Electro spun dual structured and three-dimensional biodegradable scaffolds for bone and cartilage tissue engineering

Vidya Chamundeswari Narasimhan

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NANYANG
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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

4th August 2017

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Abstract

Tissue engineering (TE) is envisaged to play a vital role in improving the quality of life by restoring, maintaining or enhancing tissue and organ functions. TE scaffolds that are two dimensional (2D) in structure suffer from undesirable issues, such as pore blockage, and do not closely mimic the native extra-cellular matrix (ECM) in tissues. Significant efforts have therefore been channeled to fabricate structurally diverse scaffolds using various techniques, especially electrospinning. Electrospinning is a cost effective, reliable, versatile and scalable technique that has widely been explored for fabrication of tissue engineered scaffolds. The aim of this study is to investigate the efficacy of a dual structured and 3-Dimensional (3D) polymeric scaffold to support and enhance tissue differentiation into desired lineage. The dual structured scaffold contains micro-particles electro sprayed onto a fibrous mesh like network. The entire structure resembles a sandwich in appearance and both the micro-particles and the nanofibers are loaded with different bioactive molecules prior to electrospinning. This study makes use of single electrospinning equipment to electro spray and electro spin the dual structured scaffold. The polymeric scaffold is made from a combination of PLGA/PCL (poly lactide co glyceride/poly capro-lactone) blend in a ratio of 1:1. The scaffold releases up to three different biomolecules simultaneously. The fate of mesenchymal stem cells (MSCs) towards differentiating into osteogenic or chondrogenic lineage has been evaluated using different combinations of bioactive molecules. Further, a modified one-step electrospinning process to arrive at a three-dimensional (3D) scaffold with highly interconnected pores was investigated. Using a blend of hydrophobic and hydrophilic polymers, this mechanically viable, sponge-like 3D scaffold exhibited sufficiently large pores and enabled cell penetration beyond 500 μm . The release study was initially carried out with an anti-inflammatory agent – Dexamethasone (Dex). Dex loaded fibers exhibited a sustained release for up to 30 days depending on the polymer blend used for fabrication. Further, the potential of this Dex-loaded 3D scaffold was evaluated for upregulation of osteogenic genes with MSCs. The as-produced Dex-loaded 3D scaffold possesses a unique intertwined sub-micron fibrous morphology that can be tailored for use in bone tissue engineering and beyond. The efficacy of this 3D scaffold was finally evaluated for sustained release of two hydrophilic biomolecules

that have applications in the arenas of bone and cartilage tissue engineering. For directing bone tissue regeneration, the scaffold was loaded with Ascorbic acid (AA) and β - Glycerophosphate (β -Gly) and electro spun prior to MSC seeding. For inducing cartilage formation, the biomolecules of interest were AA and Proline (Pro). The scaffolds were seeded with MSCs and cultured for three weeks at the end of which gene upregulation and immunohistochemical studies were conducted. Hence using a single technique, two morphologically different scaffold systems were established for addressing issues of sustained release of multiple bioactive agents, cell penetrability and for directing the fate of MSCs into desired lineage. These scaffold systems (dual structured and 3D) can be tailored to suit different applications for engineering the ECM and reducing the ever-increasing gap between demand and supply of tissue engineered constructs.

Acknowledgements

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विद्यारम्भं करिष्यामि सिद्धिर्भवतु मे सदा II

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Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
AA	Ascorbic acid
ACN	Acetonitrile
ANOVA	One-way analysis of variance
BEI	Back scattered Electron Image
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow Stem Cells
CAD	Computer Aided Design
CaP	Calcium Phosphate
CHL	Chloroform
Col I	Collagen Type I
Col II	Collagen Type II
CT	Threshold cycle
CS	Chondroitin Sulphate
DCM	Dichloromethane
Dex	Dexamethasone
DMF	Dimethyl Formamide
DSC	Differential scanning calorimetry
ECM	Extra Cellular Matrix
EE	Encapsulation Efficiency
FDA	Food and Drug Administration – USA
FDM	Fused Deposition Modeling
FGF	Fibroblast growth factor
FTIR	Fourier Transform Infrared Spectroscopy
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
HA	Hydroxyapatite
HFIP	1,1,1,3,3,3hexafluoro-2-propanol
HPLC	High Performance Liquid Chromatography
I-TGF	Insulin like transforming growth factor Beta
MeOH	Methanol

MSC	Mesenchymal Stem Cell
PBS	Phosphate Buffered solution
PCL	Poly Capro- ϵ -lactone
PEO	Poly (Ethylene Oxide)
PGA	Poly (glycolic acid)
PLA-PEG	Polylactide-Polyethylene Glycol
PLGA	Poly Lactic Acid Co Glyceride
PLLA	Poly(L-lactide)
Pro	Proline
RBCs	Red blood cells
RP	Rapid Prototyping techniques
RT PCR	Real time Polymerase Chain Reaction
SEI	Secondary Electron Image
SEM	Scanning Electron Microscopy
SLA	Stereo-Lithography
SLS	Selective Laser Sintering
β -Gly	β -Glycerophosphate
TE	Tissue Engineering
TGF- β	Transforming growth factor- Beta
THF	Tetrahydrofuran
TIPS	Thermally Induced Phase Separation
UV- Vis	Ultra Violet Visible Spectroscopy
XRD	X-ray Diffraction

Chapter 1

Introduction

“Imagine a world where transplant patients do not wait for a donor; a world where burn victims leave the hospital without disfiguring scars.

Imagine implant materials that can ‘grow’, reshape themselves, or change their function as the body requires” – Professor M V Sefton.

This chapter presents the rationale for conducting a study in the field of musculoskeletal tissue engineering. The chapter begins with a background to musculoskeletal disorders followed by the identification of problem statements. Current clinical treatment options are discussed which are followed by tissue engineered approaches for bone and cartilage tissue regeneration. The need for a scaffold to serve as an engineered ECM is discussed. Next, the hypothesis and the specific objectives of this thesis are listed. An overview of the entire dissertation is presented in section 1.6. Finally, the novel outcomes of the research conducted are summarized as the last section of this chapter.

1.1 Background and problem statements

Musculoskeletal diseases encompass a wide spectrum of conditions which include arthritis, osteoporosis, joint pain, spinal and hip injuries, fractures, other degenerative conditions and tumors. The impact of musculoskeletal disorders around the globe is pervasive and constitutes a major socioeconomic burden. In 2013, a study on the Global Burden of Disease and the worldwide impact of all diseases reported that musculoskeletal disorders affected more than 1.7 billion people worldwide. They are the second greatest cause of physical disability, and have the 4th greatest impact on the overall health of the world population[1]. As the world population ages during the next 25 years, musculoskeletal disorders will steadily increase owing to their prevalence in the older segments of the population. In the US alone, an emergency room encounters more than 2 million fall injuries, 25% of which lead to diagnosis of prevailing osteoporosis conditions predominantly in women[2]. These conditions predominantly affect women from an earlier age in comparison to men. It is a huge concern that the number of surgical procedures for bone and cartilage substitutes conducted annually in the US has doubled from 1,110,000 in 2005 to 2,100,000 in 2015 and the costs for these have crossed 74 billion USD in 2015 [3]. In Singapore, there has been a 28.5% increase in musculoskeletal diseases from 2004 to 2010 as reported by the Ministry of Health[4]. Injuries to the bone and cartilage often occur as a result of trauma, sports injuries or due to degenerative conditions exhibited by the tissues. Of all the degenerative conditions, osteoporosis, arthritis and cartilage inflammation are the leading causes of medical expenditure.

Electrospinning is a versatile technique to fabricate fibrous mesh like networks that mimic the extra cellular matrix (ECM) to serve as engineered ECMs. Electro spun fibers possess unique advantages such as high surface to volume ratio for tissue engineering applications [5]. The structural similarity of the electro spun fibers with that of the native ECM of the tissues has opened a huge potential for the usage of these fibers in tissue engineering [6]. It is also a simple, scalable and robust technique that can be employed industrially [7]. **Despite these promising benefits, the interconnected pores produced as a result of conventional 2D electrospinning are usually blocked through layering of fibers. This results in small pore sizes that restrict effective penetration of cells into the scaffold, and often results in cell**

layering on one side of the electro spun fibrous scaffold with minimal infiltration [13]. Many techniques such as salt leaching [8], post-processing[9], or template-assisted collection [10] have been employed to induce porosity into the scaffold structure. However, the **complexity of these processes, the multitude of steps involved, and their poor repeatability are major limitations**. The concept of blending two or more synthetic polymers to form a fibrous sheet of scaffold is a subject of interest in the last few years owing to a rise in demand and dearth in supply[11]. This helps to combine the desirable properties of two polymers for an intended application. Further these synthetic scaffolds evade immune rejection problems exhibited by natural polymeric scaffolds and can be specifically tailored to suit versatile applications. While the characteristic of ECM-like scaffolds is one consideration in TE, undifferentiated cells would still require cues to guide them towards the appropriate cellular lineage, e.g. mature bone, cartilage or tendon cells. To this end, bioactive molecules have been extensively explored and utilized in tissue engineering application for stem cell differentiation [12-16]. Growth factors such as transforming growth factor betas (TGF- β s), bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) are known to influence stem cell differentiation [32, 33]. For instance, ascorbic acid is known to increase collagen production [17]. These bioactive molecules are often supplemented *in vitro* into culture medium to induce stem cell differentiation, and their effects will cease when the cell-laden scaffolds are transplanted into patients [12, 18, 19]. It has been known that the sustained release of biomolecules and other growth factors could be achieved in electro spun fibrous system [20, 21]. The controlled release of these bioactive molecules from scaffolds provides immense benefits for tissue regeneration by delivering biochemical stimuli to cells after transplantation. However, for bone regeneration, the main bioactive molecules that are extensively studied are the BMP superfamily of peptides. These peptides are required in minute quantities (nano molar concentrations) within the body, are very expensive and induce extreme toxicity if supplied in excess. Their bioactivity is drastically reduced as a result of the fabrication technique used for encapsulating them into desired structures. The same is the case with Dexamethasone (Dex), which is an inducer of osteogenesis. Many research groups have tried to achieve sustained release of Dex from the 2D scaffolds with moderate success rates [22-24]. However, there are other cost-effective inducers of osteogenesis and chondrogenesis such as Ascorbic acid, β -Glycerophosphate and Proline that are not

studied extensively in the scientific community. These molecules require an effective carrier/ scaffold and can be tuned to release in a controlled manner *in vitro*. **Therefore, there is a need to enable simultaneous controlled release of multiple bioactive molecules within the transplanted tissue grafts for providing biochemical stimuli to the cells.**

1.2 Current Treatment Options

1.2.1 For severe cases of bone and cartilage degeneration, surgical intervention is the golden standard in addition to prescriptions of anti-inflammatory drugs and pain killers. There is insufficient evidence to show that surgical grafts could heal injuries without causing adverse side effects such as increased inflammation, lack of biocompatibility and antigenic reactions. Limited supply and donor site morbidity of autografts is a significant problem that prevents it from being a long-term solution. Allografts, on the other hand, are expensive, and are not devoid of the complications arising from immune reactions and transmission of diseases. Post-surgical recovery of the patient depends on many external factors and in most cases the tissue does not regain its complete functionality. Further, the minimum healing time for the tissues to restore part of their functionality falls between 8 – 10 weeks. This adversely affects the day to day activities in the case of general population and the livelihood of athletes.

1.2.2 Oral medications which include calcium and vitamin supplements are widely prescribed to patients which are to be usually taken twice a day. These medications, to an extent help to avoid further damage, but act at a very slow rate and do not give any hopes in providing full functional recovery of the affected regions.

1.2.3 Steroidal injections are administered mostly at the knee joint, spinal disc and hip areas for people suffering from osteoporosis, disc degenerative diseases and arthritis respectively. Even though these procedures avoid surgical costs, they only provide relief for a short period of time and do not aid in permanent alteration of the prevailing condition. These are not free of side effects such as complete numbness or weakness of the affected areas. In the situations when such conservative treatments do not provide relief even after a few months, surgery is required.

1.3 Tissue Engineering approaches

The field of healthcare is being revolutionized by the concept of artificial tissues. The design of bone scaffolds has received a lot of attention in the recent years as it has emerged as an alternative to bone substitutes including auto grafts, allografts and xenografts[25]. Tissue engineering (TE) of the bone and cartilage has emerged in recent times with the primary aim to overcome the limitations of all conventional treatments offered. The clinical applications in the field of bone and cartilage tissue engineering constitute a wide spectrum ranging from bone and cartilage regeneration, tumors, reconstructive surgeries and arthritis treatments [26]. The general strategy in creating a functional bone or cartilage tissue requires the incorporation of living cells into matrices or scaffolds, as a structural support for cell adhesion, while influencing the fate of immature cells through bioactive molecules and/or physical cues [27]. The key considerations to be considered while designing scaffold materials for bone and cartilage are biocompatibility, biodegradability, mechanical requirements in accordance with the tissue, architecture of the scaffolds and the fabrication technology [28]. In a cellular environment, the ECM provides the appropriate physicochemical stimuli to cells, which are essential for cell attachment, proliferation and lineage specification. In addition, it provides a conducive environment that facilitates the transport of metabolites and nutrients [5]. Thus, there is immense interest within the scientific community to develop ECM-mimicking scaffolds to serve these functions. Nano and micro fibrous structures have gained prime importance in tissue engineering applications as scaffolds. Of all the fabrication techniques available, electrospinning has demonstrated the innate ability to create different types of scaffolds ranging from three-dimensional (3D) structures to multilayered nano/micron fibers using a broad range of polymers, from synthetic to natural and even a combination of both. Using electrospinning as the fabrication technique, the feasible carrier morphologies are fibers (single layer, aligned and core shell), particles (micro/nano and capsules (eg: Alginate)), and 3D structures (cotton ball morphology) [24, 29-32]. The morphology of the drug carrier plays a crucial factor in determining the final release kinetics. Fibers are relied upon to provide sustained release over a longer period of time whereas capsules and particles (micro/nano) are well known for their burst release of the encapsulated molecules. The tissue engineered bone/ cartilage scaffolds should exhibit a perfect synergy of releasing appropriate osteogenic/chondrogenic signals for

progenitor cells and a supportive scaffold matrix in order to mimic the native ECM. A lot of research has been focused on fine tuning of the carrier morphology in order to arrive at desired delivery of the bioactive molecules and to preserve their functionality [33-35].

1.4 Hypothesis

My hypothesis is to evaluate the potential of dual structured sandwich like and 3D scaffold to act as a reservoir for loading and sustained release of combination of growth factors and small molecules to promote lineage specific differentiation of mesenchymal stem cells into bone and cartilage tissues.

The key activities performed to validate this are listed below:

- 1) Fabrication, characterization and evaluation of a dual structured “sandwich” scaffold comprising of nanofibers and micro-particles to host and release multiple bioactive agents.
- 2) A 3D scaffold system that exhibits enhanced cell penetration, permits commendable mechanical strength and provides sustained release of biomolecules for musculoskeletal tissue engineering.

1.5 Specific Objectives

The specific objectives are classified into two sub divisions to address the desired goals for the scaffold systems hypothesized above:

1. Sandwich System containing micro-particles and fibers loaded with bioactive molecules
 - To identify the optimal electrospinning parameters to obtain well controlled particle and fiber morphology.
 - Carry out material characterizations and load biomolecules into electro spun polymer fibers and micro-particles.
 - Study *in vitro* release of single and multiple biomolecules from the scaffold system.
 - Evaluating the effect of the sandwich scaffolds on supporting differentiation capabilities of MSCs into osteo and chondro lineage.

2. 3D scaffolds for sustained release of bioactive molecules

- To investigate the correlation between electrospinning parameters to obtain intertwined 3D fibers and arrive at the best polymer concentration and ratios to fabricate 3D scaffolds.
- Carry out material characterizations for all the 3D scaffolds.
- Load biomolecules into 3D scaffold systems and study the *in vitro* release patterns of single and multiple biomolecules.
- Evaluate the effect of 3D scaffolds on the differentiation potential of MSCs into osteo and chondro lineage.

1.6 Novelty of this study

This research has led to several novel outcomes such as

- a) Establishing a dual structured scaffold with microparticles and nanofibers for release of multiple biomolecules at different rates that is controlled by the scaffold material properties.
- b) Release of Dexamethasone in a controlled manner and ensuring that the release does not exceed the therapeutic dosage levels at any time point.
- c) Simultaneous release of three biomolecules from a single scaffold system without any post processing treatments.
- d) Successful demonstration of a mechanically strong 3D scaffold system whose applications can be tailored to suit different branches of tissue engineering depending on the polymer blends used.
- e) The 3D scaffolds exhibit cell penetration depths of about 500 μm which has not been reported in the scientific literature so far.

1.7 Dissertation Overview

There is a need for increased awareness in the medical community and to introduce effective interventions which can lead to betterment of the quality of life and prevention of disability among the globe's citizens. This thesis addresses the need for biodegradable scaffolds in the reign of musculoskeletal tissue engineering and evaluates two structurally different scaffold systems for bone and cartilage tissue

engineering. The aim of delivering drug loaded scaffold moieties into the site of injuries has been explored. The loaded drugs are expected to serve as molecules that attract and recruit stem cells to the site of injury thus enhancing tissue repair and regeneration. The emphasis here is on the regeneration of musculoskeletal tissues such as bone and cartilage using a combination of cells, osteogenic/chondrogenic signals, and different scaffold systems to suit to a wide range of applications. The focus is laid on using simple cost-effective bioactive molecules to accomplish bone and cartilage regeneration sans the use of expensive moieties such as BMPs and high-end fabrication techniques.

Chapter 1 provides a brief rationale for the research and outlines the specific objectives and novelty of this study. *Chapter 2* reviews the literature concerning bone and cartilage tissue engineering. Various fabrication techniques for producing biodegradable polymeric scaffolds have been discussed with special focus on electrospinning. Electrospinning is the technique employed throughout the course of this study for fabrication of all the structurally diverse scaffolds systems. The relevance of electro spun scaffolds in the context of bone and cartilage tissue engineering and literature reports on this topic have been summarized in this chapter. *Chapter 3* discusses the principles underlying the fabrication/characterization/cell studies employed for all scaffolds and the methods of data analysis and imaging. Brief working principles of equipment used during the course of this thesis are listed with the specific application for this study. This enables the reader to appreciate the significance of using a technique/instrument for a specific process. *Chapter 4* elaborates the results pertaining to the dual structured (particle on fiber) scaffolds laying emphasis on the fabrication and optimizations that had to be conducted in order to arrive at the desired morphology. This chapter then progresses to discuss the release of various bioactive molecules from the dual structured (sandwich) scaffolds. *Chapter 5* elaborates the fabrication, optimization and release studies conducted for the 3D scaffold systems. *Chapter 6* is a summary of all the in vitro experiments carried out for all the scaffolds pertaining to the scope of this thesis. *Chapter 7* is a final summary of all the findings during the course of this thesis. Some reconnaissance studies that did not warrant a complete chapter have been discussed in brief in here. The implications of my area of research and potential applications of the scaffolds systems presented are discussed with opportunities and strategies for future work.

Summary

This chapter begins with a brief insight into tissue engineering field and the requirements of scaffolds for the repair and regeneration of bone and cartilage are discussed. Gaps in literature such as need for scaffolds with enhanced cell penetration, requirement for a cost scaffold system to simultaneously release multiple bioactive molecules in a controlled manner and the importance of a cost effective, scalable and reliable fabrication technique are highlighted. The chapter further lists the specific objectives and the scope of this thesis and provides the reader with a brief overview of the dissertation. The original findings are listed as the novel outcomes in the last section.

References

- [1] D.J. Hunter, D. Schofield, and E. Callander, *Nature Reviews Rheumatology*, 2014. **10**(7): p. 437-441.
- [2] V. Vennu and S.M. Bindawas, *Clinical interventions in aging*, 2014. **9**: p. 793.
- [3] S.M. Kurtz, K.L. Ong, J. Schmier, F. Mowat, K. Saleh, E. Dybvik, J. Kärrholm, G. Garellick, L.I. Havelin, and O. Furnes, *JBJS*, 2007. **89**(suppl_3): p. 144-151.
- [4] C.J. Murray, T. Vos, R. Lozano, M. Naghavi, A.D. Flaxman, C. Michaud, M. Ezzati, K. Shibuya, J.A. Salomon, and S. Abdalla, *The lancet*, 2013. **380**(9859): p. 2197-2223.
- [5] S. Schlie-Wolter, A. Ngezahayo, and B.N. Chichkov, *Experimental cell research*, 2013. **319**(10): p. 1553-1561.
- [6] J. Yu, A.-R. Lee, W.-H. Lin, C.-W. Lin, Y.-K. Wu, and W.-B. Tsai, *Tissue engineering Part A*, 2014. **20**(13-14): p. 1896-1907.
- [7] W.-E. Teo, R. Inai, and S. Ramakrishna, *Science and technology of advanced materials*, 2011. **12**(1): p. 013002.
- [8] O.D. Schneider, F. Weber, T.J. Brunner, S. Loher, M. Ehrbar, P.R. Schmidlin, and W.J. Stark, *Acta biomaterialia*, 2009. **5**(5): p. 1775-1784.
- [9] I.K. Shim, W.H. Suh, S.Y. Lee, S.H. Lee, S.J. Heo, M.C. Lee, and S.J. Lee, *Journal of Biomedical Materials Research Part A*, 2009. **90**(2): p. 595-602.
- [10] D. Zhang and J. Chang, *Nano letters*, 2008. **8**(10): p. 3283-3287.
- [11] R. Thomson, M. Wake, M. Yaszemski, and A. Mikos, *Biopolymers II*, 1995: p. 245-274.
- [12] Y.J. Chuah, Y. Zhang, Y. Wu, N.V. Menon, G.H. Goh, A.C. Lee, V. Chan, Y. Zhang, and Y. Kang, *Acta Biomater*, 2015. **23**: p. 52-62.
- [13] F. Langenbach and J. Handschel, *Stem Cell Res Ther*, 2013. **4**(5): p. 117.
- [14] M. Yuasa, T. Yamada, T. Taniyama, T. Masaoka, W. Xuetao, T. Yoshii, M. Horie, H. Yasuda, T. Uemura, A. Okawa, and S. Sotome, *PLoS One*, 2015. **10**(2): p. e0116462.
- [15] M. Schuldiner, O. Yanuka, J. Itskovitz-Eldor, D.A. Melton, and N. Benvenisty, *Proc Natl Acad Sci U S A*, 2000. **97**(21): p. 11307-12.
- [16] N.S. Hwang, S. Varghese, and J. Elisseeff, *Adv Drug Deliv Rev*, 2008. **60**(2): p. 199-214.
- [17] Y.J. Chuah, Y.T. Koh, K. Lim, N.V. Menon, Y. Wu, and Y. Kang, *Scientific reports*, 2015. **5**: p. 18162.
- [18] Y.J. Chuah, Y.T. Koh, K. Lim, N.V. Menon, Y. Wu, and Y. Kang, *Sci Rep*, 2015. **5**: p. 18162.
- [19] Y.J. Chuah, S. Kuddannaya, M.H. Lee, Y. Zhang, and Y. Kang, *Biomater Sci*, 2015. **3**(2):

p. 383-90.

- [20] M. Rodríguez-Évora, E. García-Pizarro, C. del Rosario, J. Pérez-López, R. Reyes, A. Delgado, J.C. Rodríguez-Rey, and C. Évora, *Biomacromolecules*, 2014. **15**(4): p. 1311-1322.
- [21] J. He, T. Qin, Y. Liu, X. Li, D. Li, and Z. Jin, *Materials Letters*, 2014. **137**: p. 393-397.
- [22] L.B. Rodrigues, H.F. Leite, M.I. Yoshida, J.B. Saliba, A.S.C. Junior, and A.A. Faraco, *International journal of pharmaceutics*, 2009. **368**(1): p. 1-6.
- [23] S.L. Fialho, F. Behar-Cohen, and A. Silva-Cunha, *European Journal of Pharmaceutics and Biopharmaceutics*, 2008. **68**(3): p. 637-646.
- [24] Y. Su, Q. Su, W. Liu, M. Lim, J.R. Venugopal, X. Mo, S. Ramakrishna, S.S. Al-Deyab, and M. El-Newehy, *Acta biomaterialia*, 2012. **8**(2): p. 763-771.
- [25] M.M. Stevens, *Materials today*, 2008. **11**(5): p. 18-25.
- [26] M.L. Ferrone and C.P. Raut, *Surgical oncology clinics of North America*, 2012. **21**(2): p. 201-213.
- [27] S.P. Hoo, Q.L. Loh, Z. Yue, J. Fu, T.T. Tan, C. Choong, and P.P. Chan, *Journal of Materials Chemistry B*, 2013. **1**(24): p. 3107-3117.
- [28] F.J. O'brien, *Materials today*, 2011. **14**(3): p. 88-95.
- [29] F. Yang, R. Murugan, S. Wang, and S. Ramakrishna, *Biomaterials*, 2005. **26**(15): p. 2603-2610.
- [30] S. Heydarkhan-Hagvall, K. Schenke-Layland, A.P. Dhanasopon, F. Rofail, H. Smith, B.M. Wu, R. Shemin, R.E. Beygui, and W.R. MacLellan, *Biomaterials*, 2008. **29**(19): p. 2907-2914.
- [31] P. Yilgor, K. Tuzlakoglu, R.L. Reis, N. Hasirci, and V. Hasirci, *Biomaterials*, 2009. **30**(21): p. 3551-3559.
- [32] D. Aussawasathien, C. Teerawattananon, and A. Vongachariya, *Journal of membrane science*, 2008. **315**(1): p. 11-19.
- [33] J.A. Champion, Y.K. Katare, and S. Mitragotri, *Journal of Controlled Release*, 2007. **121**(1): p. 3-9.
- [34] Y.-Y. Yang, T.-S. Chung, X.-L. Bai, and W.-K. Chan, *Chemical Engineering Science*, 2000. **55**(12): p. 2223-2236.
- [35] J. Park, M. Ye, and K. Park, *Molecules*, 2005. **10**(1): p. 146-161.

Chapter 2

Literature Review

This chapter begins with an overview on bone and cartilage tissue engineering with a discussion on the structure and functions of the bone and cartilage. The chapter progresses to discuss on the specific requirements for tissue engineered bone and cartilage. Section 2.2 provides insights on the various fabrication techniques used widely for obtaining the tissue engineered scaffolds. After a brief overview of the techniques, the focus shifts to understanding the concept of electrospinning and electro spraying and their utility in fabrication of biodegradable scaffolds for tissue engineering. Section 2.3 focusses on electrospinning in the context of bone and cartilage tissue engineering.

2.1 Bone and Cartilage Tissue Regeneration

2.1.1 Bone – Structure and Function

The skeletal system of a human adult can be sub classified into appendicular skeleton (126 bones), axial skeleton (74 bones) and six bones belonging to the auditory ossicles. Bone can be defined as mineralized connective tissue which undergoes constant changes throughout our life in order to adapt, repair and restore the functionality of the skeletal system. Bones can be morphologically classified into four general categories namely long bones, short bones, flat bones and irregular bones (**figure 2.1**).

Figure 2.1 Morphological classification of bones [1] reproduced by permission.

The long bones are composed of a diaphysis (shaft) and epiphysis (growth plate). Common examples are the bones of the limbs, ribs, phalanges, metacarpals and metatarsals. Bones of the hands and feet have no shaft and comprise the short bones. Bones of the skull fall under the category of flat bones and irregular bones make up the spinal cord with no two bones resembling each other. Strong but light weight, bone is a dynamic ever-changing structure. In terms of gross structure, bones are classified as compact or spongy (**figure 2.2**) and developmentally they are classified as membranous or cartilaginous bones[1]. Compact or cortical bones are made up of concentric arrangement of lamellae surrounding a harversian canal at the center. Volkmann's canals are responsible for the penetration of blood vessels, lymphatic

vessels and nerves from the periosteum. The combination of a single harversian canal and the surrounding lamellae is known as osteon. Rings of hard, calcified matrix are present around the rings of the lamellae and the area between the osteons contains interstitial lamellae. Spongy bones do not contain osteons. They are comprised of irregular lattices of plates called trabeculae. The spaces between the trabeculae are filled with red marrow that produces the red blood cells (RBCs). Spongy bones are also known as cancellous bones.

Figure 2.2 Structural classification of bone[1] reproduced by permission.

The components of bone are classified into organic, inorganic and water. The organic phase is the collagen, non-collagenous proteins and lipids which roughly make up 30% of the bone tissue. Collagen type I (Col I) is the predominant collagen fibril that makes up the bone tissue. Hydroxyapatite crystals which comprise of calcium and phosphate make up the inorganic phase of the bone while the third major component is water. Collagen fibers provide tensile strength while the inorganic salts incorporate compression tolerance in the bone[2]. The process by which bone is formed is known as ossification. This process begins around 6th week of embryonic life and continues throughout adulthood. Bone formation occurs through;

Intramembranous ossification is the direct formation of bone within the fibrous connective tissue membranes. At the site of bone development, MSCs vascularize, cluster and differentiate into osteoblasts. Osteoblasts secrete organic matrix of the

bone and later calcium and other minerals are deposited followed by tissue calcification. The bone matrix develops into trabeculae which fuse together to form the spongy bone. Connective tissue in between these spaces differentiates into red bone marrow. On the outside, periosteum is developed and some spongy bone is replaced by cortical bone which is remodeled to reach its final size.

Endochondral ossification requires hyaline cartilage for bone construction. The mesenchymal cells differentiate to form a hyaline cartilage. This cartilage grows and chondrocytes in the middle region are calcified. The periosteum gives rise to the bone collar and the capillaries grow into calcified cartilage. The osteoblasts, red marrow cells, osteoclasts and blood vessels are known as the periosteal bud which is the primary ossification center. Secondary ossification centers develop around the time of birth[3].

Bones function in maintaining the framework of the body by providing structural support and protecting organs. They also enable the movement of various body parts; play a role in attachment of muscle to tendon and in hematopoiesis[4]. The bone cells can be classified into four major categories namely: osteoprogenitor cells (differentiate into osteoblasts), osteoblasts (responsible for osteogenesis), osteocytes (mature bone cells) and osteoclasts (bone resorption)[5]. Knowledge about the different bone cells is vital in developing tissue engineering strategies for bone tissue regeneration.

2.1.2 Cartilage – Structure and Function

Cartilage can be described as a dense connective tissue that functions to provide support, bear weight and withstand torsion, tension and bending. Cartilage supports those regions of the body which require more flexibility. It serves as a model for formation and growth of long bones and to support the soft tissues. Cartilage is an avascular tissue which derives its nutrition supply from blood capillaries and adjacent tissues of the perichondrium[6]. The development of cartilage occurs through the differentiation of mesenchymal stem cells into chondroblasts and begins the process of chondrogenesis. The main constituents of cartilage are chondroblasts and chondrocytes in addition to abundant extra cellular matrix (ECM). Chondroblasts are the matrix forming cells found at the periphery of the cartilage. Chondrocytes are the mature cartilage cells which are present at the interior regions and appear rounded.

The ECM of cartilage is covered by perichondrium and is composed of majorly water, ground substances like sugar moieties and collagen and elastin fibers. Aggrecans make up major part of the proteoglycans and they form large aggregates with hyaluronan[6]. These aggregates are responsible for holding water due to their negative charge. The collagen found in the cartilage is type II (Col II). Elastin fibers are also present in certain cartilage tissues (**figure 2.3**).

Figure 2.3 Summary of different types of cartilage tissues.

Unlike bone, cartilage does not undergo rapid self-healing and hence exhibits limited repair potential. Hence any damage caused to the cartilage is difficult to heal. The main causes for cartilage damage are trauma and degenerative conditions such as arthritis. As the process of aging begins, there are no major changes in the cartilage matrix. The chondrocytes become less efficient in renewing the matrix leading to reduced water content which affects the compression properties of the cartilage[7]. The proteoglycans produced in older individuals are smaller when compared with those in young adults. These changes will reflect in the injuries which cause inflammation and all the symptoms of arthritis follow.

2.1.3 Advancements in tissue engineering of the bone and cartilage

Tissue engineering is the field which applies the principle of biology and engineering to the development of functional substitutes for damaged tissues[8]. TE holds immense potential for replacement therapy where damaged tissues and organs such as liver, connective tissues, bone, cartilage and muscles can be regenerated or replaced if they are beyond repair. One of the primary goals of TE lies in developing methods to construct organs in the laboratory that can be used subsequently in medical applications. TE of the musculoskeletal system has garnered significant interest due to

the wide spectrum of diseases and damages that occur in bones, cartilages and tendons. The need for TE is due to the ever-increasing gap between demand and supply of organs, tissues and bones. The general principles involve combining the living cells with a support (scaffold) that is biodegradable in order to build a three dimensional living construct that is functionally, structurally and mechanical better than or equal to the tissue that is being replaced[9]. Thus, the major requirements for bone and cartilage tissue engineering are the perfect synergy between osteogenic/chondrogenic signals, suitable scaffolds and cells[10]. The signals constitute various biochemical cues, growth factors or biomolecules that are essential for the differentiation into desired lineage. Cells constitute osteoblasts, chondroblasts or mesenchymal stem cells (MSCs) which direct the differentiation process into respective lineage. MSCs are easily available and the protocols for their extraction and culture are well documented in literature. They can be characterized by their ability to develop into osteogenic or chondrogenic lineages in response to external stimuli and expression markers that can be supplemented[11]. Lastly, scaffolds play a vital role of a supportive matrix in formation of a tissue engineered bone or cartilage. Since the last few decades, extensive research has been carried out in the fields of bone and cartilage tissues. The clinical applications for these fields constitute a wide spectrum ranging from bone replacement, bone tumors, arthritis treatment, osteoporosis, maxillofacial and reconstructive surgeries and cartilage damage and inflammation[12]. It has been estimated that the number of surgical procedures related to bone substitutes performed every year in the United States increased drastically from 700,00 in 1998 to 1,100,000 in 2005 and has hiked up the costs involved to 74 billion USD in 2015[13]. There is insufficient evidence to show that grafts could fully heal injuries, without causing issues like increased inflammatory response, antigenic reactions, fixation site failure and lack of long term biocompatibility which has limited their application in treating bone and cartilage regeneration[14]. The design of scaffolds has received a lot of attention in the recent years as it has emerged as an alternative to bone substitutes including auto grafts, allografts and xenografts[15]. Nano fibrous structures have gained prime importance in tissue engineering applications in the form of scaffolds. Based on their morphology and processing techniques, they have multifarious applications in the nano-bioengineering domain. As there are variations in structure within a bone (compact and spongy) and cartilage (lamellae and trabeculae), the definition of an ideal scaffold is difficult for these

applications. The key factors to consider while fabricating an ideal scaffold are (a) pore size [macro- $>100\ \mu\text{m}$), micro- $<20\ \mu\text{m}$] and interconnected open porosity for in vivo tissue in-growth; (b) mechanical properties combined with controlled degradation kinetics, (c) ability to survive through all the physical forces in vivo; and (d) maintenance of a sterile environment condition for cell seeding onto the scaffold[10, 16]. **Figure 2.4** summarises the major requirements for an ideal scaffold system in musculoskeletal tissue engineering arena.

Figure 2.4 Requirements of an ideal scaffold system for musculoskeletal tissue regeneration.

High 3D porosity with interconnected pores is essential to facilitate cell growth, transport of nutrients and removal of metabolic wastes. Biocompatibility and bioresorption with controllable degradation rates are required to be in accordance with the cell model of interest. Conductive surface properties for cell proliferation, attachment and detachment are essential followed by commendable mechanical properties to ensure proper load transfer[17]. Scaffolds, in the context of musculoskeletal tissue regeneration are temporary support structures that induce the formation of desired lineage. They also act as carriers of drugs and other growth factors and bioactive molecules that enable osteogenesis or chondrogenesis to occur. To this end, scaffolds are broadly classified into three categories namely ceramics, polymers and metals. Demineralized bone matrix, collagen composites, fibrin, calcium phosphate, polylactide (PLA), poly(lactide-co-glycolide)(PLGA),

polylactide-polyethylene glycol(PLA-PEG), hydroxyapatite(HA), dental plaster and titanium are few of the currently approved materials used extensively for bone tissue regeneration approaches[18]. Metals and ceramics pose the issue of lack of biodegradation in a biologic environment and possess limited process ability and tenability. Polymeric scaffolds, on the other hand, have gained significant attention as they can be tailored to closely mimic the extra cellular matrix (ECM) of the native tissues. They are biodegradable and exhibit excellent design flexibility. This is determined by the user based on the material chosen, composition and the ratios of the polymers which can be tailored for different applications. Polymers used in bone and cartilage TE are required to be biocompatible thus avoiding any immune responses from the host tissue. Their degradation rate can be controlled to suit the rate of tissue regeneration and degradation products are excreted through metabolic pathways. Polymers are sub classified into naturally derived (eg: collagen and fibrin), synthetic polymers (eg: poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymers (PLGA). Natural polymers exhibit immunogenicity and do not provide flexibility over mechanical properties. In addition, batch to batch consistency, risk of pathogen infection and biodegradability are issues of concern. Synthetic polymers are reproducible in large quantities without any variations arising from different batches. They possess negligible immunogenicity and properties such as microstructure, degradation rate and mechanical strength can be tailored according to user needs[19]. Various research groups have focussed on composite materials that involve both natural and synthetic polymers [20-22]. Synthetic hydrogels are being explored widely for devising an injectable biodegradable scaffold system. Collagen and alginate in combination with PEG polymers are the primary focus of this investigation which can lead to a minimally invasive means of delivery of cell laden scaffolds to the site of injury [23-25]. However, these hydrogels do not exhibit mechanical strength which limits their utility. TE of bone and cartilage can be divided into six phases as stated by Dietmar [26] (**Table 2.1**).

The objectives stated in the table are interdisciplinary. Beginning with the raw polymer material chosen to the scaffold architecture to the cell seeded in it and the transplantation process, each step must be planned carefully to accomplish the final goal.

Table 2.1 Six phases of Classification of Tissue Engineering of Bone and Cartilage.

I Fabrication of bioresorbable scaffold.
II Seeding of osteoblasts/chondrocytes populations into the scaffold.
III Growth of premature tissue in a dynamic environment.
IV Growth of mature tissue in a physiological environment.
V Surgical transplantation.
VI. Tissue engineered transplant assimilation/remodeling.

The focus of my study is restricted to synthetic polymers as they have a long history of use as degradable sutures for surgery. They are biocompatible and FDA approved for human use. They degrade by non-enzymatic hydrolysis processes mainly by ester bond breakage. The degradation products of PGA, PLA, PCL and PLGA are not toxic and the body eliminates them as carbon dioxide and water. By altering parameters such as chemical composition (polymer ratios), molecular weight and crystallinity can be varied in addition to the degradation rates of these polymers.

Despite extensive research on synthetic polymers, there still exists a dire need to improve their functionality to expand their applications. Along with the scaffolds, the choices of cells that are seeded on the scaffolds are an important aspect of achieving an engineered tissue. My studies are confined to MSCs as they are multi potential cells with the ability to differentiate into both bone and cartilage in response to the cues provided to them. Their marked plasticity provides the researcher with good flexibility to use MSCs for multiple cellular differentiation lineages to fabricate a multi-phasic tissue[27]. MSCs are derived from the bone marrow and do not pose any ethical concerns in terms of usage. They can be easily expanded *in vitro* and there are vast amounts of literatures which provide strong evidence for their differentiation *in vitro* and *in vivo* [11, 27-29]. On the other hand, biomaterial scaffolds exhibit several attractive qualities for serving as a carrier for MSCs, such as providing good mechanical integrity and a 3D ECM mimicking environment. Previous reports have utilized various synthetic biodegradable materials which were fabricated into fibers,

sponge like, and gel-based structures for culturing MSCs from different species [30-33]. The following section discusses the various fabrication techniques widely employed in the field of bone and cartilage tissue regeneration.

2.2 Fabrication techniques for Bone and Cartilage Scaffolds

A bio material is “any substance or a combination of substances either natural or synthetic in origin, which can be used for any period of time, as a whole or as a part of any system which treats, augments, or replaces any tissue, organ or function of the body”[34]. Synthetic scaffolds belong to the sub class of polymeric biomaterials. The goal of a scaffold is to provide a 3D environment for cells to grow on. For a biomimetic scaffold to form bone or cartilage, the model tissue is the extra cellular matrix (ECM). ECM of bone and cartilage are composed primarily of Col I and Col II fibrils respectively which are mineralised and form the basic building blocks of the tissue. Bone and cartilage scaffolds have moved towards exhibiting 3D features in the recent past. However, issues such as cost of production and control over scaffold properties still attract researchers to utilize film like 2D models and sheets for preliminary understanding of release patterns of bioactive molecules and in *in vitro* studies[35]. **Figure 2.5** summarises all the current fabrication techniques that have been actively used to produce 2D films and 3D scaffolds. The basic working principle, advantages and disadvantages of each of these are briefly explained in this section.

2.2.1 Thermally Induced Phase Separation (TIPS)

TIPS methods were first applied to tissue engineering of scaffolds in 1990's and it is a five-step process[36]. It involves polymer dissolution, phase separation, extraction of solvent, freezing and freeze drying. Several research labs have fabricated 3D nano-fibrous structures using this technique [36-39]. In a polymer like PLLA, under ambient conditions, the polymer rich phase forms the nano-fibrous matrix while the polymer deficient phase is extracted with the solvent resulting in nano fibers in 50-500 nm regimes.

While the advantages of the technique include highly porous and narrow pore size

distribution in the resulting scaffold combined with high reproducibility and simplicity of the process, the disadvantages such as need for additives and formation of a dense skin layer on the scaffold with remnant solvent remaining in the end product are major concerns for using this method to fabricate scaffolds for clinical applications[40].

Figure 2.5 Summary of all the fabrication techniques used in the formation of a scaffold for bone and cartilage tissue engineering.

2.2.2 Solvent Casting and Particulate Leaching

This technique involves the addition of salt particles of a specific diameter to a polymer solution to form a uniform suspension. The steps are incorporation of salt to a polymer solvent combination and casting the solution to enable solvent evaporation followed by vacuum drying. This scaffold is then immersed in water to leach off all the salt thus inducing the formation of pores. Porosity values of about 86% have been achieved through this technique with pore sizes exceeding 100 μm [41]. While attaining high porosity is an advantage of this technique, it is limited in terms of use as only thin membranes or 3 mm thick scaffolds can be fabricated by this method[42].

2.2.3 Melt Molding

This process involves filling a mold with polymer melt or powder and obtaining the shape of the mold. There are two variations of this technique namely compression molding and injection molding[43]. In the work published by Thompson *et al*, compression molding was employed and Teflon was the mold used. PLGA particles and gelatin micro spheres were added to the mold and heated above the glass transition temperature of PLGA, simultaneously applying pressure to the mixture. This led to the particles to bond together. Once the mold cooled, gelatin was leached out by immersion in water and the scaffold was dried[20]. Poor mechanical integrity and lack of structural stability limit the application of this technique in tissue engineering domain despite its ability to achieve uniformly porous structures.

2.2.4 Gas Foaming

Gas foaming includes the aspects of both melt molding and particulate leaching. High porosities of about 95% can be achieved by this technique with pore sizes in the range of 200-400 μm [44]. Open porous scaffolds can be fabricated by this technique. The fabrication process involves the addition of ammonium bicarbonate to the polymer solution in an organic solvent such as chloroform. The resulting mixture possesses high viscosity and is shaped using a mold. This is followed by solvent evaporation and finally vacuum drying or immersion in hot water[45]. The undesirable surface skin layer obtained in other techniques is not observed in gas foaming and this technique can be used in the future for various tissue engineering applications. The fabricated scaffold exhibits high porosity combined with good cell viability[46]. However, the mechanical properties of the scaffold fabricated through this method need to be tailored for clinical usage.

2.2.5 Freeze Drying

In this technique, the scaffold material is frozen and surrounding pressure is increased along with addition of heat to allow the water content in the frozen scaffold to sublime. This results in direct transfer of solid phase to a gaseous phase. Yannas *et al* have developed a collagen scaffold by freezing a solution of collagen and then freeze

drying it[47]. The pore sizes can be tailored by changing the freezing temperature and also with changes in pH[48]. Lack of structural stability is a drawback of this simple technique.

2.2.6 Fiber Bonding

This technique involves the casting of a solution of one polymer over fibers of another polymer mesh. Solvent evaporation takes place and the result is a non-bonded second polymer deposited on mesh like network of the first polymer. Remnant solvent is selectively removed from polymer 1. This method is generally considered as a post treatment for fibers that are fabricated through other techniques[49]. Fiber bonding is not suitable for cases which require precise control over porosity and cannot be used for polymers that dissolve in the same solvent.

2.2.7 Rapid Prototyping Techniques (RP)

RP constitute a family of processing techniques that rely on prototypes and Computer Aided Design (CAD) models for fabricating scaffolds (figure 2.5). There are several techniques that fall under the RP umbrella. They result in the formation of a 3D scaffold/pattern formed by layer by layer assembling of the virtual designs[50]. These techniques were restricted to surgical planning in the bone and cartilage tissue regeneration arena until Griffith and co-workers reported using one of the RP techniques called Stereo-Lithography (SLA) to fabricate ceramic components[51]. Complex scaffold architecture with a vast array of polymeric materials can be designed using any of these techniques or a combination of RP techniques. 3-dimensional printing (3DP) was first introduced by Brecht *et al*[52]. The advantages of this RP technique included independent control over pore size and porosity, ability to achieve pore sizes in the range of 45 -500 μm , high surface area to volume ratio and the choice of materials that could be used for this process [53]. However, the disadvantages of having to use toxic organic solvents and lack of mechanical strength over-shadow the benefits posed by this technique.[54]. Stereo lithography (SLA) employs the usage of a UV laser to photo crosslink and polymerize a photo curable monomer which is the raw material. The complexity of the steps involved and limitations in choice of photo curable raw materials are concerns posed by this

technique. The advantages include the relative ease to achieve minute features in the resulting scaffolds[55]. Selective Laser Sintering (SLS) requires high processing temperatures to sinter heat fusible powders to a CAD geometry model one layer at a time. While this processing technique boasts of complete control over pore geometry, is solvent free and gives good compressive strength, the heat involved in sintering process makes it undesirable for loading growth factors or any bioactive molecules for tissue engineering of the bone and cartilage[56]. Fused deposition modeling (FDM) uses a moving nozzle to extrude a polymeric fiber from which a physical model is built layer by layer. Macro shape control, solvent free fabrication and high porosity are the benefits of this technique which also suffers from concerns pertaining to limited material range, high processing temperatures and inconsistent pore openings[57]. Organ printing is advancement in ink jet printing except that the print gels are thermo responsive. Cells are directly sprayed onto the solidifying thin layer of polymer solution. This technique, if perfected can help in bridging the gap between demand and supply of cell laden scaffolds for tissue regeneration. However, the current concerns that require fine tuning are limitations with the choice of materials and the issue of formation of cell aggregates within the droplets[58].

While all the above methods have been extensively studied by the scientific community, it is a well-known fact that the cellular interaction with the ECM is both a dynamic and a demanding process. Hence the next generation of TE scaffolds focused on the attractive benefits that nanotechnology posed in designing scaffolds with precision and good mechanical integrity for use as bone and cartilage replacements[59]. The two major nano fabrication techniques for bone and cartilage TE are Self -Assembly and Electrospinning.

2.2.8 Self-Assembly

Self-assembly involves the spontaneous organization of individual components into an ordered and stable structure with pre- programmed non-covalent bonds. Only a few polymer configurations such as di block, tri blocks and dendrimers are fabricated using this technique as it is a complex laboratory procedure[60]. This complexity combined with low productivity restrict the large-scale utilization of this technique for precise TE applications[61].

2.2.9 Electrospinning

The first description of electrospinning dates back to 1902 when J F Cooley filed a United States patent entitled ‘Apparatus for electrically dispersing fibers’[62]. However major commercialization of this process began only in the 1930’s after the reports of Anton Formhals and Norton[63, 64]. The theoretical basis for electrospinning was developed in 1960’s by Sir Geoffrey Ingram Taylor through the Taylor cone model[65]. The concept of electrospinning is not a new one and it received much attention after integrating it with the field of nanotechnology in late 1990’s. It is a simple, reliable, robust and scalable technique that finds wide applicability in research labs and in industries. The combination of factors such as tailorable mechanical properties, wide array of choice of polymeric materials, cost effectiveness and formation of structurally diverse nanofibers have added on to the increased interest in this fabrication process[66-68]. Further control over thickness and composition along with the porosity of the fibrous meshes can be achieved using a simple experimental set up.

2.3 Working Principle of Electrospinning

The principle of this process is that electrostatic force is applied in order to drive fiber formation. An electric field creates a charged jet of polymer solution. This charged jet forms a conical shape (Taylor cone) at the tip of the needle. This is formed due to electrostatic repulsions from within the surface charges and electrostatic attractions from the oppositely charged collector. Once the voltage crosses a threshold value, the electrostatic repulsions overcome the surface tension and thus the solution is forced out of the tip as a jet[69]. As this jet travels in air, the solvent evaporates leaving behind a charged fiber that is collected on a grounded metal screen[70].

A lot of parameters can be varied to achieve desired dimensions of the final product. The most common parameters to take into consideration for electrospinning are classified into solution and processing parameters. Solution parameters include all variations that can be done with the polymer source namely its concentrations,

viscosity, electrical conductivity, molecular weight of the polymer and ratio of the polymers. Processing parameters are the machine's variable parameters like flow rate, high voltage, distance between tip of needle and collector geometry and composition. Variations in each of these parameters have an effect on the fibers that are spun. For instance, the fiber diameter increases with increase in concentration of the polymer solution and also with increasing flow rate from the tip to collector when all the other parameters are maintained constant[68]. The fiber diameter is known to decrease with increasing distance between the needle and collector or with increasing electrical potential that is applied[71]. Temperature inside the chamber, air velocity and humidity are classified under ambient conditions for Electrospinning[72].

Figure 2.6 Illustrates the working principle of an electrospinning set up. Adapted from [73] and reproduced with permission.

2.3.1 Electrospinning Set Up

Typical set up for electrospinning requires a polymer source, syringe pump, high voltage supply and a collector. Based on the type of collector used, random (flat collector) or aligned (rotating drum collector) and oriented fibers can be electro spun. Aligned fibers find applications in tendon tissue engineering or neural tissue engineering[74]. The two most widely used electrospinning setups are the horizontal and vertical setups. When the syringe pump is stationed parallel to the floor and the collector is stationed perpendicular to the floor, then the resultant electric field vector is parallel to the floor. This is the scenario in horizontal set up. When the resultant electric field is perpendicular to the floor, then the setup is known as a vertical electrospinning set up[75].

2.3.2 Variations in Electrospinning

Initially simple non-woven mats were the major end products for electrospinning. In the recent three decades, this scenario has changed and electrospinning is no longer restricted to simple mats. **Figure 2.7** provides an insight on the different structural variations that can be achieved with electrospinning.

Figure 2.7 List of different structural variations that can be achieved with electrospinning

2.3.3 Applications of Electrospinning

The applications of electrospinning are vast and spread over multiple fields. Electro spun nanofibers have been used as membrane filters, nanocomposite reinforcements

in construction work, in protective clothing, as filters for metal recovery and as templates for many processes [76-80]. In the arena of bio medical engineering, electro spun fibers have found extensive applications in both nano and micron regime. Many synthetic electro-spun polymers were investigated for applications including but not limited to cosmetic skin, drug delivery carriers, to make tissue engineered scaffold grafts, as surgical sutures, haemostatic devices, tubes for blood vessels and also as 3D scaffolds for bone and cartilage regeneration[81, 82].

2.4 Electrospinning in the Context of Bone and Cartilage Tissue Engineering.

Porosity and mechanical strength are the most important characteristics of any tissue engineered bone or cartilage[83]. In case of cartilage, mechanical stress endurance of 0.5MPa – 2MPa is sufficient however, structural integrity of the scaffold material must be intact throughout the intended course of utilization [84]. Being a versatile field, there are ongoing works around the globe for enhancing the material properties used for bone regeneration, cartilage damage and to fabricate drug loaded scaffolds for restoring the functions in degenerative conditions such as osteoarthritis and cartilage inflammation[85]. The final goal of scaffolds is to closely mimic the ECM of the native bone and cartilage; hence recently efforts are being channelled to fabricate scaffolds that can serve as engineered ECM [86, 87]. Osteochondral tissue engineering has gained significant importance with the advent of engineered ECMs to repair osteo and cartilage defects. Moreover, ECMs are attractive biomedical platforms for taking tissue engineering forward. Engineered ECMs promote constructive remodelling which means it enables specific tissue to form at site of implantation rather than an inferior scar tissue [88]. However, the final outcome is affected by several factors such as growth factor retention, degradation of polymers, biomechanical tolerance and complete evasion of the immune system by the ECM scaffolds [89]. Collagen fibers are the building blocks of the bone and cartilage and the dimensions of these fibers vary between 100 nm to 2000 nm. Bone is predominantly composed of Col I and hydroxyapatite which confers it the mineralized structure [90]. Cartilage, on the other hand, is composed of large amounts of proteoglycans and Col II which is oriented in a random manner in the central region of the cartilage tissue [91]. Electro spun fibers can be fabricated to mimic the native

ECM of the tissue to be engineered owing to their tailorable structure (nano/micro dimensions), high surface to volume ratio and their ability to promote cell adherence and proliferation. The clinical significance in the areas of bone and cartilage tissue engineering urges the need to investigate different strategies. The advancements in electrospinning with different types of polymers are overwhelming in the recent decade. 2D and 3D structures have been extensively investigated to replace conventional grafts that are composed of metals or exhibit immunogenicity when implanted *in vivo*[92].

2.4.1 Electrospinning for Bone Tissue Engineering

There have been multiple attempts to mimic the natural ECM of the bone tissue using nanofibers and nanocomposites fabricated by electrospinning [93]. Modulating the microenvironment of the ECM further by promoting encapsulation of bioactive molecules is also being investigated extensively [94-96]. Micron and nano dimensional materials have more crystal grains and more number of atoms which contribute to their higher surface to volume ratio in comparison to conventional materials[97]. The following table summarises some significant studies conducted using electrospinning by many research groups.

Table 2.2 Summary of research conducted using electrospinning technique for bone tissue engineering.

Polymer Material		Fiber diameter (nm)	Cell line used	Reference
Gelatin/PLLA PGA core	on	704 ± 100	MC3T3-E1	[98]
PCL/Col/HA		300 ± 135	Osteoblasts	[93]
PLLA/Col		319 ± 120	MG 63	[99]

PLGA/Col/HA	100 – 350	GFP hMSC	[100]
PCL/CS	102 ± 24	Osteosarcoma cells	[101]
PLGA/HA	266 ± 8	MC3T3-E1	[102]
PLLA/HA	2500-3500	MC3T3-E1	[103]
PCL	400 ± 200	MSCs	[104]
Gelatin- siloxane	NA	MSCs	[105]
PCL coated with CaP	1500	NA	[106]
PCL coated with Gelatin	1200	MC3T3-E1	[107]
Silk/PLLA/BMP-2	500 ± 76	MSCs	[108]
PCL/TCP	NA	MSCs	[109]
PLCL/Gelatin	400 - 600	hMSCs	[110]

PLGA	2000-4000	BMSCs	[111]
PCL/ PEG-b-PCL	248 $\pm$ 27	MC3T3-E1	[112]
PCL/PLGA	500-1200	MC3T3-E1	[113]
PCL/Gelatin	250-1300	MC3T3-E1	[113]
PLGA/BMP-2	1200 $\pm$ 80	<i>In vivo</i> studies	[114]

As the structure of bone consists of both organic and inorganic phases, bio ceramics have been a popular choice for attaining the desired structure of the scaffold. Bio ceramic particles have been mixed with polymer solutions and electro spun into nano/micron scaffold mats. However, these particles do not mix well with synthetic scaffolds and hence result in poor beaded electro spun end products [94]. Ravichandran *et al* reported a scaffold for bone tissue engineering which was electro spun with PLLA/Col and then dipped in solution of Ca-P thus depositing nano HA on the surface of the fibers. In another study conducted by Nandakumar *et al*, they coated a layer of HA on top of electro spun PBT and the scaffold was validated for osteogenic studies [115]. Gelatin/PLLA core shell nanofibers mimicked the osteon structure precisely and exhibited higher bioactivity [116]. Yunos *et al* fabricated electro spun PDLLA fibers which exhibited enhanced bioactivity after bioglass pellets were deposited on it as a post processing treatment [98]. Other researches on MSCs have illustrated the importance of fiber diameter on the growth, proliferation and cell attachment. This was found out by fabricating electro spun fibers using synthetic polymers in different diameter ranges from 50 -1000 nm. The cells showed better viability and migration when the fibers were in between 500 -1000 nm in diameter

and expressed upregulated osteogenic marker expression when compared to other diameter ranges [117]. The concept of using stem cells seeded on scaffolds to direct osteogenesis is considered a golden strategy. Various research groups have investigated this concept with excellent results in terms of direction of MSCs fate towards osteogenesis using a combination of growth factors delivered through the scaffold [118-122].

2.4.2 Electrospinning for Cartilage Tissue Engineering.

Until recent past, chondrocytes were the main cell sources for cartilage regeneration. However, their reduced doubling potential is an issue of concern. Further, when cultured as monolayers, after a few passages, the cells differentiate into fibroblasts and do not produce chondrogenic ECM molecules [123]. Multipotent MSCs have been studied for their differentiation potential into various lineages including cartilage [124]. Hence further studies were predominantly focussed on using MSCs to enable cartilage repair and regeneration. Through preliminary studies, the need for a three-dimensional micro-environment for directing the chondrogenic differentiation of MSCs was understood. This kind of environment enhanced cell-cell interactions and polymeric nano/micro fibers have been explored to direct the fate of MSCs towards chondrogenic induction which was validated with the presence of cartilage gene expression markers [125]. Sasa *et al* have combined the use of polymer nanofibers seeded with MSCs and external moulding set up to create a 3D tissue formation. They were able to produce an engineered cartilage that expressed robust chondrogenic phenotype by culturing in a spinneret flask under optimized conditions [126]. PLLA was electro spun with transforming growth factor I (TGF-I) and insulin like TGF (I-TGF) and evaluated for cartilage tissue engineering. These growth factor loaded scaffolds revealed significant deposits of proteoglycans and also demonstrated a high expression of hydroxyproline after in vitro culturing with MSCs [126]. Tubular PLGA structures were fabricated by Toyokawa using electrospinning to repair cartilage defects. The results were validated with studies using white rabbits and showed that the structure of the scaffold aided in formation of cartilaginous tissue with full thickness [127]. Polyvinyl alcohol-methacrylate was mixed with chondroitin sulphate (CS) to obtain electro spun scaffolds to study chondrogenesis process. This study was conducted by Coburn *et al* and after a period of 40 days, a hyaline cartilage like tissue

was formed from BMSCs that were seeded on the scaffold [128]. The abundant expression of Col II indicated the presence of hyaline cartilage tissue. Even though MSCs demonstrate the potential to differentiate into cartilage cells and have a longer life span than the chondrocytes, the optimal micro environment to arrive at a hyaline cartilage model needs fine tuning. Care must be taken that the cartilage formed does not undergo ossification at later stages. To effectively address cartilage defects, the location of the defect is to be taken into consideration before devising a scaffold system for tissue regeneration.

2.5 Bridging the Gap

The rationale for conducting this research is to explore electrospinning further as a fabrication technique that can help to create structurally diverse scaffolds to suit a wide array of applications. Various bioactive molecules such as BMP superfamily moieties and small peptides have been electro spun as scaffolds and studied for their efficacy in promoting osteogenesis and chondrogenesis as mentioned in the sections above. However, these substances are required in very minute quantities in clinical trials, are very expensive and the fabrication protocols lead to wastage of the moieties. To add on to this, many of these substances lose their bioactivity because of the post processing steps that are carried out to enhance aspects such as porosity or surface adhesion after electrospinning. Hence, despite the significant advancements in this field there is a dire need for a one step cost effective fabrication technique and affordable bioactive molecules to bridge the gap between demand and supply. The bioactive molecules used throughout this study are cost effective, readily available molecules that are required by the human body as supplements to induce the formation of bone or cartilage tissue. During this study, electrospinning was used to create fiber mats in the diameter range of 500 -800 nm, intertwined fibers which exhibited average diameter of about 30 μm and to electro spray micro-particles which are about 10 μm in diameter onto the thin fibrous sheet to form a bi layered scaffold. A simple one step strategy to fabricate a mechanically stable three-dimensional scaffold without the need for any complex post processing steps will play a major role in bridging the gap between the demand and supply of scaffolds for this field. Tuning this scaffold to release some cost-effective biomolecules that can direct the fate of the seeded cells towards a desired lineage is an added benefit. Further, even though electrospinning has

been in existence for many decades and is widely employed to fabricate scaffolds for tissue engineering, there are no reports in literature of dual structured scaffold system fabricated through simultaneous electrospinning and electro spraying technique. Similarly, there is no evidence of electrospinning bio molecule encapsulated intertwined fibers exhibiting a 3D architecture, acceptable porosity and mechanical stability. This has been achieved in my work using a single step electrospinning technique with mere manipulation of the solution and processing parameters. These structurally different scaffold systems if fabricated successfully can play a pivotal role in reducing the ever-increasing gap between demand and supply of functional scaffolds for bone and cartilage TE applications.

References

- [1] D.C. Rizzo, Fundamentals of anatomy and physiology 2015: Cengage Learning.
- [2] P. Garnero, Calcified tissue international, 2015. **97**(3): p. 229-240.
- [3] M.R. Allen and D.B. Burr, *Bone modeling and remodeling*, in *Basic and Applied Bone Biology*. 2013, Elsevier Inc.
- [4] A.J. Salgado, O.P. Coutinho, and R.L. Reis, Macromolecular bioscience, 2004. **4**(8): p. 743-765.
- [5] M.R. Urist, Science, 1965. **150**(3698): p. 893-899.
- [6] A.J. Sophia Fox, A. Bedi, and S.A. Rodeo, Sports health, 2009. **1**(6): p. 461-468.
- [7] H. Watanabe, Y. Yamada, and K. Kimata, The journal of biochemistry, 1998. **124**(4): p. 687-693.
- [8] D.J. Mooney and J.P. Vacanti, Transplantation Reviews, 1993. **7**(3): p. 153-162.
- [9] K. Lee, E.A. Silva, and D.J. Mooney, Journal of the Royal Society Interface, 2011. **8**(55): p. 153-170.
- [10] S. Bose, M. Roy, and A. Bandyopadhyay, Trends in biotechnology, 2012. **30**(10): p. 546-554.
- [11] L. Meinel, V. Karageorgiou, R. Fajardo, B. Snyder, V. Shinde-Patil, L. Zichner, D. Kaplan, R. Langer, and G. Vunjak-Novakovic, Annals of biomedical engineering, 2004. **32**(1): p. 112-122.
- [12] M.L. Ferrone and C.P. Raut, Surgical oncology clinics of North America, 2012. **21**(2): p. 201-213.
- [13] S.M. Kurtz, K.L. Ong, J. Schmier, F. Mowat, K. Saleh, E. Dybvik, J. Kärrholm, G. Garellick, L.I. Havelin, and O. Furnes, The Journal of Bone & Joint Surgery, 2007. **89**(suppl 3): p. 144-151.
- [14] K.F. Bader and J.W. Curtin, Archives of Surgery, 1968. **97**(3): p. 406-411.
- [15] M.M. Stevens, Materials today, 2008. **11**(5): p. 18-25.
- [16] J. Rouwkema, N.C. Rivron, and C.A. van Blitterswijk, Trends in biotechnology, 2008. **26**(8): p. 434-441.
- [17] T. Cordonnier, J. Sohier, P. Rosset, and P. Layrolle, Advanced Engineering Materials, 2011. **13**(5): p. B135-B150.
- [18] N.K. Harakas, Clinical orthopaedics and related research, 1984. **188**: p. 239-251.
- [19] X. Liu and P.X. Ma, Annals of biomedical engineering, 2004. **32**(3): p. 477-486.
- [20] R. Thomson, M. Wake, M. Yaszemski, and A. Mikos, Biopolymers Ii, 1995: p. 245-274.
- [21] M. Swetha, K. Sahithi, A. Moorthi, N. Srinivasan, K. Ramasamy, and N. Selvamurugan,

- International journal of biological macromolecules, 2010. **47**(1): p. 1-4.
- [22] N.-T. Dai, M.R. Williamson, N. Khammo, E.F. Adams, and A.G. Coombes, *Biomaterials*, 2004. **25**(18): p. 4263-4271.
- [23] Q. Shang, Z. Wang, W. Liu, Y. Shi, L. Cui, and Y. Cao, *Journal of Craniofacial Surgery*, 2001. **12**(6): p. 586-593.
- [24] R. Kenley, L. Marden, T. Turek, L. Jin, E. Ron, and J. Hollinger, *Journal of Biomedical Materials Research Part A*, 1994. **28**(10): p. 1139-1147.
- [25] C.K. Kuo and P.X. Ma, *Biomaterials*, 2001. **22**(6): p. 511-521.
- [26] D.W. Hutmacher, *Biomaterials*, 2000. **21**(24): p. 2529-2543.
- [27] R.S. Tuan, G. Boland, and R. Tuli, *Arthritis Res Ther*, 2002. **5**(1): p. 32.
- [28] M.B. Murphy, K. Moncivais, and A.I. Caplan, *Experimental & molecular medicine*, 2013. **45**(11): p. e54.
- [29] Y. Qi, D. Jiang, A. Sindrilaru, A. Stegemann, S. Schatz, N. Treiber, M. Rojewski, H. Schrezenmeier, S. Vander Beken, and M. Wlaschek, *Journal of Investigative Dermatology*, 2014. **134**(2): p. 526-537.
- [30] W.-J. Li, R. Tuli, C. Okafor, A. Derfoul, K.G. Danielson, D.J. Hall, and R.S. Tuan, *Biomaterials*, 2005. **26**(6): p. 599-609.
- [31] J.S. Boo, Y. Yamada, Y. Okazaki, Y. Hibino, K. Okada, K.-I. Hata, T. Yoshikawa, Y. Sugiura, and M. Ueda, *Journal of Craniofacial Surgery*, 2002. **13**(2): p. 231-239.
- [32] U. Nöth, R. Tuli, A.M. Osyczka, K.G. Danielson, and R.S. Tuan, *Tissue engineering*, 2002. **8**(1): p. 131-144.
- [33] J.M. Murphy, K. Dixon, S. Beck, D. Fabian, A. Feldman, and F. Barry, *Arthritis & Rheumatology*, 2002. **46**(3): p. 704-713.
- [34] D. Williams, *Journal of Materials science*, 1987. **22**(10): p. 3421-3445.
- [35] S. Bosi, R. Rauti, J. Laishram, A. Turco, D. Lonardon, T. Nieu, M. Prato, D. Scaini, and L. Ballerini, *Scientific reports*, 2015. **5**: p. 9562.
- [36] H. Matsuyama, H. Okafuji, T. Maki, M. Teramoto, and N. Kubota, *Journal of membrane science*, 2003. **223**(1): p. 119-126.
- [37] Y.S. Nam and T.G. Park, *Journal of biomedical materials research*, 1999. **47**(1): p. 8-17.
- [38] H. Do Kim, E.H. Bae, I.C. Kwon, R.R. Pal, J. Do Nam, and D.S. Lee, *Biomaterials*, 2004. **25**(12): p. 2319-2329.
- [39] T. Tanaka and D.R. Lloyd, *Journal of membrane science*, 2004. **238**(1): p. 65-73.
- [40] J.F. Kim, J.H. Kim, Y.M. Lee, and E. Drioli, *AIChE Journal*, 2016. **62**(2): p. 461-490.
- [41] R. Huang, X. Zhu, T. Zhao, and A. Wan, *Materials Research Express*, 2014. **1**(4): p. 045403.
- [42] A. Prasad, M.R. Sankar, and V. Katiyar, *Materials Today: Proceedings*, 2017. **4**(2): p. 898-907.
- [43] R. Scaffaro, F. Lopresti, L. Botta, S. Rigogliuso, and G. Gherzi, *Macromolecular Materials and Engineering*, 2016. **301**(2): p. 182-190.
- [44] J.J. Yoon and T.G. Park, *Journal of Biomedical Materials Research Part A*, 2001. **55**(3): p. 401-408.
- [45] L.D. Harris, B.-S. Kim, and D.J. Mooney, 1998.
- [46] Y.S. Nam, J.J. Yoon, and T.G. Park, *Journal of biomedical materials research*, 2000. **53**(1): p. 1-7.
- [47] F.J. O'Brien, B.A. Harley, I.V. Yannas, and L. Gibson, *Biomaterials*, 2004. **25**(6): p. 1077-1086.
- [48] M.-H. Ho, P.-Y. Kuo, H.-J. Hsieh, T.-Y. Hsien, L.-T. Hou, J.-Y. Lai, and D.-M. Wang, *Biomaterials*, 2004. **25**(1): p. 129-138.
- [49] G. Chen, T. Ushida, and T. Tateishi, *Macromolecular Bioscience*, 2002. **2**(2): p. 67-77.
- [50] S. Yang, K.-F. Leong, Z. Du, and C.-K. Chua, *Tissue engineering*, 2002. **8**(1): p. 1-11.
- [51] M.L. Griffith and J.W. Halloran, *Journal of the American Ceramic Society*, 1996. **79**(10): p. 2601-2608.
- [52] E. Sachs, A. Curodeau, T. Fan, J.F. Bredt, M. Cima, and D. Brancazio, *Three dimensional printing system*. 1998, Google Patents.
- [53] B. Leukers, H. Gülkan, S.H. Irsen, S. Milz, C. Tille, M. Schieker, and H. Seitz, *Journal*

- of Materials Science: Materials in Medicine, 2005. **16**(12): p. 1121-1124.
- [54] B. Berman, Business horizons, 2012. **55**(2): p. 155-162.
- [55] F.P. Melchels, J. Feijen, and D.W. Grijpma, Biomaterials, 2009. **30**(23): p. 3801-3809.
- [56] J.M. Williams, A. Adewunmi, R.M. Schek, C.L. Flanagan, P.H. Krebsbach, S.E. Feinberg, S.J. Hollister, and S. Das, Biomaterials, 2005. **26**(23): p. 4817-4827.
- [57] H. Chim, D. Hutmacher, A. Chou, A. Oliveira, R. Reis, T.C. Lim, and J.-T. Schantz, International journal of oral and maxillofacial surgery, 2006. **35**(10): p. 928-934.
- [58] V. Mironov, T. Boland, T. Trusk, G. Forgacs, and R.R. Markwald, Trends in biotechnology, 2003. **21**(4): p. 157-161.
- [59] Y. Kumashiro, M. Yamato, and T. Okano, Bio-Nanotechnology: A Revolution in Food, Biomedical and Health Sciences, 2012: p. 124-140.
- [60] R.G. Ellis-Behnke, Y.-X. Liang, S.-W. You, D.K. Tay, S. Zhang, K.-F. So, and G.E. Schneider, Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(13): p. 5054-5059.
- [61] F. Gu, L. Zhang, B.A. Teply, N. Mann, A. Wang, A.F. Radovic-Moreno, R. Langer, and O.C. Farokhzad, Proceedings of the National Academy of Sciences, 2008. **105**(7): p. 2586-2591.
- [62] J.F. Cooley, *Apparatus for electrically dispersing fluids*. 1902, Google Patents.
- [63] F. Anton, *Process and apparatus for preparing artificial threads*. 1934, Google Patents.
- [64] C.L. Norton, *Method of and apparatus for producing fibrous or filamentary material*. 1936, Google Patents.
- [65] C. Hendricks and J. Hogan, AIAA Journal, 1965. **3**(2): p. 296-301.
- [66] I.S. Chronakis, Journal of materials processing technology, 2005. **167**(2): p. 283-293.
- [67] M. Bognitzki, W. Czado, T. Frese, A. Schaper, M. Hellwig, M. Steinhart, A. Greiner, and J.H. Wendorff, Advanced Materials, 2001. **13**(1): p. 70-72.
- [68] A. Frenot and I.S. Chronakis, Current opinion in colloid & interface science, 2003. **8**(1): p. 64-75.
- [69] D. Li, J.T. McCann, Y. Xia, and M. Marquez, Journal of the American Ceramic Society, 2006. **89**(6): p. 1861-1869.
- [70] T. Subbiah, G. Bhat, R. Tock, S. Parameswaran, and S. Ramkumar, Journal of Applied Polymer Science, 2005. **96**(2): p. 557-569.
- [71] Y. Shin, M. Hohman, M. Brenner, and G. Rutledge, Polymer, 2001. **42**(25): p. 09955-09967.
- [72] N. Bhardwaj and S.C. Kundu, Biotechnology advances, 2010. **28**(3): p. 325-347.
- [73] D.H. Reneker, A.L. Yarin, H. Fong, and S. Koombhongse, Journal of Applied physics, 2000. **87**(9): p. 4531-4547.
- [74] F. Yang, R. Murugan, S. Wang, and S. Ramakrishna, Biomaterials, 2005. **26**(15): p. 2603-2610.
- [75] D. Rodoplu and M. Mutlu, Journal of Engineered Fibers and Fabrics, 2012. **7**(2): p. 118-123.
- [76] S. Koombhongse, W. Liu, and D.H. Reneker, Journal of Polymer Science Part B: Polymer Physics, 2001. **39**(21): p. 2598-2606.
- [77] C.S. Ki, E.H. Gang, I.C. Um, and Y.H. Park, Journal of Membrane Science, 2007. **302**(1): p. 20-26.
- [78] M. Bognitzki, H. Hou, M. Ishaque, T. Frese, M. Hellwig, C. Schwarte, A. Schaper, J.H. Wendorff, and A. Greiner, Advanced Materials, 2000. **12**(9): p. 637-640.
- [79] R.A. Caruso, J.H. Schattka, and A. Greiner, Advanced Materials, 2001. **13**(20): p. 1577-1579.
- [80] P. Gibson, H. Schreuder-Gibson, and D. Rivin, Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2001. **187**: p. 469-481.
- [81] A.J. Meinel, O. Germershaus, T. Luhmann, H.P. Merkle, and L. Meinel, European Journal of Pharmaceutics and Biopharmaceutics, 2012. **81**(1): p. 1-13.
- [82] Y. Zhang, C.T. Lim, S. Ramakrishna, and Z.-M. Huang, Journal of Materials Science: Materials in Medicine, 2005. **16**(10): p. 933-946.

- [83] S. Liao, R. Murugan, C.K. Chan, and S. Ramakrishna, *Journal of the mechanical behavior of biomedical materials*, 2008. **1**(3): p. 252-260.
- [84] V.C. Mow, S. Kuei, W.M. Lai, and C.G. Armstrong, *J Biomech Eng*, 1980. **102**(1): p. 73-84.
- [85] J.K. Sherwood, S.L. Riley, R. Palazzolo, S.C. Brown, D.C. Monkhouse, M. Coates, L.G. Griffith, L.K. Landeen, and A. Ratcliffe, *Biomaterials*, 2002. **23**(24): p. 4739-4751.
- [86] K.E. Benders, P.R. van Weeren, S.F. Badylak, D.B. Saris, W.J. Dhert, and J. Malda, *Trends in biotechnology*, 2013. **31**(3): p. 169-176.
- [87] D.A. Bichara, N.A. O'Sullivan, I. Pomerantseva, X. Zhao, C.A. Sundback, J.P. Vacanti, and M.A. Randolph, *Tissue engineering. Part B, Reviews*, 2012. **18**(1): p. 51-61.
- [88] S.F. Badylak, *Biomaterials*, 2007. **28**(25): p. 3587-3593.
- [89] S. Tottey, S.A. Johnson, P.M. Crapo, J.E. Reing, L. Zhang, H. Jiang, C.J. Medberry, B. Reines, and S.F. Badylak, *Biomaterials*, 2011. **32**(1): p. 128-136.
- [90] K.A. Hing, *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, 2004. **362**(1825): p. 2821-2850.
- [91] M.B. Goldring, K. Tsuchimochi, and K. Ijiri, *Journal of cellular biochemistry*, 2006. **97**(1): p. 33-44.
- [92] P. Brun, F. Ghezzi, M. Roso, R. Danesin, G. Palù, A. Bagno, M. Modesti, I. Castagliuolo, and M. Dettin, *Acta biomaterialia*, 2011. **7**(6): p. 2526-2532.
- [93] M.P. Prabhakaran, J. Venugopal, and S. Ramakrishna, *Acta biomaterialia*, 2009. **5**(8): p. 2884-2893.
- [94] S.-S. Kim, M.S. Park, O. Jeon, C.Y. Choi, and B.-S. Kim, *Biomaterials*, 2006. **27**(8): p. 1399-1409.
- [95] J.M. Holzwarth and P.X. Ma, *Biomaterials*, 2011. **32**(36): p. 9622-9629.
- [96] J.-H. Jang, O. Castano, and H.-W. Kim, *Advanced drug delivery reviews*, 2009. **61**(12): p. 1065-1083.
- [97] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, P. Balasubramanian, G. Jin, A. Valipouri, and S. Ramakrishna, *Journal of nanoscience and nanotechnology*, 2013. **13**(7): p. 4656-4671.
- [98] T. Andric, A. Sampson, and J. Freeman, *Materials Science and Engineering: C*, 2011. **31**(1): p. 2-8.
- [99] J. Paletta, F. Mack, H. Schenderlein, C. Theisen, J. Schmitt, J. Wendorff, S. Agarwal, S. Fuchs-Winkelmann, and M. Schofer, *Eur Cell Mater*, 2011. **21**: p. 384-395.
- [100] M.V. Jose, V. Thomas, Y. Xu, S. Bellis, E. Nyairo, and D. Dean, *Macromolecular bioscience*, 2010. **10**(4): p. 433-444.
- [101] K. Shalumon, K. Anulekha, K. Chennazhi, H. Tamura, S. Nair, and R. Jayakumar, *International journal of biological macromolecules*, 2011. **48**(4): p. 571-576.
- [102] L. Lao, Y. Wang, Y. Zhu, Y. Zhang, and C. Gao, *Journal of Materials Science: Materials in Medicine*, 2011. **22**(8): p. 1873-1884.
- [103] B. Chuenjitkuntaworn, P. Supaphol, P. Pavasant, and D. Damrongsri, *Polymer International*, 2010. **59**(2): p. 227-235.
- [104] H. Yoshimoto, Y. Shin, H. Terai, and J. Vacanti, *Biomaterials*, 2003. **24**(12): p. 2077-2082.
- [105] L. Ren, J. Wang, F.-Y. Yang, L. Wang, D. Wang, T.-X. Wang, and M.-M. Tian, *Materials Science and Engineering: C*, 2010. **30**(3): p. 437-444.
- [106] F. Yang, J. Wolke, and J. Jansen, *Chemical Engineering Journal*, 2008. **137**(1): p. 154-161.
- [107] X. Li, J. Xie, X. Yuan, and Y. Xia, *Langmuir*, 2008. **24**(24): p. 14145-14150.
- [108] C. Li, C. Vepari, H.-J. Jin, H.J. Kim, and D.L. Kaplan, *Biomaterials*, 2006. **27**(16): p. 3115-3124.
- [109] M.T. Arafat, C.X. Lam, A.K. Ekaputra, S.Y. Wong, C. He, D.W. Hutmacher, X. Li, and I. Gibson, *Soft Matter*, 2011. **7**(18): p. 8013-8022.
- [110] N.G. Rim, J.H. Lee, S.I. Jeong, B.K. Lee, C.H. Kim, and H. Shin, *Macromolecular bioscience*, 2009. **9**(8): p. 795-804.
- [111] D. Zolfagari, G. Kaka, S.S.H. Sadraee, and G. Herfehdoost, *Journal of Mazandaran University of Medical Sciences*, 2014. **24**(117): p. 65-73.

- [112] Y. Ji, L. Wang, D.C. Watts, H. Qiu, T. You, F. Deng, and X. Wu, *Dental Materials*, 2014. **30**(11): p. 1263-1273.
- [113] A.R. Padalhin, N. Thuy Ba Linh, Y. Ki Min, and B.-T. Lee, *Journal of Biomaterials Science, Polymer Edition*, 2014. **25**(5): p. 487-503.
- [114] C. Del Rosario, M. Rodríguez - Evora, R. Reyes, A. González - Orive, A. Hernández - Creus, K.M. Shakesheff, L.J. White, A. Delgado, and C. Evora, *Journal of Biomedical Materials Research Part A*, 2015. **103**(9): p. 2998-3011.
- [115] A. Nandakumar, L. Yang, P. Habibovic, and C. van Blitterswijk, *Langmuir*, 2009. **26**(10): p. 7380-7387.
- [116] M. Mastrogiacomo, A. Corsi, E. Francioso, M.D. Comite, F. Monetti, S. Scaglione, A. Favia, A. Crovace, P. Bianco, and R. Cancedda, *Tissue engineering*, 2006. **12**(5): p. 1261-1273.
- [117] Y.R.V. Shih, C.N. Chen, S.W. Tsai, Y.J. Wang, and O.K. Lee, *Stem Cells*, 2006. **24**(11): p. 2391-2397.
- [118] L.A. Smith, X. Liu, J. Hu, P. Wang, and P.X. Ma, *Tissue Engineering Part A*, 2009. **15**(7): p. 1855-1864.
- [119] L.T. Nguyen, S. Liao, S. Ramakrishna, and C.K. Chan, *Nanomedicine*, 2011. **6**(6): p. 961-974.
- [120] N. Binulal, M. Deepthy, N. Selvamurugan, K. Shalumon, S. Suja, U. Mony, R. Jayakumar, and S. Nair, *Tissue Engineering Part A*, 2010. **16**(2): p. 393-404.
- [121] J. Ma, X. He, and E. Jabbari, *Annals of biomedical engineering*, 2011. **39**(1): p. 14-25.
- [122] M. Shin, H. Yoshimoto, and J.P. Vacanti, *Tissue engineering*, 2004. **10**(1-2): p. 33-41.
- [123] R. Mayne, M.S. Vail, P.M. Mayne, and E.J. Miller, *Proceedings of the National Academy of Sciences*, 1976. **73**(5): p. 1674-1678.
- [124] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak, *science*, 1999. **284**(5411): p. 143-147.
- [125] W.J. Li, K.G. Danielson, P.G. Alexander, and R.S. Tuan, *Journal of Biomedical Materials Research Part A*, 2003. **67**(4): p. 1105-1114.
- [126] S. Janjanin, W.-J. Li, M.T. Morgan, R.M. Shanti, and R.S. Tuan, *Journal of Surgical Research*, 2008. **149**(1): p. 47-56.
- [127] N. Toyokawa, H. Fujioka, T. Kokubu, I. Nagura, A. Inui, R. Sakata, M. Satake, H. Kaneko, and M. Kurosaka, *Arthroscopy: The Journal of Arthroscopic & Related Surgery*, 2010. **26**(3): p. 375-383.
- [128] J.M. Coburn, M. Gibson, S. Monagle, Z. Patterson, and J.H. Elisseeff, *Proceedings of the National Academy of Sciences*, 2012. **109**(25): p. 10012-10017.

Chapter 3

Experimental Methodology

This chapter summarizes the experimental principles applied during this thesis and is split into six sections. The first and second sections reveal the techniques of electrospinning and electro spraying used in fabrication of all the scaffold systems. Scaffold Characterization techniques are discussed in the third section. These techniques include Scanning Electron Microscopy for morphological analyses, incorporation of the biomolecules was analysed through Fourier transform Infrared Spectroscopy and Differential Scanning Calorimetry, evaluation of the mechanical properties of the scaffolds was done using MTS C42 and Goniometer was used to determine the degree of hydrophilicity/hydrophobicity of the as-fabricated scaffolds with precision. The fourth section throws insights into the methods employed for evaluation of release kinetics of the encapsulated biomolecules which include High Performance Liquid Chromatography (HPLC) and Ultraviolet-Visual Spectroscopy (UV-Vis). To evaluate the cell compliance and potential of the scaffolds in directing the differentiation of MSCs into different lineages of the musculoskeletal system, a series of cell culture studies were conducted and the techniques used are described in detail in the fifth section on cell culture. Statistical analysis was done to demonstrate whether there were significant differences among and between groups of specimens. The methods used for statistical analysis are described in the last section.

3.1 Electrospinning and Electro spraying

Electrospinning is a versatile, scalable and reliable technique that has been in use for over four decades for fabrication of a wide spectrum of scaffold materials that are mostly fibrous in nature. Due to its cost effectiveness and scalability, the use of electrospinning ranges from academia all the way to industrial large scale applications in the fields of tissue engineering, drug delivery, food packaging, cosmetics and wound dressings[1]. Metallic, ceramic, composite, bioglass, natural, synthetic and hybrid scaffolds can be fabricated by manipulation of the electrospinning parameters. The scope of this thesis is restricted to synthetic biodegradable polymeric scaffolds that have obtained the Food and Drug Administration (FDA) approval.

3.1.1 Working Principle

The basic principle of electrospinning is that an electric field creates a charged jet of polymer solution. When the electrostatic repulsion pressure of charges exceeds the surface tension of the liquid, the liquid surface becomes unstable and erupts into a jet giving rise to a Taylor cone. The polymer chain entanglements within the solution prevent the liquid jet from breaking up into droplets. As the liquid jet stretches and travels through air, the solvent gets evaporated leaving a charged jet of fibers to be deposited on a grounded metal collector. **Figure 2.6** illustrates the schematics of an electrospinning set up as postulated by Doshi *et al*[2]. Electrospinning is precisely controlled by solution parameters, processing parameters and ambient parameters[3]. These are listed in **table 3.1**.

Table 3.1 Summary of various parameters that control the process of electrospinning.

Category	List of parameters
Solution properties	Solution concentration, solution viscosity, molecular weight, dielectric effect of the solvent, solution conductivity, surface tension.
Processing parameters	Voltage applied flowrate, diameter of the needle, distance between the tip of the needle and the collector.
Ambient conditions	Temperature and humidity

Electro spraying or “electro hydro dynamic atomization” differs from electrospinning in the aspect that the liquid jet that formed as a result of electric field, breaks up into small droplets. These droplets get sprayed onto the collector which can be an aluminium foil or water/ethanol placed in a boat shaped aluminium tray. **Figure 3.1** illustrates the concept of electro spraying, (rightmost) illustrates the formation of a liquid cone with a thin jet at its apex depicting forces acting on a cone-jet adapted from Hartman *et al*[4].

Figure 3.1 Electro spraying process is illustrated in with SEM image of the particles obtained from the process; The schematics on the right depicts the process of formation of a liquid cone jet and the forces acting upon it at a time.

There are various modes of electrospray depending on processing parameters and solution parameters. They can be divided into two major groups. The first group is characterized by small fragments ejecting from the nozzle including dripping, micro-dripping, spindles and ramified meniscus modes. The second group consists of cone-jet, multi-jet, oscillating-jet, ramified-jet and precession modes as they all form a jet first followed by jet disintegration[5]. **Figure 3.2** depicts the various modes used for electro spraying.

Figure 3.2 Different modes of Electro spraying as postulated by Jaworek *et al* and

reproduced with permission.

Electrospinning Set Up: The electrospinning set up used for all the experiments is MECC NANON 01-A system. The voltage range of this equipment is 0 – 30 kV and the flow rate can be adjusted manually. The collector is an aluminium foil placed on top of a plate collector and the distance between the tip of the needle to the collector can be varied between 8 – 15 cm. The transverse speed can be adjusted from 0-300 mm/s and syringes of either 5 mL or 10 mL can be loaded onto the system. All the experiments were carried out with a 22-gauge needle fitted to the clip spinneret unless otherwise mentioned. The variations in temperature and humidity inside the chamber were minimized using a continuous supply of air throughout the duration of spinning. **Figure 3.3** shows the image of the electrospinning set up that was used throughout the duration of this study.

Figure 3.3 MECC Nanon 01 A electrospinning Apparatus

3.1.2 Materials

Poly(lactide co-glyceride) [PLGA (Resomer LG 824 S) and PDLG5010] with an inherent viscosity of 1.7-2.6 (dL/g) were purchased from EVONIK industries. Polycaprolactone [PCL] with an average molecular weight of 80KDa, Dexamethasone (Dex), β -Glycerophosphate (β -Gly), Proline (Pro) and Ascorbic Acid 2 Phosphate (AA) were purchased from Sigma Aldrich. The tubing and plastic collectors for electrospinning were purchased from Cole Palmer Scientific. The solvents used in this

study namely chloroform (CHL), methanol (MeOH), dichloromethane (DCM), dimethyl Formamide (DMF), ethanol were obtained from Sigma Aldrich.

PLGA is a linear copolymer that can be prepared at different ratios between its constituent monomers, lactic (LA) and glycolic acid (GA)[6]. Different synthesis mechanisms are used to obtain PLGA and the processing parameters play a vital role in shaping the final product. PLGA degrades by hydrolysis of its ester linkages, through bulk erosion, in aqueous environments. Briefly, three stages can summarize its degradation: (i) hydration (ii) cleavage of covalent bonds (iii) solubilizing [7]. In my study, the degradation of the PLGA was rapid when compared to PCL owing to its amorphous nature and the presence of a higher number of ester bonds.

PCL, a semi-crystalline aliphatic polyester, is synthesized by ionic and metal catalyzed ring-opening polymerization (ROP) of the cyclic monomer ϵ -caprolactone [8]. The structure of PCL comprises a repeating unit of one ester group and five methylene groups. PCL has an excellent biocompatibility and slow degradation rate [9]. Its ester linkages can be hydrolysed and excreted under normal physiological conditions. The degradation rate of PCL is slower than other aliphatic polyester [10].

3.1.3 Electrospinning of 2D fibers for Sandwich Structure

The polymers and solvents were mixed together in the ratios stated below to form a homogenous solution that was subjected to mechanical stirring overnight before spinning into thin fibrous layers. In brief, a known quantity of PLGA and PCL were added to a 20 mL glass bottle and pre-calculated quantity of the solvents was then pipetted in to the bottle to arrive at the final desired concentration. For fabrication of PLGA/PCL fibers, DCM/MeOH was the preferred solvent. The solutions containing all biomolecules were also prepared in a similar manner by first dissolving the drug in a small quantity of solvent and then introducing this solution to the polymer solution followed by overnight stirring. Preliminary experiments with varying concentrations of polymers were instrumental in tailoring the final concentration of the particles on fibers system. By varying the solvent composition ratios, the morphology of the obtained scaffold varied drastically. The variation with respect to the percentage of drug loaded will be discussed in detail at a later section. The various concentrations of the polymers are listed in **table 3.2**.

Table 3.2 Polymer concentrations and solvent ratios used for electrospinning of PLGA/PCL fibers

PLGA PCL Fibers					
Polymer concentration PLGA/PCL	Solvent ratio [DCM/ MeOH]				
100:0	9:1	8:2	1:1	2:8	1:9
80:20	9:1	8:2	1:1	2:8	1:9
50:50	9:1	8:2	1:1	2:8	1:9
20:80	9:1	8:2	1:1	2:8	1:9
0:100	9:1	8:2	1:1	2:8	1:9

Finally, a 1:1 PLGA: PCL concentration with 12% (w/w) of PLGA/PCL in 4:1 ratio of DCM/MeOH was optimized for achieving the fibers for the sandwich structure.

3.1.4 Electro spraying of Particles

For electro spraying of 5 - 10 micron sized PLGA particles onto the bottom layer of fibrous sheet, the preferred solvent of choice was CHL/DMF solution. The ratio of the polymers to the solvents tested was same as those presented in **Table 3.2**. After initial optimizations, a 3% (w/w) of PLGA in 4:1 ratio of CHL/DMF system was chosen for spraying particles.

3.1.5 Fabrication of the Sandwich Structure

The parameters for electrospinning and electro spraying can be classified broadly as solution parameters and processing parameters. The processing parameters pertain to the tenability offered by the machine. Voltage, distance between the collector and the tip of the needle and flowrate are the major parameters in play as far as the sandwich system is concerned. The effects caused by the variations of these are discussed in detail in section in 4.1.1. The solution parameters comprise of the inherent viscosity of the polymer solution, solution concentration and conductivity of the solution. All these directly affect the spin ability and spraying of the polymer solution into fibers

and particles respectively. These are detailed in section 4.1.2. It is of vital importance to understand the roles played by each of the mentioned parameters as the final morphology and dimensions of the fibers and particles are the result of a synergistic effect of the solution and processing parameters.

PLGA/PCL fibers were first electro spun and vacuum dried before the particles were deposited on them following another thin layer of fiber deposition. A fibrous scaffold mat with dimensions of $150 \times 50 \times 0.8 \text{ mm}^3$ was achieved through electrospinning and dried overnight before spraying particles onto it and finally spinning another thin fibrous sheet over the particles to prevent risking their loss during characterization and further studies. The optimized parameters for spinning were 18kV, 14cm needle to target distance and 1.5mL/h flowrate with a constant spinneret speed of 20mm/s. For electro spraying onto the pre-spun fiber layer, a voltage of 15kV with tip to target distance of 10cm and flow rate of 0.5 mL h^{-1} was the desired set of parameters (**figure 3.4**). The flow rate, however, could be tailored in accordance with the fiber to particle ratio needed. For preparing drug loaded fibrous mats and particles, Dex was loaded into the fibers solution and AA was added to 1% (w/v) of particle solution before spinning and spraying respectively. After electrospinning and electro spraying, all the specimen scaffolds were subjected to vacuum drying for 48 hours before further characterization. After preliminary characterizations, the percentage of loaded biomolecules was reduced to 1% polymer weight each due to their excellent encapsulation efficiencies.

Figure 3.4 Illustration of the process of electrospinning and electro spraying.

3.2 Fabrication of 3D Scaffolds by Electrospinning

3.2.1 Materials

Polycaprolactone [PCL, molecular weight = 80 000 Da; Sigma Aldrich]; poly(-L lactic acid) [PLLA, intrinsic viscosity = 6.5 dl g⁻¹; Purac Biomaterials] and polyethylene oxide [PEO, molecular weight = 4 x 10⁶ Da; Sigma Aldrich] were used to fabricate the scaffolds. Laboratory chemicals and solvents were purchased from Sigma Aldrich (Singapore); cell culture reagents were purchased from Thermo Fisher Scientific (Singapore).

3.2.2 Porous 3D Fibrous Scaffolds

Briefly, the polymer was prepared in 1,1,1,3,3,3hexafluoro-2-propanol (HFIP) and homogenized overnight with magnetic stirring. The electrospinning process was then conducted using the MECC NANON Systems (MECC Co. Ltd., Fukuoka, Japan) with a 22-gauge blunt needle for 4 h per sample under ambient temperature and humidity. The fibrous scaffolds collected on the aluminum foil were then dried in a vacuum over 72 h to remove any residual organic solvent. In this study, the 3D fibrous scaffolds were optimized by varying both the polymer blend solution (i.e. polymer concentration and composition) and electrospinning parameters (i.e. flow rate, voltage, tip to target distance and spinneret speed). The effect of the polymer concentration on the formation of 3D scaffold was first investigated with fixed a PLLA: PCL: PEO ratio (66.5:28.5:5) for different polymer concentrations (2% w/v, 5% w/v and 8% w/v)..

The electrospinning parameters were tailored after exploring a range of values as presented in chapter 5. The optimized parameters used for fabrication of all 3D scaffolds are - 15 kV, 1 mL h⁻¹ flowrate, 2 mm s⁻¹ spinneret speed and a 12-cm distance between needle tip and the collector. The optimized polymer concentrations investigated are summarized in **table 3.3**.

Table 3. 3 A list of fibrous scaffolds developed from varying the polymer concentration or their composition.

Scaffold	Concentration (% w/v)	PLLA (%)	PCL (%)	PEO (%)
Variation of polymer concentration				
2%	2	66.5	28.5	5
5%	5	66.5	28.5	5
8%	8	66.5	28.5	5
Variation of polymer composition				
A	8	66.5	28.5	5
B	8	70.0	30.0	0
C	8	28.5	66.5	5
D	8	95	0	5
E	8	100	0	0

3.3 Materials Characterization

3.3.1 Morphological Assessment

Scanning Electron Microscope (SEM) is a powerful and versatile imaging technique utilized in the arenas of biology, metallurgy, physics, medicine, geology and for other scientific applications. SEM is often used in conjunction with Energy-Dispersive X-ray Microanalysis (EDX, EDS, EDAX) which in turn provides information on the composition of individual crystals. The working principle of an SEM is that when accelerated primary electrons strikes the sample, it produces secondary electrons. These secondary electrons are collected by a positive charged electron detector which in turn gives a 3D image of the sample. There are different types of electron images. The two most common are the secondary electron image (SEI) and the backscattered electron image (BEI). SEI is used mainly to image fracture surfaces and gives a high-resolution image whereas BEI is used to image a polished section. **Figure 3.5** is a digital image of the JEOL SEM 6360.

Figure 3.5 SEM 6360A (Jeol JSM, USA)

EDX is complementary to the SEM and operates on the principle that the electron beam generates X-rays within the specimen. These X-rays have characteristic energies of the elements that emitted them. So, if one can measure the energy of the X-rays, the elements present in the specimen can be determined. There are three principal components to a basic EDX system: The X-ray detector; a box of electronics called the "pulse processor" that measures the voltage pulses corresponding to the X-

ray energies, and a computer. In my work, the morphology of the electro spun and electro sprayed scaffolds were analyzed using the Scanning Electron Microscope (SEM; JEOL JSM 6360A, USA) with an accelerating voltage of 3-5 keV at a magnification of 500x and above. Samples were first gold coated at 20 mA for predetermined time interval. EDX analysis was used to detect the presence of calcium ($K\alpha = 3.619$) and phosphorus ($K\alpha = 2.013$) in the cell seeded scaffolds after 21 days of cell culture followed by freezing of the cell laden scaffolds.

3.3.2 FTIR and Raman Analysis

Fourier transform infrared (FT-IR) spectrometers obtain infrared spectra by first collecting an interferogram of a sample signal using an interferometer. An FT-IR spectrometer collects and digitizes the interferogram, performs the FT function, and displays the spectrum. The working principle of an FTIR is based on the experimental set up of the Michelson Interferometer[11].

Raman spectroscopy is of great potential in a broad range of applications from pharmaceuticals, materials sciences to gem identification and forensics. In the field of materials science, it is employed widely for research pertaining to polymers, fuel cells, minerals and mining industry, semiconductor arena and in textile and fabric industries. Raman is a vibrational spectroscopy that can provide chemical and component information with high spatial resolution[12]. The working principle of Raman is that when light hits a sample, the sample is excited and is forced to move and vibrate. These vibrations are measured in Raman spectroscopy. The vibrational information which is specific to the chemical bonds and symmetry of molecules provides a fingerprint by which the molecule can be identified. It is both qualitative and a quantitative technique. A molecule which undergoes change in polarizability when subjected to vibration is known to be Raman active[13]. In other words, a laser source causes in-elastic scattering of light whereby photons are initially absorbed on the sample's surface and later re-emission occurs with a minor shift in frequency. This effect is termed as Raman Effect. The instrumentation comprises of a laser source, sampling optics, wavelength selector and detector as shown in **figure 3.6**.

Figure 3.6 Raman Spectrometer Instrumentation reproduced with permission from [13].

In this work, the chemical structure of the blank and the drug loaded scaffolds were analyzed using attenuated reflectance FTIR Spectroscopy (Perkin Elmer Frontier, USA) as shown in **figure 3.7a**. All samples were measured over a wavelength range between 4000 to 600 cm^{-1} and the spectral resolution was 4 cm^{-1} with an average accumulation of 32 spectrums per sample. Further Raman spectra were also obtained to confirm the chemical composition of the as synthesized scaffolds. Raman spectra was recorded using WiTec Alpha 300SR (**figure 3.7b**) under a wavelength of 633nm generated using a He-Ne laser source. Data was acquired over a period of 3 s and averaged for 32 accumulations.

Figure 3.7 Digital Images of (a) Perkin Elmer FTIR Spectrometer and (b) WiTec Alpha 300SR Raman Spectrometer.

3.3.3 Differential Scanning Calorimetry

In any reaction, the quantity of heat exchanged is measured using calorimetry. Polymers undergo thermal transitions because of heating. Differential scanning calorimetry (DSC) is a simple and reliable technique that helps the user to evaluate what happens to a polymer when it is heated. This is measured using the differences in heat flow between two pans; one of them is an empty reference pan while the other has the polymer sample sealed in it. Both these pans are placed on a heater and when phase transitions occur, either endothermic or exothermic reactions takes place in order to maintain the same temperature in both the pans. DSC is useful in determining characteristic properties of polymers such as its melting point (T_m), crystallization point and glass transition temperature (T_g). This knowledge is indicative of a polymeric sample's purity or the efficacy of a blend formation when two or more different polymers are involved[14]. The incorporation of a drug inside a polymer can also be evaluated qualitatively using DSC if the T_m of the drug and the polymer are known. In my work, DSC analyses were conducted using TA Instrument (DSC Q20, USA) analyzer (**figure 3.8**). The heat flow and temperature calibration was done with aluminium standard reference. The weight of all the scaffolds was maintained at 3-4mg range. A dry nitrogen atmosphere with a flowrate of 50 mL/h was maintained for all the thermal analyses experiments.

Figure 3.8 Digital image of the Differential Scanning Calorimetry Q20

3.3.4 Mechanical testing

It is of vital importance for any scaffold material to exhibit mechanical properties that can match the specific application it is intended for. Hence mechanical testing, in particular tensile testing is a significant and essential tool that can provide information on the applicability of the scaffold as such. Scaffolds should ideally have sufficient mechanical strength during in vitro culturing to resist the physiological mechanical environment in regenerating load-bearing tissues (cartilage, bone) at the desired implantation site[15]. The mechanical strength of the scaffolds was measured using MTS Criterion 40 series (C42 system **figure 3.9**) after recording the sample thickness length and width using a Vernier caliper.

Figure 3.9 MTS Criterion 40 series - C42 system

Samples were tested in tensile mode by increasing strain at a rate of $10 \text{ mm minute}^{-1}$ until they broke. Samples that exhibited necking behavior were not taken into consideration. The distance between the clamp grips was 25 mm. The ultimate tensile strength and strain at break were determined from the stress-strain curves generated from three independent experiments for each specimen. The Young's modulus was determined by calculating the gradient of the initial linear slope of the stress-strain curve. For reference, the tensile strength of plain PCL, PLGA, PLLA, PLLA/PCL and PLGA/PCL scaffold mats were also measured under the same conditions. Similarly the homogenous compression tests were performed at a crosshead speed of 5 mm

minute⁻¹ and each sample's thickness was 0.7 cm (n=3). The final compression value post testing was recorded followed by calculation of compressive strength and breakage strain values.

3.3.5 Contact Angle measurement

Contact angle indicates the degree of wetting when a solid and liquid interact. When the wettability is high, contact angle $< 90^\circ$ and vice versa. More specifically, a polymeric scaffold with a contact angle less than 90° is favorable for cell studies as the fluid will spread over a large area on the surface; while contact angles $\gg$ than 90° indicate that the polymer is unfavorable hence the fluid will minimize its contact with the surface and impede adhesion and spreading of cells[16].

Figure 3.10 FTA200 Dynamic Contact Angle Analyzer

Water contact angle for fibrous mats of plain scaffolds and drug loaded scaffolds were determined through a goniometer (FTA200 Dynamic Contact Angle Analyzer **figure 3.10**). This method of calculating the contact angle is referred to as the direct contact method and its advantages lie in its simplicity and requirement of few microliters of liquid drop. The instrumentation precisely consists of a horizontal stage with an illumination source, camera to take pictures of the liquid drop on the surface of the specimen and lastly a motor driven syringe that dispenses required volume of liquid onto the sample surface. In brief, 6 μ L of deionized water was carefully dispensed onto the surface of each scaffold and the direct microscopic measurement of contact angle was carried out using the sessile drop mode. The mean contact angle was calculated for n=5 for each scaffold mat/cast film of polymer blend.

3.3.6 Shear rate-dependent viscosity

Rheology studies provide firsthand information pertaining to the flow and deformation of materials. In other words, deformation and flow are referred as strain or strain rate respectively, hence rheology is referred to as study of stress-strain relationship of materials. Viscosity is the measure of the internal friction contained in a material, in this case a fluid (polymer solution). Viscosity measurements are quick, accurate and most reliable way to analyze some of the most important factors like presence of batch to batch variation or consistency of the polymer solution. Rheological relationships help us to understand the fluids we are working with in order to enable the user to manipulate its properties according to need. In general, fluids are classified as Newtonian and non-Newtonian based on their flow properties. Polymers fall into the category of non-Newtonian fluids and thus the relationship between shear stress and shear strain is non-linear. A rheometer is effective in containing the specimen in a geometric configuration, controlling the environmental factors around it, and also measuring changes in stress and strain. In the course of my study, the intrinsic viscosity of the polymers used for the sandwich scaffold system was constant and only the solution viscosity for the 3D scaffolds was measured in the controlled shear rate mode, using cone-plate geometry (CP-60) with an Physica Rheometer MCR 301 (Anton Paar, Singapore) equipped and PTD 200 Peltier temperature control device set at $25.0 (\pm 0.1) ^\circ\text{C}$ (**figure 3.11**).

Figure 3.11 Physica Rheometer MCR 301 Anton Paar, Singapore

The shear rate was set to increase from 0.1 to 10,000 s⁻¹ logarithmically and with a data point gradient of 10. Data was obtained and further analyzed with the help of Rheoplus v2.66 software. Triplicates were performed for each polymer solution prior to electrospinning.

3.4 Encapsulation of Biomolecules

The biomolecules that were studied during this dissertation are listed below with the scaffold system that they were used alongside with. To evaluate the efficacy of the fabricated scaffold systems, either single or multiple biomolecules were loaded onto the fibers/particles/3D scaffolds to study their individual and simultaneous release profiles, how their burst release patterns varied and their respective encapsulation efficiencies in the different scaffold systems.

For the study pertaining to the dual structured “sandwich” scaffold dexamethasone (Dex) was the preferred drug of choice for the fibrous sheet and the loading percentages of Dex was 4 wt% initially and was finally brought down to 1 wt%. The particles were loaded with multiple biomolecules either individually or in combination. For scaffolds that were intended for use for differentiation of MSCs into osteogenic lineage, the particles were loaded with 1 wt%. of β -Gly and 1 wt%. of AA. For scaffolds that were intended for use to study the differentiation potential of MSCs into chondrogenic lineage, 1 wt%. of Proline and 1 wt%. AA were the biomolecules encapsulated into the particles that were sprayed on top of the fibrous sheet containing Dex. The topmost layer of fibrous sheet was solely to ensure that the particles did not drop off from the fibers and to help to achieve the goal of a sandwich structured scaffold system. No drugs were loaded onto this layer which was a very thin electro spun mat of the same polymer that constituted the bottom most layer of the scaffold.

For the study comprising of the 3D scaffolds, Dex was the chosen drug of interest which was loaded onto the PLLA and then mixed with other polymers. Based on the promising results of the sandwich system with AA, Proline and β -Gly, a second set of experiments with 3D fibers containing AA + Pro in one scaffold and AA + β -Gly in another scaffold was also planned and conducted. The drug loading for all the 3D scaffolds remained constant at 2 wt% for all the fabricated scaffolds.

3.4.1 Methods for Detection of Biomolecules

High Performance Liquid Chromatography (HPLC)

Chromatography separates mixtures of substances into their components taking into account their molecular structure and composition. This technique involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase generally flows through the stationary phase thus carrying the components of the mixture with it. Components that display stronger interactions with the stationary phase progress more slowly through the column than components with weaker interactions. This difference in rates causes the separation of various components. Chromatographic separations can be carried out using a variety of stationary phases, including immobilized silica on glass plates (thin-layer chromatography), volatile gases (gas chromatography), paper (paper chromatography) and liquids (liquid chromatography). HPLC is a powerful analytical technique used for effective identification, separation and quantification of each compound that is present in a solution mixture[17]. The system used for the analysis of different molecules for the duration of this study was the Agilent 1100 HPLC system (**figure 3.12**).

Figure 3.12 Agilent 1100 HPLC system

The instrumentation mainly comprises of a sampler, pumps and a detector (**figure 3.13**). The auto-sampler (G1313A-1100) functions to extract the sample which is passed to interact with the mobile phase. A high-pressure Quaternary pump (G1311A-1100) ensures that the mobile phase is pumped into the system. The mobile phase together with the sample is flushed into the stationary phase which is the column. The column used in this study is a C18 column with the following dimensions: 5 μm , 250 mm x 4.6 mm purchased from Agilent. The good maintenance of the column is vital for avoiding false positive results and unwanted peaks. This can be achieved by following simple precautions while preparing the sample by first filtering the mobile phase or using HPLC grade (filtered) mobile phases, avoiding excess salt concentrations in the mobile phase which may get stuck to the column and reduce its lifetime and subject the analyte to contamination or false positive peaks. The sample to be quantified was also filtered using a 0.22 μm filter before introducing it into the HPLC vials and sealing them. The column is to be washed with mobile phase before and after the experimental run every time. This is also to avoid clogging of the column by the salt/particles present in the mobile phase buffer solution. Based on factors such as partition coefficient, velocity of the elements in the sample and the characteristic retention time, the elution time can be calculated for each sample peak to appear.

Figure 3.13 Schematic of working components of HPLC reproduced with permission from [17].

The detector utilized is an UV-Vis and its principle is based on the Beer-Lambert Law[18]. Each analyte selectively absorbs at a wavelength. This information is translated into an electrical signal, recorded and transferred to a recorder. Then, a unique UV peak appears on the chromatography at its corresponding elution time point. The peak area is proportional to the analyte concentration. The UV-Vis detector

is typically used as it is responsive to most organic compounds, with broad detection range and high sensitivity. Once the run for each experiment is complete, the data with peak information is collected and analyzed offline.

UV Vis Spectroscopy (UV-Vis)

The concentration of AA during predetermined time points was quantified with the help of a UV-Vis spectrophotometer (UV-Vis NIR Cary 5000 **figure 3.14**). The working principle of this technique is in accordance with Beer Lambert law as stated above. However, using UV-Vis eliminates the tedious steps of having to prepare a mobile phase and column maintenance which are vital for obtaining reproducible results through HPLC. A standard calibration curve is first plotted for AA with different concentrations and then the unknown concentrations are found with the help of the standard curve. This instrument is reliable; however, it is not as sensitive as HPLC and hence is not employed for the detection of Dex, Proline and other biomolecules which are present in micromolar (μM) concentrations. In contrast AA is present and detected in millimolar (mM) concentrations; hence UV-Vis is used for quantification. For all experiments and measurements, a solution volume of 2 mL of the AA standards and release media were used. The peak for AA appeared at 262 nm and the reference solution used for all release study samples was deionized water ($\text{DI H}_2\text{O}$).

Figure 3.14 UV-Vis NIR Cary 5000

3.4.2 Encapsulation Efficiency of the Biomolecules

Encapsulation efficiency is generally defined as the ratio of actual to theoretical drug loading within the scaffolds[19]. The calculation equation is as follows:

$$\begin{aligned} & \text{Encapsulation Efficiency (\%)} \\ &= \left\{ \frac{\text{actual dex loaded into the scaffold}}{\text{theoretical dex loaded into the scaffold}} \right\} * 100\% \end{aligned}$$

3.4.2.1 Quantitative Assessment of Dexamethasone Release Profile

Briefly, 2 wt% Dex in HFIP was mixed with the polymer and stirred overnight to form a homogenous solution. This polymer solution was electro spun into a 3D fibrous scaffold, and dried in vacuum. The encapsulation efficiency was calculated as by first recording the dried weight of Dex loaded 3D scaffold which was dissolved in dichloromethane (DCM). Once the scaffold disintegrated, the encapsulated Dex extracted using a known quantity of phosphate buffered saline (PBS) and subjected to High Performance Liquid Chromatography (HPLC) with a flow rate of 1.0 mL h^{-1} at a wavelength of 246 nm. The mobile phase used for the detection of Dex was 2 mM acetate buffer solution ($\text{pH } 4.8 \pm 0.82$) and the entire process was carried out using C18 column ($5 \mu\text{m}$, $250 \text{ mm} \times 4.6 \text{ mm}$) where the peak for Dex was observed at 4.05 minutes. The encapsulation efficiencies (EE) were calculated for $n=3$. The same wavelength was also used to calculate the release profile for Dex. The scaffolds were immersed in 1 x PBS, and incubated at 37°C in a shaking incubator at a rate of 100 strokes per minute. Known quantities of PBS was removed and replaced at different time intervals and the harvested samples were stored at 4°C till further characterization. The retention time for each sample in the column was 12 minutes. The peak for Dex appeared in between 3.8 - 4.2 minutes. Triplicates were analyzed for this assay. The same protocol was followed for quantifying Dex from the sandwich scaffolds too.

3.4.2.2 Quantitative Assessment of Ascorbic Acid

For the 3D scaffold, briefly, 2 wt% AA in DI H_2O was mixed with the polymer and stirred overnight to form a homogenous solution. This polymer solution was electro spun and dried in vacuum. The EE was calculated by first recording the dry weight of AA loaded 3D scaffold which was then dissolved in DCM. Once the scaffold disintegrated, the encapsulated AA was extracted using a known quantity of DI H_2O and subjected to UV-Vis analysis at a wavelength of 262 nm. A standard calibration

plot is essential for determining the concentration of the drug and this was prepared by serial dilution of known quantity of AA in DI H₂O and a set of six readings were taken from which the calibration curve for quantifying AA was obtained. The same wavelength was also used to calculate the release profile for AA loaded samples collected at predetermined time intervals and stored at +4°C. Triplicates were analyzed for this assay.

For the determination of AA encapsulated in the particles in the sandwich system, the protocol remains the same as mentioned above. In the case of the sandwich system, the total concentration of AA loaded into the particles was 1 wt % with respect to the polymer.

3.4.2.3 Quantitative Assessment of β -Gly

For the 3D scaffold system, briefly, 2 wt% β -Gly in DI H₂O was mixed with the polymer and stirred overnight to form a homogenous solution. This polymer solution was electro spun into a 3D fibrous scaffold, and dried in vacuum. The EE was calculated by extracting the drug in a similar manner to AA extraction and the solution was subjected to HPLC analysis flow rate of 0.5 mL h⁻¹ at 254 nm. The mobile phase used for the detection of β -Gly was 10 mM ammonium acetate containing 10 % MeOH solution and the entire process was carried out using C18 column (5 μ m, 250 mm x 4.6 mm) where the peak for β -Gly was observed at 2.2 - 2.4 minutes. A standard calibration plot was prepared by serial dilution of known quantity of β -Gly in DI H₂O with six readings. The same wavelength was also used to calculate the release profile for β -Gly loaded samples collected at predetermined time intervals and stored at 4°C. Triplicates were analyzed for this assay.

For the determination of β -Gly encapsulated in the particles in the sandwich system, the protocol remains the same as mentioned above. In the case of the sandwich system, the total concentration of β -Gly loaded into the particles was 1 wt% with respect to the polymer.

3.4.2.4 Quantitative Assessment of Proline

For the 3D scaffold system, 2 wt% Pro in DI H₂O was mixed with the polymer and stirred overnight to form a homogenous solution. This polymer solution was electro spun into a 3D fibrous scaffold, and dried in vacuum. The encapsulation efficiency

was calculated by first recording the dried weight of Pro loaded 3D scaffold which was dissolved in DCM. Once the scaffold disintegrated, the encapsulated drug was extracted using a known quantity of DI H₂O: TFA in the ratio of 20:1 and subjected to HPLC analysis flow rate of 1.0 mL h⁻¹ at 210 nm. The mobile phase used for the detection of Pro was water (10%), ACN (90%), 0.5% H₃PO₄, 0.1% TFA and the entire process was carried out using ABI C8 RP-HPLC column (5 µm, 220 mm x 4.6 mm) where the Pro separated out at 25 - 28 minutes[20]. A standard calibration plot is essential for determining the concentration of the drug and this was prepared by serial dilution of known quantity of Proline in DI H₂O: TFA in the ratio of 20:1 and a set of six readings were obtained. The same wavelength was also used to calculate the release profile for Proline loaded samples collected at predetermined time intervals and stored at 4°C. Triplicates were analyzed for this assay.

For the determination of Proline encapsulated in the particles in the sandwich system, the protocol remains the same as mentioned above. In the case of the sandwich system, the total concentration of Proline loaded into the particles was 1 wt% with respect to the polymer.

3.5 Cell Culture

Materials: Human Mesenchymal Stem Cells (MSCs) (RoosterBio, MD, USA) were cultured with expansion medium comprising of low glucose DMEM, penicillin (100 U mL⁻¹)-streptomycin (100 µg mL⁻¹), 10% fetal bovine serum (FBS) and 2 mM GlutamaxTM with its expansion medium changed every two to three days. Prior to cell seeding, scaffolds were UV-sterilized for 10 min and rinsed twice with 1xPBS. Subsequently, cells were trypsinized, re-suspended in complete DMEM, counted and pipetted onto the different scaffolds at a seeding density of 2×10⁶ cells and cultured overnight in a humidified atmosphere at 37°C with 5 % CO₂. All these chemicals were acquired from Life Technologies, Singapore, except stated otherwise. The effect of various drug loading within the scaffold to enhance expression of osteogenic markers and chondrogenic markers were investigated by subjecting the cell laden scaffolds to normal expansion medium for three weeks.

3.5.1 Cell Metabolic Activity Quantification and Live/Dead Assay

The metabolic activity of the cells seeded on the scaffold was measured using the Alamar Blue (Invitrogen) assay[21]. To measure the metabolic activity, culture

medium was replaced by a modified medium which contained 10% Alamar Blue reagent without FBS. The absorbance of the supernatant was measured at wavelengths of 570 and 600 nm after 4 hours of incubation at 37°C, 5% CO₂. Live/dead staining assay was used to evaluate the cell distribution and viability. Cultured samples on scaffolds were washed with PBS and incubated in a solution of 20 µg/mL fluorescein diacetate (Sigma Aldrich, Singapore) for 15 minutes (observation of green stain for live cells) while the dead cells were stained red with 100 µg mL⁻¹ propidium iodide (Sigma Aldrich, Singapore) for 5 min. Samples were then washed again in PBS and transferred onto microscope slides for imaging under a confocal laser microscope (Zeiss LSM 710, Carl Zeiss Pte. Ltd., Singapore) at excitation/emission values of 494/517 nm using a 20X objective.

3.5.2 Gene Expression Analysis

The quantitative real-time RT-PCR technique (qPCR) has emerged as a powerful tool which is sensitive to detect mRNA quantitatively. In brief, the expression of the target gene is analyzed with respect to a reference gene. This is done to normalize template amount first and aid in estimating the target gene's relative expression [22]. The expression of the target gene of interest (for instance Col I, Col II, etc.) is then calculated as relative to the reference gene. In most cases, this normalization is conducted with glyceraldehyde-triphosphate dehydrogenase (GAPDH), β -actin, or an rRNA (ribosomal) gene. These genes are referred to as housekeeping genes as they are not affected by the status of the experiment [23].

Flowchart of technical steps followed in Quantitative RT PCR

The cultured tissue construct was initially homogenized with TRIzol Reagent to obtain the RNA aqueous phase. The collected RNA was further purified with PureLink™ RNA Mini Kit (Life Technologies, Singapore). 100ng of total RNA was reverse transcribed to complementary DNA with the use of SensiFAST™ cDNA Synthesis Kit (Bioline Singapore, Singapore). Real time polymerase chain reaction (qPCR) was carried out to quantify the targeted gene of tissue construct after 3 weeks of osteo and chondrogenic differentiation with the use of SYBR PCR Master Mix Kit (Life Technologies, Singapore) and StepOnePlus™ Real Time PCR Systems (Life Technologies, Singapore). A schematic flowchart of the technical steps is mentioned above. The lists of most of the primer sequence used for qPCR are listed below in **Table 3.5**. qPCR was initiated at 95° C for 10 min, followed by 40 cycles of amplification comprising of a denaturation step (95° C for 15 s) and an extension step (60° C for 1 min) respectively.

The obtained qPCR result was then analyzed by Livak method whereby the expression level of targeted genes was first normalized to GAPDH mRNA level and later expressed as the relative expression of targeted gene of the tissue construct as compared to the MSCs prior to experimental treatment (day 0). Firstly the threshold cycle (CT) for each sample is identified and subtracted from that of the housekeeping gene's value. This gives us the Delta CT value. The $2^{(-\Delta\Delta CT)}$ was the formula that has been throughout this study to analyze the relative changes in gene expression from q PCR experiments.

Table 3.5 Primer sequences used in real time PCR

Gene	Accession number	Primer sequence (both 5'-3')	Product size
Collagen Type I (COL 1)	NM_000088	F:CAGCCGCTT CACCTACAGC R:TTTTGTATTCAATCACTGTCTTGCC	83
Collagen Type 2 (COL 2)	NM_001844	F:GGCAATAGCAGGTTACGTACA R: CGATAACAGTCTTGCCCCACTT	79
<u>Aggrecan</u> (AGG)	NM_001135	F: ACTTCCGCTGGTCAGATGGA R: TCTCGTGCCAGATCATCACC	111
Sox9	NM_000346	F: AGCGAACGCACATCAAGAC R: CTGTAGGCGATCTGTTGGGG	85
Glyceraldehyde 3- phosphate dehydrogenase (GADPH)	NM_002046	F: ATGGGGAAGGTGAAGGTCG R: TAAAGCAGCCCTGGTGACC	70

3.5.3 Histological Sectioning and Staining

The cell loaded scaffolds were fixed with 10% formalin overnight and washed with PBS prior to histochemical studies. Osteogenic and chondrogenic pellets were embedded in Tissue Freezing Medium (Leica Microsystems, Singapore) and frozen at -20°C overnight. Sections of $8\text{ }\mu\text{m}$ thickness were sliced for each scaffold with a Leica CM1900 cryostat (Leica Microsystems, Singapore) and collected on Menzel-Glaser Superfrost Plus Slides (Thermo Scientific, Singapore).

To evaluate the calcium deposition capability of osteogenic cell lineage, alizarin red solution (Sigma, Singapore) was introduced to the cells for five min, and gently washed multiple times with DI H_2O to remove any non-specific staining.

To evaluate the expression of glycosaminoglycans, cryo-sections of the chondrogenic cell laden scaffolds were stained with 1.0% alcian blue (Sigma–Aldrich, Singapore) in 0.1 M HCl for 10 min and further stained with Safranin O (Sigma–Aldrich, Singapore) for 3 min followed by series of washes with DI H_2O and mounted on slides.

The evaluation of collagen type I and II within the scaffolds was carried out with an immunohistochemical staining kit using UltraVision™ Quanto Detection System HRP DAB (Thermo Scientific, Singapore). Initially, cryo-sections were treated with 0.5% pepsin (Sigma, Singapore) for 20 min prior at 37°C . This was followed by blocking with hydrogen peroxide for 10 min. Then monoclonal antibodies of collagen types I and II (Merck, Singapore) of dilution factor 1:500 in PBS were used to flood the slides for 1 h, followed by incubation with primary antibody Amplifier Quanto for 10 min[22]. HRP polymer Quanto was subsequently added for 10 min (in the dark) and DAB Quanto Chromogen + Substrates were used as a chromogenic agent for collagen detection. Lastly, counterstaining was done with hematoxylin solution (Sigma Aldrich, Singapore). Alizarin red stained cells and histological stained slides were imaged with a Canon 50D DSLR Camera (Canon, Singapore) and an Olympus IX71 inverted bright-field microscope (Olympus, Singapore), respectively.

3.6 Statistical Analysis

All data of representative experiments carried out in triplicate are presented as mean $\pm$ standard deviations. One-way analysis of variance (ANOVA) is a statistical technique

used to determine if the means are different between three or more samples. If the variance between groups is larger than the variance within the group, it's likely that the mean differs. Generally, the samples are considered to be independent, the variances of populations should be equal and the response variable residuals are normally distributed while one-way ANOVA is executed. One-way ANOVA was employed followed by Student t-test (two samples assuming equal variance). Bonferroni correction was done on the two-tail P value from the t-test to determine statistical significance differences between experimental groups[23]. Corrected P-value <0.05 was considered as statistically significant.

Summary

A fundamental understanding of the fabrication technique is required in order to effectively manipulate the parameters to arrive at the structural variations. The working principles of characterization techniques and the various optimizations conducted have been explained in this chapter. The materials characterization section provides an understanding of the different characterization techniques used throughout the course of this study. Morphological analysis, detection of functional groups, thermal behavior, response to stress applied and the extent of hydrophilicity of the fabricated scaffolds have been evaluated. Next, the performance of the scaffold in response to drug loading and the release profiles are important in determining the final applications for the scaffold material. This is evaluated using HPLC and UV spectroscopy techniques and all the bioactive molecules loaded into the scaffold have been quantified precisely. Finally the scaffolds that exhibited excellent encapsulation of all the biomolecules and good mechanical integrity have been subjected to MSC culture for three weeks. The culture conditions used and the various analysis techniques employed have been briefly explained with rationale for choosing them. ANOVA and Student's t test are the statistical methods for quantifying if the results reveal significant differences between various groups.

References

- [1] Q.P. Pham, U. Sharma, and A.G. Mikos, Tissue engineering, 2006. **12**(5): p. 1197-1211.
- [2] J. Doshi and D.H. Reneker, Journal of electrostatics, 1995. **35**(2-3): p. 151-160.
- [3] P. Lu and B. Ding, Recent patents on nanotechnology, 2008. **2**(3): p. 169-182.

- [4] R. Hartman, J.-P. Borra, D. Brunner, J. Marijnissen, and B. Scarlett, Journal of electrostatics, 1999. **47**(3): p. 143-170.
- [5] A. Jaworek and A. Krupa, Journal of Aerosol Science, 1999. **30**(7): p. 975.
- [6] P. Gentile, V. Chiono, I. Carmagnola, and P.V. Hatton, International journal of molecular sciences, 2014. **15**(3): p. 3640-3659.
- [7] C. Engineer, J. Parikh, and A. Raval, Trends in Biomaterials & Artificial Organs, 2011. **25**(2).
- [8] B.D. Ulery, L.S. Nair, and C.T. Laurencin, Journal of polymer science Part B: polymer physics, 2011. **49**(12): p. 832-864.
- [9] M.A. Woodruff and D.W. Hutmacher, Progress in polymer science, 2010. **35**(10): p. 1217-1256.
- [10] K. Rezwani, Q. Chen, J. Blaker, and A.R. Boccaccini, Biomaterials, 2006. **27**(18): p. 3413-3431.
- [11] H. Susi and D.M. Byler, Methods in enzymology, 1986. **130**: p. 290-311.
- [12] R.S. Das and Y. Agrawal, Vibrational Spectroscopy, 2011. **57**(2): p. 163-176.
- [13] N. Colthup, Introduction to infrared and Raman spectroscopy 2012: Elsevier.
- [14] C. Schick, Analytical and bioanalytical chemistry, 2009. **395**(6): p. 1589-1611.
- [15] R.F. Landel and L.E. Nielsen, Mechanical properties of polymers and composites 1993: CRC Press.
- [16] Y. Yuan and T.R. Lee, *Contact angle and wetting properties*, in *Surface science techniques*. 2013, Springer. p. 3-34.
- [17] L.M. Nollet and F. Toldrá, Food analysis by HPLC 100. 2012: CRC Press.
- [18] G.R. Grimsley and C.N. Pace, Current protocols in protein science, 2004: p. 3.1. 1-3.1. 9.
- [19] S. Jain and M. Datta, Applied Clay Science, 2015. **104**: p. 182-188.
- [20] K.D. O'NEAL, M.V. CHARI, C.H. McDONALD, L.-y. YU-LEE, J.D. MORRISETT, and T.W. SHEARER, Biochemical Journal, 1996. **315**(3): p. 833-844.
- [21] E. Vega-Avila and M.K. Pugsley. *An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells*. in *Proc West Pharmacol Soc*. 2011.
- [22] Y.J. Chuah, Y. Zhang, Y. Wu, N.V. Menon, G.H. Goh, A.C. Lee, V. Chan, Y. Zhang, and Y. Kang, Acta biomaterialia, 2015. **23**: p. 52-62.
- [23] J. Cohen, Current directions in psychological science, 1992. **1**(3): p. 98-101.

Chapter 4

Dual Structured Sandwich Scaffold System – Fabrication, Characterization and Release of Bioactive Molecules.

This chapter presents all the findings related to the preliminary characterizations conducted for optimization of parameters towards achieving the dual structured sandwich scaffold system and their release patterns. The rationale of using techniques of electrospinning and electro spraying followed by optimization of the processing and solution parameters for all the scaffolds are explained in the first section. The next section throws light into the characterization of all scaffolds for evaluation of its material properties. This is followed by identifying the release patterns exhibited by various biomolecules that were encapsulated into the particles and fibers. Initially, Dexamethasone (Dex) was used as the hydrophobic drug and was loaded into the fibers. The hydrophilic biomolecules that were analyzed in this thesis are Ascorbic acid(AA), β - Glycerophosphate (β - Gly) and Proline (Pro). The sandwich scaffolds exhibited controlled and sustained release of hydrophobic and hydrophilic molecules. The final section discusses the release profiles of all the biomolecules loaded either alone or in combination into the dual structured scaffolds.¹

¹ Some results from this chapter have been submitted to Journal of Materials Chemistry B and is under peer review post first revision.

4.1 Electrospinning and Electro spraying - Optimization of Parameters

4.1.1 Processing Parameters

The advantages and limitations of the electrospinning system need to be carefully considered while planning experiments to ensure reproducibility throughout the course of the study. MECC Nanon system was chosen for all electrospinning and electro spraying experiments after evaluating its voltage range, maximum distance between collector and tip of needle and its feed rate input range variation. A maximum volume of 10 mL syringes could only be loaded on this system. Hence predominantly 5 mL and 10 mL syringes were used for the entire duration of this project. The first processing parameter of interest is the voltage. There have been quite a few studies pertaining to examining the effect of voltage on the biodegradable polymers used for medical purposes [1-3]. From previous studies by various research groups, it has been found out that the voltage range for spinning of PLGA and PCL is between 8 kV to 25 kV [4, 5]. Voltage is one of the important factors that determines the shape of the initiating drop in addition to factors like viscosity and solution flow rate [6]. It has been postulated that voltage predominantly affects the beading of the fibers for synthetic polymers such as PLGA, PLLA and PCL[7]. A micro ammeter is used to measure the electric current for any electrospinning process. The circuit is considered closed when charge is transported by the droplets (electro spray) or fibers (electro spun) all the way to the grounded metal collector. In the case of electrospray, a change in spraying is generally accompanied by an increase in frequency of drop formation and a corresponding change in the measured electrospray current [8]. This current goes through changes when there is an increase in the voltage and this in turns affects the jet initiation patterns. For electrospinning, the electric current due to the ionic conduction of charge in the polymer solution is usually assumed small enough to be negligible and hence the sole mechanism of charge transport is the flow of polymer from the tip to the target [6]. Thus, when the electrospinning current is increased so is the flow rate from the capillary tip to the grounded target. This is also with the assumption that the other variables namely feed rate of solution, dielectric constant and solution conductivity are maintained constant. These observations were further confirmed through our experiments too as evidenced in **figure 4.1**. When the voltage was at 10 kV excessive beading of the fibers was observed, and as it was gradually

increased to 18 kV (PCL) and 25 kV (PLGA), uniform fibers were obtained which were subjected to further characterizations.

Figure 4. 1 Electro spun fibers of PCL (top row) and PLGA (bottom row) at various voltages showing the gradual disappearance of beading to form uniform fibers.

4.1.2 Solution Parameters

The solution parameters to be taken into consideration are the polymer concentration, viscosity of the solution and the conductivity of the solution. The solvent that is used to dissolve the polymer is to be chosen carefully after examining its conductivity values. As such, conductivity affects the quality of the fibers. It also helps in reducing the critical voltage required for the electrospinning process[9]. A highly conductive solution has good spin-ability and prevents the formation of bead in the fibers. Addition of salts is known to increase the conductivity of a polymeric solution. In the case of PLGA and PCL the main solvents that were employed during this thesis are chloroform (CHL), dichloromethane (DCM), tetrahydrofuran (THF), dimethyl formamide (DMF) and methanol (MeOH) alone or in combination. DMF was the preferred solvent of choice when conductivity of the solution needed to be increased.

Viscosity of the polymer solution plays a significant role in determining whether the solution can be stretched into fibers or not. Highly viscous solutions generally suffer from low flow ability from the syringe to the collector. This is due to lack of

sufficient strength generated by the electrical charges which prevents the fibers from stretching out. The chain entanglements of the polymer molecules are the major determinant of the viscosity of the solution. For a dilute solution, the polymer chains are placed far apart and hence there is no overlap between the molecules. The concentration at which the polymer chains begin to overlap is known as the critical concentration c and the formation of beads occur at this regime[10]. If a solution is so viscous that it is almost "gel-like", it may be necessary to reduce the concentration of the solution. The solvent used for this system is DCM:MeOH in the ratio of 4:1. The concentration of the polymers was denoted in w/v and was maintained at a constant value of 12% for all fiber fabrication process and 3% for all particles spraying. This concentration was arrived at after many preliminary experiments.

There is a complex interplay of all the solution parameters towards obtaining a uniform fibrous morphology and particles of sub-micron size range. The complete evaluation of these is beyond the scope of this thesis. Thus, in summary, the voltage used for fabrication of fibers was between 15 kV to 25 kV, flow rate varied between 0.8 mL to 1.5 mL and the distance between the needle tip and the collector was constant at 12 cm. Solution parameters were 12% polymer concentration. For the fabrication of particles that were electro sprayed on top of the fibrous mesh, the processing parameters were 12- 15 kV with 10 cm distance and 0.2 - 0.5 mL flowrate of the solution from the needle tip towards the collector.

4.2 Material Characterization

4.2.1 Electro spun Fibers

The scanning electron micrograph in **figure 4.2** reveals the morphology of the electro spun PLGA/PCL fibers fabricated with the following parameters: voltage 18kV, Flowrate -1mL hour⁻¹, 120mm tip to target distance and 12%w/v polymer concentration. The fibers were uniform with a diameter of $0.89 \pm 0.22 \mu\text{m}$ ($n=50$). It can be observed that the fibers were homologous, cylindrical and distinct. Fibers with these features exhibit a morphologic similarity to the extracellular matrix (ECM) of natural tissue, which is characterized by a wide range of pore diameter distribution, high porosity, and effective mechanical properties[11]. All these criteria are hall mark

characteristics of an ideal engineered scaffold. The presence of both PLGA and PCL was confirmed in the blend scaffold using FTIR analysis as shown in **figure 4.3**.

Figure 4. 2 Scanning Electron Micrograph of electro spun PLGA/PCL fiber mat.

Figure 4. 3 FTIR spectrum of PLGA, PCL and its physical blend (PLGA/PCL).

FTIR revealed the characteristic peaks of PLGA at 3432 cm^{-1} (O-H stretching), $2948\text{--}2999\text{ cm}^{-1}$ (Stretching of C-H), 1758 cm^{-1} (C=O group), $1133\text{--}1213\text{ cm}^{-1}$ (Stretching of C-O saturated ester) and $860\text{--}875\text{ cm}^{-1}$ (OH out of plane bending vibration). PCL

exhibited characteristic peaks at 2866-2943 cm^{-1} (Stretching of C-H), 1728 cm^{-1} (Stretching of C=O) and 1168-1242 cm^{-1} (Stretching of C-O saturated ester). The peaks of PLGA and PCL predominantly overlap each other except for the peak at 865 cm^{-1} which is solely attributed to the stretching vibration of C - O in PLGA.

Table 4.1 IR spectral regions for PLGA and PCL revealing their characteristic functional groups

Sample	Wave number (cm^{-1})	Functional groups
PCL	2866–2943.2	Stretching of C–H
	1728.1	Stretching of C=O carbonyl
	1168.8–1242.1	Stretching of C–O saturated ester
PLGA	3432	O–H
	2948–2999	Stretching of C–H
	1758	C=O carbonyl
	1387–1459	Bending of C–O–H
	1133–1213	Stretching of C–O saturated ester
	860-875	O-H out of plane bending vibration
PLGA/PCL	2873–3001	Stretching of C–H
	1759	C=O carbonyl
	1387–1458	Bending of C–O–H
	1134–1214	Stretching of C–O saturated ester
	860-875	O-H out of plane bending vibration

There was no observed shift in any of the bands of the polymers which leads to the inference that merely a physical blend is formed without any visible changes to the functional groups. The water contact angle of the electro spun blend fibers (**figure 4.4**) indicated that PLGA/PCL blend was more hydrophilic than neat PCL. The disadvantage of neat PCL is its super hydrophobic nature which prevents cell adhesion onto it and requires additional surface modification protocols to enhance cell attachment even though it exhibits excellent mechanical strength. PLGA on the other hand exhibits excellent cell adhesion and promotes cellular attachment onto the scaffold[12]. However, PLGA suffers from inherent shrinkage issues when introduced

into media for a long duration. This affects the mechanical integrity of the scaffolds. To address all these issues, the formation of a blend polymer was chosen as the approach for fabricating the sandwich scaffolds. The two polymer solutions were formulated in a 1:1 ratio and spun into fiber mats. Using co electro-spinning of two single biopolymers to overcome the limitations associated with a single polymer was achieved, and in addition this resulted in formation of a polymer blend without requirement of any new synthesis.

Figure 4. 4 Water contact angle measurements of neat and blend polymers (n = 5).

The scaffolds were subjected to mechanical testing and compared with pure PLGA and pure PCL scaffold mats. **Figure 4.5** plots the tensile strength of the PLGA/PCL blend scaffold mat along with its neat polymers. The amorphous fractions of the polymer chains within the fibers influence the strength and elastic modulus of the fibers. Changes in the fibers' structural formation took place while spinning. These may be crystallinity and molecular orientation changes which impart physical uniqueness to the material and play an important role in the deformation behavior of the fibers[13]. Research by Duan *et al.* reports of electro spun PLGA mats with stress of 9 MPa and strain of 30%[14]. The stress of the PLGA/PCL fibers was dependent on both the amount of PLGA and the uniformity of the electro spun fibers mats.

Figure 4. 5 Tensile stress vs strain (%) plotted for neat PLGA, PCL and PLGA/PCL blend.

PCL is very flexible and when added to the brittle PLGA enhanced its mechanical strength. The tensile strength values are 5.2 MPa with strain % value of about 500%. This fits well into the regime of scaffolds used for bone tissue regeneration. In general, the scaffolds that have been fabricated through electrospinning for bone tissue engineering till date fall into the tensile moduli range of 0.5MPa to 60 MPa[15].

4.2.2 Electro spraying of Particles

PLGA is a copolymer of lactic acid and glycolic acid and exhibits desirable characteristics of PLA and PGA thus combining the best of both into one functional polymer. Owing to its tailor able degradation properties, mechanical strength and bio compatibility, it is a good choice for clinical applications such as in sutures and has more prospective in the field of tissue engineering. Indeed, PLGA is currently widely used as scaffold material for bone repair. There are multiple factors that govern the degradation rate of PLGA. The biodegradable acids are metabolized entirely without getting accumulated in any of the vital organs[16]. In this section, the detailed

optimization towards obtaining PLGA micro-particles are discussed with respect to different solvent systems and the processing parameters of electro spray technique. PLGA solution was prepared in various concentrations by weight with respect to the solvent volume. The different solvents employed in this study are chloroform (CHL), dichloromethane (DCM), and tetrahydrofuran (THF). Briefly, solutions of polymer concentration of 2%, 3% and 4% were prepared and stirred under ambient temperature for several hours before subjecting them to electro spraying. The processing parameters that were optimized are feed rate, solution concentration, voltage and the distance from the tip of the needle (the region of the formation of the tailor cone) to the collector (aluminium foil). The following images are the Scanning electron micrographs of the micro-particles obtained for each solvent system. The samples were all gold coated at 18 mA for 80 s and imaged at an accelerating voltage of 5 kV. **Figure 4.6** shows the micro-particles obtained in DCM system. The particles are not of uniform size and are highly porous. Some of the particles were flattened and such morphology is not suitable for loading of biomolecules and studying their release patterns.

Figure 4. 6 PLGA microspheres electro sprayed using DCM as the solvent

Figure 4.7 represents the micro-particles prepared using THF as solvent. The average size of the particles was 5-7 μ m and the polymer concentration was 2% (**4.7 A and B**). Increasing the solution concentration to 3% led to the formation of thin platelet like structures as seen in **figure 4.7 C and D** This can be attributed to the rate of change in solvent evaporation and instability of the Taylor cone. During electro spraying it was observed that there were multiple spray jets which also caused the resulting samples

to exhibit flattened morphology. Further, solvent saturation at the collector attributed to the formation of platelets. While using CHL as the solvent, the micro-particles obtained were of about $6.15 \pm 1.4 \mu\text{m}$ in diameter as evidenced in **figure 4.8**. CHL:DMF was thus finalized as the preferred solvent of choice owing to the uniformity of the micro-particles produced

Figure 4.7 PLGA microspheres using THF as the solvent system (A-B) 2% w/v and (C-D) 3% w/v.

Figure 4. 8 PLGA microspheres using CHL:DMF as the solvent system (left) with the particle diameter distribution graph n=30 (right).

There is currently no method to validate the mechanical strength of these particles. The particles were chosen to be fabricated only from PLGA as the degradation rate for PLGA is faster than PCL. In this sandwich scaffold system, we require the particles to release the biomolecules loaded into them within two weeks of exposure to the liquid media and degrade thereafter. Keeping this in mind the polymer, its concentration and appropriate solvent system for fabrication was chosen.

4.2.3 Sandwich System Fabrication and Characterization

After carefully tailoring the properties and morphology of individual fibers and micro-particles, the sandwich system was fabricated through electrospinning and electro spraying techniques. A homogenous solution of 12 % w/v PLGA/PCL in the ratio of 1:1 was spun into a uniform fibrous mesh for two hours followed by electro spraying of 3% PLGA solution on top of the mesh like fibrous mat at a controlled flow rate of 0.5 mL h^{-1} . After about thirty minutes of drying in ambient conditions of temperature and humidity within the electrospinning chamber, a third layer of thin fibrous mesh was spun on top of the existing particle layer to prevent particle loss during further characterizations and release studies. The scanning electron micrograph of a sandwich scaffold mat is shown in **figure 4.9**.

Figure 4. 9 SEM Image of sandwich scaffold containing a thin fibrous layer of fibers spun on top of a uniform layer of particles on top of a fibrous mesh.

The mechanical strength of the sandwich scaffolds was investigated using a MTS

Criterion C40 series C42 system with a load cell of 500 Newton (N) with a constant rate of 10 mm min^{-1} (tensile testing). **Figure 4.10a** is the image of an experiment in progress and 4.12b illustrates the value of tensile strength of the sandwich scaffold which was $2.9 \pm 0.9 \text{ MPa}$. The values were compared with that of blank fibers of PLGA/PCL as shown in the **figure 4.10 B**. It is to be noted that even though the tensile strength value of the sandwich scaffold is lesser than the fibers alone, it still fits into the regime of scaffolds currently approved and fabricated by various researchers for use as bone regeneration material[17]. In the field of bone tissue engineering, porosity is a vital parameter for validation of the use of any new scaffold material. The porosity of the sandwich structure was tested using the liquid displacement method with cyclohexane as the solvent. The porosity value of the scaffold was calculated to be 58%. This is higher than the porosity value of 2D fibers alone which was 34%. The porosity of the sandwich system is within the range of scaffolds currently employed for bone tissue engineering [18]. These are fabricated with secondary post processing steps such as sintering to yield a porosity range of 36 – 85%.

Figure 4. 10 (A) Measurement in progress during experiment in a MTS C42 system (B) Graph illustrating the tensile strength of polymeric fibers versus the sandwich structure.

The degradation of the sandwich scaffolds was studied by completely immersing a known weight of the scaffold with the dimension of 2 cm x 1 cm in 10 mL of PBS solution. The scaffolds were taken out from PBS at pre-determined time points, their pH was measured followed by drying overnight SEM imaging to study the

morphological changes and the confirm the degradation of the constituent polymers that made up the scaffold. This study (**figure 4.11**) was important as it provided valuable insights on which biomolecule could be loaded into the particle and fiber based on its degradation rate.

Figure 4. 11 Degradation of the sandwich scaffold in PBS until Day 48.

It can be observed that on day 16, the particles have started to degrade as they are contained PLGA as their sole polymer constituent and lose their morphological integrity. At day 48, the particles have totally disintegrated and the fibers have also begun to disintegrate. Further pH of the release medium was tested on day 0, day 15 and day 45. The values decreased to about 6.9 on day 15 and reduced further to ~5.6 on day 45. This is due to the rapid degradation of PLGA and the release of acidic by-products into the release media. Based on these observations, the drug release studies were carried out for a maximum of 45 days for all the scaffolds as after that they exhibit loss of mechanical integrity (results not shown) and break down into smaller pieces in PBS solution.

4.2.4 Effect of Particle loading on the fibers

Four different ratios of particles loaded onto the fibers were studied in this work and they varied on the basis of weight ratios electro sprayed or electro spun. They are 1:1, 1:3, 1:5 and 1:10 Particle: fiber. The SEM images for these ratios are shown in **figure 4.12**. The release profiles for all the four ratios were calculated to arrive at the most suitable ratio for loading of multiple drugs. For ratio of 1:1, the particles disintegrated from the fibers during mechanical characterization and also when the scaffold was immersed in PBS for a long period of time. In many cases, the particles were observed to be stuck to one another and the loss of the particles could not be accounted for. The ratio of 1:3 exhibited non-uniform particle sizes and hence was not

considered for further studies. 1:10 ratio had very less particles scarcely deposited on the fibers and hence reproducibility could not be precisely achieved for this system as we could not exert control over the location of the particles that got sprayed on the fibers. The ratio of 1:5 was considered the most suitable for further release studies and evaluation of single and multiple drug studies. This is due to the presence of adequate number of particles on the fibers and the mechanical strength exhibited by the scaffold.

Figure 4.12 Scanning electron micrograph showing particles sprayed on top of fibers at different ratios of (a) 1:1; (b) 1:3; (c) 1:5; (d) 1:10.

Figure 4.13 Tensile testing of the sandwich scaffolds at different ratios of particles: fibers

The particles did not tear off from the scaffold when subjected to tensile testing. The graph (**figure 4.13**) summarizes the mechanical behavior of the different ratios of particles on the fibers (sandwich scaffold). The values of the tensile behavior are in accordance with the inference mentioned above (n=3). Further the release of Dexamethasone from all these scaffolds has been discussed in the next section.

4.3 Release of Dexamethasone from sandwich scaffold

4.3.1 Optimization for Dexamethasone

The final concentration of the polymer for fabrication of particles was 3% w/v and 12 % w/v for fibers. These concentrations were maintained constant throughout the course of this study. Dexamethasone was chosen as the drug of interest owing to its anti-inflammatory properties and its role in differentiation of mesenchymal stem cells [19]. It was important to establish the sandwich system with a drug which plays roles in multiple organ systems to illustrate the broad applicability of this work. For osteogenesis and chondrogenesis, it is of paramount importance for Dex to be released in minute quantities (ng to μ g levels). Dex is well known for its potential toxicity when released in higher concentrations[20]. Hence the efficacy of this scaffold system to exhibit a controlled release of Dex not exceeding therapeutic dosage levels was the primary issue to be addressed. Taking the above factors into consideration and after referring to various literature sources, 1 wt % of Dex was chosen for optimization of release study of the drug with respect to the weight of the polymer. The cyto-compatibility values of Dex vary drastically in human when compared to rodent models[21]. Also, the therapeutic dosage of Dex is directly dependent on bone morphogenetic proteins (BMPs). BMPs are the effector peptides that facilitate the differentiation of MSCs towards osteo lineage in conjunction with Dex. However, in the case of humans, the amount of BMP required to induce osteogenesis is at least six orders of magnitude higher than a corresponding rodent model[22]. BMP is not cost effective and such high concentrations lead to adverse side effects. Hence scaffold formulations that involve localized and sustained delivery of Dex alone or in combination with other osteogenic inducers such as Ascorbic acid (AA) and β -Glycerophosphate (β -Gly) are the primary focus of this chapter. The

concentration of Dexamethasone that is supplemented in cell media for osteogenesis to occur is in the range of 100 nM – 250 nM per 10 mL of medium prepared[23]. This amount needs to be supplemented every time the media is replaced. However, quantifying the release of such a minute quantity using technique like HPLC has limitations with regards to the sensitivity of the equipment and detection limits. Hence, quantification was carried out with 1wt % Dex with respect to polymer concentration. The drug was directly added to the polymer before addition of solvents and stirred to achieve homogeneity overnight followed by electrospinning. Post-spinning, all the scaffolds were dried completely to remove any residual solvent remnants and subjected to further characterizations.

4.3.2 Encapsulation Efficiency of Dex

After complete drying of the scaffold (fibers) Dex was quantified by taking a known weight of the scaffold and dissolving it in organic solvent to extract Dex from it using methanol (MeOH).

Table 4.2 lists the Encapsulation Efficiency of Dex (1 wt %) when encapsulated alone (n=6).

Weight of the scaffold (mg)	Theoretical amount of encapsulated Dex (μ g)	Actual Dex detected (μ g)	Encapsulation Efficiency (%)
20	200	163.82	78.04 $\pm$ 3.36
20	200	151.75	
20	200	152.69	
25	250	214.77	86.37 $\pm$ 2.70
25	250	223.19	
25	250	209.83	

4.3.3 Release of Dex from Particles, Fibers and Sandwich scaffold

Firstly, the above-mentioned concentration of Dex was encapsulated into three different structured systems namely a) micro-particles (5 -8 μ m in diameter)[Scaffold M] , b) fibers (400-800nm in diameter) [Scaffold F] and c) bi-layered dual structured scaffold in both the particles and in the fibers [Scaffold MF]. Thus, the total

concentration of Dex encapsulated in each of the systems was 1 wt %. Scaffolds M, F and MF were weighed and immersed in PBS. Known volumes of PBS was removed and replaced at pre-determined intervals. The samples were stored at 4 ° C until analysis through HPLC. The graph (**figure 4.14**) reveals the release profiles of Dex from all the three scaffolds. The ratio of particles on the fibers used for this study was 1:5 particles: fibers. This concentration of the particles was ideal for drug loading as there was no loss of particles observed during the entire duration of release study. The burst release of Dex from the scaffold M was 56%, which was significantly higher than that of scaffold F at 19%, ($P < 0.01$) and MF at 41%, ($P < 0.01$). Complete release of encapsulated Dex was recorded at day 14 for scaffold M, day 60 for scaffold F and day 35 from MF. While several studies have reported the encapsulation of Dex within PLGA microparticles using techniques such as solvent emulsion, the EE achieved was very low (~4% compared to their initial loading of 20% (w/w)) [24]. Here, we showed that by encapsulating Dex within electrosprayed microparticles we could achieve an EE of $72 \pm 5.1\%$, which is at least four times higher than the solvent emulsion technique (**table 4.2**) and with a simpler technique.

To elucidate the release kinetics, the individual release profiles were fitted into mathematical models (**Figure 4.15**), after excluding the initial burst release values from each scaffold. For scaffold M, the data plot (until day 10) reveals a high correlation coefficient ($r^2=0.96$) for zero-order that suggests Dex was released at a near constant rate that is independent of its initial concentration (**Figure 4.15 A**). The release kinetics exhibited by scaffold F clearly followed first order kinetics with a correlation coefficient (r^2) of 0.99 (**Figure 4.15 B**). In the case of MF, the plotted data exhibited first order kinetics with $r^2=0.98$ (**Figure 4.15 C**)

Figure 4.14 The release profiles of Dex from M (red), F (black) and from MF(blue)

Figure 4.15 Kinetics of Dex release from all three scaffold systems. (A) The release of Dex from scaffold M follows zero order kinetics with $r^2 = 0.96$. (B) Scaffold F follows a first order kinetic profile exhibiting a correlation coefficient $r^2 = 0.99$. (C) Release of Dex from MF exhibits a first order kinetics with a $r^2 = 0.98$.

Thus, having achieved the sustained release of a hydrophobic drug from the sandwich scaffold, we further explored the loading of multiple biomolecules into this system to evaluate its efficacy for sustained drug release.

4.4 Release of Multiple Biomolecules from Sandwich Scaffold.

After observing the efficacy of the sandwich scaffold in controlled release of Dex, I further advanced my studies to simultaneously release multiple biomolecules that are essential in bone and cartilage regeneration. Dex was the only hydrophobic drug and hence was always loaded into the fibers. Moreover, the sustained release of Dex was required for the longest period owing to its anti-inflammatory property[25]. For an *in vivo* condition, the suppression of chronic and acute inflammation is the major requirement when a scaffold material is introduced into the body. There are scientific reports of Dex encapsulated into PLGA micro-particles (10-20 μm diameter)[26]. However, the disadvantage of having a single micro particle system for release of Dex is the unaccountability of the release of Dex due to loss of particles during handling. Also in order for Dex to be released continuously for a period of one month, Hickey *et al* have proposed a mixture of freshly prepared and pre-degraded micro-particles[20]. These add on to the complexity in the process in comparison to our simple one step electro spinning of Dex loaded polymer solution. The burst release of Dex is significantly reduced when encapsulated into fibers when compared to particles as observed from Figure 4.17. The other biomolecules used during this study are predominantly hydrophilic, were loaded into the micro-particles and were required within the first two weeks for osteogenic or chondrogenic induction. These biomolecules were mixed with the PLGA solution that was used to form the electro sprayed particles. The concentration of these biomolecules was initially 4wt % of the polymer. After optimizations, the final concentrations used for all biomolecules were 1 wt %. For osteogenic differentiation to occur, the biomolecules that were loaded into the particles were Ascorbic acid (AA) and β -Glycerophosphate (β -Gly). For chondrogenesis to occur, β -Gly was replaced by an amino acid Proline (Pro) and loaded alongside AA into the particles.

4.4.1 Simultaneous Release of Hydrophobic and Hydrophilic Drug from Sandwich Scaffold.

Initially the release study was conducted with Dex in the fibers and AA in the particles only. This system was quantified for effective release of a single hydrophobic and hydrophilic drug simultaneously.

Table 4.3 Encapsulation Efficiencies of all biomolecules incorporated in this study.

Biomolecule	Incorporated into	
	Particle	Fiber
	Encapsulation Efficiency (EE)%	
Dexamethasone	n/a	76.7 ± 1.54
Ascorbic acid	65 ± 7.5	n/a
β-Gly	72 ± 3.3	n/a
Proline	88 ± 0.51	n/a

Further, AA was quantified using UV-Vis spectroscopy while Dex was quantified using the above-mentioned method of HPLC with a standard calibration curve for reference. Firstly, the encapsulation efficiency of this system was evaluated. The percentage of hydrophilic biomolecules encapsulated into the particles was higher when the fabrication technique employed was electro spraying. This was verified by fabricating the particles by solvent emulsion technique using an organic phase to dissolve the polymer and an aqueous phase to dissolve the drug. The protocol was followed from the research of Yang *et al* [27]. Other research groups have also successfully encapsulated proteins such as Bovine Serum Albumin (BSA) into micro-particles/microspheres made of PLGA or PCL. The particles when made only from PLGA present the problem of high shrinkage. This can be combated by increasing the polymer concentration. Further, there is unavoidable burst release of the encapsulated drugs from these microspheres due to presence of the drug on the outer surface of the spheres[28]. AA was chosen for this study as the primary hydrophilic drug owing to its importance in both osteogenesis and chondrogenesis. Under *in vivo* conditions, AA is responsible for collagen synthesis which is essential for bone and cartilage tissue engineering[29]. The sandwich scaffold was formed with Dex loaded into the bottom fibrous layer and AA loaded into the micro-particles. These scaffolds were weighed and immersed in PBS for the release study. Known volume of PBS was removed and replaced in the sample tube at pre-determined time intervals and the release of Dex was quantified by HPLC-UV at 246 nm with 50:50 40mM acetate buffer: Acetonitrile (ACN) as the mobile phase. The pH of acetate buffer was maintained at 4.8 and the peak for Dex appeared at 3.8 – 4.2 min. AA was quantified with UV Vis at 262 nm with DI H₂O as the reference. The concentration of AA present was quantified with the help of a standard curve which was obtained by serially diluting AA from

concentration of $100 \mu\text{g mL}^{-1}$ to $1 \mu\text{g mL}^{-1}$ in DI H_2O . The standard calibration curve for AA is shown in **figure 4.16**. A standard calibration was done every time AA sample was analyzed under UV Vis to avoid variations from one batch to another.

Figure 4.16 Standard calibration curves for Ascorbic acid quantification through UV–Vis spectroscopy

Figure 4.17 Cumulative Dex release profiles for Dex and AA from a sandwich scaffold.

The burst release percentage and the cumulative drug released were calculated. The cumulative release amount of both the drugs was proportional to the actual drugs

encapsulated in the scaffold. The release profile of dex from the fibrous scaffolds exhibited linear release pattern at an early stage and slower release pattern at a later stage, typical of a biphasic diffusion system[30]. At the later stages, the slow release can be attributed to the gradual depletion of Dex in the polymer phase. It is of vital importance to ensure that the drug released at any time point should not exceed the therapeutic dosage concentration. Hence the initial burst release of Dex and AA at various time points was calculated and tabulated in **table 4.4**.

Table 4.4 Cumulative release of Dex and AA in the first 24 hours of interaction with PBS.

Bio molecule	Cumulative Drug released at specific time points (μg per 2 mL of media)				%Drug released by 24 hours
	0 - 4 h	4 - 8 h	8 - 12 h	12 - 24 h	
<u>Dex</u>	57.9	44.88	49.23	65.16	30%
AA	29.12	13.83	30.03	66.43	53%

For Dex, the results revealed that in the first 4 hours, only 8% of the encapsulated drug had released from the scaffold. Dex did not exhibit burst release when encapsulated within the fibers. The burst release for Dex after 24 hours in PBS was estimated to be 30 % of the total drug encapsulated in the fibers and for AA it was 53% of the total drug encapsulated successfully in the particles. These values indicate that the burst release of both the biomolecules are significantly less when compared to similar studies conducted by other research groups[31]. These values were achieved by only tailoring the polymer and drug concentrations sans any additional steps like crosslinking[32]. In the case of Ascorbic acid, the total amount released in the first 4 hours was 16% of the total encapsulated AA in the particles. AA is essential for the synthesis of collagen, helps in maintaining cell viability and plays a vital role in DNA biosynthesis[33]. Thus, the presence of AA is of prime importance for bone and cartilage formation[34]. However, AA cannot be synthesized by human and non-human primates and has to be supplied exogenously[35]. The amount of AA required

for collagen synthesis varies between 20-160 $\mu\text{g mL}^{-1}$ for a period of 2 weeks. The daily requirement of AA is around 20-30 μM [36]. From the release profile of the sandwich system, the total amount of encapsulated AA was calculated to be 182 μg and this was released in a sustained manner over a period of 16 days. While a number of literatures report the sustained release of Dex from various structures such as capsules, fibers and particles, there are no convincing reports of controlled release of AA for a time period of 2-3 weeks[37]. Hence this sandwich scaffold system bridges the knowledge gap in achieving a sustained release of hydrophilic biomolecule from particles. It was verified that the release of Dex and AA do not interfere with each other as there were no new peaks found during the course of the release study. Each study was repeated thrice to ensure reproducibility of the obtained profiles and EE values.

4.4.2 Simultaneous Release of One Hydrophobic and Two Hydrophilic Drugs.

After we validated the release pattern for a hydrophilic and hydrophobic molecule, we explored the release of two hydrophilic molecules and a single hydrophobic molecule (Dex). The rationale for this study was to examine the possibility of simultaneously releasing multiple growth factors for directing the stem cell fate towards osteogenesis and chondrogenesis to occur without adding any cues in the cell culture media. AA remained as the primary hydrophilic biomolecule due to its multiple roles in promoting bone and cartilage formation[38, 39]. We introduced two biomolecules as the second hydrophilic drug and thus formulated two sandwich systems that exhibit simultaneous release of three drugs. The first system included β -Glycerophosphate (β -Gly) in addition to Dex and AA. β -Gly is generally added as a supplement in the cell medium (osteogenic media) for differentiating stem cells towards osteogenesis. There are no reports in literature on the controlled release of β -Gly and its effect on osteogenic differentiation. Hence, we have added β -Gly alongside AA and fabricated micro-particles that encapsulated AA and β -Gly. The drugs were loaded into different micro-particles and not mixed within a single micro particle to avoid any residual interference between the two biomolecules. The concentration of β -Gly was maintained at 4 wt % with respect to the polymer (same as AA). The quantification of β -Gly was done with HPLC and the mobile phase used was 10mM ammonium acetate with 10% MeOH. A standard calibration curve for β -Gly was used to determine the

concentration of β -Gly in the unknown scaffold samples. **Figure 4.18** shows the release profile of all the three biomolecules from the sandwich structure simultaneously. The encapsulation efficiencies for each biomolecule were calculated and are presented below as table 4.5.

Table 4.5 Encapsulation efficiencies of all 3 the biomolecules that were released simultaneously for bone formation.

EE of Dex (%)	EE of AA (%)	EE of β -Gly (%)
79 ± 4.3	61 ± 1.8	72 ± 3.3

Figure 4.18 Release profiles of Dex, AA and β -Gly from sandwich scaffold.

From the release pattern, we can conclude that β -Gly and AA exhibited a similar profile and released in a sequential manner. The burst release of β -Gly was higher than AA as it is more hydrophilic. The release of Dex was not affected in the presence of two hydrophilic biomolecules. The release of both β -Gly and AA exhibited zero order kinetics while Dex exhibited a bi-phasic release which included a linear initial release followed by a slower release after week 1 in response to the polymer degradation rate. Next, we replaced β -Gly with Proline (Pro), another hydrophilic amino acid molecule that is essential for cartilage formation along with AA and Dex. 1 wt % Pro was added to the micro particle forming polymer solution before electro spraying it on top of the fibrous mesh like network. Proline was quantified using HPLC analysis with a flow rate of 1.0 mL h^{-1} at 210 nm. The mobile phase used for the detection of Proline was

Water (10%), ACN (90%), 0.5% H_3PO_4 , 0.1% TFA and the entire process was carried out using ABI C8 RP-HPLC column (5 μm , 220 mm x 4.6 mm) where the Proline separated out at 25 - 28 minutes[40]. Encapsulation Efficiency for Proline was calculated to be $88 \pm 0.5\%$ and the presence of Proline did not interfere with the release of AA and Dex. **Figure 4.19** reveals the release profiles of all the three biomolecules from the scaffold. The burst release percentage of Pro was $40 \pm 2.3\%$. Proline is essential in minute quantities for chondrogenesis and hence HPLC was the preferred method of detection owing to its high sensitivity. The encapsulation efficiencies for each biomolecule were calculated and are presented below as table 4.6. This initial burst release is within the acceptable therapeutic range for chondrogenesis to occur. There is no evidence of any toxic levels of Pro that is caused by over dosage. The recommended dosage of Proline in combination with vitamin C is generally 500 -1000 mg per day for healthy individuals. Intracellularly, Proline is present within in the 100 -200 μM range[41]. In a study related to Alzheimer's, proline rich peptides (PRP) were administered to patients orally at a dosage of 100 μg per day and this caused no toxicity to the patients[42].

Figure 4.19 Release profiles of Dex, AA and Pro from sandwich scaffold.

Summary

This chapter provides an insight into the fabrication technique for arriving at a dual structured scaffold with micro-particles and fibers. This sandwich system has not been reported yet in the scientific literature. The roles of various processing parameters are of prime importance for arriving at the final desired morphology. Bioactive molecules were loaded into the micro-particles and the fibers and their release profiles were evaluated. The release kinetics, encapsulation efficiencies and initial burst release percentages have been quantified for the hydrophobic Dex and the hydrophilic Ascorbic acid, β -Gly and Proline. The effect of simultaneous loading and release of these molecules was explored and understood.

References

- [1] J. Deitzel, N. Beck Tan, J. Kleinmeyer, J. Rehrmann, D. Tevault, D. Reneker, I. Sendijarevic, and A. McHugh, *Army Research Laboratory Technical Report*, 1999, ARL-TR-1989.
- [2] X. Li, Z. Li, L. Wang, G. Ma, F. Meng, R.H. Pritchard, E.L. Gill, Y. Liu, and Y.Y.S. Huang, *ACS applied materials & interfaces*, 2016. **8**(47): p. 32120-32131.
- [3] N. Okutan, P. Terzi, and F. Altay, *Food Hydrocolloids*, 2014. **39**: p. 19-26.
- [4] N.T. Hiep and B.-T. Lee, *Journal of Materials Science: Materials in Medicine*, 2010. **21**(6): p. 1969-1978.
- [5] R. Murugan and S. Ramakrishna, *Tissue engineering*, 2006. **12**(3): p. 435-447.
- [6] P.K. Baumgarten, *Journal of colloid and interface science*, 1971. **36**(1): p. 71-79.
- [7] J.M. Deitzel, J. Kleinmeyer, D. Harris, and N.B. Tan, *Polymer*, 2001. **42**(1): p. 261-272.
- [8] D.-R. Chen, D.Y. Pui, and S.L. Kaufman, *Journal of Aerosol Science*, 1995. **26**(6): p. 963-977.
- [9] W.K. Son, J.H. Youk, T.S. Lee, and W.H. Park, *Journal of Polymer Science Part B: Polymer Physics*, 2004. **42**(1): p. 5-11.
- [10] D.H. Reneker, A.L. Yarin, H. Fong, and S. Koombhongse, *Journal of Applied physics*, 2000. **87**(9): p. 4531-4547.
- [11] S. Ramakrishna, K. Fujihara, W.-E. Teo, T. Yong, Z. Ma, and R. Ramaseshan, *Materials today*, 2006. **9**(3): p. 40-50.
- [12] J.Y. Kim and D.-W. Cho, *Microelectronic Engineering*, 2009. **86**(4): p. 1447-1450.
- [13] S.-C. Wong, A. Baji, and S. Leng, *Polymer*, 2008. **49**(21): p. 4713-4722.
- [14] B. Duan, X. Yuan, Y. Zhu, Y. Zhang, X. Li, Y. Zhang, and K. Yao, *European Polymer Journal*, 2006. **42**(9): p. 2013-2022.
- [15] Z.-M. Huang, Y.-Z. Zhang, M. Kotaki, and S. Ramakrishna, *Composites science and technology*, 2003. **63**(15): p. 2223-2253.
- [16] R. Kulkarni, K. Pani, C. Neuman, and F. Leonard, *Archives of Surgery*, 1966. **93**(5): p. 839-843.
- [17] H.J. Shin, C.H. Lee, I.H. Cho, Y.-J. Kim, Y.-J. Lee, I.A. Kim, K.-D. Park, N. Yui, and J.-W. Shin, *Journal of Biomaterials Science, Polymer Edition*, 2006. **17**(1-2): p. 103-119.
- [18] V. Karageorgiou and D. Kaplan, *Biomaterials*, 2005. **26**(27): p. 5474-5491.
- [19] N.R. Jørgensen, Z. Henriksen, O. Sørensen, and R. Civitelli, *Steroids*, 2004. **69**(4): p. 219-226.

- [20] T. Hickey, D. Kreutzer, D. Burgess, and F. Moussy, *Biomaterials*, 2002. **23**(7): p. 1649-1656.
- [21] C. Bellows, J. Heersche, and J. Aubin, *Developmental biology*, 1990. **140**(1): p. 132-138.
- [22] R.F. Service, *Science* (New York, NY), 2000. **289**(5484): p. 1498.
- [23] J.B. Baker, G.S. Barsh, D.H. Carney, and D.D. Cunningham, *Proceedings of the National Academy of Sciences*, 1978. **75**(4): p. 1882-1886.
- [24] Z.-X. Su, Y.-N. Shi, L.-S. Teng, X. Li, L.-x. Wang, Q.-F. Meng, L.-R. Teng, and Y.-X. Li, *Pharmaceutical development and technology*, 2011. **16**(4): p. 377-384.
- [25] S. Tsurufuji, K. Sugio, and F. Takemasa, *Nature*, 1979. **280**(5721): p. 408-410.
- [26] M. ElHag, K. Coghlan, P. Christmas, W. Harvey, and M. Harris, *British Journal of Oral and Maxillofacial Surgery*, 1985. **23**(1): p. 17-23.
- [27] Y.-Y. Yang, T.-S. Chung, and N.P. Ng, *Biomaterials*, 2001. **22**(3): p. 231-241.
- [28] I.D. Rosca, F. Watari, and M. Uo, *Journal of Controlled Release*, 2004. **99**(2): p. 271-280.
- [29] S. Murad, D. Grove, K. Lindberg, G. Reynolds, A. Sivarajah, and S. Pinnell, *Proceedings of the National Academy of Sciences*, 1981. **78**(5): p. 2879-2882.
- [30] M. Zilberman, N. Schwade, R. Meidell, and R. Eberhart, *Journal of Biomaterials Science, Polymer Edition*, 2001. **12**(8): p. 875-892.
- [31] J.J. Yoon, J.H. Kim, and T.G. Park, *Biomaterials*, 2003. **24**(13): p. 2323-2329.
- [32] A.J. Thote, J.T. Chappell Jr, R. Kumar, and R.B. Gupta, *Drug development and industrial pharmacy*, 2005. **31**(1): p. 43-57.
- [33] P.F. Choong, T.J. Martin, and K.W. Ng, *Journal of orthopaedic research*, 1993. **11**(5): p. 638-647.
- [34] Y.J. Chuah, Y. Zhang, Y. Wu, N.V. Menon, G.H. Goh, A.C. Lee, V. Chan, Y. Zhang, and Y. Kang, *Acta biomaterialia*, 2015. **23**: p. 52-62.
- [35] H. Moteki, Y. Shimamura, M. Kimura, and M. Ogihara, *European journal of pharmacology*, 2012. **683**(1): p. 276-284.
- [36] Z.-G. Piao, J.-S. Kim, J.-S. Son, S.-Y. Lee, X.-H. Fang, J.-S. Oh, J.-S. You, and S.-G. Kim, *Tissue Engineering Part A*, 2014. **20**(23-24): p. 3322-3331.
- [37] G.P. Mishra, R. Kinser, I.H. Wierzbicki, R.G. Alany, and A.W. Alani, *European Journal of Pharmaceutics and Biopharmaceutics*, 2014. **88**(2): p. 397-405.
- [38] T.L. MCCARTHY, M. CENTRELLA, and E. CANALIS, *Endocrinology*, 1989. **124**(1): p. 301-309.
- [39] P.S. Leboy, L. Vaia, B. Uschmann, E. Golub, S. Adams, and M. Pacifici, *Journal of Biological Chemistry*, 1989. **264**(29): p. 17281-17286.
- [40] K.D. O'NEAL, M.V. CHARI, C.H. McDONALD, L.-y. YU-LEE, J.D. MORRISETT, and T.W. SHEARER, *Biochemical Journal*, 1996. **315**(3): p. 833-844.
- [41] R. Smith, A. Reddi, and J. Phang, *Calcified tissue international*, 1979. **27**(1): p. 275-279.
- [42] A. Gladkevich, F. Bosker, J. Korf, K. Yenkovyan, H. Vahradyan, and M. Aghajanyan, *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, 2007. **31**(7): p. 1347-1355.

Chapter 5

3D scaffolds – Fabrication, Characterization and Release of Multiple Biomolecules for Musculoskeletal Regeneration.

Chapter 5 focuses on 3D scaffolds that were fabricated through electrospinning technique. The first section sheds insights into the various parameters that were optimized to incorporate 3D morphology to the scaffold. These parameters include voltage, distance between tip to collector, polymer concentration, viscosity and spinneret speed among others. This was followed by the characterizations carried out for the 3D scaffolds in the second section. Cross-sectional images of the 3D and 2D scaffolds were taken and the porosity of these scaffolds was evaluated using the liquid displacement method. Five formulations of polymer blends were initially taken into consideration and after extensive optimizations and characterizations; this was tunneled down to final two blends. The third section presents the release patterns exhibited by various biomolecules that were encapsulated into the 3D scaffolds. Initially, Dexamethasone (Dex), a hydrophobic drug was used for preliminary optimizations. The hydrophilic biomolecules whose release profiles were evaluated are Ascorbic acid, β - Glycerophosphate and Proline.²

² A part of this chapter has been published to Journal of Bio Medical Materials. Chamundeswari, Vidya N., et al. "Sustained releasing sponge-like 3D scaffolds for bone tissue engineering applications." *Biomedical Materials* 13.1 (2017): 015019.

5.1 Fabrication of Electro spun 3D Scaffolds

In this study, I have tailored specific electrospinning parameters to achieve sponge-like 3D scaffolds with sufficiently large pore sizes. The schematic of this study is illustrated in **figure 5.1**. The first section focuses on the optimization of the processing and solution parameters to achieve 3D morphology and the various characterization techniques that were used to characterize the fibrous scaffold.

Figure 5. 1 Experimental scheme illustrating the development of a drug loaded 3D fibrous scaffold.

The influence of polymer solution concentration (2 % to 8 % w/v) was first investigated. For consistency, the composition used across the samples was 68.5:26.5:5 (PLLA: PCL: PEO). This composition was chosen as the starting material of investigation for preliminary optimization due to its combined factors of hydrophilicity (due to PEO) which helps in cell attachment and favorable mechanical properties which are conferred by PCL and PLLA.

Figure 5.2 shows the SEM micrographs of the scaffolds obtained from different polymer solution concentrations. At 2 % w/v, a thin 2D sheet-like film was obtained with minute pore sizes that would limit cell penetration. Increasing solution concentration to 5 % w/v, led to the formation of non-uniform fibers with beading. The concept of beading is related to chain entanglements of the polymers and is a commonly encountered issue in highly viscous solutions. For a dilute solution, the polymer chains are placed far apart and hence there is no overlap

between the molecules. The concentration at which the polymer chains begin to overlap is known as the critical concentration ‘c’ and the formation of beads occur at this regime[1]. At 5 % w/v the polymer solution had reduced spin-ability due to its “gel-like” texture, and increasing the voltage alone did not resolve the beading condition as solution viscosity, net charge density carried by the electrospinning jet and surface tension of the solution are the other factors in play. Higher viscosity favours formation of fibers without beads and it has been postulated that increase in polymer concentration leads to viscosity increment[2]. Hence, we increased the polymer blend concentration to 8 % w/v. This gave a 3D structural scaffold with large pore sizes and intertwined fibrous morphology. Future studies were conducted taking the 8 % w/v concentration into account and modifications to the processing parameters were made to arrive at the best 3D scaffold with structural integrity.

The electrospinning parameters were thus tailored after exploring a range of values as presented in **figure 5.2 B-M**. The flow rate of the solutions plays a crucial role in conferring 3D features to the scaffold. Experiments were conducted whereby the flowrate was gradually increased from 0.5 mL h⁻¹ to 2 mL h⁻¹. As the flow rate was increased beyond 1.5 mL h⁻¹, the formation of 3D fibers decreased and a flat deposition was observed (**figure 5.2 D**), hence an ideal flow rate to achieve 3D texture was fixed at 1 mL h⁻¹. Initial 3D morphology formation is followed by flattening of the scaffold when we increase the flowrate from 0.8 up to 2 mL gradually. Also the fibers become thicker as the flow rate increases from 0.5 to 1 mL h⁻¹[3]. The distance between the collector plate and the needle tip plays a role in the drying pattern of the obtained scaffold. A broad range of 8cm to 15 cm was taken into consideration for optimizing the final values. **Figure 5.2 E F G** shows the images of the scaffolds obtained at 8cm, 12cm and 14 cm respectively. At 8cm, the scaffolds exhibited ribbon like morphologies due to remnant solvent presence [4]. At 12 to 14cm, a thoroughly dried intertwined morphology was obtained. The effect of voltage was not very prominent in the formation of the 3D scaffolds. An ideal voltage range is 12-18 kV depending on the formulations being spun (**5.2 K, L, M**). When low voltage of <10kV was applied, lesser electric field was produced to the polymer jet, hence resulting in a less organized structure being formed. On the other hand, when a higher voltage >18kV was applied, the higher electric field

caused a higher volume of solution being dispensed and hence the fibers were collected which were not fully dried. Fibers did not have sufficient time to dry up properly; therefore, they aggregated at the centre of the collector. The optimized parameters used for fabrication of all **3D scaffolds are - 15 kV, 1 mL h⁻¹ flowrate, 2 mm s⁻¹ spinneret speed and a 12-cm distance between needle tip and the collector**. While numerous reports in the literature describe how different types of spinneret can affect electrospinning of nanofibers, the effects of spinneret speed are not well documented [5]. Hence, we have laid emphasis on investigation of the spinneret speed as another processing parameter. Spinneret speed is essential in determining how the fibers arrange themselves either above or adjacent to one another.

Figure 5. 2 (A) Digital and SEM micrographs of the electro spun 3D scaffolds at different polymer concentrations. Figure 2B-M: Digital and SEM images (inserts (scale bar 100 μm) of the 3D scaffold at flowrate of B. 0.5ml/h, C. 1mL/h, D. 1.5mL/h; effect of distance can be observed from E.-8cm, F. 12cm and G. 14cm; the spinneret speed was varied from H. 0mm to I. 2mm until J. 10mm; with voltage applied ranging from K. 7kV to L. 15kV to M. 20kV.

To investigate its effect on 3D scaffold formation, we investigated different spinneret speeds and observed the structure of the final electro spun products. At static (0 mm s⁻¹), a fluffy structure was produced that can be attributed to the accumulation of electrical charges. At a range between 2 – 10 mm s⁻¹, the aggregated fibers oriented themselves adjacent to the existing fibers throughout the entire 3D structures (**Figure 5.2 H, I, J**). When spinneret speeds that exceed 20 mm s⁻¹, extensive clumping was observed that limited the formation of a 3D

fibrous scaffold. Thus, in summary, the most important parameters for 3D fibrous system are the polymer composition ratios and the spinneret speed, variation of which results in drastic changes in the morphology of the obtained scaffold.

5.2 Characterization of Electro spun 3D Scaffolds

The scanning electron micrographs for the 3D scaffolds are presented in **figure 5.2A**. The primary fibers had a mean diameter of $4.41 \pm 0.8 \mu\text{m}$, intertwined to form larger secondary fibers of diameter $30 \mu\text{m}$. Pores measured from this 3D scaffold revealed sizes in the range of $343.19 \pm 71.9 \mu\text{m}$ (figure 5.2; 8% w/v), which are sufficiently large for the infiltration of numerous cell types [6]. The above observation implies that higher polymer concentrations tend to favor 3D scaffold formation with larger pores. **Figure 5.3** plots the viscosity measurements of the three polymer solutions. A scaffold prepared from a 2 % w/v polymer solution had a viscosity of 2.72 ± 0.38 Pascal seconds (Pa. s), 5 % w/v was 3.5 ± 0.24 Pa. s, whereas the 3D scaffold had a polymer solution viscosity of 4.73 ± 0.19 Pa.s. At 2 % w/v, a thin 2D sheet-like film was obtained with minute pore sizes that would limit cell penetration. Increasing solution concentration to 5 % w/v, led to the formation of non-uniform fibers with beading. The concept of beading is related to chain entanglements of the polymers and is a commonly encountered issue in highly viscous solutions. For a dilute solution, the polymer chains are placed far apart and hence there is no overlap between the molecules. The concentration at which the polymer chains begin to overlap is known as the critical concentration and the formation of beads occur at this regime[1]. At 5 % w/v the polymer solution had reduced spin-ability due to its “gel-like” texture, and increasing the voltage alone did not resolve the beading condition. Solution viscosity, net charge density carried by the electrospinning jet and surface tension of the solution are vital factors that affect beading. Higher viscosity favors formation of fibers without beads and it has been postulated that increase in polymer concentration leads to viscosity increment[2]. These results postulate that viscosity played a significant role in determining the outcome of the scaffold, i.e. 2D or 3D, where higher viscosities tend to favor the formation of a 3D structure. Hence, we increased the polymer blend concentration to 8 % w/v and this led to the formation of a 3D structural scaffold with large pore sizes and intertwined fibrous morphology. We further

investigated the influence of viscosity values of other 8 % w/v polymer solution concentrations by varying the polymer composition (**Table 3.4**). Apart from scaffold C, similar 3D structures were obtained despite changes in the polymer composition. It can be further inferred that the minimal viscosity range to achieve a 3D morphology through electrospinning would be above 4 Pa.s. On the other extreme, any formulation above 6 Pa. s led to poor electrospinning control due to a reduced flow of the very viscous solution into the tubing. A 3D structure was therefore empirically determined to be achievable between a viscosity range of 4 to 5.5 Pa. s for PLLA/PCL polymer blends.

Figure 5. 3 Viscosities of all the polymer solutions recorded prior to spinning (A) presents the viscosity of the different concentrations of polymer blends (B) presents the variations in viscosities of different blends with same final polymer concentration of 8% w/v ($P < 0.05$).

To investigate why a 3D structure was not obtained for scaffold C, the ratio of PLLA/PCL/PEO blend was further varied (**table 5.1**). The results confirmed that achieving a 3D structure was possible only when the PLLA content was above 60% of the scaffold composition. Viscosity measurements for polymer solutions less than 60% PLLA were found to be below the range of 4 Pa.s (scaffold C). This determines that PLLA played a critical role in conferring 3D architecture to electro spun scaffolds by increasing viscosity of the polymer solution, a pre-requisite for 3D electro spun structures.

Table 5.1. Variation of polymer blend ratios to determine the minimum PLLA required for formation of 3D fibers.

PLLA (%)	PCL (%)	PEO (%)	3D scaffold formed
100	0	0	Yes
95	0	5	Yes
70	30	0	Yes
66.5	28.5	5	Yes
50	50	0	No
28.5	66.5	5	No
0	95	5	No
0	100	0	No

Next, the mechanical properties of 3D scaffolds were evaluated. While scaffold E exhibited a 3D structure, it was too brittle for handling as PLLA was its only polymer component. Even though PLLA has attractive biomedical applications, its inherent brittleness restricts its application in conditions that require high toughness and tensile property. It has been previously reported that physical blending with polymers like PCL and poly-butylene succinate (PBS) resulted in significant improvements to its toughness [7, 8]. It was also observed that scaffold D (without PCL), was not suitable for long term applications as it disintegrated into smaller pieces within just a few days. This is highly undesirable as these implantable scaffolds are required to maintain their mechanical properties *in vivo* until the regenerated biological tissues are adequately developed. This further validates the importance of PCL in producing mechanically useful scaffolds.

Scaffolds A and B were subsequently characterized for their mechanical properties, through tensile and compressive tests (**figure 5.4**). Scaffold A (PEO containing) exhibited relatively lower mechanical strength as compared to scaffold B ($P < 0.01$, ANOVA). This is in agreement with an earlier study that the addition of PEO to polymer blends can compromise mechanical strengths [9]. The tensile strength (TS) of scaffold A was 0.54 ± 0.13 MPa, while its compressive strength (CS) was 0.55 ± 0.21 MPa. Scaffold B, on the other hand, exhibited ~166% and

~500% higher TS and CS, respectively. The compressive was conducted with starting sample thickness of 0.7 cm. The scaffolds exhibited a compression of about 71% before breaking down. As scaffold B exhibited mechanical integrity of practical relevance, it is therefore an ideal candidate for musculoskeletal tissue engineering applications. Subsequent studies were therefore conducted on Scaffold B, unless stated otherwise.

Figure 5. 4 Mechanical testing for 3D Scaffolds (A and B) without and with Dex illustrating the compressive and tensile strength

Further the porosity of the 3D scaffold was evaluated using the liquid displacement method. Hexane was employed as the solvent since the polymers do not dissolve in it. It was inferred that the porosity of the 3D scaffold was $67 \pm 3.5\%$ whereas the 2D scaffold exhibited a porosity of $34 \pm 0.6\%$. Three readings were taken for each scaffold and the individual values are presented in **table 5.2**. This value is on the higher spectrum when compared with the current scaffolds fabricated by other research groups for bone tissue regeneration. Their porosity values range from 36 – 90% after undergoing extensive post processing steps such as sintering[10].

Table 5.2 Porosity values of 3D and 2D scaffolds calculated using hexane displacement method (n=3).

Scaffold	V ₁ (mL)	V ₂ (mL)	V ₃ (mL)	Porosity (%)
3D B s 1	5	5.18	4.57	70.4
3D B s 2	5	5.25	4.34	72.5
3D B s 3	5	5.28	4.45	66.2
2D S 1	5	5.04	4.97	42
2D S 2	5	5.05	4.98	28.5
2D S3	5	5.06	4.97	33.3

To compare the pore distribution between 2D and 3D scaffolds, cross sectional images of the scaffolds was inspected and are presented in **figure 5.5**. The 2D scaffold was a very compact structure with less visible pores being observed. In comparison, the 3D scaffold exhibited pores greater than 100 μm which are essentially a pre-requisite for the scaffold to be used in bone tissue regeneration.

Figure 5. 5 Cross Section SEM image of 2D (left) and 3D scaffold (right).

The degradation of the 3D scaffold was studied by completely immersing a known weight of the scaffold in PBS solution for 45 days. At pre-determined time intervals, a sample of the scaffold was removed from PBS, dried and imaged under SEM to inspect its morphological changes with respect to time. The various time points for this study were Day 0, Day 10, Day 20, Day 30 and Day 45. **Figure 5.6** depicts the morphology of the scaffolds at the above-mentioned time points. The gradual disintegration of ribbon like intertwined morphology can be observed from day 20 onwards.

Figure 5. 6 Degradation study of 3D scaffold depicting the SEM micrographs at day 0, 10, 20, 30 and day 45.

FT-IR analysis of the drug-loaded scaffold B revealed peaks corresponding to PCL and PLLA (**Figure 5.7 A**). Standard peaks of Dex at 1706, 1621 and 1662 cm^{-1} which represents the -C=O bonding, were also observed in the PLLA/PCL-Dex scaffold [11, 12]. The absence of any new peaks confirmed that the drug is physically mixed with the polymer matrix. Also, the incorporation of Dex into the PLLA/PCL blend was prominently visible through Raman spectroscopy. The characteristic peak of Dex at 1650 cm^{-1} (**Figure 5.7 B**) was observed in both the pure Dex as well as PLLA/PCL Dex samples.

Figure 5. 7 (A) FTIR (B) Raman of scaffold B with and without Dex.

Figure 5. 8 DSC spectrum of scaffold B with Dex revealing the melting point of each polymer and the drug.

The blank scaffold and Dex loaded scaffold were subjected to thermal analysis using DSC. The aim of this study was to confirm their respective melting points (T_m). DSC measurements were carried out by (1) Heating from room temperature to 200°C at a rate of 10°C/min; (2) Cooling to 30°C at 50°C/min; (3) Maintaining for 3 min at 30°C; (4) Repeating the above. If the polymers formed only a physical

blend, they will not exhibit any changes in their T_m values. This was the observation here too as the melting points of PLLA and PCL did not change in the blank scaffold and corresponded to 175 ° C and 54° C respectively [13, 14]. The melting point of Dex is in the range of 249-254 ° C which was also observed in the pure Dex and from the Dex loaded scaffold thus confirming the presence of the drug within the scaffold qualitatively (**figure 5.8**).

The water contact angle of the 3D scaffolds was difficult to obtain post spinning due to the rough surface of the scaffold and the fibrous nature of the mesh. Hence the homogenous blend solution was vacuum dried for 48 hours and contact angle measurements of the dried polymer solution were measured. The contact angle values for individual PLLA, PCL and the PLLA/PCL blend polymers are presented in **figure 5.9 and table 5.3**. From the values, it can be inferred that the addition of PCL to the hydrophobic PLLA aided in increasing the hydrophilicity of the blend solution. The PLLA that I have used for my study has a very high intrinsic viscosity of 6.5 dl/g. (Mol weight ~700,000 Daltons). The enthalpy values of 100% crystalline PLLA and PCL were accounted from literature sources [15-17]. The percentage of crystallinity of PLLA used in this study was calculated to be 68% compared to PCL whose crystallinity was ~15.9%. From these values, we can infer that higher percentage of crystallinity is one of the influencing factors that results in higher hydrophobicity values of the PLLA polymer[18]. Hence addition of PCL to the PLLA in this condition resulted in a pronounced reduction of the water contact angle values of the polymer blend (PLLA/PCL). Addition of Dex to the polymer solution had no effect on the water contact angle. To illustrate the effect of adding in PEO in the blend (scaffold A), the water contact angle of PLLA/PCLPEO scaffold was also measured (**figure 5.9 C**). PEO aided in improving the hydrophilicity of the PLLA/PCL blend by 46% approximately. Thus, scaffold A is viable for applications in TE which require a high degree of cell attachment and negligible mechanical strength.

Figure 5. 9 Contact angle images for (A) PLLA, (B) PLLA/PCL and (C) PLLA/PCLPEO.

Table 5.3 Contact angle measurements of the cast films of the polymer blends as measured using a goniometer (n=5).

Sample	Contact angle n=5 (degrees)
PLLA	124 ± 6.1
PLLA/PCL	89 ± 3.9
PLLA/PCLPEO	67 ± 4.6

5.3 Release study of biomolecules from 3D Scaffolds

5.3.1 Dexamethasone Release profiles

Depending on the polymer concentration ratios, five formulations of the 3D scaffolds were taken into considerations for studying the release pattern of Dex. The concentration of Dex for these studies was maintained constant as 2 wt % of total polymer concentration. Table 5.5 illustrates the compositions of all the five formulations of 3D scaffolds that were electro spun for the Dex release study.

Table 5.4 Polymer composition of PLLA, PCL and PEO

PLLA (%)	PCL (%)	PEO (%)	3D scaffold Formation
66.5	28.5	5	Scaffold A
70	30	0	Scaffold B
28.5	66.5	5	Scaffold C
95	0	5	Scaffold D
100	0	0	Scaffold E

Dex was mixed along with 0.5 mL of HFIP and added to the polymer solution and homogenized overnight through constant mechanical stirring. As mentioned earlier,

scaffold C did not exhibit a 3D morphology which may be attributed to the 28% PLLA concentration which directly affected the viscosity of the solution. The SEM images of scaffold A-E and Dex loaded scaffold are shown in **figure 5.10**. While scaffold E exhibited a 3D structure, it was too brittle for handling as PLLA was its only polymer component. Even though PLLA has attractive biomedical applications, its inherent brittleness restricts its application in conditions that require high toughness and tensile property. It has been previously reported that physical blending with polymers like PCL and poly-butylene succinate resulted in significant improvements to its toughness [7, 8]. It was also observed that scaffold D (without PCL), was not suitable for long term applications as it disintegrated in PBS into smaller pieces within just a few days. This is highly undesirable as these implantable scaffolds are required to maintain their mechanical properties *in vivo* until the regenerated biological tissues are adequately developed. This further validates the importance of PCL in producing mechanically useful scaffolds. The disintegration of scaffold D after a few days in liquid media is further effected by its degradation mechanism[19]. PLLA undergoes bulk degradation i.e mass loss occurs and the molecular chains are broken down in a way that they diffuse out of the polymer matrix and undergo drastic weight loss and structural integrity[20]. Subsequently scaffold C and E were eliminated from further studies and the release study of scaffolds A, B and D were conducted. The mechanical properties of scaffolds A and B were previously evaluated and their performance to tensile stress was within the acceptable regime for bone and cartilage tissue regeneration studies.

Further, the 3D scaffolds (both A and B) retained their fibrous morphology even after 45 days in 1xPBS (**figure 5.6**). Hence, the scaffolds preserve their structural integrity for almost two months, which indicates that it has potential for musculoskeletal regeneration.

Figure 5. 10 shows the scanning electron micrographs of (a) Scaffold A; (b) Scaffold B; (c) Scaffold C; (d) Scaffold D; (e) Scaffold E; (f) Dex loaded 3D scaffold.

The release of Dex from Scaffold A, B and D are compiled as **figure 5.11**. The high encapsulation efficiency of Scaffold B is due to the polymer composition. PLLA/PCL (scaffold B) is more hydrophobic, and hence could host in and retain significantly more Dex when compared to PLLA/PCL/PEO (scaffold A). Scaffold A contains PEO whereas scaffold B doesn't and this may attribute to a higher initial burst release exhibited by scaffold A. In the case of scaffold D, the reduced EE can be attributed to its polymer composition (PLLA/PEO). Eventhough this scaffold could release the encapsulated Dex for the longest duration; its mechanical integrity was compromised due to PEO and its disintegration of into

smaller pieces within the media posed challenges in determining the time duration for studying complete release of Dex. Hence was not an ideal candidate for musculoskeletal tissue regeneration applications. It was observed that scaffold B (**Figure 5.11 A**) exhibited a higher EE of about $86 \pm 2.1\%$ when compared with scaffolds A and D (**figure 5.11 B**). Burst release of the drug was within 33% of the total encapsulated Dex. This control over the burst release can be attributed to the unique intertwined morphology of the scaffolds. The ability to control the initial burst release is an added advantage as Dex is known to cause systemic toxicity at higher concentrations [21].

Figure 5. 11 Release pattern of hydrophobic Dex from (A) scaffold B; (B) scaffolds A and D.

Table 5.5 Burst release percentages, Encapsulation efficiency and the number of days for complete release for all the 3D scaffold systems when loaded with 2% Dex

Scaffold	Encapsulation efficiency of Dex (%)	Burst release in 24 hours	Duration for complete release
A	81	33	50
B	86	28	30
D	63	35	N/A

We plotted the release kinetics for scaffold A and B (**figure 5.12**) and it was observed that the 3D fibers released Dex in a controlled manner and this release profile closely resembled zero-order kinetics. Such a release profile will ensure consistent release of Dex and ensuring that the release amount does not exceed the

therapeutic dosage levels at any point in time. A nearly zero order kinetics also indicates that the release of the drug is independent of the concentration of drug loaded in the polymer solution [22]. In this case, this remains true when we varied the drug loading from 1% to 4 wt % of the polymer (results not shown).

Figure 5. 12 The kinetics of Dex release from Scaffold A and B follows nearly zero order.

5.3.2 Simultaneous release of Dex and Ascorbic acid (AA) from the 3D scaffold

The simultaneous release of a single hydrophobic and hydrophilic drug from the 3D scaffold was evaluated next. Dex and AA both play significant roles in differentiation of stem cell fate towards osteogenesis or chondrogenesis. While Dex is an anti-inflammatory agent, it also plays a vital role in directing the fate of mesenchymal stem cells towards desired lineage. The release profile for Dex and AA from the 3D scaffold B revealed a very low EE value for both the drugs. Dex being hydrophobic was always incorporated into PLLA and AA was incorporated into PCL before mixing both the polymer solutions in a 7:3 ratio to form a homogeneous blend. The EE values for Dex and AA were 55 % and 35 % respectively. Further, AA exhibited a burst release of 63% in the first 24 hours. Hence, we have eliminated further studies on this polymer drug combination as more than 120 μM of AA released because of the burst release. The desired quantity of AA per day ranges from 20 -30 μM [23].

5.3.3 Simultaneous Release of two Hydrophilic Biomolecules from Scaffold B

Hydrophilic biomolecules such as Proline and β - Glycerophosphate are essential for MSC differentiation towards chondro or osteo lineage respectively. These molecules are supplemented through the cell media initially and their supply is not possible *in vivo*. Further, we wanted to establish the release of two hydrophilic biomolecules from the as-fabricated 3D scaffolds that find applications in the musculoskeletal arena. Hence, we studied the sequential release of two 3D systems each containing a different combination of hydrophilic bioactive molecules.

Firstly, we investigated the effect of loading AA and B Gly together in the 3D scaffold. Both the molecules were mixed with 0.5 mL of DI H₂O and added to the PLLA/PCL blend solution before electrospinning. This combination was explored for studying the potential of MSCs towards osteogenesis. AA is responsible for collagen synthesis while β - Gly is an osteogenic inducer molecule. The loading concentration for each of the drugs was 2 wt % with respect to the polymer.

Figure 5.13 reveals the release profiles of both these molecules from the 3D scaffold.

Figure 5. 13 Release profiles for AA and β - Gly from scaffold B.

The EE, burst release percentages and the quantity of the molecules released at every time point was calculated. It was observed that the EE of both the molecules

was higher than the previous case where Dex and AA were encapsulated together. AA exhibited 61% EE while about 55% of β – Gly was successfully encapsulated. Both the biomolecules released within 20 days and exhibited $50 \pm 12\%$ burst release during the first 24 hours. However, the amount of the biomolecules that was released during this phase did not exceed the therapeutic dosage limits and hence these scaffolds were further evaluated for their efficacy in directing the fate of MSCs towards osteogenesis. Similarly, for evaluating the efficacy of MSCs towards chondrogenesis, β – Gly was replaced by Proline (Pro), an amino acid. Proline is a highly hydrophilic molecule and is supplemented into the cell culture media for inducing the formation of cartilage tissue. In the case of this study, the 3D scaffold was loaded with 2wt % Pro alongside AA and the release profiles of this combination were evaluated (**figure 5.14**).

Figure 5. 14 Release profiles for AA and Proline from scaffold B.

The EE % of Pro was calculated to be 78% and that of AA was around 60 %. Unlike many hydrophilic molecules, Proline's burst release was capped at 12% and it released completely over a period of 18 days. This scaffold was also subjected to *in vitro* experiments with MSCs to validate their application for cartilage regeneration.

Summary

A simple one step spinning strategy for fabrication of 3D scaffolds has been reported in this chapter. The scaffolds formed exhibit a unique intertwined fibrous 3D architecture without the need for any post processing procedures. The effect of various solution and processing parameters are discussed and manipulated to arrive at the final, mechanically stable scaffold (scaffold B). This scaffold exhibited excellent cell penetration depths of more than 500 μm which has not been reported in the scientific literature so far using a one-step fabrication protocol. Various bioactive molecules – hydrophobic (Dex) and hydrophilic (AA, β -Gly and Pro) were loaded into this scaffold and their release profiles were evaluated. Finally, the gene expression studies and immunohistochemical analyses were carried out for various gene markers of the osteogenic and chondrogenic lineage which are discussed in the next chapter. This further confirms the importance of controlled release of the biomolecules to direct the fate of MSCs to differentiate towards the desired lineage

References

- [1] D.H. Reneker, A.L. Yarin, H. Fong, and S. Koombhongse, *Journal of Applied physics*, 2000. **87**(9): p. 4531-4547.
- [2] H. Fong, I. Chun, and D. Reneker, *Polymer*, 1999. **40**(16): p. 4585-4592.
- [3] V. Milleret, B. Simona, P. Neuenschwander, and H. Hall, *Eur Cell Mater*, 2011. **21**: p. 286-303.
- [4] A. Greiner and J.H. Wendorff, *Angewandte Chemie International Edition*, 2007. **46**(30): p. 5670-5703.
- [5] B. Lu, Y. Wang, Y. Liu, H. Duan, J. Zhou, Z. Zhang, Y. Wang, X. Li, W. Wang, and W. Lan, *Small*, 2010. **6**(15): p. 1612-1616.
- [6] C.M. Murphy, M.G. Haugh, and F.J. O'Brien, *Biomaterials*, 2010. **31**(3): p. 461-466.
- [7] R. Wang, S. Wang, Y. Zhang, C. Wan, and P. Ma, *Polymer Engineering & Science*, 2009. **49**(1): p. 26-33.
- [8] M. Broz, D.L. VanderHart, and N. Washburn, *Biomaterials*, 2003. **24**(23): p. 4181-4190.
- [9] S. Zivanovic, J. Li, P.M. Davidson, and K. Kit, *Biomacromolecules*, 2007. **8**(5): p. 1505-1510.
- [10] V. Karageorgiou and D. Kaplan, *Biomaterials*, 2005. **26**(27): p. 5474-5491.
- [11] L.B. Rodrigues, H.F. Leite, M.I. Yoshida, J.B. Saliba, A.S.C. Junior, and A.A. Faraco, *International journal of pharmaceutics*, 2009. **368**(1): p. 1-6.
- [12] W.H. Hoidy, E.A.J. Al-Mulla, and K.W. Al-Janabi, *Journal of Polymers and the Environment*, 2010. **18**(4): p. 608-616.
- [13] C.-C. Chen, J.-Y. Chueh, H. Tseng, H.-M. Huang, and S.-Y. Lee, *Biomaterials*, 2003. **24**(7): p. 1167-1173.

- [14] M. Todo, S.-D. Park, T. Takayama, and K. Arakawa, *Engineering Fracture Mechanics*, 2007. **74**(12): p. 1872-1883.
- [15] B. Gupta and A.R. Ray, *Journal of applied polymer science*, 2012. **123**(4): p. 1944-1950.
- [16] T. Patrício and P. Bártolo, *Procedia Engineering*, 2013. **59**: p. 292-297.
- [17] E. Can, G. Udenir, A.I. Kanneci, G. Kose, and S. Bucak, *Aaps Pharmscitech*, 2011. **12**(4): p. 1442-1453.
- [18] A. Areias, C. Ribeiro, V. Sencadas, N. García-Giralt, A. Diez-Perez, J.G. Ribelles, and S. Lanceros-Méndez, *Soft Matter*, 2012. **8**(21): p. 5818-5825.
- [19] S. Li, H. Garreau, and M. Vert, *Journal of Materials Science: Materials in Medicine*, 1990. **1**(4): p. 198-206.
- [20] R.L. Kronenthal, *Biodegradable polymers in medicine and surgery*, in *Polymers in medicine and surgery*. 1975, Springer. p. 119-137.
- [21] D.-H. Kim and D.C. Martin, *Biomaterials*, 2006. **27**(15): p. 3031-3037.
- [22] Q. Wang, J. Wang, Q. Lu, M.S. Detamore, and C. Berkland, *Biomaterials*, 2010. **31**(18): p. 4980-4986.
- [23] X. Zhao, Y.S. Lui, P.W.J. Toh, and S.C.J. Loo, *Materials*, 2014. **7**(11): p. 7398-7408.

Chapter 6

Cell Studies for Dual Structured and 3D Scaffolds Systems

This chapter sheds insights into the in vitro experiments conducted for all the fabricated scaffold systems. All the in vitro studies for the dual structured systems have been discussed first followed by the cellular studies conducted with 3D scaffolds. For the dual structured scaffolds, gene expression studies were conducted for Dex and AA loaded into the fibers and particles respectively and the effect of the loading concentrations were inferred. A third bio molecule was further added to the micro-particle solution to direct the MSCs to differentiate into osteo (β - Gly) or chondro (Pro) lineage. In the case of 3D scaffolds, the effect of a single hydrophobic drug (Dex) on MSCs proliferation was conducted followed by addition of AA, β - Gly and Pro loaded scaffolds to direct MSCs to form desired tissue. The various genes that were analyzed in this chapter are Col I, Col II, aggrecans, OCN, RunX2 and ALP. Immuno-histochemical staining was carried out for confirming the presence of Col I, Col II and aggrecans for the scaffolds. Alizarin red staining and EDX were employed to confirm the presence of calcium and phosphorous in the scaffolds three weeks post MSC seeding.³⁴

³ A part of this chapter has been published to Journal of Bio Medical Materials. Chamundeswari, Vidya N., et al. "Sustained releasing sponge-like 3D scaffolds for bone tissue engineering applications." *Biomedical Materials* 13.1 (2017): 015019.

⁴ Some results from this chapter have been submitted to Journal of Materials Chemistry B and are under peer review post first revision.

Introduction

The multi-potency of stem cells has been a growing interest in the field of tissue engineering which can be directed to certain lineage differentiation for tissue regeneration. For instance, mesenchymal stem cells (MSCs) have been explored for use in various branches of tissue regeneration from bone to vertebral discs under appropriate culture conditions. Their multi-potency, ease of availability, negligible immune-rejection, accessibility, and ethical sources for harvest make them attractive candidates amongst others for translational medicine applications [1-3]. Many studies have investigated the effects of MSCs as alternative strategies for bone and cartilage regeneration [4]. The combination of various properties of MSCs has made them ideal candidates for regenerative and reparative tissue engineering strategies.

6.1 Bio Molecules Induced MSC Differentiation on Sandwich Scaffolds.

The efficacy of the sandwich scaffolds in promoting bone formation was evaluated initially with two biomolecules (Dex and AA) at 4 wt% and 1 wt% concentration each. It was observed that the encapsulation efficiency (EE) of Dex and AA was $79 \pm 3.4 \%$ and $68 \pm 6.1 \%$, respectively. For the cell studies, hMSCs were cultured in an expansion medium comprising of low glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, penicillin (100 U mL^{-1}), streptomycin ($100 \mu\text{g mL}^{-1}$) mixture, and 2 mM glutamaxTM at 37°C under a humidified atmosphere with 5% CO₂. Culture medium was changed thrice a week. 1.0×10^6 hMSCs per cm² were seeded on scaffolds that were uniformly cut into circular shape with 8 mm diameter. The scaffolds (containing 4 wt% of each drug) were classified into four groups namely (i) Blank, (ii) Dex only, (iii) AA only and (iv) Dex and AA. They were subjected to the expansion medium for a period of three weeks and later analysed for a late marker of osteogenesis – Osteocalcin (OCN). The scaffolds were also evaluated to detect the upregulation of Col type II which is an indicator of cartilage tissue formation. Interestingly, it was observed that the amount of biomolecules released had a negative effect towards inducing osteogenesis and chondrogenesis; instead they down regulated all the gene markers. This is represented in **figure 6.1**.

Figure 6. 1 Relative gene expression of (A) Osteocalcin (OCN) and (B)Col type II (Col II) after three weeks of MSC seeding.

It can be observed that in some cases, the presence of one of the biomolecules upregulated the relative gene expression; however the combination of both the biomolecules did not induce the desired lineage formation[5]. Also, the blank scaffolds without any biomolecules was shown to upregulate the expression of Col II as observed in **figure 6.1B**. This validates the potential of the scaffold to enhance the expression of chondrogenic markers. However, the significant down regulation of markers when biomolecules are present at a 4 wt% concentration may be attributed to the fact that the initial release of Dex and or AA exceeded the therapeutic dosage levels thus not aiding further in the differentiation of MSCs into desired lineage. To address this issue and validate this theory, the drug loading was reduced to 1 wt% of the polymers to observe its efficacy in promoting osteogenesis and chondrogenesis. For this condition, the EE for the biomolecules was 76.7 ± 1.4 and 65 ± 7.5 % for Dex and AA respectively. With this loading rate, the burst release of Dex was 28.9 μg per mL of the expansion medium and for AA it was 14.5 μg per mL of the expansion medium. The total expansion medium used for each scaffold was 200 μL which was changed at an interval of three days. The benefits of loading AA and Dex together is to firstly utilize the efficacy of Dex in preventing inflammatory reactions as well as to provide cues to the MSCs to differentiate into osteo and chondrogenic lineages[6]. While Dex releases in a controlled manner for more than 30 days, AA releases completely from the particles within two weeks. AA plays vital role in

wound healing, maintains the immunity of the body and also helps in tissue repair by inducing the post translational modification of collagen molecules and the biosynthesis of collagen[7-9]. It is widely utilized in the expansion medium as a stimulatory agent for lineage specific differentiation of mesenchymal stem cells[7, 10]. It also plays a critical role in cell sheet engineering[11].

Cell sheet formation could be observed for the drug loaded scaffolds. **Figure 6.2A-B** illustrates the synergistic effect of loading both Dex and AA into the scaffold which was validated with the upregulation of osteogenic markers (Col I and ALP). Further, the scaffold was subjected to immunohistochemical staining for Col I as shown in **figure 6.2 C**. Vit C indicates a blank scaffold with AA supplemented through the medium rather than sequential release from the scaffolds.

Figure 6. 2 Relative gene expression studies of (A) Col I and (B) ALP. The immuno histochemical staining for Col I primary antibody is presented in (C).

6.2 Multiple Biomolecules Induced MSC Differentiation on Sandwich Scaffolds

The dual bio molecule loaded scaffold system was further tailored to simultaneously release three biomolecules. Dex and AA remained constant in both the systems while the third biomolecule was varied to direct the fate of MSCs towards bone or cartilage. Firstly MSCs adhesion to the scaffold plays a critical role in its differentiation

potential. Hence this was studied on the blank and drug loaded scaffolds (**figure 6.3**). It was observed that single or multiple drug loading had no negative impact on the adhesion of MSCs when compared to a blank bi-layered scaffold ($P=0.767$).

Figure 6.3 MSC adhesion on blank and drug(s) loaded scaffolds.

For induction into bone formation the third biomolecule loaded into the particles was β - Glycerophosphate (β - Gly). For differentiation into cartilage tissue, Proline (Pro) was introduced into the particle solution before electro spraying along with Dex loaded fibers and AA loaded particles. The concentration of both of these molecules was 1 wt % each with respect to the polymer. 0.5 mil hMSCs per cm^{-2} of the scaffold were seeded and cultured in expansion medium for a period of three weeks. The results pertaining to osteogenesis are summarised in 6.2.1 and the findings pertaining to chondrogenesis are presented in 6.2.2.

6.2.1 Osteogenic Differentiation of MSCs

The encapsulation efficiency of β - Gly was calculated to be $72 \pm 3.3\%$ and it was encapsulated into the particle solution at 1 wt % loading before electro spraying onto the Dex loaded fibrous mesh. Runt-related transcription factor 2 (Runx2) is a transcription factor with an important regulatory role in MSCs osteogenic differentiation and mineralization[12]. Osteogenic medium (OM) signifies that the biomolecules were added in solution form directly to the expansion medium while all the scaffolds were blank. This was done to prove the need for sustained and controlled release of the biomolecules over a period of time rather than adding a significant amount thrice a week to the expansion medium. **Figure 6.4** presents the results pertaining to upregulation of osteogenesis markers namely ALP, Col I and RunX2 which was quantified using q PCR.

Figure 6. 4 Gene upregulation studies of osteogenesis markers (A) ALP, (B) Col I and (C) RunX2.

It was observed that the markers for osteogenesis were significantly upregulated in the scaffold loaded with all the three biomolecules in comparison to the blank scaffold seeded with MSCs. Also, Alizarin red stain was utilized to evaluate presence of Ca^{++} on the scaffold and to confirm if mineralization has been induced (**figure 6.5**). The entire scaffolds were stained with alizarin red complex for five minutes followed by series of washing steps to remove any temporary staining. It was established that when scaffolds were used as reservoirs and carriers of the biomolecules, the calcium (Ca^{++}) deposition on the scaffolds was increased in comparison to OM.

Figure 6. 5 Alizarin red complex formation reaction on the scaffolds. OM denotes the blank scaffold supplemented with all the drugs through the medium.

This further proves that more mineralization occurred in the case of drug loaded scaffolds. An EDX study was conducted on the blank and the drug loaded scaffolds to detect presence of calcium and phosphorus (P). These elements essential for mineralization to occur *in vitro*[13, 14]. The EDX graph is presented as **figure 6.6**. The levels of Ca and P secreted were not quantitatively measured in this study.

However, from the graph, it can be inferred that both Ca and P are present in elevated concentrations in the biomolecules loaded scaffold while the blank scaffold does not reveal any presence of Ca or P. We can also observe the presence of P in the OM scaffold, as it contains some β - Gly (2 mM per 200 μ L) within. However, alizarin staining gave a brown coloration for the OM scaffold and a bright red coloration for the multidrug loaded scaffold. No Ca could be detected in the OM scaffold. This reiterates the importance of sequential release of β - Gly and other biomolecules for the mineralization to occur.

Figure 6. 6 EDX analysis of the drug loaded scaffold after three weeks of MSC seeding. Presence of Ca and P is observed and the K α values are mentioned.

Figure 6.7 Immuno histochemical staining for Col I primary antibody

Finally, immunohistochemical staining was conducted for the drug loaded and blank scaffolds after cutting them into 8 μ m sections onto a glass slide[15]. Col type I primary antibody was used to see evaluate the efficacy of the scaffold in promoting osteogenesis. A darker deposition of Col 1 was observed on the drug loaded scaffolds

when compared to the blank scaffolds as seen in **figure 6.7**.

6.2.2 Chondrogenic Differentiation of MSCs

The differentiation of MSCs into cartilage lineage was also explored by adding a third bio molecule – Proline to the system. Proline was also added at a concentration of 1 wt% and the EE % of Pro was 88 ± 0.5 %. Pro is crucial for cartilage formation and is supplemented in the expansion medium at a concentration of $40 \mu\text{g mL}^{-1}$ [16]. The scaffolds released Pro for a period of 16 days after MSC seeding. Col II is the primary marker for detecting the occurrence of chondrogenesis. Hence, the scaffolds were examined for upregulation of Col II after three weeks of differentiation.

Figure 6. 8 Relative gene expression results to quantify the expression of (A) Collagen Type II and (B) Aggrekans. Immunohistochemical staining for evaluating the presence of (C) Col II and (D) Aggrekans.

The results (**figure 6.8A and C**) revealed an enhanced upregulation of Col type II in the drug loaded scaffolds when compared to the blank scaffolds. However the presence of the drugs did not significantly affect the upregulation of aggrekans as evidenced from the quantitative results (**figure 6.8B and D**). Aggrekans are an

important component of the cartilage tissue. The globular and the glycosaminoglycan(GAGs) domains of the aggrecans plays vital roles in maintaining cartilage structure and function[17]. This showed significantly increased gene expression of Col II when compared with the blank scaffold (figure 6.8A, $P < 0.001$). While the presence of AA and Pro could enhance biosynthesis of Col II, these bioactive molecules exhibited negligible effect on aggrecan gene expression. However when the presence of aggrecans was evaluated through immunohistochemical staining with Sulfonin – O and Alcian blue it can be seen that which stained the sectioned tissues in the drug loaded scaffolds exhibited pink and blue colouration respectively (figure 6.8D). These results validate the potential role of bioactive molecules such as Proline, Ascorbic acid in directing the differentiation of MSCs towards cartilage tissue formation.

6.3 MSC Differentiation on 3D Scaffolds.

The 3D scaffolds were also evaluated as potential candidates for validation of the differentiation capability of MSCs. Human mesenchymal stem cells (hMSCs) have been widely utilized and explored in regenerative medicine due to their self-renewal abilities, and capability to differentiate into various cell lineages. These inherent properties of hMSCs thus present them as a potential source of cells for bone tissue engineering. The open pores present in the 3D scaffolds mimic the ECM closely than conventional 2D scaffold mats and may provide a base for angiogenesis to occur at later stages[18]. In this section, the MSC penetration is discussed first followed by the effect of loading a single hydrophobic molecule such as Dex and the effect of loading two hydrophilic molecules such as AA - β - Gly combination or AA – Pro combination are discussed in detail.

6.3.1 MSCs Penetration on the 3D Scaffolds

Cell penetration was evaluated with MSCs and compared with the conventional 2D scaffold mat to illustrate the cell spreading in 3D scaffold. **Figure 6.10** illustrates the cell penetration exhibited by the 3D scaffold. Cell viability was well observed within both scaffolds with minimal cell death. Conventional electrospinning often resulted in densely packed nanofibers (2D scaffolds) that limited cell penetration thus restricting

its use in tissue engineering applications. Our strategic electrospinning approach helps to develop a thick fibrous scaffold with large pore sizes which aid in cell infiltration. This facilitates tissue regeneration at a later stage. It was observed that MSCs were able to infiltrate through the 3D scaffolds with the penetration depth of more than 350 μm . MSCs were mostly confined at the top of the 2D scaffold with less than 50 μm of cell penetration. Layering of the fibers resulted in highly dense 2D scaffolds that resulted in total penetration lesser than 20% of the scaffold thickness. Similarly, other reports for electro spun 3D scaffolds had cell penetration often limiting only to the surface, with none reporting a more significant cell penetration [19, 20]. The results (**figure 6.9**) highlight the importance of high porosity with large pore sizes in permitting the easy infiltration of cells into the scaffold for tissue regeneration[21]. These observations confirmed that mechanically stable 3D scaffolds of substantial pore sizes can be obtained through a one-step electrospinning approach.

Figure 6. 9 Cell growth and distribution within electro spun 2D and 3D scaffolds using MSCs. Live dead staining was conducted and the scaffolds were visualized via confocal microscope. Z-stacked images reveal cells infiltrated into the 3D scaffolds for a thickness of 350 μm while for 2D scaffolds cells were only stationed as a planar layer. Total thickness of 2D scaffold = 0.1mm and 3D scaffold =0.7cm.

6.3.2 The Effect of Dex loading on MSC Differentiation.

Dex is widely known to play pivotal roles in bone repairs and osteogenic differentiation of hMSCs. In this study, 2 wt % Dex was incorporated into scaffold A, B and D for profiling the release from these three matrices. The release study results

evaluated the EE of Dex to be in between 63 – 89 % depending on the polymer blend used for the fabrication process. However, all the cell work for the 3D scaffolds was carried out using scaffold B due to its excellent mechanical integrity.

Both Osteocalcin (OCN) and Alkaline Phosphatase (ALP) have critical roles in the mineralization of bone tissues, and are thus usually assessed in various studies involving osteogenic differentiation [22]. ALP is implicated in the mineralization of carbonated apatite while OCN is involved in bone mineralization and calcium ion homeostasis. Both ALP and OCN are markers of osteoblasts in the transition phase from maturation to bio mineralization during MSCs osteogenesis[23]. Numerous studies have shown that the addition of Dex influences the expression of ALP and OCN genes of hMSCs during osteogenic differentiation [24]. For instance, the presence of 10^{-7} M Dex in osteogenic medium promoted the gene expression of both ALP and OCN even in the absence of other osteogenic inducing factors such as β -glycerophosphate and ascorbic acid[25]. Hence the osteogenic gene expression of hMSCs in the absence of free Dex in the osteogenic differentiation medium was evaluated and compared with the Dex loaded 3D scaffolds (**figure 6.11**).

Figure 6. 10 (A) Relative gene expression of osteogenic markers, Alkaline Phosphatase (ALP) and Osteocalcin (OCN). P-value < 0.05 shows significant difference between the scaffolds with and without Dexamethasone; (B) Alizarin red stained scaffolds with and without Dex; (C) EDX image of scaffolds with and without Dex illustrating the peaks for Ca and P.

In this study, gene expression of ALP and OCN were significantly upregulated (P value < 0.0040, Student's t-test) on Dex loaded 3D scaffolds as compared to blank 3D during the transition phase from maturation to bio mineralization (2 weeks after induction of osteogenic differentiation) (**figure 6.10 A**). Bio-mineralization of the tissue constructs was demonstrated by Alizarin red stain and EDX analysis at the end

of 3 weeks (**figure 6.10 B, C**). The presence of brick red nodules revealed biomineralization on Dex loaded scaffold while minimal staining was observed within the blank 3D scaffolds indicating poor mineralization during osteogenic differentiation. For further verification, EDX analysis confirms the high level of calcium deposition and phosphate groups in the Dex-loaded scaffolds as compared to the blank scaffold. This validates the importance of loading and substantial release of Dex in promoting mesenchymal stem cell fate towards an osteogenic lineage.

6.3.3 Hydrophilic Drug loading into 3D Scaffolds for MSC differentiation.

AA, β - Gly and Pro were the hydrophilic biomolecules of interest in this study. AA is essential for both osteogenesis and chondrogenesis to occur while β - Gly and Pro have specific roles in directing osteo and chondro lineages respectively. Hence two sets of scaffolds were fabricated for this study. Set A was scaffold loaded with AA and β - Gly which was used to explore the fate of MSCs towards osteo lineage. Set B was the scaffold loaded with AA and Pro and this combination was used to direct the fate of MSCs towards chondrogenic tissue formation. These molecules were released from the scaffolds within 20 days of exposure to the release medium as discussed in chapter 5. The role of β - Gly is essential for enhancing the formation of bone tissue[26]. The gene upregulation results and the immunohistochemical staining results are discussed in depth. **Figure 6.11** summarizes the gene expression study results for Set A. The markers that were analyzed for osteogenesis are OCN and Col type I. It can be observed that the expression of OCN was upregulated in the presence of both AA and β - Gly, whereas Col I was downregulated. Further, OCN was not significantly upregulated in this case.

Figure 6. 11 Relative Gene expression analysis of Set A for detecting osteogenesis markers - Osteocalcin (OCN left); and Collagen type I(Col I right).

Hence, immuno-staining for Col I primary antibody was conducted to observe if there were deposits of Col I on the scaffold loaded with AA and β - Gly (**figure 6.12**).

Figure 6. 12 Immunostaining for Col I antibody on Set A containing AA and β - Gly after three weeks of MSC seeding.

Interestingly, it was observed that after three weeks of culture at 37°C, the stain for Col I primary antibody was only found on the outer most surface of the 3D scaffold and had not penetrated into the scaffold. This further led to subjecting the scaffold for porosity testing which revealed that the scaffold's porosity was $47 \pm 6.9\%$ which was very less for 3D scaffolds. Thus, the conclusion for this study was that even though the scaffold exhibits potential for directing the MSCs towards osteogenesis, porosity is a critical parameter that should not be neglected[27]. Bones as such are very porous structures and hence this scaffold could not promote osteogenesis despite demonstrating controlled release of two hydrophilic drugs. Further experiments will be conducted to establish the efficacy of AA and β - Gly loading for bone tissue formation. In the case of Set B, the gene expression studies were conducted after three weeks of MSC seeding and expansion on the blank and AA, Pro loaded scaffolds. From **figure 6.13**, we can observe that collagen type II, which is a critical marker for chondrogenesis has been upregulated many folds in the biomolecules loaded scaffold. This proves that the drugs released aid in successfully directing MSCs towards becoming cartilage cells. The levels of aggrecans were also

upregulated in the biomolecules loaded scaffolds. Further, immunostaining experiments were conducted to confirm the deposition of Col II and aggrecans on the scaffolds with and without the biomolecules. **Figure 6.14** shows that Col I, Col II and aggrecans staining were more prominent for the scaffolds containing both AA and Proline in comparison to the blank scaffolds. This validates the gene expression results thus proving the potential of this scaffold in promoting chondrogenesis. The pink coloration observed for aggrecans is due to Sulfonin-O and the blue coloration is due to Alcian blue staining. These compounds are instrumental in detecting the presence of Proteoglycans which are a major component of the cartilage tissue. Col type II and type I are prominently visible in the scaffold containing both AA and Pro. They are seen in very less quantity in the blank scaffold.

Figure 6. 13 Relative gene expression of Col II (left) and Aggrecans (right) for 3D scaffold with and without AA and Proline.

Figure 6. 14 Immunostaining for Col I, Col II and Aggrecans for scaffolds with and

without AA and Proline.

Summary

This chapter summarizes all the cellular experiments conducted during this doctoral study. These experiments were vital for understanding the performance of the scaffolds when exposed to cell media. We have correlated the release profiles of the bioactive molecules to match the rate of formation of the new tissue. Thus, the molecules that have released enable the desired lineage specific differentiation to occur. MSCs which are multi potent, exhibit enhanced differentiation into osteo and chondrogenic lineage in response to the sustained release of multiple biomolecules. It is to be noted that in addition to the sustained release of the bioactive molecules and the differentiation ability of MSCs, the porosity of the scaffolds plays an important role in completing the differentiation process.

References

- [1] E.Y.S. See, S.L. Toh, and J.C.H. Goh, Journal of tissue engineering and regenerative medicine, 2012. **6**(7): p. 528-535.
- [2] M.V. Risbud, I.M. Shapiro, A.R. Vaccaro, and T.J. Albert, The Spine Journal, 2004. **4**(6): p. S348-S353.
- [3] M.N. Knight and K.D. Hankenson, Advances in wound care, 2013. **2**(6): p. 306-316.
- [4] J.-B. Park, K.-Y. Kim, W. Lee, H. Kim, and I. Kim, Tissue Engineering and Regenerative Medicine, 2015. **5**(12): p. 343-351.
- [5] L. Meinel, V. Karageorgiou, R. Fajardo, B. Snyder, V. Shinde-Patil, L. Zichner, D. Kaplan, R. Langer, and G. Vunjak-Novakovic, Annals of biomedical engineering, 2004. **32**(1): p. 112-122.
- [6] B. Weiser, F. Sommer, M. Neubauer, A. Seitz, J. Tessmar, A. Goepferich, and T. Blunk, Cells Tissues Organs, 2009. **189**(6): p. 373-381.
- [7] F. Langenbach and J. Handschel, Stem cell research & therapy, 2013. **4**(5): p. 117.
- [8] J.C. Geesin, D. Darr, R. Kaufman, S. Murad, and S.R. Pinnell, Journal of Investigative Dermatology, 1988. **90**(4): p. 420-424.
- [9] O. Chow and A. Barbul, Advances in wound care, 2014. **3**(1): p. 46-53.
- [10] F. Wei, C. Qu, T. Song, G. Ding, Z. Fan, D. Liu, Y. Liu, C. Zhang, S. Shi, and S. Wang, Journal of cellular physiology, 2012. **227**(9): p. 3216-3224.
- [11] Y.J. Chuah, Y. Zhang, Y. Wu, N.V. Menon, G.H. Goh, A.C. Lee, V. Chan, Y. Zhang, and Y. Kang, Acta biomaterialia, 2015. **23**: p. 52-62.
- [12] A.W. James, Scientifica, 2013. **2013**.
- [13] Y.L. Chang, C.M. Stanford, and J.C. Keller, Journal of Biomedical Materials Research Part A, 2000. **52**(2): p. 270-278.
- [14] P. Duheyne, J. Beight, J. Cuckler, B. Evans, and S. Radin, Biomaterials, 1990. **11**(8): p. 531-540.
- [15] T. Ono, R. Yokoi, and H. Aoyama, Experimental Animals, 1996. **45**(4): p. 347-352.
- [16] I. Sekiya, J.T. Vuoristo, B.L. Larson, and D.J. Prockop, Proceedings of the National Academy of Sciences, 2002. **99**(7): p. 4397-4402.
- [17] H. Watanabe, Y. Yamada, and K. Kimata, The journal of biochemistry, 1998. **124**(4): p. 687-693.
- [18] H. Li, S.M. Oppenheimer, S.I. Stupp, D.C. Dunand, and L.C. Brinson, Materials

Transactions, 2004. **45**(4): p. 1124-1131.

[19] W.-J. Li, R. Tuli, C. Okafor, A. Derfoul, K.G. Danielson, D.J. Hall, and R.S. Tuan, Biomaterials, 2005. **26**(6): p. 599-609.

[20] W.-J. Li, R. Tuli, X. Huang, P. Laquerriere, and R.S. Tuan, Biomaterials, 2005. **26**(25): p. 5158-5166.

[21] P.X. Ma and J.-W. Choi, Tissue engineering, 2001. **7**(1): p. 23-33.

[22] H. Orimo, J Nippon Med Sch, 2010. **77**(1): p. 4-12.

[23] B. Kulterer, G. Friedl, A. Jandrositz, F. Sanchez-Cabo, A. Prokesch, C. Paar, M. Scheideler, R. Windhager, K.-H. Preisegger, and Z. Trajanoski, BMC genomics, 2007. **8**(1): p. 70.

[24] S.M. Kim, Y.G. Kim, J.W. Park, J.M. Lee, and J.Y. Suh, J Periodontal Implant Sci, 2013. **43**(4): p. 168-76.

[25] M. Yuasa, T. Yamada, T. Taniyama, T. Masaoka, W. Xuetao, T. Yoshii, M. Horie, H. Yasuda, T. Uemura, A. Okawa, and S. Sotome, PLoS One, 2015. **10**(2): p. e0116462.

[26] L. Buttery, S. Bourne, J. Xynos, H. Wood, F. Hughes, S. Hughes, V. Episkopou, and J. Polak, Tissue engineering, 2001. **7**(1): p. 89-99.

[27] R. McCalden, J. McGeough, and M. Barker, JBJS, 1993. **75**(8): p. 1193-1205.

Chapter 7

Conclusions and Future Recommendations

This chapter summarizes all the findings reported in this thesis and recommends some future directions for readers. The first section of this chapter pulls together all the threads of the various studies conducted for validating the hypotheses listed in chapter 1. The second section presents some reconnaissance studies that was conducted initially, with a bioactive molecule namely – epigallocatechin-3-gallate (EGCG). The third and final section of this thesis lists some future recommendations for the fabricated scaffold systems in different branches of tissue engineering and beyond.

7.1 Conclusions

Multi-structural polymeric scaffold systems were fabricated and evaluated in this study using the technique of electrospinning. The aim was to investigate the efficacy of the fabricated scaffold systems as potential materials for inducing lineage specific tissue regeneration. There has been a lot of focus on developing multi layered/structured scaffolds that serve as an engineered ECM for musculoskeletal applications in the last few years using a variety of fabrication techniques such as electrospinning [1], micro-embossing [2], fiber bonding [3], needle punching [2], 3D-printing [2, 4], lithography [5], etc. Out of all these, electrospinning is the most versatile, reproducible, scalable and cost effective technique. However, there has been no convincing evidence of a dual structured or porous 3D scaffold system for bone and cartilage that has been fabricated with a single equipment without employing post-processing steps [6, 7]. Thus, combining the knowledge obtained in the field of electrospinning and drug delivery techniques, I have successfully fabricated a dual structured scaffold demonstrating controlled release of multiple biomolecules and a 3D scaffold exhibiting optimal cell penetrability and mechanical integrity.

The first set of objectives was to fabricate two structurally different scaffold systems to serve as engineered ECMs. The first system that was fabricated was a dual structured “sandwich” scaffold system with PLGA/PCL fibers and PLGA micro-particles. The fibers were electro spun whereas the particles were electro sprayed on top of the fibrous mesh (**figure 7.1**). To meet the first objective, various processing and solution parameters were investigated and manipulated to arrive at the final scaffold dimensions. **The optimized electrospinning parameters that were used for the fabrication of the fibrous layers are: high voltage range between 18 kV to 25 kV, flow rate of 0.8 mL to 1.5 mL and the needle tip to collector distance of 12 cm. Solution parameters were finalized as 12% polymer concentration for fibers. For the fabrication of particles that were electro sprayed on top of the fibrous mesh, the processing parameters were 15 kV with 10 cm distance and 0.2 - 0.5 mL flowrate of the solution from the needle tip towards the collector with the final concentration of 3 % polymer concentration.**

Figure 7. 1 Schematic illustration of the entire electrospinning and spraying process to arrive at the final dual structured scaffold.

With the fine tuning of these parameters, the first objective of this scaffold system **“To identify electrospinning parameters to obtain well controlled particle and fiber morphology”** was achieved. The morphology of dual structured scaffold was confirmed using a SEM. The formation of a physical polymer blend and encapsulation of drugs was verified by FTIR and DSC. The tensile properties of the scaffold were compared to neat polymers and sheets of polymeric fibers using an MTS C42 system. Further porosity is an important aspect for a scaffold to be eligible to treat any disorder in the musculoskeletal system. This dual structured scaffold system exhibited 60.7% more porosity in comparison to conventional 2D fibers. Initially, preliminary drug loading and encapsulation studies were conducted using Dexamethasone (Dex) as the model drug. This further progressed to confining Dex to the fibers and loading few other biomolecules that have specific roles in induction of osteogenesis and chondrogenesis into the particles. These biomolecules are Ascorbic acid (AA), β -Glycerophosphate (β - Gly) and Proline (Pro). The loading ratios and efficacies of these molecules were calculated in accordance with the permitted therapeutic dosage levels. Thus, I accomplished the objective to **“Carry out material characterizations and load biomolecules into electro spun polymer fibers and micro-particles”**. The third objective of **“evaluating the release patterns of all the encapsulated biomolecules”** was achieved using the techniques of HPLC and UV-

Vis spectroscopy. These techniques enabled the accurate quantification of the amount of each bio molecule that released from the scaffold at every pre-determined time interval. The encapsulation efficiency of the drugs was also calculated and the loading concentration was accordingly tuned. The initial loading concentrations of the biomolecules were 4 wt % with respect to the constituent polymers. However, this concentration was too high for *in vitro* experiments and hence the loading was reduced to 1 wt % with respect to the polymers. Finally, to accomplish the last objective for this scaffold system, MSCs were seeded on the drug loaded scaffolds. Dex was confined to the fibers owing to the need for its controlled and sustained delivery for a longer period of time. AA was confined to the particles as it was required during the first two weeks of MSC proliferation to help in inducing bone and cartilage formation. For the scaffolds to promote the differentiation of MSCs towards osteogenesis (bone formation), β - Gly was added as the third biomolecule. For directing MSCs to differentiate into chondrogenic lineage, Proline was the ideal candidate that was sprayed as microparticles onto the fibrous sheet. The entire drug loaded scaffolds exhibited potential in directing the fate of MSCs towards desired lineage specific to the musculoskeletal system. These findings were further confirmed with qPCR experiments for detecting the presence and upregulation of osteogenic and chondrogenic gene markers namely OCN, ALP, Col type I, RunX2, Col II and aggrecans. Also, immuno-staining was conducted to confirm the deposits of Col I, aggrecans and Col II. Hence the first set objectives of this work were met with the successful completion of these experiments to validate the proposed hypothesis. Thus, **using a single set up, I have successfully demonstrated the fabrication of structurally different moieties that were combined into one dual structured scaffold system for controlled release of multiple biomolecules. These scaffolds successfully enabled the differentiation of MSCs into desired lineages of the musculoskeletal system.**

In the second part of this work, electrospinning was employed to fabricate spongy 3D scaffolds with PLLA, PCL, PEO and their combinations. For the first time, a know-how method to fabricate an intertwined fibrous 3D structure with diameter of 1-4 μm each fiber and about 30 μm for each intertwined bundle was fabricated. The first objective of **“evaluating the correlation between electrospinning parameters to obtain intertwined 3D fibers and arrive at the best polymer concentration and**

ratios to fabricate 3D scaffolds” was met by manipulating the solution and processing parameters. The hydrophilicity and hydrophobicity of the polymers come into play in determining the final properties of the scaffold combinations. Initial experiments started off with testing different polymer concentrations at fixed ratio of PLLA/PCL and established that 8% w/v was the ideal concentration that resulted in a 3D architecture. Next, maintaining the concentration as constant, the polymer ratios were varied to arrive at the combination that successfully forms a 3D structure. In this process, various electrospinning parameters were manipulated. The importance of spinneret speed is often an unnoticed parameter when compared to the other processing parameters for electrospinning such as voltage applied, flow rate of the solution and the distance between the needle-tip to the collector. The spinneret speed was vital in order to achieve non-fluffy mechanically stable 3D scaffolds and it also determined how the fibers oriented themselves. Finally, out of the different combinations, the study was narrowed down to two scaffolds namely PLLA/PCL (70/30 ratio) which was termed as scaffold B and PLLA/PCL/PEO (66.5/28.5/5) denoted as scaffold A. The optimized parameters used for fabrication of all **3D scaffolds are - 15 kV, 1 mL h⁻¹ flowrate, 2 mm s⁻¹ spinneret speed and a 12-cm distance between needle tip and the collector**. In addition, the inherent viscosity (IV) of the polymer blend solution played a crucial role in conferring 3D features to the final structure. A viscosity range of 4-5.5 Pa. s was determined to be ideal for arriving at a 3D morphology. The successful optimizations of the solutions and the processing parameters led to the fulfilment of the first part of the objective set to fabricate 3D scaffolds. The second objective of this study was **“to carry out material characterizations for the 3D scaffolds”**. All the characterization studies were predominantly confined to scaffold B as all the other scaffold combinations did not exhibit the tensile and compression values to fit into musculoskeletal applications (specifically bone and cartilage formation). The mechanical integrity required for a scaffold material to function as bone and cartilage tissue falls within the regime of 0.5 – 60 MPa. It is inferred that even though the tensile strength of the 3D scaffolds is lesser than the conventional 2D fibers, its compression strength is 200 times higher than 2D fibers.

Figure 7. 2 Schematic illustration of the process of formation of 3D scaffold and studies conducted with the scaffolds.

The reported values still fit into the regime of scaffolds currently approved and fabricated by various researchers for use as bone regeneration material[8]. Porosity is an important aspect for bone and cartilage scaffolds and scaffold B exhibited an increase of 97% in porosity than conventional 2D scaffolds and 16% more than the dual structured scaffold system discussed earlier. The 3D scaffolds started degrading slowly during the course of fifty days when exposed to water and PBS. The cell penetration capability of 3D scaffolds was demonstrated with the help of MSCs and compared to conventional 2D fibers. The 3D scaffolds penetrated more than 90% of the scaffold thickness in comparison to the conventional 2D fibers that only penetrated to 20% of the total scaffold thickness. The third part of the objectives was **“to load different biomolecules into the 3D scaffolds and study the release profiles of biomolecules from this scaffold”**. Dex was employed as the initial molecule of interest owing to its anti-inflammatory properties. The amount of Dex released during the initial burst release did not exceed the therapeutic dosage values which may be attributed to the intertwined morphology of the fibers. Further, Dex released in a sustained manner within five weeks which demonstrates the efficacy of this Dex loaded 3D scaffold for controlled release of encapsulated biomolecules. Three hydrophilic biomolecules namely AA, β - Gly and Pro were also loaded later

into the 3D scaffold to validate its efficacy in directing osteogenesis (AA and β - Gly combination) and chondrogenesis (AA and Pro combination). The hydrophilic biomolecules exhibited higher burst release when compared to hydrophobic Dex, however, they released in a sustained manner over a period of two weeks and their burst release did not exceed the accepted therapeutic dosage limits.

Finally, **“to evaluate the differentiation potential of MSCs into Osteo and Chondro lineage”**, MSCs were cultured and seeded on the 3D scaffolds. It was observed that the cells penetrated about 500 μ m deep into the scaffold and proliferated throughout the entire thickness of the scaffold. Such a finding is being reported for the first time in scientific literature. Moreover, Dex was found to enhance the expression of osteogenic markers such as ALP, OCN and Collagen Type I. The upregulation of these markers supports my hypothesis that Dex directs the fate of MSCs towards osteogenesis and fulfils an objective. Then AA and β - Gly were used as the model biomolecules to validate the efficacy of this 3D scaffold system in enabling osteo specific differentiation. AA and β - Gly promoted MSC proliferation, attachment and also played a role in enhancing the expression of osteogenic markers such as Col I, ALP and OCN. These results are supported with immunohistochemical staining results for detecting the presence of Col I. To broaden the utility of this scaffold system, β - Gly was substituted with Pro which is an effector of chondrogenesis. The combination of AA and Pro in the 3D scaffolds led to excellent encapsulation efficiencies for both the hydrophilic molecules and also directed MSCs towards differentiating into cartilage cells. This was confirmed by upregulated expression of Collagen type II and aggrecans which are the major components of a cartilage tissue. Further immuno-staining was also conducted to confirm the presence of Col II and Proteoglycan deposition on the scaffold sections. Further studies, to improve cellular interactions and increasing the bioavailability of encapsulated drugs can be conducted to validate these scaffolds. This is mentioned in the recommendation section. In summary, both the scaffold systems demonstrated optimal mechanical strength and controlled released of single and multiple bioactive molecules. They are effective in directing the fate of MSCs towards differentiating into desired lineage by releasing the encapsulated biomolecules sustainably for more than one month. This scaffold can be used as a platform for inducing MSCs to differentiate into different cell types for applications in other fields of tissue

engineering such as tendon and adipose tissues.

Table 7.1 Comparison of conventional 2D, dual structured and 3D scaffolds fabricated during this study.

Scaffold	Advantages	Disadvantages
Conventional 2D scaffolds	Conventional electrospinning setup Fast process. Adaptable to a wide range of materials (natural and synthetic) Mechanically strong	Does not mimic the ECM Not suitable for <i>in vivo</i> studies
Dual structured scaffold system	Conventional electrospinning setup Multi structural morphology. Simultaneous release of multiple biomolecules. Adaptable to a wide range of polymers.	No precise control over particle to fiber ratio. Time consuming as two structures are involved. Mechanical strength is affected due to presence of particles.
3D self-standing scaffolds	Mimics the ECM closely. Excellent cell penetration with access to nutrition uptake. Commendable encapsulation efficiency and negligible burst release.	Only possible for selective polymers. Mechanical strength needs to be improved. Formation of 3D features depends on the viscosity to a great extent. Release profiles for multiple drugs to be fine-tuned. Slow process.

7.2 Reconnaissance studies

EGCG is poly phenolic compound extracted from green tea and exhibits properties such as wound healing ability, reducing oxidative stress, anti-inflammatory compound, anti-oxidant, used in cancer therapy and is known to have negligible toxicity even at milligram quantities[9-13]. In these studies, EGCG was the first model hydrophilic drug used for the dual structured scaffold system. EGCG was encapsulated in the micro-particles and Dex was loaded into the fibers. The successful loading of EGCG was confirmed with FTIR and DSC studies (results not shown here). The concentration of EGCG loading was varied from 1 - 4 wt % with respect to the PLGA polymer (figure 7.3) and release profiles were obtained.

Figure 7. 3 Release profiles for different loading concentrations of EGCG.

Figure 7. 4 Release profile for EGCG from PLGA micro-particles

It was observed that the loading concentration had negligible effect on the final encapsulation efficiency of the drug in the particles. The entire drug was seen to be completely released from the system within the first two weeks of release study (figure 7.4). EGCG is known to promote joint and bone health and green tea supplements are advised to patients suffering and recovering from conditions such as osteoarthritis and osteoporosis. While the effect of Dex on bone tissue regeneration has been reported before, there is no scientific evidence to prove EGCG's effect on improving joint and bone degenerative disorders. Hence this opens up new scope for innovation and advancement in knowledge in this field. With this aim in mind, Dex and EGCG were loaded into the same scaffold system into fibers and particles respectively hoping for a synergistic effect to occur due to the combination of these biomolecules releasing simultaneously.

Figure 7. 5 Release profiles for EGCG and DEX loaded into the sandwich scaffold system.

Figure 7. 6 SEM images of all the fibrous scaffold mats with and without EGCG

The release profile for this system is observed in **figure 7.5**. The encapsulation efficiency of Dex was calculated to be 76% and that of EGCG at 4% loading concentration was 63.65%. The initial burst release of EGCG did not have any side effects as the acceptable levels in the body are in milligram quantities. But, in contrast to the hypothesis, the combination of EGCG and Dex had a negative effect on MSCs proliferation and the scaffold downregulated the differentiation of MSCs to any desired lineage (results not shown here). This scenario did not change even when the bio molecule loading concentration was reduced from 4 wt % to 1 wt % with respect to PLGA. Hence this study was not further considered and EGCG was replaced by AA, Pro and β - Gly eventually.

To check if EGCG alone promoted osteogenesis or chondrogenesis, fibrous sheets of PLGA/PCL, PLGA and PCL were electro spun with and without EGCG and MSCs were seeded on them.

The loading concentration of EGCG was 4wt % with respect to the polymer and the EE varied between 70-85% for the three different polymer scaffolds. The gene expression results were analyzed after three weeks of MSC proliferation on the scaffolds and results showed that under normal conditions and with the common expansion medium, MSCs did not form bone or cartilage but in turn differentiated into tenogenic lineage (tendon cells). This finding was interesting, preliminary results are presented in figure 7.7 and more studies were conducted to study the mechanism

behind this occurrence. However, this study is beyond the scope of this thesis and hence is not discussed in detail here.

Figure 7.7 Gene expression results for tenogenic markers for different scaffolds loaded with EGCG.

For the 3D systems, the results pertaining to loading and release of Dex alone and loading and release of two hydrophilic molecule combinations have been discussed in detail in the previous chapters. However, the simultaneous release and loading of a hydrophilic and hydrophobic bio molecule combination deserves a mention. Dex and AA were the biomolecules of interest for this study. Dex is hydrophobic and AA is hydrophilic and was loaded into PLLA and PCL respectively to form homogenous solutions. These solutions were mixed and stirred overnight before being spun into scaffolds with a 3D architecture. However, the encapsulation efficiency of both these biomolecules decreased drastically when loaded together and the values was as low as 15% and 8% respectively for Dex and AA. Even though the release profiles matched nearly zero order kinetics (results not shown here), loss of more than 80% of the drugs during initial mixing stage is a disadvantage and hence this study was not pursued further for *in vitro* experiments[14].

7.3 Future Recommendations

Bone and cartilage scaffolds have moved towards exhibiting 3D features in the recent past. However, issues such as cost of production and control over scaffold properties still attract researchers to utilize film like 2D models and sheets for preliminary understanding of release patterns of bioactive molecules and in *in vitro* studies[15]. The aim of this PhD was to bridge the gap between 2D and 3D scaffolds by fabrication and validation of a dual structured scaffold. Based on the studies conducted during this study, the writer has put forth some recommendations for future studies for the readers to pursue. For the ease of understanding, this section is divided into two sub sections discussing a) Dual scaffold systems and b) 3D scaffolds.

a) Dual Scaffold System

In this system, it will be interesting to create a gradient in the particle layer using electrospinning thus reducing/increasing the effective concentration of a particular bio molecule and to correspondingly evaluate the behavior of MSCs and other cell lines [16]. Throughout the course of this work, the upper layer of fibrous sheet was a blank layer whose sole purpose was to hold the micro-particles together in place. This layer can be made into another bio molecule incorporated layer thus promoting the scaffold system to study cellular behavior on both the fibrous layers (top and bottom). Multiple layers of fibers and particles can be electro spun and electro sprayed on top of each other to arrive at a thicker scaffold that can be used as model system for cellular differentiation[17]. These approaches will contribute towards the clinical translation of this technology. The degradation rates of the particles and fibers can be drastically varied by changing the constituent polymer blends for different applications. However, the mechanical stability and integrity of this scaffold can be further improved by tailoring the particle spraying volume and the thickness of the top (blank) fiber layer thickness. *In vitro* studies need to be supplemented with *in vivo* assessments for establishing the efficacy of this system in promoting differentiation of MSCs. As such this system can be applied in different branches of tissue engineering ranging from neural to vascular and soft tissues with a different combination of biomolecules[18].

b) 3D Scaffolds

The 3D scaffolds can further be fine-tuned with different combinations of polymers

such as PLLA/PLGA for applications in the neural and cardiovascular arenas. Preliminary studies were conducted in this direction and PCL from scaffold B was replaced by PLGA. The release profile of PLLA/PLGA scaffold for 2 wt % Dex is presented in figure 7.8.

Figure 7.8 Release profile for Dex from PLLA/PLGA scaffold system.

The burst release of Dex was 25% and the scaffold demonstrated sustained release of Dex for up to 35 days. Further mechanical testing and loading of different biomolecules are to be explored for this system with different biomolecules according to the desired application. Also, efforts can be directed towards increasing the hydrophilicity of the entire scaffold system without compromising on the mechanical integrity of the scaffold. From this study, the inference is that as PEO is added to the existing PLLA/PCL blend, the hydrophilicity of the scaffold increases however, when subjected to mechanical testing, the scaffold shows decreased compression and tensile strength compared to the PLLA/PCL scaffold. This may be attributed to the excessive porosity that is created by addition of PEO. Other hydrophilic polymers can be taken into consideration to overcome this issue.

Next, the 3D scaffolds can be integrated with micro-particles to form a 3D particle entrapped fibrous system. The applications for this scaffold are not well defined yet,

but the particles can be tuned to release certain biomolecules while the fibers provide controlled release of drugs to enable processes such as osteogenesis and beyond. Aligned fibers have been extensively studied in tissue engineering for applications in tendon, muscular and neural branches of TE [19-21]. Until now, the alignment has been reported only in 2D structures and there are limited scientific reports of 3D aligned scaffolds. Since the 3D scaffolds mimic the ECM more closely in comparison to their 2D counterparts, the concept of aligned 3D fibers holds immense potential in the above fields.

References

- [1] D. Han and P.-I. Gouma, *Nanomedicine: Nanotechnology, Biology and Medicine*, 2006. **2**(1): p. 37-41.
- [2] Y. Yang, S. Basu, D.L. Tomasko, L.J. Lee, and S.-T. Yang, *Biomaterials*, 2005. **26**(15): p. 2585-2594.
- [3] S. Yang, K.-F. Leong, Z. Du, and C.-K. Chua, *Tissue engineering*, 2001. **7**(6): p. 679-689.
- [4] S. Bose, S. Vahabzadeh, and A. Bandyopadhyay, *Materials Today*, 2013. **16**(12): p. 496-504.
- [5] G. Vozzi, C. Flaim, A. Ahluwalia, and S. Bhatia, *Biomaterials*, 2003. **24**(14): p. 2533-2540.
- [6] O.D. Schneider, F. Weber, T.J. Brunner, S. Loher, M. Ehrbar, P.R. Schmidlin, and W.J. Stark, *Acta biomaterialia*, 2009. **5**(5): p. 1775-1784.
- [7] D. Zhang and J. Chang, *Nano letters*, 2008. **8**(10): p. 3283-3287.
- [8] H.J. Shin, C.H. Lee, I.H. Cho, Y.-J. Kim, Y.-J. Lee, I.A. Kim, K.-D. Park, N. Yui, and J.-W. Shin, *Journal of Biomaterials Science, Polymer Edition*, 2006. **17**(1-2): p. 103-119.
- [9] J.-G. Leu, S.-A. Chen, H.-M. Chen, W.-M. Wu, C.-F. Hung, Y.-D. Yao, C.-S. Tu, and Y.-J. Liang, *Nanomedicine: Nanotechnology, Biology and Medicine*, 2012. **8**(5): p. 767-775.
- [10] G.L. Tipoe, T.M. Leung, E.C. Liong, T.Y.H. Lau, M.L. Fung, and A.A. Nanji, *Toxicology*, 2010. **273**(1): p. 45-52.
- [11] Y. Zhong, Y.-S. Chiou, M.-H. Pan, and F. Shahidi, *Food chemistry*, 2012. **134**(2): p. 742-748.
- [12] S. Valcic, A. Muders, N.E. Jacobsen, D.C. Liebler, and B.N. Timmermann, *Chemical research in toxicology*, 1999. **12**(4): p. 382-386.
- [13] S. Shankar, S. Ganapathy, S.R. Hingorani, and R.K. Srivastava, *Frontiers in bioscience: a journal and virtual library*, 2008. **13**: p. 440-452.
- [14] X. Zhao, Y.S. Lui, P.W.J. Toh, and S.C.J. Loo, *Materials*, 2014. **7**(11): p. 7398-7408.
- [15] S. Bosi, R. Rauti, J. Laishram, A. Turco, D. Lonardoni, T. Nieuws, M. Prato, D. Scaini, and L. Ballerini, *Scientific reports*, 2015. **5**: p. 9562.
- [16] A.P. Kishan, A.B. Robbins, S.F. Mohiuddin, M. Jiang, M.R. Moreno, and E.M. Cosgriff-Hernandez, *Acta biomaterialia*, 2017. **56**: p. 118-128.
- [17] T.J. Levingstone, A. Ramesh, R.T. Brady, P.A. Brama, C. Kearney, J.P. Gleeson, and F.J. O'Brien, *Biomaterials*, 2016. **87**: p. 69-81.
- [18] O.S. Manoukian, R. Matta, J. Letendre, P. Collins, A.D. Mazzocca, and S.G. Kumbar, *Biomedical Nanotechnology: Methods and Protocols*, 2017: p. 261-278.
- [19] A. Cooper, N. Bhattarai, and M. Zhang, *Carbohydrate Polymers*, 2011. **85**(1): p. 149-156.
- [20] I.-C. Liao, J.B. Liu, N. Bursac, and K.W. Leong, *Cellular and molecular bioengineering*, 2008. **1**(2): p. 133-145.
- [21] L. Di Marcantonio, V. Russo, R. Wyrwa, T. Walter, M. Schnabelrauch, and B. Barboni, 2016.