate-leaching, gas foaming, rapid prototyping, phase separation, melt moulding, emulsion freeze drying, solution casting, freeze drying and electrospinning are a few of the recent techniques investigated. More details about these techniques can be found in the reviews from Sachlos and Czernuszka (2003) and Yang et al. (2001). The electrospinning technique will be described in Section 2.5.

2.4.3 Scaffolds for osteochondral TE

Approaches to the fabrication of osteochondral constructs have recently been reviewed by Martin et al. (2007) and Hutmacher (2000). These involve the use of both homogeneous and heterogeneous scaffolds. Since the criteria that the engineered matrix must satisfy are very different for the bone and the cartilage, the creation of heterogeneous

biphasic constructs has become a promising strategy. Various ways of achieving biphasic scaffold have been identified in the literature, and an important goal is to obtain a strong adherence between the different parts of the multiphasic scaffold. The different approaches fall into three categories, that are illustrated in Figure 2.5.

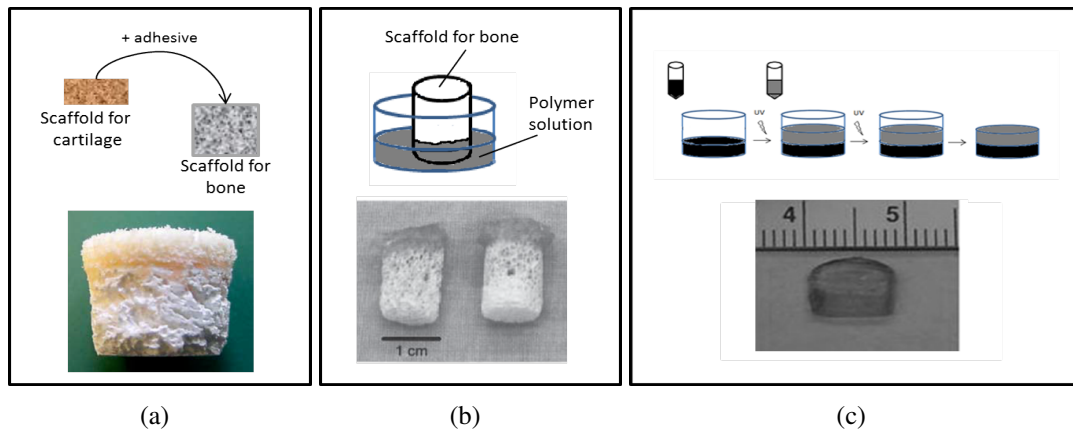


Figure 2.5: Illustration of the current strategies used to build heterogeneous osteochondral scaffolds : (a) Poly(ethylene glycol)-terephthalate/Poly(butylene)-terephthalate foam glued on calcium phosphate block (Martin et al., 2007), (b) partial modification of a ceramic block by soaking its extremity in a gelatin solution (Chang et al., 2004a), (c) multi-layering of photopolymerised hydrogels (Kim et al., 2003).

The most common and straightforward approach involves the unification of different homogeneous scaffolds, designed and produced separately for each tissue type. For example, Schaefer et al. (2000) reported a method for preparing tissue construct with both hard and soft tissues by using a microfibrinous matrix made of Poly(glycolic acid) for the cartilage, and a foam made of Poly(lactic-co-glycolic) acid (PLGA) and Poly(ethylene glycol) for the bone. Both tissues were grown separately and then artificially bound by suturing. Jiang et al. (2007) fabricated a biphasic cylindrical porous plug using a

PLGA sheet for the chondral phase and a rigid disk made of PLGA tricalcium phosphate for the osseous phase. Autologous chondrocytes were seeded into the upper, chondral phase of the plug, which was inserted by press-fitting to fill defects surgically created in mini-pigs femur. Swieszkowski et al. (2007) reported the use of either fibrin and Poly- ϵ -caprolactone (PCL) or PCL and PCL/tricalcium phosphate scaffolds. For each construct, the two phases were prepared separately and integrated into a single construct using fibrin glue before implantation.

The second strategy involves the partial modification of homogeneous scaffolds. Chang et al. (2004a) created biphasic scaffolds by soaking a calcium phosphate block in a gelatin solution and then studied the behaviour of porcine chondrocytes seeded into the gelatin-modified part of the scaffold. Their results show good integration of cartilage in the calcium-phosphate layer, although no zonal organisation was observed. In a similar way, Chen et al. (2006) prepared a biphasic cylinder made of collagen and PLGA-collagen by freeze-drying a collagen solution gelled on the top of and extending into the pores of a PLGA sponge.

The third key approach builds biphasic constructs by using a layering method (Kim et al., 2003). Holland et al. (2005) reported the use of photo-polymerisable hydrogels, based on Poly(ethylene glycol) fumarate, to repair osteochondral defects in rabbits. These scaffolds may potentially serve as carriers of bioactive agents, but may suffer from poor mechanical properties. Guo et al. (2009) used crosslinked Poly(ethylene glycol) fumarate and gelatin microparticles to fabricate a bilayered osteochondral construct, consisting of a chondrogenic layer and an osteogenic layer. This scaffold was used to investigate the

differentiation of rabbit MSCs.

Although the aforementioned approaches have shown potential for the repair of osteochondral defects, none of them offer a complete satisfactory solution. Few authors take into account the characteristics of the interface between the bone and cartilage when designing the scaffolds. As described above (section 2.1.4), the soft transition between bone and cartilage, as well as a strong interaction between both tissues has been suggested as critical considerations to obtain a successful osteochondral graft. Recently, Harley et al. (2009) and Lynn et al. (2009) have reported the design of a layered osteochondral scaffold with continuous interfaces, using a co-precipitation technique. However, the potential of this scaffold for osteochondral repair remains to be investigated. Sherwood et al. (2002) have also described a similar scaffold. They used a three-dimensional printing process and progressively changed the density and nature of the polymer during the lamination.

A tendency in TE scaffold design is to mimic more and more the structure and biology of the extracellular matrix (ECM) surrounding the cells in the body (Srouji et al., 2008; Li et al., 2005). The ECM is a fibrous network made of insoluble polymer trapping water, solutes and other biomolecules. ECM fibre diameters are typically between 50 and 500nm. So far, the production of polymeric nanofibres has been achieved with different manufacture techniques, such as drawing, template synthesis, phase separation, self-assembly and electrospinning. These techniques are described in detail by Ramakrishna et al. (2005). The electrospinning technique is a promising approach, and other techniques are unable

to compete in terms of versatility, flexibility and ease of fibre production (Li et al., 2006; Schindler et al., 2005).

2.5 Electrospinning

2.5.1 Principle of electrospinning

The process of electrospinning was first patented in 1934 by Anton Formhals for the fabrication of textile yarns. It was only later in the 90's that electrospinning became popular, after Reneker's group demonstrated that many polymers could be electrospun into micro- and nanofibres (Doshi and Reneker, 1995). Since then, the number of publications about the technique has been increasing dramatically, as shown in Figure 2.6.

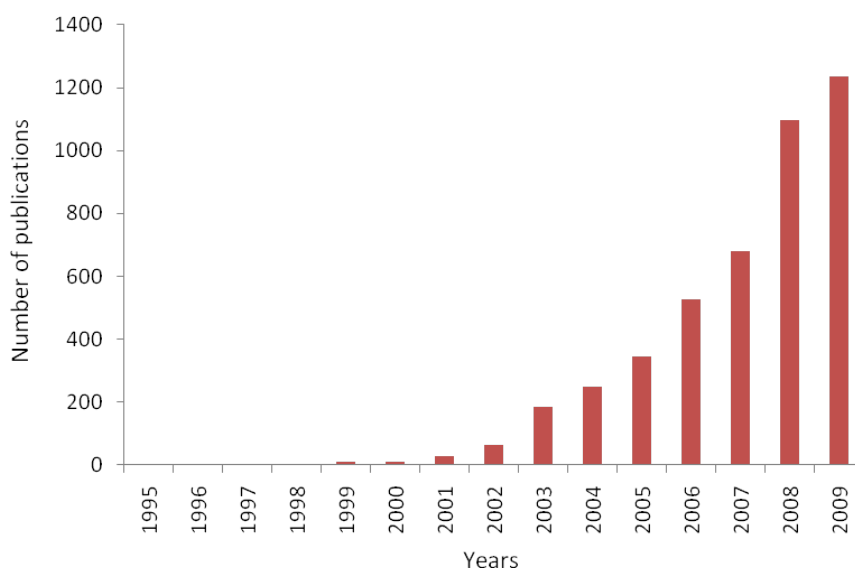


Figure 2.6: Papers published on the electrospinning topic over the last years (research performed with Web of Knowledge).

Electrospun fibres are formed by applying a high voltage to a polymer solution. The principle is similar to the electrospraying technique, that employs electricity to disperse liquid solution into a fine aerosol. The difference between the two is determined by the interplay between the surface tension and the viscosity of the solution. If the viscosity is high, entanglements between polymer chains prevents jet break-up due to surface tension and cause the liquid solution to remain as a jet that will later become the solidified fibres due to solvent evaporation. For instance, Figure 2.7 shows that, when increasing the concentration of Poly(lactic-co-glycolic acid) (ratio 75:25, Mw: 66,000-107,000) in hexafluoroisopropanol, the initial electrospraying phase shifts to electrospinning. If the concentration of the polymer is too low, entanglement between the polymer chains will not occur, resulting in a spray. As a result, the jet cannot be maintained in the electrical field and will break-up into small droplets (Figure 2.7a). This phenomenon is known as the Rayleigh instability (Yarin, 1993). At higher concentrations, entanglements between the polymer chains will happen, preventing the break-up of the jet and allowing fibres to be formed (Figure 2.7b and 2.7c). However, if the concentration is below a critical value (Figure 2.7b), the Rayleigh instability will still occur and beads will appear along the fibres. Although molten polymers have been used, in most electrospinning experiments the liquid solution is a polymer dissolved in a solvent at concentrations between 5% and 15% and the molecular weights of the polymers are mostly between 50kDa and 150kDa. Besides the polymer, the selection of the solvent is also crucial for the electrospinning process. Indeed, the solvent must not only dissolve the polymer but also has to be highly volatile and conductive.

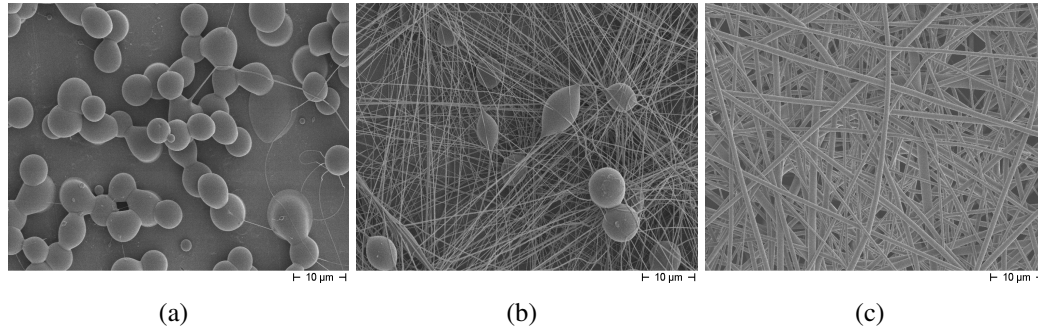


Figure 2.7: Effect of the polymer solution concentration on the resulting electrospun membranes: (a) 5% PLGA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), (b) 10% PLGA in HFIP, (c) 15% PLGA in HFIP (w/v). Electrospinning was conducted in a N_2 atmosphere using a solution flow rate of 1ml/hr, a tip-collector distance of 14 cm, a needle inner diameter of 8mm and a voltage of about 9.5kV.

The four main components found in a typical electrospinning setup, as shown in Figure 2.8, are:

- a high voltage power supply (typically up to 20kV)
- a charged capillary tube used as the first electrode (e.g. needle)
- a grounded metal collector, used as the second electrode
- a syringe pump

Variations of these components are commonly found in the literature, for example the use of dynamic collectors of various shapes, coaxial nozzles and multiple nozzles (Teo and Ramakrishna, 2006). Also, other components can be found in more advanced setups. These include the use of additional electrodes that are placed between the tip and the collector in order to control the electrospinning jet and the fibre deposition. Also, gas flow

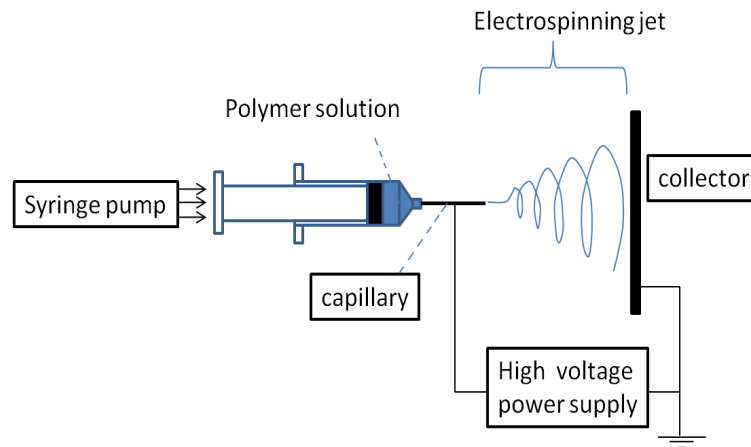


Figure 2.8: A typical electrospinning set-up, using a grounded static collector.

can be used in a closed environment to control the atmosphere surrounding the process.

The formation of electrospun fibres can be broken down into four different steps:

(i) Charging of the fluid

Typically, a high voltage (usually in the range of 10kV to 20kV) is applied to a polymer fluid, such that charges are induced in the fluid. The excess charges (or uncompensated ions) move towards the surface of the fluid in response to the electric field, generated between the needle and the grounded electrode.

(ii) Formation of the Taylor cone

In a routine electrospinning experiment, the fluid is pushed through the orifice of the capillary tube and a drop is formed at the tube tip due to interactions of surface tension and viscoelastic stresses. When a sufficient electrical potential is applied between the tube and a collector, the charges within the fluid reach a critical level and the force generated

by the repulsive charges at the surface of the fluid becomes larger than its surface tension. The drop then approaches a conical shape known as the Taylor cone and an electrically charged jet erupts from the tip of the cone (Taylor, 1969).

(iii) Thinning of the jet in the electric field

The path of the jet in the electrical field is illustrated in Figure 2.9. The jet follows a path that begins with a linear segment. The fluid in this segment accelerates as the Coulomb forces pull it toward the collector, acting against the surface tension and viscoelastic forces. This linear part of the jet soon becomes unstable in response to the Coulomb repulsion of the charges and elongates in the electrical field. The bending perturbations begin and grow rapidly into a coil of increasing diameter. The bent part of the jet is elongated and reduces in diameter while the polymer jet keeps its integrity due to entanglements taking place between the polymer molecules. After several turns are formed, a new electrical bending instability forms a smaller coil on a turn of the larger coil. This process is repeated until the elongation stops, usually with solidification of the thin jet due to solvent evaporation in the surrounding environment. It is important to note that the jet shape and elongation process follow a chaotic behaviour which makes them very difficult to model and predict. Whipping, thinning, and bending of electrospinning jets have been described by Reneker and Yarin (2008) and Reneker et al. (2000). The electrospinning jet and fibre formation can be visualised using a high speed video camera.

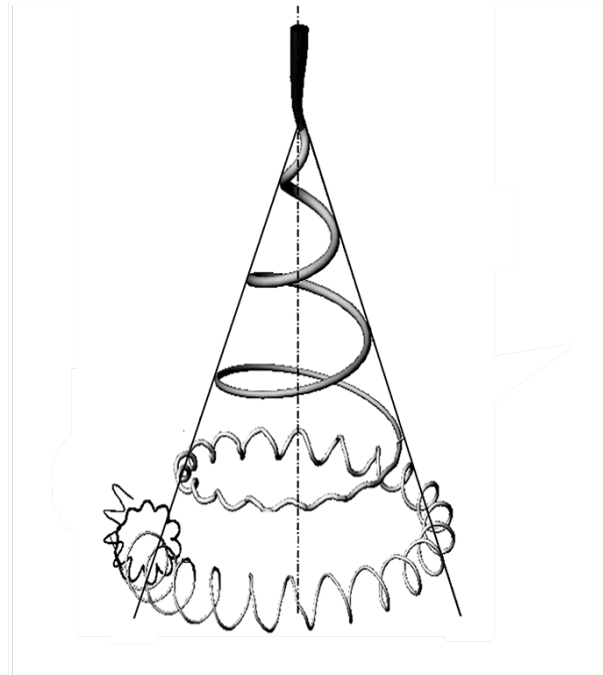


Figure 2.9: A model electrospinning jet undergoes a thinning process in the electric field, during a typical electrospinning experiment (Reneker and Fong, 2005). The electrospinning jet corresponds to the segment labelled in Figure 2.8.

(iv) Collection of the resulting fibres on the target

The resultant solidified and continuous fibre, which arrives on the collector is usually collected in the form of a nonwoven mesh, although the use of a rapidly moving target can be used to align the fibres. The fibre diameter is rarely uniform throughout the whole mesh and measurements can often be described by a normal or a gamma distribution (Weissmueller, 2010). Initial collection of solidified fibres usually shows different deposition patterns compared to later fibres. When the jet hits the bare surface of the collector, it undergoes a more important compression than when the surface is already partially covered with fibres. This forms easily distinguishable shapes due to the buckling. The deposited buckling patterns include sinuous, zigzag-like, figures-of-eight, recurring curves, coiled

and other structures that resemble patterns created by uncharged jets of highly viscous fluids hitting the hard flat surface (Han et al., 2007). An example of the buckling pattern is given in Figure 2.10.

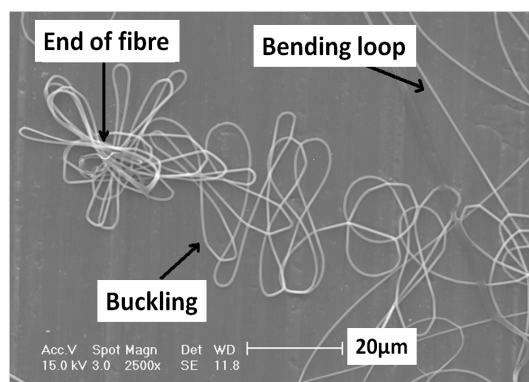


Figure 2.10: Buckling observed at one end of polyvinyl alcohol fibre after the electrospinning of a 8% polyvinyl alcohol/water solution (Mouthuy, 2008)

Many parameters can influence the properties of the resultant electrospun fibres. Those parameters are usually classified in two categories: (i) system parameters, such as polymer molecular weight, molecular weight distribution, molecular structure (branched, linear etc.) and solution properties (viscosity, conductivity and surface tension), and (ii) process parameters such as electric potential, solution flow rate, needle-to-collector distance, diameter of the needle, ambient parameters (temperature, humidity and air velocity in the chamber), and finally, motion of the collector (Huang et al., 2003). Varying any of these parameters will most likely affect the properties of the fibres, and influence the response of the cells to the final electrospun biomaterial. The electrospinning process has been comprehensively reviewed in the literature. For further information, see papers from Teo and Ramakrishna (2006), Reneker and Yarin (2008), Huang et al. (2003), Li et al.

(2006) and the book by Ramakrishna et al. (Ramakrishna et al., 2005).

A consequence of the sub-micron nature of the fibres is that electrospun materials also possess a high surface area-to-volume ratio (or specific surface area) which contributes to their success as biomaterials (Frenot and Chronakis, 2003). Electrospun fibres have mainly attracted interest from the field of regenerative medicine. They can mimic the morphology of the natural sub-micron fibres found in human tissues and organs in organized hierarchical structures: they have found applications as scaffolds for tissue engineering, wound healing devices, drug delivery systems, fillers for composites biomaterials, and surface modifiers of biomedical devices.

Electrospun materials have also attracted interest from many other fields, with possible applications including colloids filters (aerosols and sols), gas filters, chemical sensors, immobilisation membranes for catalysts, electronic devices (e.g.: transistors, circuits, sensors), energy storage devices, protective clothing and other textiles, and reinforcement matrices for composite materials (Huang et al., 2003; Li and Xia, 2004b; Ramakrishna et al., 2005).

2.5.2 Electrospun materials: a wide range of possibilities

The versatility of electrospinning makes it possible to process many categories of materials: polymers, ceramics, metals and composites.

To date, a wide variety of polymers have been successfully electrospun (Ashammakhi et al., 2007; Li et al., 2006). These include biodegradable polymers such as Poly(lactic acid), Poly(lactic-co-glycolic acid), Poly- ϵ -caprolactone, Polyhydroxybutyrate, and other synthetic polymers, such as Poly(ethylene-co-vinyl acetate), Poly(ethylene terephthalate), Polyurethane, Polyethylene oxide, Poly(vinyl alcohol). Natural polymers such as collagen, gelatin, chitosan, silk protein, hyaluronan, fibrinogen and elastin, have also been electrospun. Blends of polymers have been widely used in order to combine properties from the different constituents. For instance, blends of natural and synthetic polymers combine both good cell response and good mechanical properties (Li et al., 2005).

Although the polymer phase is required to provide the entanglement necessary to obtain a jet, ceramic and metal electrospun fibres can be produced by adding precursors (salts or particles) into the polymer solution and performing a further processing step which is typically a thermal treatment (Wu et al., 2004; Tadjiev et al., 2007; Kim and Kim, 2006). Ceramic and metal electrospun scaffolds have the potential to be used as biomaterials for hard tissue replacement and regeneration, and also as a reinforcement matrix for existing biomaterials.

The combination of different types of materials, for instance a polymer matrix containing hydroxyapatite nanoparticles, is a promising approach for the production of biomimetic electrospun materials with functional activity. Various morphologies and structures can also be obtained easily by changing either the process or system parameters and/or com-

binning it with other processing technologies (thermal treatment, freeze drying, simulated body fluids, metal coating, etc.).

The fibre diameter is affected by many parameters, but is predominantly controlled by varying the voltage and concentration of the polymer solution. Misadjustments of these parameters can result in the formation of beads along the fibre length, often seen as a defect (see Section 2.5.1). Figure 2.11 shows fibres with surface nanostructures created by controlling the evaporation of the solvent and the subsequent fibre solidification (Bognitzki et al., 2001).

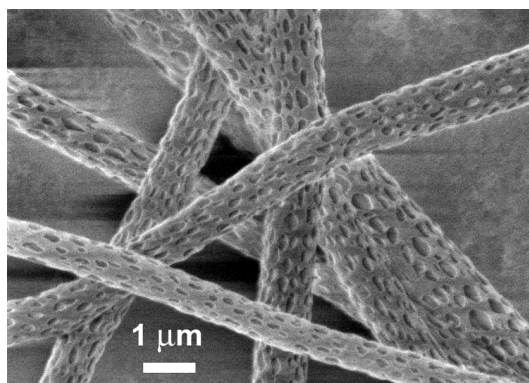


Figure 2.11: Nanostructures created by controlling the evaporation of the solvent and the subsequent fibre solidification in electrospun fibres (Bognitzki et al., 2001)

The fibre organisation can easily be controlled using an appropriate collector. With a basic static collector, a random mesh will be obtained. Dynamic collectors, such as rotating drums or disks, can be used to obtain aligned fibres, as shown in Figure 2.12. One of the current challenges in electrospinning is to precisely control the organisation and structure of the fibres in a 3D arrangement by using collectors and additional electrodes

to control the jet (Teo and Ramakrishna, 2006).

Multi-fluid electrospinning setups have the potential to produce a wide range of electrospun structures. In particular, the use of a co-axial nozzle allows the production of core-shell structures that have many possible applications as biomaterials. Figure 2.13 shows a typical hollow fibre that can be obtained via co-axial electrospinning (Li and Xia, 2004a; Li et al., 2006). Core-sheath electrospun structures can potentially serve to incorporate drugs or nutrients into the fibres for controlled delivery, reinforce a fibre to improve its mechanical properties and produce multi-component fibres with the most bio-compatible material at the surface. It has also been used to produce electrospun fibres made of non-electrospinnable materials (placed in the core). Co-axial electrospinning has been recently reviewed by Moghe and Gupta (2008).

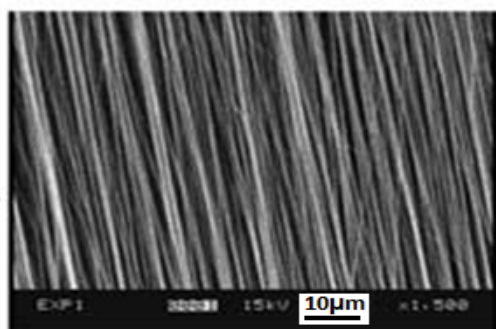


Figure 2.12: Aligned electrospun fibres using a dynamic collector (Ma et al., 2005)

Further processing steps can be performed to affect the electrospun biomaterial morphology and organisation. For instance, calcium-phosphate crystals can be grown on the

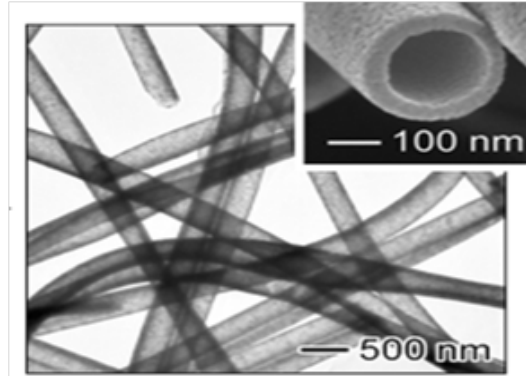


Figure 2.13: Ceramic nanotubes created by coaxial electrospinning (Li and Xia, 2004a)

surface of the fibres by immersion of the fibres in simulated body fluid (Figure 2.14). This should help to further optimise biomimetic scaffolds according to their target locations. In other cases, meshes can be patterned using a laser beam, as shown in Figure 2.15 (Choi et al., 2007). Such scaffolds might help to improve our understanding of cell-cell and cell-material interactions since it could be used to direct cell growth, study interactions between various cell types, and study cell growth on multi-polymeric networks.

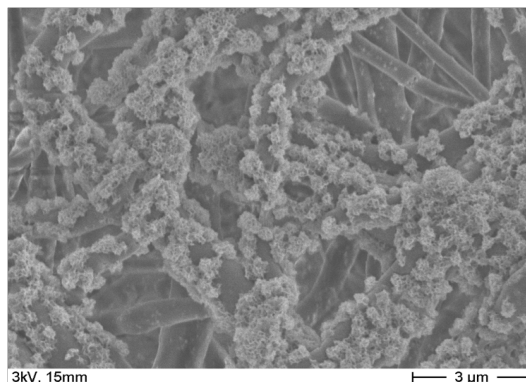


Figure 2.14: Formation of calcium phosphate crystals on electrospun fibres after immersion in simulated body fluid (Mouthuy and Ye, 2011)

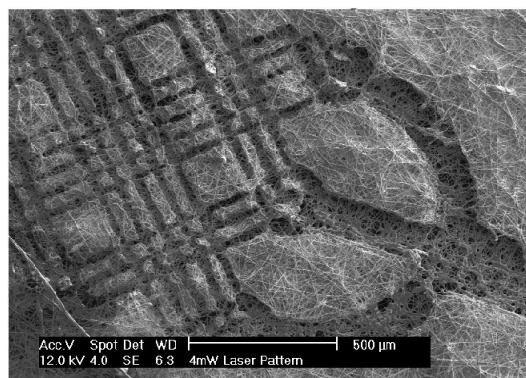


Figure 2.15: Laser-machined pattern in electrospun poly- ϵ -caprolactone mesh (Choi et al., 2007)

2.5.3 Biocompatibility of electrospun materials

Biocompatibility is a key concept in scaffold design. It can be defined as the ability of a material to induce an appropriate host response for a specific application (Williams, 2003). Besides the intrinsic biocompatibility of a material, due to its chemistry, the success of a biomaterial depends mainly on the processing methods used to produce its final structure, organisation and shape. Amongst many processing methods, electrospinning is a simple and versatile technique, able to manufacture most classes of materials into a fibrous structure with a micro- or nano-scale fibre diameters. Compared to conventional methods of processing, the unique geometric features offered by electrospinning strongly affect the biocompatibility of a biomaterial. As described in the following sections, changes can be seen in terms of protein adsorption, cell fate and cytotoxicity.

2.5.3.1 Protein adsorption

When a biomaterial is placed in a physiological environment, its surface undergoes the competitive adsorption of biomolecules (blood proteins, lipids, sugars, etc.) long

before cell adhesion occurs. As a result, the biomaterial response is governed by the interactions of the molecules on its surface. A change in surface properties alters its interaction with molecules and subsequently affects cell fate. Surface nanostructures are well known to influence the quantities, densities, distributions, conformation and orientations of biomolecules adsorbed and their importance in cell response has been reported (Palin et al., 2005; Latour, 2005).

Besides nano-architecture, electrospun biomaterials also possess high specific surface areas that are believed to promote the adsorption of greater amounts of protein from the culture medium. The development of electrospun biomaterials with surface chemistries promoting the adsorption of specific proteins should offer new possibilities in terms of controlling cell interactions with the biomaterials itself (Grafahrend et al., 2008). For instance, the use of hydrophilic electrospun membranes are known to be preferred in most tissue engineering applications, as well as in protein separation, as the adsorption of non-specific proteins is minimised. It has been reported that, compared with biomaterials with solid pore walls, approximately four times more serum proteins and more than twice the quantity of fibronectin and vitronectin were adsorbed on an electrospun matrix (Li et al., 2006).

2.5.3.2 Cell adhesion, proliferation and differentiation

When used as biomaterials, it is assumed that electrospun fibres will come into contact with cells. In some cases, cell attachment is not desirable. However, in general, elec-

trospun biomaterials are tailored to offer good cell adhesion as well as supporting and inducing a desired tissue response. Many studies have investigated the cell response to electrospun biomaterials in terms of adhesion, spreading, proliferation and differentiation. Although behaviours are cell-type dependent, several reports have demonstrated that matrices of nanofibres are more favourable than microfibres for cellular activity, suggesting that cell activities can be regulated by the fibre width (Yang et al., 2005; Kwon et al., 2005). The specific mechanisms are largely unknown but recent studies have identified the role of integrin binding, and subsequent regulation of focal adhesion kinase (FAK) and Rho kinase pathways (Nur-E-Kamal et al., 2005). Cells cultured on electrospun biomaterials display three-dimensional adhesion behaviours, showing less defined patterns of FAK compared to flat culture on glass. A lower expression of FAK is also observed for cells in natural tissue suggesting that electrospun biomaterials create an environment closer to *in vivo* conditions (Nur-E-Kamal et al., 2005; Schindler et al., 2005). By analogy, adhesions on electrospun biomaterials are referred to as "three-dimensional matrix adhesions" to distinguish them from "focal adhesions" formed in two-dimensional culture. Moreover, the high specific surface area of electrospun materials is believed to promote the deposition of secreted matrix from the cells and may lead to an increase of the number of cell-matrix contacts (Alavi and Stupack, 2007).

Studies have also shown that cells seeded onto electrospun biomaterials tend to spread nicely, attaching at multiple focal points and in some cases extending filopodia along the length of nanofibres. Some authors have reported that the Rho kinase pathway is more

activated in cells cultured on electrospun matrix than on standard tissue culture plates, resulting in enhanced spreading and proliferation (Ashammakhi et al., 2007).

It is well-known that matrix materials which support stem cells affect their differentiation (see Section 2.3.3). However, there are very few studies related to the effect of electrospun biomaterials on stem cell differentiation, although differentiation of human mesenchymal stem cells into adipocytes, chondrocytes and osteoblasts has been successfully achieved with materials like Poly- ϵ -caprolactone or Poly(lactic-l-glycolic acid) (Li et al., 2005; Liao et al., 2008). Poly(l-lactic acid) nanofibrous scaffolds have also been shown to support efficient human mesenchymal stem cell differentiation into chondrocytes (Janjanin et al., 2008). Moreover, Xie et al. (2009) have found that mouse embryonic stem cells cultured on Poly- ϵ -caprolactone electrospun fibres differentiate into neural lineage while Christopherson et al. (2009) have reported that the diameter of fibres made of Poly(ethersulfone) plays an important role in regulating differentiation and proliferation of rat neural stem cells. These few results indicate that multilineage differentiation of stem cells within nanofibrous scaffolds can be supported, suggesting the potential of electrospun biomaterials in the development of multiphasic tissue constructs.

2.5.3.3 Degradation

The ability of a biomaterial to degrade over time is an important characteristic in many applications, such as tissue regeneration or drug delivery. Therefore, the degradation properties of a material must be taken into consideration when designing a biomaterial.

Often it is necessary to have a certain control over the degradation in space and time. For instance, a rapid degradation may cause the loss of mechanical integrity of the engineered construct which can compromise the healing of critical defects (Schlichting et al., 2008). Control over the degradation process is also important to ensure that the compounds released have a low toxicity and are metabolised quickly. The degradation of a material not only depends on its chemistry or environmental conditions, but also on morphological variables and internal organisation (crystallinity and polymer chain orientation). Therefore, due to their unique properties, the degradation behaviour of electrospun biomaterials is expected to be different from those observed in bulk samples or thin films.

The high porosity of electrospun biomaterials should result in better diffusion of the degradation by-products, reducing their accumulation locally and improving their elimination. Also, reports indicate that the size of the fibres influences the degradation, i.e. smaller fibre widths increase the degradation rate (Ohkawa et al., 2004). This is an interesting design consideration as it suggests that the fibre diameter can be tailored to adjust the rate of degradation of an electrospun device. Another parameter that could decrease the degradation rate is the high alignment and crystallinity of the polymer chain resulting from the electrospinning process (You et al., 2005). However, more research is needed as the study of the rate of degradation of electrospun biomaterials has been insufficiently investigated so far.

2.5.3.4 Solvent retention

A major drawback of the high specific surface area in electrospun biomaterials is the retention of solvents which have not been fully evaporated during spinning. As the solvent is used to dissolve the polymer in solution, it is still strongly retained in the final solidified electrospun biomaterials. Organic solvents are used in most electrospinning experiments and the presence of remaining solvent in the fibre mesh can have a toxic effect on cell growth (Nam et al., 2007b). Before using electrospun biomaterials for further applications, a vacuum is usually applied to remove most of the solvent residing in the polymer and reduce the risk of cytotoxicity. Further research is required to investigate the retention of solvents in electrospun biomaterials and their cytotoxic effect.

2.5.4 Electrospun fibres for three dimensional tissue regeneration

Most studies using electrospun biomaterials for tissue regeneration have been carried out in two-dimensions and not in three-dimensions. Today's considerations must be brought to bear at a three-dimensional level to truly assess the potential of electrospun biomaterials for tissue engineering by providing the cells with the three-dimensional environment found in natural tissue. Three-dimensional scaffolds are essential to create realistic tissues and organs for regenerative medicine. With regards to this, the most important challenges are the assimilation and expansion of cells within the entire scaffold and the provision of an optimal supply of nutrients. Seeding of cells into the scaffold is the first step in establishing a three-dimensional culture and is crucial for the tissue development.

When working with electrospun constructs, the diffusion of nutrients and waste are facilitated by the high porosity of the scaffold. However, the incorporation of cells is a challenge. Indeed, the pores usually have a size much smaller than the cell dimensions, making the migration of cells within the scaffold difficult. Also, the mesh obtained by electrospinning is generally very thin and thus has a low volume capacity.

Different strategies can be used to incorporate cells into an electrospun biomaterial. The basic way is to seed the cells on the surface of the membrane and allow the cells to migrate into the scaffold. However this is only suitable for relatively thin scaffolds with large pore sizes, as the cells need enough space to move within the construct. Cell penetration can be improved by modifying the pore size of the scaffold using techniques such as enzyme degradation, laser treatment and co-spinning with salt particles followed by leaching (Nam et al., 2007a; Kim et al., 2008). Alternatively, cells can be seeded onto the prepared electrospun fibres and then centrifuged together to obtain a three-dimensional fibrous scaffold with trapped cells (Li et al., 2008).

Cells can also be directly incorporated inside the electrospun fibres by mixing the cells with the initial polymer solution and then electrospinning the resulting suspension (van Aalst et al., 2008). This technique is often referred to as "cell electrospinning". A major issue is that the viability of the cells is strongly affected by the high voltage used to produce the fibres. However, the use of a coaxial nozzle seems promising. With such

a setup, the inner flow can contain the cell suspension, and the outer flow the polymer solution (Townsend-Nicholson and Jayasinghe, 2006). However, cell electrospinning still faces limitations, mainly due to the low availability of non-toxic solvents (such as water) that allow for the electrospinning process.

Multilayering is probably the most attractive way to obtain three-dimensional constructs made of electrospun fibres and cells. For instance, cells can be seeded in suspension onto thin electrospun membranes that are then stacked together (Srouji et al., 2008). However, although the concept of this approach is simple, inoculating a cell suspension onto thin membranes remains challenging due to practical issues. These include floating and folding of the membranes in presence of a liquid as well as poor attachment between the different layers of the construct. Other methodologies for producing such three-dimensional multilayered assemblies have been proposing to switch regularly between fibre production and cell inoculation (the so called "layer-by-layer" approach). Cells are incorporated between the fibrous layers with a pipette (Yang et al., 2008). In more complex setups, cells can be electrosprayed for better distribution throughout the scaffold. The scaffold fabrication thus consists of switching between electrospinning and electrospraying (Stankus et al., 2006). In this case, the use of liquid collectors would be more advantageous, by spraying and spinning directly into cell growth medium. A major problem with the "layer-by-layer" approaches is that cellular damages can be caused by the residual solvent and by the high voltages.

The recent development of the "cell sheet engineering" technology (Yamato, 2004; Yang et al., 2005) might provide a very attractive and novel solution to engineer, together with electrospun materials, high density multilayered tissue constructs. Briefly, the technology uses a change in temperature to detach a cell layer from a tissue culture dish. The surface of the dish is grafted with a temperature-responsive polymer, Poly(N-isopropylacrylamide), which has a hydrophobic behaviour at physiological temperature, and become hydrophilic at room temperature. When the temperature is reduced, hydration of the polymer occurs, allowing detachment of the cell layer covering the polymer. These cell sheet could be collected by electrospun membranes and used for TE purposes in a layered arrangement. An additional advantage is the excellent adhesive properties of those cell sheet, since the ECM is conserved during the separation from the dish. This approach has not been investigated yet, and therefore it is an exciting opportunity to consider in this research.

Although their high porosity is favourable for the diffusion of nutrients, electrospun materials also are confronted to a common challenge, also faced by all three-dimensional scaffolds, which is the need of a delivery system of nutrients through the entire construct. Indeed, most cells *in vivo* are situated no more than a few hundred micrometers away from the nearest capillary, due to diffusion limitations (Alavi and Stupack, 2007). The same problem occurs *in vitro* when the tissue is thicker than few hundred micrometers. With the exception of a few tissues, such as avascular cartilage, tissues and organs require blood vessels to supply cells with nutrients and oxygen. Therefore, the development of

delivery system for three-dimensional electrospun biomaterials is an important issue. Various strategies have been developed to create vascularisation networks inside engineered scaffolds. Although they have been used for other types of scaffolds, similar approaches can be used for electrospun biomaterials. These may include channelling (e.g. via large interconnected pores or hollow fibres), growth factors released from the scaffold to stimulate the in-growth of blood vessels and utilisation of cells to create blood vessels inside the construct (Rouwema et al., 2008; Ko and McFarland, 2007).

Together with the need for supplying the system with nutrients and oxygen comes the requirement to have a proper control over the tissue growth environment, which can be provided by a bioreactor. It is universally acknowledged that culturing cells three-dimensionally in a bioreactor is a challenge in tissue engineering, requiring many more considerations than when working with two-dimensional systems (Yamada and Cukierman, 2007). The primary objectives of bioreactors are to establish spatially uniform cell distributions on three dimensional scaffolds, to help to maintain desired concentrations of gases and nutrients in the culture medium, and to expose developing tissue to appropriate physical stimuli (Korossis et al., 2005). Many other biological issues have to be considered for the design of bioreactors, such as growth conditions, harvesting time, storage, and sterility. Additionally, typical engineering aspects such as reliability, reproducibility, flexibility, scalability and safety are design considerations. Each type of tissue construct requires an individualized cultured environment that is designed on the basis of a global understanding of the biology of the tissue, as well as on the basis of engineering aspects

(Martin et al., 2004; Portner et al., 2005). The development of bioreactors is another important aspect in the emergence of three-dimensional electrospun tissue engineered constructs.

2.5.5 Electrospun materials for bone and cartilage TE

2.5.5.1 Conventional electrospun materials for bone and cartilage TE

As mentioned in Section 2.5.2, a variety of polymers have been electrospun in the context of TE. Of particular interest, PLGA and PCL electrospun fibres have shown particular promise in both bone and cartilage tissue engineering (Puppi et al., 2010). They have been shown to support adhesion, proliferation and differentiation of stem cells. Yoshimoto et al. (2003) have reported that PCL scaffolds provide an environment that supports mineralized tissue formation and may be a suitable candidate for the treatment of bone defects. In another paper, Wutticharoenmongkol et al. (2006) studied the response of human osteoblasts on PCL electrospun fibres containing nanoparticles of calcium carbonate or hydroxyapatite. Their results revealed that better cell adhesion was observed on the fibres compared to film membrane. Moreover, the authors found out that cells cultured on PCL fibres containing hydroxyapatite nanoparticles had a higher alkaline phosphatase activity in comparison to cells cultured on calcium carbonate and in the absence of nanoparticles. With regard to applications for cartilage, Shin et al. (2006) demonstrated that electrospun PLGA scaffolds were non-toxic, and that cell proliferation and extracellular matrix (ECM) formation was superior to those in membrane-type scaffolds. Moreover, they reported that hydrostatic pressure applied to the construct increased chondrocyte prolifer-

ation and ECM formation.

2.5.5.2 Calcium phosphate electrospun fibres

Although several authors report the use of polymers such as PCL, PLGA and collagen to engineer mineralised cartilage and bone (Puppi et al., 2010; Porter et al., 2009; Yoshimoto et al., 2003; Jose et al., 2009; Wutticharoenmongkol et al., 2006), synthetic hydroxyapatite (HA) and similar calcium phosphate (CaP) materials are often described as the most promising candidates for the repair of mineralised tissues (Suominen et al., 1996). CaP materials are now known to support bone ingrowth and to promote bone integration (Kokubo and Takadama, 2006). They have been produced under various forms. Bulk bodies have been used on many occasions but usually face the problem of unsuitable mechanical properties (*e.g.* too brittle). Increasingly, applications have made use of CaP coatings for improving the surface properties of biomaterials. This was initially to improve the biological integration of metal and ceramic biomaterials, however, coating of polymer scaffolds becomes more and more common. A very successful approach to achieve calcium phosphate deposition is the immersion of the scaffold in simulated body fluids (see Section 2.5.5.3). Also, CaP granules (*e.g.* short fibres, spherical particles and other shapes) have been shown to be very successful for filling defects in the damaged tissue. Furthermore, they have been dispersed in polymer matrices for reinforcement purposes or for improved biocompatibility.

CaP fibres have been produced through several routes including spinning, extrusion,

precipitation, sol-gel and pyrolysis (Ramanan and Venkatesh, 2004; Tanahashi et al., 1992; Nakahira et al., 1999; Kolos et al., 2003; Ota et al., 1998). Although electrospinning has been used to produce many different types of ceramic fibres (Sigmund et al., 2006; Ramaseshan et al., 2007), little work has been done on producing calcium phosphate fibres. Wu et al. (2004) have applied the usual approach to make ceramic fibres by electrospinning. They used a precursor mixture of $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $(\text{C}_2\text{H}_5\text{O})_3\text{PO}$ and blended it to the polymer solution. After electrospinning, they performed a thermal treatment on the collected fibres at 600°C for 1 hour and demonstrated that HA microfibrils were obtained. The generation of HA nanofibrils has been investigated by Kim et al. (2006). They have based their strategy on the use of sol-gel precursors of the apatites and by adjusting the concentration of the sols, they could vary the diameter of the fibres in a range of a few micrometers to hundreds of nanometers. Calcination was performed at 700°C for 2 hours. Also, Tadjiev et al. (2007) have used the same precursors as Wu et al. (2004) and heat treatments between 500 and 800°C to produce β -tricalcium phosphate (β -TCP) ceramic nanofibrils.

2.5.5.3 Coating of electrospun fibres with simulated body fluids

By definition, a simulated body fluid (SBF) describes a metastable CaP solution with inorganic ion concentrations similar to those found in human blood plasma. Many different SBF solutions can be found in the literature. To ease the preparation, most conventional SBF solutions usually use higher Cl^- concentrations and lower HCO_3^- concentrations than those in the blood plasma. Of particular interest for this study, ten times concen-

trated SBF solution (SBF10) can also be used to increase the rate of coating (Yang et al., 2008). Historically, SBF was used to assess the biocompatibility of biomaterials destined for calcified tissues by looking at their ability to form apatite at their surface (Kokubo and Takadama, 2006). Nowadays, it is also used to create a biomimetic mineral layer onto biomaterials for increasing their chance of integration in osseous tissues (Leon and Jansen, 2009). In most cases, apatite coating is performed on metallic or ceramic biomaterials. The coating of polymeric scaffolds such as PLGA or PCL is usually very difficult, due to a lack of nucleation sites on the material surface and to problems of degradation during the process. Besides increasing their biocompatibility, the coating may provide further advantages such as improvement of the mechanical properties and the possibility of creating scaffolds with gradient of apatite, which might be useful for creating osteochondral grafts (Li et al., 2009).

With regards to electrospun scaffolds, several authors have already used SBF to assess their bioactivity for bone tissue engineering. For instance, Chen et al. (2006) successfully coated Poly(l-lactic acid) electrospun scaffolds with a full apatite layer after immersion in SBF for 4 weeks. Madurantakam et al. (2009) assessed the mineralization of various types of SBF solutions, by immersion of polydioxanone electrospun fibres with different concentrations of hydroxyapatite nanoparticles (nHAp) in SBFs at 37°C in 5% CO₂ atmosphere for 14 days. They showed that fibres containing 50% nHAp, incubated in SBF solutions containing all ions at blood plasma concentrations, gave the maximum mineralisation. Yang et al. (2008) achieved complete coating of PCL electrospun fibre surface

after immersion in SBF10 for only a few hours, in comparison to the few weeks necessary with other SBF solutions. Similarly, Jin et al. (2009) mineralised PLGA/gelatin electrospun mats by incubating in SBF10 for only 4 hours. They have also reported that nucleation and growth of HA crystals occurs faster on PLGA/gelatin fibre mats in comparison to PLGA membranes, due to the presence of gelatin. SBF mineralisation is reported to improve the bioactivity of electrospun scaffolds in the context of bone tissue engineering. For instance, Mavis et al. (2009) studied the osteoblastic activity on PCL nanofibres coated with apatite. They demonstrated that the mineral layer increases the levels of both alkaline phosphatase activity and osteocalcin compared to the uncoated PCL nanofibres, suggesting that mineralisation supports osteoblastic differentiation. Also, Nandakumar et al. (2010) successfully coated Poly(ethylene oxide terephthalate)-Poly(buthylene terephthalate) electrospun fibres with CaP and reported that subcutaneous implantation in mice showed new bone formation in the calcium phosphate coated samples, while no bone formation was observed in uncoated samples.

2.6 Drug delivery

2.6.1 Drug delivery from TE scaffolds

In the field of TE, therapeutic agents can be incorporated into the matrix in order to increase the rate of tissue repair. This approach aims to enhance the delivery efficiency of signalling molecules by increasing their residence time within tissue, maintaining locally high concentration and protecting them against degradation through interactions with ma-

materials (Jang et al., 2004). However, where the active factor is a protein, a major inconvenience with this approach is the loss of its native conformation within the scaffold, which can result in decreasing its therapeutic effect, or even cause immunogenic response due to exposure of non-native epitopes (Ji et al., 2010; Langer, 1992). With the requirements of TE scaffolds to repair multiphasic tissues, the encapsulation of different types of growth factor in specific parts of the scaffold may facilitate local differentiation of stem cells into a particular phenotype. This can lead to multilineage differentiation inside the same construct. Such a strategy would be particularly useful for the engineering of osteochondral grafts, since BMP can be encapsulated in the part of the scaffold designed for bone, while TGF can be incorporated into the cartilage region in order to differentiate hMSCs into either osteoblasts or chondrocytes.

2.6.2 Drug delivery from electrospun fibres

The high specific surface area of electrospun materials makes them an ideal candidate for the creation of drug delivery systems. Although drug delivery in the field of biomaterials is under investigation, it is still in the early stage of research with respect to electrospun materials.

There are several ways to load a therapeutic agent into an electrospun materials (Huang et al., 2003; Sill and von Recum, 2008). The drug can be coated onto the surface of the fibres after collection of the electrospun material (post-treatment). This coating can be performed either by adsorption or by covalent bonding (this last approach may require

functionalisation of the polymer). Also, the drug can be mixed with the initial polymer solution to be later dispersed into the polymer matrix of the fibre. In this case, the polymer matrix can play the role of a carrier. Alternatively, with a proper choice of solvents, the drug can be incorporated in the polymer solution in the form of micelles. This approach is often referred to as "emulsion electrospinning" (Angeles et al., 2008). Using multi-fluid electrospinning, many different structures have been created. With a nozzle containing concentric needles, the drug and the carrier can either be encapsulated in the core or in the sheath around the polymer fibre. This technique can also be used to produce fibres of non-electrospinnable materials by combining the drug with a carrier, if the sheath uses an electrospinnable polymer that is later dissolved to expose the other drug-loaded material.

The release profile of the drug will depend on the approach used to incorporate the drug in the material. However, a typical profile in polymer-based scaffold exhibits an important initial "burst" release, followed by a plateau phase. Minimising the burst release is often one of the objectives during the optimisation of drug delivery systems.

Several authors have reported that signalling molecules released from electrospun fibres are able to induce desired cellular responses (Li et al., 2008; Cui et al., 2006). Although this suggests that the activity of each released compound is conserved to some extent, it does not mean that the drug's behaviour is similar to its native protein analogue (Ji et al., 2010). In fact, many factors may be harmful to encapsulated protein activity during the scaffold preparation process, such as the high voltage and the contact with or-

ganic solvents. Also, the residence within the fibre and the degradation of the scaffold can affect protein stability. Although it is hard to quantify the loss of activity due to the electrospinning process, the structural integrity can be examined by fluorescent spectroscopy (Zeng et al., 2005).

In order to minimize the interaction between protein and organic phase and improve the preservation of activity, the addition of hydrophilic additives such as polyethylene glycol or hydroxyapatite was reported (Ji et al., 2010). Also, the choice of solvent is crucial, as denaturation of the drug should be avoided. So far, the choice of solvent has been limited to allow for the electrospinning process, i.e. they must have high volatility, high conductivity and dissolve the components to be electrospun.

2.6.3 Calcium phosphate materials for drug delivery

CaP materials have also been shown to be suitable for drug delivery (Bose and Tarafder, 2011; Habraken et al., 2007). In addition to their biocompatibility, calcium phosphates have excellent surface and dissolution properties, which are adequate for drug adsorption and subsequent release (Shinto et al., 1992). In addition to their osteoconductive properties that make them useful to coat metal implants, calcium phosphates can also become osteoinductive by retaining and slowly releasing cell-derived signalling molecules to stimulate the surrounding tissue. Besides adsorption, drugs can also be incorporated in CaP materials by co-precipitation with the CaP, which might offer higher storage capacity and steadier release profiles (Wang et al., 2011).

When the ceramic material is combined into a polymer matrix (ceramic-polymer composites), various release profiles can be obtained, depending where the drug has been incorporated. If the drug is in (or bound to) the polymer phase, the presence of CaP is expected to decrease the initial burst release due to the interaction of the bioactive compound with CaP active surface. If the drug is initially linked to the CaP phase, the polymer matrix may act as a barrier against the diffusion of the drug, also resulting in a more sustained drug release.

2.7 Summary

A comprehensive literature review of the current state of the art indicates that there is still no widely accepted approach for the complete repair of osteochondral defects and that further development of the strategies is needed. In this context, the TE approach offers very encouraging solutions. Moreover, it seems clear that the eventual goal of osteochondral tissue engineering is to replicate the zonal structure of the cartilage and the underlying bone, and to obtain a strong interface between the two tissues. To this end, electrospinning may offer a promising solution.

Electrospinning has recently attracted much interest in the field of TE because it produces scaffolds with features that have sizes in proportion to the natural extracellular matrix. It is also possible to produce a wide range of different fibres compositions, in-

cluding composite and ceramic fibres. Thin membranes with random fibre orientation can easily be produced, and can be used in a novel layering approach combining them with cell sheets to build 3D tissue constructs.

As a cell source, the use of mesenchymal stem cells has a tremendous potential. They have the inherent ability to self-renew, and can later differentiate into many different tissue specific cell types, including bone and cartilage. The high specific surface area of electrospun materials suggests that growth factors can be incorporated into the fibrous scaffolds to have a controlled and localised drug release. This approach could enable the development of multiphasic tissues for regenerative medicine, such as implants for the osteochondral defects.

Chapter 3

Materials and Methods

3.1 Materials and equipment

The materials used for this study are listed in Table 3.1. Similarly, the equipment used in the studies is listed in Table 3.2.

Table 3.1: List of materials used for the present work.

Materials	Purchased from
Trypsin/EDTA solution, Phalloidin texas red, Live/Dead viability assay	Gibco BRL, Invitrogen Ltd., Paisley, UK
Mesenchymal Stem Cell Growth Medium Bulletkit, hMSC Chondro Bulletkit, Human Mesenchymal Stem Cells, TGF- β 3	Lonza, Basel, Switzerland
Alamar Blue	Biosource, London, UK
Cell culture flask, 24-well plates, 96-well-plates, needles, syringes, PE membranes	Falcon Fahrenheit, Milton Keynes, UK

Materials (cont.)	Purchased from
Poly(lactic-co-glycolic acid) (75:25) (Mw : 66,000-107,000), Phosphate buffered saline (PBS), Dimethyl sulfoxide (DMSO), Hoechst 33258, Teflon Tubing, Glycerol (99%), Poly- ϵ -caprolactone (Mw : 70,000-90,000) Primers and probes (for details, see Tables 3.5 and 3.6), Hydroxyapatite nanoparticles, Monoclonal anti-human collagen type X, 3,3'-diaminobenzidine tetrahydrochloride (DAB), DPX reagent, Albumin-fluorescein isothiocyanate conjugate (BSA-FITC, named BSA by default),	Sigma-Aldrich Chemical Company Ltd., Dorset, UK
Collagen rat tail type I	First Link UK Ltd., Birmingham, UK
Hexafluoroisopropanol (HFIP)	Apollo Scientific Limited, Cheshire, UK
Adhesive carbon tabs, Aluminium specimen stubs,	Agar Scientific, Essex, UK
Nunc UpCell Surface, Haematoxylin (Shandon Harris), Verso cDNA synthesis Kit, NucleoSpin RNA II extraction kit, Absolute Blue QPCR mix (with ROX),	Thermo Scientific UK Ltd., Leicestershire, UK
Guava ViaCount assay	Millipore UK Ltd., Durham
Monoclonal antibodies anti-CD29, -CD34, -CD44, -CD45, Monoclonal anti-human collagen type I, Polyclonal anti-human collagen type II	Abcam, Cambridge, UK
Monoclonal antibodies anti-CD14, - CD105 and -CD106 -CD105 and -CD106, Monoclonal anti-human aggrecan, DuoSet ELISA Development kit	R&D Systems Europe Ltd., Abingdon, UK
EnVision+ Dual Link System-HRP	Dako Ltd., Ely, UK
Live/Dead Cell Viability Assay	Invitrogen, Paisley, UK
Hydrogen peroxide Histoclear	Fisher Scientific Ltd., Loughborough, UK

Table 3.2: List of equipment used for the present work, with origin.

Equipment	Origin
Syringe pump	Harvard Apparatus Ltd., Kent, UK
High voltage supply (model 73030DC)	Genvolt, Shropshire, UK
Electrospinning glove box	IBME workshop
RotorGene 6000	Corbett LifeScience
Multiphoton microscope	Mira-coherent system, UK
Spectrophotometer GENios	Tecan, Jencons, UK
Fourier transform infrared (FTIR) spectrometer	Bruker Optics Ltd., Ettlingen, Germany
Tensile testing machine (load cell of 20 N)	ElectroForce Systems Group, Bose Corporation, Minnesota, USA
Microplate reader	Perkin Elmer Ltd., Beaconsfield, UK
Nikon Eclipse 80i microscope	Nikon Instruments Inc., New York, USA
Flow cytometry	FACSCalibur, Becton Dickinson, UK
Electron microscope JEOL JSM 840F	JEOL Ltd., Tokyo, Japan
Cressington 208 HR sputter coater	Vortex Control Systems Inc., Texas, USA
Vibracell ultrasonicator	Sonics & Materials Inc., Newtown, USA
Contact Angle	Teclis, Marseille, France

3.2 Electrospinning

3.2.1 Electrospinning apparatus

The electrospinning apparatus used for this study is shown in Figure 3.1 and Figure 3.2. This apparatus was manufactured in the IBME workshop and uses a high voltage power supply (model 73030DC, Genvolt, Shropshire, UK), a syringe pump (Harvard Apparatus Ltd., Kent, UK), and a gas-tight, sterile glove box containing the electrospinning nozzle and the electrodes. A gas supply system (BOC Gases, Manchester, UK) was also used to provide a constant flow of nitrogen to maintain an inert atmosphere inside the chamber.



Figure 3.1: Electrospinning setup showing the HV power supplier (A), the syringe pump (B), and the glove box where the fibre meshes are produced (C).

The HV power supply system was used to create a potential difference of up to 30kV

between the electrode located at the end of the insulator post (the anode) and the grounded collector (the cathode). The electrodes are shown in Figure 3.2. The polymer solution was supplied to the nozzle with the pump via a Teflon tube. Most experiments were carried out using a single channel nozzle, as shown in Figure 3.3(a). The polymer solution was pushed through nozzle, where it was electrically charged through contact with a brass anode before reaching a Teflon tip for the jet formation. Both the tip and the tubing had an internal diameter of 0.8 mm and external diameter of 1 mm. When the coaxial nozzle was used (Figure 3.3(b)), inner and outer polymer solutions were individually supplied to the electrode. Both solutions were charged as they passed through the stainless steel needles which were connected to the brass anode inside the nozzle. The inner needle internal diameter was 0.3 mm and external diameter was 0.6 mm, while the outer needle had an internal diameter of 0.8 mm and an external diameter of 1 mm. The cathode collector was a flat plate made of stainless steel (316 grade) and the distance to the tip of the nozzle was maintained at 14 cm for both single and dual channel systems.

For all experiments, a constant flow of nitrogen (oxygen-free, 5L/min) was provided inside the glove box to create an inert atmosphere. The glove box was connected to the fume cupboard via a long hose to evacuate the vapours of solvent released during the fibre solidification.

For electrical safety, microswitches were placed at the upper and the front lids of the glove box, preventing the user from applying the voltage whilst accessing the electrodes.

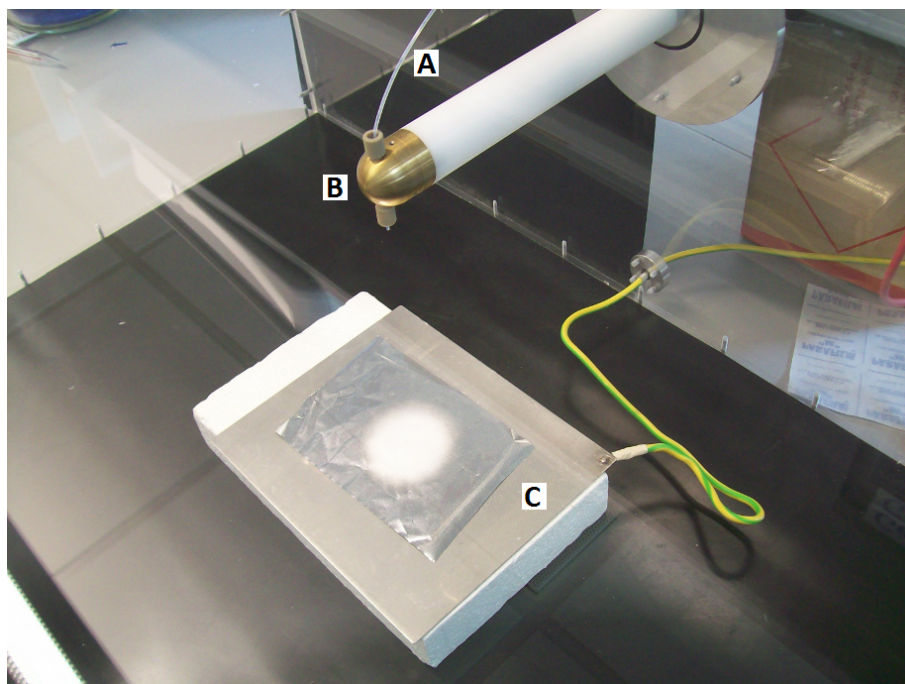


Figure 3.2: View showing the inside of the glove box with the capillary tubing (A), the charging electrode(B), and the collector(C). The area where the fibre mesh is collected appears as a white disk on the piece of aluminium foil covering the stainless steel plate.

3.2.2 Preparation of the electrospinning apparatus

The experiments were carried out in aseptic conditions, where possible. The inner walls and the gloves of the box were thoroughly cleaned with 70% ethanol prior to each experiment. Pieces of aluminium foil (about 200x200 mm), tweezers, and other objects being introduced into the glove box were also cleaned with 70% ethanol. The polymer solution was transferred to a syringe (generally 1 ml or 5 ml volume) and the air bubbles were carefully removed before positioning the syringe into the pump. The fluid was then pushed through the tube until it reached the electrode.

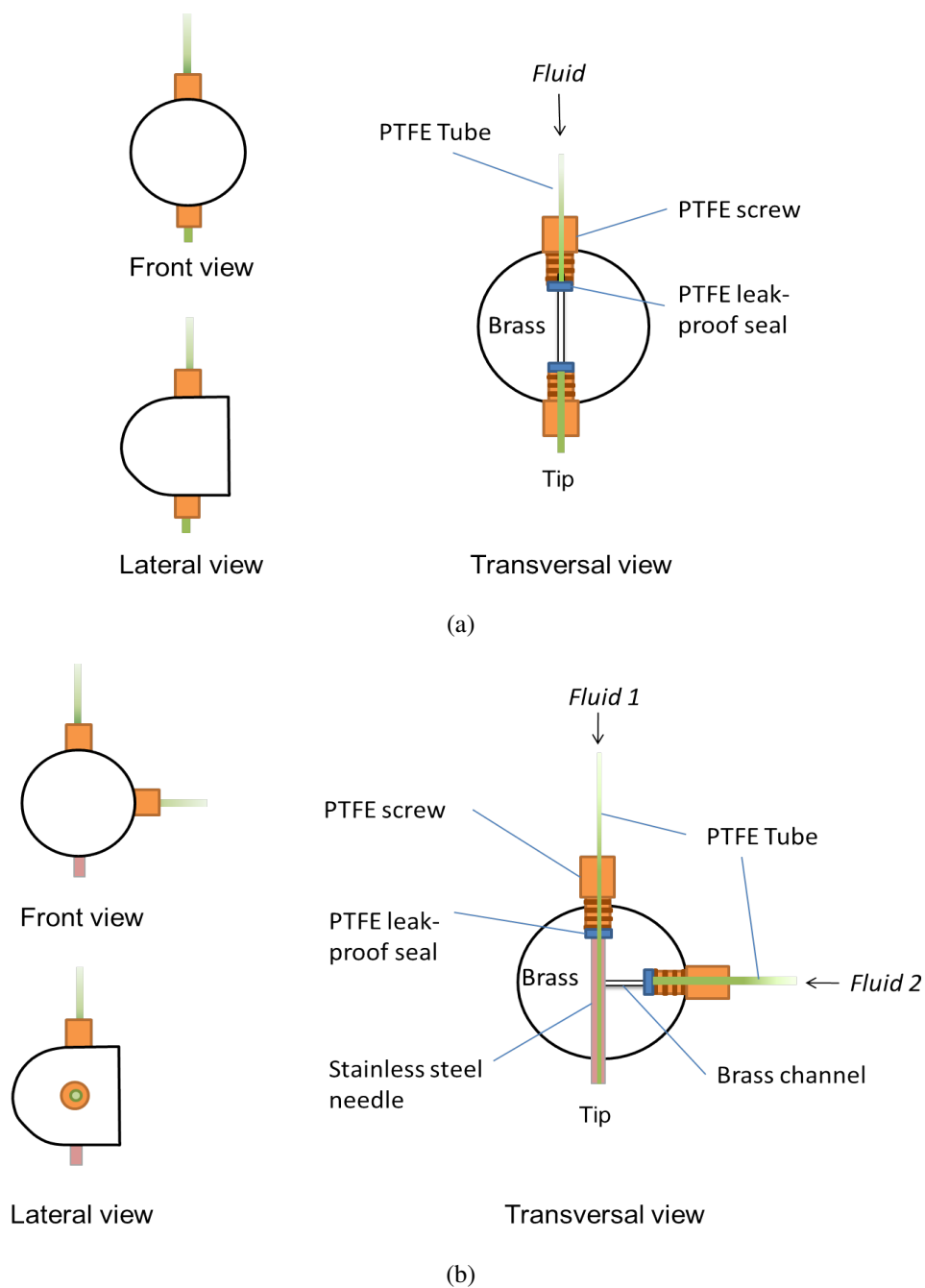


Figure 3.3: Front, lateral and transversal schematic views showing: (a) the single channel nozzle (also pictured in Figure 3.2) and (b) the co-axial nozzle used in the studies.

3.2.3 Preparation of electrospinning solutions

Polymer solutions were prepared by dissolving Poly(lactic-co-glycolic acid) (PLGA, ratio 75:25, Mw: 66-107 kDa, Sigma-Aldrich Chemical Company Ltd., Dorset, UK) or Poly- ϵ -caprolactone (PCL, Mw: 70-90 kDa, Sigma Aldrich) into 1,1,1,3,3,3-hexafluoroisopropanol (HFIP, Apollo Scientific Limited, Cheshire, UK). The structure of these polymers is represented in Figure 3.4. For composite fibres, hydroxyapatite synthetic nanopowder (nHAp) with an average particle diameter of around 93 nm (Sigma Aldrich) was added to the polymer solution at concentrations varying between 1% and 50%(w/v). Lyophilised rat tail collagen type I (First Link UK Ltd., Birmingham, UK) was also dissolved together with the polymer and nHAp at various ratios. Solutions were agitated at room temperature on a roller for at least 24 hours to allow for complete dissolution of the polymers. Each solution was then homogenised on ice using a vibracell ultrasonicator (130 W, 20 kHz; Sonics Materials Inc., Newtown, USA) prior to electrospinning.

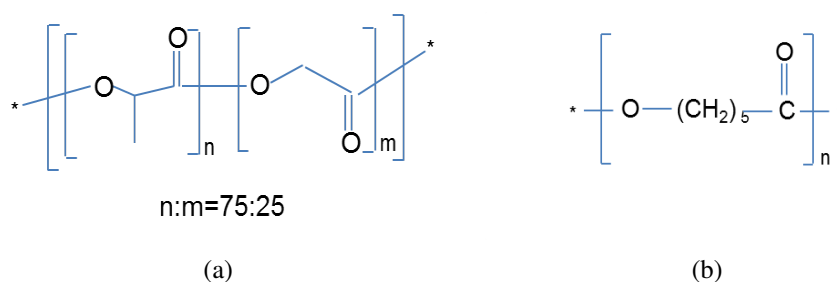


Figure 3.4: Schematic representation of the molecular structure of the synthetic polymers used in this study: (a) Poly(lactic-co-glycolic acid) and (b) Poly- ϵ -caprolactone.

3.2.4 Preparation of electrospun membranes for TE applications

The polymer solution was transferred to a 1 ml or 5 ml syringe and positioned in the pump for the electrospinning process. All experiments were carried out with a flow rate of 1ml/hr. The voltage was adjusted to obtain a stable jet and the duration of electrospinning was adjusted to control the thickness of the fibrous mat. The electrospun fibres were collected in the form of a random mesh on top of a thin layer of sterile phosphate buffer saline (PBS) solution spread on the collector. The membrane floating on top of the fluid was easily removed by use of a Petri dish (60mm or 100mm diameter depending on the mesh diameter). The open face of the dish was placed onto the membrane and the membrane's coronal edges were pulled up onto the dish's sidewalls. After removal from the collector, the samples were dried in a laminar flow hood overnight. Further drying was achieved within a vacuum chamber to aid removal of remaining HFIP from the membranes. All samples were stored in a desiccator.

For the studies described in Chapter 4, Group 1 is used to refer to PLGA membranes containing hydroxyapatite nanoparticles at different concentrations while Group 2 is used to refer to PLGA membranes containing both collagen type I and hydroxyapatite nanoparticles at different ratios. The top and bottom sides of the membranes, referred as the upper and lower sides, respectively describe the face in contact with the air and the lower face in contact with the PBS solution, during electrospinning. In Chapter 5, the terms PLGA-c and PCL-c refer to PLGA and PLC-based membranes, each containing both collagen type I and hydroxyapatite nanoparticles at fixed concentrations.

3.2.5 Preparation of calcium phosphate fibres

The electrospun composite samples were folded to fit into an alumina ceramic crucible and the container was placed in the middle of a furnace (University of Oxford, Materials Department, Parks Road). The heat treatment was applied in a 0.5 bar Argon atmosphere at 1100°C for one hour. The furnace was brought to a temperature with a heating rate of 16°C/min and was cooled at a rate of 6°C/min. The samples were collected the next day and were removed for characterisation.

3.3 Physico-chemical characterisation

3.3.1 Scanning electron microscopy (SEM)

From each electrospun membrane, pieces were cut and mounted on an aluminium stub using a carbon adhesive disk. For large sample such as HA disks, silver paint was also used to make electrical contact from the surface of the sample onto the carbon disk. Samples were then coated with a 2.5nm layer of platinum using a Cressington 208 HR Sputter coater (Vortex Control Systems Inc., Texas, USA) and high resolution images of the scaffolds were taken using a JSM 840F scanning electron microscope (JEOL, Tokyo, Japan). Three different areas were observed randomly on each sample at magnifications of 400x, 2000x, and 10000x. Fibre diameters were measured on each picture using ImageJ software for statistical analysis. In addition to morphology, the thickness of each membrane was also measured by SEM, after mounting the samples at an angle of 90° on the aluminium stub.

For the preparation of biological samples for SEM imaging, membranes with attached cells were fixed in glutaraldehyde (2.5% v/v in deionised water) overnight. The fixative was removed and membranes were rinsed twice in PBS before undergoing sequential dehydration in a graded ethanol series - 40%, 70%, 90%, 95%, to 100% ethanol in deionised water - for 10 minutes each step. Cells were further dehydrated using hexamethylsilazane and samples were left inside the fume cupboard overnight for complete drying. After platinum coating (described above), biological samples were observed under SEM.

3.3.2 Fourier transform infrared spectroscopy (FTIR)

The surface chemistry of the membranes was analysed by using a FTIR spectrometer (Bruker Optics Limited, Ettlingen, Germany). Each spectrum was acquired and averaged with a resolution of 4 cm^{-1} by accumulation of 128 scans and the signal was measured in a range between 400 and 4000 cm^{-1} .

3.3.3 Tensile testing

Mechanical properties of the scaffold were assessed using a tensile testing machine (ElectroForce Systems Group, Bose Corporation, Minnesota, USA) with a load cell of 20N. A template with standard dimension (BS 2782-2,1996) was used to cut the specimens to a dog-bone shape. The extension rate applied to all samples was fixed at $50\mu\text{m/s}$. A minimum of 6 samples were tested for each type of membrane fabricated.

3.3.4 Contact angle measurements

An approximation of surface hydrophobicity was assessed by measuring static contact angle formed by a drop of deionised water on the surface of the sample by using a CCD camera assisted with digital measurements, as described by Benjamins et al. (1996). Experiments were performed on fibres collected on aluminium foil in order to maintain the scaffolds in a flat position.

3.3.5 Simulated body fluids (SBF)

The SBF solution was prepared according to the method described by Oyane et al. (2003). NaCl, NaHCO₃, Na₂CO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, HEPES, CaCl₂ and Na₂SO₄ were dissolved in deionised water as described in Table 3.8, filtered and stored at 4°C. The fluid was adjusted to a final pH of 7.40 at 37°C. All chemicals were purchased from Sigma Aldrich (Dorset, UK). After incubation in the final SBF solution, the membranes were rinsed twice in dH₂O and set to dry in the fume hood for 24 hours.

Table 3.3: List of the different amounts of salts used to prepare 500mL of SBF solution.

Reagent	Amount (g)
NaCl	2.70
NaHCO ₃	0.37
Na ₂ CO ₃	1.02
KCl	0.11
K ₂ HPO ₄ ·3H ₂ O	0.12
MgCl ₂ ·6H ₂ O	0.16
HEPES	5.96
CaCl ₂	0.15
Na ₂ SO ₄	0.04

3.3.6 X-Ray diffraction (XRD)

The sample was prepared by grinding the fibres into a powder and spreading the powder onto a crystal of silicon covered with a layer of silicone grease. Excess powder was removed and the crystal and powder were exposed to an X-ray beam. XRD was carried out using a fully automated Siemens D5000 powder diffractometer, employing copper $k\alpha$ radiation ($\lambda=0.15406\text{nm}$) and a secondary monochromator. The samples were continuously spun during data collection and scanned using a step size of $0.05^\circ 2\theta$ over the range of 5° - $75^\circ 2\theta$, with a count time of 12 seconds per step.

3.3.7 X-Ray photoelectron spectroscopy (XPS)

The samples were mounted on a stub using a double sided adhesive tab before being placed into the ultrahigh vacuum (2×10^{-9} mbar) analysis chamber of the spectrometer. XPS analysis was carried out using a VG Clam 4 MCD analyser system (Begbroke Science Park) with X-ray radiation from the Al $K\alpha$ band ($h\nu= 1486.7$ eV). Recording was performed with an analyser energy of 100eV and a takeoff angle of 90° . Charge accumulation was prevented by bombarding the surface of the sample with an electron beam (10eV). The spectra was analysed using Microcal Origin 6.0 software and the assignment of the peaks was performed with reference to the UKSAF and NIST databases.

3.3.8 Statistical analysis

All statistical analysis was carried out using the Student's t-test with a confidence interval of 95%.

3.4 Biological characterisation

3.4.1 hMSCs culture

Human mesenchymal stem cells (hMSCs) were purchased from Lonza (Basel, Switzerland). Cells were thawed and cultured according to the manufacturer's instructions. Briefly, cells were seeded in tissue culture flasks (T75, treated polystyrene, Corning Inc., New York, USA) with mesenchymal stem cell growth medium (MSCGM, Lonza) which contains 10% mesenchymal cell growth supplement (MCGS, Lonza), 2% L-Glutamine (Lonza), and GA-1000 (Gentamicin-sulfate, amphotercin-B, Lonza). Flasks were placed in a 37°C humidified 5% CO₂ incubator (HERA cell150, Thermo Scientific, Leicestershire, UK). The medium was changed every two or three days until the cells reached confluence. Cells were then trypsinised and seeded onto temperature-responsive Petri dishes (Nunc UpCell Surface, Thermo Scientific, Leicestershire, UK) at high density (10^4 cells/cm²). The hMSCs were cultured in the Upcell dishes under the same initial conditions and were ready for use when at confluence.

3.4.2 Cell adhesion study

To study the cell adhesion to the membranes, about 5×10^3 /cm² hMSCs were seeded onto each membrane tested, cultured in 6-well plates, and fixed at the following time points: 3 hours, 1 day, 3 days, 7 days. The side of each membrane seeded with cells was chosen randomly. Flat polyester (PE) membranes with small pores were used as controls and all tests were performed in triplicate. For each time point, cell were fixed with

a solution of 4% formaldehyde in PBS for 15 minutes. To observe cell morphology on the membranes, actin filaments were stained using a phalloidin texas red dye (Invitrogen, UK). Samples were washed (3x) with PBS and 0.1% Triton X-100 was added for 5 minutes to permeabilise the cells. After three more washing steps using PBS, 200 μ l of a PBS solution containing 1% bovine serum albumin was added to each sample. Each 200 μ l of the PBS solution was also supplemented with 5 μ L of a 20 μ M phalloidin texas red stock solution. After an incubation time of 20 minutes at room temperature, samples were washed again (3x) with PBS and mounted on a glass slide by using Mowiol (Sigma, Dorset, UK). Samples were then observed using a fluorescent microscope (Nikon Eclipse 80i, Nikon Instruments Inc., New York, USA).

3.4.3 Cell proliferation study

The alamarBlue (AB; Biosource, London, UK) assay was used to assess the proliferation of the cells on PLGA, PLGA-Col, and PLGA-Col-nHAp electrospun membranes. Test samples were punched from the electrospun membranes with a diameter of 6mm, sterilised with UV light (20 minutes each side), and placed into the wells of 96-well plates. Samples were tested at 1, 2, 3, 5, and 7 days after inoculation of hMSCs at 5×10^3 cells/well. Seeded samples were transferred to new 96-well plates after one day of culture. The side of the membrane seeded with cells was chosen randomly. The control used for this experiment was the bottom of the 96-well plate. Six measurements were recorded for each type of membrane.

AB was added into culture medium to obtain a final concentration of 5%. 200 μ l of this solution was used to replace the medium in each 96-well containing cells and membranes. As a negative control, medium with AB was also added to empty wells. The plate was placed in an incubator for four hours at 37°C, and then 100 μ l were transferred from each 96-well to a new 96-well plate for measurement. During incubation, resazurin, with a maximum absorption of 605nm (dark blue), is reduced by cells into resorufin, which has a maximum absorption of 573nm (pink). Accumulation of reduced AB into the culture medium was determined using a spectrophotometer Tecan GENios (Jencons, UK) by measuring absorbance of the test and control wells at wavelengths of 570nm and 600nm. The number of viable cells correlates with the magnitudes of dye reduction and is expressed as percentage of AB reduction. The calculation of the percentage of AB reduction is shown in the following equation according to the manufacturer's instruction:

$$\%AB \text{ Reduction} = \frac{(\epsilon_{ox,600nm} \times A_{570nm} - \epsilon_{ox,570nm} \times A_{600nm})}{(\epsilon_{red,570nm} \times A'_{600nm} - \epsilon_{red,600nm} \times A'_{570nm})} \times 100$$

where

$\epsilon_{ox,600nm}$	=	117,216 (molar extinction coefficient of oxidized AB at 600 nm)
$\epsilon_{ox,570nm}$	=	80,586 (molar extinction coefficient of oxidized AB at 570 nm)
$\epsilon_{red,600nm}$	=	14,652 (molar extinction coefficient of reduced AB at 600 nm)
$\epsilon_{red,570nm}$	=	155,677 (molar extinction coefficient of reduced AB at 570 nm)
A_{570nm}	=	absorbance of the sample at 570 nm
A_{600nm}	=	absorbance of the sample at 600 nm
A'_{570nm}	=	absorbance of negative control wells without cells at 570 nm
A'_{600nm}	=	absorbance of negative control wells without cells at 600 nm

3.4.4 Cell sheet engineering and multilayering

A schematic diagram showing the different steps involved in the preparation of multilayered constructs is shown in Figure 3.5. Electrospun membranes were prepared from two sets of polymer solution (see Table 3.4). The first set of membranes, named PLGA-c, was electrospun for 6 minutes at 14.4 ± 0.4 kV from a mixture of 10% (w/v) of hydroxyapatite nanoparticles (nHAp) in a polymer solution containing 15% (w/v) of a blend of Poly(lactic-l-glycolic acid) and rat tail collagen type I in ratio 8:2 (w/w) in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). The second set of membranes, named PCL-c, was electrospun for 4 minutes at 13.5 ± 0.1 kV from a mixture of 10% (w/v) nHAp in a polymer solution containing 8% (w/v) of a blend of Poly- ϵ -caprolactone and rat tail collagen type I in ratio 8:2 (w/w) in HFIP.

Table 3.4: Composition of electrospinning solutions and voltage used for the electrospinning process.

	Polymer phase	Ceramic phase	Solvent	Voltage (kV)
PLGA-c	PLGA:Collagen 15% (%w/v) (ratio 8:2, %w/w)	nHAp 10% (w/v)	HFIP	14.4 ± 0.4
PCL-c	PCL:Collagen 8% (%w/v) (ratio 8:2, %w/w)	nHAp 10% (w/v)	HFIP	13.5 ± 0.1

After electrospinning, disks of 18 mm diameter were punched from the membranes (either PLGA-c or PCL-c). Each side of the disk was sterilised by exposure to UV light for 20 minutes. Temperature-responsive UpCell Petri dishes (35mm diameter, Thermo Scientific Nunc Surface) were used for the production of cell sheets. After 4 to 6 days of culture, when cells had formed a confluent cell layer, the culture medium was removed

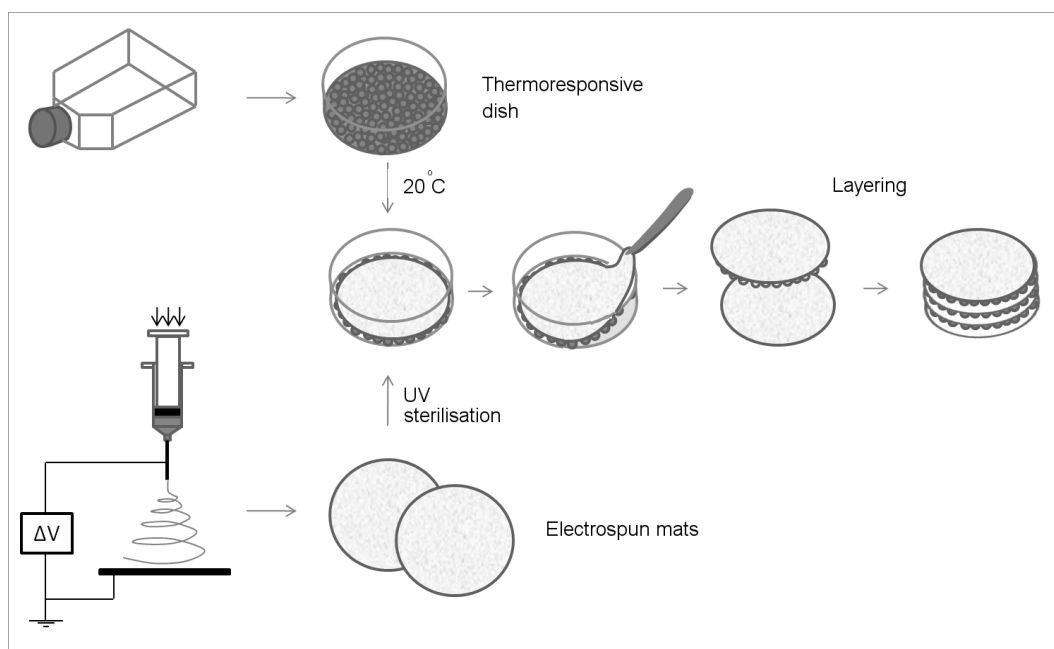


Figure 3.5: Schematic diagram showing the different steps involved in the preparation of multi-layered constructs. Electrospun membranes are used to pick up the cell sheets from the thermoresponsive dishes, forming a unit block. These are then stacked together to form the 3D assemblies comprising a total of three layers of cells.

from the Upcell dishes and cells were left for 20 minutes at 20°C with $50\mu\text{l}$ of fresh medium. After that time, each cell layer became detached and was removed with an electrospun disk (see Figure 3.5). The resulting unit blocks were left for 30 minutes at 37°C with $50\mu\text{l}$ of fresh medium. A construct was then built by stacking together three unit blocks of the same composition (either PLGA-c or PCL-c) onto a pre-incubated electrospun disk in culture medium (see Figure 3.5). Each multilayered assembly was left for 3 hours in a 6-well plate with $150\mu\text{l}$ of medium to allow for cell attachment between the different layers. Finally 2ml of fresh growth medium was added to each well and the samples were left for 3 days prior differentiation.

3.4.5 Assessing the cell coverage after transfer of the cell sheet

To assess the cell coverage of the membrane, cells sheets were cultured on electrospun membranes for 3 days after transfer. Samples were then fixed with a solution of 4% paraformaldehyde solution for 15 minutes in PBS, stained with phalloidin texas red dye (see paragraph 3.4.2) and mounted on a glass slide in Mowiol(Sigma, Dorset, UK). The fluorescence images revealing the cells were converted to binary mode (after adjusting the threshold) by using the ImageJ software (Abramoff et al., 2004) and the ratio black to white was used to assess the fraction of the area of the membranes covered by the cells.

3.4.6 Differentiation of hMSCs in 3D constructs

The tissue constructs were cultured for 21 days in chondrogenic medium (Lonza, Basel, Switzerland) to induce chondrocyte differentiation of hMSCs. Serum-free basal medium was supplemented with dexamethasone, ascorbate, penicillin, streptomycin, sodium pyruvate, proline, and L-glutamine (Lonza). The growth factor TGF- β 3 (10 ng/ml) was also added to stimulate chondrogenic differentiation of the stem cells, and the medium was replaced every 2-3 days.

3.4.7 Viability test

The number of viable, apoptotic and dead cells was measured by using the Guava ViaCount assay (Millipore, Durham, UK). Cells were detached from the construct using concentrated trypsin (2x), resuspended in culture medium at a density of 10^5 cells/mL, stained for 10 minutes with the Guava ViaCount reagent by adding 450 μ l to each 50 μ l of

cell suspension. Cells were then sorted by flow cytometry (FACSCalibur, Becton Dickinson, UK). Additionally, the cell viability was assessed *in situ* within the construct with the Live/Dead viability assay (Invitrogen, Paisley, UK): the samples were washed twice with PBS and incubated in PBS at 37°C for 30 minutes with 1 μ l/ml of calcein AM (1mM) and with 2 μ l/ml of propidium iodide (10 μ M). Images were recorded using a multiphoton microscope (Mira-Coherent System, Ely, UK).

3.4.8 hMSCs surface markers

To analyse surface expression markers of hMSCs after culture on electrospun membranes, cells were trypsinised, washed and incubated with complete medium on a rotator (40 rpm, Blood Tube Rotator, Stuart Scientific SB1, UK) for 3 hours (37°C, 5% CO₂). Cells (1x10⁴, 300 μ l) were then dispensed into a 96 V-bottom well plate, and spun down at 300g, 4°C for 5 minutes. Cells were resuspended in 100 μ l of culture medium with the following monoclonal antibodies (1:100 in dilution) anti-CD29, -CD34, -CD44, -CD45 (Abcam, Cambridge, UK), -CD14, -CD105 and -CD106 (RD Systems, Abingdon, UK) conjugated with phycoerythrin. The populations of stained cells were then detected by flow cytometry, with isotype controls being prepared in the same manner.

3.4.9 RT-PCR

Total cellular RNA was extracted using the NucleoSpin RNA II extraction kit (Thermo Scientific UK Ltd., Leicestershire, UK) according to manufacturer's instructions. Reverse transcription was then performed with the Verso cDNA synthesis Kit (Thermo Scientific

UK Ltd., Leicestershire, UK) and the nanodrop technique (ND-1000 spectrophotometer, Labtech International, Ringmer, UK) was used to evaluate and adjust the concentration of cDNA in each sample. The cDNA was then mixed with probes, primers, and absolute blue QPCR mix with ROX (Thermo Scientific UK Ltd., Leicestershire, UK). Primers and probes were all purchased from Sigma Aldrich (Dorset, UK). Their sequences are presented in Table 3.5 and Table 3.6 respectively. Real time PCR was carried out with the RotorGene 6000 (Qiagen Ltd., West Sussex, UK). The following PCR profile was used: 40 cycles at 95°C for 9 seconds, 60°C for 60 seconds after activation of polymerase at 95°C for 15 minutes. Volumes and concentration of PCR reagents used for each 20 μ l of mixture are indicated in Table 3.7.

Table 3.5: Primer sequences for GAPDH, Collagen II, Collagen X, Sox9 and Aggrecan

Gene	Forward sequence	Reverse sequence
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC
Type II collagen	GGCAATAGCAGGTTACGTACA	CGATAACAGTCTTGCCCCACTT
Type X collagen	CAAGGCACCATCTCCAGGAA	AAAGGGTATTTGTGGCAGCATATT
Sox9	CACACAGCTCACTCGACCTTG	TTCGGTTATTTTTAGGATCATCTCG
Aggrecan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA

Table 3.6: Probe sequences for GAPDH, Collagen II, Collagen X, Sox9 and Aggrecan

Gene	probe sequence
GAPDH	CGCCCAATACGACCAAATCCGTTGAC
Type II collagen	CCGGTATGTTTCGTGCAGCCATCCT
Type X collagen	TCCCAGCACGCAGAATCCATCTGA
Sox9	CCCACGAAGGGCGACGATGG
Aggrecan	ATGGAACACGATGCCTTTCACCACGA

Table 3.7: Volumes of PCR reagents used for the real time PCR study.

Reagent	Volume
Absolute Blue QPCR mix + Rox	12.5 μ l
Forward primer (100 μ M)	0.25 μ l
Reverse primer (100 μ M)	0.25 μ l
Probe (10 μ M)	0.5 μ l
Molecular grade water	6.5 μ l

3.4.10 Paraffin embedding and sectioning

After culture, the multilayered constructs were fixed in a 4% paraformaldehyde solution at 4°C overnight and were demineralised in a 10% EDTA solution (pH 7.4) for 2 weeks. The samples were then placed in agar, dehydrated in increasing grades of ethanol and xylene, and were finally embedded in paraffin. The paraffin blocks were cut into sections (4 μ m) by using a microtome (Leica Microsystems Ltd., Milton Keynes, UK) and were then collected on adhesive slides (Colorfrost Plus, Thermo Shandon Ltd., Runcorn, UK).

3.4.11 Immunohistochemistry

Tissue sections were dewaxed in xylene and ethanol before being rehydrated in water. For epitope retrieval, sections were placed in a citrate buffer solution (pH6) at 98°C for 25 minutes. Samples were then incubated for 2 hours at room temperature in the presence of the primary antibodies. Tissue sections were stained with anti-human collagen type I (monoclonal, Abcam, Cambridge, UK), anti-human collagen type II (polyclonal, Abcam, Cambridge, UK), anti-human collagen type X (monoclonal, R&D Systems, Abingdon, UK), and anti-human aggrecan (monoclonal, R&D Systems, Abingdon, UK). Following

these procedures, the EnVision+Dual Link System-HRP (Dako Ltd., Ely, UK), containing horseradish peroxidase-labelled secondary antibodies, was applied to each section for 30 minutes at room temperature. Specific staining was revealed by placing the samples into a Tris-buffered saline (TBS) solution with 0.05% of 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma, Dorset, UK) and 0.009% hydrogen peroxide (Fisher Scientific Ltd., Loughborough, UK) for 10 minutes. Sections were counterstained with haematoxylin (Shandon Harris, Thermo Scientific UK Ltd., Leicestershire, UK), dehydrated in ethanol and HistoClear (Fisher Scientific Ltd., Loughborough, UK), and mounted on coverslip using DPX reagent (Sigma, Dorset, UK). Images were observed using an optical microscope (Nikon eclipse 80i, Nikon Instruments Inc., New York, USA) and recorded using a Nikon camera (Digital sight DS-5M, Nikon Instruments Inc., New York, USA).

3.5 Drug delivery from electrospun fibres

3.5.1 Preparation of drug releasing membranes

The fibres designed for the drug delivery experiments were created with the coaxial nozzle described in subsection 3.2.1. For the preparation of membranes loaded with BSA, a core solution was consisting of 8% (w/v) PCL and 5% (w/v) Bovine Serum Albumin-fluorescein isothiocyanate conjugate (BSA-FITC, shortened BSA in this thesis, Sigma Aldrich) in HFIP was prepared. The sheath solution was made by mixing PLGA and collagen at a total concentration of 15% (w/v) and a ratio 8:2 (w/w) in HFIP with. For a number of studies, 10% of nHAp (w/v) was also added to the polymer solution.

For the membranes loaded with growth factors, the core solution was prepared by adding 250 μl of a 0.2mg/ml solution of human recombinant Transforming Growth Factor- $\beta 3$ (TGF- $\beta 3$, PromoKine GmbH, Heidelberg, Germany) to each 750 μl of PCL solution in HFIP. The amount of PCL dissolved in HFIP was chosen to achieve a final concentration of 8% (w/v). The sheath solution was made from a blend of PLGA and collagen at a ratio 8:2, as indicated in the previous paragraph.

The inner and outer polymer solutions were transferred to 3 ml syringes, positioned in the syringe pumps and the flow were set up at a steady rate of 0.33ml/hr and 0.66ml/hr respectively. The voltage was adjusted to obtain a stable jet. As described in the Section 3.2.4, the fibres were collected onto a thin layer of phosphate buffer saline (PBS) solution. After 10 minutes of electrospinning, the resulting mats were quickly removed from the liquid surface by use of cast Petri dish (an 18 mm hole was created at the bottom of the dish). Membranes were then washed with deionised water and left overnight to dry in the fume cupboard. After a further drying step in a vacuum chamber, BSA-loaded membranes were stored at 4°C while TGF- $\beta 3$ -loaded membranes were kept at -20°C.

3.5.2 Preparation of the SBF solution

A ten times concentrated body fluids solution (SBF10) was prepared according to the method described by Tas and Bhaduri (2004). A solution of NaCl, KCl, $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and NaH_2PO_4 (pH4.3 at room temperature) was prepared in deionised water

as described in Table 3.8, filtered and stored at 4°C. Shortly before use, the solution was activated by adding 10mM NaHCO₃ (pH6.5 at room temperature) and vortexed for 3 minutes. All chemicals were purchased from Sigma Aldrich (Dorset, UK).

Table 3.8: List of salts used to prepare 500mL of SBF10 solution.

Reagent	Amount (g)
NaCl	29.22
KCl	0.18
CaCl ₂ .H ₂ O	1.84
MgCl.6H ₂ O	0.51
NaH ₂ PO ₄	0.6
NaHCO ₃	0.42

3.5.3 Membrane mineralisation

For fully coating the fibres, the samples (still attached to the Petri dish) were immersed in 7 mL of SBF10 for 3 hours at room temperature. Control samples (no mineralisation) were immersed for three hours in 7 mL of deionised water. The membranes, once removed from the SBF10 solution, were rinsed twice in dH₂O and set to dry in the fume hood for 24 hours. The SBF10 solution and deionised water used for the experiment were collected to measure the loss of BSA during the mineralisation step.

3.5.4 BSA release study

Mineralised membranes were punched into small disks of 18mm diameter and were placed into 2 mL Eppendorf tubes containing 1 mL of PBS solution. A minimum of four membranes were tested per sample. The tubes were inserted onto a rotating plat-

form (20rpm, VWR, Dublin, Ireland) which was placed into an incubator maintaining a temperature of 37°C. The PBS solution was collected for measurement and each time replaced by fresh PBS solution. A minimum of eleven time points were taken over a duration of 4 days: 0.5,1,1.5,2,3,4,6,12,24,48,92. At the end of the experiment, the incubated membranes were dissolved in 1ml HFIP each for a minimum of 48 hours and the resulting solutions were diluted 1:1 with dionised water before measurement. The sum of the measurements were used to calculate the initial C_0 concentrations.

The amount of BSA in each sample was assessed by exciting the fluorescent marker at a wavelength of 495 nm and measuring the emission at 525 nm (at room temperature) in microplate reader (Perkin Elmer Ltd., Beaconsfield, UK). To correlate the fluorescent signal to the amount of BSA, a standard curve was measured from successive dilutions of a stock solution of BSA in PBS, for which the concentration was already known.

3.5.5 TGF release study

Study of the release profile

The membranes loaded with the growth factor were punched into disks of 18mm diameter and placed into Eppendorf tubes containing 1ml of PBS solution. Six membranes were tested per sample. Incubation was performed as described in the previous section. A total of 14 time points were measured over a duration of 4 days.

Detection of the TGF- β 3 released in the PBS solution was performed using the DuoSet ELISA Development kit (R&D systems, UK). The optical density was measured using a microplate reader set to 450 nm (Perkin Elmer Ltd., Beaconsfield, UK). These values were then correlated to the actual TGF- β 3 concentration after generating a standard curve according to the manufacturer's instructions.

Differentiation study

A brief investigation of the effect of the release of growth factors on the differentiation of hMSCs into chondrocytes was also carried out. A cell sheet was transferred onto a drug loaded membrane using the approach described in Section 3.5. Prior to differentiation, the cell sheet was incubated for three hours with 150 μ l of growth medium, to allow for cell attachment to the membrane. Cells were cultured for a week in 1mL of chondrogenic medium depleted in soluble TGF- β 3. The negative control consisted of cells cultured in 1mL of growth medium on PCL/PLGA-col fibres, whilst the positive control consisted of cells cultured in 1mL of chondrogenic supplemented with soluble TGF (10ng/mL) on PCL/PLGA-col fibres. For all samples, the medium was replaced twice over the week, at day 3 and 5. mRNA levels of expression for Collagen II and Collagen X were then assessed by RT-PCR according to the protocol described in Section 3.4.9.

Chapter 4

Functional Electrospun Scaffolds for Bone and Cartilage Tissue Engineering

4.1 Introduction

As mentioned in Chapter 2, a number of approaches have been proposed for the fabrication of osteochondral grafts, including the use of homogenous scaffolds (Schlichting et al., 2008; Yaylaoglu et al., 1999; Holland et al., 2005; Suominen et al., 1996; Wang and Hsieh, 2008) and heterogeneous biphasic scaffolds (Jiang et al., 2006; Schaefer et al., 2000; Chang et al., 2004b). However, a major drawback of these methods is that they fail to produce a functional interface between bone and cartilage that is able to ensure proper exchanges of gases and nutrients as well as appropriate transmission of mechanical stresses. Therefore, it has been suggested that the characteristics of the interface between the bone and cartilage is a crucial consideration in scaffold design in order to

obtain a successful osteochondral graft (Sharma and Elisseeff, 2004; Sharma et al., 2007; Woodfield et al., 2005; Harley et al., 2009; Lynn et al., 2005).

In vivo, the bone-cartilage interface is characterised by a gradual transition that occurs both in term of composition and architecture between unmineralized cartilage and mineralized bone. For example, in articular cartilage, calcium-phosphate content progressively increases from the surface, across the zone of calcified cartilage to reach high levels in the subchondral bone. Type II collagen content dramatically decreases from the high levels found in cartilage to disappear and be replaced by type I collagen in the subchondral bone (Mow and Hayes, 1997). Besides their composition, zones near the interface also vary in distribution and arrangement of components and as a consequence each zone of tissue contains significantly different mechanical properties (Yang and Temenoff, 2009).

4.2 Objective and methodology

The aim of this chapter is to investigate the potential of electrospinning as a technique to build a three-dimensional construct that is able to mimic the zonal matrix of the bone-cartilage interface. In that context, a range of Poly(lactic-co-glycolic acid) (PLGA) membranes with different proportions of collagen and hydroxyapatite nanoparticles were obtained by electrospinning. This chapter presents mainly a physico-chemical characterisation of the different membranes in terms of composition, morphology, surface properties

and mechanical properties. A short section will also study the adhesion and proliferation of human mesenchymal stem cells onto the electrospun fibres.

Two groups of polymer solutions were prepared for this study, as presented in Table 4.1. Polymer solutions from Group 1 were prepared by dissolving PLGA into 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) overnight at room temperature. Hydroxyapatite synthetic nanopowder (nHAp) was added to the polymer solution at different concentrations (w/v): 0%, 1%, 5%, 10%, 20%, and 50%. For the polymer solutions from Group 2, lyophilised rat tail collagen type I was dissolved together with PLGA at different ratio: 7:3, 8:2 and 9:1 (w/w ratio PLGA:collagen). nHAp was added to the polymer solution at different concentrations (w/v): 0%, 5%, and 10%.

Table 4.1: Detailed compositions of Group 1 and Group 2 membranes.

	PLGA:collagen ratio	nHAp content (% w/v)
Group 1	10:0	0
		1
		5
		10
		20
		50
Group 2	7:3	0
		5
		10
	8:2	0
		5
		10
	9:1	0
		5
		10

4.3 Results

4.3.1 Membrane morphology

Figure 4.1 presents typical morphologies and fibre diameter distributions observed at the upper (a and b) and lower (c and d) sides of a pure PLGA membrane (Group 1, 0% nHAp). The morphology appears to differ from one side to the other: the fibres are relatively straighter at the upper side and very tortuous at the lower side. The diameter distributions appear to be slightly different although the change is not significant (Figure 4.1(b) and 4.1(d), $714\pm181\text{nm}$ and $940\pm273\text{nm}$ respectively). Figure 4.2 shows the morphologies and fibre diameter distributions observed at the upper (a and b) and lower (c and d) sides of a typical PLGA:collagen membrane at ratio 7:3 (Group 2, 0% nHAp). In contrast with pure PLGA membranes, the morphology does not appear to differ from one side to the other. Moreover, the diameter distributions of the fibres are very similar (near 450nm) and the standard deviation is relatively small (about 100nm). When compared to PLGA membranes, the substitution of PLGA by collagen results in a significant decrease of the average diameter. A similar observation has been reported by others ((Jose et al., 2009)).

4.3.2 nHAp incorporation

The effect of the addition of nHAp on the morphology of the resulting PLGA fibres is shown in Figure 4.3. As the concentration increases, nanospots progressively appear

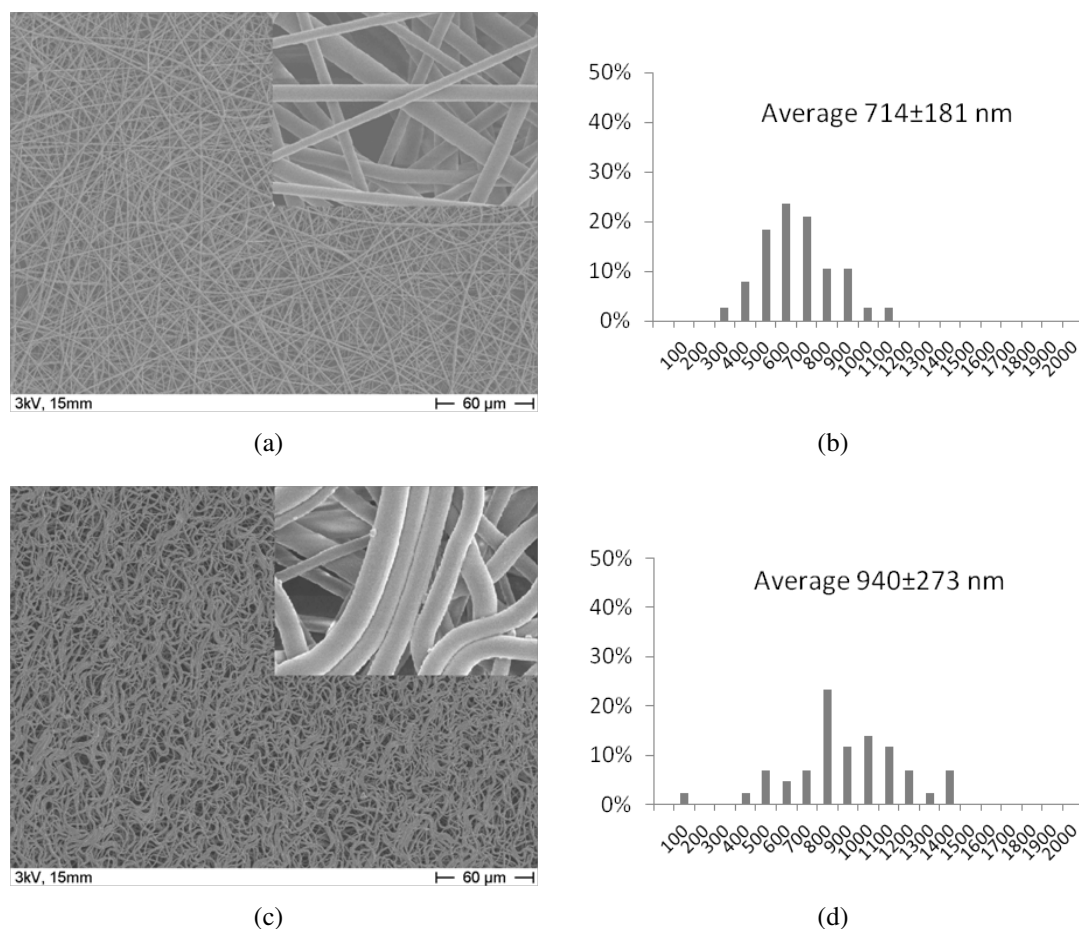


Figure 4.1: Morphologies and fibre diameter distributions of typical PLGA membranes (Group 1, 0% nHAp) collected on PBS solution. (a), (b) upper side; (c), (d) lower side. Higher magnification images are at the top right of (a) and (c).

at the surface of the fibres. Further characteristics regarding the membranes of Group 1 are presented in Table 4.2. The average diameter of the fibres is observed at 0% of nHAp is 714 ± 181 and no significant change is observed at higher contents, except at 50% of nHAp, at which content the average diameter is 914 ± 435 nm. The voltages used to produce the fibres were similar in all cases (about 9.6kV), except again for the solution containing 50% of nHAp, for which a voltage of about 12kV was required. Also, as the content of nHAp increase from 0 to 50%, the thickness of the membranes increases

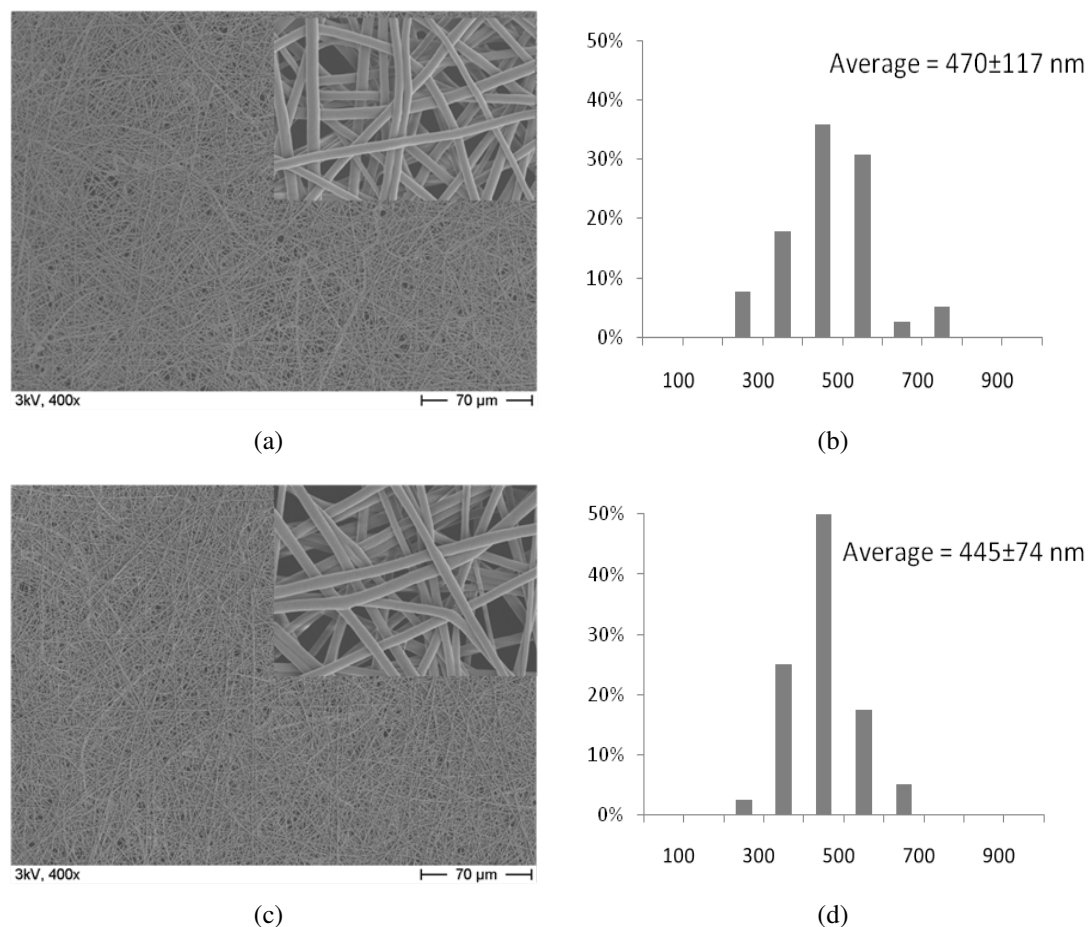


Figure 4.2: Morphologies and fibre diameter distributions of typical PLGA-collagen membranes (Group 2, ratio 7:3, 0% nHAp) collected on PBS solution. (a), (b) upper side; (c), (d) lower side. Higher magnification images are at the top right of (a) and (c).

exponentially, from $18.4 \pm 1.7 \mu\text{m}$ to $752.8 \pm 65.4 \mu\text{m}$. Figure 4.4 and Table 4.3 show the effect of the incorporation of nHAp on the morphology of PLGA:collagen fibres (Group 2). Again, as the concentration increases, nanospots progressively appear at the surface of the fibres. However, in this case, the fibre diameter distribution is strongly affected by the incorporation of nHAp. Fibres with average diameters below 100nm appear in the scaffold when collagen is substituting PLGA. This population of nanofibres (Pn), can be distinguished from the initial population of microfibers (Pm) with diameters greater

than 100nm. The presence of these nanofibres becomes more evident when both the proportion of collagen and the concentration of nHAp increase, as reported in Table 4.3 by the ratio between the number of Pn fibres and the number of Pm fibers ($\#Pn/\#Pm$ increases from 0.05 to 2.5 at a ratio 7:3). Also, wider fibre diameter were usually observed after addition of nHAp (see Figure 4.4 and Table 4.3). The voltages required to produce the PLGA:collagen fibres were usually higher than the values reported to make PLGA fibres and were generally increasing with the content of nHAp. Finally, Table 4.3 also shows that the thickness of the PLGA:collagen membrane generally increases significantly from values close to $15\mu m$ up to $30\mu m$ when the content of nHAp is increased from 0 to 10%.

Table 4.2: Characteristics of Group 1 membranes. When varying the nHAp content, no significant change was observed either in the voltage required for electrospinning or in the resulting average diameter, excepted for a nHAp content of 50% (w/v). The membrane thicknesses indicated in the table were obtained after 90 seconds of electrospinning.

nHAp content (w/v)	Voltage (kV)	Fibre diameter (nm)	Membrane thickness (μm)
0%	9.5 ± 0.1	714 ± 181	18.4 ± 1.7
1%	9.6 ± 0.1	665 ± 236	21.4 ± 3.1
5%	9.7 ± 0.2	605 ± 206	22.4 ± 2.9
10%	9.6 ± 0.1	621 ± 227	30.3 ± 2.8
20%	9.6 ± 0.1	701 ± 242	73.4 ± 12.0
50%	12.2 ± 0.8	914 ± 435	752.8 ± 65.0

4.3.3 Fourier transform infrared spectroscopy (FTIR)

The spectra of Group 1 membranes are shown in Figure 4.5. From the spectrum of pure PLGA membrane (Figure 4.5(a), spectrum A), C=O stretching at 1765cm^{-1} and C-O characteristic bands in the region $1065\text{-}1280\text{ cm}^{-1}$ can be seen. On the other hand, in the spectrum of a pure hydroxyapatite powder (Figure 4.5(a), spectrum G), characteristics of

Table 4.3: Characteristics of Group 2 membranes. Both the ratio PLGA:collagen and the amount of nHAp in solutions affect the voltage required for electrospinning and the morphology of the membranes. Two populations of fibres were observed: Pn represents the population of fibres with a diameter around and below 100nm (nanofibres) and Pm is the population of fibres with higher diameters (microfibres). Pn/Pm is the ratio between the number of nanofibres and the number of microfibres observed in the membrane. The membrane thicknesses indicated in the table were obtained after 360 s of electrospinning.

Ratio; nHAp content (w/v)	Voltage (kV)	Fibre diameter, Pm (nm)	Fibre diameter, Pn (nm)	#Pn/#Pm	Membrane thickness (μm)
7 : 3; 0%	13.6 $\pm$ 0.1	458 $\pm$ 97	47 $\pm$ 32	0.05	10.9 $\pm$ 1.0
7:3; 5%	12.8 $\pm$ 0.2	511 $\pm$ 104	51 $\pm$ 25	0.2	14.6 $\pm$ 2.3
7:3; 10%	15 $\pm$ 0.1	929 $\pm$ 435	66 $\pm$ 35	2.5	32.9 $\pm$ 2.9
8:2; 0%	11.1 $\pm$ 0.1	627 $\pm$ 79	None	<0.01	13.4 $\pm$ 2.2
8:2; 5%	11 $\pm$ 0.1	952 $\pm$ 284	71 $\pm$ 50	0.3	18.1 $\pm$ 1.7
8:2; 10%	15 $\pm$ 0.1	804 $\pm$ 346	80 $\pm$ 58	2.5	30.5 $\pm$ 2.3
9:1; 0%	11.9 $\pm$ 0.1	557 $\pm$ 68	None	<0.01	15.3 $\pm$ 2.0
9:1; 5%	11 $\pm$ 0.1	1195 $\pm$ 261	68 $\pm$ 45	0.2	20.0 $\pm$ 3.7
9:1; 10%	15 $\pm$ 0.1	623 $\pm$ 223	63 $\pm$ 30	0.4	28.0 $\pm$ 2.5

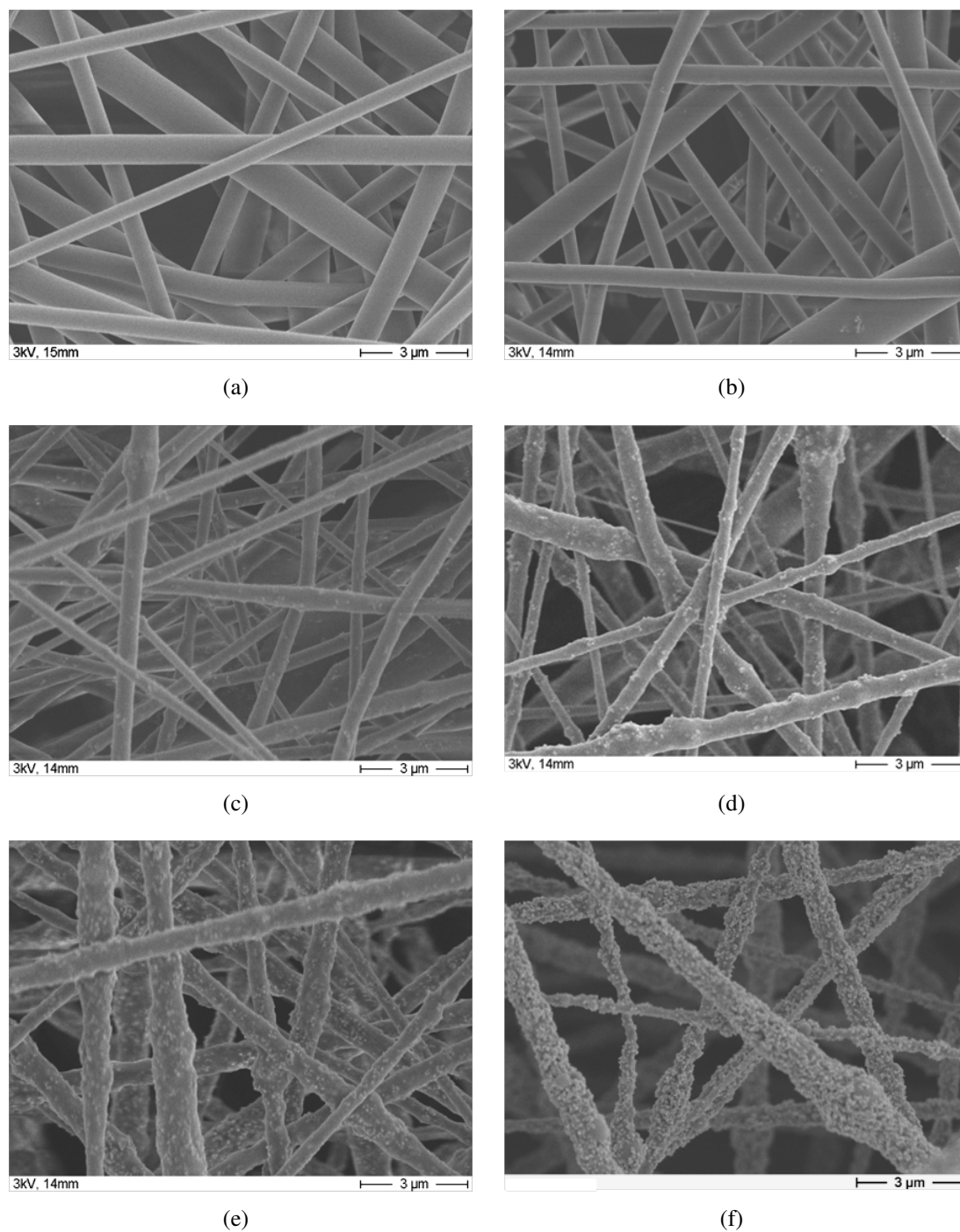


Figure 4.3: SEM images of electrospun fibres obtained from a solution of 15% PLGA in HFIP with different concentrations of hydroxyapatite nanoparticles (Group 1). (a): 0% nHAp; (b): 1% nHAp; (c): 5% nHAp; (d): 10% nHAp; (e): 20% nHAp; (f): 50% nHAp (% w/v in HFIP).

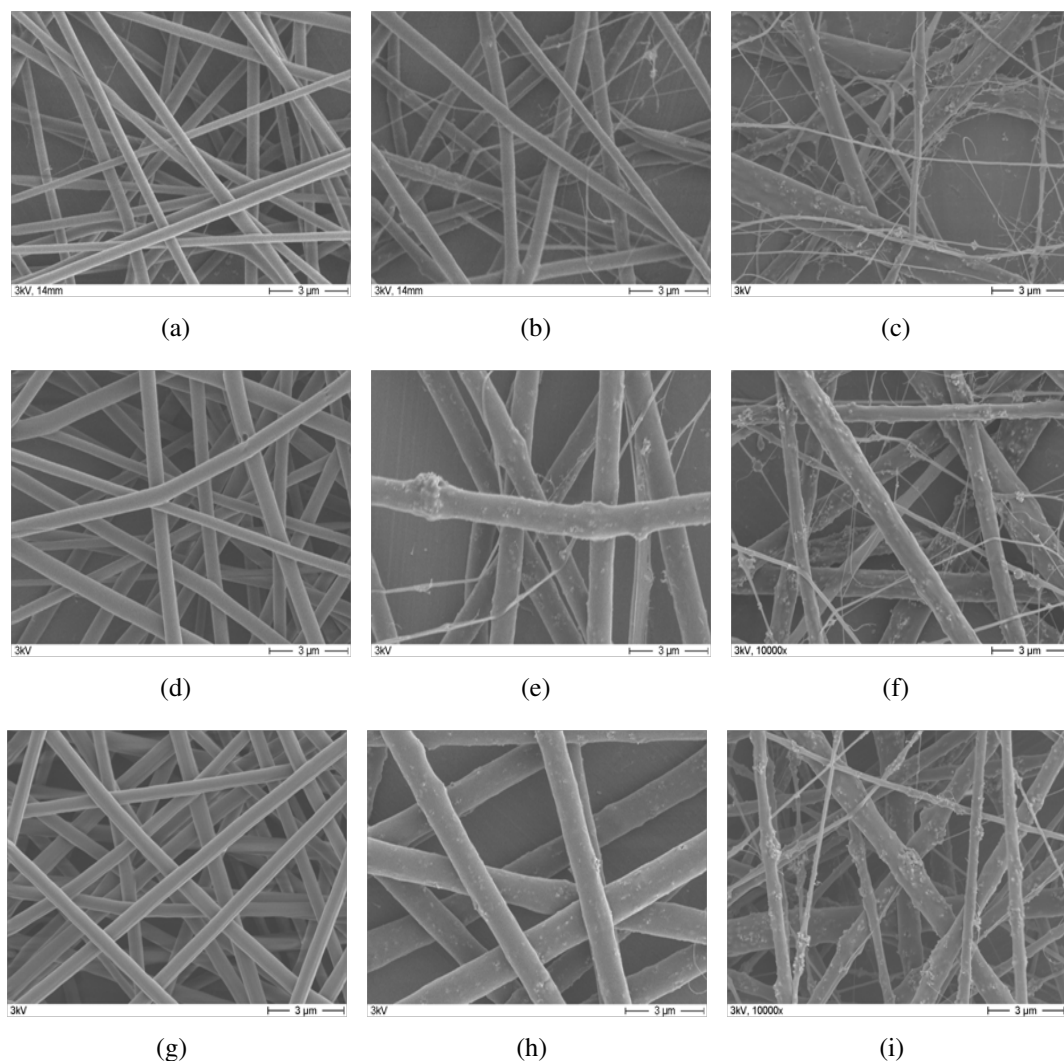


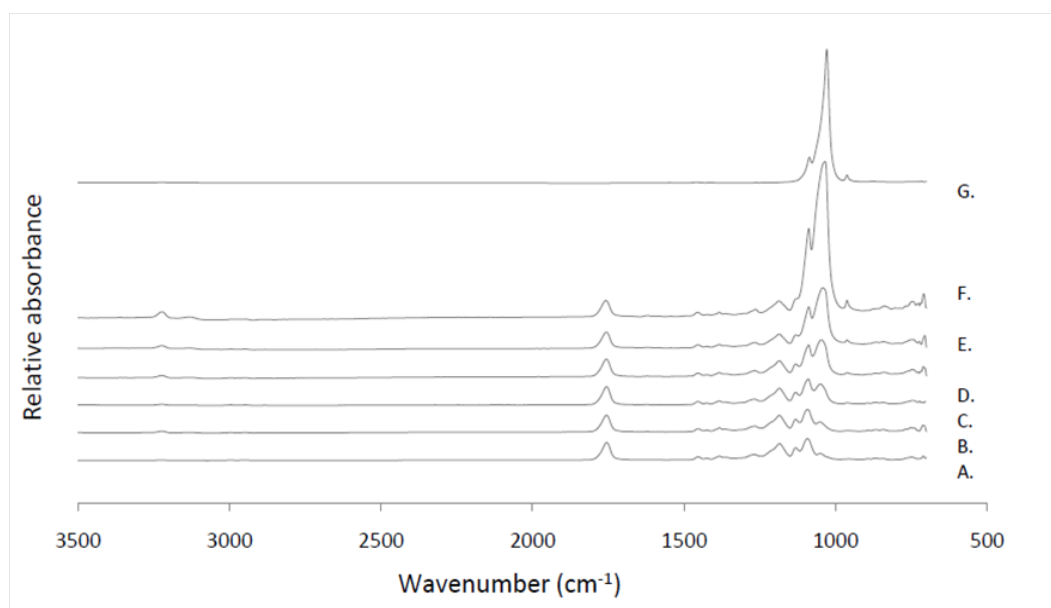
Figure 4.4: SEM images of electrospun fibres obtained from a solution of 15% PLGA:collagen in HFIP at different ratios PLGA:collagen and with different concentrations of hydroxyapatite nanoparticles. (a): ratio 7:3 with 0% nHAp; (b): ratio 7:3 with 5% nHAp; (c): ratio 7:3 with 10% nHAp; (d): ratio 8:2 with 0% nHAp; (e): ratio 8:2 with 5% nHAp; (f): ratio 8:2 with 10% nHAp; (g): ratio 9:1 with 0% nHAp; (h): ratio 9:1 with 5% nHAp; (i): ratio 9:1 with 10% nHAp. Added together to PLGA, collagen and nHAp lead to the formation of two populations of fibres, one with an average fibre diameter above 100nm (Pm fibres) and the other with an average fibre diameter below 100nm (Pn fibres).

PO_4^{3-} are observed: ν_1 vibration mode at 964 cm^{-1} , ν_3 vibration mode at 1031 cm^{-1} and 1091 cm^{-1} (asymmetric). The presence of nHAp in the PLGA membranes (Figure 4.5(a), spectra B to F) is confirmed as the characteristics of PLGA and hydroxyapatite

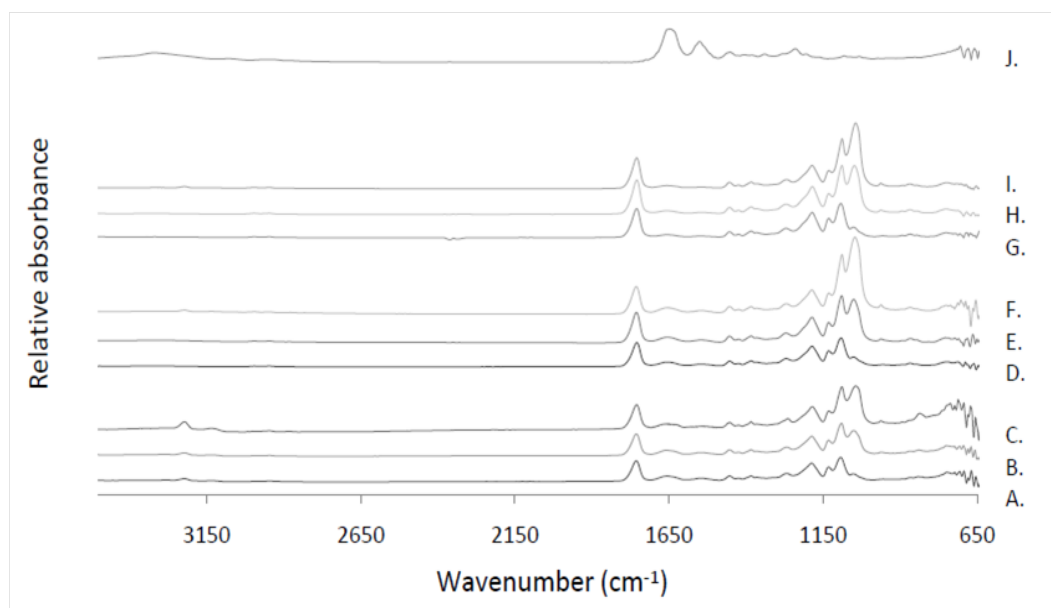
overlap in the region $964\text{--}1143\text{ cm}^{-1}$ with an intensity proportional to the concentration of nHAp. The spectra of Group 2 membranes are shown in Figure 4.5(b). The spectrum of pure collagen (Figure 4.5(b), spectrum J) shows characteristic bands at 1660cm^{-1} , assigned to amide I, and 1560 cm^{-1} , assigned to amide II (combined bands of N-H bending vibration and C-H). Spectra A to I in Figure 4.5(b) show both characteristics of PLGA and collagen. Distinction between PLGA-collagen blend at ratio 7:3 (A to C), 8:2(D to F), and 9:1 (G to I) can also be made according to the relative peak intensity, the signal measured for amide I and II being progressively attenuated as the ratio increases. As expected, spectra of membranes containing nHAp (Figure 4.5(b), spectra B, C, E, F, H and I) also show the characteristics of hydroxyapatite in the region $964\text{--}1143\text{ cm}^{-1}$ according to its concentration.

4.3.3.1 Contact angles

Contact angle values are presented in Figure 4.6(a) (Group 1) and Figure 4.6(b) (Group 2). As shown in Figure 4.6(a), high contact angles are observed for pure PLGA membranes. Incorporation of nHAp results in a small decrease of the contact angle. When adding collagen to PLGA, the contact angle is diminished by half, as shown in Figure 4.6(b). However, the addition of nHAp to PLGA-collagen membranes results in an increase of the angle. The ratio PLGA:collagen also affects the contact angle: the angle is usually higher at higher ratio. Interestingly, contact angles measured in Group 1 and Group 2 tend to reach a plateau at similar values when increasing nHAp content.



(a)



(b)

Figure 4.5: FTIR spectra of Group 1 and Group 2 electrospun scaffolds. (a) FTIR spectra of electrospun membranes obtained from 15% PLGA solutions with different concentrations of nHAp (Group 1). A: 0% nHAp; B: 1% nHAp; C: 5% nHAp; D: 10% nHAp; E: 20% nHAp; F: 50% nHAp (% w/v). The spectrum of pure nHAp is shown in G. (b) 15% PLGA-collagen solutions at different ratios PLGA:collagen and with different concentrations of nHAp (Group 2). A: ratio 7:3 with 0% nHAp; B: ratio 7:3 with 5% nHAp; C: ratio 7:3 with 10% nHAp; D: ratio 8:2 with 0% nHAp; E: ratio 8:2 with 5% nHAp; F: ratio 8:2 with 10% nHAp; G: ratio 9:1 with 0% nHAp; H: ratio 9:1 with 5% nHAp; I: ratio 9:1 with 10% nHAp (% w/v). The spectrum of pure collagen is shown in J.

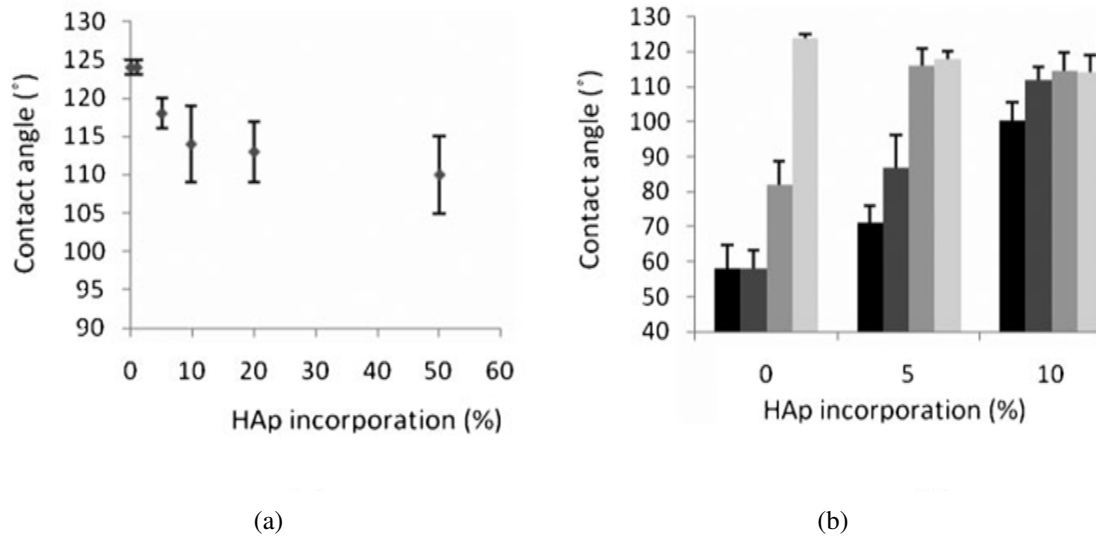


Figure 4.6: Effect of the nHAp content (% w/v) on the contact angle measured at the surface of the resultant membranes: (a) PLGA membranes (Group 1); (b) PLGA-collagen membranes (Group 2), where black represents the ratio 7:3, dark grey represents the ratio 8:2, medium grey represents the ratio 9:1, and light grey represents the ratio 10:0.

4.3.4 Tensile testing

The Young's modulus (Y_m), the ultimate tensile stress (U_{max}) and the ultimate tensile strain (ϵ_{max}) of each membrane presented in this study are shown in Figures 4.7 and 4.8. For Group 1 membranes (Figure 4.7), a decrease in all properties is observed when nHAp is added to PLGA. Indeed, when the content of nHAp increases from 0% to 50%, Y_m drops off from 34.3 ± 1.8 MPa to 0.103 ± 0.056 MPa, U_{max} diminishes from 2.7 ± 0.6 MPa to 0.019 ± 0.008 MPa and ϵ_{max} decreases from a value higher than 40% (highest elongation allowed by the tensile machine) to $5 \pm 3\%$. For Group 2 membranes (Figure 4.8), the mechanical properties vary differently when the content of nHAp is increased from 0 to 10%, depending on the ratio PLGA:collagen. At a ratio 7:3, Y_m increases from 41.9 ± 7.9 MPa to 63.9 ± 6.1 MPa, while U_{max} and ϵ_{max} decrease from 4.0 ± 0.3 MPa to 2.1 ± 0.2 MPa

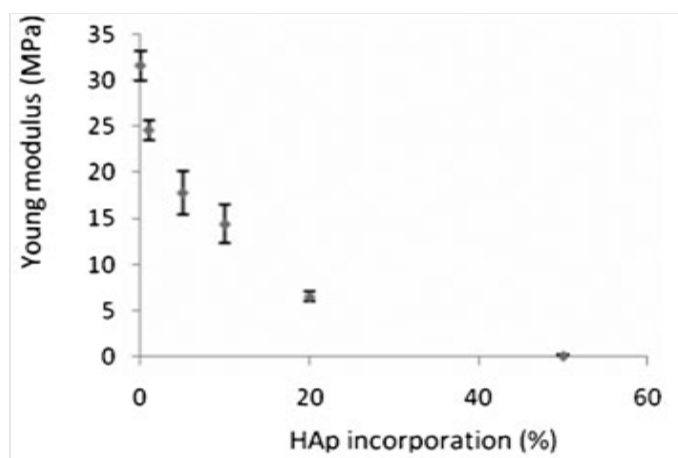
and $20\pm 2\%$ to $5\pm 1\%$, respectively. At a ratio 8:2, Y_m increases from 36.4 ± 3.2 MPa to 69.9 ± 6.6 MPa while U_{max} , and ϵ_{max} decrease from 3.6 ± 0.2 MPa to 1.7 ± 0.3 MPa and $>40\%$ to $7\pm 3\%$, respectively. Finally, at a ratio 9:1, Y_m , U_{max} and ϵ_{max} all show a drop in values from 50.0 ± 3.9 MPa to 20.0 ± 4.9 MPa, 5.6 ± 0.5 MPa to 1.7 ± 0.6 MPa and $>40\%$ to $25\pm 4\%$, respectively. Compared to Group 1, the presence of collagen in Group 2 generally has a positive effect on Y_m and U_{max} but negative on ϵ_{max} .

4.3.5 Biological characterisation

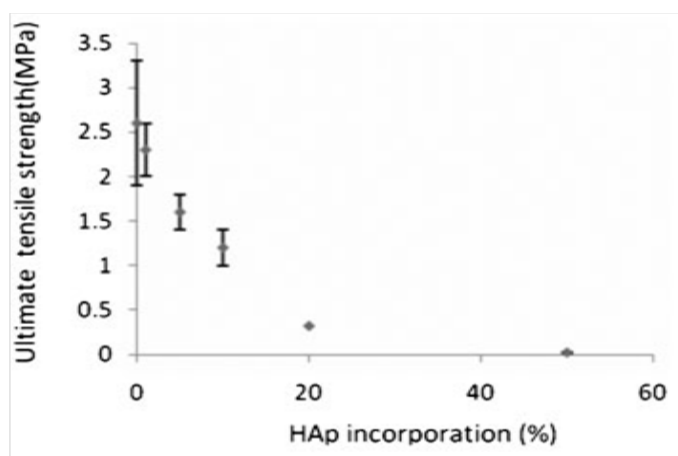
4.3.5.1 Adhesion study

Studying the adhesion of hMSCs has been the first step towards the biological characterisation of electrospun membranes. Figure 4.9 shows the morphologies of hMSCs on electrospun mats and on the control (polyester membranes, PE) at different times of culture: 3 hours, day 1, day 3 and day 7.

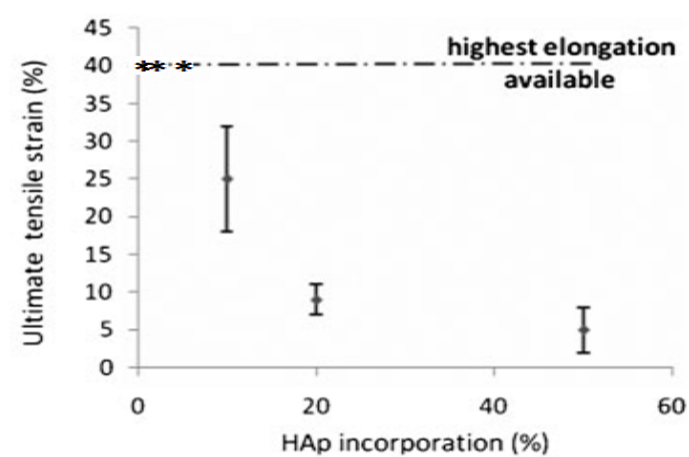
In general, a low number of cells was observed on pure PLGA scaffolds (Group 1, 10:0) with comparison to the membranes containing collagen and the control. However, a relatively high number of cells was seen on the PLGA matrix after 3 hours. On the contrary, a low number of hMSCs seem to have attached to the control membrane after 3 hours, although the cells proliferate well afterwards. The highest number of cells was in general observed on the PLGA-Col membrane (Group 2, 7:3, 0%nHAp).



(a)

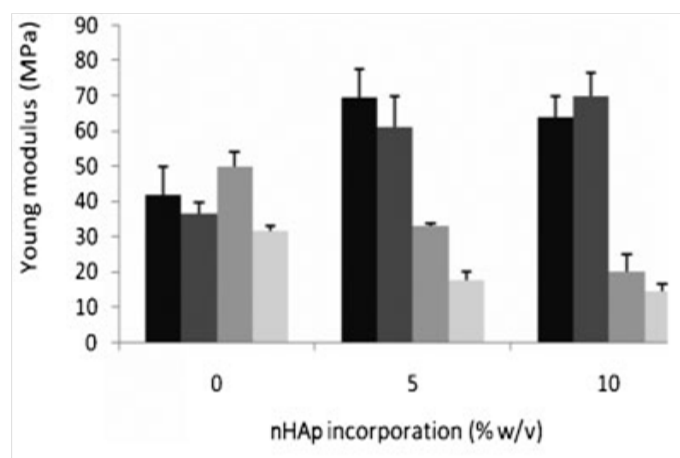


(b)

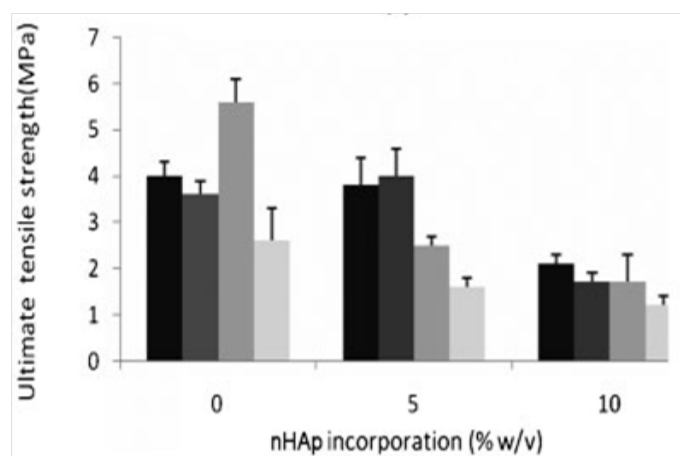


(c)

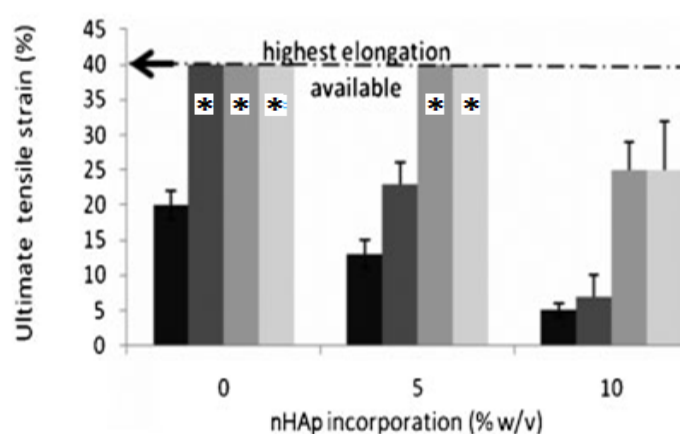
Figure 4.7: Relationship between the amount of nHAp incorporated into 15% PLGA solutions and the mechanical properties of the resultant electrospun membranes: (a) Young's modulus; (b) ultimate tensile strength; (c) ultimate tensile strain (*value above 40%).



(a)



(b)



(c)

Figure 4.8: Relationship between the amount of nHAp incorporated into 15% PLGA-collagen solutions and the mechanical properties of the resultant electrospun membranes: (a) Young's modulus; (b) ultimate tensile strength; (c) ultimate tensile strain (*value above 40%), where black represents the ratio 7:3, dark grey represents the ratio 8:2, medium grey represents the ratio 9:1, and light grey represents the ratio 10:0.

When looking at the cell morphology, hMSCs seem to have spread very well on the control and the PLGA-Col in comparison to the PLGA membranes where they show mostly an elongated shape. Differences can already be seen after only 3 hours, as cells seem to have spread well on membranes containing collagen, while they generally keep a small and round shape on pure PLGA matrices. Similar observations have been made under the electron microscope (Figure 4.10).

4.3.5.2 Proliferation study

Figure 4.11 shows the percentage of AB reduction for different hMSCs densities after 4 hours of incubation. The reduction of AB occurs linearly as a function of the cell number. The trendline is characterised by the following slope equation: $y = 0.0008x + 7.761$. The coefficient of determination, $R^2=0.982$, supports the linear model.

Figure 4.12 shows the percentage of AB reduction after hMSCs on electrospun membranes at day 1, 2, 3, 5, and 7. The percentage of AB reduction is related to a cell number by the standard curve. When looking at the general trend, an increase in the cell number is observed between day 1 and day 5 although a slight drop of activity can be seen at a day 2, probably due to the adaptation of the cells to their new environment. According to the t-test (95% confidence interval), significant differences are observed between PLGA sample and all the other ones. No significant differences were observed between PLGA-col, PLGA-col-nHAp and the control membranes.

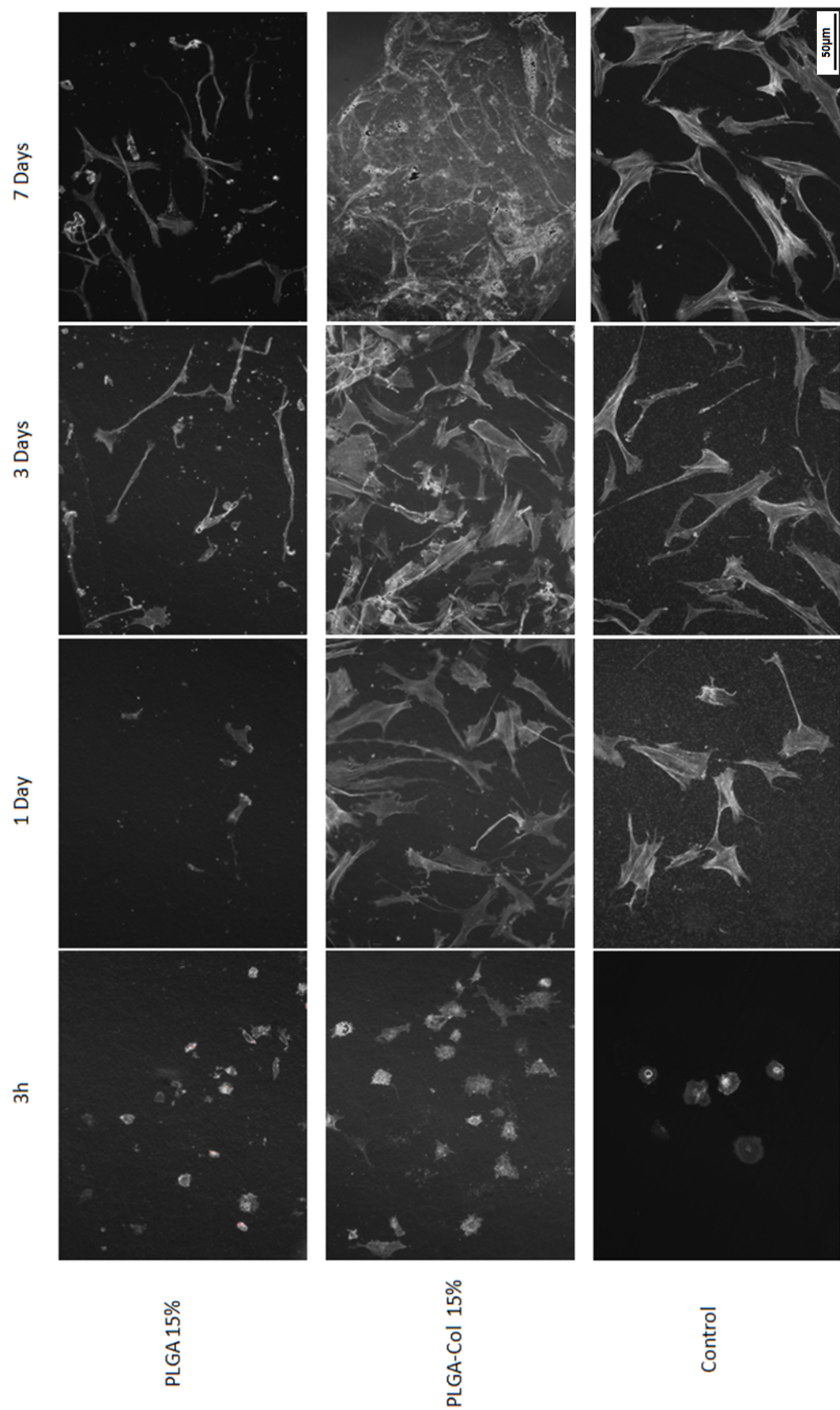


Figure 4.9: Morphologies of hMSCs cultured on PLGA electrospun membranes, PLGA-col electrospun membranes and PE films (control) revealed by fluorescence microscopy after staining of the actin with phalloidin Texas Red. Observations were carried out at different times of culture: 3 hours, day 1, day 3 and day 7. PLGA-col membranes show better cell adhesion and proliferation than pure PLGA membranes. A low number of cells seem to attach to the PE film initially although the cells proliferate well afterwards. The scale bar is indicated in the bottom right corner.

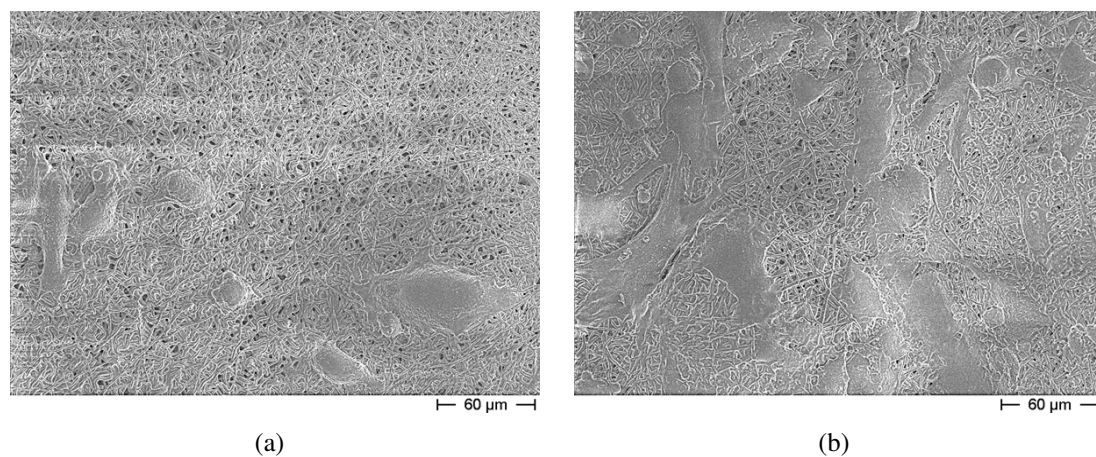


Figure 4.10: Adhesion of hMSC after 3 hours of culture on electrospun membranes shown by electron microscopy: (a) PLGA 15% (Group 1, 10:0) and (b) PLGA-Col 15% (Group 2, 7:3, 0%nHAp).

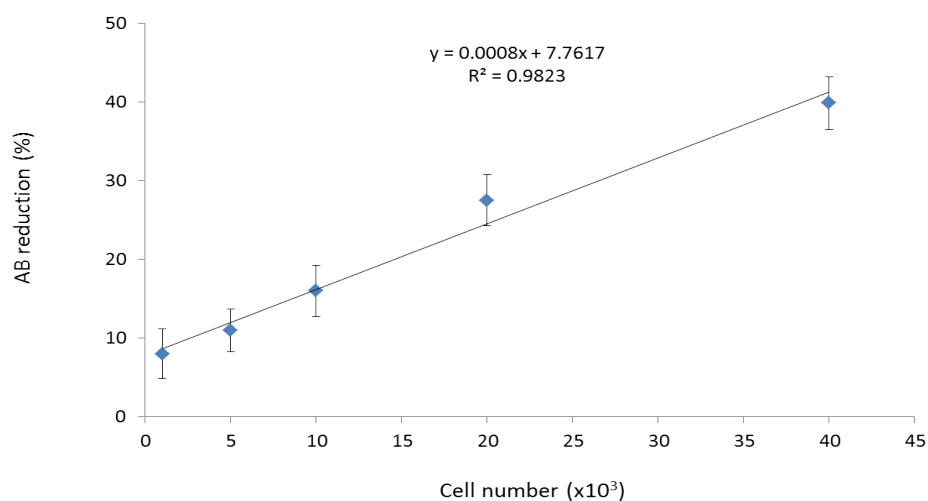


Figure 4.11: Standard curve relating the number of hMSCs to the Alamar Blue reduction (percent) after 4 hours of incubation.

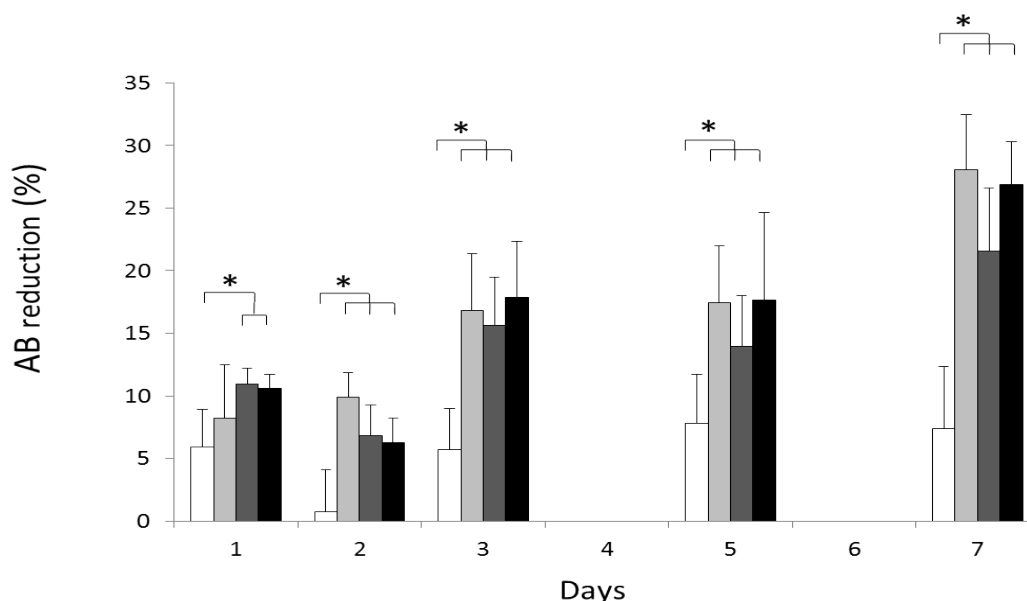


Figure 4.12: Alamar Blue reduction (percent) by hMSCs growing on electrospun membranes, measured at different times of culture. White: PLGA (Group 1, 0%nHAp); grey: PLGA-Col (Group 2, ratio 7:3, 0%nHAp); dark grey: PLGA-Col-nHAp (Group 2, ratio 7:3, 10%nHAp); black: control. The error bar represent the standard deviation (* $p < 0.05$).

4.3.6 Simulated body fluids

In this chapter, SBF is used to predict the *in vivo* bone bioactivity of the electrospun membranes, as suggested by Kokubo and Takadama (2006). Figure 4.13 indicates the presence of a dense layer of minerals at the surface of PLGA-nHAp fibres after 72 hours of incubation in SBF at 37°C. In contrast, pure PLGA samples exhibited none or very poor deposition at the surface of the fibres.

Moreover, Figure 4.14 shows the morphology of PLGA-nHAp samples observed after different incubation times in SBF: 1h, 6h, 12h, 24h, 72h, and 14 days. Crystals seem to appear on PLGA-nHAp fibres from 12 hours of incubation. Growth of the crystals was

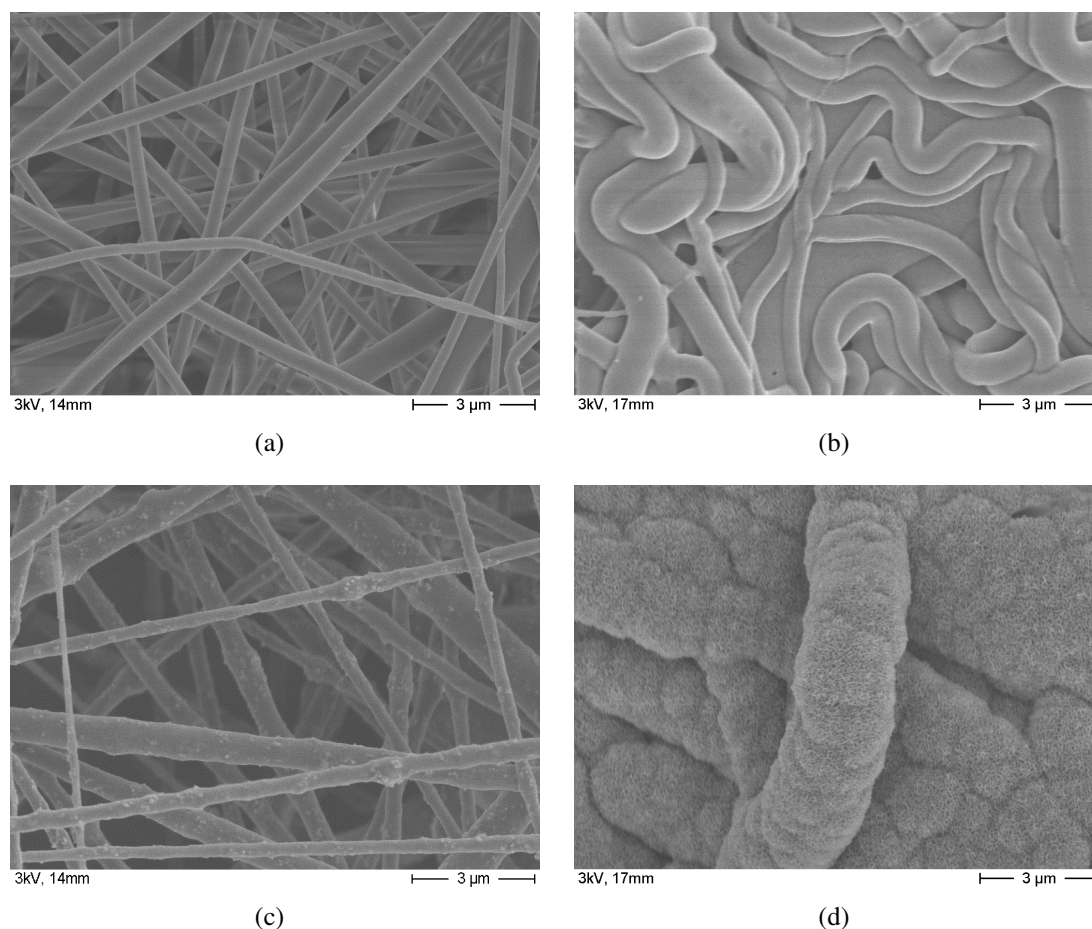


Figure 4.13: Influence of nHAp incorporation in PLGA electrospun fibres on the deposition of hydroxyapatite from SBF: (a) 0% nHAp, before incubation; (b) 0% nHAp, after 72h of incubation; (c) 10% nHAp, before incubation; (d) 10% nHAp, after 72h of incubation.

leading to full coating of the fibres after only 72 hours of incubation.

4.4 Discussions

Fibrous membranes produced by electrospinning are of increasing interest because they may mimic the extracellular matrix (ECM) surrounding cells *in vivo* and therefore can be used as a support for tissue culture (Ashammakhi et al., 2007). Moreover they can

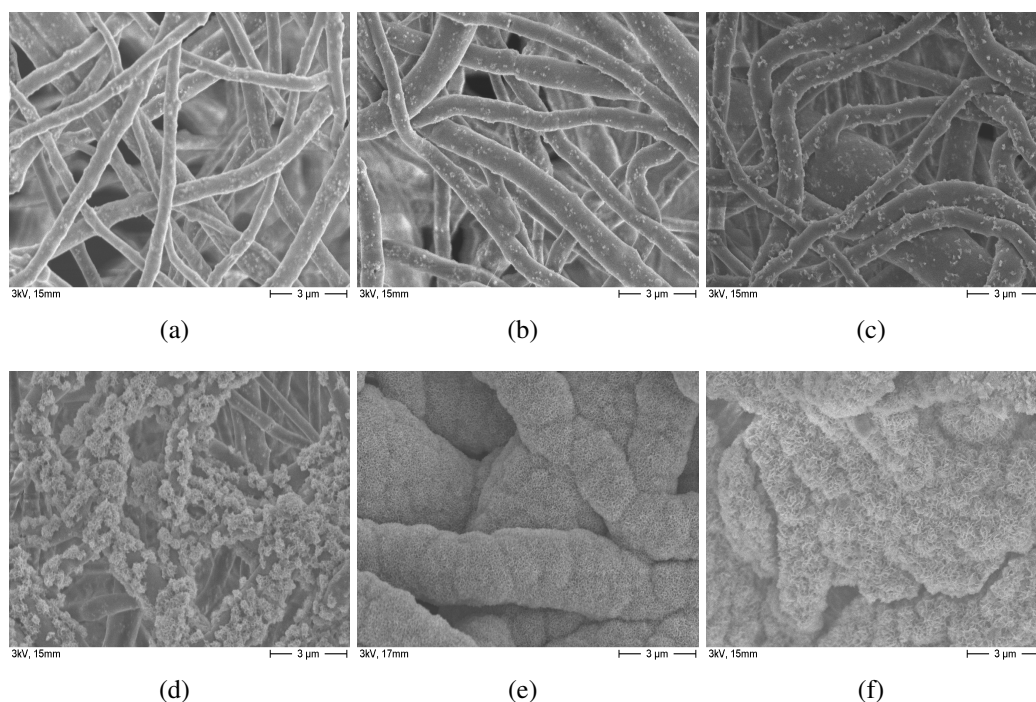


Figure 4.14: Influence of the incubation time in SBF on the deposition of hydroxyapatite onto PLGA-nHAp fibres:(a) 1h, (b) 6h, (c) 12h, (d) 24h, (e) 72h,(f) 14 days.

be tailored in terms of architecture and composition in order to optimise beneficial cell responses and subsequently be used in a multilayered arrangement for three-dimensional tissue formation (Venugopal et al., 2008; Srouji et al., 2008). Furthermore, the creation of electrospun membranes with gradual change in properties could serve to engineer zonal tissue organisations such as those observed at the bone-cartilage interface.

4.4.1 Membrane collection

The collection of the membrane resulting from the electrospinning process is often problematic, with difficulties of detachment and recovery of the membrane from the collector,

especially for very thin layers. Therefore, in this study, fibres were collected on a layer of PBS spread on the electrospinning collector. This approach was an efficient way to obtain membranes with controlled thicknesses. With that technique, ultrafine membranes made of only a few layers of fibres could easily be obtained. However, according to the composition of the polymer solution, the use of a liquid collector may affect the morphology of the side of the membrane directly in contact with the liquid, as seen for the PLGA membrane in Figure 4.1. The hydrophobicity of the material might well be responsible for this phenomenon. PLGA fibres are hydrophobic (see contact angle values in Figure 4.6(a)) and could organise themselves to decrease the contact area with the PBS solution (hydrophilic) and increase contacts between each other, resulting in their tortuous aspect. The fact that some solvent might still reside inside the fibres may contribute to this effect, as it should give them more flexibility. When blended with collagen, the fibres become hydrophilic and this could explain the fact that the morphology does not change from side to side.

4.4.2 Homogenisation and distribution of nHAp

The conditions in which the particles are dispersed in the solution are known to influence the resulting morphology of electrospun fibres (Luong et al., 2008). In this study, ultrasonic homogenisation of the electrospinning solution containing nHAp was a crucial step to obtain a very stable electrospinning process and as a consequence, a uniform membrane. This ultrasonic homogenisation allowed successful electrospinning of PLGA

solutions containing up to 50% (w/v) of nHAp. However, it is worth noting that the actual mineral content in the resulting fibre is different to the initial concentration within the solution. 50% of nHAp (w/v) in 15% PLGA solution actually give rise to a scaffold with a mineral content of about 75% (w/w), as the solvent evaporates during the fibre formation. Therefore, the solution containing 10% of nHAp (w/v) may be more appropriate for bone TE as it corresponds to about 40% (w/w) of mineral content in the resulting scaffold which is close to the proportion of calcium and phosphate found in the natural bone matrix (Driessens and Verbeeck, 1990).

FTIR measurements have confirmed the nature of the polymers and the presence of nHAp at different concentration into the membranes. Although it is believed that the particles are evenly distributed inside the fibres, as discussed later, the morphologies observed by SEM raise the question of whether or not the nHAp observed on the fibres are located directly at their surface or rather embedded into a very fine polymer matrix. The later hypothesis is probably more likely to be true as the contact angles shown in Figure 4.6(a) do not decrease as much as expected when the concentration of nHAp increases. Indeed, contact angle for pure hydroxyapatite have been reported to be about 90° (Teixeira et al., 2006). The exposure of mineral to the cells may be an important parameter governing cell behaviour but, because the polymers used are biodegradable, the particles would be exposed sooner or later to the cells.

4.4.3 Morphological characteristics

The thickness of a membrane (either for Group 1 and Group 2), measured after a defined duration of electrospinning, is shown to be related to the amount of nHAp initially present in the polymer solution. However, at very high concentration (50% nHAp, w/v), results have shown that the membrane is thicker than expected, suggesting the contribution of an electrostatic effect. As the quantity of nHAp increases, the number of charges within the fibres increases and the repulsion between the fibres becomes more important, augmenting consequently the thickness of the membrane. This is consistent with the SEM image shown in Figure 4.3(f), where most of the fibres appear out of focus, due to the large space between the fibres. As a result, the porosity and the mean pore size of that membrane are expected to be higher than at lower concentrations of nHAp.

Other morphological analysis have shown that PLGA:collagen membranes exhibit two discrete populations of fibre diameters, referred to as Pn and Pm. The nanofibres (Pn) are most probably created by ramification of the jet during the electrospinning process. Indeed, PLGA and collagen are likely to form two different phases, one polymer being hydrophobic and the other hydrophilic, which may favour the formation of secondary jets. The presence of nHAp may also help to initiate the branching by creating local charge instabilities and this would explain the increase of Pn/Pm (observed in Table 4.3). In order to identify the actual nature of the Pn and Pm fibres and to verify this hypothesis, further physicochemical analysis, using techniques such as the atomic force microscopy, should be carried out. However, this is not within the scope of this project.

4.4.4 Surface properties

The properties observed at the surface of a tissue engineered scaffold are well-known to influence cell behaviour, and especially the process of cell adhesion. For instance, a hydrophobic surface will usually result in a poor cell attachment (Guelcher and Hollinger, 2005). Measurement of the angle formed between a drop of water and the surface of the material is a straightforward way to characterise its hydrophobicity. However, in this case, fibrous membranes are not perfectly flat surfaces and therefore values obtained must be considered with caution. Contact angles presented for Group 1 (Figure 4.6(a)) have shown high contact angles for pure PLGA, confirming its high hydrophobicity. The drop in hydrophobicity due to the incorporation of nHAp may be due to the chemistry of hydroxyapatite, for which values of contact angles are reported to be about 90° , as stated before. The drop in the contact angle observed when collagen is added to PLGA (Figure 4.6(b)) was expected, as collagen is known to be very hydrophilic. The increase of the angle observed when nHAp is added to PLGA-collagen membranes can be explained by the less hydrophilic nature of nHAp compared to collagen. Also, results have shown a ratio effect (between 7:3, 8:2 and 9:1) as the hydrophobicity is higher at higher ratio, according to the proportion of hydrophobic/hydrophilic material.

4.4.5 Mechanical properties

Mechanical properties of any scaffold to be used in Tissue Engineering are very important as they have to ensure the integrity of the engineered tissue in vivo. The decrease of all mechanical properties observed for the PLGA scaffolds indicates that the interaction between nHAp particles and the polymer matrix is weak. Similar observations have been reported in the literature (Nichols et al., 2007). Because the particles and the polymer matrix are two different categories of materials, they cannot form covalent bonds between them, compromising the mechanical strength of the composite membrane. With regard to the previous discussion about the particle distribution within the fibres, this decrease in the mechanical properties also suggests that the particles are probably well-dispersed within the fibres, instead of being just at the surface. Interestingly, the substitution of PLGA by collagen results in a stiffer (Y_m higher) although more brittle (ϵ_{max} smaller) scaffold. In fact, it has recently been reported that stiff scaffolds improve osteochondral repair in the femoral condyles of sheep when compared to soft scaffolds, suggesting that stiffness is a more important characteristic to take into consideration for the scaffold design than flexibility (Schlichting et al., 2008). Moreover, the addition of nHAp amplifies this effect at a ratio 7:3 and 8:2. This could be related to the increasing number of nanofibres among the microfibrils (see Table 4.3) that may contribute to the stiffness of the scaffold. At a ratio 9:1, a decrease of all mechanical properties is observed when nHAp is added, showing a behaviour similar to PLGA membranes (i.e. ratio 10:0). Therefore, in a multilayered arrangement, a gradient of composition would be automatically accompanied by a gradient of mechanical properties.

4.4.6 Biocompatibility

4.4.6.1 Effect of collagen incorporation

The biological characterisation confirmed the fact that the incorporation of collagen in PLGA electrospun membranes improves both attachment and proliferation of hMSCs. This behaviour was expected since collagen contains peptide sequences, such as the sequence Arginine-Glycine-Aspartic acid (RGD), that are specifically recognised by the cell receptors to stimulate the cell adhesion. Moreover, the addition of collagen also caused a dramatic drop in hydrophobicity which might have been favourable for the cell attachment. This change in surface properties not only results in better wetting of the scaffold during inoculation but it also alters the adsorption of the biomolecules from the culture medium onto the fibres in terms of quantities, densities, distributions, conformation and orientations (see Section 2.5.3). The changes might have contributed to the better cell attachment. Indeed, hydrophilic electrospun membranes are usually preferred in tissue engineering applications as the non-specific adsorption of proteins is often minimised (Li et al., 2006).

No significant differences could be observed in terms of proliferation between PLGA-col and control membranes. However, the adhesion study has revealed that less cells were attaching to the surface of the control membrane when comparing to the PLGA-col samples (see Figure 4.9). This observation most probably results from the fact that the

inoculated cells rolled down to the bottom of the plate more easily from the smooth plastic film compared to the rough fibrous and porous surface of electrospun mats.

4.4.6.2 Effect of nHAp incorporation

The biological characterisation did not show any significant difference between the PLGA-col mats (Group2, ratio 7:3, 0%nHAp) and the PLGA-col-nHAp mats (Group2, ratio 7:3, 10%nHAp) suggesting that nHAp has no particular influence on adhesion and early proliferation. However, incubation of PLGA-nHAp mats (Group 1) in SBF have shown accelerated mineralisation compared to pure PLGA fibres. Moreover, total mineralisation of PLGA-nHAp mats occurred in less than 72 hours. This is a dramatic increase in terms of rate of mineralisation since pure PLLA fibres would require up to 4 weeks of incubation to complete the mineralisation (Chen et al., 2006). These results support the hypothesis that incorporation of nHAp during electrospinning makes the PLGA fibres more osteoconductive since immersion in SBF is known as a method to assess the biocompatibility of biomaterials destined for calcified tissues. Similar observations were made recently by Madurantakam et al. (2009).

The rich deposition observed on PLGA-nHAp fibres can be explained by the fact that nanoparticles present at the surface of the fibres serve as nucleation sites for the crystal formation, speeding up the coating process of mineralisation. The availability of sites promoting nucleation coupled with local supersaturation of ions are well-known to be crucial for crystal formation. The spontaneous mineralization at the surface of PLGA

fibres is prevented by the existence of a high energy barrier for nucleation. That barrier is sufficiently reduced by the presence of nHAp particles to allow nucleation. After the formation of stable nuclei, the growth of mineral aggregates can easily take place and occur as long as the concentration gradient for the ions exists.

4.5 Conclusions

Membranes containing different proportions of PLGA, collagen and nHAp have been successfully produced by electrospinning by using a thin layer of phosphate buffer saline solution as a collector. Physico-chemical characterisation has shown that a change in composition also results in significant changes in terms of morphology, surface properties, and mechanical properties. Biological characterisation has shown the importance of collagen for the adhesion and proliferation of hMSCs. The consequences (trends) of the addition of collagen and/or nHAp are summarised in Figure 4.15. The presence of collagen and nHAp in Group 2 membranes, increases the biocompatibility of the scaffolds and also results in a membrane stiffer and more hydrophilic, with interesting nanofeatures. Therefore, Group 2 membranes have a great potential to serve as a scaffold for both bone and cartilage.

In conclusion, this work shows the potential of electrospinning as a technique to produce easily a wide range of membranes from which some can be selected in order to create, with the help of a multilayered arrangement, a 3D scaffold with a gradient of

properties recapitulating the zonal matrix of the bone-cartilage interface. Moreover, it is worth mentioning that such membranes and such approach could also be used for other tissue interfaces, such as the bone-ligament, bone-tendon, and bone-meniscus transitions.

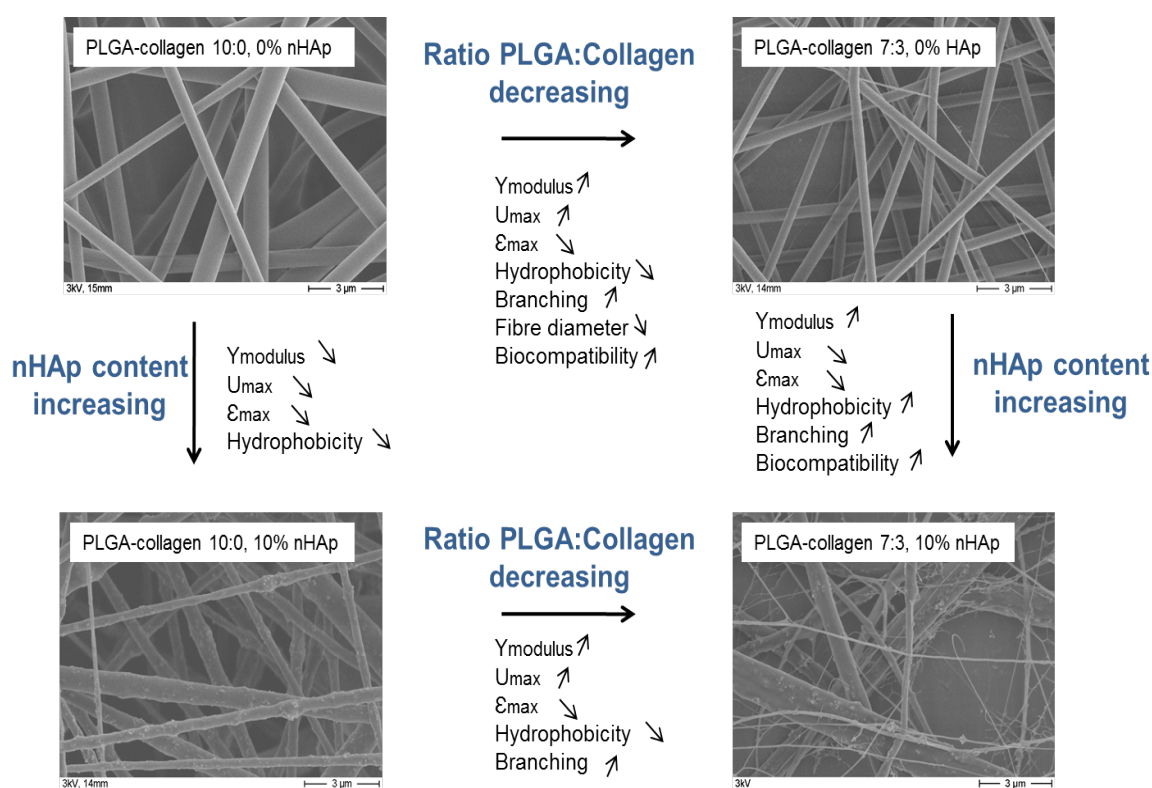


Figure 4.15: Diagram summarising the changes of the membrane properties generally observed when the content of collagen and/or of nHAp is increased.

Chapter 5

Electrospinning for Cartilage Tissue Engineering

5.1 Introduction

Tissue engineering is well-recognised as one of the most promising approaches towards developing a treatment to repair osteochondral defects. Although significant improvements have been achieved during the last decade, many challenges remain. In particular, the engineered matrix must satisfy both the requirements of bone and cartilage allowing for the repair of both tissues. A simple strategy to achieve this is to create biphasic scaffolds that mimic the bone-cartilage interface in order to obtain functional osteochondral grafts, i.e. allowing proper diffusion of gas and nutrients, and suitable transmission of mechanical stresses (Sharma and Elisseeff, 2004; Harley et al., 2009; Lynn et al., 2005). It is important that a soft transition is achieved between both parts of the biphasic scaffold

in terms of fibre arrangement, composition and mechanical properties. Also, a sufficient bond between the different parts of the construct needs to be established in order to avoid any possible loss of integrity during applications *in vivo*.

As described in Chapter 4, electrospinning can be successfully performed to produce fibres of various composition. Although the study focussed on PLGA-based materials, the same protocol can be applied to other polymers such as PCL. PLGA and PCL are synthetic and biodegradable polymers that are popular as biomaterials. The electrospinning of these polymers has been well investigated and both electrospun materials have been shown to support adhesion, proliferation and differentiation of stem cells (Puppi et al., 2010). PLGA and PCL have also been spun with collagen to improve the biocompatibility of the fibres (Choi et al., 2008; Jeong et al., 2007; Schnell et al., 2007). Although PCL, PLGA and collagen have been used to engineer bone tissue (Porter et al., 2009; Yoshimoto et al., 2003; Jose et al., 2009; Wutticharoenmongkol et al., 2006), synthetic hydroxyapatite (HA) is often described as the most promising candidate for the repair of mineralised tissues (Suominen et al., 1996). Synthetic HA has been found to support bone ingrowth and to promote scaffold integration in bone (Kokubo and Takadama, 2006).

One of the main challenges facing the development of three dimensional electrospun constructs is to find an appropriate seeding technique, as mentioned in Chapter 2, Section 2.5.4. Multilayering approaches are a very convenient way to obtain three-dimensional constructs made of electrospun fibres and cells. In this context, the recent "cell sheet engi-

neering" technology (Yamato, 2004; Yang et al., 2005), which uses a change in temperature to detach a layer of cells from a thermoresponsive plate, was recognised as a suitable tool to engineer seeded multilayered tissue constructs. Based on a thorough search of the literature, this thesis is the first to describe the combination of electrospinning and cell sheet engineering.

5.2 Objectives and methodology

The work described in this chapter investigates the potential of combining electrospinning and cell sheet engineering to create three-dimensional osteochondral grafts. To assess the capability of creating three-dimensional constructs with electrospun membranes and cell sheets, the focus is placed on homogeneous fibre arrangements only, *i.e.* no gradient of composition has been used. Two membrane compositions are tested to build layered constructs. These are termed PLGA-c and PCL-c, as they refer to PLGA and PCL-based membranes, each containing both Collagen I and hydroxyapatite nanoparticles at fixed concentrations. Characterisation includes evaluation of the pluripotency, viability and chondrogenic differentiation of hMSCs seeded within the engineered constructs.

A complementary section to this chapter can be found in the Appendix. That additional section briefly considers the possibility of binding these multilayered scaffolds onto calcium phosphate disks to obtain biphasic osteochondral constructs. Heterogeneous con-

structs will not be discussed in the current chapter.

5.3 Results

5.3.1 Membrane characteristics

The composite membranes fabricated from PLGA or PCL display similar morphologies, as seen in Figure 5.1 and Table 5.1. In both cases, fibres exhibit a wide distribution of diameters centring on an average diameter of about 430nm. The initial thicknesses of both scaffolds are of about $7\mu\text{m}$. However, PLGA-c mats undergo shrinkage of $70\pm 3\%$ after immersion in a phosphate buffered solution at 37°C for three days, whilst no significant shrinkage was observed for PCL-c membranes. Also, the mechanical properties are significantly different, as shown in Table 5.2. PLGA-c membranes have higher Young's modulus (Y_m) than the PCL-c membranes, 69.9 ± 6.6 MPa against 38.0 ± 7.3 MPa, respectively. However, the maximum tensile strength (U_{max} , 1.7 ± 0.3 MPa against 3.3 ± 0.4 MPa) and the maximal elongation (ϵ_{max} , $7\pm 3\%$ against $80\pm 4\%$) are both significantly higher for the PCL-c mats.

Table 5.1: Morphological properties of PLGA-c and PCL-c mats. Values indicated are the means with the standard deviations.

Membrane	Fibre diameter (nm)	Thickness (μm)	Surface shrinkage at 37°C (in %)
PLGA-c	421 ± 208	7.3 ± 1.4	70 ± 3
PCL-c	435 ± 168	6.7 ± 1.3	0

Table 5.2: Mechanical properties of PLGA-c and PCL-c membrane. Values indicated are the means with the standard deviations.

Membrane	Y_m (MPa)	U_{max} (MPa)	ϵ_{max} (%)
PLGA-c	69.9 ± 6.6	1.7 ± 0.3	7 ± 3
PCL-c	38.0 ± 7.3	3.3 ± 0.4	80 ± 4

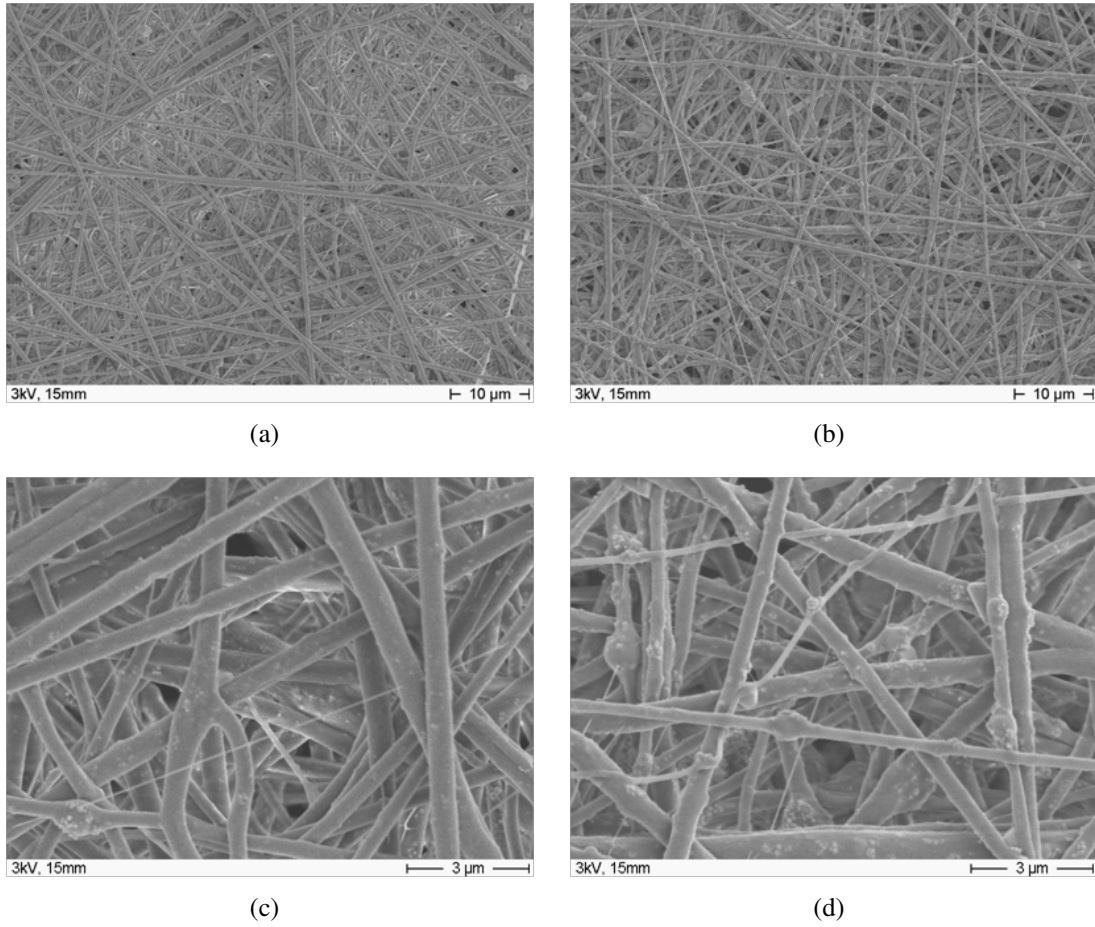


Figure 5.1: SEM images of electrospun mats obtained from: (a) and (c) a mixture of 10% (w/v) of nHAp in polymer solution containing 15% (w/v) of a blend of PLGA and Collagen I in ratio 8:2 (w/w) in HFIP; (b) and (d) a mixture of 10% (w/v) nHAp in polymer solution containing 8% (w/v) of a blend of PCL and Collagen I in ratio 8:2 (w/w) in HFIP.

5.3.2 Culture of cell sheet on electrospun membranes

5.3.2.1 Cell coverage after transfer of the cell sheet

The coverage of the electrospun membranes by cells was observed three days after the cell sheet transfer, using actin staining and SEM imaging. Although the cell sheets were intact directly after their transfer (data not shown), they did not appear to entirely cover the membranes after 3 days of culture, as shown in Figure 5.2. As indicated in Table 5.3, the area covered by hMSCs was significantly larger on PLGA-c mats in comparison to PCL-c mats.

Table 5.3: Fraction of the membrane covered by hMSCs three days after the transfer of a cell sheet (p value $< 2 \times 10^{-4}$).

Membrane	Surface coverage (%)
PLGA-c	71 ± 20
PCL-c	30 ± 18

5.3.2.2 Phenotype characterisation

To determine the capability of hMSCs to retain their pluripotency after being transferred onto electrospun membranes, cells were cultured for seven days in growth medium on the different membranes and were characterised for the expression of specific cell-surface markers by use of flow cytometry. As shown in Figure 5.3, in all cases (control flask, PLGA-c and PCL-c membranes), hMSCs were positive for CD29 (integrin $\beta 1$), CD44 (hyaluronic acid receptor), CD105 (endoglin) and CD 166 (activated leukocyte cell

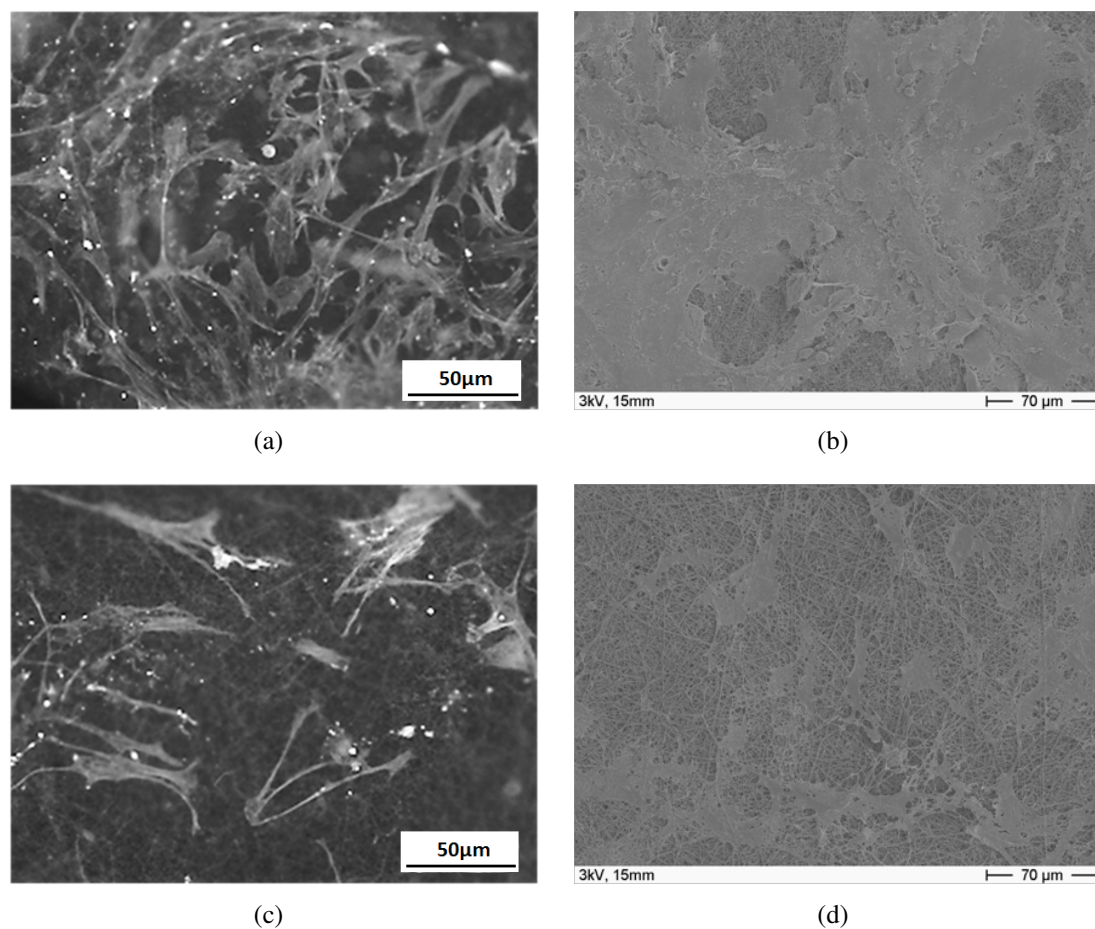


Figure 5.2: Fluorescence imaging (left) and SEM pictures (right) of hMSCs cultured on electrospun membranes three days after transfer of the cell sheet: (a, b) PLGA-c, and (c, d) PCL-c.

adhesion molecule). In contrast, cells were negative for CD14 (receptor for endotoxin), CD34 (hematopoietic cell adhesion molecule) and CD45 (protein tyrosine phosphatase, receptor C).

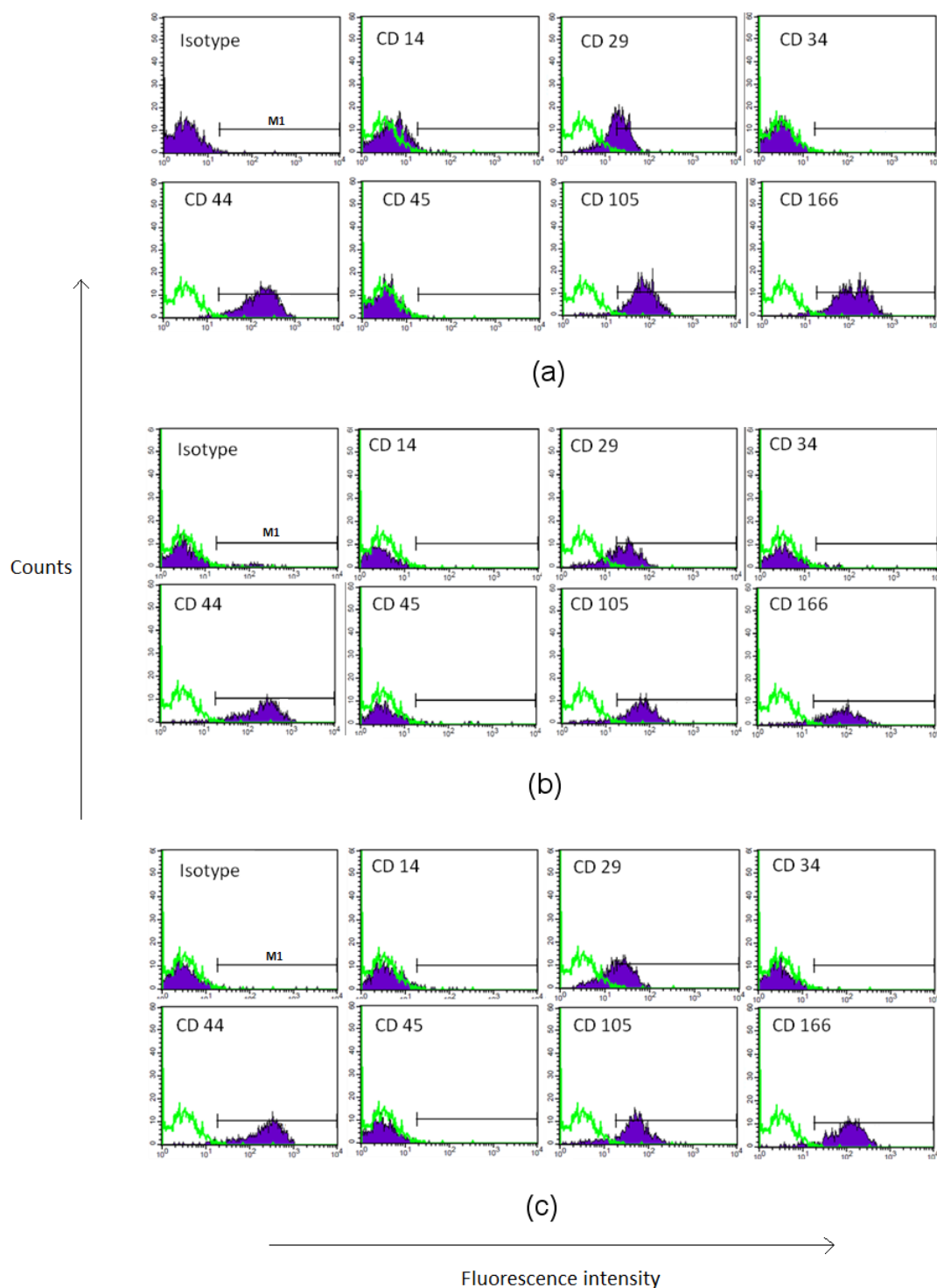


Figure 5.3: Flow Cytometry analysis of CD14, CD29, CD34, CD44, CD45, CD105, and CD166 expression on hMSCs cultured 7 days on (a) tissue culture flasks (control), (b) PLGA-c membranes, (c) PCL-c membranes.

5.3.3 Monophasic layered constructs

5.3.3.1 Morphology and mechanical properties

Both differentiated and undifferentiated (control) PLGA-c constructs underwent shrinkage after 3 weeks in culture, in either growth medium or differentiation medium. Conversely, PCL-c constructs maintained their initial size and shape (see Figure 5.4). In general, after culture, the different layers could be detached from each other without major damage on the electrospun membranes. This was not the case for the differentiated PLGA-c constructs, where attachment between layers was strong.

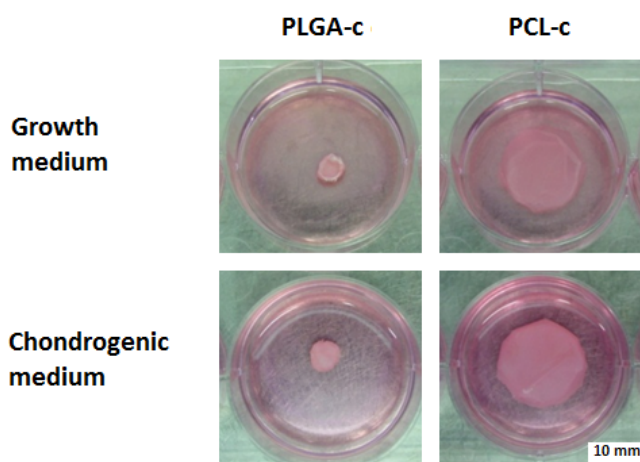


Figure 5.4: Morphology of the layered arrangement observed after 21 days of culture in either growth medium or differentiation medium. Top left: control PLGA-c constructs; bottom left: differentiated PLGA-c constructs; top right: control PCL-c constructs; bottom right: differentiated PCL-c constructs.

5.3.3.2 Viability

The percentages of viable, apoptotic and dead cells measured after seven days of culture in the layered arrangements are indicated in Table 5.4. The results show that there is no significant difference in terms of viability between hMSCs cultured in flasks and those cultured in the layered constructs.

Table 5.4: Viable, apoptotic and dead hMSCs detected using the Viacount assay after seven days of culture in flask (control) and in the layered constructs.

Membrane	Viable (%)	Apoptotic (%)	Dead (%)
Flask	91.1 $\pm$ 5.7	3.6 $\pm$ 1.6	4.9 $\pm$ 3.3
PLGA-c	94.3 $\pm$ 2.2	3.9 $\pm$ 0.6	1.5 $\pm$ 2.0
PCL-c	91.0 $\pm$ 1.6	2.5 $\pm$ 0.9	5.1 $\pm$ 0.3

5.3.3.3 Cell morphology: 2D Vs. 3D

Figure 5.5 shows the size and granularity of hMSCs cultured at the surface of PLGA-c and PCL-c membranes (two dimensions, 2D) and within the layered construct (three dimensions, 3D) as determined by flow cytometry analysis. The data reveals that when hMSCs are cultured in 2D, the morphology and granularity of the cells remain similar to the hMSCs cultured in flask (control). However, in 3D, within the PLGA-c and PCL-c layered constructs (Figure 5.5(b)), both the size and the granularity of hMSCs were decreased in comparison to cells cultured in flasks and to cells cultured on surface of the electrospun mats.

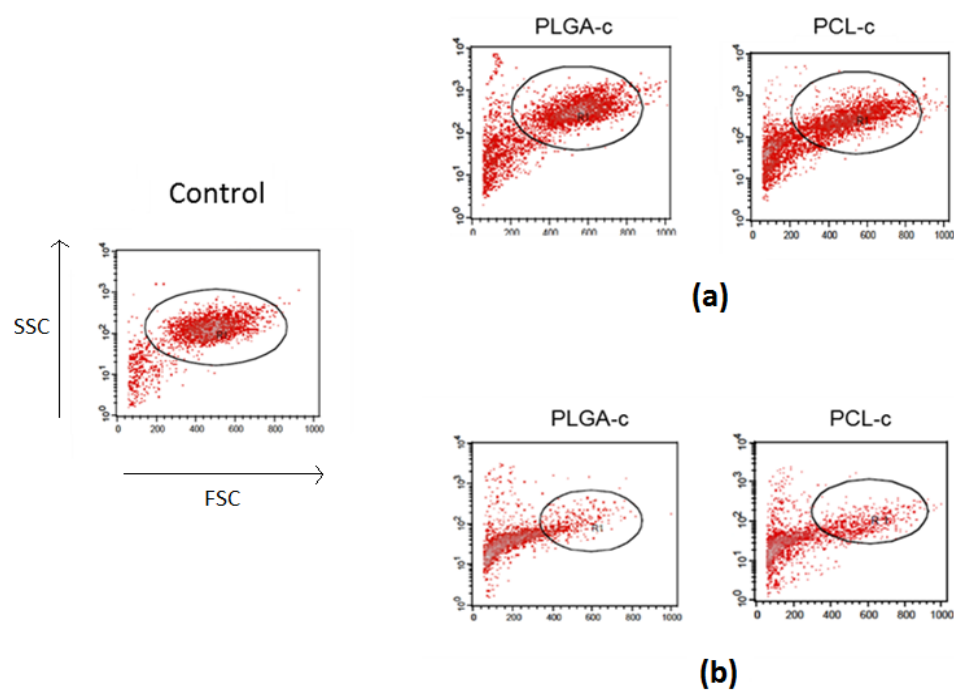


Figure 5.5: Size and granularity of hMSCs cultured a) at the surface of PLGA-c and PCL-c membranes (2D) and b) within the layered construct (3D). The control (left) corresponds to cells cultured in flasks.

5.3.3.4 Gene transcription

Following the culture of the layered constructs in the presence of chondrogenic medium, RT-PCR analysis was used to detect the relative expression of RNA of selected cartilage-specific genes. Results reveal that RNA from Collagen II, Aggrecan and Sox9 genes were found in both the PLGA-c and PCL-c multilayered constructs. Apart from Collagen II in the PLGA-c constructs, no significant difference was observed between the two scaffolds. In both types of samples, Collagen X mRNA was significantly increased in the differentiated constructs in comparison to controls. Also, the increase in Collagen X transcription was found to be significantly higher in PLGA-c compared to the increase observed for PCL-c samples (see Figure 5.6 and Figure 5.7; note the scale for Collagen X in Figure 5.6).

5.3.3.5 Gene expression

The presence of extensive extracellular matrix within the differentiated PLGA-c constructs after 21 days of culture was revealed by second harmonic generation (SHG) with multiphoton microscopy (Figure 5.8). Little or no SHG signal was recorded in other samples. Viable cells (green) and dead cells (red) were revealed by Live/Dead staining. The high cell density in PLGA-c compared to PCL-c assemblies is evident.

The expression of Collagen I, II, X and Aggrecan was further examined by immunohistochemistry (IHC) on vertical cross-sections of the layered constructs. Results are

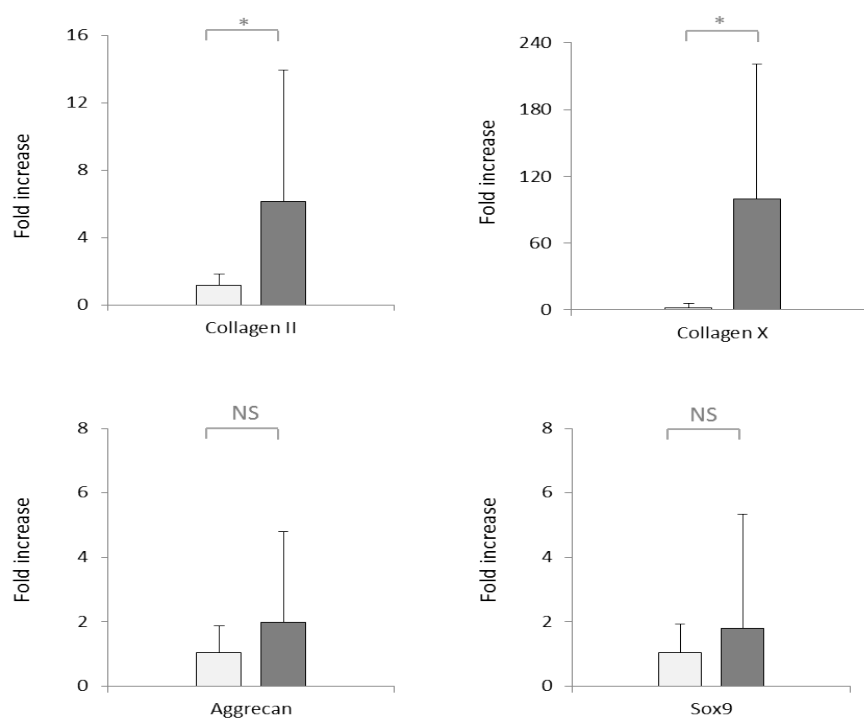


Figure 5.6: Fold increase in gene transcription as determined by RT-PCR analysis observed for the differentiated PLGA-c constructs (dark grey) normalised to levels of mRNA found in undifferentiated PLGA-c samples (light grey) after 21 days of culture. ΔC_t were corrected by the reference housekeeping gene GAPDH. The error bars represent the standard deviation (n=3; each experiment was performed in triplicate; *p < 0.05; NS=not significant).

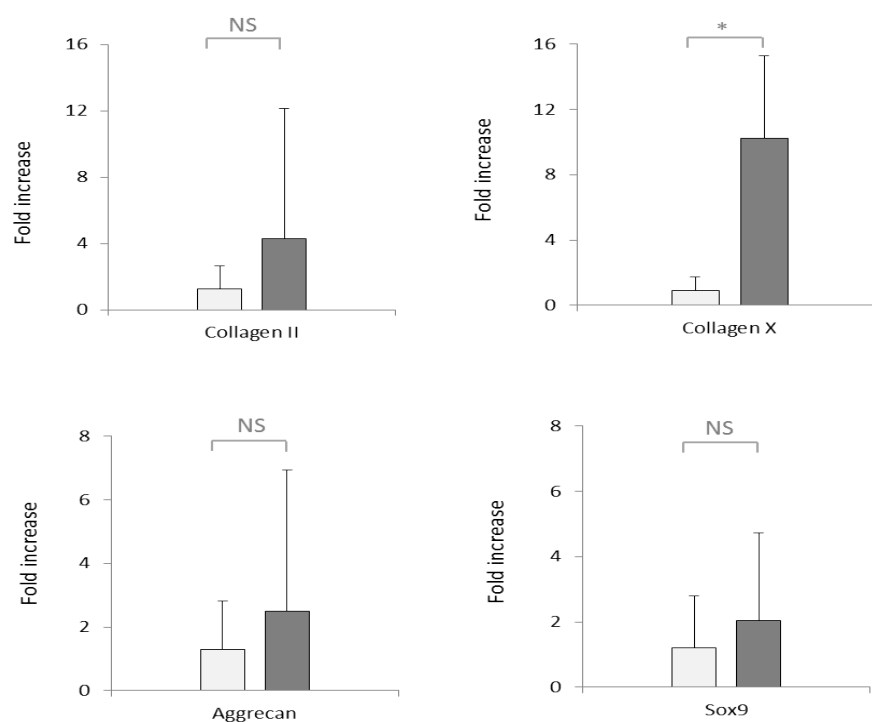


Figure 5.7: Fold increase in gene transcription observed for the differentiated PCL-c constructs (dark grey) normalised to levels of mRNA found in undifferentiated PCL-c samples (light grey) after 21 days of culture. ΔC_t were corrected by the reference housekeeping gene GAPDH. The error bars represent the standard deviation (n=3; each experiment was performed in triplicate; *p < 0.05; NS=not significant).

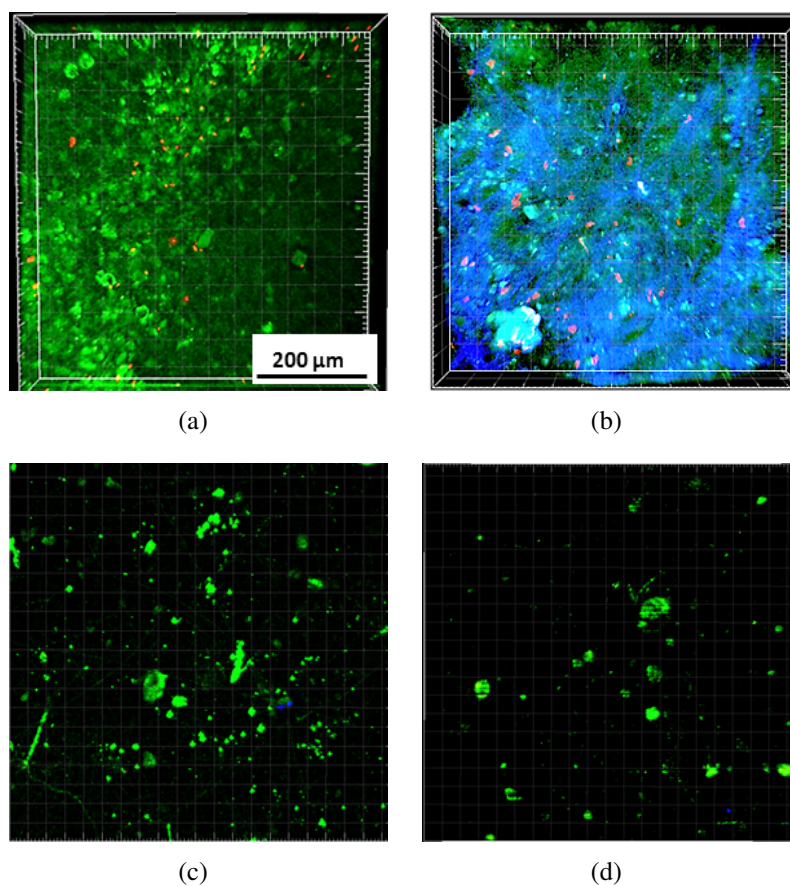


Figure 5.8: Multiphoton microscope images of tissue constructs cultured for 21 days (a) PLGA-c undifferentiated, (b) PLGA-c differentiated, (c) PCL-c undifferentiated and (d) PCL-c differentiated. Blue: extracellular matrix; Green: living cells; Red: dead cells. The scale bar is represented in (a).

presented in Figure 5.9 for PLGA-c and Figure 5.10 for PCL-c. The staining reveals the presence of Collagen II and Aggrecan in both differentiated constructs. However, Collagen I and Collagen X were more strongly observed on the differentiated PLGA-c constructs.

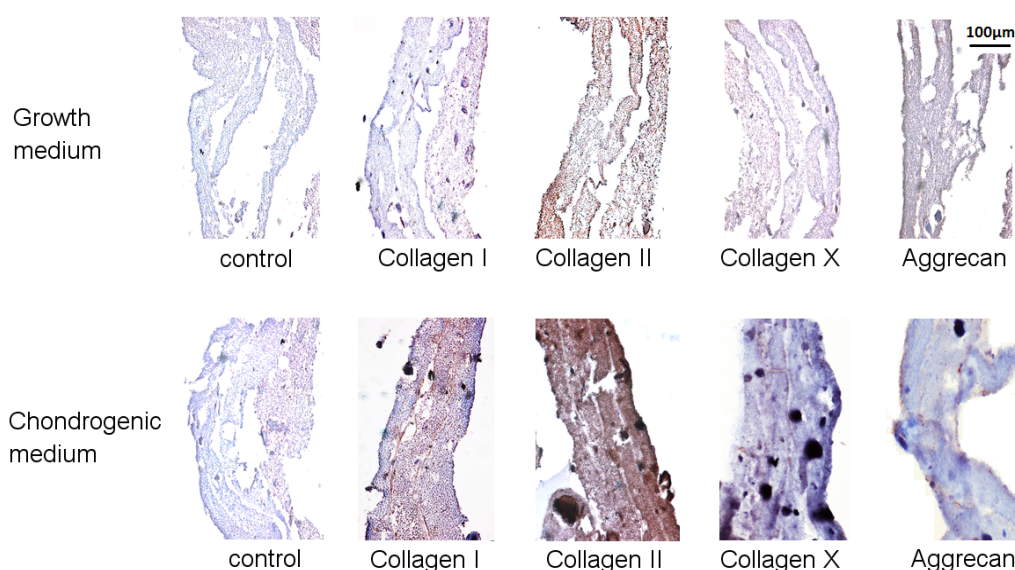


Figure 5.9: Immunohistochemistry performed on cross-sections of undifferentiated (top) and differentiated (bottom) PLGA-c tissue constructs: (a) control stain (no primary antibody), (b) Collagen I, (c) Collagen II, (d) Collagen X and (e) Aggrecan. The scale bar is represented in the right corner of the figure.

5.4 Discussions

5.4.1 Membrane characteristics

PLGA and PCL are both FDA-approved biodegradable synthetic polymers that have been investigated widely for the preparation of scaffolds for bone and cartilage tissue

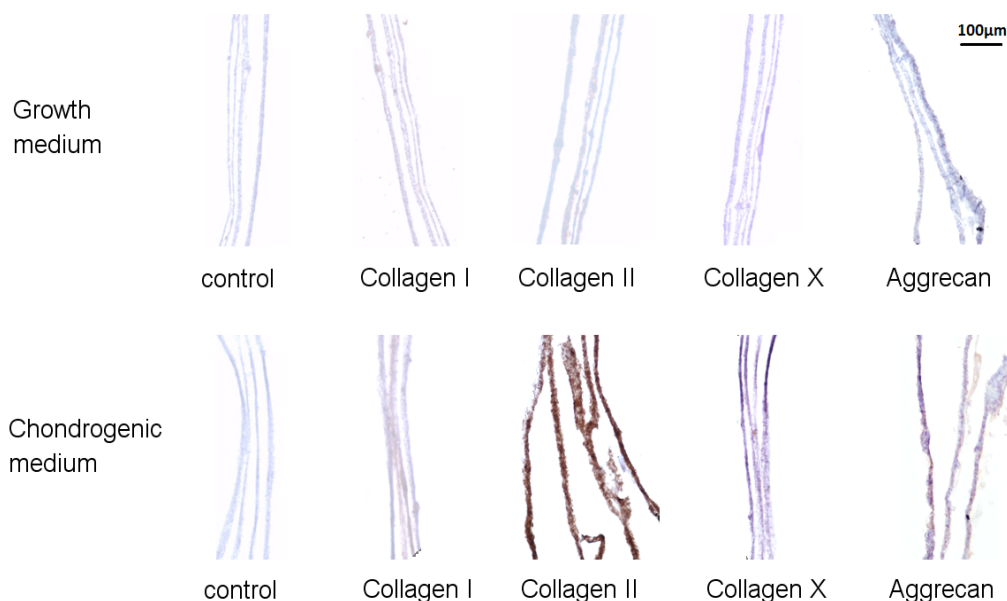


Figure 5.10: Immunohistochemistry performed on cross-sections of undifferentiated (top) and differentiated (bottom) PCL-c tissue constructs: (a) control stain (no primary antibody), (b) Collagen I, (c) Collagen II, (d) Collagen X and (e) Aggrecan. The scale bar is represented in the right corner of the figure.

engineering (Puppi et al., 2010). They can be easily electrospun and their rate of degradation through the hydrolysis of ester bonds and their mechanical properties are readily adjustable over a wide range by changing the molecular weight and copolymer ratios. PLGA and PCL based electrospun scaffolds are both known to be capable of supporting cellular viability, attachment, proliferation and differentiation during *in vitro* culture. However, PCL is often preferred to PLGA, as PLGA electrospun membranes shrink dramatically at physiological temperature, compromising the architecture of the initial scaffold. It is thought that the shrinkage is caused by the reorganisation of the polymer chains from a highly aligned structure found in electrospun fibres to a more relaxed state, provoking an important change in the scaffold volume. The glass transition of PLGA should only occur at temperature above 45°C but the presence of residual solvent within the fibres seems to

facilitate motion of the chains, making the glass transition possible at a lower temperature (Yoshimoto et al., 2003). It has also been observed that PLGA scaffolds are subject to further shrinkage due to contractive stresses caused by the extracellular matrix produced by adherent cells and this phenomenon was also observed in the present study. Besides shrinkage, PLGA scaffolds release acidic degradation products which may cause inflammatory responses in the host tissues (Li et al., 2005).

In order to increase the biocompatibility of both materials, collagen was added to each synthetic polymer. This addition is known to increase cell binding and to modify the physicochemical properties of the scaffold. Used on its own, collagen has high degradation rate and poor mechanical properties. Hydroxyapatite nanoparticles were also added to investigate the possibility of constructing mineral gradient scaffolds. Hydroxyapatite is the main inorganic component of bone and is also found at the interface and transition between bone and mineralised cartilage. Synthetic hydroxyapatite is well-known to be a bone bioactive material (Thomson et al., 1998; Zhang et al., 2009; Oliveira et al., 2006). Moreover, nanoparticles at the surface of the fibres act as nucleation sites, encouraging the formation of hydroxyapatite crystals in simulated body fluid environments (see Chapter 4).

PLGA-c and PCL-c membranes were found to have very different mechanical properties, despite similar fibre morphology. PLGA-c membranes are more stiff and brittle while PCL-c membranes are more flexible and can withstand higher compressive and ten-

sile strengths. Mechanical measurements were carried out on dried membranes at room temperature but compare favourably to general observation when handling wet samples at physiological temperature.

5.4.2 Electrospun membrane maintain the hMSCs phenotype

In this study, all cells seeded were selected before passage 10 to avoid possible abnormalities in phenotypic markers that arise in the long term culture (El-Sherbini, 2011). After being cultured for 7 days on electrospun membranes, cells were still positive for the hMSC markers CD29, CD44, CD105 and CD 166 and negative for CD14, CD34 and CD45, indicating that hMSCs maintained their stem cell phenotype. This expression profile matches the hMSCs identification criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici et al., 2006).

5.4.3 Cell coverage after transfer of the cell sheet

After transfer of the cell sheet to the 3D-like electrospun membranes, the cells were seen to reorganise, and discontinuities were observed to be present in the developing cell layers. The high degree of membrane coverage by cells on the PLGA-c membranes (compared to PCL-c membranes) is thought to be a consequence of the shrinkage of the PLGA. This created zones with high cell densities. These observations made on membranes were confirmed for 3D constructs as shown in the MPM images (see Figure 5.8).

5.4.4 Integrity of the multilayered construct

Use of electrospun membranes for the collection of cell sheets was shown to be a very straightforward and rapid way to build 3D constructs. During the assembly, cell sheets acted as adhesive between the different layers of the construct, making multilayering possible. The adhesive properties of the cell sheet are given by the ECM proteins, which are preserved intact during the sheet removal (Yamato, 2004). The binding between the different layers, observed after 21 days of culture in chondrogenic differentiation conditions, was much stronger for PLGA-c than for PCL-c constructs and the latter were relatively easy to disassemble into separate sheets. This was observed during manipulation of the constructs and can also be seen by the spacing between the layers in histological analysis (see Figures 5.9 and 5.10).

5.4.5 Viability in the multilayered construct

The cell viability was found to be maintained in 3D culture, showing no significant difference between the multilayered constructs and the original flask cell culture. The high porosity of the electrospun mats is thought to enhance viability by permitting good diffusion of nutrients within the multilayered constructs. Also, it was observed that the membranes were not entirely covered by hMSCs after the transfer of a cell sheet, which would have allowed better diffusion of nutrients and gases.

5.4.6 Cell morphology in the multilayered construct

In our studies, the size and granularity of hMSCs were found to decrease when switching from 2D to 3D culture. Cells are indeed known to adopt smaller sizes in 3D compared to 2D, in part due to the differences in the development of the cytoskeleton (Ochsner et al., 2010).

5.4.7 Gene transcription and expression

The gene expression profiles, as determined by mRNA expression of selected constituents, were investigated after 21 days of differentiation. The levels of mRNA of Collagen X were significantly higher in the PLGA-c and PCL-c constructs, as compared to their corresponding controls. The increase in Collagen X transcription was also found to be significantly higher in the PLGA-c scaffold than in the PCL-c scaffolds. The low levels observed for mRNA for Collagen II, Sox9 and Aggrecan may be due to the fact that these moieties are early markers of differentiation and that mRNA expression levels had decreased by day 21. IHC results support this conclusion and show the presence of Collagen II protein and Aggrecan detected, indicating that differentiation of hMSCs into chondrocytes occurred within both types of multilayered constructs.

Ordered deposition of collagen fibres was observed within the differentiated PLGA-c constructs after 21 days of culture, as indicated by SHG with multiphoton microscopy. Although other tissue components have been identified as sources of SHG, collagen has been shown to be the most efficient and likely source of SHG in tissues (Zoumi et al.,

2002; Zipfel et al., 2003). As Collagen X is a non-fibrillar protein, it is probable that this framework contains both Collagen II and Collagen I, as indicated by the IHC results.

Collagen X was observed in the PLGA-c constructs by IHC whilst little signal was seen in PCL-c samples. This seems to be in agreement with the above comments concerning the differences in observed increases in mRNA levels for Collagen X between PLGA-c and PCL-c samples. These observations, together with the results of SHG, suggest that hMSCs are at a further stage of differentiation in PLGA-c compared with the PCL-c constructs. As Collagen X is a marker for cell hypertrophy (Shapiro et al., 2008), these results suggest that hMSCs differentiated into chondrocytes in PLGA-c favour hypertrophy in comparison to PLC-c. Two causes may be behind the hypertrophy of the differentiated hMSCs. Firstly, it may be due to the intrinsic nature of hMSCs and the factors controlling their differentiation. It has been reported in the literature that chondrocytes differentiated from hMSCs have a rather unstable phenotype and may take on a hypertrophic phenotype under chondrogenic induction, later resulting in calcification after transplantation (Pelttari et al., 2006). Others have demonstrated that mesenchymal stem cells can rapidly differentiate into chondrocytes with a high expression of the Collagen type X gene and that these chondrocytes appear to play an important role in endochondral bone formation and regression of hyperostosis in the grafted tissue (Nagamoto et al., 1993). Secondly, the choice of materials used to culture hMSCs may influence the hypertrophic state. In this study, the scaffolds used have both a polymer phase and a ceramic phase. As mentioned earlier, PLGA scaffolds release acidic degradation products which

may cause inflammatory responses *in vivo* (Li et al., 2005). The lactic and glycolic acidosis created within the engineered tissue may promote the cell hypertrophy. Indeed, lactic acid is known to provoke osmotic pressure changes that causes increases in cell volume and consequently cell hypertrophy (Shapiro et al., 2008). Spadaccio et al. (2009) have demonstrated that the hMSCs differentiate into chondrocytes in PLLA/nHAp constructs but did not report the expression of the Collagen X gene. However, Kwarciak et al. (2007) have identified high Collagen X expression when normal chondrocytes were cultured in Polyglycolic acid (PGA) constructs suggesting that PGA could be used to generate hypertrophic chondrocytes. On the other hand, PCL does not release acidic compounds during its degradation in the culture medium (Lam et al., 2008). Li et al. (2005) reported the possibility of multilineage differentiation of hMSCs on PCL electrospun scaffolds. They observed that Collagen X was consistently down-regulated on PCL scaffolds compared to the undifferentiated cells, suggesting that hMSCs can be differentiated into non-hypertrophic chondrocytes on PCL constructs. Furthermore, Kim et al. (2008) also reported on the potential of PCL scaffolds to improve chondrogenesis of MSCs, and avoid hypertrophy.

Another important difference between PLGA and PCL that may influence differentiation is the shrinkage of PLGA membranes during culture, which increases the density of adherent cells. This effect may be favourable for the differentiation of hMSCs into chondrocytes since a pellet of cells is usually required in standard protocols. Hydroxyapatite has also been shown to influence stem cell differentiation towards bone and cartilage lin-

eage (Suominen et al., 1996) but the effect of HA on cell hypertrophy remains unknown. However, the present study seems to indicate that the polymer phase of the scaffold has a more important impact on the state of differentiation than the ceramic phase.

The above information strongly suggests that hMSCs reach the state of hypertrophic chondrocytes in PLGA-based constructs, whilst maintaining the chondrocyte phenotype in PCL-c matrices. These observations should be taken into consideration for the design of scaffold for a bone-cartilage interface or for the creation of osteochondral grafts. They suggest that membranes containing both polymers, with different ratios of PLGA to PCL and located at different positions in the construct, should be employed in order to obtain chondrocytes at different stages of differentiation in the same tissue construct. However future research is required to investigate this possibility.

5.5 Conclusions

Through the combination of cell sheet engineering and electrospun membranes, this chapter presents a straightforward way to build 3D tissue constructs. Culture of hMSCs in growth medium on PLGA-c and PCL-c membranes did not show any change in the hMSC phenotype. High viability was also found to be maintained in the multilayered construct. Under chondrogenic conditions, it was shown that PLGA-c and PCL-c membranes supported different states of hMSCs differentiation, and it is suggested that the first scaffold lead to hypertrophic chondrocytes and the second to stable chondrocytes. This suggests

that PLGA and PCL-based materials could be used at different locations in the same engineered construct in order to copy the phenotype transition observed in native articular cartilage. Nevertheless, further investigations are still necessary to allow better comparisons between the PLGA and PCL-based materials. In particular, more fundamental work is required with regard to the study of hMSC adhesion, proliferation, cytotoxicity, and differentiation onto the electrospun membranes.

Chapter 6

Protein Delivery from Mineralised Electrospun Fibres

6.1 Introduction

Amongst the different signals required to maintain the characteristics of stem cells or to guide them towards a certain phenotype, chemical cues are well-known to play a major role (see Chapter 2, Section 2.3.3). While both their nature and concentration are important, their delivery mode is also crucial (Moses et al., 2003). In a typical laboratory experiment, signalling molecules are provided to the cells as a soluble compound in the culture medium. However, this approach is time consuming and expensive since proteins such as growth factors tend to lose their activity quickly, thus requiring regular replacement of the culture medium. Various alternative delivery methods and systems have been proposed and much work is still required to optimise the drug release dynamics.

Electrospun materials are ideal candidates for the creation of drug delivery systems, due to their high specific surface area and the multitude of morphologies that can be fabricated. This aspect of electrospinning has been relatively undeveloped and is of growing interest, as mentioned in Chapter 2, Section 2.6.2.

Among ceramics, calcium phosphate materials have also shown potential as drug delivery systems. Besides their biocompatibility, they have excellent surface properties and dissolution behaviours, which are suitable for drug adsorption and subsequent release (see Section 2.6.3). Therefore, both incorporation of nHAp in the polymer matrix and precipitation from SBF at the surface of drug-loaded electrospun fibres may affect the release profile of a particular drug. Indeed, further interactions can take place between the drug and the ceramic material, which may reduce the important initial burst and long term release kinetics.

6.2 Objectives and methodology

In this chapter, the possibility of using mineralised electrospun fibres as a delivery system for TGF- β 3 is investigated. TGF molecules are growth factors required during the differentiation of hMSCs into chondrocytes (see Chapter 5). The methodology used in this chapter is based on the approach first developed by Mr Nikolas Weissmueller in the

Tissue Engineering group (Institute of Biomedical Engineering, University of Oxford).

For his research project, Mr Weissmueller investigated the effect of mineralisation on the release of bovine albumin serum protein (BSA) from PCL-BSA(core)/gelatin(sheath) co-axial electrospun membranes (Weissmueller, 2010). To achieve this, different levels of mineralisation were achieved by varying the incubation time in SBF10 (10x concentrated SBF) between 0 and 3 hours. SEM and alizarin red staining have shown progressive mineralisation of the scaffolds while XPS and FTIR characterisation techniques have confirmed that the quality of the hydroxyapatite deposited on the fibres during the immersion in SBF10 was suitable for bone tissue engineering. It was demonstrated that the mineralisation of the gelatin sheath had a significant effect on the release of BSA. A computational model extrapolating from six discrete levels of mineralisation was also developed to visualise the drug release profile of a continuous mineral gradated electrospun scaffold.

In this work, we study the effect of a sheath made of PLGA and collagen (PLGA-col), the impact of the presence of hydroxyapatite nanoparticles in the polymer matrix, and the influence of mineralisation by immersion in SBF10 on the release profiles of BSA delivered from a PCL core. To prevent interference between the fluorescence signals of unlabelled BSA and of molecules such as collagen and gelatin (an issue identified during the work of Mr Weissmueller), BSA conjugated with fluorescein isothiocyanate (FITC) was used. The presence of a fluorescent marker also allows easier, faster, more precise quantifications as well as lower detection limits.

A preliminary work will investigate the encapsulation and release of TGF- β 3 from PCL-TGF- β 3(core)/PLGA-Collagen(sheath) membranes. It will also attempt to compare the differentiation of hMSCs using the TGF- β 3-loaded scaffold to the conventional approach using TGF- β 3 solubilised in the chondrogenic medium (standard method used in Chapter 5).

6.3 Results and discussions

6.3.1 BSA study

6.3.1.1 Morphology of fibres: mineralisation and degradation

Figure 6.1 and Figure 6.2 depict the morphology of the coaxial electrospun fibres loaded with BSA. Figures 6.1(a) and 6.1(c) show the aspect of the PCL-BSA(core)/PLGA-col(sheath) fibres before and after total mineralisation in SBF10, respectively. Figures 6.1(b) and 6.1(d) show the morphologies of same fibres after two weeks of incubation at 37°C in PBS. Figure 6.2 shows similar information for PCL-BSA(core)/PLGA-col-nHAp(sheath) fibres (nHAp has been incorporated in the sheath). Table 6.1 summarises the main parameters used and the average diameters measured for the two fibre compositions. Data for PCL-BSA/gelatin fibres (Weissmueller, 2010) have also been added to this table.

The SBF10 solution was used to ensure the coating of the fibres within only 3 hours,

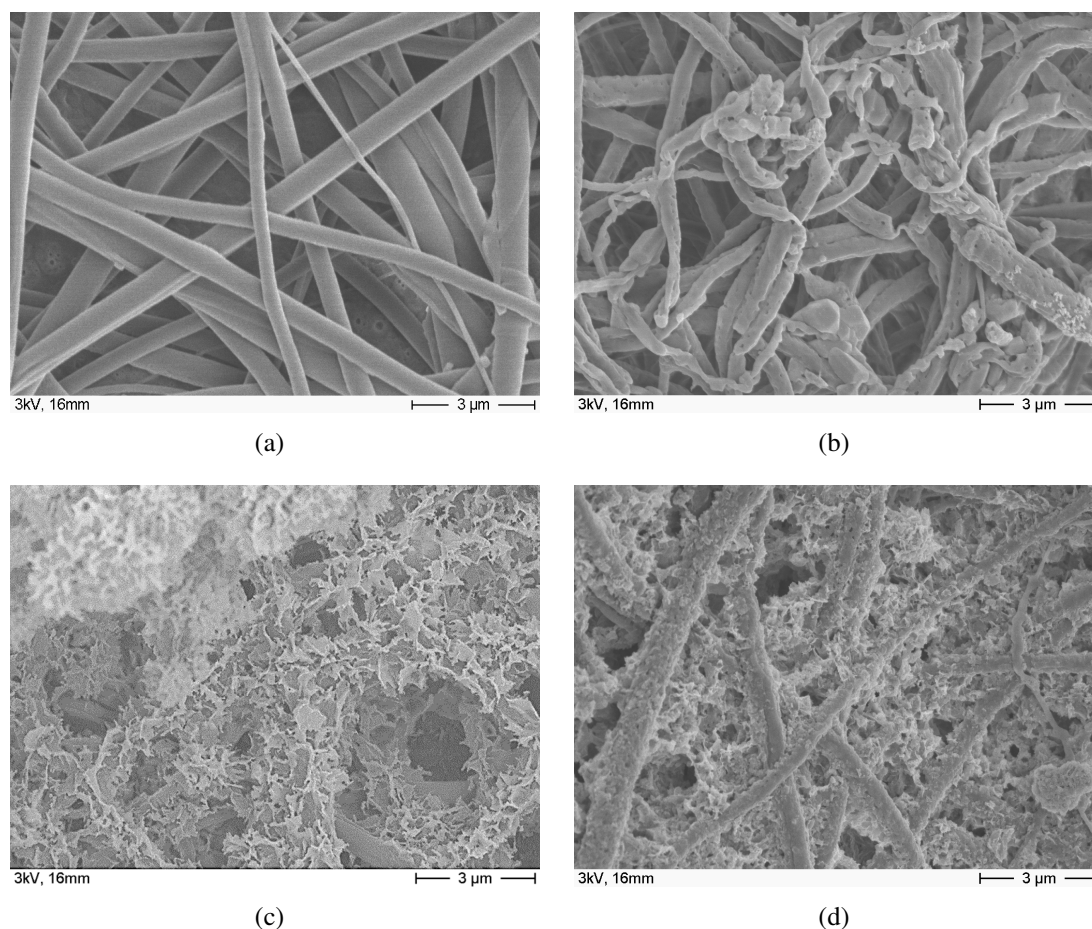


Figure 6.1: Morphology of PCL-BSA(core)/PLGA-col(sheath) electrospun fibres observed by SEM. (a) without mineralisation, before the release experiment; (b) without mineralisation, after two weeks of release; (c) with total mineralisation, before the release experiment; (d) with total mineralisation, after two weeks of release.

Table 6.1: Average fibre diameters observed for different coaxial fibre compositions. Voltages and flow rates used for the electrospinning process are also indicated. F_{core} = flow rate of the core solution; F_{sheath} = flow rate of the sheath solution.

Composition (core/sheath)	Voltage (kV)	F_{core} (ml/hr)	F_{sheath} (ml/hr)	Average fibre diameter(nm)
PCL-BSA/PLGA-col	13.2 ± 0.7	0.33	0.66	520 ± 156
PCL-BSA/PLGA-col-nHAp	13.6 ± 0.7	0.33	0.66	535 ± 202
PCL-BSA/gelatin	11.5 ± 0.5	0.33	0.66	508 ± 159

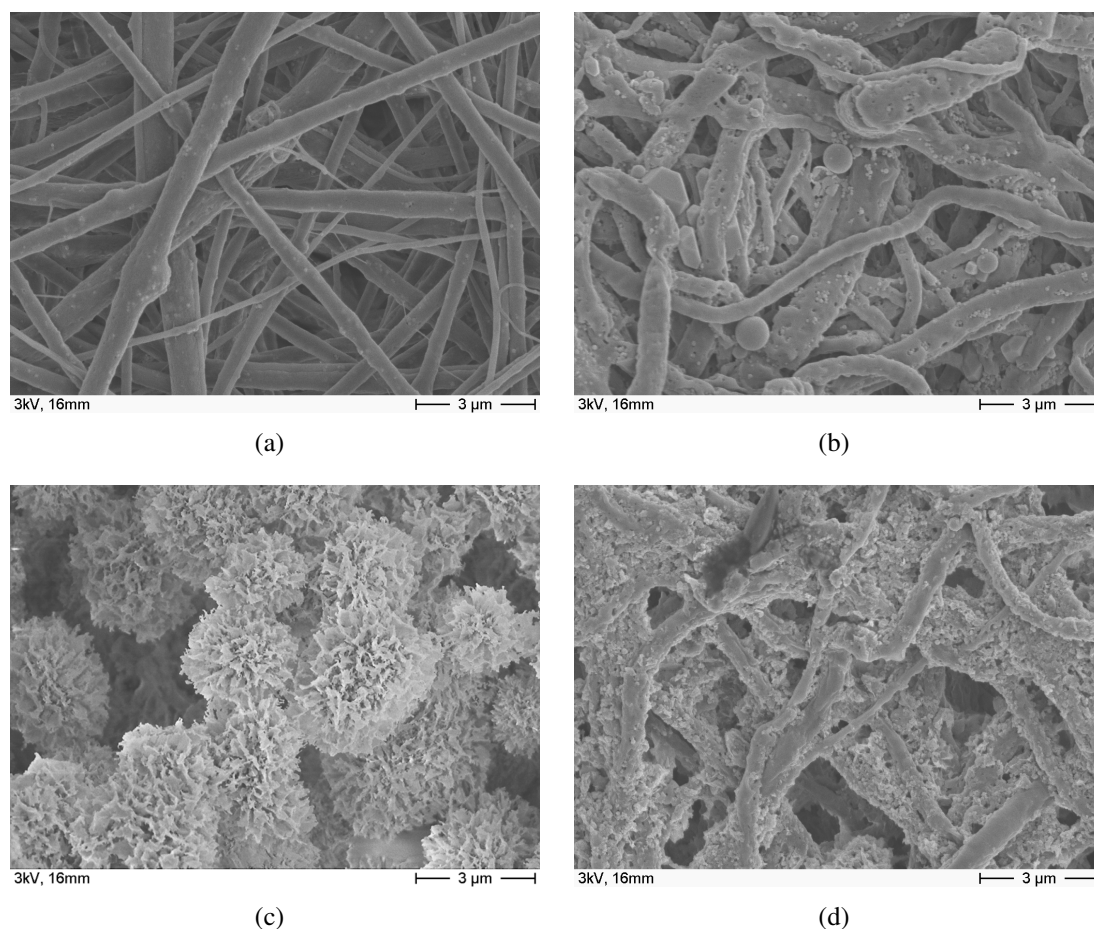


Figure 6.2: Morphology of PCL-BSA(core)/PLGA-col-nHAp(sheath) electrospun fibres. (a) without mineralisation, before the release experiment; (b) without mineralisation, after two weeks of release; (c) with total mineralisation, before the release experiment; (d) with total mineralisation, after two weeks of release.

compared to the minimum 72 hours of incubation required for full mineralisation with normal SBF (see Chapter 4, Section 4.3.6). This is crucial in the context of drug delivery, since the short incubation in SBF10 reduces the loss of the bioactive molecule through dissolution during the mineralisation.

It is interesting to note that, although PCL and BSA were added into the core of the

PLGA-col fibres, fibre diameters similar to the original PLGA-col fibres were obtained for the selected electrospinning parameters (see Table 6.1). However, from the SEM images alone (see Figures 6.1a and 6.2a), it is not possible to determine whether or not the fibres have a proper co-axial structure.

Homogeneous mineralisation of fibres was observed on both PCL-BSA/PLGA-col and PCL-BSA/PLGA-col-nHAp fibres. Through visual inspection mineralisation did not appear higher on the samples containing nHAp, as it was suggested from earlier findings (see Chapter 4, Section 4.3.6). The rapid and efficient mineral deposition by SBF10 may be one reason explaining that no difference was observed between the two samples. Moreover, the presence of collagen may also have promoted CaP deposition, since such effect has been seen for gelatin, a hydrolysed form of collagen (Gupta, 2009). The homogeneous mineralisation observed supports the prevalence of a uniform coating of PCL with PLGA and collagen. In the work of Mr Weissmueller, gelatin coating had led to non-homogeneous mineralisation which was attributed to inconsistent coating during electrospinning. However, it is likely that the coating was partially lost by dissolution during the immersion in PBS and SBF10 solutions, as gelatin is soluble in water.

Remarkably, visual inspection suggest that the mineralised membranes retained a significant amount of minerals after two weeks of incubation, although evident degradation of the biomaterials was observed, as shown by the breaks and pores appearing within the meshes (see Figures 6.1d) and 6.2d)).

In addition to the SEM images, Figure 6.3 reveals the morphology of the fibres using fluorescent microscopy. Although fluorescence from the FITC-labelled BSA was observed along the direction of the fibres, it remains difficult to conclude about their possible core-sheath structure because it was not possible to visualise the sheath and the core simultaneously.

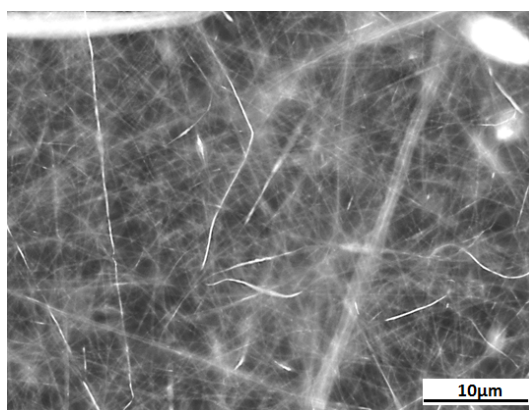


Figure 6.3: PCL-BSA(core)/PLGA-col(sheath) coaxial electrospun fibres observed by fluorescence microscopy. The signal (white) was given by the FITC molecules that are conjugated to BSA, revealing the core of the coaxial fibres. Weaker signal may also have come from the sheath layer, in which the BSA molecules may have already diffused.

6.3.1.2 Efficiency of the coaxial encapsulation

Images presented above could not determine if the encapsulation was successful or not. In order to assess the efficiency with which the core material was surrounded by the outer polymer blend, nHAp was placed in the core solution, instead of BSA, and the fibres were observed using SEM.

Figures 6.4 (a) and (b) reveal that nHAp can be observed at the surface of some of the fibres within the mesh. This suggests that the encapsulation was not totally achieved as the nanoparticles should remain hidden inside the fibres. Also, a clear boundary is seen, delimited by the appearance of the nanoparticles at the surface of the fibres (especially seen in Figure 6.4(b)). This indicates that the two solutions (inner and outer) remain separate during the stretching and solidification of the electrospun jet. These observations suggest that future work could reconsider the selection of the solvents and flow rates for the inner and outer solutions (*e.g.* immiscible solvents and smaller inner flow rates). It would also be worthy to study further the effect of the design of the coaxial nozzle (presented in Chapter 3, Figure 3.3). However, this optimisation aspect is out of the scope of the current research.

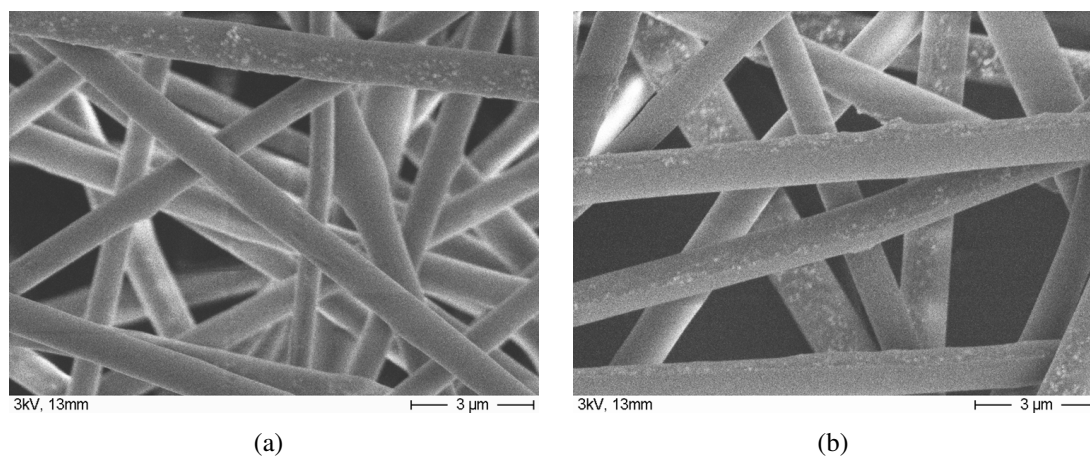


Figure 6.4: SEM images showing the morphology of PCL-nHAp(core)/PLGA-col(sheath) electrospun fibres at random locations within the membrane. HFIP was used as a solvent for both the inner and outer polymer solutions. The images reveal that nHAp (provided with the core solution) can be observed at the surface of some fibres within the mesh, suggesting that total encapsulation of the core material was not achieved.

6.3.1.3 Release profile

Details on the protocol used for the investigation of the rate of drug release are found in Chapter 3, Section 3.5.4. The standard curve for BSA in PBS is presented in logarithmic values in Figure 6.5. The "S" curve indicates a linear region of interest between 0.1 $\mu\text{g/ml}$ and 0.1 mg/ml which can be described by the power equation displayed on the graph.

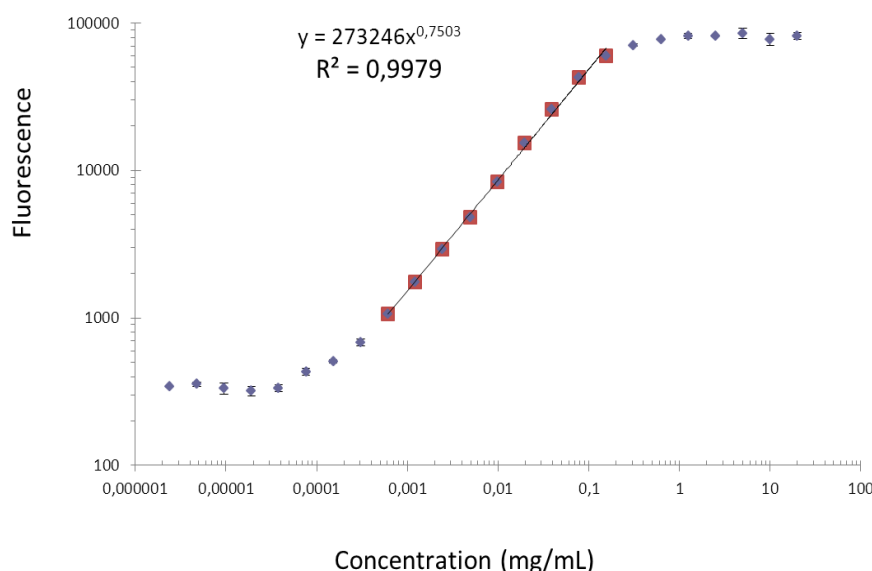


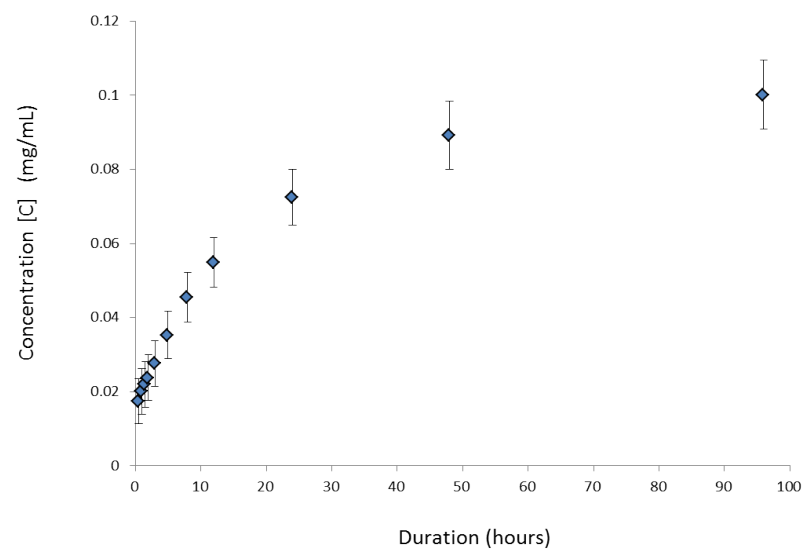
Figure 6.5: Standard curve for BSA measured in PBS solution. The graph uses logarithmic axis. The region of interest is shown in red.

The accumulative curve obtained for the release of BSA from PCL-BSA(core)/PLGA-col(sheath) fibres over 4 days is depicted in Figure 6.6 (concentration profile and C/C_0 profile are both represented). All concentrations measured were in the linear part of the standard curve. The release profile suggests an initial burst release during the first 24 hours followed by a reduced, more steady rate of release over the following days. The

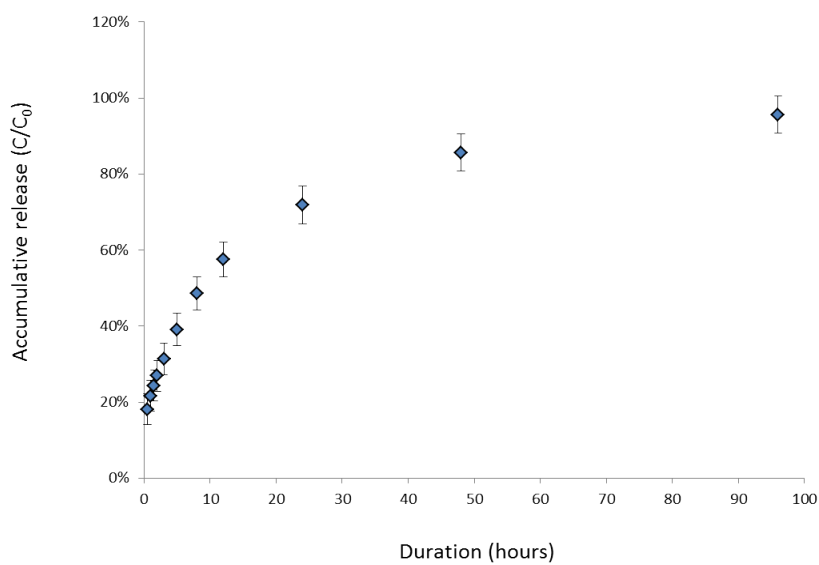
release profile PCL-BSA/PLGA-col fibres is compared to the profile of PCL-BSA/gelatin fibres in Figure 6.7. PCL-BSA/gelatin fibres exhibit a more pronounced release than when PLGA-col sheath is covering the fibres, indicating the importance of the polymer sheath. T-tests performed at different time points indicated that the difference between the two curves is significant ($p < 0.01$) until 48 hours of incubation.

These results were expected since gelatin is, unlike collagen, soluble in water. The gelatin layer is thought to swell when placed in contact with water and dissolve rapidly with agitation increasing the rate of dissolution, explaining the rapid initial release of BSA. On the other hand, PLGA and collagen are both insoluble in water and therefore are expected to act as a stronger barrier against the diffusion of BSA. Also, tests performed with unloaded scaffolds confirmed that the fluorescence from gelatin or collagen was not interfering with the BSA-FITC signal at the concentration used and at the excitation/emission wavelengths selected.

The release profile PCL-BSA/PLGA-col fibres is also compared to the profile of PCL-BSA/PLGA-col-nHAp fibres in Figure 6.8 to investigate the effect of nHAp on the release of bioactive molecules. T-tests showed that no significant difference was observed between the two curves at any time point. This results implies that, in a multilayered scaffold with a gradient of nHAp between the layers, the release dynamics of BSA would not depend on the amount of nHAp in the sheath (for the range of concentrations studied). However, as mentioned in Section 6.3.1.2, the sheath may not have totally encapsulated



(a)



(b)

Figure 6.6: Accumulative curves obtained for the release of BSA from PCL-BSA(core)/PLGA-col(sheath) fibres: (a) concentration profile, (b) C/C_0 profile. C_0 correspond to the concentration measured after total release of BSA from the fibres. The error bars represent the standard deviation ($n=3$, each experiment was performed in triplicate).

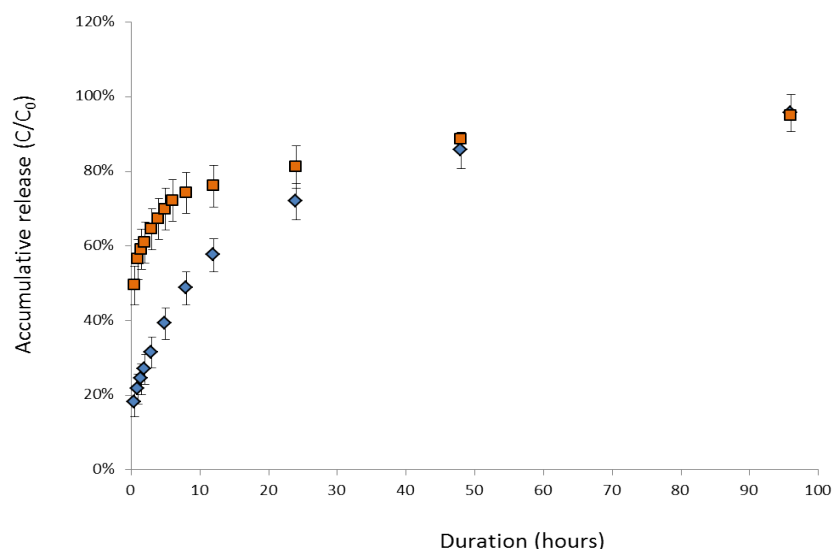


Figure 6.7: BSA release profile from PCL-BSA(core)/gelatin(sheath) fibres (orange squares) and PCL-BSA/PLGA-col fibres (blue diamonds). The error bars represent the standard deviation ($n=3$, each experiment was performed in triplicate). T-tests performed at different time points indicated that the difference between the two curves is significant ($p<0.01$) until 48 hours of incubation.

the PCL-core and this may have influenced the results.

The effect of the mineralisation in SBF10 on the release profile of BSA from PCL-BSA/PLGA-col fibres is shown in Figure 6.9. The initial burst release (after 30 minutes of incubation) was found to be decreased from about 18% to about 10% (C/C_0). T-tests performed at different time points showed that a significant difference ($p<0.02$) can be seen until three hours after the initiation of the release. The effect of the mineralisation on the initial release of BSA is in agreement with the work of Mr Weissmueller, which demonstrated that the scaffold's protein release was significantly affected by its level of mineralization (Weissmueller, 2010). This phenomenon may be due to the excellent binding surface properties of CaP materials (see Chapter 2, Section 2.6.3), causing further chemical interactions of the drug with the ceramic phase while being released from the

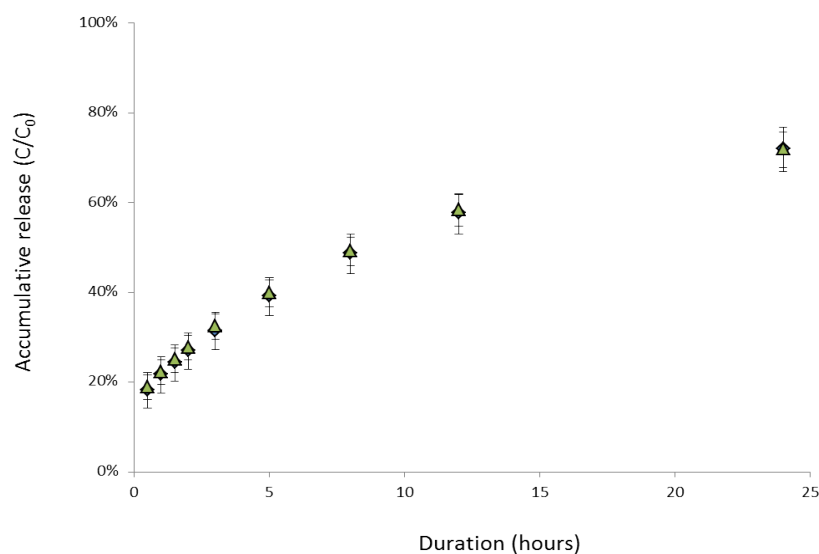


Figure 6.8: BSA release profile from PCL-BSA(core)/PLGA-col(sheath) fibres (blue diamonds) and PCL-BSA/PLGA-col-nHAp fibres (green triangles). The error bars represent the standard deviation ($n=3$, each experiment was performed in triplicate). T-tests showed no significant difference between the two curves at any time point.

polymer matrix. The mineral layer may also simply act as a physical barrier to delay the diffusion of the protein.

No significant difference was observed between the release profile of mineralised PCL-BSA/ PLGA-col fibres and the profile of mineralised PCL-BSA/PLGA-col-nHAp fibres at any time point (Figure 6.10). This may be due to the fact that the amount of mineralisation was the same on both samples. As mentioned earlier in Section 6.3.1.1, the highly concentrated SBF solution and the presence of collagen may explain the strong mineral deposition on both PCL-BSA/PLGA-col and PCL-BSA/PLGA-col-nHAp membranes.

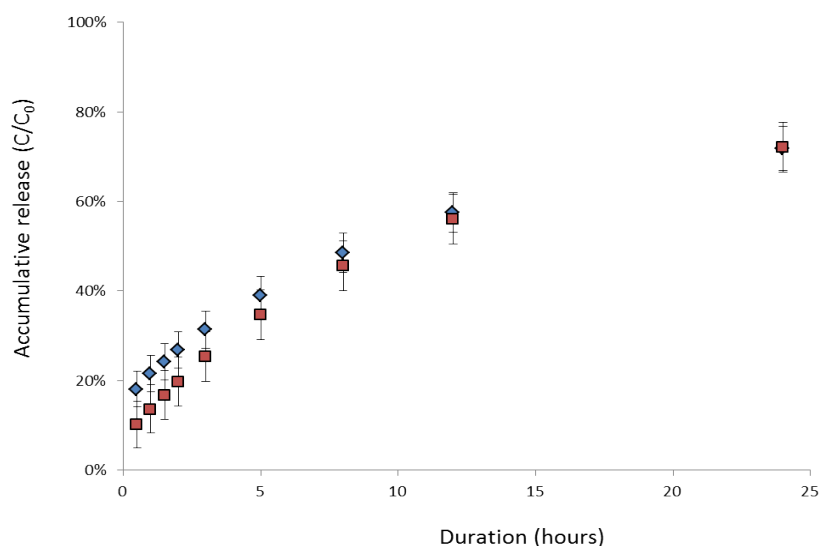


Figure 6.9: BSA release profile from unmineralised PCL-BSA(core)/PLGA-col(sheath) fibres (blue diamonds) and mineralised PCL-BSA/PLGA-col fibres (red squares). The error bars represent the standard deviation ($n=3$, each experiment was performed in triplicate). T-tests performed at different time points showed that a significant difference ($p<0.02$) can be seen until three hours after the initiation of the release.

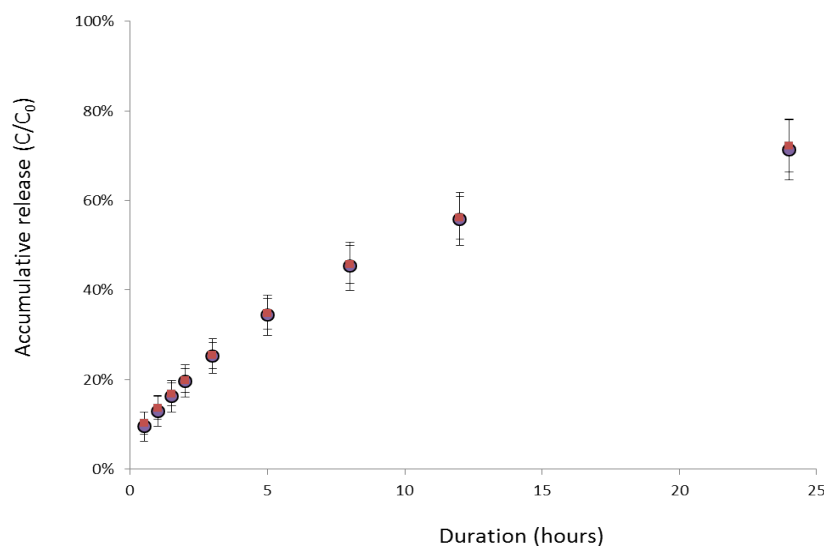


Figure 6.10: BSA release profile from mineralised PCL-BSA(core)/PLGA-col(sheath) fibres (red squares) and mineralised PCL-BSA/PLGA-col-nHAp fibres (purple circles). The error bars represent the standard deviation ($n=3$, each experiment was performed in triplicate). T-tests showed no significant difference between the two curves at any time point.

6.3.1.4 Stability of the protein

As mentioned earlier, a successful drug release from the scaffold does not mean that the protein maintains its activity. Its structural integrity may have been compromised during the electrospinning process or through the exposure to scaffold degradation products. It has been shown that electrostatic interactions of protein and solvent can have deep effects on the tertiary structure and function of a protein (Vivian and Callis, 2001). Since HFIP is a strong polar solvent that exhibits hydrogen bonding properties, an analysis of the conformation of the released BSA after dissolution in HFIP and electrospinning with PCL was carried out by Mr Weissmueller. Fluorescent spectroscopy analysis has shown that BSA was reasonably intact after suspension in HFIP, electrospinning and release from PCL electrospun membranes, suggesting that protein functions would be retained (see Figure 6.11). However, the effect of the scaffold composition and its degradation remains to be investigated. Particularly in the current study, the presence of PLGA in the sheath may affect the protein activity. Indeed, during its degradation, PLGA liberates acidic compounds that could damage protein integrity.

6.3.2 TGF- β 3 release study: a preliminary work

6.3.2.1 Membrane morphology

Figure 6.12 and Table 6.2 indicate that the average fibre diameter and the morphology of PCL-TGF- β 3/PLGA-col membrane exhibit similar properties to the membranes pre-

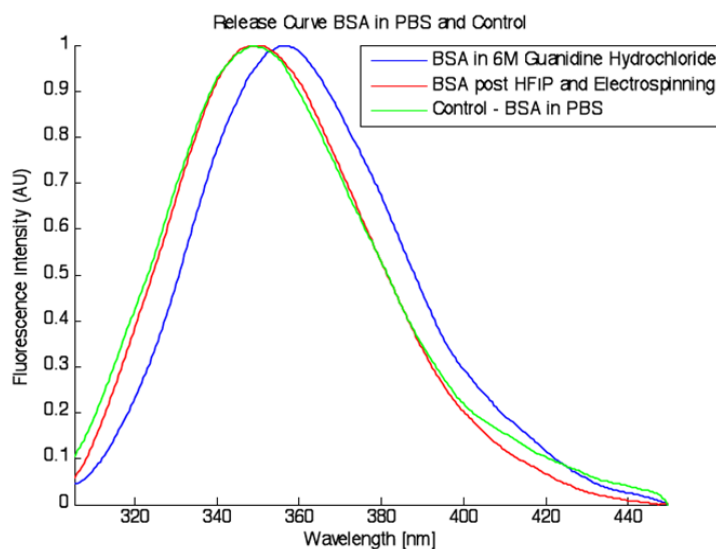


Figure 6.11: Fluorescence spectra of BSA (not conjugated to FITC) obtained to assess its tertiary structure in the following conformation: natural (PBS), denatured (guanidine hydrochloride) and post release (HFIP and electrospinning) (Weissmueller, 2010).

viously obtained with BSA. Voltage and flow rates used for the electrospinning process, indicated in Table 6.2, were also similar.

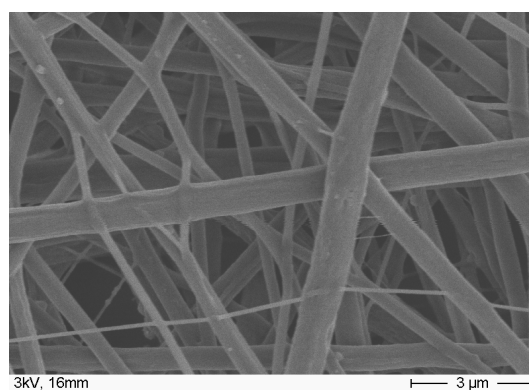


Figure 6.12: Typical observations of PCL-TGF- β 3(core)/PLGA-col(sheath) fibres under SEM.

Table 6.2: Average fibre diameters observed for PCL-TGF- β 3(core)/PLGA-col(sheath) fibres. Voltage and flow rates used for the electrospinning process are also indicated. F_{core} = flow rate of the core solution; F_{sheath} = flow rate of the sheath solution.

Composition (core/sheath)	Voltage (kV)	F_{core} (ml/hr)	F_{sheath} (ml/hr)	Average fibre diameter(nm)
PCL-TGF- β 3/PLGA-col	13.1 ± 0.6	0.33	0.66	557 ± 224

6.3.2.2 Release profile

Details about the protocol used for this study can be found in Chapter 3, Section 6. The standard curve for TGF- β 3 in PBS is presented in logarithmic values in Figure 6.13. The data was linearised by plotting the log of the concentrations versus the log of the optical density (O.D.). The region of interest ranges approximately from 100 pg/ml to 1000 pg/ml and a good fit is given by the power equation, displayed on the graph.

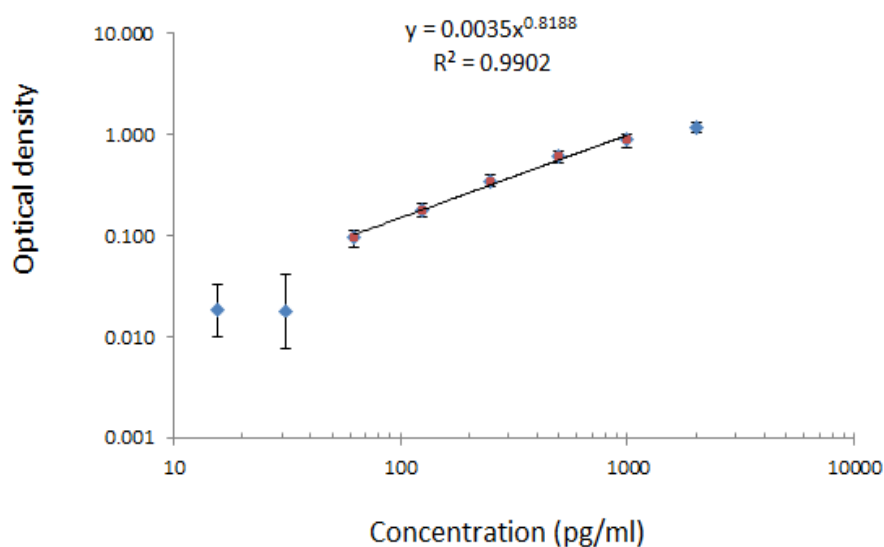


Figure 6.13: Standard curve for TGF- β 3 measured by elisa in PBS solution. The graph uses logarithmic ($\log_{10}$) axis. The region of interest is shown in red.

The concentration profile obtained for the release of TGF- β 3 over 4 days from PCL-TGF- β 3/PLGA-col electrospun fibres is similar to the curve obtained for the BSA release (see Figure 6.14). The curve shows a pronounced burst release during the first 12 hours, which was followed by a reduced, more steady rate of release over the following days. Compared to the BSA release curve from PCL-BSA/PLGA-col fibres (see Figure 6.6), the initial burst release of TGF- β 3 appears much more pronounced. This was expected, since TGF- β 3 is a smaller protein (TGF- β 3: 25kDa; BSA: 67kDa), resulting in a faster diffusion.

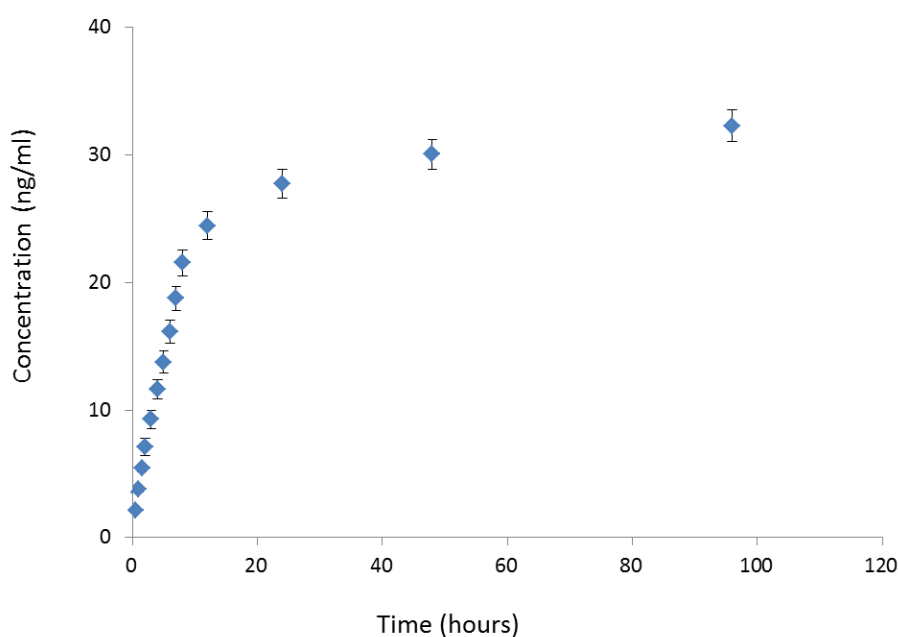


Figure 6.14: TGF- β 3 release profile from PCL-TGF- β 3(core)/PLGA-col(sheath) fibres measured by elisa. The release experiment was performed on 6 samples but was not repeated (n=1).

6.3.2.3 TGF- β 3 activity

Given the successful release of TGF- β 3 from the membrane, the differentiation of hMSCs into chondrocytes using the drug releasing membranes was carried out for a duration of 7 days to assess the activity of the protein and the potential of the approach. The experimental protocol is described in Chapter 3, Section 3.5.5. For the positive control, TGF- β 3 is provided in the culture medium, while not contained in the fibres. For the negative control TGF- β 3 is not provided neither in the culture medium nor from the fibres. The values of fold increase measured for the mRNA expression of Collagen II and Collagen X are indicated in Figure 6.15. Collagen X mRNA expression in both differentiated samples (positive control and TGF- β 3 releasing sample) exhibits significantly higher values than the negative control (undifferentiated). The high expression level of Collagen X is consistent with the findings presented in Chapter 5, which suggested that the acidic degradation products of PLGA cause chondrocyte hypertrophy, characterised by high expression of Collagen X. For Collagen II mRNA expression, results show a significant difference for the positive control when comparing to both the treatment group and the negative control. There is no significant difference between the treatment group and the negative control. Collagen II mRNA expression in cells cultured on the drug releasing scaffolds was not significantly higher than the negative control. This experiment was performed on a small sample set (three samples for each condition) and these preliminary results suggests that the differentiation using the scaffold as a delivery system was not successful for the differentiation period considered (one week).

The poor activity of TGF- β 3 may be due to two main reasons: chemical or mechanical denaturation of the protein and/or improper concentration profile of the bioactive molecule. First, the protein structure (secondary or/and tertiary) might have been damaged at some point during the experiment. As mentioned earlier in Section 6.3.1.4, this could have occurred during dissolution and encapsulation of TGF- β 3, since a highly polar solvent and strong electrical fields were used. However, damage of the protein may also have been caused by the acidic environment created by the degradation products of PLGA during release. Such negative effect from acid compounds has been reported in the literature (Estey et al., 2006; Houchin and Topp, 2009). In order to avoid damage of the growth factor from the acidic environment, maintaining the pH within the system using poorly water-soluble basic salts such as $\text{Mg}(\text{OH})_2$ has been suggested (Zhu et al., 2000).

Second, the concentration profile of TGF- β 3 to which the cells were exposed may not have been suitable for proper differentiation. As mentioned earlier in the introduction of the chapter, the concentrations of the signalling molecules in the environment of the cells are crucial. According to the release profile shown in Figure 6.14 the membrane releases about 35 ng of growth factor over 4 days of incubation. Although values were provided from dynamic conditions, it can be approximated that a similar amount would be released over a week of culture in static condition (the rate of release is expected to be lower though). The amount lost during incubation for cell attachment (3 hours in growth medium) is estimated to less than 8ng meaning that a release of about 30ng is expected over the week in chondrogenic conditions. This corresponds to the amount of

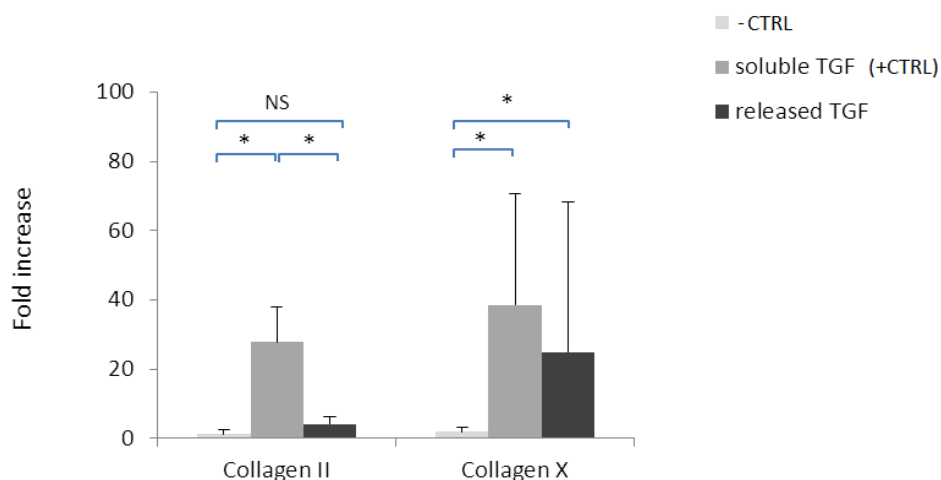


Figure 6.15: Fold increase in gene transcription observed for the cells culture in chondrogenic medium depleted in TGF- β 3 on PCL-TGF- β 3(core)/PLGA-col(sheath) membranes (black) and for cells cultured on PCL/PLGA-col membrane in presence of chondrogenic medium supplemented with TGF- β 3 (dark grey). The values normalised to levels of mRNA found in undifferentiated samples (light grey) after 7 days of culture on PCL/PLGA-col membranes. Δ Ct were corrected by the reference gene GAPDH. The error bar represents the standard deviation (test performed with three samples for each condition; *p < 0.05; NS, not significant).

TGF- β 3 provided into the medium for the positive control (three times 1mL of chondrogenic medium containing 10ng/mL of TGF- β 3). However, although similar amounts of growth factors are believed to have been provided in both methods of differentiation, the concentration profile resulting from the two approaches are very different. The conventional approach provides a large amount of fresh growth factors into the medium and the drug releasing system provides a slow release of factors. It may be that a concentration threshold must be reached to trigger differentiation. This suggests that supplying TGF- β 3 continuously through the use of a perfusion bioreactor may serve as a better control.

Besides assessing the effect of TGF- β 3 on the differentiation of hMSCs into chondrocytes, it seems evident that complementary methods of characterisation should be carried

out to confirm its loss of activity. In particular, ultraviolet circular dichroism can provide useful information on the changes occurring in both the secondary and tertiary structures of the protein (Kelly and Price, 2000). However, this is out of the scope of this research and it is only suggested as future work.

6.4 Conclusions

Drug-loaded fibres were fabricated to have diameters in the range of interest for tissue engineering (50-500nm) while combining the properties of PCL, PLGA and collagen. Immersion of the membranes in SBF10 led to the formation of a homogeneous layer of hydroxyapatite on the surface of the fibres. Also, the incorporation of nHAp during electrospinning revealed that the efficiency of incorporating the core material inside the fibres was not ideal since nanoparticles were observed at the surface of some fibres. However the coating of the fibres with a PLGA-col sheath did result in a slower release of BSA compared to a coating of gelatin, suggesting that the coaxial electrospinning had an effect on the drug release. The incorporation of nHAp in the sheath material did not show any significant effect on the release profile of BSA. On the other hand, the mineralisation of the fibres by SBF10 was found to cause a significant decrease in the rate of release of the model protein compared to the unmineralised fibres.

Preliminary results carried out with TGF- β 3 indicated that the release profile of the

TGF- β 3 had a more pronounced burst release than BSA. This is believed to be related to the smaller molecular size of the growth factor which is likely to lead to a higher diffusion rate. Chondrogenic differentiation of hMSCs using the TGF- β 3-loaded scaffold was not successful compared to controls, suggesting a possible damage of the protein activity by the electrospinning process (voltage and/or solvent) or by the acidity of the PLGA degradation product. Another possibility is that the release dynamics were not suitable for activating the chondrogenic differentiation. The loss of activity of TGF- β 3 suggests that other methods to incorporate growth factors into electrospun scaffolds should be investigated. Post-electrospinning treatments, such as coating the fibres with TGF- β 3 by adsorption (see Chapter 2, Section 2.6.2), may preserve the protein bioactivity better than co-axial electrospinning.

Chapter 7

Creation of Calcium Phosphate Fibres through Electrospinning and Sintering

7.1 Introduction

Calcium phosphate (CaP) materials are known to support bone ingrowth and to promote bone integration (Kokubo and Takadama, 2006). They are also non-toxic, biodegradable and can easily adsorb bioactive molecules at their surface. These characteristics make them useful for both TE and drug delivery applications (Bose and Tarafder, 2011; Habraken et al., 2007). Among CaP materials, synthetic hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) and β -tricalcium phosphate (β -TCP, $\text{Ca}_3(\text{PO}_4)_2$) are most widely used. Hydroxyapatite is the main form of calcium-phosphate crystal found in human bone. Usually, the degradability of CaP materials varies according to the Ca/P ratio, with β -TCP being more soluble than HA (Klein et al., 1990). Therefore, mixing CaP materials may help

to control, to some extent, the degradation of the TE scaffold. In particular, HA/ β -TCP ceramic scaffolds have been shown to be more effective in bone repair than pure HA or pure β -TCP (Nery et al., 1992).

CaP solid bodies have been used for many applications but they usually have the issue of being too brittle. The poor mechanical properties of CaP ceramic materials have severely obstructed their clinical applications (Ducheyne, 1987). Fibres and other types of particle are generally more successful for filling bone defects and helping to repair the damaged tissue. Furthermore, they can be dispersed in polymer matrices for mechanical reinforcement purposes and for improved biocompatibility, as described in Chapter 4.

CaP fibres have been produced through several routes including spinning, extrusion, precipitation, sol-gel and pyrolysis (Ramanan and Venkatesh, 2004; Tanahashi et al., 1992; Nakahira et al., 1999; Kolos et al., 2003; Ota et al., 1998). Electrospinning has previously been used to produce many different types of ceramic fibres (Sigmund et al., 2006; Ramaseshan et al., 2007), however, little work has been carried out to produce calcium phosphate fibres. As mentioned in Chapter 2, Section 2.5.5.2, the existing approach uses an electrospinning solution mixing polymer and precursors, and a further calcination step is performed on the sample after electrospinning. An unexplored approach is the combination of electrospinning and sintering. Indeed, the composite fibres containing hydroxyapatite nanoparticles, presented in Chapter 4, suggest the possibility of creating ceramic fibres through the simultaneous decomposition of the polymer matrix and sin-

tering of the nanoparticles.

Sintering of pure HA particles is usually reported to occur above 1000°C. However, the choice of the sintering temperature is important as it has an effect on the properties of the resulting sample. Most investigators agree that pure HA (ratio CaP = 1.67) is stable in an air and argon atmosphere at temperatures up to 1200°C (Ye et al., 2009; Zhou et al., 1993; Muralithran and Ramesh, 2000). However, decomposition of HA at temperatures as low as 800°C has been observed for calcium deficient hydroxyapatites samples (DHA) (Zhou et al., 1993).

7.2 Objectives and methodology

This chapter investigates the possibility of applying a thermal treatment to the previously manufactured PLGA-nHAp composite electrospun fibres in order to obtain CaP fibres. In Chapter 4, we demonstrated that up to 50% (w/v) of nHAp could be incorporated into a fibrous PLGA matrix. The high concentration of nHAp present within the solidified fibres suggests that a heat treatment at sintering temperatures could be used to create aggregations of the nanoparticles and lead to the production of ceramic fibres. The polymer matrix would undergo thermal decomposition and therefore disappear at such temperature.

This is an original approach to create hydroxyapatite fibres. Instead of using of chemi-

cal precursors, hydroxyapatite is directly incorporated as a powder in the polymer solution and, therefore, is already present before the heat treatment. In the context of the creation of multiphasic scaffold for osteochondral repair, producing a HA fibrous scaffolds is a very promising approach as it can enable the creation of a biphasic scaffold with a ceramic fibrous part for bone repair, together with a polymer-ceramic composite fibrous part for cartilage repair.

7.3 Results and discussions

7.3.1 Morphology of HA fibres

Figure 7.1 indicates the morphology of fibres electrospun from a PLGA solution containing 50% nHAp (left side), and the same fibres after the treatment at 1100°C (right side). Interestingly, sintered fibres, having similar diameters to those observed in the untreated PLGA-nHAp scaffold, were observed after the thermal step. However, a close look at the morphology of the two samples (Figures 7.1(e) and 7.1(f)) reveals that sintered fibres exhibit smoother surface with no evidence of nanoparticles by opposition to PLGA-nHAp scaffold. These observations suggest that sintering of the nanoparticles has occurred while the polymer matrix was thermally decomposed and evaporated, leaving pure ceramic fibres behind. Moreover, the fibres seem segmented into larger aggregates. This observation is typical for the sintering process, during which the grain growth follows the Ostwald ripening process (small grains dissolve and redeposit onto larger grains). In order to assess the nature of the sintered fibres, further characterisation involving FTIR,

XPS and XRD is presented in the following sections.

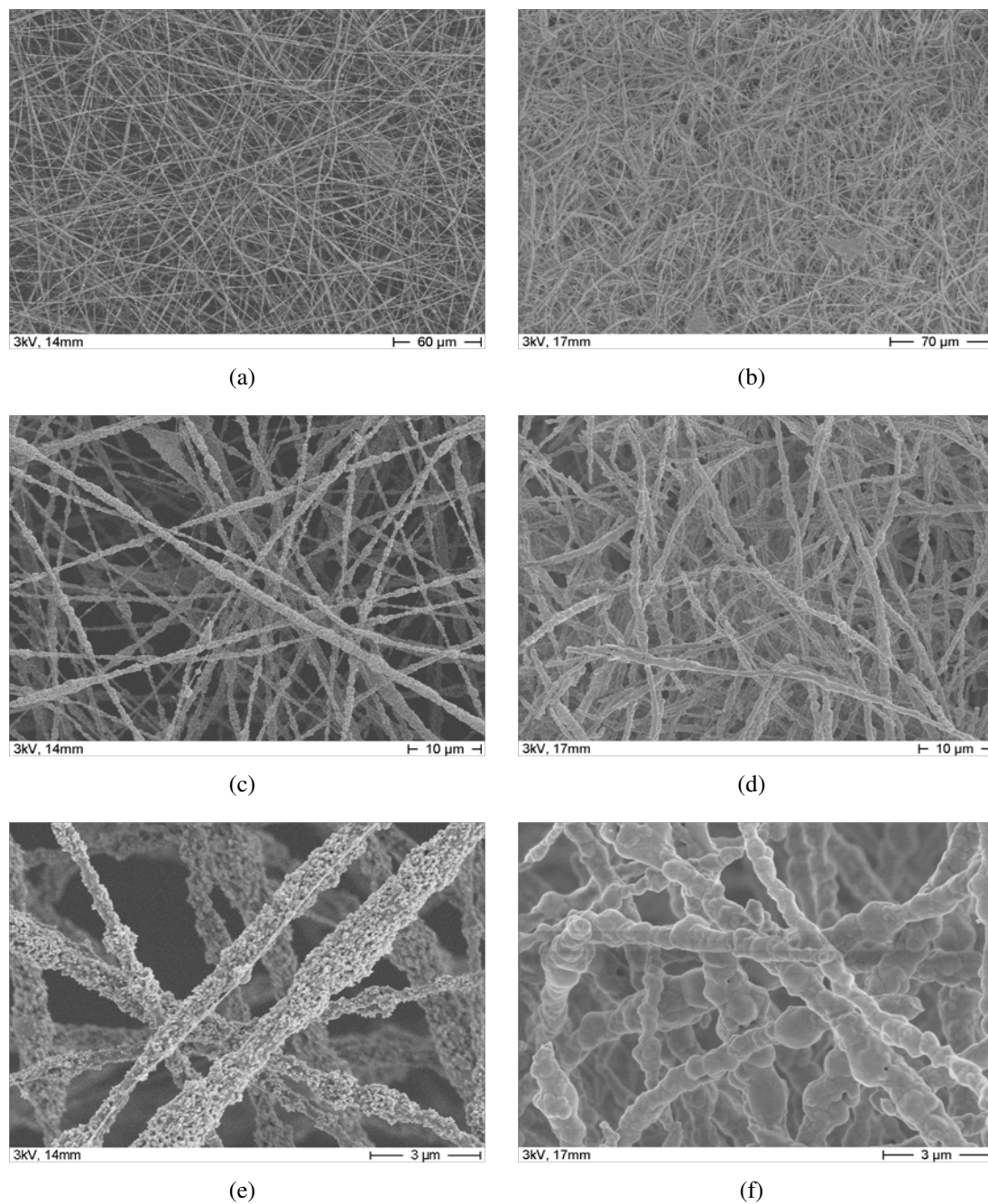


Figure 7.1: SEM images showing the morphology of fibres electrospun from a PLGA solution containing 50%nHAp (left side) and the morphology of the same fibres after the treatment at 1100°C (right side). Images are shown at different magnifications to reveal surface structures.

7.3.2 FTIR analysis

Figure 7.2 indicates the main characteristics of the FTIR spectra for the different samples. For the pure PLGA spectrum, the C-O characteristic bands in the region 1065-1280 cm^{-1} can be seen. On the other hand, the spectrum of pure HA powder reveals the characteristic peak assigned to PO_4^{-3} : ν_1 vibration mode at about 964 cm^{-1} , ν_3 vibration mode at 1031 cm^{-1} and 1091 cm^{-1} (asymmetric). The spectrum of untreated PLGA-nHAp50% displays both characteristics from PLGA and HA, as described in Chapter 4, Section 4.3.3. On the other hand, the spectrum of sintered fibres seems to have lost the characteristics of PLGA (C=O and C-O bands). However, the aspect of the curve is slightly different from the one recorded for the pure HA powder. Indeed, weak absorption can be detected at about 993, 1043 and 1067 cm^{-1} around the strong peaks caused by the ν_3 vibration mode of PO_4^{-3} (1031 cm^{-1} and 1091 cm^{-1}). Another small shoulder could be observed at about 950 cm^{-1} near the ν_1 vibration mode characteristic (964 cm^{-1}).

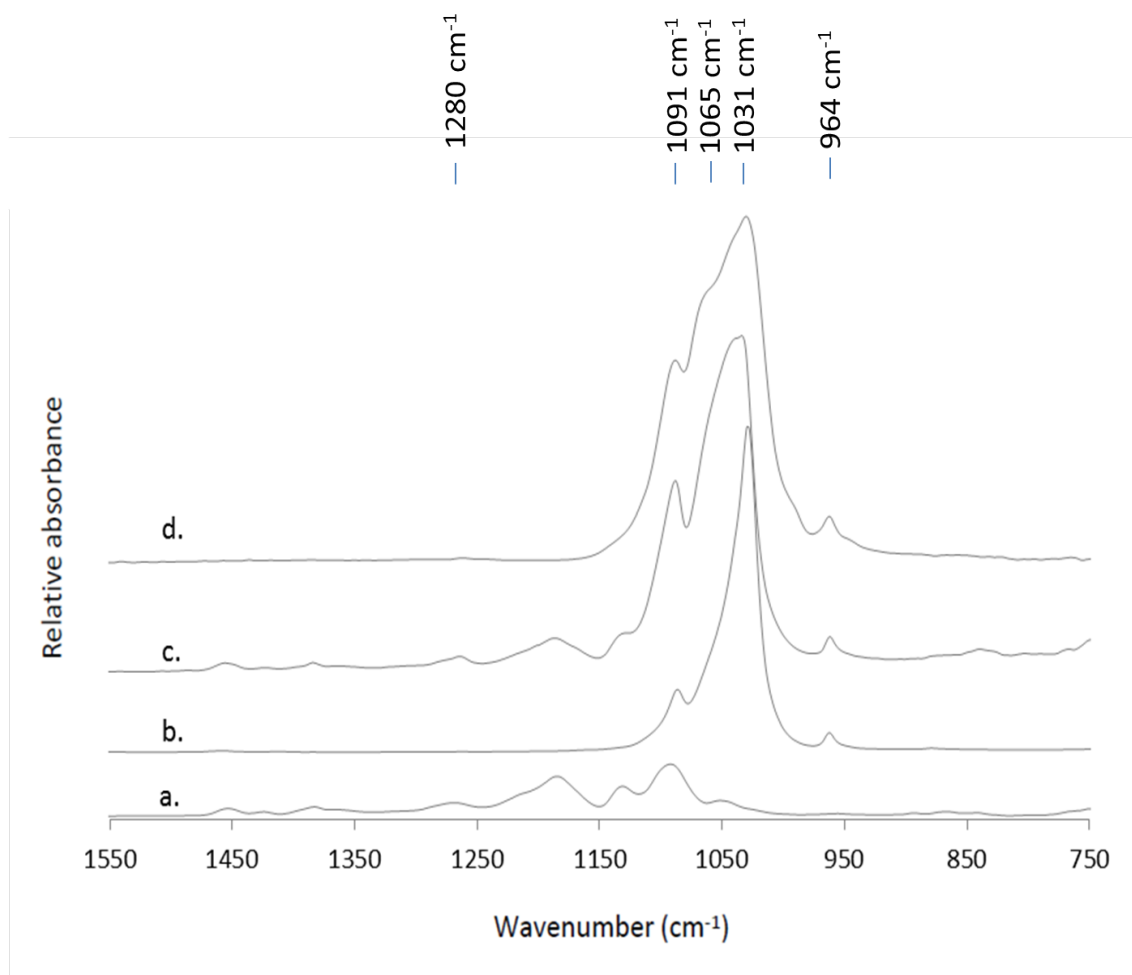


Figure 7.2: FTIR spectra of (a) PLGA fibres, (b) pure HA powder, (c) PLGA-HA fibres, and (d) sintered PLGA-HA fibres. The pure PLGA spectrum shows the C-O characteristic bands in the region 1065-1280 cm^{-1} . The spectrum of pure HA powder reveals the characteristic peak assigned to PO_4^{-3} : ν_1 vibration mode at about 964 cm^{-1} , ν_3 vibration mode at 1031 cm^{-1} and 1091 cm^{-1} (asymmetric). The spectrum of untreated PLGA-nHAp50% displays both characteristics of PLGA and HA. However the spectrum of sintered fibres shows the loss of the characteristics of PLGA (C-O bands), suggesting the succesful thermal degradation of the polymer, while the characteristics of HA (PO_4^{-3}) remain present.

7.3.3 XPS analysis

The general spectra obtained using XPS for the PLGA-HA fibres, HA powder, and sintered fibres are presented in Figures 7.3, 7.4, and 7.5 respectively. The experimental binding energies of the O 1s, C 1s, P 2p, and Ca 2p electrons and proportions of the corresponding atoms are indicated in Tables 7.1, 7.2, and 7.3. In PLGA-HA membranes, C 1s was found at 284.4 eV, O 1s at 530.7 eV, Ca 2p at 345.5 eV, and P 2p at 130.2 eV. Carbon was the dominant element (51%) while only 2.37% of Ca and 2.95% of P were detected in the sample (ratio Ca/P = 1.24). In HA powder, O was found to be the dominant element with 50.87%, while Ca and P reached values as high as 13.94% and 10.13% respectively (ratio Ca/P = 1.37). Ca 2p and P 2p were found at slightly higher binding energies: 347.5 eV and 133.3 eV, respectively. Sintered fibres showed very similar observation when compared to the pure powder in terms of binding energies and percentages of elements. Although the ratio O/C is slightly lower, 1.49 against 2.03 for the HA powder, O was still the major element with 46.41%. Ca and P reached values of 12.78% and 9.6%, respectively (ratio Ca/P = 1.33).

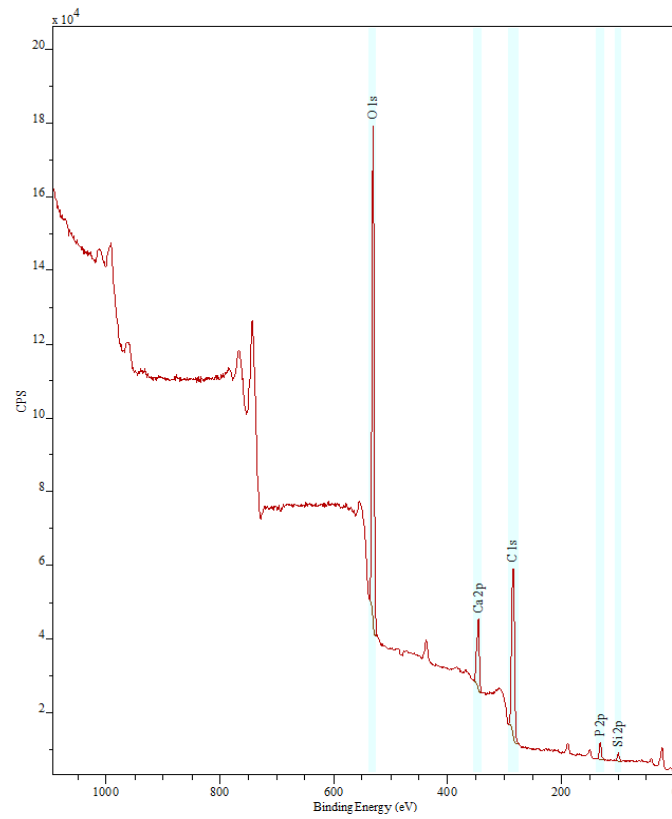


Figure 7.3: XPS spectrum obtained for PLGA-HA fibres.

Table 7.1: Experimental binding energies of the O 1s, C 1s, P 2p, and Ca 2p electrons and proportions of the corresponding elements measured in PLGA-HA fibres by XPS analysis.

Element	Binding Energy (eV)	Proportion (%)
O 1s	530.704	41.78
C 1s	284.404	51.28
P 2p	131.217	2.37
Ca 2p	345.478	2.95

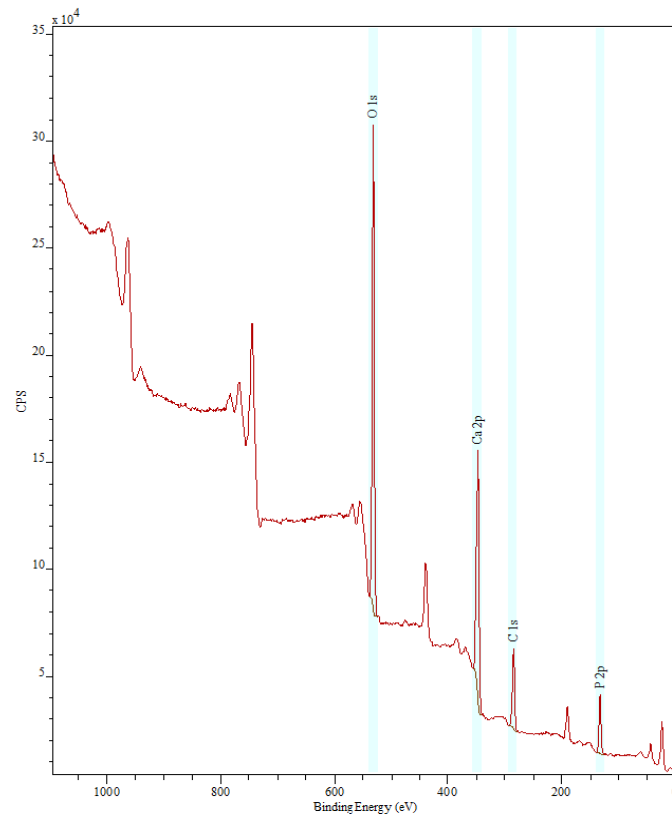


Figure 7.4: XPS spectrum obtained for HA powder.

Table 7.2: Experimental binding energies of the O 1s, C 1s, P 2p, and Ca 2p electrons and proportions of the corresponding elements measured in HA powder by XPS analysis.

Element	Binding Energy (eV)	Proportion (%)
O 1s	531.744	50.87
C 1s	284.442	25.07
P 2p	133.258	10.13
Ca 2p	347.519	13.94

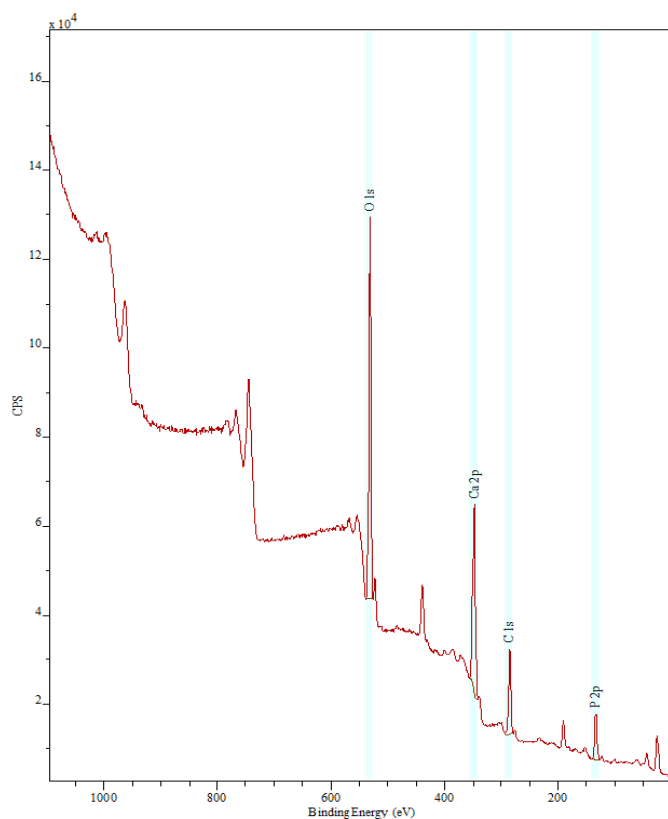


Figure 7.5: XPS spectrum obtained for sintered fibres.

Table 7.3: Experimental binding energies of the O 1s, C 1s, P 2p, and Ca 2p electrons and proportions of the corresponding elements measured in sintered fibres by XPS analysis.

Element	Binding Energy (eV)	Proportion (%)
O 1s	531.485	31.21
C 1s	285.185	46.41
P 2p	133	9.60
Ca 2p	347.261	12.78

7.3.4 XRD analysis

Finally, Figures 7.6 7.7 and 7.8 depict the x-ray diffraction (XRD) patterns for the same set of samples. The chemical identification was performed by comparing the experimental X-ray patterns to standards compiled by the International Centre for Diffraction Data (ICDD). The analysis show that the diffractogram obtained for the PLGA-HA sample and HA powder identified synthetic hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) with a probability above 99% for both. The pattern observed for sintered fibres identified hydroxyapatite with a probability of 81% with small amounts of whitlockite ($\text{Ca}_9(\text{Mg,Fe}^{++})(\text{PO}_4)_6(\text{PO}_3\text{OH})$) and β -TCP ($\text{Ca}_3(\text{PO}_4)_2$) with probabilities of 14% and 11% respectively.

7.4 Discussions

FTIR analysis has confirmed that the polymer was burnt out and evaporated during the thermal treatment since no characteristic of PLGA was observed in the spectrum of sintered fibres. Moreover, no band corresponding to carbonates was observed in the range $1400\text{-}1550\text{cm}^{-1}$, which suggests that carbonate ions were only present in trace quantities. This may be due to the volatile nature of the carbonate group. Also, the emerged peaks from the splitting of the ν_1 and ν_3 vibration modes are characteristic of tricalcium phosphate (Jillavenkatesa and Condrate Sr., 1998). This suggests that the decomposition of HA started during the heat treatment. Moreover, consultation of the literature indicates that the spectra observed for sintered fibres is typical for a mixture of HA and

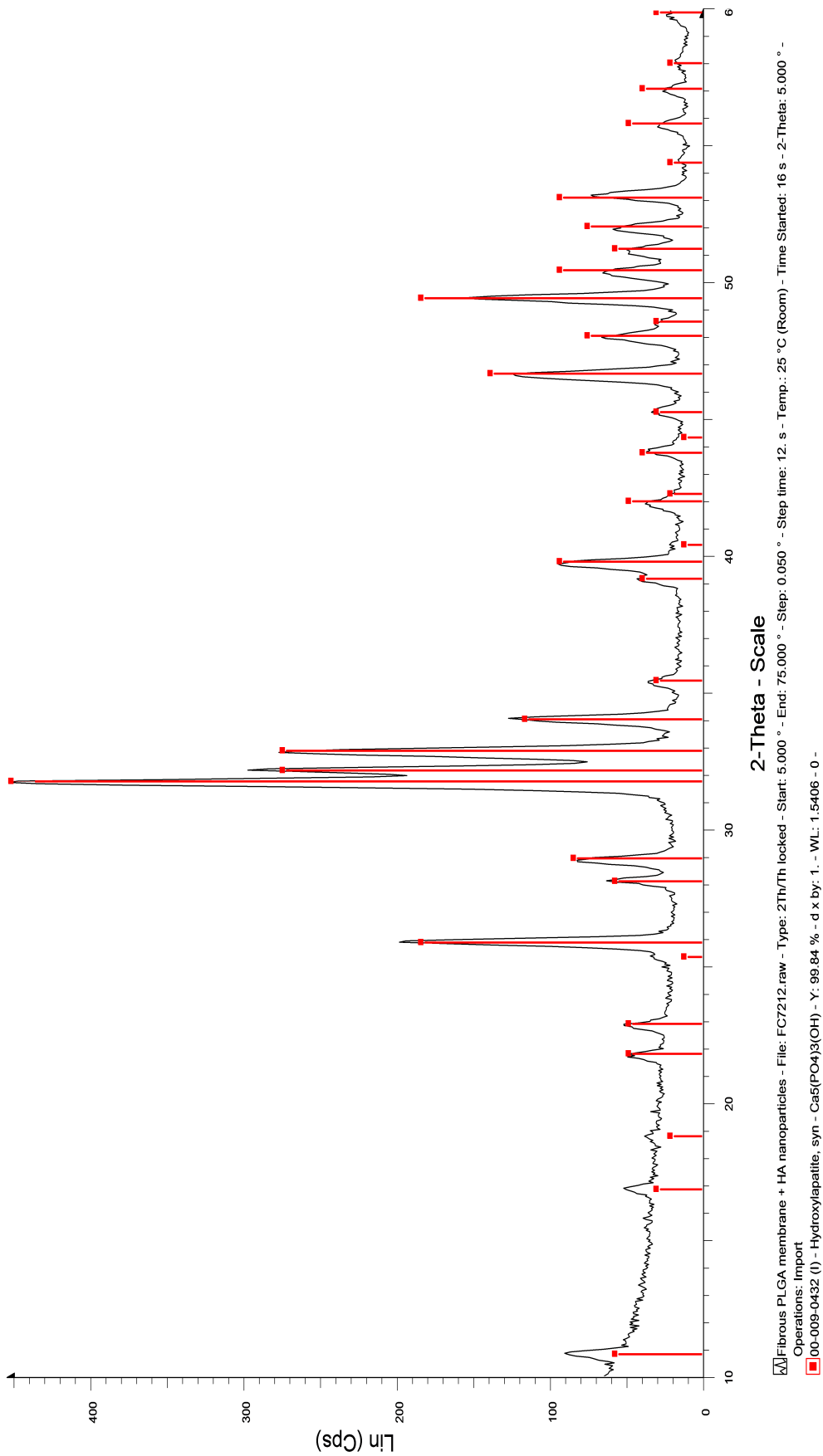


Figure 7.6: XRD patterns obtained for PLGA-HA fibres. Hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, $p=99.84\%$) was identified at significant levels.

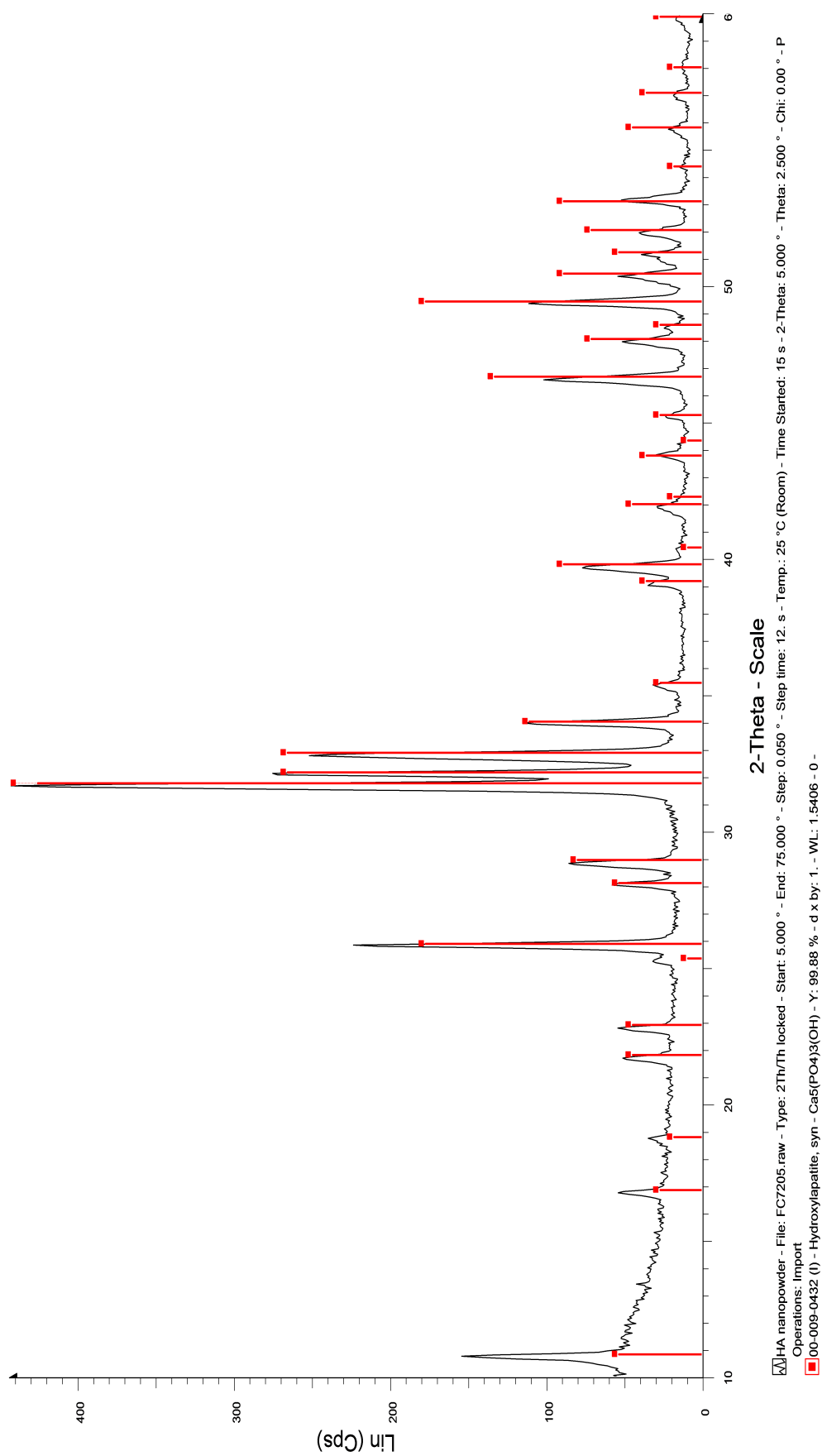


Figure 7.7: XRD patterns obtained for HA powder. Hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, $p=99.88\%$) was identified at significant levels.

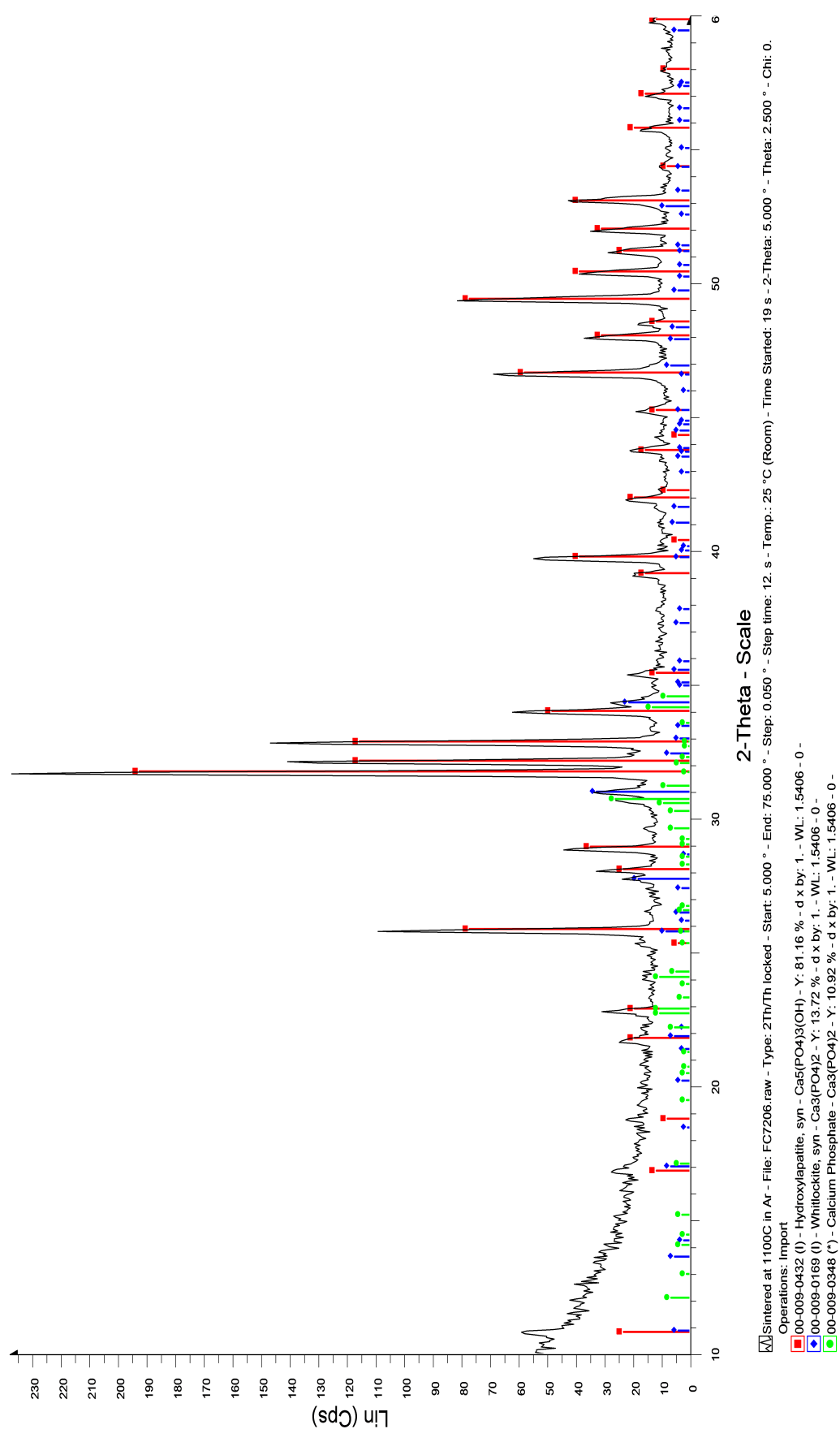
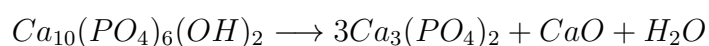


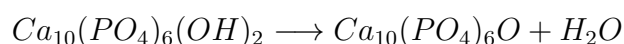
Figure 7.8: XRD patterns obtained for sintered fibres. Hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$, $p=81.16\%$), whitlockite ($\text{Ca}_3(\text{Mg,Fe++})(\text{PO}_4)_2$ (PO_3OH), $p=13.72\%$) and β -TCP ($\text{Ca}_3(\text{PO}_4)_2$, $p=10.92\%$) were identified at significant levels.

β -tricalcium phosphate (LeGeros et al., 2003; Slosarczyk et al., 1997; Lin et al., 1998). This last observation is in agreement with the outcome of the analysis of the XRD diffractograms. Indeed, although the analysis identified HA as the main phase of the sintered fibres, β -tricalcium phosphates (β -TCP) and whitlockite (a Mg-containing β -TCP) were also identified. On the other hand pure synthetic HA was the only phase in both HA powder and the inorganic part of PLGA-HA fibres. Many interesting observations can be made from the XPS data. First of all, the Ca/P ratio clearly indicates that the initial HA is deficient in calcium (the ratio= 1.37). This would explain why decomposition occurs before 1200°C. Indeed, as mentioned in the chapter introduction, deficient hydroxyapatites (DHA) start their decomposition at temperatures lower than pure HA (ratio= 1.67) (Zhou et al., 1993). In the literature, this decomposition is described as partial although the reason remains unknown. The commonly accepted decomposition reaction for DHA is:



A second observation made from the XPS analysis is that low calcium phosphate levels were found in the PLGA-HA sample, although the fibres were filled with a high concentration of particles. Since XPS is only recording from the surface of the material (about 10nm in depth), the weak signal suggests that the nanoparticles lying at the surface of the fibres are embedded in a thin layer of polymer. The presence of carbon observed by XPS in HA powder is probably due to contamination from the atmosphere by CO₂

and volatile fatty acids. The high specific surface area of electrospun biomaterials may also favour the adsorption of atmospheric compounds. In the sintered fibres, an additional reason for the presence of carbon might be the existence of residual atoms from the degradation-evaporation of the PLGA polymer. This would explain the higher proportion of carbon when compared to the native HA powder and why the ratio oxygen to carbon is lower. Finally, the slightly lower percentage of oxygen in sintered fibres, compared to the HA powder, may be due to the loss of OH groups (dehydroxylation) that can occur at high temperature. For pure HA, this reaction is known to occur at sintering temperatures below 1200°C with a conversion degree of 0.4 to 0.5 (Cihlar et al., 1999). A simple way to present the reaction formula for dehydroxylation would be:



However, the resulting oxyhydroxyapatite could not be detected by XRD due to the high similarity of its crystalline structures with hydroxyapatite. It could not be seen by FTIR either, since the band characteristic of -OH was observed below 400cm⁻¹ which is below the range explored in this study.

As mentioned earlier, systems comprising both HA and TCP phases have been intensively investigated and have shown potential for bone tissue engineering (Barinov, 2010; Lin et al., 1998; Zhang et al., 2009). β -TCP has exceptionally good tissue compatibility, directly bonding to regenerated bone without intermediate connective tissue, and encour-

aging faster bone regeneration and resorbability than HA. HA/TCP systems have been developed in order to regulate the resorption kinetics through varying the ratio HA/TCP. For similar reasons, the chemistry of the HA/TCP fibres is likely to induce an excellent response from bone tissue. However, the mechanical properties of the produced HA-TCP electrospun fibres are currently insufficient for use in any biological applications. Measurements on mechanical robustness remain to be conducted, but initial inspection indicated profound scaffold fragility, leading to material failure during handling and transport.

7.5 Conclusions

The presented results have shown that HA/ β -TCP fibres could be prepared by electrospinning and subsequent sintering. The polymer phase was evaporated during the heat treatment and the nanoparticles were sintered without losing the fibrous morphology of the scaffold. The initial hydroxyapatite nanoparticles were shown to be deficient in calcium. Hydroxyapatite was identified to be the main phase in the sintered scaffold along with additional tricalcium phosphates. β -TCP production resulted from dehydroxylation and decomposition of DHA that occurred at high temperature. Although the chemistry of the new fibres holds great potential for bone tissue engineering, the mechanical properties of the scaffold fell short of the required characteristics, impeding its use as a bulk material for tissue culture applications. However, one potential application is to use a modified version of these scaffolds as a coating material for other ceramic or metal implants, to increase osteoconduction and incorporation of the implant into the native bone tissue.

Chapter 8

Conclusions and Future Work

8.1 Conclusions

Articular cartilage (AC) morbidity presents a substantial burden to global and public health and continues to afflict millions of people worldwide. Among the different treatment strategies proposed for AC repair, the field of tissue engineering offers some of the most promising solutions. In particular, electrospun materials have recently been shown to have an excellent suitability to serve as tissue culture matrices. The present work demonstrates that electrospun materials also have the potential to be used for engineering tissue interfaces and, in particular, for creating osteochondral grafts. According to a throughout search in the literature, this work is the first attempt to use the electrospinning as a core technology to recapitulate an orthopedic tissue interface.

Chapter 4 describes the fabrication of a wide range of fibrous membranes containing

different proportions of PLGA, collagen and nHAp by electrospinning. The electrospinning protocols were effectively developed to provide membranes thin enough to enable the formation of layered arrangement that mimic the zonal matrix of the bone-cartilage interface. Physico-chemical characterisation was used to reveal that gradual changes in composition can cause significant gradual changes in terms of morphology, surface properties, mechanical properties and biocompatibility. The addition of collagen and nHAp to PLGA polymeric fibres improved the biocompatibility of the scaffolds. This was demonstrated by the enhanced adhesion and proliferation of stem cells and by the ability of the scaffold to deposit calcium phosphate minerals in presence of SBF. Collagen and nHAp were also found to increase dramatically the branching of electrospun fibres, resulting in stiffer scaffolds.

The preparation method of electrospun fibres offered a simple approach for the efficient incorporation of large amount of nHAp (up to 50%w/v in the initial polymer solution). This allowed for a novel way of producing pure ceramic electrospun fibres by simultaneously achieving thermal decomposition of the polymer and sintering of the nHAp during a heat treatment at 1100°C. Chemical analysis of the sintered fibres identified a main phase made of hydroxyapatite, with significant amounts of β -TCP. Biomaterials with such composition have been reported to be very desirable for bone applications (Barinov, 2010; Lin et al., 1998; Zhang et al., 2009). However, sintered samples were too fragile for direct use and further development is required to improve their mechanical properties.

The possibility of creating layered constructs was investigated in Chapter 5 by combining the electrospinning technique together with the cell sheet engineering approach. Tissue constructs were created by stacking electrospun mats seeded with cell sheets. This unique strategy was found to be very effective and straightforward for seeding cells into electrospun materials and for engineering three-dimensional tissue. Results have shown that high cell viability was maintained in such arrangements, for constructs containing three cell sheets. Culture of hMSCs in growth medium, on PLGA and PCL-based membranes, revealed that the cells retained pluripotency. Immunostaining and mRNA expression suggested that hMSCs successfully differentiated into chondrocytes when cultured in the constructs under chondrogenic conditions. It also showed that the choice of scaffold material affects the chondrocyte phenotype. Hypertrophic chondrocytes were observed in PLGA-based scaffolds, as depicted by the high expression level of Collagen X, whilst chondrocytes differentiated in the PCL-based material did not show signs of hypertrophy. Since hypertrophic chondrocytes and collagen X are typically found *in vivo* in the calcified zone of the articular cartilage (*i.e.* at the interface between the cartilage and the underlying bone), this finding suggests that PLGA and PCL-based materials could be used at different locations in the engineered construct in order to stimulate the desired chondrocyte phenotype at given coordinates. However, it would be desirable to carry out further investigations to expand on the results obtained. In particular, the results suggest that more fundamental work should be carried out to study the interaction between the cells and the supportive material. Such work should include two-dimensional hMSC cultures

onto electrospun membranes which are characterised in terms of adhesion, proliferation, cytotoxicity, and multilineage differentiation. This should allow better comparisons between the PLGA and PCL-based materials.

In a complementary section to Chapter 5 found in the Appendix, the study was enhanced and brought further towards the engineering of osteochondral graft by investigating the feasibility of combining layered electrospun materials with a ceramic block. Tight attachment between the two parts of the biphasic scaffold was achieved by electrospinning the first fibrous layer directly onto the calcium phosphate disk. Moreover, differentiation of hMSCs into chondrocytes was successfully induced in the layered polymeric part of the biphasic construct, which supports the approach.

Delivering proteins directly from electrospun fibres could also help in the successful outcome of osteochondral grafts. Indeed, the nature of the growth factor could be varied according to the region of the scaffold to stimulate multi-differentiation in the same construct (*e.g.* cartilage and bone). To investigate this aspect, a protocol was developed to study the release of BSA from coaxial electrospun fibres (Chapter 6). The presence of calcium phosphate nanoparticles in the polymer matrix did not play a significant role in the release profile of BSA. However, minerals deposited onto the fibres from simulated body fluids significantly affected the initial burst release, suggesting CaP coatings can be used to control the release of bioactive molecules. Our investigation also show that the PLGA-col sheath induced a slower release of BSA compared to gelatin, highlighting

the importance of the composition of the sheath layer on the release rate of a therapeutic agent. Following the successful release of BSA from co-axial electrospun fibres, a similar approach was used to investigate the release dynamics of TGF- β 3, a growth factor required for the differentiation of stem cells into chondrocytes. The TGF- β 3 release profile exhibited a higher initial burst release compared to BSA, which was most likely related to the smaller molecular size of the growth factor and its higher diffusion rate. Pilot studies using TGF- β 3-loaded membranes to differentiate hMSCs into chondrocytes suggested that protein activity was negatively impacted at some point during the experiment, since the levels of collagen II mRNA were significantly lower in comparison with control where TGF- β 3 was supplied in the culture medium. Possible causes explaining the loss of bioactivity include denaturation by the organic solvent or by the high voltage used for the electrospinning process, as well as structural degradation in the acidic environment created by the degradation of PLGA during culture. Also, it may be that the slow release of TGF- β 3 did not reach the threshold required to differentiate the cells. The loss of activity of TGF- β 3 suggests that growth factors should be incorporated into electrospun scaffolds by other means, such as through post-electrospinning surface modifications.

To conclude, although much work is still to be done in order to achieve a functional tissue graft, it seems evident that using electrospinning as a core technology for the generation of osteochondral grafts is a powerful strategy. Moreover, this approach could easily be extended to other tissue orthopedic interfaces, such as bone-ligament, bone-tendon, and bone-meniscus.

8.2 Future work

This research supports the use of electrospun materials for building tissue interfaces and suggests a way to achieve functional bone-cartilage engineered constructs. However, it does not offer a final solution for the fabrication of grafts for the repair of osteochondral defects. This section discusses possible future research directions to address some of the remaining challenges towards achieving this goal.

Many suggestions for future work concern the scaffold design. First of all, the composition of the scaffold could be revised and optimised. Inherent limitations of PLGA include problems of shrinkage and problems of cell inflammation and degradation of therapeutic agents due to the acidity of its degradation products. However, PLGA could be useful in blended materials. It has excellent mechanical properties and was found to possibly induce hypertrophy of chondrocytes. It is also worth considering the use of other natural polymers such as collagen II and proteoglycans, specifically found in hyaline cartilage. Collagen II may stimulate the chondrocyte differentiation while proteoglycans can be used to re-create the gradient of compressive moduli found in AC (see Section 2.1.2). Regarding the inorganic part of the scaffolds, improvements can be achieved by modifying the surface of hydroxyapatite nanoparticles to improve the bonding strength between nano-hydroxyapatite and degradable polymer matrix. This should create stronger composite fibres. Investigations aiming to increase the pore size should also be carried out. Indeed, it can be beneficial to encourage cell migration between different layers of tissue constructs. Different strategies to enlarge the pores of an electrospun scaffold were

presented in Section 2.5.4. Another important challenge pertaining to the electrospinning process itself, is to achieve a better control over the fibre orientation. Enhanced control would enable the manufacture of scaffolds with preferential fibre orientation as it can be found in the extracellular matrix of a particular AC region (see Section 2.1.2).

With regard to the characterisation of composite fibres, further mechanical testing to assess the compressive moduli of the membranes should be carried out. Also further biological characterisation could be undertaken to study the cytotoxicity of nHAp and of the degradation products of the polymers used. Studying the degradation behaviour of the electrospun scaffolds should also be considered.

A substantial amount of work remains concerning the incorporation of therapeutic agents into the electrospun fibres. In particular, the possibility of creating better core-sheath structures needs to be investigated. This may require reconsideration of the nozzle design and the parameters in use, such as the flow rate and the choice of solvent. Also, other methods of encapsulation, such as the use of emulsions, could be considered. Approaches using emulsions have the advantage of using single channel nozzle, but it remains challenging to keep an emulsion stable during the electrospinning process. Post-electrospinning treatments, such as coating the fibres with the growth factors by adsorption, should also be considered as they may provide better preservation of the protein bioactivity. In order to ensure that the core material has been entirely covered by a sheath, better tools of characterisation may be necessary. These could include techniques such as

transmission electron microscopy (TEM) or confocal microscopy (allowing 3D imaging of the fibres with high resolution). Studies of how drug release profiles are affected by varying electrospinning parameters will help to further minimise burst release. For instance, a thicker sheath will most probably slow down the drug release. With regard to the conservation of the biomolecule activity, further work should look at the effect of the solvent, the voltage and the degradation products from the scaffold. This should involve tools that are able to characterise the changes in the protein structure such as circular dichroism, fluorescence spectroscopy and FTIR. This will help to postulate and select better formulations for scaffold materials which may also include drug carrier and stabiliser.

Another part of the project which is worth examining in more depth concerns the CaP electrospun fibres. This material could indeed be very useful for bone tissue engineering applications, given its mechanical properties can be improved. To achieve this, various strategies can be considered. In some preliminary work that was not presented in this thesis, we investigated the use of carbon nanotubes (CNT's) to reinforce the CaP fibres: up to 10% CNTs were incorporated into the core of PLGA-HA fibres before sintering. Although slight improvements were observed, mechanical properties of the final sintered fibres were still insufficient for TE applications. Other approaches may involve metallic precursor to reinforce the fibres with metal oxides. Alternatively, a thin metallic layer could be deposited onto the CaP fibres. The mechanical properties of the ceramic scaffolds should be investigated using techniques such as, but not limited to, indentation

analysis.

A number of improvements to the tissue constructs arise from the cell culture investigations carried out. Besides modifying scaffold pore size and the choice of materials, increasing the cell density between the fibrous layers should produce better tissue constructs. This can be done by inserting two or more cell sheets between each successive electrospun mat. Other ways to obtaining cell sheets could also be considered, since the technology using thermoresponsive plates remains expensive. An interesting research question is how the number of layers (electrospun mats seeded with cell sheets) affects the viability of cells in the multilayered arrangement. Further work carried out with the biphasic construct could quantify the effect of the CaP disk on the differentiation of hMSCs into chondrocytes. Also, characterisation of the physical and chemical bond between the two materials (polymer and ceramic) could be investigated. Improvements of the interface between polymer and ceramic disk might be achieved through better penetration of the fibres within the CaP disk and through CaP deposition from SBF.

The tissue engineering experiments presented in this report were performed with homogenous layered scaffolds. It is suggested that, once an optimal arrangement is found, investigations of layered construct with a gradient of properties should be envisaged. In particular, PLGA and PCL-based materials could be used at different locations in the engineered construct in order to stimulate the desired chondrocyte phenotype at given coordinates. Furthermore, culture of the three-dimensional arrangement should be con-

ducted in a perfusion bioreactor, rather than in a static environment. Applying mechanical loading during differentiation of the construct will also be an interesting consideration, in order to improve the mechanical and tissue quality of the engineered graft (see Chapter 2, Section 2.3.3). Eventually, the suggestions presented in this section may lead to a functional engineered osteochondral graft that can be taken to pre-clinical trials to assess the true potential of electrospinning as a core technology in AC repair.

Bibliography

- Abramoff, M., P. Magelhaes, and S. Ram (2004). Image processing with ImageJ. *Bio-photonics International* 11, 36–42.
- Alavi, A. and D. G. Stupack (2007). Cell survival in a three-dimensional matrix. *Methods in Enzymology* 426, 85–101.
- Alford, J. W. and B. J. Cole (2005a). Cartilage restoration, part 1: Basic science, historical perspective, patient evaluation, and treatment options. *The American Journal of Sports Medicine* 33, 295–306.
- Alford, J. W. and B. J. Cole (2005b). Cartilage restoration, part 2: Techniques, outcomes, and future directions. *The American Journal of Sports Medicine* 33, 443–460.
- Angeles, M., H.-L. Cheng, and S. S. Velankar (2008). Emulsion electrospinning: composite fibers from drop breakup during electrospinning. *Polymers for Advanced Technologies* 19, 728–733.
- Arthritis and Musculoskeletal Alliance (Ed.) (2004). *Standards of care for people with osteoarthritis*.
- Arthritis Research UK (Ed.) (2008). *Arthritis in the UK - key facts*.
- Ashammakhi, N., A. Ndreu, A. Piras, L. Nikkola, T. Sindelar, H. Ylikauppila, A. Harlin, E. Chiellini, V. Hasirci, and H. Redl (2007). Biodegradable nanomats produced by electrospinning: expanding multifunctionality and potential for tissue engineering. *Journal of Nanoscience and Nanotechnology* 6, 2693–2711.
- Barinov, S. M. (2010). Calcium phosphate-based ceramic and composite materials for medicine. *Russian Chemical Reviews* 79, 13–29.
- Benjamins, J., A. Cagna, and E. Lucassen-Reynders (1996). Viscoelastic properties of triacylglycerol/water interfaces covered by proteins. *Colloid and Surfaces* 114, 245–254.

- Bianco, P. and P. G. Robey (2001). Stem cells in tissue engineering. *Nature* 414, 118–121.
- Bognitzki, M., W. Czado, T. Frese, A. Schaper, M. Hellwig, M. Steinhart, A. Greiner, and J. H. Wendorff (2001). Nanostructured fibers via electrospinning. *Advanced Materials* 13, 70–72.
- Bose, S. and S. Tarafder (2011). Calcium phosphate ceramic systems in growth factor and drug delivery for bone tissue engineering - a review. *Acta Biomaterialia*. In Press, Accepted Manuscript.
- Buckwalter, J. A. and H. J. Mankin (1998). Articular cartilage: tissue design and chondrocyte-matrix interactions. *Instructional Course Lectures* 47, 477–486.
- Caplan, A. I. (1991). Mesenchymal stem cells. *Journal of Orthopaedic Research* 9, 641–650.
- Caplan, A. I. (2007). Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *Journal of Cellular Physiology* 213, 341–347.
- Chang, C. H., F. H. Lin, C. C. Lin, C. H. Chou, and H. C. Liu (2004a). Cartilage tissue engineering on the surface of a novel gelatin-calcium-phosphate biphasic scaffold in a double chamber bioreactor. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 15, 313–321.
- Chang, C.-H., F.-H. Lin, C.-C. Lin, C.-H. Chou, and H.-C. Liu (2004b). Cartilage tissue engineering on the surface of a novel gelatin-calcium-phosphate biphasic scaffold in a double-chamber bioreactor. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 71, 313–321.
- Chen, G., T. Sato, J. Tanaka, and T. Tateishi (2006). Preparation of a biphasic scaffold for osteochondral tissue engineering. *Materials Science and Engineering C* 26, 118–123.
- Chen, J., B. Chu, and B. S. Hsiao (2006). Mineralization of hydroxyapatite in electrospun nanofibrous poly(l-lactic acid) scaffolds. *Journal of Biomedical Materials Research - Part A* 79, 307–317.
- Choi, H. W., J. K. Johnson, J. Nam, and D. F. Farson (2007). Structuring electrospun polycaprolactone nanofiber tissue scaffolds by femtosecond laser ablation. *Journal of Laser Applications* 19, 225–231.

- Choi, J. S., S. J. Lee, G. J. Christ, A. Atala, and J. J. Yoo (2008). The influence of electrospun aligned poly(ϵ -caprolactone)/collagen nanofiber meshes on the formation of self-aligned skeletal muscle myotubes. *Biomaterials* 29, 2899–2906.
- Christopherson, G. T., H. Song, and H.-Q. Mao (2009). The influence of fiber diameter of electrospun substrates on neural stem cell differentiation and proliferation. *Biomaterials* 30, 556–564.
- Cihlar, J., A. Buchal, and M. Trunec (1999). Kinetics of thermal decomposition of hydroxyapatite bioceramics. *Journal of Materials Science* 34, 6121–6131.
- Cohen, D. M. and C. S. Chen (2008). Mechanical control of stem cell differentiation. In T. S. C. R. Community (Ed.), *StemBook*, pp. 1–16.
- Cui, W., X. Li, X. Zhu, G. Yu, S. Zhou, and J. Weng (2006). Investigation of drug release and matrix degradation of electrospun poly(dl-lactide) fibers with paracetamol inoculation. *Biomacromolecules* 7, 1623–1629.
- Currey, J. (Ed.) (2002). *Bones: Structure and Mechanics*. Princeton University Press.
- Davisson, T., S. Kunig, A. Chen, R. Sah, and A. Ratcliffe (2002). Static and dynamic compression modulate matrix metabolism in tissue engineered cartilage. *Journal of Orthopaedic Research* 20, 842–848.
- Deans, R. J. and A. B. Moseley (2000). Mesenchymal stem cells: biology and potential clinical uses. *Experimental Hematology* 28, 875–884.
- Dominici, M., I. Mueller, I. Slaper-Cortenbach, D. Krause, A. Keating, D. Prockop, and E. Horwitz (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. the international society for cellular therapy position statement. *Cytotherapy* 8, 315–317.
- Doshi, J. and D. H. Reneker (1995). Electrospinning process and applications of electrospun fibers. *Journal of Electrostatics* 35, 151–160.
- Driessens, F. C. M. and R. M. H. Verbeeck (Eds.) (1990). *Biominerals*. CRC Press, Florida.
- Ducheyne, P. (1987). Bioceramics: material characteristics versus in vivo behavior. *Journal of Biomedical Materials Research* 21, 219–236.

- El-Sherbini, Y. (2011). *Genetic modification of human mesenchymal stem cells using HSV-1 amplicons to improve their therapeutic potential*. Ph. D. thesis, University of Oxford, Department of Clinical Pharmacology.
- Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher (2006). Matrix elasticity directs stem cell lineage specification. *Cell* 126, 677–689.
- Estey, T., J. Kang, S. P. Schwendeman, and J. F. Carpenter (2006). BSA degradation under acidic conditions: a model for protein instability during release from PLGA delivery systems. *Journal of Pharmaceutical Sciences* 95, 1626–1639.
- Even-Ram, S., V. Artym, and K. M. Yamada (2006). Matrix control of stem cell fate. *Cell* 126, 645–647.
- Fouillard, L., A. Chapel, D. Bories, S. Bouchet, J. M. Costa, H. Rouard, P. Herve, P. Gourmelon, D. Thierry, M. Lopez, and N. C. Gorin (2007). Infusion of allogeneic-related HLA mismatched mesenchymal stem cells for the treatment of incomplete engraftment following autologous haematopoietic stem cell transplantation. *Leukemia* 21, 568–570.
- Frenot, A. and I. S. Chronakis (2003). Polymer nanofibers assembled by electrospinning. *Current Opinion in Colloids and Interface Science* 8, 64–75.
- Gooch, K. and T. C. J. (1997). Chondrocytes. In T. C. Gooch KJ (Ed.), *Mechanical Forces: Their effects on cells and tissues*, pp. 79–100. Landes Bioscience.
- Grafahrend, D., J. L. Calvet, K. Klinkhammer, J. Salber, P. D. Dalton, M. Moller, and D. Klee (2008). Control of protein adsorption on functionalized electrospun fibers. *Biotechnology and Bioengineering* 101, 609–621.
- Grassel, S. and N. Ahmed (2007). Influence of cellular microenvironment and paracrine signals on chondrogenic differentiation. *Frontiers in Bioscience* 12, 4946–4956.
- Guelcher, S. A. and J. O. Hollinger (Eds.) (2005). *An introduction to biomaterials*. Taylor and Francis Group.
- Guo, X., H. Park, G. Liu, W. Liu, Y. Cao, Y. Tabata, F. K. Kasper, and A. G. Mikos (2009). In vitro generation of an osteochondral construct using injectable hydrogel composites encapsulating rabbit marrow mesenchymal stem cells. *Biomaterials*, 1–12.

- Gupta, D. Venugopal, J. M. S. G. V. R. R. S. (2009). Nanostructured biocomposite substrates by electrospinning and electrospraying for the mineralization of osteoblasts. *Biomaterials* 30, 2085–2094.
- Habraken, W., J. Wolke, and J. Jansen (2007). Ceramic composites as matrices and scaffolds for drug delivery in tissue engineering. *Advanced Drug Delivery Reviews* 59, 234–248.
- Han, T., D. H. Reneker, and A. L. Yarin (2007). Buckling of jets in electrospinning. *Polymer* 48, 6064–6076.
- Harley, B. A., A. K. Lynn, Z. Wissner-Gross, W. Bonfield, I. V. Yannas, and L. J. Gibson (2009). Design of a multiphase osteochondral scaffold iii: Fabrication of layered scaffolds with continuous interfaces. *Journal of Biomedical Materials Research Part A*. Published online.
- Hellman, K. (1995). Biomedical applications of tissue engineering technology: Regulatory issues. *Tissue Engineering* 1, 203–210.
- Holland, T. A., E. W. H. Bodde, L. S. Baggett, Y. Tabata, and J. A. Mikos, A. G. and Jansen (2005). Osteochondral repair in the rabbit model utilizing bilayered, degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds. *Journal of Biomedical Materials Research Part A* 75, 156–167.
- Houchin, M. L. and E. M. Topp (2009). Chemical degradation of peptides and proteins in PLGA: a review of reactions and mechanisms. *Journal of Pharmaceutical Sciences* 97, 2395–2404.
- Hu, J. C. Y. and A. K. A. (2003). Structure and function of articular cartilage. In Y. H. An and K. L. Martin (Eds.), *Handbook of Histology Methods for Bone and Cartilage*, pp. 73–95. Humana Press.
- Huang, C. Y., A. Stankiewicz, G. A. Ateshian, and V. C. Mow (2005). Anisotropy, inhomogeneity, and tension–compression nonlinearity of human glenohumeral cartilage in finite deformation. *Journal of Biomechanics* 38, 799–809.
- Huang, Z. M., Y. Z. Zhang, M. Kotaki, and S. Ramakrishna (2003). A review on polymer nanofibers by electrospinning and their applications in nanocomposites. *Composites Science and Technology* 63, 2223–2253.

- Hui, J., H. W. Ouyang, D. W. Hutmacher, J. C. Goh, and E. H. Lee (2005). Mesenchymal stem cells in musculoskeletal tissue engineering: a review of recent advances in National University of Singapore. *Annals Academy of Medicine Singapore* 34, 206–212.
- Hutmacher, D. W. (2000). Scaffolds in tissue engineering bone and cartilage. *Biomaterials* 21, 2529–2543.
- Ito, Y., N. Adachi, A. Nakamae, S. Yanada, and M. Ochi (2008). Transplantation of tissue-engineered osteochondral plug using cultured chondrocytes and interconnected porous calcium hydroxyapatite ceramic cylindrical plugs to treat osteochondral defects in a rabbit model. *Artificial Organs* 32, 36–44.
- Iwasa, J., L. Engebretsen, Y. Shima, and M. Ochi (2009). Clinical application of scaffolds for cartilage tissue engineering. *Knee Surgery, Sports Traumatology, Arthroscopy* 17, 561–577.
- Jang, J.-H., T. L. Houchin, and L. D. Shea (2004). Gene delivery from polymer scaffolds for tissue engineering. *Expert review. Medical devices* 1, 127–138.
- Janjanin, S., W.-J. Li, M. T. Morgan, R. M. Shanti, and R. S. Tuan (2008). Mold-shaped, nanofiber scaffold-based cartilage engineering using human mesenchymal stem cells and bioreactor. *Journal of Surgical Research* 149, 47–56.
- Jeong, S. I., S. Y. Kim, S. K. Cho, M. S. Chong, K. S. Kim, H. Kim, S. B. Lee, and Y. M. Lee (2007). Tissue-engineered vascular grafts composed of marine collagen and PLGA fibers using pulsatile perfusion bioreactors. *Biomaterials* 28, 1115–1122.
- Jeong, W. K., S. H. Oh, J. H. Lee, and G. I. Im (2008). Repair of osteochondral defects with a construct of mesenchymal stem cells and a polydioxanone/poly(vinyl alcohol) scaffold. *Biotechnololy and Applied Biochemistry* 49, 155–164.
- Ji, W., Y. Sun, F. Yang, J. J. J. P. van den Beucken, M. Fan, Z. Chen, and J. A. Jansen (2010). Bioactive electrospun scaffolds delivering growth factors and genes for tissue engineering applications. *Expert review. Pharmaceutical Research*.
- Ji, W., F. Yang, J. P. vandenBeucken, Z. Bian, M. Fan, Z. Chen, and J. A. Jansen (2010). Fibrous scaffolds loaded with protein prepared by blend or coaxial electrospinning. *Acta Biomaterialia* 6, 4199–4207.
- Jiang, C.-C., H. Chiang, C.-J. Liao, Y.-J. Lin, T.-F. Kuo, C.-S. Shieh, Y. Y. Huang, and R. S. Tuan (2007). Repair of porcine articular cartilage defect with a biphasic osteochondral composite. *Journal of Orthopaedic Research*, 1277–1290.

- Jiang, X. S., C. Chai, Y. Zhang, R. X. Zhuo, H. Q. Mao, and K. W. Leong (2006). Surface-immobilization of adhesion peptides on substrate for ex vivo expansion of cryopreserved umbilical cord blood CD34+ cells. *Biomaterials* 27, 2723–2732.
- Jillavenkatesa, A. and R. A. Condrate Sr. (1998). The infrared and raman spectra of β - and α -tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$). *Spectroscopy Letters* 31, 1561–1570.
- Jin, Z., Z. YuPing, Z. Wei, Y. XiaoYan, and Y. KangDe (2009). Preparation and mineralization of PLGA/Gt electrospun fiber mats. *Chinese Science Bulletin* 54, 1328–1333.
- Jose, M. V., V. Thomas, D. R. Dean, and E. Nyairo (2009). Fabrication and characterization of aligned nanofibrous PLGA/Collagen blends as bone tissue scaffolds. *Polymer* 50, 3778–3785.
- Keating, A., L. Berkahn, and R. Filshie (1998). A phase I study of the transplantation of genetically marked autologous bone marrow stromal cells. *Human Gene Therapy* 9, 591–600.
- Kelly, S. M. and N. C. Price (2000). The use of circular dichroism in the investigation of protein structure and function. *Current Protein and Peptide Science* 1, 349–384.
- Kim, H. J., J. H. Lee, and G. I. Im (2008). Chondrogenesis using mesenchymal stem cells and PCL scaffolds. *Journal of Biomedical Materials Research Part A* 92A, 659–666.
- Kim, H. W. and H. E. Kim (2006). Nanofiber generation of hydroxyapatite and fluorohydroxyapatite bioceramics. *Journal of Colloid and Interface Science* 77, 323–328.
- Kim, T. G., H. J. Chung, and T. G. Park (2008). Macroporous and nanofibrous hyaluronic acid/collagen hybrid scaffold fabricated by concurrent electrospinning and deposition/leaching of salt particles. *Acta Biomaterialia* 4, 1611–1619.
- Kim, T. K., B. Sharma, C. G. Williams, M. A. Ruffner, A. Malik, E. G. McFarland, and J. H. Elisseeff (2003). Experimental model for cartilage tissue engineering to regenerate the zonal organization of articular cartilage. *OsteoArthritis and Cartilage* 11, 653–664.
- Klein, C. P. A. T., J. M. A. de Bleeck-Hogervorst, J. G. C. Wolke, and K. de Groot (1990). Studies of the solubility of different calcium phosphate ceramic particles in vitro. *Biomaterials* 11, 509–512.
- Ko, H.C.H., B. K. M. and C. D. McFarland (2007). Engineering thick tissues - the vascularisation problem. *European Cells and Materials* 14, 1–19.

- Kokubo, T. and H. Takadama (2006). How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 27, 2907–2915.
- Kolos, E. C., A. J. Ruys, and G. J. Roger (2003). A novel biomimetic approach for the manufacture of hydroxyapatite fibres. *Key Engineering Materials: Bioceramics* 15, 47–50.
- Kon, E., M. Delcogliano, G. Filardo, D. Pressato, M. Busacca, B. Grigolo, G. Desando, and M. Marcacci (2010). A novel nano-composite multi-layered biomaterial for treatment of osteochondral lesions: technique note and an early stability. Pilot clinical trial. *The American Journal of Sports Medicine* 41, 1180–1190.
- Korossis, S. A., F. Bolland, J. N. Kearney, J. Fisher, and E. Ingham (2005). Bioreactors in tissue engineering. In N. Ashammakhi and R. L. Reis (Eds.), *Topics in Tissue Engineering, Volume 2, E-book*, pp. 1–23. Expertissues.
- Kwarciak, A., A. Crawford, I. Brook, and P. Hatton (2007). Engineering of hypertrophic cartilage with and without poly(glycolic acid) scaffold. *European Cells and Materials* 14, 73.
- Kwon, I. K., S. Kidoaki, and T. Matsuda (2005). Electrospun nano- to microfiber fabrics made of biodegradable copolyesters: structural characteristics, mechanical properties and cell adhesion potentia. *Biomaterials* 26, 3929–3939.
- Lam, C. X. F., M. M. Savalani, S. H. Teoh, and D. W. Hutmacher (2008). Dynamics of in vitro polymer degradation of polycaprolactone-based scaffolds: accelerated versus simulated physiological conditions. *Biomedical Materials* 3, 1–15.
- Langer, R. (1992). New methods of drug delivery. *Science* 249, 1527–1533.
- Lanza, R. (Ed.) (2006). *Essentials of stem cell biology*. Elsevier Academic Press.
- Latour, R. A. (2005). Biomaterials: Protein-surface interactions. In Taylor and Francis (Eds.), *Encyclopedia of Biomaterials and Biomedical Engineering*.
- Lee, C. and Grad, S., M. Wimmer, and M. Alini (2006). The influence of mechanical stimuli on articular cartilage tissue engineering. In N. Ashammakhi and R. L. Reis (Eds.), *Topics in Tissue Engineering*, pp. 1–32.
- Lee, S.-H. and H. Shin (2007). Matrices and scaffolds for delivery of bioactive molecules in bone and cartilage tissue engineering. *Advanced Drug Delivery Reviews* 59, 339–359.

- LeGeros, R. Z., S. Lin, R. Rohanizadeh, D. Mijares, and J. P. LeGeros (2003). Biphasic calcium phosphate bioceramics: preparation, properties and applications. *Journal of Materials Science: Materials in Medicine* 14, 201–209.
- Leon, B. and J. A. Jansen (Eds.) (2009). *Thin Calcium Phosphate Coatings for Medical Implants*. Springer.
- Li, D., J. T. McCann, Y. Xia, and M. Marquez (2006). Electrospinning: A simple and versatile technique for producing ceramic nanofibers and nanotubes. *Journal of the American Ceramic Society* 89, 1861–1869.
- Li, D. and Y. Xia (2004a). Direct fabrication of composite and ceramic hollow nanofibers by electrospinning. *Nano Letters* 4, 933–938.
- Li, D. and Y. Xia (2004b). Electrospinning of nanofibers: Reinventing the wheel. *Advanced Materials* 16, 1151–1170.
- Li, M., M. Mondrinos, X. Chen, and P. I. Lekes (2005). Electrospun blends of natural and synthetic polymers as scaffolds for tissue engineering. *Proceedings of the 2005 IEEE: Engineering in Medicine and Biology 27th Annual Conference*, 5858–5861.
- Li, W., Y. J. Jiang, and R. S. Tuan (2008). Cell-nanofiber-based cartilage tissue engineering using improved cell seeding, growth factor, and bioreactor technologies. *Tissue Engineering, Part A* 14, 639–648.
- Li, W.-J., R. Tuli, X. Huang, P. Laquerriere, and R. S. Tuan (2005). Multilineage differentiation of human mesenchymal stem cells in a three-dimensional nanofibrous scaffold. *Biomaterials* 26, 5158–5166.
- Li, W.-L., R. M. Shanti, and R. S. Tuan (2006). Electrospinning technology for nanofibrous scaffolds in tissue engineering. In C. S. S. R. Kumar (Ed.), *Tissue, Cell and Organ Engineering*, Volume 9, pp. 135–187. Nanotechnologies for the Life Sciences.
- Li, X., J. Xie, J. Lipner, X. Yuan, S. Thomopoulos, and Y. Xia (2009). Nanofiber scaffolds with gradations in mineral content formimicking the tendon-to-bone insertion site. *Nano Letters* 9, 2763–2768.
- Liao, S., C. K. Chan, and S. Ramakrishna (2008). Stem cells and biomimetic materials strategies for tissue engineering. *Materials Science and Engineering C* 28, 1189–1202.

- Lim, J. Y. and H. J. Donahue (2007). Cell sensing and response to micro- and nanostructured surfaces produced by chemical and topographic patterning. *Tissue Engineering* 13, 1879–1891.
- Lin, F. H., C. J. Liao, K. S. Chen, and J. S. Sun (1998). Preparation of high-temperature stabilized beta-tricalcium phosphate by heating deficient hydroxyapatite with $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ addition. *Biomaterials* 19, 1101–1107.
- Luong, N. D., I. S. Moon, D. S. Lee, Y. K. Lee, and J. D. Nam (2008). Surface modification of poly(l-lactide) electrospun fibers with nanocrystal hydroxyapatite for engineered scaffold applications. *Materials Science and Engineering: C* 28, 1242–1249.
- Lynn, E. F., G. Allen, and J. Lieberman (Eds.) (2005). *Nanotechnology: Science, Innovation, and Opportunity*. Prentice Hall Professional Technical Reference.
- Ma, Z., M. Kotaki, R. Inai, and S. Ramakrishna (2005). Potential of nanofiber matrix as tissue-engineering scaffolds. *Tissue Engineering* 11, 101–109.
- Madurantakam, P. A., I. A. Rodriguez, C. P. Cost, R. Viswanathan, D. G. Simpson, M. J. Beckman, P. C. Moon, and G. L. Bowlin (2009). Multiple factor interactions in biomimetic mineralization of electrospun scaffolds. *Biomaterials*, 1–9.
- Martin, I., S. Miot, A. Barbero, M. Jakob, and D. Wendt (2007). Osteochondral tissue engineering. *Journal of Biomechanics* 40, 750–765.
- Martin, I., S. Miot, M. Barbero, Jakob, and D. Wendt (2007). Osteochondral tissue engineering. *Journal of Biomechanics* 40, 750–765.
- Martin, I., D. Wendt, and M. Heberer (2004). Review. the role of bioreactors in tissue engineering. *Trends in Biotechnology* 22, 80–86.
- Mason, C. and P. Dunnill (2008). A brief definition of regenerative medicine. *Regenerative medicine* 3, 1–5.
- Mavis, B., T. T. Demirtas, M. Gumusderelioglu, G. Gunduz, and U. Colak (2009). Synthesis, characterization and osteoblastic activity of polycaprolactone nanofibers coated with biomimetic calcium phosphate. *Acta Biomaterialia* 5, 3098–3111.
- Mendonça, G., D. B. S. Mendonça, L. G. P. Simoes, A. L. Araujo, E. R. Leite, W. R. Duarte, F. J. L. Aragao, and L. F. Cooper (2009). The effects of implant surface nanoscale features on osteoblast-specific gene expression. *Biomaterials* 30, 4053–4062.

- Mente, P. and J. Lewis (1994). Elastic modulus of calcified cartilage is an order of magnitude less than that of subchondral bone. *Journal of Orthopedic Research* 12, 637–647.
- Moghe, A. K. and B. S. Gupta (2008). Co-axial electrospinning for nanofiber structures: Preparation and applications. *Polymer Reviews* 48, 353–377.
- Moses, M. A., H. Brem, and R. Langer (2003). Advancing the field of drug delivery: Taking aim at cancer. *Cancer Cell* 4, 337–341.
- Mouthuy, P.-A. (2008). Electrospinning of polymer solutions: Effect of charge density on the process and the morphology of spun fibres. Master Thesis, University of Birmingham.
- Mouthuy, P.-A. and C. H. Ye (2011). Biomaterials: electrospinning. In M. Moo-Young (Ed.), *Comprehensive Biotechnology, 2nd Edition*, Volume 5, pp. 23–36. Elsevier.
- Mow, V. and M. Rosenwasser (1988). Articular cartilage: Biomechanics. In S. Woo and J. Buckwalter (Eds.), *Injury and Repair of the Musculoskeletal Soft Tissues*, pp. 427–463.
- Mow, V. C. and W. C. Hayes (Eds.) (1997). *Basic Orthopaedic Biomechanics*. Lippincott-Raven.
- Muralithran, G. and S. Ramesh (2000). The effects of sintering temperature on the properties of hydroxyapatite. *Ceramics International* 26, 221–230.
- Nagamoto, N., K. Iyama, M. Kitaoka, Y. Ninomiya, H. Yoshioka, H. Mizuta, and K. Takagi (1993). Rapid expression of collagen type X gene of non-hypertrophic chondrocytes in the grafted chick periosteum demonstrated by in situ hybridization. *Journal of Histochemistry and Cytochemistry* 41, 679–684.
- Nakahira, A., K. Sakamoto, S. Yamaguchi, M. Kaneno, S. Takeda, and M. Okazaki (1999). Novel synthesis method of hydroxyapatite whiskers by hydrolysis of alpha-tricalcium phosphate in mixtures of water and organic solvent. *Journal of American Ceramic Society* 82, 2029–2032.
- Nam, J., Y. Huang, S. Agarwal, and J. Lannutti (2007a). Improved cellular infiltration in electrospun fiber via engineered porosity. *Tissue Engineering* 13, 2249–2257.
- Nam, J., Y. Huang, S. Agarwal, and J. Lannutti (2007b). Materials selection and residual solvent retention in biodegradable electrospun fibers. *Journal of Applied Polymer Science* 3, 1547–1554.

- Nandakumar, A., L. Yang, P. Habibovic, and C. van Blitterswijk (2010). Calcium phosphate coated electrospun fiber matrices as scaffolds for bone tissue engineering. *Langmuir* 26, 7380–7387.
- National Joint Registry (Ed.) (2011). *8th Annual report*. UK.
- Nery, E. B., R. Z. LeGeros, K. L. Lynch, and K. Lee (1992). Tissue response to biphasic calcium phosphate ceramic with different ratios of HA/bTCP in periodontal osseous defects. *Journal of Periodontology* 63, 729–735.
- NHS (2008). National Institute for Health and Clinical Excellence. the use of autologous chondrocyte implantation for the treatment of cartilage defects in knee joints. *Technology Appraisal* 89 16, pp. 28.
- Nichols, H. L., N. Zhang, J. Zhang, D. Shi, S. Bhaduri, and X. Wen (2007). Coating nanothickness degradable films on nanocrystalline hydroxyapatite particles to improve the bonding strength between nanohydroxyapatite and degradable polymer matrix. *Journal of Biomedical Materials Research Part A* 82, 373–382.
- Nur-E-Kamal, A., I. Ahmed, J. Kamal, M. Schindler, and S. Meiners (2005). Three-dimensional nanofibrillar surfaces induce activation of Rac. *Biochemical and Biophysical Research Communications* 331, 428–434.
- Ochsner, M., M. Textor, V. Vogel, and M. L. Smith (2010). Dimensionality controls cytoskeleton assembly and metabolism of fibroblast cells in response to rigidity and shape. *PLoS ONE* 5, 1–12.
- Oegema, T., R. Carpenter, F. Hofmeister, and R. Thompson (1997). The interaction of the zone of calcified cartilage and subchondral bone in osteoarthritis. *Microscopy Research and Technique* 37, 324–332.
- Ohkawa, K., K. Hakyong, and L. Keunhyung (2004). Biodegradation of electrospun poly(e-caprolactone) non-woven fabrics by pure-cultured soil filamentous fungi. *Journal of Polymers and the Environment* 12, 211–218.
- Oliveira, J. M., M. T. Rodrigues, S. S. Silva, P. B. Malafaya, M. E. Gomes, C. A. Viegas, I. R. Dias, J. Azevedo, J. F. Mano, and R. L. Reis (2006). Novel hydroxyapatite/chitosan bilayered scaffold for osteochondral tissue-engineering applications: Scaffold design and its performance when seeded with goat bone marrow stromal cells. *Biomaterials* 27, 6123–6137.

- Ota, Y., T. Iwashita, T. Kasuga, and Y. Abe (1998). Novel preparation method of hydroxyapatite fibers. *Journal of the American Ceramic Society* 81, 1665–1668.
- Oyane, A., H. Kim, T. Furuya, T. Kokubo, T. Miyazaki, and T. Nakamura (2003). Preparation and assessment of revised simulated body fluids. *Journal of Biomedical Materials Research Part A* 65A, 188–195.
- Palin, E., H. Liu, and T. J. Webster (2005). Mimicking the nanofeatures of bone increases bone-forming cell adhesion and proliferation. *Nanotechnology* 16, 18280–1835.
- Peltari, K., A. Winter, E. Steck, K. Goetzke, T. Hennig, B. G. Ochs, T. Aigner, and W. Richter (2006). Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in scid mice. *Arthritis Rheumatism* 54, 3254–3266.
- Pereira, R. F., K. W. Halford, M. D. O'Hara, D. B. Leeper, B. P. Sokolov, M. D. Pollard, O. Bagasra, and D. J. Prockop (1995). Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proceedings of the National Academy of Sciences* 23, 4857–4861.
- Perin, E. C., H. F. Dohmann, R. Borojevic, S. A. Silva, A. L. Sousa, G. V. Silva, C. T. Mesquita, L. Belem, W. K. Vaughn, F. O. Rangel, J. A. Assad, A. C. Carvalho, R. V. Branco, M. I. Rossi, H. J. Dohmann, and J. T. Willerson (2004). Improved exercise capacity and ischemia 6 and 12 months after transendocardial injection of autologous bone marrow mononuclear cells for ischemic cardiomyopathy. *Circulation* 110, 213–238.
- Porter, J. R., A. Henson, and K. C. Popat (2009). Biodegradable poly(e-caprolactone) nanowires for bone tissue engineering applications. xxx, 1–9.
- Porter, J. R., T. T. Ruckh, and K. C. Popat (2009). Bone tissue engineering: A review in bone biomimetics and drug delivery strategies. *Biotechnology Progress* 25, 1539–1560.
- Portner, R., S. Nagel-Heyer, C. Goepfert, P. Adamietz, and N. M. Meenen (2005). Bioreactor design for tissue engineering. *Journal of Bioscience and Bioengineering* 100, 235–245. Review.
- Puppi, D., F. Chiellini, A. M. Piras, and E. Chiellini (2010). Polymeric materials for bone and cartilage repair. *Progress in Polymer Science* 35, 403–440.
- R., L. and J. Vacanti (1993). Tissue engineering. *Science* 260, 920–926.

- Ramakrishna, S., K. Fujihara, W.-E. Teo, T.-C. Lim, and Z. Ma (Eds.) (2005). *An introduction to Electrospinning and Nanofibers*. World Scientific.
- Ramanan, S. R. and R. Venkatesh (2004). A study of hydroxyapatite fibers prepared via sol-gel route. *Materials Letters* 58, 3320–3323.
- Ramaseshan, R., S. Sundarrajan, R. Jose, and S. Ramakrishna (2007). Nanostructured ceramics by electrospinning. *Journal of Applied Physics: Focussed Review* 102, 1–17.
- Reneker, D. H. and H. Fong (Eds.) (2005). *Polymeric Nanofibers*. ACS Symposium Series.
- Reneker, D. H. and A. L. Yarin (2008). Electrospinning jets and polymer nanofibers. *Polymer* 49, 2387–2425.
- Reneker, D. H., A. L. Yarin, H. Fong, and S. Koombhongse (2000). Bending instability of electrically charged liquid jets of polymer solutions in electrospinning. *Journal of Applied Physics* 87, 4531–4547.
- Rodrigues, M. T., M. E. Gomes, and R. L. Reis (2011). Current strategies for osteochondral regeneration: from stem cells to pre-clinical approaches. *Current Opinion in Biotechnology* 22, 1–8.
- Rouwkema, J., N. C. Rivron, and C. A. van Blitterswijk (2008). Vascularization in tissue engineering. *Trends in Biotechnology* 26, 434–441.
- Sachlos, E. and J. T. Czernuszka (2003). Making tissue engineering scaffolds work. Review: the application of solid freeform fabrication technology to the production of tissue engineering scaffolds. *European Cells and Materials* 5, 29–40.
- Sarraf, C. E. and M. Eastwood (2005). Stem cells, tissue engineering and the mechanical environment. VIII stem cells. In E. N. Ashammakhi and R. Reis. (Eds.), *Topic in Tissue Engineering*, Volume 2, pp. 1–51.
- Schaefer, D., I. Martin, P. Shastri, R. F. Padera, R. Langer, L. E. Freed, and G. Vunjak-Novakovi (2000). In vitro generation of osteochondral composites. *Biomaterials* 21, 2599–2606.
- Schinagl, R., D. Gurskis, A. Chen, and R. Sah (1997). Depth-dependent confined compression modulus of fullthickness bovine articular cartilage. *Journal of Orthopedic Research* 15, 499–506.

- Schindler, M., I. Ahmed, J. Kamal, A. Nur-E-Kamal, T. H. Grafe, H. Young Chung, and S. Meiners (2005). A synthetic nanofibrillar matrix promotes in vivo-like organization and morphogenesis for cells in culture. *Biomaterials* 26, 5624–5631.
- Schlichting, K., H. Schell, R. U. Kleemann, A. Schill, A. Weiler, G. D. Duda, and D. Epari (2008). Influence of scaffold stiffness on subchondral bone and subsequent cartilage regeneration in an ovine model of osteochondral defect healing. *The American Journal of Sports Medicine* 36, 2379–2391.
- Schnell, E., K. Klinkhammer, S. Balzer, G. Brook, D. Klee, P. Dalton, and J. Mey (2007). Guidance of glial cell migration and axonal growth on electrospun nanofibers of poly- ϵ -caprolactone and a collagen/poly- ϵ -caprolactone blend. *Biomaterials* 28, 3012–3025.
- Shapiro, I., C. Adams, V. Srinivas, and T. Freeman (2008). Chondrocyte hypertrophy and apoptosis at the cartilage-bone interface. *Bone and Osteoarthritis. Topics in Bone Biology* 4, 109–129.
- Sharma, B. and J. H. Elisseeff (2004). Engineering structurally organized cartilage and bone tissues. *Annals of Biomedical Engineering* 32, 148–159.
- Sharma, B., C. G. Williams, T. K. Kim, D. Sum, A. Malik, M. Khan, K. Leong, and J. H. Elisseeff (2007). Designing zonal organization into tissue-engineered cartilage. *Tissue Engineering* 13, 405–414.
- Sherwood, J. K., S. L. Riley, R. Palazzolo, S. C. Brown, D. C. Monkhouse, M. Coates, L. G. Griffith, L. K. Landeen, and A. Ratcliffe (2002). A three-dimensional osteochondral composite scaffold for articular cartilage repair. *Biomaterials* 23, 4739–4751.
- Shin, H. J., C. H. Lee, I. H. Cho, Y.-J. Kim, Y.-J. Lee, I. A. Kim, K.-D. Park, N. Yui, and J.-W. Shin (2006). Electrospun PLGA nanofiber scaffolds for articular cartilage reconstruction: mechanical stability, degradation and cellular responses under mechanical stimulation in vitro. *Journal of Biomaterials Science, Polymer Edition* 17, 103–119.
- Shinto, Y., A. Uchida, F. Korkusuz, N. Araki, and K. Ono (1992). Calcium hydroxyapatite ceramic used as a delivery system for antibiotics. *Journal of Bone Joint Surgery* 74, 600–604.
- Sigmund, W., J. Yuh, H. Park, V. Maneeratana, G. Pyrgiotakis, A. Daga, and J. C. Taylor, J. and Nino (2006). Processing and structure relationships in electrospinning of ceramic fiber systems. *Journal of the American Ceramic Society* 89, 395–407.

- Sill, T. J. and H. A. von Recum (2008). Electrospinning: Applications in drug delivery and tissue engineering. *Biomaterials* 29, 1989–2006.
- Slosarczyk, A., C. Paluszkiwicz, M. Gawlicki, and Z. Paszkiewicz (1997). The FTIR spectroscopy and QXRD studies of calcium phosphate based materials produced from the powder precursors with different Ca/P ratios. *Ceramics International* 23, 297–304.
- Spadaccio, C., A. Rainer, M. Trombetta, G. Vadala, M. Chello, E. Covino, V. Denaro, Y. Toyoda, and J. A. Genovese (2009). Poly-l-lactic acid/hydroxyapatite electrospun nanocomposites induce chondrogenic differentiation of human MSC. *Annals of Biomedical Engineering* 37, 1376–1389.
- Srouji, S., T. Kizhner, E. Suss-Tobi, and E. Livne (2008). 3-d nanofibrous electrospun multilayered construct is an alternative ecm mimicking scaffold. *Journal of Materials Science: Materials in Medicine* 19, 1249–1255.
- Stankus, J. J., J. Guan, K. Fujimoto, and W. R. Wagner (2006). Microintegrating smooth muscle cells into a biodegradable, elastomeric fiber matrix. *Biomaterials* 27, 735–744.
- Strem, B. M., K. C. Hicok, M. Zhu, I. Wulur, Z. Alfonso, R. E. Schreiber, J. K. Fraser, and M. H. Hedrick (2005). Multipotential differentiation of adipose tissue-derived stem cells. *Keio Journal of Medicine* 54, 132–141.
- Suominen, E., A. J. Aho, E. Vedel, I. Kangasniemi, E. Uusipaikka, and A. Yli-Urpo (1996). Subchondral bone and cartilage repair with bioactive glasses, hydroxyapatite, and hydroxyapatite-glass composite. *Journal of Biomedical Materials Research* 32, 543–551.
- Swieszkowski, W., B. H. S. Tuan, K. J. Kurzydowski, and D. W. Hutmacher (2007). Repair and regeneration of osteochondral defects in the articular joints. *Biomolecular Engineering* 24, 489–495.
- Sykova, E., P. Jendelova, L. Urdzikova, P. Lesny, and A. Hejcl (2006). Bone marrow stem cells and polymer hydrogels - two strategies for spinal cord injury repair. *Cellular and Molecular Neurobiology* 26, 1113–1129.
- Tadjiev, T. R., S. S. Chun, H. M. Kim, I. K. Kang, and S. Y. Kim (2007). Preparation and characterization of β -tricalcium phosphate nanofibers via electrospinning. *Key Engineering Materials: Bioceramics* 342, 817–820.

- Tanahashi, M., K. Kamiya, T. Suzuki, and H. Nasu (1992). Fibrous hydroxyapatite, grown in the gel system: Effects of pH of the solution on the growth rate and morphology. *Journal of Material Science: Materials in Medicine* 3, 48–53.
- Tanaka, T., H. Komaki, M. Chazono, and K. Fujii (2005). Use of a biphasic graft constructed with chondrocytes overlying a β -tricalcium phosphate block in the treatment of rabbit osteochondral defects. *Tissue Engineering* 11, 331–339.
- Tas, A. and S. Bhaduri (2004). Rapid coating of Ti6Al4V at room temperature with a calcium phosphate solution similar to 10x simulated body fluid. *Journal of Materials Research* 19, 2742–2749.
- Taylor, G. (1969). Electrically driven jets. *Proceedings of the Royal Society of London A: Mathematical, Physical Engineering Sciences* 313, 453–475.
- Teixeira, S., F. J. Monteiro, M. P. Ferraz, R. Vilar, , and S. Eugenio (2006). Laser surface treatment of hydroxyapatite for enhanced tissue integration: Surface characterization and osteoblastic interaction studies. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 81, 920–929.
- Teo, W.-E., W. He, and S. Ramakrishna (2006). Electrospun scaffold tailored for tissue-specific extracellular matrix. *Biotechnology Journal* 1, 918–929.
- Teo, W. E. and S. Ramakrishna (2006). A review on electrospinning design and nanofibre assemblies. *Nanotechnology* 17, 89–106.
- Thomson, R. C., M. J. Yaszemski, P. J. M., and A. G. Mikos (1998). Hydroxyapatite fiber reinforced poly(alpha-hydroxy ester) foams for bone regeneration. *Biomaterials* 19, 1935–1943.
- Townsend-Nicholson, A. and S. N. Jayasinghe (2006). Cell electrospinning: a unique biotechnique for encapsulating living organisms for generating active biological microthreads/scaffolds. *Biomacromolecules* 7, 3364–3369.
- Tuan, R. S., G. Boland, and R. Tuli (2002). Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Research and Therapy* 5, 32–45.
- van Aalst, J. A., C. R. Reed, L. Han, T. Andradý, M. Hromadka, S. Bernacki, K. Kollappa, J. B. Collins, and E. G. Lobo (2008). Cellular incorporation into electrospun nanofibers: retained viability, proliferation, and function in fibroblasts. *Annals Plastic Surgery* 60, 577–583.

- Venugopal, J., S. Low, A. T. Choon, and S. Ramakrishna (2008). Interaction of cells and nanofiber scaffolds in tissue engineering. *Journal of Biomedical Materials Research - Part B Applied Biomaterials* 84, 34–48.
- Vivian, J. and P. Callis (2001). Mechanisms of tryptophan fluorescence shifts in proteins. *Biophysical Journal* 80, 2093–2109.
- Wang, G., L. Zheng, H. Zhao, J. Miao, C. Sun, N. Ren, J. Wang, H. Liu, and X. Tao (2011). In vitro assessment of the differentiation potential of bone marrow-derived mesenchymal stem cells on genipin-chitosan conjugation scaffold with surface hydroxyapatite nanostructure for bone tissue engineering. *Tissue Engineering: Part A* 17, 1341–1349.
- Wang, X., A. Ito, X. Li, Y. Sogo, and A. Oyane (2011). Signal molecules-calcium phosphate coprecipitation and its biomedical application as a functional coating. *Biofabrication* 3, 1–11.
- Wang, Y. and Y. L. Hsieh (2008). Immobilization of lipase enzyme in polyvinyl alcohol (PVA) nanofibrous membranes. *Journal of Membrane Science* 309.
- Weiner, S. and H. D. Wagner (1998). The material bone: structure-mechanical function relations. *Annual Review of Materials Science* 28, 271–298.
- Weissmueller, N. T. (2010). Drug delivery from a mineral graded electrospun nanofiber scaffold. *MSc thesis, Department of Engineering Science, University of Oxford*, pp. 61.
- Williams, D. F. (2003). Revisiting the definition of biocompatibility. *Med Device Technol.* 14, 10–13.
- Woodfield, T. B., C. A. Van Blitterswijk, J. De Wijn, T. J. Sims, A. P. Hollander, and J. Riesle (2005). Polymer scaffolds fabricated with pore-size gradients as a model for studying the zonal organization within tissue-engineered cartilage constructs. *Tissue Engineering* 11, 1297–1311.
- World Health Organisation (2010). Hip and knee replacement. In *Health at a glance: Europe 2010*, pp. 96–97.
- Wu, Y., L. L. Hench, Du, J., K. L. Choy, and J. Guo (2004). Preparation of hydroxyapatite fibers by electrospinning technique. *Journal of the American Ceramic Society* 87, 1988–1991.

- Wutticharoenmongkol, P., N. Sanchavanakit, P. Pavasant, and P. Supaphol (2006). Novel bone scaffolds of electrospun polycaprolactone fibers filled with nanoparticles. *Journal of Nanoscience and Nanotechnology* 6, 514.
- Xie, J., S. M. Willerth, X. Li, M. R. Macewan, A. Rader, S. E. Sakiyama-Elbert, and Y. Xia (2009). The differentiation of embryonic stem cells seeded on electrospun nanofibers into neural lineages. *Biomaterials* 30, 354–362.
- Xue, D., Q. Zheng, C. Zong, Q. Li, H. Li, S. Qian, B. Zhang, L. Yu, and Z. Pan (2010). Osteochondral repair using porous poly(lactide-coglycolide)/nano-hydroxyapatite hybrid scaffolds with undifferentiated mesenchymal stem cells in a rat model. *Journal of Biomedical Materials Research Part A* 94, 259–270.
- Yamada, K. M. and E. Cukierman (2007). Modeling tissue morphogenesis and cancer in 3D. *Cell* 130, 601–610.
- Yamato, M. and Okano, T. (2004). Cell sheet engineering. *Materials Today* 7, 42–47.
- Yang, F., R. Murugan, S. Wang, and S. Ramakrishna (2005). Electrospinning of nano/micro scale poly(l-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials* 26, 2603–2610.
- Yang, F., J. G. C. Wolke, and J. A. Jansen (2008). Biomimetic calcium phosphate coating on electrospun poly (ϵ -caprolactone) scaffolds for bone tissue engineering. *Chemical Engineering Journal* 137, 154–161.
- Yang, J., M. Yamato, C. Kohno, A. Nishimoto, H. Sekine, F. Fukai, and T. Okano (2005). Cell sheet engineering: Recreating tissues without biodegradable scaffolds. *Biomaterials* 26, 6415–6422.
- Yang, P. J. and J. S. Temenoff (2009). Engineering orthopedic tissue interfaces. *Tissue Engineering: Part B* 15, 127–141.
- Yang, S., K.-F. Leong, Z. Du, and C.-K. Chua (2001). The design of scaffolds for use in tissue engineering. part I. traditional factors. *Tissue Engineering* 7, 679–689.
- Yang, X., J. D. Shah, and H. Wang (2008). Nanofiber enabled layer-by-layer approach toward three-dimensional tissue formation. *Tissue Engineering: Part A* 14, 1–12.
- Yarin, A. L. (Ed.) (1993). *Free Liquid Jets and Films: Hydrodynamics and Rheology*. Longman Scientific Technical.

- Yaylaoglu, M. B., C. Yildiz, F. Korkusuz, and V. Hasirci (1999). A novel osteochondral implant. *Biomaterials* 20 (1999) 20, 1513–1520.
- Ye, H., X. Y. Liu, and H. Hong (2009). Characterization of sintered titanium/hydroxyapatite biocomposite using FTIR spectroscopy. *Journal of Materials Science: Materials in Medicine* 20, 843–850.
- Yim, E. K. F., S. W. Pang, and K. W. Leong (2007). Synthetic nanostructures inducing differentiation of human mesenchymal stem cells into neuronal lineage. *Experimental Cell Research* 313, 1820–1829.
- Yoon, S. H., Y. S. Shim, Y. H. Park, J. K. Chung, J. H. Nam, M. O. Kim, H. C. Park, S. R. Park, B. H. Min, E. Y. Kim, B. H. Choi, H. Park, and Y. Ha (2005). Treatment of complete spinal cord injury patients by autologous bone marrow cell transplantation and administration of granulocyte-macrophage colony stimulating factor. *Tissue Engineering* 11, 913–922.
- Yoshimoto, H., Y. M. Shin, H. Terai, and J. P. Vacanti (2003). A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering. *Biomaterials* 24, 2077–2082.
- You, Y., S. W. Lee, J. H. Youk, B. M. Min, S. J. Lee, and W. H. Park (2005). In vitro degradation behaviour of non-porous ultra-fine poly(glycolic acid)/poly(L-lactic acid) fibres and porous ultra-fine poly(glycolic acid) fibres. *Polymer Degradation and Stability* 90, 441–448.
- Zeng, J., A. Aigner, F. Czubayko, T. Kissel, J. H. Wendorff, and A. Greiner (2005). Poly(vinyl alcohol) nanofibers by electrospinning as a protein delivery system and the retardation of enzyme release by additional polymer coatings. *Biomacromolecules* 6, 1484–1488.
- Zhang, L., N. Hanagata, M. Maeda, T. Minowa, T. Ikoma, H. Fan, and X. Zhang (2009). Porous hydroxyapatite and biphasic calcium phosphate ceramics promote ectopic osteoblast differentiation from mesenchymal stem cells. *Science and Technology of Advanced Materials* 10, 1–9.
- Zhou, J., X. Zhang, and J. Chen (1993). High temperature characteristics of synthetic hydroxyapatite. *Journal of Materials Science: Materials in Medicine* 4, 83–85.
- Zhu, G., S. R. Mallery, and S. P. Schwendeman (2000). Stabilization of proteins encapsulated in injectable poly (lactide-co-glycolide). *Nature Biotechnology* 18, 52–57.

Zipfel, W., R. M. Williams, and W. W. Webb (2003). Nonlinear magic: multiphoton microscopy in the biosciences. *Nature Biotechnology* 21, 1369–1377.

Zoumi, A., A. Yeh, and B. J. Tromberg (2002). Imaging cells and extracellular matrix in vivo by using second-harmonic generation and two-photon excited fluorescence. *Proceedings of the National Academy of Sciences* 99, 11014–11019.

APPENDIX

Engineering biphasic osteochondral grafts - A preliminary study

A. Introduction

This appendix is a complementary section to Chapter 5 which considers briefly the possibility of binding such multilayered arrangement onto a calcium phosphate disk to obtain a biphasic osteochondral construct. To ensure attachment between the polymer and the ceramic, a first layer of electrospun fibres will be deposited directly onto the ceramic scaffold.

B. Materials and methods

Calcium phosphate (CaP) disks, made of 60% hydroxyapatite and 40% other CaP, were kindly provided by Professor James Triffitt, from the Botnar center. Prior to electrospinning, the disks were autoclaved, rinsed with deionised water and then placed onto a thin layer of deionised water spread on the collector. The fibres were then electrospun on CaP disks for 30 seconds. The extra fibres deposited around the disk were used to wrap it completely. Finally, the samples were rinsed with deionised water, dried in a sterile hood, and left in a vacuum chamber overnight to aid removal of remaining HFIP.

Biphasic constructs were created by building a layered arrangement onto the prepared ceramic disk. Three unit blocks using PLGA-c membranes (see Section 3.5, Chapter 3) were stacked on the top of a CaP material. The diameter of the CaP disk was selected to fit the diameter of the membranes measured after shrinkage had occurred. The biphasic assemblies were left for 3 hours in a 6-well plate with 150 μ l of medium added on the top to allow further cell attachment between the different fibrous layers. 2 ml of fresh medium were finally added and the samples were left for 3 days prior to initiating differentiation.

C. Results

a. Morphology of fibres electrospun on CaP disk

Figure 1 shows that the 0.1-1mm sized pores in the CaP disk are progressively masked by fibres as the duration of electrospinning increases. Fibres were found to completely cover the disk after 120 seconds. Figure 2 reveals the existence of intimate contacts between the electrospun fibres and the surface of CaP disks.

b. Morphology of the biphasic constructs

Figure 3 shows the macroscopic aspect of a PLGA-c/CaP biphasic construct seeded with hMSCs obtained after 21 days of culture in chondrogenic conditions. A cross-section through the biphasic construct is shown in Figure 4, where the upper part of the image reveals the three electrospun membranes stacked on the top of the CaP block.

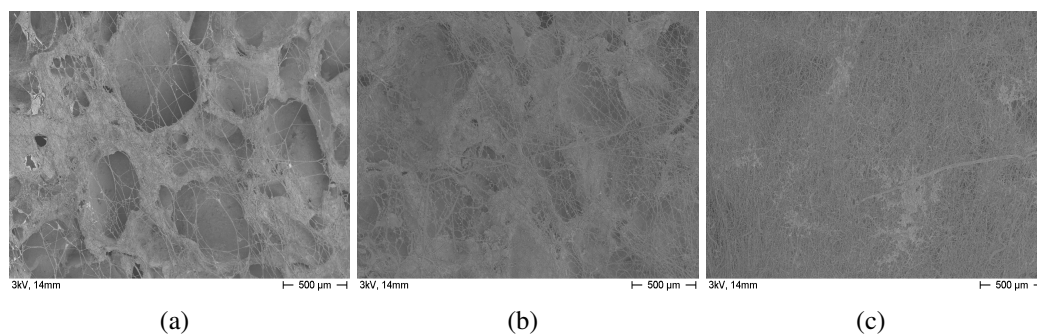


Figure 1: Influence of the duration of the electrospinning process on the fibre deposition observed at the surface of CaP blocks: (a) 15 seconds, (b) 60 seconds, (c) 120 seconds.

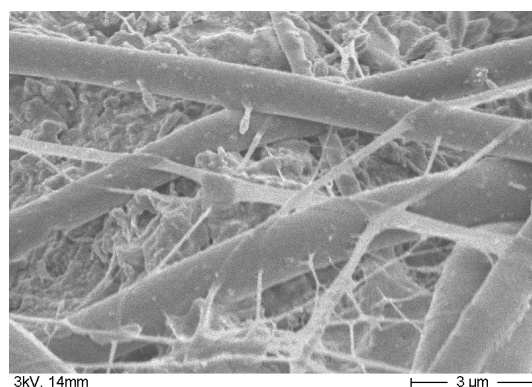


Figure 2: Intimate adhesion of the PLGA-c electrospun fibres onto the CaP disks

c. Gene expression

The expression of Collagen I, II, X and Aggrecan were examined by immuno-histochemistry on cross-sections of the PLGA-c/CaP biphasic construct. Results are presented in Figure 5. The staining indicates the presence of all proteins in the differentiated biphasic construct.

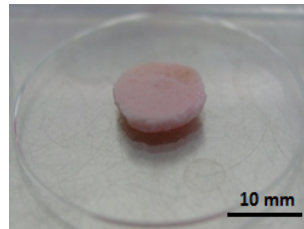


Figure 3: Morphology of the osteochondral construct observed after 21 days of culture in chondrogenic conditions. The construct was made from a porous CaP disk covered with electrospun fibres onto which were stacked PLGA-c electrospun membranes seeded with cell sheets.

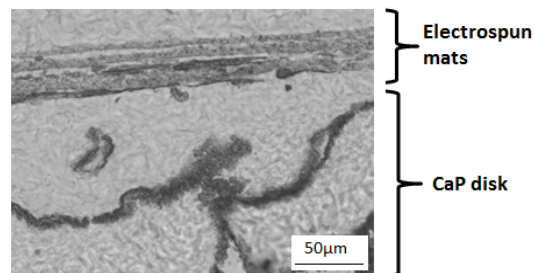


Figure 4: Cross-section through the multilayered construct observed by light microscopy. The upper part reveals the three electrospun membranes stacked on the top of the CaP disk. The cells seeded in between the membranes are not visible in this image.

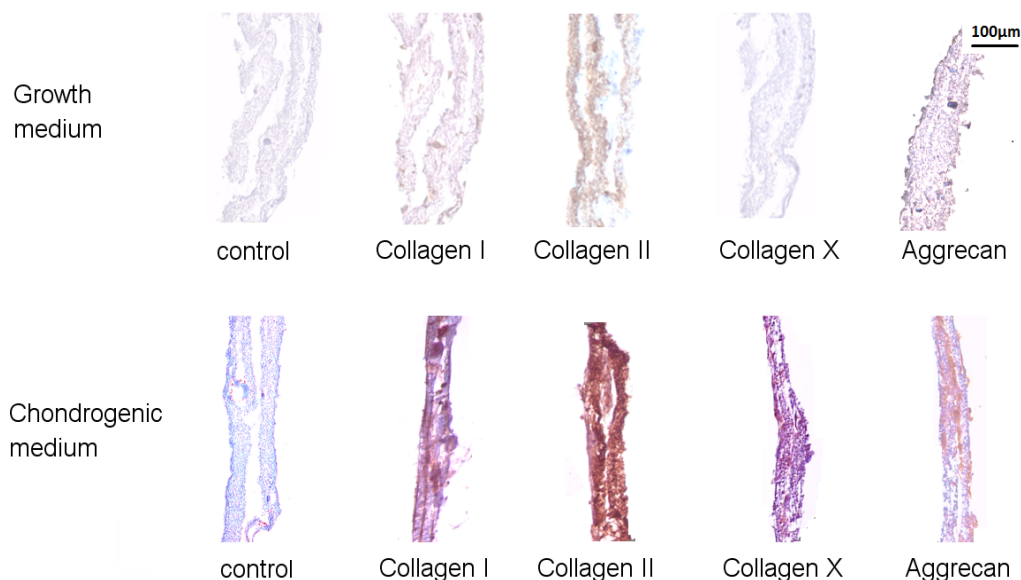


Figure 5: Immunohistochemistry performed on cross-sections of a biphasic construct: control stain (no primary antibody), Collagen I, Collagen II, Collagen X and Aggrecan. The CaP phase can not be observed in those sections due to its removal during the sample preparation. The scale bar is represented in the right corner of the figure.

D. Discussions

Results demonstrated that fibres can be collected at the surface of CaP disks. The coverage of the disk by the fibres can be easily controlled by the duration of the collection. Also, as depicted in Figure 1, the electrospun fibres did not penetrate the pores of the ceramic material, due to the rigidity of the solidified fibres and to the apparent fibre length being far longer than the width of the pores.

Intimate interactions were observed between the fibres and the surface of the ceramic material, which might help for the adhesion between the polymer and ceramic phases of the scaffold. It was observed that the electrospun mats remained attached to the disks after incubation in PBS at 37°C for at least two weeks.

There was no loss of adhesion at the interface between the two materials after culture of the layered biphasic construct for 21 days. Although shrinkage of the fibrous PLGA membranes was observed (it happens mostly before the layering step), cross-sections through the membranes revealed a flat morphology (see Figure 4), unlike the one observed in Figure 5.9. This suggests that the tendency of PLGA to shrink during the culture was partially limited by the presence of the CaP disk.

Preliminary IHC investigations (unrepeated) have revealed evidence of Collagen I, II, X and Aggrecan, which suggest the presence of hypertrophic chondrocytes and confirms the results presented earlier in Chapter 5, Section 5.4.7.

E. Conclusions

This preliminary work involving the integration of CaP porous disks with the multilayered constructs suggests that biphasic osteochondral constructs could potentially be built using the same layering approach as presented in Chapter 5. Indeed, the biphasic construct showed chondrocyte differentiation in the seeded polymer region of the arrangement and no detachment between the polymer and the ceramic was observed after 21 days of culture.