Parametric studies of polycaprolactone-based composites for tissue engineering and regenerative medicine

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**PARAMETRIC STUDIES OF
POLYCAPROLACTONE-BASED COMPOSITES
FOR TISSUE ENGINEERING AND REGENERATIVE
MEDICINE**

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Doctor of Philosophy

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Preface

This thesis is submitted for the degree of Doctor of Philosophy in the School of Chemical and Biomedical Engineering (Division of Bioengineering) at the Nanyang Technological University, under the supervision of Professor Teoh Swee-Hin and Dr. Mark Chong Seow Khoon. No part of this thesis has been submitted for other degree at other universities and/or institutions. To the author's best knowledge, all the work presented in this thesis is original unless reference is made to other works. Parts of this thesis have been published in the international journals and conferences listed in the **List of Publications, Patents**.

List of Publications, Patents

Journal Publications (accepted):

1. **J Lim** et al., Effects of Polycaprolactone-based Scaffolds on the Blood-Brain Barrier and Cerebral Inflammation. *Tissue Engineering(A)*. 2014, *in press*.
2. Tan, A; Farhatnia, Y; **Lim, J**; Rajadas, J; Alavijeh, M; Seifalian, A; (2014) A nano-inspired multifunctional polymer platform for surgical applications. In: BRITISH JOURNAL OF SURGERY. (pp. 29 - 29).
3. EZ Cai, EY Teo, **J Lim**, YP Koh, SQ Tan, F Wen, JWK Lee, ECH Hing, YL Yap, H Lee, CN Lee, S-H Teoh, J Lim, TC Lim. Bio-conjugated Polycaprolactone Membrane: A Novel Wound Dressing. *Archives of Plastic Surgery*. 2014; *in press*
4. Chong M, **Lim J**, Goh J, Sia MW, Chan JKY, Teoh SH. Co-culture of Mesenchymal Stem Cells and Endothelial Cells as an Organotypic Model of Prostate Cancer Metastasis. *Mol Pharm*. 2014.
5. **Lim J**, Chong MS, Chan JK, Teoh SH. Polymer Powder Processing of Cryomilled Polycaprolactone for Solvent-free Generation of Homogeneous Bioactive Tissue Engineering Scaffolds. *Small*. 2014;17:201302389.
6. Liu Y, **Lim J**, Teoh S-H. Review: Development of clinically relevant scaffolds for vascularised bone tissue engineering. *Biotechnology Advances*. 2013;31:688-705.
7. Wang ZY, Teo EY, Chong MSK, Zhang QY, **Lim J**, Zhang ZY, et al. Biomimetic Three-Dimensional Anisotropic Geometries by Uniaxial Stretch of Poly(epsilon-Caprolactone) Films for Mesenchymal Stem Cell Proliferation, Alignment, and Myogenic Differentiation. *Tissue Eng Part C-Methods*. 2013;19:538-49.
8. Wang Z-Y, **Lim J**, Ho YS, Zhang QY, Chong MS, Tang M, et al. Biomimetic three-dimensional anisotropic geometries by uniaxial stretching of poly (ε-caprolactone) films: Degradation and mesenchymal stem cell responses. *Journal of Biomedical Materials Research Part A*. 2013.

9. Tan A, Farhatnia Y, Goh D, Natasha G, de Mel A, **Lim J**, et al. Surface modification of a polyhedral oligomeric silsesquioxane poly (carbonate-urea) urethane (POSS-PCU) nanocomposite polymer as a stent coating for enhanced capture of endothelial progenitor cells. *Biointerphases*. 2013;8:1-18.
10. Tan A, Goh D, Farhatnia Y, Natasha G, **Lim J**, Teoh S-H, et al. An Anti-CD34 Antibody-Functionalized Clinical-Grade POSS-PCU Nanocomposite Polymer for Cardiovascular Stent Coating Applications: A Preliminary Assessment of Endothelial Progenitor Cell Capture and Hemocompatibility. *PloS one*. 2013;8:e77112.
11. **Lim J**, Chong MSK, Teo EY, Chen GQ, Chan JKY, Teoh SH. Biocompatibility studies and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)/polycaprolactone blends. *Journal of Biomedical Materials Research Part B-Applied Biomaterials*. 2013;101B:752-61.
12. Choy DKS, Nga VDW, **Lim J**, Lu J, Chou N, Yeo TT, et al. Brain tissue interaction with three-dimensional, honeycomb polycaprolactone-based scaffolds designed for cranial reconstruction following traumatic brain injury. *Tissue Eng*. 2013.

Journal Publications (submitted, in preparation):

1. **J Lim**, Y Gao, SH Teoh, CJ Xu. Inorganic Nanocomposites for Tissue Engineering, **submitted to: *Nanomedicine: Nanotechnology, Biology and Medicine (Elsevier)***
2. Y Gao, **J Lim**, SH Teoh, CJ Xu. Emerging Translational Research on Magnetic Nanoparticles for Regenerative Medicine, **submitted to: *Chemical Society Reviews***
3. **J Lim** et al. Degradation behavior and osteogenic evaluation of cryomilled bioactive polycaprolactone/tricalcium phosphate composites, **submitted to: *Journal of Biomedical Materials Research (A)***

4. **J Lim** et al. The influence of exogenous magnesium on mesenchymal stem cell proliferation and early osteogenic activity (in preparation)

Patents

1. SH Teoh, J Lim, MSK Chong (Singapore Provisional Patent)

Book Chapters:

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2. **J Lim**, SH Teoh. Composite Biomaterials in Medicine in *Engineering Materials for Biomedical Applications*. World Scientific Publishing (in preparation).

Conferences:

1. **J Lim**, MSK Chong, JKY Chan, SH Teoh. Polymer Powder Processing Techniques for the Generation of Polymer Matrices with High Tricalcium Phosphate Content. bone-tec 2013
2. **J Lim**, MSK Chong, JKY Chan, SH Teoh. Magnesium as a Regulator of Mesenchymal Stem Cell Proliferation and Differentiation for Bone Regeneration. bone-tec 2013
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Evaluation of Bioactive Polycaprolactone-based Films for Guided
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Abstract

Bone is a composite structure that is made up of collagen (organic), calcium phosphates and trace metal elements (inorganic). Together with strict mechanical requirements, many scaffold-based tissue engineering strategies are now focused on developing composite systems that provide sufficient mechanical stability and biological stimulation to dictate osteogenic events *in vivo*. On this note, processing techniques for bioresorbable composites for bone tissue engineering have traditionally involved solvents and heat, which may not be ideal due to toxicity and material degradation, respectively. As such, alternative processing methods are actively pursued and evaluated. In this thesis, we hypothesized that cryomilling is a potential alternative processing technique for composites.

The effectiveness of cryomilling to refine particle size was first demonstrated using PCL and PLGA. Strength improvement was demonstrated, particularly in the case of PLGA. By subjecting composite materials to temperatures sufficiently below glass transition, effective attrition into fine powders (micron-range) was achieved. In addition, this attrition process facilitated distribution of the fillers (tricalcium phosphate (TCP) and magnesium (Mg)), resulting in homogeneous PCL/TCP and PCL/Mg powders. This homogeneous distribution was retained in further post-processing techniques. A mechanistic explanation for this phenomenon may be that of solid-state diffusion, where effective interfacial interactions are created, resulting in the retention of their homogeneous distributions.

The implications of this phenomenon were demonstrated through a variety of *in vitro* studies, with notable advantages in composites processed with cryomilling (such as mechanical and biological responses). *In vivo*, PCL/TCP films were able to influence

bone regeneration by preventing soft tissue invasion into the defect site (in pigs and rabbits). PCL/Mg films were also biocompatible, with no cases of rejection in pigs. When fabricated into 3D scaffolds with applications in cranioplasty, their biocompatibility with brain tissue was established in rat models.

In conclusion, we have demonstrated in this thesis that cryomilling will be a suitable processing method that is entirely solvent-free, and maintains homogeneity of composites processed in this manner. This culminated in robust in vitro and in vivo responses as described. Going forwards, we believe that cryomilling may become clinically translatable. At the same time, we aim to develop cryomilling as a versatile platform for the incorporation of drugs, antibiotics, and growth factors.

(361 words)

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1. Introduction[†]

1.1 Tissue engineering and bone tissue replacement

Tissue engineering and regenerative medicine has come a long way since it was first coined by Langer and Vacanti in 1993.¹ The need for tissue engineered scaffolds and tissues, comes from the lack of autologous grafts and donors. As such, the field is driven by the need to provide medical solutions to relief this burden. In the early 2000s, Teoh presented five tenets of tissue engineering: scaffolds, cells, growth factors, bioreactors, and bioimaging.² In general, regenerative techniques may be broadly based on these strategies, although it is not uncommon for strategies to be based on two or more of these tenets.

Among all organs and tissues that may be regenerated through tissue engineering techniques, bone is the second most transplanted. With a reported 15 million fractures³ worldwide, there is an urgent need to provide tissue engineering solutions to ensure that patients receive appropriate levels of healthcare to maintain/improve their quality of life. To this end, the field has invariably seen the transition from the initial use of inert biomaterials that provide restoration of form over function⁴, to the use of bioactive scaffolds that elicit certain desired responses from host tissue.^{5, 6} Current state-of-art for scaffold development involves the use of 'smart' scaffolds that degrade in response to tissue ingrowth, termed bioresorbables that are also either bioactive, and/or which may be used as vehicles for the delivery of bioactive molecules over a period of time.⁷⁻⁹ In order for these scaffolds to have an impact to healthcare, successful clinical translation needs to be achieved. However, very few of these lab-based developments are successfully commercialized, owing to reasons not within the scope of this thesis (e.g. regulatory barriers, financial difficulties etc.).

[†] Partially published in Y Liu, **J Lim**, SH Teoh., *Biotechnology Advances*, 2013

Table 1-1 Scaffold criteria and function required for tissue engineered scaffolds for directing bone tissue regeneration.¹⁰ Table reproduced from Liu et al. with permission.

Criteria	Function
Biocompatibility	Ability to perform its function in the host tissue without eliciting any immune response
Biodegradability	Tunable rate of degradation to match growth of new bone tissue as scaffold gets replaced by new bone
Mechanical properties	Sufficient mechanical strength to provide temporary support to the defect region and withstand in vivo loading forces
Microarchitecture	Interconnected scaffold structures to uniformly distribute stresses throughout scaffold
Osteoinductivity	Osteoinductive properties to recruit and differentiate osteoprogenitors to the defect region
Porosity	Large surface area: volume and pore size to allow for tissue in-growth, neovascularisation, mass transport and osteogenesis
Surface properties	Appropriate chemical and topographical properties for influencing cellular adhesion, proliferation and differentiation

Scaffolds for bone replacement have certain requirements as listed in Table 1-1. Certain criteria such as mechanical properties, microarchitecture, porosity and surface properties may be influenced by fabrication techniques, such as those listed in Table 1-2. In general, these techniques employ the use of heat or solvents to achieve the desired mechanical properties and microarchitecture (including porosity). However, these techniques face significant fabrication challenges when composite structures are required, as they are plagued with issues of composite inhomogeneity and in some cases, just being unable to be fabricated in a reproducible fashion.

Table 1-2 Fabrication techniques for scaffolds developed for clinical use in bone tissue regeneration/replacement.¹⁰

Fabrication technique	Total publications	Advantages	Disadvantages	References
Extrusion-based	54	-Fast, automated -CAD/CAM defines microarchitecture -Solvent-free	-Requires melt extrusion	11-13
Gas foaming	48	-Able to fabricate porous scaffolds (up to 95%)	-Most pores are not interconnected	14
Particulate leaching	90	-Able to fabricate porous scaffolds	-Uses toxic solvents	15, 16
Selective laser sintering	55	-Fast, automated -CAD/CAM defines microarchitecture	-Uses toxic solvents -Requires heat	17-19

Low temperature processing of biomaterials (further explained in Chapter 2.2)

As such, there appears to be a need for the development of an alternative form of manufacturing in order to ensure homogeneous distribution of the composite while achieving the criteria listed in Table 1-1. Within the review, a low temperature attrition method that comminutes large particles to small, homogenously sized powders, known as cryomilling, was suggested. Using this low-temperature approach has certain advantages: apart from being able to incorporate composites in a homogeneous fashion, cryomilling has made possible the addition of heat-sensitive materials, drugs, and biologics without affecting their microstructure, activity, as well as function. Therein, the author of this thesis demonstrated this by employing magnesium (Mg), a metal that is unstable at elevated temperatures. Mg was successfully incorporated into a polymer matrix through cryomilling, and exhibited unique compressive yield strength (Figure 1-1).¹¹

Figure 1-1 (A) Hardness-Vickers values of various PCL/TCP/Mg compositions, with increased Mg demonstrating greater hardness and yield strength.¹⁰

1.2 Tissue engineered scaffolds – Past to present

Scaffolds play an important role in tissue engineering, particularly in hard tissue applications such as bone, where structural support is a necessary element. The development of scaffolds may be traced back to the beginning of regenerative medicine in 1902 by Alexis Carrel, in the field of cardiovascular and transplant surgery.²⁰ Bone replacements such as bone grafts, and gold plates were probably one of the first synthetic materials that were used as early as the 16th century.⁴ Since then, the field of regenerative medicine has progressed significantly, owing to the improved understanding of tissue and organ epidemiology²¹⁻²³, as well as disease etiology and traumatology. The contributions of various institutions around the world is evident of the progress the field has made (Figure 1-2).¹¹ Although the gold standard of replacement remains the autograft, the lack of appropriate autografts combined with the increasing demand for tissue replacements fuels the need for the development, and clinical translation of biomaterials.

Figure 1-2 Leading countries in scaffolds and bone tissue engineering, represented by the percentage of publications per country. Published by the author of this thesis in 2013.¹¹

Among the various biomaterials used for BTE, polymers dominate (Figure 1-3). Due to the biochemical nature of physiological bone, it is by no coincidence that polymer-ceramic composites form the predominant fraction among biocomposites for BTE (Figure 1-3).

Figure 1-3 Representation of the number of publications for polymers, metals, and ceramics in BTE (left), followed by composites (right). Published by the author of this thesis in 2013.¹¹

1.3 Polymers

Biodegradable polymers have been used with increased popularity in tissue engineering.²⁴⁻³² The main advantage of using biodegradable polymers in BTE would be their ability to support tissue growth and remodelling over their functional lifespan before being resorbed by the body.³³ Natural polymers such as collagen, chitosan, hyaluronic acid have low immunogenic potential, and bioactivity thus rendering them capable of interacting favorably with host tissue.³⁴ Synthetic polymers have an added advantage due to their tailorable biodegradation rates, controllable physical properties, better lot-to-lot consistency³³ and thus could be easily fabricated and shaped accordingly.

Both natural and synthetic polymers are biodegradable and hence ideal for use as scaffolding structures for regenerative medicine. Some regulatory approved polymers include collagen, polyglycolide (PGA), polylactides (PLLA, PDLA) and PCL. However, polymers have limited capabilities in achieving strong bonding and

integration with bone, and have weak mechanical properties. Polymers exhibit an elastic moduli typically between 7MPa for elastic materials and 4GPa for stiff polymers, which is lower than that of cortical bone and cancellous bone that have an elastic moduli of approximately 17GPa and between 0.1 to 2 GPa respectively.³⁵ In addition to their low elastic moduli, polymers are prone to a deformation mechanism called creep³⁶, which can be significant even at room temperature and at stresses below their individual yield strengths.³⁵ Hence, polymers are often reinforced with other materials and used as composites for BTE.

1.4 Ceramics

Ceramics are used as BTE scaffolds primarily due to their good biocompatibility. Of the many ceramics available, the calcium phosphate (CaP) group consisting of HA and tricalcium phosphate are the most commonly used.^{28, 37-42} However, ceramics have low fracture toughness (high brittleness) with a comparatively high Young's modulus of between 7 and 234GPa² (higher than cortical bone), and are therefore used more often in compressive loading conditions⁴³⁻⁴⁵. Ceramics have the tendency to undergo creep (the tendency to slowly deform permanently under the influence of stress) like that of polymers and metals, but less likely under physiological conditions when the conditions are about 40%-50% of their melting temperatures.³⁵ An important property of ceramics will be their tribology. As compared to metals and polymers, ceramics have very low wear rates (ceramic-on-ceramic articulating surfaces have a wear rate of 0.003mm/year⁴⁶), a result of their high hardness values. Consequently, ceramic-based materials are commonly used on articulating surfaces.^{47, 48}

1.5 Composites

Composites combine desirable properties of different materials in order to achieve a synergistic effect in their resultant properties.³⁵ This unique property of composites has led to their application in BTE to circumvent various short-comings of such as creep-induced failure, fracture toughness and biocompatibility issues associated to the use of pure polymer, ceramic, or metallic biomaterials.

Briefly, composites comprise of two phases: the matrix and the dispersed phase. The dispersed phase is usually harder and stiffer for preventing the movement of the matrix phase, whereas the matrix phase functions primarily as a stress transfer medium.³⁵ Composites can be broadly categorised into particle-reinforced, fiber-reinforced and structural composites. A prediction of the combined properties can be estimated via the “rule of mixtures” equations (explained in Chapter 2), which represent the upper and lower bound of the elastic modulus respectively.

An increase in the volume percentage of the dispersed phase will result in higher stiffness due to combined elasticity. In BTE, composite scaffolds are commonly fabricated using polymer as a matrix (polymer-matrix composites [PMC]) and then combined with ceramics, demonstrated to have improved compressive load-bearing capabilities, improved bioactivity and enhanced host-implant interface interactions.^{43, 49-55} Additionally, the use of ceramic-matrix composites (CMC) combined with metals have been found to be physiologically relevant due to the presence of trace elements within the natural bone tissue.⁵⁶⁻⁶³ The addition of metals has also shown significant improvements in the compressive strength of the scaffold, osteogenesis, and enhanced bone-implant interface interactions.⁶⁴ Few have explored the utility of polymer-matrix incorporated with metals for BTE⁶⁵, likely due to limitations in fabrication technologies and inability in retaining desired properties.

1.6 Motivation – Composites for BTE

At the present moment, composite scaffolds are suitable tissue engineered replacements for bone. However, limitations in fabrication techniques meant that current scaffolds are unable to achieve a composition similar to that of physiological bone. In addition, the distribution of the dispersed phase is still suspect, and might not be homogeneous, leading to inconsistent biological responses as well as compromised mechanical properties.

Therefore, there is a need to develop a processing method for generating well-dispersed composites that has the potential to achieve a composition similar to physiological bone. In addition, it is in the interest of this thesis to ensure that the processing technique is clinically translatable.

1.7 Aims and hypothesis

1.7.1 Aims

The main aim of this thesis is to develop bioactive composites based on polycaprolactone (PCL), via a solvent-free, low temperature fabrication process. More precisely:

Specific Aim 1: Develop homogenous composites based on the technique of cryomilling by incorporating inorganic elements and trace metal ions.

Specific Aim 2: Evaluate physical properties of PCL-based composites and in vitro mineralization events.

Specific Am 3: Demonstrate biocompatibility and regenerative capabilities of PCL composites in vivo.

In line with the specific aims listed above, the following working hypotheses were formulated:

1.8 Hypotheses

Hypothesis 1: We hypothesize that cryomilling improves the distribution of the fillers (inorganic elements and trace metal ions), leading to advantages in fabrication.

Hypothesis 2: We hypothesize that PCL-based composites present desirable physical properties, robust cellular responses, and regenerative capabilities.

Hypothesis 3: We hypothesize that PCL-based composites are biocompatible with both soft (cerebral) and hard (bone) tissues.

1.9 Novelty and clinical implications

The novelty of this thesis lies in the use of cryomilling as a primary, important composite processing step that allows incorporation of physiologically relevant fillers for tissue engineering and regenerative medicine. Importantly, this method has considerable clinical importance due to its solvent-free approach. Critically, we believe and would like to emphasize that cryomilling significantly improves the distribution and homogeneity of the dispersed phase, which may not be obvious.

1.10 Scope of thesis

Chapter 1 provides a brief overview of the field of tissue engineering, with a focus on scaffolds. The aims and hypotheses are also delineated here.

Chapter 2 provides a literature review focused on cryomilling, and its application to polymers and various polymer-based systems. A mechanistic view is also provided.

Chapter 3 describes the cryomilling of PCL and PLGA and their associated physical properties, degradation responses, and cytocompatibilities.

Chapter 4 discusses the application of cryomilling to PCL/TCP composites, evaluating the distribution of TCP. It also provides extensive in vitro materials characterization and cell response. Finally, the biocompatibility and osteogenic response of PCL/TCP composites are presented.

Chapter 5 demonstrates the incorporation of trace metal ion Mg to PCL. In vitro osteogenic events were first evaluated, followed by in vivo evaluation of the biocompatibility of PCL/Mg composites.

Chapter 6 evaluates brain tissue compatibility of PCL/TCP composites designed for the delivery of therapeutic agents.

Chapter 7 concludes this thesis, and presents recommendations on future work, with preliminary literature review and data.

2. Literature Review – Polymer composite processing for biomaterials[‡]

2.1 Fabrication with solvents and heat

Traditionally, the approach to biomaterial fabrication has been direct, that is, using the materials as received, and applying either solvent-based⁶⁶⁻⁷⁰ or heat-based techniques⁷¹⁻⁷⁴ to fabricate the required films and scaffolds for achieving their desired functions. While this does not pose significant challenges in single component systems (i.e. only the use of one polymer), the same cannot be said about multi-component systems like polymer blends and polymer-based composites, which require extensive mechanical or chemical interactions⁷⁵ between phases in order to achieve a synergistic effect. In the case of solvent-based fabrication of multi-component polymer-based biomaterials, selection of solvent for the dissolution and subsequent mixing is straightforward considering that most polymers are soluble exclusively in organic solvents. Significant challenges lie however, in homogenously dispersing the second and/or subsequent phases within the first. While inherent chemical configurations prevent intimate interactions among the phases, thermodynamic driving forces also contribute to immiscibility of polymer systems, which has been identified since 1975^{76, 77} until now^{78, 79}. A recent miscibility study was conducted between PCL and poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), and immiscibility in majority of the blend proportions was observed through cross-polarized light microscopy⁸⁰ (Figure 2-1). As seen in the polarized light microscopy images (PLM), clear segregation and aggregation of PHBHHx and PCL could be observed due to their different crystallizing abilities, with PCL showing

[‡] Partially published in Y Liu, **J Lim**, SH Teoh., *Biotechnology Advances*, 2013; J Lim et al., *JBMR(B)*, 2013.

stronger ability to form spherulites. To address the issue of immiscibility, various researchers have proposed the use of surfactants, which has brought about significant improvements in miscibility^{81, 82}.

Figure 2-1 Cross-polarized light microscopy images of various PHBHHx/PCL blends, observed over a 14-day degradation period. The characteristic Maltese cross is most clearly seen in the 0/100 pure PCL. Published by the author of this thesis.⁸⁰

The issue of immiscibility is not restricted to polymer blends: polymer composites are also subject to miscibility issues, although it is more commonly referred to as non-homogenous distribution. As identified by Wang et al., non-homogeneity poses a significant challenge in stress analysis⁸³, consequently leading to significant difficulties in predicting mechanical behavior. Segurado and team have developed computational models for evaluating the effect of particle agglomeration on the eventual mechanical properties⁸⁴. Despite these potential challenges, composites

offer the advantage of improving stiffness and mechanical strength^{85, 86} while the addition of bioactive salts confers bioactivity to the otherwise inert polymers^{87, 88}. Particularly in the area of bone tissue engineering, where implant stiffness⁸⁹ and bioactivity^{90, 91} are two major determinants of functional outcome, the need for well-designed biomaterials is always present. On this note, various research groups have worked on developing bioactive composites for bone tissue engineering. For example, Catledge et al.⁹² showed that by incorporating HA into both a PCL and collagen matrix, the resulting stiffness was considerably higher than without. Recent work by Phipps et al.⁹³ described similar results. Coating polymeric matrices with bioactive molecules were also attempted, as a way to avoid miscibility issues. Whited et al.⁹⁴ demonstrated that apatite-coated PLLA nanofibers were able to direct osteogenic differentiation of MC3T3-E1 pre-osteoblasts. In summary, solvent-based techniques have been employed to fabricate composite films and scaffolds, evaluated for bone tissue engineering.

The use of solvents, though attractive, have been shown to influence cytotoxic effects observed in the livers and kidneys of mice⁹⁵. It has also been suggested to be carcinogenic according to a study by Golden et al.⁹⁶. Therefore, although solvent-based techniques are relatively easy to master, they are unsuitable for clinical translation⁹⁷. In this light, this and other groups have developed solvent-free techniques based on heat, for the generation composites for BTE. Teoh and team developed a rapid prototyping technique known as fused deposition modeling (FDM) in 2001^{11, 13}, and showed that a layer configuration of 0/60/120° provided significant mechanical advantages while maintaining 61% porosity. From this platform, bioactive TCP was incorporated into PCL and fabricated into 3D scaffolds^{51, 98-100}. Mechanical stiffening of PCL/TCP scaffolds was recorded¹⁰¹. The mechanical and

biological success of these FDM-fabricated scaffolds have been successfully translated into clinics¹⁰². Apart from extrusion-based fabrication (i.e. FDM), other solid free-form fabrication (SFF) techniques like stereolithography, selective laser sintering, and 3D printing have been used for the fabrication of polymers and composites¹⁰³. For example, Tan et al.¹⁰⁴ fabricated composite scaffolds with polyetheretherketone (PEEK) and HA using a SLS platform, incorporating up to 40 wt% of HA. Wiria et al.¹⁰⁵ also demonstrated using the SLS platform that PCL/HA composites of up to 30 wt% HA could be fabricated. From literature, it is apparent that the limit of CaP incorporation via heat-based techniques was at 40 wt%, and important reasons that could explain this phenomenon lies in rheology and second phase (CaP) distribution. These are important concepts that will be discussed in greater detail.

2.1.1 The influence of polymer rheology and filler distribution on composite processing and fabrication

Successful composite fabrication relies on polymer (matrix) rheology and filler distribution, and also determined by the interfacial interactions between both phases¹⁰⁶⁻¹⁰⁸. We shall now present the concepts behind polymer rheology, and discuss its effect and implication on composite fabrication.

Rheology is a physical property that describes the ability of a material to flow in response to applied mechanical forces such as shear, compression, and torsion. More precisely, rheology is associated with soft matter that has complex microstructures (e.g. polymers), and with the capacity to present both solid and fluid characteristics. By extending continuum mechanics to the study of fluid mechanics, we are able to understand and predict the behavior of these materials in response to

an applied force¹⁰⁶. These information are particularly important in the fabrication of composites, particularly in the melt.

Depending on their microstructures, their flow properties may be described as Newtonian or non-Newtonian. Newtonian fluids may be characterized by a single coefficient of viscosity at a specified temperature, which is independent of strain rate. However, few materials display such characteristics and as such, the study of rheology is commonly applied to non-Newtonian fluids.

The study of non-Newtonian fluids is complex, due to the number of functions that are involved in describing their flow properties in relation to changing strain levels. There is a group of materials that exhibit shear-thinning behavior^{109, 110}, and another group that exhibits shear-thickening behavior¹¹¹. On this note, Giesekus¹¹² presented an equation based on deformation-dependent tensorial mobility to represent the flow properties of non-Newtonian fluids. The approach begins with a set of constitutive equations (E1) and (E2) combined to relate the tensors S_k of excess stresses (which describes the motion of polymer units in relation to their surroundings) with configurational tensors C_k (describes configuration of polymer structure in the solution or melt). The final equation after simplification and removal of trivial terms is represented by E3.

$$S_k + \eta_k \frac{\partial C_k}{\partial t} = 0 \quad (E1) \quad S = \sum_k S_k \quad (E2)$$

$$\beta S + \check{\eta} \frac{\partial C}{\partial t} = 0 \quad (E3)$$

S: tensor of excess stresses

C: configurational state of structures in solution or melt

η : material constant with properties of zero viscosity

β : relative mobility tensor

To present concept above in a more simplified manner, consider the most important factor in polymer rheology: viscosity, as represented by η , which describes the ratio of shear stress (τ) to shear rate ($\dot{\gamma}$). This is represented mathematically by E4, and from there, it is clear that viscosity is inversely related to shear rate.

$$\eta = \frac{\text{shear stress}}{\text{shear rate}} = \frac{F/A}{V/h} = \frac{\tau}{\dot{\gamma}} \quad (\text{E4})$$

F: applied force

A: surface area

V: velocity

h: gap height

Therefore, an increase in shear rate would result in a corresponding decrease in viscosity, which implies better rheological properties.

Experimentally, changes in viscosity lead to implications in material processability as described in literature¹¹³⁻¹¹⁵. Specifically to the strategy of adding bone-related fillers to polymeric matrices, Sun et al.¹¹⁵ described the shear flow behavior of needle-like hydroxyapatite (HA) nanoparticles in polymeric matrices of polycaprolactone (PCL) and methylethylketone (MEK), and showed that the addition of PCL reduced hysteresis (Figure 2-2) and resulted in a stable composite suspension, which favored homogenous HA distribution. In another study, Liang et al.¹¹⁶ studied the effects of adding calcium carbonate to PCL through the melt, and found that at low filler levels (between 1 – 4%), the melt shear viscosity was not significantly affected. Across the

range of shear rates tested, the melt shear viscosity of composites increased with increasing filler content, while at the same time decreased with increasing shear rate. This effect is due to the phenomenon of shear thinning, which arises because of significant polymer chain disentanglement and orientation.

Figure 2-2 (A) Dependence of viscosity on shear rate, between MEK and HA. (B) Dependence of viscosity on shear rate when PCL is added. The hysteresis loops becomes significantly smaller upon the addition of PCL. Images adapted with permission from Sun et al.¹¹⁵

Here, some of the factors that affect polymer viscosity, as described in Vlachopoulos and Strutt¹¹⁷, are introduced. Firstly, molecular weight (M_w) is directly correlated with viscosity. This may be explained by the bulky conformation of high M_w polymers, leading to resistance in chain mobility that is manifested as high viscosity. Equation E5 shows mathematically that a power law function relates viscosity to M_w .

$$\eta = kM_w^{3.4} \quad (\text{E5})$$

where k is a constant

Secondly, polymer viscosity is sufficiently influenced by the amount of fillers, the two quantities being directly correlated. To this end, there are many studies that have demonstrated that an increase in the amount of fillers lead to increasingly solid-like

behavior, which impeded the ease of composite fabrication. For example, Li et al.¹¹⁸ demonstrated recently that the viscosity of poly(propylene)/graphene composites was significantly higher when the amount of graphene (filler) was more than 15%. Bigg¹¹⁹ also demonstrated the increased viscosity of polypropylene when the proportion of stainless steel spheres (filler) increased.

In the reports described previously, the materials were used as received, and physically blended with their respective second phases during the fabrication process. For example, PCL and HA were physically blended in a mixing/grinding roller¹⁰⁵, as were PEEK and HA¹²⁰, for varying times depending on the amount of HA incorporated. Both groups^{105, 120} reported homogenous HA (second phase) distribution. In FDM manufactured PCL/TCP scaffolds^{51, 98-100}, millimeter sized PCL pellets were placed together with micron sized TCP particles into a hopper, heated and extruded through an orifice. The reported limit for these scaffolds were 20 wt% of TCP¹²¹, beyond which resulted in processing issues. Presumably, these processing issues could arise as a consequence of impeded rheology, although the reason was not clearly stated.

The rheological properties of the matrix in polymer-based composites have long been known to play a role in composite fabrication¹²², with a compromise between second phase loading and processability. The effect of extensive incorporation of a second phase results in a drastic changes in melt viscosity, leading to significant challenges in processing. Zhang et al.¹²³ demonstrated that low filler addition into a polymer matrix resulted in a solid-like behavior from the fabricated composite. On the other hand, Saengsuwan et al.¹²⁴ demonstrated that the incorporation of a second phase could lead to improvements in processing, and distribution. Summarizing

above, the following properties of the second phase influence composite processing:

(1) Amount incorporated; (2) Chemical properties; (3) Interfacial properties.

2.2 Alternative composite processing – Low temperature conditions

Considering the challenges involved in processing composite materials via heat, solvent-free approaches, significant second phase loading of polymer matrices are a major challenge. In the case of BTE where 70 wt% of CaP exists within a network of collagen fibrils, engineering a replacement with the same composition of CaP, without the use of solvents or excessive heat appears to be impossible. Recently, a new field of biomaterials processing has emerged, using low temperature conditions¹²⁵⁻¹²⁷. Bergmann et al.¹²⁷ combined TCP with PEG and applied SFF through extrusion at room temperature. They managed to fabricate scaffolds with consistent pore sizes that were also capable of directing bony repair in vivo. Bernstein et al.¹²⁶ employed cold sintering (room temperature) at high pressure (1-3 GPa) to consolidate PCL/TCP particles, and showed comparable strength to its hot forged counterpart^{91, 128}. Makarov et al.¹²⁹ synthesized HA in situ within a PCL matrix, and the powders were then cold sintered (room temperature) at 2.5 GPa to form 1 mm high scaffolds that supported cellular proliferation. More importantly, Makarov et al. demonstrated the possibility of incorporating unusually high volume fractions of HA (80-95 vol%). These reports demonstrated the potential advantages of low temperature processing: (1) High second phase incorporation, (2) Avoidance of heat during processing. Apart from these advantages, low temperature also facilitates the incorporation of heat-sensitive biomaterials, drugs, and biologics.

As mentioned in the earlier section, successful composite processing is influenced by the distribution of the second phase, as well as the mechanical and/or chemical interactions between the matrix and the second phase. Although the above-

mentioned reports have shown homogenous distribution, little was said about the interactions between phases. Here, it is noteworthy to highlight the fact that high pressure sintering was applied¹²⁹. Even in the case of extrusion, the mechanical shear experienced at the extruding orifice would possibly have exerted significant mechanical forces on the material^{126, 127}. These mechanical forces may be particularly influential in multi-component systems with distinctly different chemical properties. Nonetheless, the potential of achieving significant breakthrough in the loading capacity of composites is obvious, and this thesis shall henceforth present a low temperature, solvent-free method of processing biomaterials (in particular composites) for use as scaffolds in tissue engineering.

2.3 Cryomilling

Solid-state mixing^{130, 131} is an alternative strategy for processing blends and composites by minimizing phase separation¹³² (which is responsible for second phase agglomeration). To achieve solid-state mixing, sufficient mechanical forces have to be applied for the following reasons: (1) to improve size compatibility; (2) to enhance interfacial interactions. This processing method, which is well known in the field of metal alloying^{133, 134}, is termed milling or attrition^{130, 132, 135, 136}. Milling results in the generation of heat, which may be non-ideal for processing materials such as plastics due to their low melting temperatures. In fact, interest in the use of cryomilling began as early as 1990, though not as a means to process materials for subsequent fabrication purposes. It was originally employed as a way of creating well-homogenized, small particulate tissues

As such, cryogenic milling (cryomilling) was developed as a low temperature, mechanical alloying¹³⁷ method for multi-component systems. For example, cryomilling was extended to ceramics¹³⁸, ceramic-matrix metal composites¹³⁹⁻¹⁴² and

polymer-matrix metal composites¹⁴³. Smith and team^{132, 144-148} have made significant contributions over the years for developing polymer blends with little phase separation. Zhu et al.^{149, 150} focused on the structural and thermal behavior changes in poly(ethylene terephthalate). More recently, Allaf et al.¹⁵¹ demonstrated that cryomilling may be employed as a means of improving blend compatibility between immiscible PCL and poly(ethylene oxide) (PEO). In the area of scaffold development specifically for tissue engineering applications, they were probably the first to demonstrate the feasibility of cryomilling on a polymer blend system.

Given the wide applicability of cryomilling, a mechanistic understanding of this process is needed. In this section, a systematic review on the physical, chemical, and structural effects of cryomilling on polymers and polymer-based composites is presented.

2.3.1 Physical changes – Particle size reduction, crystallinity, molecular weight

Cryomilling leads to changes in the physical properties of the materials, which may influence downstream processing efficiencies. Here, the physical influences that cryomilling exerts by discussing with respect to particle size, melting temperature, glass transition, crystallinity and molecular weight are discussed.

As mentioned earlier, cryomilling promoted homogenous blending of polymer blends, as well as homogenous filler distribution in various composite systems. One of the reasons may be the improved particle size compatibility between the matrix and dispersed phases. As such, it is important to study the achievable particle size reduction with the cryomilling process when compared to other mechanical attrition methods such as ambimilling (ambient temperature milling).

For the cryomilling of polymer-based systems, the low temperature environment is maintained either through a constant flow of liquid nitrogen, or through immersing the milling jar within liquid nitrogen (Figure 2-3). As a result, the temperature of polymers will be lowered and maintained beyond their T_g , which is a transitional point below which polymer chain mobility is restricted and as a result, displays a 'glassy' state and exhibit brittle properties. Therefore, by processing polymers below their T_g , the efficiency of particle size reduction may be influenced¹⁵².

Figure 2-3 Commercially available cryomill systems, including a ball cryomill from Retsch®, and a magnetic impeller cryomill from Spex®. Both systems immerse their cryomilling jars in liquid nitrogen throughout the entire milling process.

From available literature, the effect of ambimilling would see the initial increase in particle size due to plastic deformation, before they are broken up into smaller entities. This has been observed and reported by Zhu et al.^{149, 150}, where an initial increase in particle sizes of poly(ethylene terephthalate) (PET) over the first 2 hours. This initial increase is mostly due to the plastic deformation of most polymers at room temperature. In the same study conducted, PET was put through a cryomilling process, and the particle size reduction was found to be more efficient, with larger size reduction (Figure 2-4). Zhu et al.¹⁵³ cryomilled poly(ethylene terephthalate)

(PET) and SiO_2 to form polymer-inorganic composites. They found that particle size was a function of cryomilling time, with the most significant change occurring within the first 2-5 h.

Figure 2-4 Size of PET particles with respect to time, when cryomilled and ambimilled. Significantly efficient particle size reduction could be observed, particularly at short milling times. Figure taken from Zhu et al.¹⁵⁰, with permission from Rightslink.

The physical reduction in particle size suggested that on a molecular and structural level, significant changes have taken place. On this note, Martin and Kander¹⁵² found significant improvements in poly(etheretherketone) (PEEK) rheology after 10 h of cryomilling, which was attributed to the reduction in molecular weight (M_w). In their system (polycarbonate (PC)-PEEK), PEEK became largely amorphous after 10 h of cryomilling, as validated by x-ray diffraction methods. Smith et al.¹⁵⁴ studied the effect of cryomilling on the changes in M_w , rheology, crystallinity, and glass transition of poly(methyl methacrylate), polyisoprene and poly(ethylene-alt-propylene). Interestingly, the effects differed among these polymers, suggesting that the effects of cryomilling were unique.

Here, it is important to point out that the thermodynamics of chain scission is critical to the understanding of M_w changes during the milling process. In the solid state, chain scission may only occur due to mechanical degradation. The high impact energy of the milling process provides sufficient activation energy to break intermolecular bonds. A highly dynamic competition between chain scission and crosslinking takes place during this cryomilling process, and this may be represented by the Charlesby-Pinner equation (E6), which was originally used to represent the molecular evolution of insoluble polymers as a result of extensive crosslinking after exposure to irradiation, as described by these classic studies by Charlesby and team¹⁵⁵⁻¹⁵⁷.

$$S + S^{0.5} = \frac{2}{\delta} = \frac{p}{q} + \frac{1}{qNr} \quad (E6)$$

S = soluble fraction of polymer

δ = number of crosslinks per initial weight-average molecule

p = fracture density per unit radiation dose

q = crosslink density per unit radiation dose

N = number-average degree of polymerization

r = number of chain scission events

Smith et al.¹⁵⁴ altered the equation slightly, to relate the events of both chain scission and crosslinking due to milling. By replacing “ r ” with “ t_m ”, and by plotting $S+S^{0.5}$ against $1/t_m$, Smith found out that in the case of polyisoprene (PI), crosslinking was more likely to occur than chain scission, by about 50%. This relation appeared to be valid for “long” milling durations (longer than 2h).

Given the molecular changes that may occur during the milling process, another physical property that may subsequently be influenced is that of crystallinity. Common knowledge suggests that polymer chain packing and folding efficiencies^{158, 159}, and side-groups¹⁶⁰ affect the crystallinity of the polymer. To this end, the effects of cryomilling on crystallinity have been reported by Bai et al.¹³⁷. PET, which is a semi-crystalline polymer, was put through both cryomilling and ambimilling from as short as 30 mins to as long as 16 hours. Their results suggested amorphous PET showed increase in crystallinity after 16 hours, while semi-crystalline PET showed decrease crystallinity within the first hour of cryomilling.

In short, this section has illustrated the potential effects of cryomilling on polymeric systems, by focusing on its ability to reduce particle size, induce changes in M_w , and also influence crystallinity.

2.3.2 Generation of multi-component systems via cryomilling

Given the unique characteristics of cryomilling as discussed above, much effort has been put into employing cryomilling as either a material processing or fabrication method, particularly in multi-component systems. This is mainly due to its ability to homogeneously disperse the second phase (filler) and improve miscibility between phases through an entirely solvent-free, solid-state approach. This section reviewed the literature specifically for multi-component systems prepared using cryomilling specifically for tissue engineering applications (Table 2-1). To this end, few have focused on evaluating cryomilling as a processing method, apart from significant contributions from Allaf^{151, 161, 162}.

Table 2-1 Summary of cryomilled systems developed for tissue engineering applications.

Materials/Ratio (if any)	Duration/Frequency	Particle size	References
Polyetherimide (PEI)	8h/400	20 μ m	Parquette et

		(original: 350um)	al. ¹⁶³
Poly(ϵ-caprolactone)	36 min/?	40-60 um	Allaf et al. ^{151,}
(PCL) and Poly(ethylene oxide) (PEO)/50:50	90 min/?	55-1215 um (original unknown)	^{161, 162}
Decellularized adipose tissue (DAT)	2min/2500 rpm	(original unknown)	Cheung ¹⁶⁴

As seen from the table, a wide variety of cryomilling protocols exist, and reasonably so due to the inherent properties of the different polymers. Therefore, an attempt to reconcile the differences among the various protocols would be sufficiently complex. However, that which has clearly been demonstrated would be the significant reduction in particle size, leading to changes in both the physical and possibly chemical properties of the materials^{165, 166}, which influence subsequent material fabrication success.

Traditional polymer processing is conducted above T_g for ease, which is once again related to the concept of rheology. Cryomilling however, processes polymers at extremely low temperatures, easily below their T_g , to overcome challenges faced in the melt (above their T_g)¹⁶⁷. To date, work is still being done in trying to overcome rheological challenges when processing molten polymers^{168, 169}. The potential advantage that cryomilling has over melt processing would be the avoidance of rheological impediments, and instead facilitate more interaction and homogenous distribution through solid-state diffusion¹⁷⁰ mechanisms and particle size compatibilization.

This theory will be continually explored throughout this thesis, in relation to the multitude of systems developed and presented here.

3. Development and characterization of cryomilled polycaprolactone and poly(D,L-lactic-co-glycolic acid)[§]

3.1 Introduction

Polymeric thin films have wide applicability in the field of tissue engineering as potential muscle sheet constructs (author's unpublished data), and vascular constructs^{171, 172}. However, many solution-based strategies of fabricating and/or processing biomaterials have faced significant challenges in attaining regulatory approval for use in clinics, due to the potential toxicity of the solvents used during the process.

On the other hand, heat-based techniques face a dynamic competition between ensuring sufficient heat to improve polymer rheology, while at the same time necessitating minimal exposure to heat due to potential degradation. This dynamic interplay between rheology and degradation has thus prompted the field to look into alternative ways of polymer processing and/or fabrication, by ideally removing both heat and solvents from the processing/fabrication process. To this end, this thesis has showed earlier that cryomilling may be a potential, viable alternative for polymer processing and fabrication.

On this note, it is prudent to mention here that cryomilling is not solely limited to polymers; it may and should also be extended to composite systems, in order to harness its true potential. The motivation for extending its applicability to composite system comes from the literature, that cryomilling has in fact been used in various metal and ceramic based systems.

[§] Partially published in **J Lim** et al., Small, 2014; ZY Wang, **J Lim** et al., JBMR(A), 2013; ZY Wang et al., TE(C), 2013.

In 1998, Agnew and Weertmen¹³³ reported on the significant softening of ultrafine grain copper produced by severe plastic deformation. Martinez-Blanco *et al.*¹³⁴ studied the changes in microstructure of iron powder samples post-milling. They postulated that the distinct magnetic behavior of the samples was a result of the microstructure. In addition, mechanical alloying or milling has also been extended to ceramics, ceramic blends (with metal components)¹³⁹⁻¹⁴² and polymer-metal composites¹⁴³. Mechanical milling of pure ceramics have also been investigated¹³⁸.

As a result of mechanical milling and alloying, particles underwent physical changes in dimension; mechanical and thermal properties (subject to post-processing treatment) are also altered. In metallic systems, Kumar *et al.* reported nanocrystalline metals and alloys processed by cryomilling (including ambient temperature milling), with particles sizes falling in the range of a few nanometers (nm) to 100 nm¹⁷³. With further investigation of yield strength, they found that fully dense face-centered-cubic (FCC) metals with grain sizes smaller than 100 nm show enhanced yield strength. Cyclic tests on metals with grain sizes less than 100 nm also show enhanced fatigue life as compared to those with bigger grain sizes¹³³. Such improvements in yield strength have been attributed to grain size effect, solid-solution hardening, dispersion strengthening, and precipitate strengthening, of which grain size effect was considered to play an important role^{174, 175}. This experimental observation has been previously espoused in theory, through the indirect correlation between mechanical strengthening and filler size (E3-1).

$$E \propto \frac{1}{d^k} \quad (\text{E3-1})$$

where

d = diameter (longest dimension of filler)

k = constant

Lavernia *et al.*¹⁷⁶ reported high thermal stability of aluminum alloys produced via cryomilling by studying their microstructure after creep tests. In polymeric systems, mechanical milling results in a decrease in molecular weight after ambimilling and cryomilling. More importantly, cryomilling has been shown to have reduced molecular degradation as compared to ambimilling¹³². Changes in glass transition temperature (T_g) changes have also been reported by Smith *et al.*¹⁴⁴ after investigating the effect of block copolymer addition via cryomilling. In addition, due to the high energy milling/alloying process, interfacial energy is reduced and as a result, the intimacy and compatibility of the composite/blend can be improved¹⁵⁰.

3.2 Biomaterials commonly used in tissue engineering – Polycaprolactone and poly(D,L-lactic-co-glycolic acid)

3.2.1.1 Polycaprolactone

Polycaprolactone (PCL) is a well-investigated biomaterial that is biodegradable, bioresorbable, and biocompatible. While the details leading to the production of PCL is not the focus of this thesis, it is important to understand and appreciate the process in order to appreciate its chemical properties. Labet and Thielemans¹⁷⁷ reviewed the various processes by which PCL was synthesized, which may be broadly categorized into four main mechanisms of ring opening polymerization (ROP): anionic, cationic, monomer-activated, and coordination-insertion (Figure 3-1).

Figure 3-1 (A) Anionic ROP¹⁷⁸; (B) Cationic ROP¹⁷⁸; (C) Monomer-activated ROP¹⁷⁹; (D) Coordination-insertion ROP¹⁷⁸.

As observed from the final chemical structure of PCL, it essentially is an aliphatic polymer chain that contains one ester bond per five-carbon alkyl. This structure with no bulky side groups ensure that it has efficient chain packing and folding, leading to its semi-crystalline properties. These properties thereby lay the foundation for its mechanical and degradation properties, as observed in many studies^{100, 180}.

PCL has thus been employed in the field of tissue engineering and regenerative medicine, initially as drug release microparticles, and more recently as potential scaffolds for bone tissue engineering applications¹⁸¹⁻¹⁸³. On this note, the history of PCL has been expertly summarized by Woodruff and Hutmacher¹⁸⁴.

Over the last few years, Teoh's group has developed new ways to take advantage of the mechanical and degradation properties of PCL for various tissue engineering

applications. For example, uniaxial stretching led to the generation of topographical features that were able to guide and direct cell alignment on the films over an extended period of time (Figure 3-2)¹⁸⁵.

Figure 3-2 (A) MSCs seeded onto control PCL films (without topographical features) showed random orientation, while those on uniaxial PCL (UXPCL) films demonstrated a high degree of alignment that was maintained for a period of 10 days. Various quantification of (B) the degree of alignment, (C) cell shape index, and (D) alignment efficiency. **, *** vs control. Scale bar represents 500 μ m. Image taken from Wang et al.¹⁸⁵ with permission from Mary Ann Liebert, Inc..

In addition, the raised ridges of the PCL film contained more crystalline regions as compared to the 'grooved' features. With this improvement in crystallinity, the raised features (ridges) were found to be more resistant to degradation¹⁷² (Figure 3-3).

Figure 3-3 Degradation SEM images of control PCL films (a, c, e, g, i, k) and UXPCL films (b, d, f, h, j, l) suggested that the ridge structures in UXPCL films were more resistant to degradation, and maintained their features clearly throughout most of the degradation. Scale bar represents 20 μm . Image taken from Wang et al.¹⁷² with permission from John Wiley & Sons, Inc..

In another study, PCL was blended with poly(3-hydroxybutyrate-co-hydroxyhexanoate) (PHBHHx), and demonstrated that blend compatibility could be achieved at a ratio of 30/70 (PHBHHx/PCL), with comparable mechanical properties to cancellous bone, while maintaining predictable degradation behavior. Its ability to support MSC proliferation also suggests that it may have applications in tissue engineering, particularly bone (Figure 3-4).

Figure 3-4 (A) MSC live (green)/dead (red) stains on PHBHHX/PCL films over a period of 5 days. 30/70 films demonstrated the highest cell viability as compared to 0/100 (PCL, control) films, as validated by AlamarBlue® assay (B). The adapted table summarized the mechanical properties of various PHBHHx/PCL films, with 30/70 films performing similarly to 0/100 (PCL). Scale bar represents 100 μ m. Image taken from Lim et al.⁸⁰, with permission from John Wiley & Sons, Inc..

3.2.2 Poly(D,L-lactic-co-glycolic acid)

Poly(D,L-lactic-co-glycolic acid) (PLGA) is another widely studied biomaterial that has been used extensively. The current state-of-art is focused on harnessing the tunable properties (mechanical, degradation) of PLGA fabricated in the form of micro-/nano-spheres, and using them as drug/biomolecule vehicles¹⁸⁶. It is well-known that PLGA may be synthesized with varying proportions of its monomers, namely poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) (Figure 3-5). By doing so, the mechanical and chemical properties of PLGA may then be altered

accordingly, as demonstrated by Xie et al.¹⁸⁷. In that study, PLGA with varying monomer ratios were evaluated for their emulsifying ability. By increasing the glycolic acid monomer from 10% to 50%, the amount of PLGA required to form stable emulsions decreased.

PLGA has also been investigated as potential matrices to be used in the field of bone tissue engineering. For example, Thomson et al.¹⁸⁸ demonstrated that PLGA is a competent conductor of new tissue growth and formation, but is inherently not osteoconductive. Moreover, the degradation rate depended on the PLA:PGA ratio. Nonetheless, they managed to obtain bony chambers when PLGA was mixed with morcelized bone grafts (MBG), a common inorganic ingredient for stimulating bone formation. On this note, it should be noted that many other groups have also employed PLGA in a similar fashion, by using it as a matrix within which bioactive components may be contained, in particular calcium phosphates¹⁸⁹⁻¹⁹².

Figure 3-5 Chemical structure of PLGA, with x and y denoting the number of repeat units for each of the monomers¹⁹³.

3.3 Schematic of workflow

Figure 3-6 Schematic of workflow for the cryomilling and characterization of PCL and PLGA.

3.4 Materials and Methods

3.4.1 Polymers: PCL and PLGA

Medical grade polycaprolactone (PCL) was purchased from Osteopore International, Singapore. 50:50 PLGA was purchased from Sigma-Aldrich, Singapore.

3.4.2 Cryomilling

PCL and PLGA were processed using cryomilling (Retsch®, Germany). PCL and PLGA were individually loaded into the cryogenic vial with a ball-to-mass ratio of 30:1 (two balls were used). The cryomilling protocol was: 6 – 8 min of pre-cooling in liquid nitrogen and 20 min of continuous milling for one cycle.

3.4.3 Scanning electron microscopy

Samples were first coated with platinum using a JFC-1600 Autofine Coater (JEOL, Japan), with a coating current of 20 mA for 60 sec. Scanning electron microscopy (SEM) was then performed using the SEM6390LA (JEOL, Japan).

3.4.4 Rheology

The rheological properties of PCL and PCL/TCP were investigated using the AR 2000ex rheometer (TA instruments, USA). Calibration of the equipment was conducted according to manufacturer's instruction. A parallel plate configuration (25 mm steel plate, 0°) was chosen, and all tests were carried out at 100°C. A frequency of 10 Hz was applied after a pre-test frequency sweep (1 – 500 Hz). The gap was maintained between 700 – 900 µm, and a normal contact force of 1.0 ± 0.5 N was maintained throughout. 30 sec was given to allow equilibration of the system, and 1 min was given to allow stabilization of the temperature.

3.4.5 Molecular weight – Gel Permeation Chromatography

Gel permeation chromatography (GPC) was performed by Supramolecular Biomaterials Lab (National University of Singapore, Singapore) using a Shimadzu SIL-10A and LC-20AD system equipped with two Phenogel 5µ 100 and 10000 Å columns (size: 300 x 4.6 mm) connected in series and a Shimadzu RID-10A refractive index detector. A solution of each sample was prepared using tetrahydrofuran (THF) as a solvent. 1.5 ml THF was added to approximately 5 mg of the sample and at least 30mins was allowed to dissolve the sample completely. The solutions were then thoroughly mixed and filtered through a 0.45 µm phobic polytetrafluoroethylene filter. The mobile phase flow-rate used was 0.3 ml/min at a temperature of 40 °C. The data was collected and analyzed using LCsolution 1.22 software (Shimadzu, Japan). Mw and Mn of the PCL were calibrated using mono-dispersed polystyrene standard samples (n=3).

3.4.6 Mechanical testing

Tensile and compressive mechanical tests were performed on the polymer composites fabricated in this thesis with an Instron 5582 (Instron, Norwood, MA). For tensile tests, films were cut into rectangular sheets and fixed onto a paper frame with

a standard gauge length of 30 mm. A crosshead moving speed of 3 mm / min was then applied, and the samples were stretched until failure. For compression tests, cylindrical blocks of polymer composites (height 10 mm) were placed in between two platens, with a crosshead moving speed of 1 mm / min.

3.4.7 Degradation studies

Degradation studies were performed in physiological and accelerated conditions. For physiological degradation, samples were pre-weighed and placed into 1 mL of phosphate buffer saline (PBS) at 37°C. At stipulated time-points, samples were retrieved, washed with deionized water (DI water) and dried for two days before being reweighed. For accelerated degradation, films were placed in 1M NaOH at 37°C, and removed at pre-determined time-points to assess mass loss, changes in morphology, crystallinity, molecular weight, and mechanical properties.

3.4.8 Mass loss

Mass loss of samples was taken using a mass balance (RADWAG, Poland). Samples were dried completely before their mass was taken at an accuracy of ± 0.1 mg.

3.5 Results

3.5.1 PCL

3.5.1.1 Cryomilling time vs. particle size

PCL pellets were put through various cryomilling times (5, 10, and 20 mins), and subsequently characterized for the particle sizes using morphological assessment (Figure 3-7). Results suggested that 20 mins of cryomilling resulted in significant reduction in PCL particle sizes as compared to 5 and 10 mins of cryomilling (***).

Figure 3-7 PCL particle morphologies and size characterization after cryomilling times of (A) 5 mins (B) 10 mins (C) 20 mins. (D) Characterization of PCL sizes revealed that particle sizes were similar between 5 mins and 10 mins of cryomilling, while 20 mins of cryomilling led to significantly smaller (***) particle sizes. Scale bar in all images represents 1mm.

3.5.1.2 PCL rheology

The rheological properties of PCL before and after cryomilling (cPCL) were investigated via rheometry (Figure 3-8). From the results, it can be seen that the viscosity of cPCL was consistently 20% lower than that of PCL (*). In addition, the ratio of elastic to loss moduli suggested that cPCL was more elastic than PCL (*).

Figure 3-8 (A) Viscosity vs frequency of PCL vs cryomilled PCL (cPCL). (B) Elastic/Loss moduli of PCL and cPCL. Table compiling the viscosity values of viscosity and viscosity ratio, as well as the ratio of elastic to loss moduli.

3.5.1.3 Thermal properties of PCL and cPCL

The thermal properties of PCL before and after cryomilling (cPCL) were investigated. Results suggested that crystallinities remained similar (Figure 3-9, Table 4-1). The thermal stability of PCL, as indicated by the melting temperature, also remained comparable (within 3°C) before and after.

Figure 3-9 Thermal profile of PCL and cPCL, indicating a similar melting peak.

Table 3-1 Thermal properties of pristine PCL and cPCL suggested minimal changes in crystallinity and peak melting temperature ($p > 0.05$). Molecular weight and number, and polydispersity index changes were also monitored. Significant reduction (20%, $*p < 0.05$) in molecular weight was recorded.

	Xc (%)	T _m (°C)	Molecular Weight (g/mol)	Molecular Number (g/mol)	n
PCL	62.3 ± 0.4	81.1 ± 0.7	118 000 ± 1030	66 900 ± 3570	1.72 ± 0.09
cPCL	60.9 ± 1.4	78.6 ± 0.9	92 300 ± 680,*	65 700 ± 690	1.40 ± 0.01

3.5.1.4 Molecular weight changes in PCL

The molecular weight changes (M_w) of PCL before and after cryomilling (cPCL) are shown in Figure 3-10 and Table 3-1. From the results, it is obvious that a significant shift in peak M_w took place after cryomilling. This change was determined to be approximately 20% (*). The average molecular weight number did not indicate significant differences from the original PCL.

Figure 3-10 Distinct peaks of M_w distribution for PCL and cPCL.

3.5.1.5 Mechanical properties of PCL and cPCL

The mechanical properties of PCL and cPCL were evaluated by uniaxial tensile stretching at room temperature, and the results are presented in Figure 3-11 and Table 3-2. It was demonstrated that the tensile strengths of PCL and cPCL were not significantly different. However the elastic properties of cPCL was found to be different from that of PCL, in terms of strain (*).

Table 3-2 Summary of the mechanical properties of PCL and cPCL.

	Yield Stress (MPa)	Young's Modulus (MPa)	Yield Strain
PCL	16.0 ± 1.4	356 ± 66	0.09 ± 0.02
cPCL	15.3 ± 0.7	305 ± 24	$0.11 \pm 0.01,^*$

Figure 3-11 Stress strain profiles of PCL and cPCL.

3.5.2 PLGA

3.5.2.1 PLGA films – melt pressed and solvent casted

PLGA films were successfully fabricated from solvent casting, and cryomilling followed by melt pressing. Melt pressing of PLGA directly from its original form without cryomilling was difficult, and did not yield consistent film structures. Gross morphological observations (Figure 3-12A, B) concurred with microscopic observations under SEM (Figure 3-12C, D), with a generally featureless appearance.

Figure 3-12 Camera images (A, B) and SEM images (C, D) of melt pressed PLGA films (A, C) and solvent casted PLGA films (B, D).

3.5.2.2 Mechanical properties of PLGA films

The mechanical properties of PLGA films are presented in Figure 3-13 and Table 3-3. Briefly, both films displayed a similar stress-strain curve profile. Between the two, melt pressed films displayed higher tensile strength and toughness ($p < 0.05$). The Young's modulus of melt-pressed films was lower than that of solvent casted films ($p < 0.05$). Maximum strain of PLGA films fabricated via a combination of cryomilling and heat pressing was one-fold higher than that fabricated via solvent casting (260% vs. 130%).

Figure 3-13 Stress-strain profile of PLGA films fabricated using a combination of cryomilling and melt pressing (dotted lines), and from solvent casting (solid line).

Table 3-3 Summary of the mechanical properties of solvent casted and melt pressed PLGA films.

Parameter	Solvent Casted	Melt Pressed
Tensile strength (MPa)	28.8 ± 2.72	35.98 ± 3.65
Modulus of Elasticity (MPa)	2460 ± 160	1720 ± 370
Maximum Strain (%)	130 ± 10	260 ± 30

3.5.2.3 Degradation studies of PLGA films

Mass loss of PLGA

The mass losses of PLGA films are depicted in Figure 3-14. It was observed that there was minimal mass loss over the first week, with significant mass loss recorded from day 10 onwards for melt pressed PLGA while solvent casted PLGA was

comparatively more resistant to degradation. Catastrophic degradation prevented further meaningful measurement and comparison beyond 14 days.

Figure 3-14 Percentage mass retention was charted over a period of 14 days. Solvent casted PLGA films (solid line) maintained their mass better than PLGA films fabricated via cryomilling and melt pressing (dotted lines).

Morphological changes of PLGA

Over the duration of the degradation period, solvent cast films remained predominantly featureless, although degradation characteristics in the form of small pits were observed on day 10 (Figure 3-15A, black arrows). Melt-pressed films displayed features indicative of degradation on day 10, with cracks appearing throughout the entire surface of the film (Figure 3-15B, black arrows). On day 14, micron-sized pits were found on the surface of PLGA melt-pressed films (Figure 3-15B, day 14 inset).

Figure 3-15 SEM images of PLGA films fabricated via (A) solvent casting and (B) melt pressing, degraded over a period of 14 days in PBS.

3.6 Discussion

3.6.1 PCL

Here, it was shown that PCL may be cryomilled (Figure 3-7) to generate powders with a micro-particulate size of $180 \pm 70 \mu\text{m}$ from their original sizes (Figure 3-7B-D), with a 60-fold increase in surface area-to-volume ratio (cPCL: 2218; PCL: 36.9)

(Figure 3-7). The ability to achieve particle size reduction has been well-studied in literature¹⁴⁴. The underlying mechanism lies in processing polymers in their glassy state. PCL has a reported T_g of -60°C ¹⁸⁴, therefore by processing at cryogenic temperature, brittle fracture of PCL occurs. As a result, a significant change in particle size could be achieved in a very short time (33 times reduction in 20 min of continuous milling), in line with similar studies describing an exponential decrease in grain sizes of cryomilled poly(ethylene terephthalate) (PET)¹⁵⁰.

The influences of cryomilling on polymer properties were then studied. Percentage crystallinity (X_c), taken as a ratio of the area under the thermogram against the theoretical 100% crystalline value of PCL (136 J/g ¹⁹⁴) indicate crystallinity to be unaffected by cryomilling (Table 3-1, $p>0.05$) and further implies that molecular chain packing and folding were not affected by cryomilling. Importantly, retaining the crystalline nature of PCL suggests that in general, strength of PCL is retained. With reference to Teoh's earlier report, the tensile strength and elastic moduli of PCL films lie within the range of cancellous bone^{80, 195, 196}, which is desirable when considering stiffness matching of biomaterials.

To address the question on effects of cryomilling on the molecular weight of PCL, it was found that gel permeation chromatography profiles of both PCL and cPCL were similarly unimodal, but a 21.7 % decrease in the peak M_w was observed in cPCL (Table 3-1, *: $p<0.05$). Molecular number (M_n) recorded minimal changes (Table 3-1). Additionally, the polydispersity index (n) for both PCL and cPCL were similar, suggesting that the distributions of M_w/M_n were not affected significantly. In their study, Smith et al. investigated the effects of cryomilling on the M_w of several polymers, including poly(methyl methacrylate) (PMMA), polyisoprene (PI) and poly(ethylene-*alt*-propylene) (PEP). Their results indicated exponential M_w decrease

profiles for PMMA and PEP after cryomilling¹³². The results here are in good agreement, with a 21.7 % decrease in M_w after cryomilling. From the representative thermographs, it can be observed that the peak melting temperatures, as observed by the vertical dotted lines, are similar for PCL and cPCL (81.1°C vs 78.6°C). It was also observed that, apart from the physical reduction in size, chain scission and/or chemical crosslinking might occur¹⁹⁷. The results here suggest that PCL underwent primarily scission without cross-linking, implying improved chain mobility and consequently, reduced viscosity.

Rheological examination of the cPCL indeed showed significantly lowered inherent viscosity. Across the wide range of oscillation frequencies tested, the viscosity of cPCL was consistently 18 % – 22 % lower than that of non-cryomilled PCL (Figure 3-8, inset Table). Additionally, loss tangent data suggested better elastic property of cPCL, and supported the claim that the inherent viscosity of cPCL was improved (Figure 3-8, inset Table). Based on the changes in M_w , which was approximately 21.7 % lower after cryomilling ($p < 0.05$), the improvement in cPCL viscosity is in good agreement with the decrease in M_w , suggesting that viscosity is directly correlated to M_w . Taken together, these data suggest cPCL to be highly mobile in the melt solution, and consequently, contribute towards reduction of fusion defects during powder processing.

A comparison between films generated from native PCL and cryomilled PCL revealed the effect of moulding artifacts (data not shown), suggesting impaired polymer flow, resulting in impaired structural integrity. These might have implications in the mechanical properties, as summarized in Table 3-2. PCL and cPCL films indicated similar stress-strain curve profiles (Figure 3-11) with similar yield stresses (PCL: 16.0 ± 1.4 vs. cPCL: 15.3 ± 0.7 MPa, $p > 0.05$). A lower Young's modulus in

cPCL (305 ± 24 MPa) resulted in an improvement in yield strain (PCL: 0.09 ± 0.02 vs. cPCL: 0.11 ± 0.01 , *: $p < 0.05$). An interesting observation was the improved elastic strain by cPCL. From the rheological results obtained earlier (Table 3-2), the lowered loss tangent of cPCL could have played a possible role in the manifestation of improved elasticity (Table 3-2, *: $p < 0.05$) and possibly tensile strength, although the reduced tensile strength in cPCL did not prove to be statistically meaningful.

3.6.2 PLGA

Cryomilling has emerged as useful method for polymer processing^{150, 198, 199}, and in particular biomaterials.¹⁶¹ It was previously described that rheological changes after cryomilling facilitated PCL fabrication, and thus this thesis has extended cryomilling to PLGA.

The results here demonstrated significant advantages in the mechanical properties of PLGA films fabricated via cryomilling and melt pressing, with a significant improvement in toughness (approximately 160%, $p < 0.05$). The UTS improved by approximately 25%, while Young's modulus decreased by approximately 30%. On this note, it is interesting to report that similar observations were reported in PCL films fabricated using the same process (pristine PCL: 356 MPa; cryomilled PCL; 305 MPa²⁰⁰).

The effect of polymer processing history on mechanical properties has been suggested and validated in this study. By cryomilling PLGA, changes in their physical properties may result, leading to the enhanced mechanical properties observed. One of the determinants of mechanical properties in amorphous polymers lie in the difference between the temperature of the conducted experiment and the T_g .²⁰¹ Here, PLGA processing via cryomilling may have altered the T_g of PLGA, causing it to

exhibit enhanced mechanical properties. However, it should be mentioned that the change in T_g is material specific, and dependent on the milling time.²⁰²

Structural refinement is another possible effect of cryomilling. To this end, studies have shown that cryomilling can lead to molecular weight changes, which occurs via random chain scission.¹⁹⁷ Earlier, the dispersity index of PCL was improved (1.72 before cryomilling, 1.40 after cryomilling), suggesting a more homogenous microstructure, leading to improved mechanical properties. The refinement on microstructure is once again material specific, and might be a contributing factor to the improved mechanical properties as compared to solvent cast PLGA films.

The degradation of PLGA is a well-understood mechanism of hydrolytic ester cleavage, accelerated by the auto-catalyzing effect due to the acidity of the degradation side-products, which typically lowers pH after the first week.²⁰³ Additionally, the extent of amorphosity plays a role in dictating the degradation behavior of polymeric films, with 50:50 PLGA showing the earliest and fastest degradation among other copolymers.^{70, 204} The results here corroborated with existing literature, with solvent-casted films indicating less than 10 % mass loss over the first 14 days. On the other hand, cryomilled and melt-pressed PLGA films recorded a significant decrease in mass ($p < 0.05$) on day 10, and by day 14, approximately 20% mass loss was recorded. By evaluating the morphological changes of the melt pressed PLGA films, the presence of pores on day 10 allowed fluid penetration into the bulk of the material, and has been suggested to influence the degradation process.⁷⁰ Significant mass loss was recorded thereafter, which may be due to the reduced molecular weight after cryomilling, as was observed when PCL underwent cryomilling.²⁰⁰ Put in perspective, sufficient structural stability is required as a platform to support tissue growth while being degraded. On this note,

in vivo degradation of PLGA scaffolds have been reported²⁰⁵, with a reported stability of between 2 – 4 weeks depending on the degree of porosity.

As mentioned earlier, the ability to support cellular and tissue growth is important. Here, it was demonstrated that both solvent cast and melt pressed PLGA were able to support HS-5 growth and proliferation, and slight advantages on HS-5 viability was recorded on day 7 ($p=0.06$). The potential cytotoxic effect of organic solvents have previously been reported^{66, 206}, and could have contributed to the slower growth of HS-5 on solvent cast films. On the other hand, cryomilling and melt pressing are completely solvent free techniques, thus do not pose cytotoxicity concerns.

3.6.3 General discussion

Cryomilling is a technique of low temperature attrition that has been demonstrated across many metal-based systems in the literature, as described earlier. In addition, studies focusing on the changes in the thermal, structural, and mechanical properties have also been conducted. As described above, the technique of cryomilling was extended to biodegradable, bioresorbable biomaterials, and showed similar findings to the literature.

To begin with, particle size reduction was the most observable change that occurred as a result of the high-energy impacting process conducted below their T_g . The manner in which the milling process was conducted facilitated rapid particle size reduction, an observation that was previously reported, particularly in techniques of achieving finely ground animal tissue.^{207, 208} That which was not obvious in those techniques would be the structural changes that occurred during this process, which may influence their physical and chemical properties. To this end, sufficient work in the literature has suggested, and this thesis has also demonstrated that cryomilling

influences the molecular weight, thermal and rheological properties. These three physical properties are also interdependent, with molecular weight possibly playing a larger role. Downstream of these changes, their effects were manifested in the fabrication of films. In the case of PCL, the viscous flow lines that were previously visible disappeared after it was put through a cryomilling process, suggesting a definite improvement in rheology. On the other hand, PLGA film fabrication via melt pressing was significantly improved upon cryomilling, leading to successful melt pressing when it was previously challenging.

From a mechanical perspective, cryomilled PCL displayed a greater potential for elastic behavior, as suggested by its tangent delta and improved elastic strain. PLGA displayed enhanced strength and toughness after cryomilling, as compared to its solvent casted form. While the mechanism behind these observations is not clear, a potential explanation could lie in structural refinement, which was demonstrated via a tighter polydispersity index. Specifically for PLGA, where molecular chain orientation is of particular importance to explain large strain deformations (or improvements), the cryomilling process may have similarly resulted in a tighter molecular weight distribution, creating similarly long polymer chains together with a tighter distribution.

Modifying the Charlesby-Pinner equation (E6, earlier in Chapter 2.3.1) could provide a mechanistic explanation for the phenomenological observations. As mentioned earlier, by substituting r with t_m (represents milling time in hours), E6 becomes:

$$S + S^{0.5} = \frac{2}{\delta} = \frac{p}{q} + \frac{1}{qNt_m} \quad (E7)$$

This suggests that by relating the soluble fraction ($S + S^{0.5}$) to the reciprocal of milling time ($1/t_m$) and attaining the line of best fit, the ratio of chain scission (p) to

crosslinking (q) may be obtained by noting the point where the best fit line meets the y-axis. Three scenarios may result from this:

- (i) $p/q > 1$: predominantly chain scission
- (ii) $p/q < 1$: predominantly crosslinking
- (iii) $p/q \sim 1$: both crosslinking and chain scission occurring at the same rate

Figure 3-16 Relation between crosslinking density and milling time, suggesting that crosslinking density was significantly lower at short milling times. Image taken from Smith et al.¹⁵⁴, with permission from Elsevier.

Although this was not studied in detail in this thesis, prior literature from Smith et al.¹⁵⁴ suggested that the crosslinking density was low at short cryomilling times (Figure 3-16), thus there is reason to believe that at low milling times, the probability of chain scission will be much higher than that of crosslinking.

3.7 Summary

As demonstrated in the results above, cryomilling was employed as a polymer powder processing technique that facilitated subsequent fabrication into films and/or scaffolds, using both semi-crystalline PCL and amorphous 50:50 PLGA. The development of a solvent-free technique that may be relevant to clinical translation has been a long-standing interest in the field, and particularly in Teoh's group.^{13, 54, 209-214} Here, by demonstrating that cryomilling may be extended to two clinically used, semi-crystalline and amorphous polymers, there is reason to believe that this technique has the potential to be developed into a reliable platform from which blends and/or composites (subsequent chapters in this thesis) may be fabricated and evaluated for tissue engineering applications with a potential clinical slant. More importantly, it has been systematically demonstrated that apart from the reduction in size of polymer particles, mechanistic explanations are available for explaining changes in the physical properties such as rheology and molecular weight, primarily due to chain scission.

4. Bioactive PCL/TCP composites: In vitro evaluation, biocompatibility and bone tissue regeneration^{}**

4.1 Introduction

Bioactive composites have been developed with the aim of achieving closer mechanical resemblance as well as biological function to host tissue. In addition, the composite nature of natural tissue structure provides further validation of synthetic composite systems. As reviewed earlier in chapter 2, there are significant challenges in composite processing that need to be addressed. For example, distribution of the filler and subsequent processability are major concerns that affect the reliability and effectiveness of the final composite. In the event that potentially suitable solutions are proposed, the clinical translatability of these techniques is also called into question (e.g. use of potentially toxic organic solvents). The proposed solution of employing cryomilling as a solvent-free and low temperature processing method has the potential to address these unmet needs. Here, the most common inorganic materials that may be incorporated with PCL are introduced.

4.2 Calcium phosphates

Calcium phosphates (CaPs) form the predominant fraction of bone, making up 70% of the inorganic phase. CaPs in bone have apatite structures and are conjugated with Ca to form hydroxyapatite (HA), tricalcium phosphate (TCP, both α and β), and calcium orthophosphates. Although each of these forms of CaPs vary in terms of their chemical composition and properties²¹⁵, they are both present in bone to provide structural support (due to their hardness and strength) as well as for biological significance, as described below:

^{**} Partially published in: J Lim et al., Small. 2014.

Focal adhesion-directed Runx2 expression

Muller et al²¹⁶ studied the gene expression profiles of MSCs when in contact with CaP surfaces. Additionally, cell-matrix interactions were also characterized. By hypothesizing that focal adhesion plays an important role in integrin mediated signal transduction for stem cell response, they showed that the size and number of focal adhesions decreased when osteoblasts were cultured on CaP surfaces, and also noted that these osteoblasts had higher motility on CaP surface than tissue culture plastic surfaces. Runx2 expression was significantly increased on CaP surfaces 3 days and 14 days after culture (vs. tissue culture plastic), while osteocalcin expression was markedly higher on day 14. Importantly, non-collagenous matrix proteins like osteocalcin have calcium and hydroxyapatite (HA) binding characteristics and could be adsorbed on the CaP surface. Taken together, the expression of Runx2 (which is a key molecule in osteogenesis) was subject to favorable cell-matrix interactions with CaP surfaces via integrin binding.

Mineralized matrix-directed osteogenesis via transport of extracellular P through SLC20a1 symporter

Chien and team²¹⁷ recently elucidated another potential molecular mechanism by which CaP surfaces induce osteogenesis: adenosine signaling. Non-degradable CaP surfaces were used in the study to prevent the interference of matrix degradation on osteogenic differentiation, but dynamic dissolution and reprecipitation of CaP was not affected and remained a possibility – they showed that the CaP components of the matrix underwent dissolution to Ca and P when exposed to a medium devoid of these ions. When hMSCs were cultured on these non-degradable surfaces in normal growth media, consistent upregulation of osteopontin and osteocalcin were observed,

together with highly-expressed SLC20a1 sodium-phosphate symporter. When SLC20a1 was knocked-down, there was a corresponding down-regulation of osteocalcin and osteopontin. Subsequently, exogenous P was added separately, and showed a similar trend with and without SLC20a1 knock-down. From this, it was clear that P played a role in osteogenesis, specifically through the SLC20a1 symporter. Since P is also a substrate for ATP synthesis, ATP production was also significantly increased. ATP can also mediate osteogenic signaling through purinergic receptors. Therefore, in order to evaluate the ability of extracellular P to induce osteogenesis from the mineralized matrices, ATP transport was inhibited with N-ethyl maleimide (NEM), and results showed abrogation of osteocalcin and osteopontin expression. Adenosine, an ATP metabolite, was also found to stimulate osteogenesis in the absence of CaP moieties. When the A2b receptor responsible for adenosine signaling was inhibited (by PSB603), expression of osteocalcin and osteopontin was down-regulated, suggesting a role for osteogenesis by the A2b receptor. Taken together, there is compelling evidence to suggest that P is able to stimulate osteogenesis through the A2b receptor, but this conclusion does not refute claims that a combinatorial CaP surface is beneficial to osteogenesis. Rather, the dynamic dissolution and reprecipitation contributed to the exogenous supply of P, thereby driving the osteogenic differentiation pathway through the A2b receptors.

Soluble Ca- and P-directed expression of BMP-2 driving osteogenesis

Chai et al.²¹⁸ studied the effect of extracellular Ca and P in modulating osteogenesis of human periosteal derived cells (hPDCs). hPDCs were seeded at a density of 4500 cells/cm² and monitored for the proliferation over a period of 28 days, and mineralization was assessed at 21 days. Persistent upregulation of BMP-2 was expressed in varying concentrations of Ca and P when compared to normal growth

media and osteogenic media. When Ca and P were added in combination, a time-dependent increase in osteocalcin, osteopontin and BMP-2 was observed from 7 to 21 days. Runx2 expression was comparable to normal growth media at days 7 and 14 with the combinatorial CaP treatment. The functional measure of osteogenesis lies in the amount of mineral deposition, and results from their study indicated a dependence on P concentrations while being independent of Ca. However, excessive P concentrations (<5mM) induced cellular apoptosis due to the failure of the mitochondrial function. ALP expression was not significantly increased regardless of Ca, P or CaP treatment. ALP is important for the conversion of extracellular pyrophosphate (ePPi) to inorganic phosphate (P)²¹⁹. Osteocalcin is involved in the binding of Ca to the ECM for regulating the size and growth of mineral crystals²²⁰, and was found to be significantly upregulated by Ca, P and CaP treatments in a specific time- and dose-dependent fashion. More importantly, the master gene of bone formation, Runx2, was significantly upregulated by P treatment only. Taken together, it appears that the molecular mechanism involved in CaP-induced osteogenesis follows that of the expression of BMP-2 by Ca and P, leading to paracrine/autocrine signaling effects to drive mineral deposition.

4.2.1 Tricalcium phosphates

As mentioned earlier, various forms of CaPs exist. Among them, TCP has shown higher resorption kinetics as opposed to HA²¹⁵, which is desirable from a tissue engineering perspective due to the preference of complete foreign material degradation and/or resorption to minimize chronic inflammation.

Expectedly, TCP has been incorporated in many composites. For example, Rakovsky et al. generated PLA-TCP composites with uniform distribution through attrition milling²²¹ and demonstrated the mechanical advantages of doing so. On the

other hand, Paik et al. electrospun PCL-TCP nanofibers²²² and showed that MC3T3-E1 osteoblasts proliferated and differentiated well on these bioactive composite matrices. Other similar studies incorporating TCP are listed in Table 4-1.

Table 4-1 Summary of selected, recent publications incorporating TCP with polymer matrices for developing bioactive (since 2010)

Polymer matrix	Fabrication technique	Selected Reference(s)
PCL	Cryomilling	200
	Electrospinning	222
	Selective laser sintering	223
	In situ formation	126, 129
PLA	Attrition milling	221
	Chloroform in water	224
PCL/PLGA	Solid freeform fabrication	225
PLCL	Extrusion	226

4.3 Bioactive composites: The importance of mechanical, thermal and degradation characteristics

Bioactive polymeric composites have been developed over the years with the aim of improving the biological function and mechanical compatibility of synthetic polymers. Conventional methods of composite fabrication employ the use of carcinogenic organic solvents^{66, 206, 227-229}, raising concerns over potential safety issues. To avoid the use of organic solvents, heat-based methods^{13, 230} have also been explored, but suffer from poor mixing and distribution due to the altered viscosity of polymer melts after incorporation of the dispersed phase. In addition, recent interests in developing drug-incorporated polymer composites have also been limited to non-thermosensitive drugs, due to the presence of elevated temperature during

processing. As such, low-temperature processing methods have been explored for the development of bioactive composites^{125, 221}.

One of the applications of polymer composites is in bone tissue engineering (BTE), where it is well understood that material bioactivity and stiffness play determining roles in the replacement and regeneration of lost tissue.⁸⁹ Given the physiological relevance of calcium phosphates (CaPs) (they make up 70 wt% of the inorganic phase) to bone, work has been done to develop composites that incorporate calcium phosphates (CaPs) into polymer matrices.^{231, 232} The main challenge however, remains in matching the physiological CaP loading capacity. To this end, Guarino et al.²³¹ studied the incorporation of 0, 13, 20, and 26 vol% of biphasic CaP and demonstrated a direct correlation between compression modulus and increasing vol% of CaP. Bleach et al.²¹⁷ incorporated up to 25 vol% of CaP into polylactide (PLA), and showed the amount of CaP was inversely correlated to tensile strength. In a unique study, Makarov et al.¹²⁹ managed to fabricate polycaprolactone (PCL)-based CaP composites with unusually high volume fractions (80-95 vol%) through in situ CaP formation.

Apart from demonstrating the dependence of moduli on varying volume fractions of CaP²²¹, Rakovsky et al. also showed that these composites were able to maintain their mechanical properties under mild degradation conditions over one month. On this note, Teoh's group has also made significant contributions to the degradation stability of PCL/CaP composites, although it was limited to tricalcium phosphate (TCP) loading of 20 wt%.¹⁸⁰

4.4 Schematic of workflow

A schematic for the results presented in this chapter are illustrated in Figure 4-1. Briefly, PCL-TCP films were put through in vitro and in vivo tests and evaluated according to their physical, degradation, and biological responses (in vitro), as well as their general biocompatibility with bone tissue, and barrier membrane properties.

Figure 4-1 Schematic of workflow for evaluating PCL/TCP composites in vitro and in vivo.

4.5 Materials and Methods

4.5.1 PCL and Tricalcium Phosphate

PCL and tricalcium phosphate (TCP) powders were purchased from Osteopore International Pte Ltd, of which the latter had a reported diameter of 2 – 10 μm .

4.5.2 Cryomilling

PCL/TCP composites were fabricated using cryomilling (Retsch®, Germany). PCL/TCP in three proportions (75/25, 65/35, 55/45) were weighed using a microbalance (Mettler Toledo, USA), and loaded into the cryogenic vial with a ball-to-

mass ratio of 30:1. The cryomilling protocol was: 6 – 8 min of pre-cooling in liquid nitrogen and 20 min of continuous milling for one cycle.

4.5.3 Fabrication of PCL/TCP films and 3D scaffold

PCL/TCP films were thermally pressed into films of thickness approximately 30 – 60 μm . Briefly, a known mass of PCL/TCP was placed between two stainless steel sheets on the Carver system (Carver Inc, USA). Temperature was elevated to 100 $^{\circ}\text{C}$ and pressure added for 30 min. The pressed film was then allowed to cool to room temperature via normal convection cooling. 75/25 3D scaffold was fabricated using the same method, with the incorporation of 50 vol% of sodium chloride, which was subsequently salt-leached in water.

4.5.4 Mechanical testing

Tensile and compressive mechanical tests were performed on the polymer composites fabricated in this thesis with an Instron 5582 (Instron, Norwood, MA). For tensile tests, films were cut into rectangular sheets and fixed onto a paper frame with a standard gauge length of 30 mm. A crosshead moving speed of 3 mm / min was then applied, and the samples were stretched until failure. For compression tests, cylindrical blocks of polymer composites (height 10 mm) were placed in between two platens, with a crosshead moving speed of 1 mm / min.

4.5.5 Thermal properties – Differential Scanning Calorimetry

The thermal properties ($n=3$) were investigated using the Differential Scanning Calorimetry (DSC) (Diamond DSC, PerkinElmer, USA). The samples were heated from room temperature to 150 $^{\circ}\text{C}$ at a rate of 20 K/min. Crystallinity (X_c) was calculated by taking a ratio of the experimentally measured enthalpy heat of melting

to the theoretical value of 100 % crystalline PCL (136 J/g²³³). Results are presented as an average of three readings and their respective standard deviations.

4.5.6 X-ray micro computed tomography (micro-CT)

High-resolution, qualitative visualization of TCP distribution in PCL matrix was conducted by micro-CT imaging (Skyscan in vivo microtomograph, 1076, Belgium). The films were placed on a sample holder and scanned through 180° at a spatial resolution of 9 µm. The image data from the scanned planes are subsequently thresholded and reconstructed to create 3-D images to visualize TCP distribution.

4.5.7 Thermogravimetric analysis

Thermogravimetric analysis (TGA) (n=3) was conducted using the TGA/DSC Mettler Toledo (Mettler Toledo, USA). PCL/TCP samples were heated at a rate of 20 K/min from room temperature until 450 °C, and held isotherm for 5 min.

4.5.8 Degradation studies

Degradation studies were performed in physiological and accelerated conditions. For physiological degradation, samples were pre-weighed and placed into 1 mL of phosphate buffer saline (PBS) at 37°C. At stipulated time-points, samples were retrieved, washed with deionized water (DI water) and dried for two days before being reweighed. For accelerated degradation, films were placed in 1M NaOH at 37°C, and removed at pre-determined time-points to assess mass loss, changes in morphology, crystallinity, molecular weight, and mechanical properties.

4.5.9 Mass loss

Mass loss of samples was taken using a mass balance (RADWAG, Poland). Samples were dried completely before their mass was taken at an accuracy of ± 0.1 mg.

4.5.10 Cell source

Human fetal bone marrow-derived stem cells (hfMSCs, Passage 7) were isolated as previously described²³⁴. Cells were seeded in a flask (75ml, Nunc, Rochester) with a cell density of 10^6 /ml in DMEM (10% Fetal Bovine Serum/1% Penicillin-Streptomycin). Non-adherent cells were removed with the first medium change on day 3. Adherent hfMSCs were recovered from primary culture after one week.

4.5.11 hfMSC seeding on films

PCL and PCL/TCP films were attached to 12 mm diameter glass coverslips soaked in 70% ethanol for 24 hours. Subsequently, the films were washed twice with phosphate buffer saline (PBS) and adherent hfMSCs (Passage 7) from primary culture were seeded with a density of 15,000 cells/cm². Samples were kept in culture for 14 days.

4.5.12 Immunohistochemical staining of Osteocalcin and von Kossa

Immunohistochemical (IHC) assay was performed to investigate the expression level of osteocalcin (OCN), a late-stage differentiation marker. Cells were fixed, penetrated and blocked. Briefly, hfMSC films were first fixed in 4% paraformaldehyde and treated with 0.1% Triton X-100 each for 15 mins, before treatment in 1% bovine serum albumin (BSA) for 24 hours. For antibody labeling, samples were first incubated with primary rabbit antibody (1:100, Abcam) followed by secondary goat anti-rabbit IgG conjugation to OCN (1:100, Molecular probes). 4'-6-diamidino-2-phenylindole (DAPI) was added last before mounting onto glass coverslips for detection of fluorescence emission at 488nm using fluorescence light microscopy (CKX-41, Olympus). To ensure consistency, images were taken using the same parameters across all groups, at 4x magnification.

For von Kossa, hfMSC films were gently rinsed twice with PBS, before being fixed in 10 % formalin for 5 min, and subsequently washed twice with water. Finally, they were stained with freshly made 2 % silver nitrate in water (w/v) for 10 min in the dark and exposed to strong light for 15 min.

4.5.13 Pig studies

Three skeletally mature female micropigs of age 2 years old (mean body weight: 30kg) were housed for three months in this study. The pigs used in this study were supplied by PWG Genetics Pte Ltd (Good Lab Practice certified, Contract Research Organization). These pigs were maintained under specific pathogen free (SPF) conditions, and the study was conducted after approval from their in-house IACUC (IACUC No. PN13011). PCL/TCP films were sterilized with 100% ethanol overnight, and washed with PBS thrice before implantation. First, the femur was exposed and two 4mm circular defects were created along the femur, apart from each other. All defect were filled with PCL scaffolds donated by Osteopore International Pte Ltd (Singapore). The surrounding periosteum was resected, and these defects were randomly assigned to receive either no films or PCL/TCP films. The animals were maintained for 3 months before sacrificing. (n=6 per group)

4.5.14 Rabbit studies

Eight male New Zealand rabbits (*Orytolagus cuniculus*), weighing 3.0- 4.0 kilograms were studied, with ethics approval from the Institutional Animal Care and Use Committee (IACUC No. INH 2013/013). A midline incision was made in the skin of the calvaria along the sagittal suture line. The soft tissue and periosteum were carefully raised. Circular osseous defects of 10 mm diameter were created using surgical bur on each side of the suture. Geistlich Bio-Oss® (Geistlich Pharma AG, Division Biomaterials, Wohlen, Switzerland) was used in this study. It is derived

from the mineralized part of bovine bone, with granule size 0.25 - 1 mm. The materials were provided in standard 0.25g sterile cylinders.

The periosteum over each bone defect was excised to ensure no role of the periosteum in the guided bone regeneration process. The defects were randomly assigned to receive either no films or PCL/TCP films. The animals were maintained for 1 and 2 months before sacrifice.

4.5.15 Hematoxylin and Eosin, Masson's Trichrome Staining

Hematoxylin and eosin (H&E) and Masson's Trichrome Staining (MTS) were performed according to standard protocols, to determine the tissue structure and cellularity of the explanted tissues.

4.6 Results

4.6.1 Homogeneous TCP distribution – SEM and macroscopic observations

This was demonstrated when PCL/TCP (65/35) powders were mixed using the following conditions and compared: cryomilling, and physical blending without cryomilling (**Figure 4-2A**). Under SEM, it could be observed that the TCP (distinguished as bright and round particles) agglomerated into isolated islands (indicated by arrows) in the control group (65/35 without cryomilling). In contrast, the cryomilled PCL/TCP (65/35) (**Figure 4-2B**) presented a homogeneous distribution into the matrix of PCL (black arrows). Consequently, condensed film structures could be produced in a highly reproducible fashion, suggesting the lack of gross defects (**Figure 4-2C, D**). Cross-sectional SEM images also showed homogeneous distribution of TCP within PCL as compared to non-cryomilled PCL/TCP (65/35) films (**Figure 4-2E, F**) Salt leached scaffolds, suitable for bone tissue engineering, were also fabricated, and exhibit similarly uniform TCP dispersion through the polymer matrix.

Figure 4-2 SEM images of (A) non-cryomilled PCL/TCP composite particles, where some TCP agglomerated into isolated islands (indicated by arrows) while some TCP were on the surface of the PCL pellet (circles); (B) cryomilled PCL/TCP particles, where TCP particles were well distributed (arrows). Gross morphological observations of films (C, D) and their corresponding cross-sectional SEM images (E, F). The effect of “spalling” was observed on non-cryomilled PCL/TCP control films.

4.6.2 Consistent PCL/TCP ratios

Thermogravimetric analysis (TGA) and micro-CT imaging (**Figure 4-3**) were then used to verify the distribution of TCP in the polymer matrix. In all cryomilled proportions of 75/25, 65/35, and 55/45 (**Figure 4-3A**), TGA analyses revealed measured PCL/TCP ratios of randomly sized samples to be identical to predicted values, suggesting homogeneous distribution of the TCP through the polymeric phase. To demonstrate processability of the mixed powder and to be representative of scaffolds in BTE, porous blocks were also generated from cryomilled 75/25 (75/25 3D). When the films and scaffold were put through a micro-CT (**Figure 4-3B**), significant improvements in TCP distribution may be observed when compared to the control group. Notably, incorporation of physiological amounts of TCP (70 wt%) was successfully attained, and which also suggested homogeneous distribution of TCP. In the control group (65/35 control), less TCP was observed mainly due to the effect of spalling as shown earlier (**Figure 4-2C**, circled). Among the other test groups (75/25, 65/35, 55/45, 30/70), a homogeneous distribution of TCP could be observed. Here, the authors also fabricated a 3D scaffold (75/25 3D) using the same method, with the inclusion of salt particles that comprised 50 vol%. Following a salt leaching process in which all salt particles were removed, micro-CT results (**Figure 4-3B**, 75/25 3D) indicated the homogeneous distribution of TCP throughout the entire geometry, supporting the claim that cryomilling is able to achieve good particulate distribution in composite fabrication.

Figure 4-3 (A) TGA analysis of the compositional make-up of PCL/TCP films. (B) MicroCT images of PCL/TCP films and scaffold. 65/35 control films refer to the non-cryomilled PCL/TCP films.

4.6.3 Homogeneous mineralization on cryomilled PCL/TCP films

In order to substantiate the point that homogeneous distribution of bioactive TCP may lead to robust cellular responses, hfMSCs were seeded on PCL films, 65/35 films or 65/35 control films and subsequently chemically induced to form bone in the presence of bone media (BM), (**Figure 4-4**). Corresponding negative control groups were conducted using culture media (D10). Due to the extremely high osteogenic potential of these cells, significant differentiation could be induced regardless of the substrate used. However, hfMSC mineralization was most pronounced and homogeneous in 65/35 (**Figure 4-4A**). 65/35 control films had uneven mineralization,

which could be due to the uneven distribution of TCP. Additionally, the expression of late-stage osteogenic marker osteocalcin (OCN) was expressed homogeneously on 65/35 films, while that of 65/35 control films resulted in hfMSC clustering and preferential differentiation around the TCP regions (**Figure 4-4B**, darker regions on 65/35 control). Taken together, it was shown that hfMSCs were able to differentiate homogeneously on 65/35 films, while non-cryomilled 65/35 control films resulted in preferential differentiation centered on the TCP regions (**Figure 4-4**).

Figure 4-4 (A) von Kossa assessment of hfMSCs on PCL and PCL/TCP films in bone media (BM) and culture media (D10). Mineralization was observed in all groups in BM, with only 65/35 showing homogeneous mineralization over the entire film surface. Scale bar represents 500 μm . (B) Immunohistochemical (IHC) staining of osteocalcin (OCN) was conducted after 14 days of osteogenic incubation. hfMSC clustering and OCN expression was highly localized on the TCP regions (darkest regions) in 65/35 control, while hfMSC distribution and OCN expression was homogeneous on the 65/35 films. Images were taken at 4x magnification. Scale bar represents 250 μm .

4.6.4 Accelerated studies of PCL-TCP degradation

4.6.4.1 PCL-TCP mechanical properties

The mechanical properties of PCL/TCP films were presented in Figure 4-5 and Table 5-2. The stress-strain profile of pristine PCL and the composite films were different, in that there was an appreciable plastic deformation in pristine PCL while composite films did not. Accordingly, the toughness of these composite films were found to be significantly lower than that of pristine PCL (composite films: 0.8 J/m^3 , PCL: 17.1 J/m^3 ; $p < 0.05$). When uniaxially stretched, the Young's modulus displayed an increasing trend when more TCP was incorporated into the PCL matrix. There was also a concomitant decrease in strain.

When the films were exposed to degradation media, the behavior of pristine PCL was different from that of the composite films: pristine PCL showed a decrease in the Young's modulus, while the composite films showed an increasing trend. 35/65 films were too fragile for meaningful mechanical testing once degraded, and thus were not represented in the table.

Figure 4-5 Stress-strain profiles of PCL/TCP films tested under tension.

Table 4-2 Summary of the mechanical properties of PCL/TCP films at 0h, 5h, and 12h.

Composition	Degradation time	Yield Stress (MPa)	Young's Modulus (MPa)	Strain (mm/mm)	Toughness (J/m ³)
PCL	0 h	9.3 ± 0.6	331 ± 31	0.061 ± 0.009	17.1
	5 h	10.0 ± 1.5	327 ± 23	0.065 ± 0.03	NA
	12 h	8.9 ± 1.7	265 ± 22	0.071 ± 0.01	
75/25	0 h	10.3 ± 0.1	378 ± 38	0.037 ± 0.003	0.8
	5 h	6.9 ± 2.9	461 ± 162	0.021 ± 0.009	NA
	12 h	11.5 ± 3.0	583 ± 198	0.028 ± 0.010	
55/45	0 h	8.4 ± 0.5	546 ± 22	0.025 ± 0.005	0.8
	5 h	6.0 ± 1.9	527 ± 382	0.017 ± 0.007	NA
	12 h	9.5 ± 1.5	869 ± 224	0.010 ± 0.004	

4.6.4.2 PCL crystallinity in PCL-TCP films

The crystallinity of PCL in pristine PCL and PCL/TCP films were evaluated and represented in Table 5-3. Pristine PCL recorded a crystallinity of approximately 60%,

while it was observed that the incorporation of TCP led to a decrease in crystallinity. In addition, the increase in TCP proportion led to a concomitant decrease in PCL crystallinity. With degradation, the crystallinities recorded in 25 wt% and 45 wt% films were different from 65 wt% films particularly over the first 12 h. The 65 wt% films exhibited close to 20% increase in crystallinity while the other films recorded a slight decrease.

Table 4-3 Summary of the crystallinity of PCL/TCP films after various degrees of degradation, as recorded at 5h, 12h, 24h, and 48h.

Composition (PCL/TCP)	Crystallinity (%X _c)				
	0 h	5 h	12 h	24 h	48 h
PCL	58.0 ± 3.3	60.6 ± 2.4	53.8 ± 3.7	56.9 ± 3.8	49.1 ± 1.0
75/25	43.5 ± 2.2	42.5 ± 8.0	33.4 ± 8.5	49.4 ± 1.2	45.0 ± 3.7
55/45	39.6 ± 3.0	25.6 ± 4.1	35.8 ± 10.4	52.6 ± 3.4	35.0 ± 4.0
35/65	36.3 ± 1.2	51.0 ± 11.0	54.4 ± 18.9	38.7 ± 7.8	NA

4.6.4.3 Mass loss in PCL-TCP films

The mass loss profiles of PCL/TCP films were illustrated in Figure 4-6. It is apparent that the incorporation of TCP accelerates the degradation process, with the rate of degradation correlating positively with the amount of incorporated TCP.

Figure 4-6 Mass loss profiles of PCL/TCP films over a period of 48h.

4.6.4.4 Osteogenic differentiation of MSCs on pre- and post-degraded PCL/TCP films

The ability of PCL/TCP films to direct osteogenic differentiation of MSCs pre- and post-degradation was evaluated with von Kossa stains on days 3, 7, and 14. From the images, PCL films did not show observable positive stains until day 14 while positive staining from PCL composite films was observed from as early as day 3 (55/45, 35/65) and day 7 (75/25) (Figure 4-7A). Importantly, all films showed robust differentiation by day 14. When degraded, all films did not appear to show observable differences among them (Figure 4-7C).

Figure 4-7 (A) von Kossa staining of PCL/TCP films seeded with MSCs, evaluated over a period of 14 days. (B) Changes in PCL/TCP ratios over time with degradation, with subsequent evaluation of their ability to induce MSC mineralization (C).

4.6.4.5 Changes in PCL/TCP ratios

The variations in the ratio of PCL to TCP were evaluated with thermogravimetric analysis. With increasing degradation time, the proportion of TCP to PCL increased steadily (Figure 4-7B).

4.6.5 Biocompatibility of PCL/TCP films

Following defect creation, PCL/TCP films were placed directly over the defect site. Some defects were filled, while others were left empty (Figure 4-8).

Figure 4-8 Camera images of the surgical process beginning from the exposure of the defect site until the implantation of films and scaffolds. F represents films, while S represents scaffolds (PCL in porcine, Bio-Oss® in rabbit). * indicates the location of the created defects.

Porcine femur

PCL-TCP films were implanted as a bioactive barrier membrane to fibrous tissue invasion in a non-critical sized femoral defect in pigs. However, due to the stiffness of the PCL-TCP films, their shapes were not adaptable to the curvature of the femur. After three months of implantation, all PCL-TCP films were observed to be well-accepted by host bone as they were firmly lodged onto the femur (Figure 4-9). The animals did not have any issues with mobility, as expected.

Rabbit calvarium

PCL-TCP films were well-accepted by the rabbit calvarium with no cases of rejection. However, due to the stiffness of the PCL-TCP films, their shapes were not adaptable to the curvature of the calvarium (Figure 4-9).

Figure 4-9 Explanted bone tissue samples of porcine femur after 3 months (left) and rabbit calvarium after 2 months (right), with dotted outlines indicating the location of the films, which directly covers the defect site.

4.6.6 Biocompatibility of PCL/TCP in porcine femur

Histological evaluation of the PCL/TCP films reported that a moderate fibrous capsule and inflammatory cells surrounded the PCL/TCP films, which extended to the adjacent periphery (Figure 4-10). Presence of inflammatory cells was observed surrounding the periphery of the films as evidenced by the multinucleated giant cells, forming a thin layer of approximately 10 μ m. Inflammation within the defect site was mild when the films were present. These findings were part of the histopathology report from a qualified pathologist who was blinded during the assessment.

4.6.7 Biocompatibility of PCL/TCP in rabbit calvarium

Histological evaluation of the PCL/TCP films reported mild fibrosis and inflammation within the defect. At an earlier time-point of 1 month (data not shown), there was less inflammation as compared to 2 months (data presented here). Without the presence of films, chronic inflammation was observed, as evidenced by the persistent observation of inflammatory cells over 2 months (Figure 4-10). These findings were

part of the histopathology report from a qualified pathologist who was blinded during the assessment.

Figure 4-10 H&E stains of the porcine femur and rabbit calvarium. (top row) Without the presence of films covering the defect region, inflammation of the defect site could be observed. (bottom row) With the films, inflammation within the defect region was markedly less, with more extensive matrix deposition. F represents films, while S represents scaffolds (PCL in porcine, Bio-Oss in rabbit). * indicates a concentrated region of inflammation.

4.6.8 Effect of PCL/TCP films as barriers to fibrous tissue invasion and bone regeneration

From histological observations of Masson's Trichrome Staining (MTS) on the defect regions (Figure 4-11), the formation of mineralized bone (B) and woven bone (WB) structures was more prominent in the groups that had a PCL/TCP film covering the

defect. On the other hand, where PCL/TCP films were not present, woven bone formation was predominant, with numerous medullary spaces and lack of remodeling. Compact bone was also observed, although ossification and remodeling were incomplete. In addition, periosteum/soft tissue regeneration appeared to be more distinct in the porcine femur group where PCL/TCP films were present.

Figure 4-11 MTS images of tissue samples taken from porcine femur and rabbit calvarium. (left column) Without the presence of films covering the defect region, a mixture of woven bone (WB) and mineralized bone (B) structures was formed in the porcine samples. Regeneration of periosteum/soft tissue (P/ST) was also less clearly defined. In the presence of films, there were significantly more mineralized bone structures than woven bone around the scaffold (S). In addition, there was a thick regenerating periosteum/soft tissue covering that clearly defined the boundary between hard and soft tissue (dotted lines). (right column) In the rabbit calvarium, the lack of a film resulted in poor bone regeneration, as observed by soft tissue infiltration with minimal regeneration. On the other hand, the presence of films resulted in markedly higher woven bone structures around the scaffold (S). * indicates a concentrated region of inflammation.

4.7 Discussion

PCL-TCP composites faced considerable limitations in terms of distribution and loading capacity. In addition, most methods of fabricating these composites were also inadmissible for regulatory approval, making their translation to clinics very challenging. This thesis explored a low temperature, solvent-free method for processing biomaterials into fine powder, which significantly enhanced their rheology as described in Chapter 4. In this Chapter, the homogeneity of TCP, added as bioactive fillers within the PCL matrix was evaluated. From the results of SEM, micro-CT imaging, and TGA analyses, TCP distribution was homogeneous post-cryomilling in the form of fine powder, and distribution was maintained up to the point where they were fabricated into films and 3D scaffolds. To verify that the homogeneity would be able to result in biological advantages, hfMSCs seeded onto PCL-TCP films demonstrated robust and homogeneous mineralization. These in vitro studies have thus shown that cryomilling is a viable and reliable processing method for inorganic PCL-TCP composites.

The use of such inorganic composites in vivo would require an understanding about its degradation responses. Due to the stability of PCL in normal degradation conditions, accelerated degradation study in NaOH was conducted. In general, degradation of any polymer can occur via hydrolytic cleavage or enzymatic action. In an in vitro scenario, it may be difficult to replicate in vivo enzymatic activity, thus hydrolysis is usually conducted. From the results, an increase in the amount of TCP loaded within the scaffolds led to faster degradation of the inorganic composites. Artefacts represented by the formation of pits^{70, 235} were observed later when the amount of TCP incorporated was lesser, while the converse is true: 65 wt% TCP films displayed signs of degradation as early as 5 h and fragmented after 24 h.

Polymer degradation mechanism proceeds by the well-known process of hydrolysis¹⁸⁴, but it is also well-understood that the proportion of crystalline and amorphous regions also influence the progression of degradation. The ordered packing structure in a crystalline region minimizes the infiltration of hydroxyl ions, causing them to be less prone to degradation.²³⁶ Based on the results presented here, where PCL recorded the highest crystallinity and the composite films showed a decreasing crystallinity with increasing TCP incorporation, there is good agreement between the rate of degradation and polymer matrix crystallinity.

Of importance to the development of these composite membranes as bioactive scaffolds for tissue engineering applications would be their ability to maintain osteogenicity while providing structural support.²³⁷ To this end, PCL/TCP films were able to direct osteogenic differentiation of hfMSCs over the course of 14 days while the films remained intact after various extents of degradation. This implies that there may be potential benefits when PCL/TCP is implanted as a bioactive membrane, as it is able to support cellular growth and differentiation while maintaining its structural integrity.

The 3-month study in the porcine femur, where the PCL/TCP films were implanted as a bioactive barrier membrane to fibrotic invasion yielded positive results. Firstly, they served their primary function in ensuring that minimal fibrotic invasion into the defect cavity occurred over the 3 month period. Secondly, the PCL/TCP film facilitated the regeneration of the periosteum beneath it. Through the prevention of fibrotic invasion coupled with the promotion of periosteal regeneration, healing within the defect cavity was facilitated, as mineralized bone and woven bone structures were seen, as compared to incomplete ossification and fibrotic tissue invasion in the groups without films.

4.8 Summary

In this chapter, PCL/TCP composites were demonstrated to maintain homogeneity after processing into powder and scaffolds (films and 3D scaffolds). In addition, homogeneous distribution resulted in added advantages for in vitro mineralization. To further characterize these films, their in vitro accelerated degradation responses in the presence of NaOH demonstrated that the rate and extent of degradation are dependent on the crystallinity of the PCL matrix and the amount of TCP added. These PCL/TCP films were then implanted into the femur of the porcine, with the aim of evaluating their ability to function as bioactive barrier membranes. PCL/TCP films were well-accepted by the host, with notable advantages in bone regeneration within the defect cavity due to the presence of PCL/TCP films, which prevented fibrotic tissue invasion while at the same time promoting periosteal regeneration. Taken together, these results suggest that cryomilling can homogeneously distribute TCP within PCL matrices, and that these resultant films are stable in vivo in the femur over a period of 3 months. In addition, the PCL/TCP films are able to function as barrier membranes, while maintaining their ability to guide periosteal regeneration over the defect.

5. Bioactive PCL scaffolds with Magnesium trace element for tissue regeneration^{††}

5.1 Introduction

Magnesium ions (Mg^{2+}) are the fourth most abundant mineral in the body²³⁸, and 60% of it is stored in bone²³⁹. As such, many have studied ways in which Mg may influence bone homeostasis²⁴⁰. From early literature, Mg^{2+} was found to influence both the metabolic activity of cells²⁴¹ and also pyrophosphate metabolism²⁴². As part of the evidence that Mg^{2+} influences calcification²⁴³, the intimal media thickness of blood vessels have been reported to be under the external influence of Mg^{2+} ²⁴⁴, with these studies suggesting that Mg^{2+} supplementation may be an option for the treatment and prevention of vascular calcification.

In the field of orthopedics²⁴⁵, studies on Mg^{2+} began following reports that magnesium alloys or magnesium-coated implants promoted osteoblast adhesion²⁴⁶ and in situ calcium phosphate precipitation²⁴⁷. However, the issue of localized corrosion^{248, 249} is still inadequately addressed despite efforts to control the rate of corrosion through the use of calcium phosphates²⁵⁰, polymers²⁴⁰, or a combination of both²⁵¹. In order to further the understanding on the action of Mg^{2+} , Yoshizawa et al.²⁵² suggested that elevated levels of Mg^{2+} (particularly at 5 mM and 10 mM) were able to induce mineralization of the extracellular matrix (ECM), possibly through hypoxia-inducible factor (HIF)-2 α in undifferentiated human bone marrow stromal cells (hBMSCs) or proliferator-activated receptor gamma coactivator (PGC)-1 α in the osteogenically differentiated state. In an effort to study the effects of corrosion by-products, Li et al.²⁵³ immersed various magnesium alloys in culture media and

^{††} Patent pending, Singapore Provisional Patent, 2014.

through the use of conditioned media (CM), demonstrated that Mg^{2+} increased human mesenchymal stem cell (hMSC) proliferation and differentiation, in a dose-dependent manner for the latter. It was also proposed that the presence of extracellular Mg^{2+} suppressed the nucleation and growth of hydroxyapatite by stabilizing and prolonging amorphous calcium phosphate (ACP).^{254, 255}

The vast and varied literature validates the understanding that Mg^{2+} acts upstream of many cellular processes.²⁵⁶ Importantly, the common theme among all these studies was the ability of extracellular Mg^{2+} to stimulate pyrophosphatase activity²⁴², which may be more commonly known as alkaline phosphatase (ALP). This would naturally lead to increased availability of inorganic phosphate (P) for the formation of CaP. However, as shown in previous studies^{254, 255}, prolonged exposure to Mg resulted in delayed formation of crystalline hydroxyapatite (HA), which is an important and necessary inorganic component in physiological bone.

5.2 Schematic of workflow

The application of Mg as a potential bioactive stimulant for directing bone regeneration has been evaluated through in vitro tests on MSCs. Prior to testing, the source of Mg was determined, followed by exposure of MSCs to varying levels of Mg, both with and without the presence of osteogenic factors. Gene and protein related studies were conducted. In addition, Mg was incorporated into PCL films through a cryomilling and heat pressing process, and evaluated for their mechanical properties, changes in morphology, degradation characteristics and ability to release Mg in the presence of PBS. Finally, biocompatibility tests were conducted in the fat tissue of pigs over a period of 3 months.

Figure 5-1 Illustration of the work presented in this chapter.

5.3 Materials and Methods

5.3.1 Human fetal MSCs isolation and culture

Human fetal MSCs (hfMSCs) were obtained as previously described²⁵⁷. Cells were seeded in a flask (T175, Nunc, Rochester) at a density of 10^6 / ml in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (pen/strep) (D10). Non-adherent cells were removed with media change on day three. The remaining adherent cells were subsequently used for this work (Passage 3 – 6).

5.3.2 Experimental culture of hfMSCs

hfMSCs that were isolated were exposed to the following conditions:. For this purpose, Mg-free DMEM (BioRev, Singapore) was supplemented with 10% FBS/1% pen/strept, and supplemented with variable amounts of magnesium chloride (Sigma Aldrich, Singapore) to achieve the following concentrations: 0.8 mM, and 8 mM. This

shall henceforth be denoted as “proliferative media”. “Osteogenic media” was prepared by supplementing various proliferative media with 10 mM β -glycerophosphate, 10^{-8} dexamethasone, and 0.2 mM ascorbic acid.

5.3.3 Cell proliferation and viability

hfMSCs were seeded at a density of $7.5 \times 10^4/\text{cm}^2$ in 6-well plates in the various proliferative and osteogenic medium. At days 3 and 7, AlamarBlue® reagent (Invitrogen, Singapore) was added according to the manufacturer’s instruction, and incubated in the dark for 1.5 hours before fluorescence reading at 590 nm with a microplate reader (Spectramax). In addition, cells were stained with fluorescein diacetate (FDA) and propidium iodide (PI) to visualize live and dead cells.

5.3.4 Alizarin red and von Kossa

hfMSCs were seeded at confluence ($20 \times 10^4/\text{cm}^2$), and cultured in both proliferative and osteogenic media for 14 days. Alizarin red stains were prepared according to the manufacturer’s instruction, and maintained at pH 4.2. hfMSCs were fixed with 4% paraformaldehyde for 5 mins, washed and stained with Alizarin red for 10 mins under gentle shaking. Subsequently, they were thoroughly washed and air-dried before visualization with a microscope. von Kossa staining was done as mentioned earlier.

5.3.5 ALP and calcium

hfMSCs cultured in both proliferative and osteogenic medium were rinsed with phosphate buffer saline (PBS) and incubated in a mixture of collagenase and trypsin for 4 hours at 37°C . Subsequently, they underwent three freeze-thaw cycles to lyse the cells, and the lysates were evaluated for ALP activity according to the manufacturer’s instructions. The pellet obtained was stored separately for evaluating calcium deposition. The pellet obtained previously was dissolved overnight in 0.5 N

acetic acid. A calcium (Ca) assay was then used to quantify the amount of Ca deposited by measuring its absorbance at 612 nm, in accordance with the manufacturer's instructions.

5.3.6 Real-time polymerase chain reaction

Real-time polymerase chain reaction (RT-PCR) was performed to study the expression of early osteogenic genes by hfMSCs cultured in 6-well plates under proliferative and osteogenic conditions. Total RNA was harvested by using a Reverse Transcription System (Promega, USA) on days 4 and 8. Next, 1 mg total RNA was reverse-transcribed to cDNA. Finally, the CFX Connect system (BioRad, Singapore) was used to conduct quantitative real-time PCR with TaqMan Universal PCR Master Mix and gene-specific PCR primers including osteocalcin (OCN), coll-1, transforming growth factor-beta (TGF- β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression was normalized to GAPDH by using the comparative $2^{-\Delta\Delta Ct}$ method. The primers used in this experiment are shown in Table 5-1. All PCRs were carried out in triplicate.

Table 5-1 List of genes and their primer sequences, annealing temperatures, and product sizes.

Gene	Primer sequence (both 5' to 3')	Annealing Temperature (°C)	Product size (basepairs)	Accession Number
GAPDH	F: CCACCCATGGCAAATTCC R: GGATTTCCATTGATGACAAGCTT	58	67	NM_00128974 6.1
ON	F: CGCGGTCTTCAGACTGCCC R: AGGCCCTCATGGTGCTGGGA	58	85	NM_003118.3
COL1A1	F: AGGACAAGAGGCATGTCTGGTT R: CCCTGGCCGCCATACTC	58	70	XM_00672170 3.1
TGFβ1	F: GGCAGTGGTTGAGCCGTGGA R: TGTTGGACAGCTGCTCCACCT	58	531	NM_000660.5

5.3.7 Osteocalcin immunocytochemical staining

For immunocytochemistry (ICC), cell sheets were fixed in 4% paraformaldehyde and stained with anti-osteocalcin rabbit polyclonal antibody (1:100) and 4',6-diamidino-2-phenylindole (DAPI) was used for cell nuclei staining. Stained sections were then viewed under the microscope (Olympus CX41, Olympus Singapore) at various magnifications (4x, 10x).

5.3.8 Fabrication of Mg-releasing PCL films

PCL was placed together with MgCl_2 in a cryomill (Retsch®, Germany). Using similar settings as described elsewhere²⁰⁰, fine powders of PCL/Mg were generated, and subsequently pressed between two stainless steel sheets to obtain PCL/Mg films with a thickness of 30 – 40 μm . 4 compositions (in terms of weight ratios) were fabricated: (PCL/Mg) 100/0, 95/5, 90/10, 80/20.

5.3.9 Pig studies

Three skeletally mature female micropigs of age 2 years old (mean body weight: 30kg) were housed for three months in this study. The pigs used in this study were supplied by PWG Genetics Pte Ltd (Good Lab Practice certified, Contract Research Organization). These pigs were maintained under specific pathogen free (SPF) conditions, and the study was conducted after approval from their in-house IACUC (IACUC No. PN13011). PCL and PCL/Mg films (80/20) were sterilized with 100% ethanol overnight, and washed with PBS thrice before implantation. First, the abdominal cavity was exposed through incision, and the fat pockets were created using blunt dissection. Thereafter, the films were inserted into these fat spaces, and tagged with a suture for identification purposes. Each animal received two films of PCL and 80/20 each (n=6 per group). The animals were maintained for 3 months before sacrificing. (n=6 per group)

5.4 Results

5.4.1 Validating the source of Mg

The sources of Mg were evaluated to determine their suitability for this study. From the results, both MgCl_2 and MgSO_4 resulted in the proliferation and differentiation of hfMSCs (Figure 5-2). All subsequent studies utilized MgCl_2 henceforth.

Figure 5-2 Validation of the sources of Mg, for the viability of MSCs. (A) Transmitted light microscopy on days 2 and 14. (B) von Kossa staining of differentiated MSCs after culture in 14 days in osteogenic induction media. All images were taken at 4x magnification.

5.4.2 Effect of various Mg levels on MSC proliferation

hfMSC proliferation in the Mg free and 8 mM groups were compared and normalized against the basal level (0.8 mM), in both proliferative and osteogenic media. In both conditions, the lack of Mg suppressed cell growth significantly (Figure 5-3; $p < 0.001$). On the other hand, 8 mM of Mg supported cell proliferation ($p < 0.001$). Live/dead (FDA/PI) imaging (Figure 5-3) corroborated well with the observed viability.

Figure 5-3 Live/Dead staining of MSCs and corresponding viability of MSCs in (A) maintenance media, and (B) osteogenic induction media. Images were taken at 4x magnification. Scale bar represents 600 μ m.

5.4.3 ALP activity and Ca deposition

ALP activity (normalized to total protein, using BSA) and Ca (normalized to total DNA) deposition were evaluated over two time-points on days 3 and 7, to determine the effects of Mg on MSCs. From the data presented (Figure 5-6), exposure of MSCs to 7 days of elevated Mg (8mM) resulted in significantly higher Ca deposition as well as an earlier peak in ALP activity on day 3. In contrast, the ALP activity of MSCs in normal conditions (0.8mM) was elevated only on day 7.

Figure 5-4 ALP activity (normalized to total protein) (left) and Ca deposition (right) of MSCs when exposed to various levels of Mg, in the presence of osteogenic inductive media.

5.4.4 Effect of Mg levels on osteocalcin protein expression

Expression of osteocalcin (OC) protein was determined using immunocytochemical staining. From the results (Figure 5-5), OC expression was clearly demonstrated in the group exposed to decreasing concentrations of Mg, while prolonged exposure to Mg resulted in suppressed expression of OC from the MSCs.

Figure 5-5 Osteocalcin protein expression of MSCs under various conditions of Mg treatment. Representative images were taken at 10x magnification; scale bar represents 200 μ m. H-L-0.8: initially high [Mg] 8mM decreased to low [Mg] 0.8m, 4 days each. H8: consistently high [Mg] 8mM, 8 days continually.

5.4.5 Effect of Mg levels on osteogenic differentiation

hfMSCs cultured under prolonged exposure to high levels of Mg (8mM) exhibited lower levels of ON, coll-1, and TGF- β expressions (Figure 5-6). Upon switching to Mg-free conditions after 4 days, hfMSCs demonstrated higher potential for osteogenic differentiation as compared to 0.8mM.

Figure 5-6 Expression of mRNA of (A) osteonectin (B) TGF-β (C) coll-1 under various conditions of Mg exposure. H-L-0.8: initially high [Mg] 8mM decreased to low [Mg] 0.8m, 4 days each. H-L-Mg-: initially high [Mg] 8mM decreased to no [Mg] 0mM, 4 days each. H8: consistently high [Mg] 8mM, 8 days continually.

5.4.6 PCL/Mg film characterization

PCL was cryomilled with MgCl_2 before being thermally pressed into PCL/Mg films.

5.4.6.1 Scanning electron microscopy (SEM)

SEM images of PCL/Mg films (Figure 5-7) below showed that the appearances of the films were similar. Upon closer observation at higher magnifications (1500x), particulate-like structures could be observed (yellow arrows), suggesting that some Mg may have been presented to the surface of the films.

Figure 5-7 SEM images of pristine PCL at 300x and PCL/Mg of each respective concentration at magnifications of 300x (insets) and 1500x.

5.4.6.2 Surface roughness – Atomic Force Microscopy (AFM)

The surface topographies of PCL/Mg films were determined using AFM (Figure 5-8). PCL films reported roughness values of 9.5nm while 80/20 films had roughness of 58nm. PCL films reported a maximum height difference of approximately 50nm while that of 80/20 was approximately 200nm.

Figure 5-8 Surface topographies of (A) PCL and (B) PCL/Mg (80/20) films. Considerable changes in roughness could be observed in the PCL/Mg films.

5.4.6.3 Thermal properties

Thermal properties of PCL/Mg films were determined using the first heating curve of the DSC thermogram, and were summarized in Table 5-2. The addition of Mg did not appear to alter the crystallinity of PCL. There were also no notable changes in terms of their melting points.

Table 5-2 Summary of PCL crystallinity with incorporation of PCL

PCL/Mg	Crystallinity, X_c (%)	Melting Temperature, T_m (°C)
100/0	50.4 ± 6.2	75.8 ± 2.7
90/10	51.0 ± 1.6	67.7 ± 1.9
85/15	51.9 ± 1.7	65.3 ± 2.9
80/20	63.9 ± 13.0	71.1 ± 1.0

5.4.6.4 Mechanical Properties

5.4.6.5 Tensile mechanical properties of PCL/Mg films

The mechanical properties of PCL/Mg films are summarized in Table 5-3. In general, the yield stresses, Young's moduli, and toughness decreased with increasing amounts of Mg.

Table 5-3 Summary of tensile mechanical properties of PCL/Mg films

PCL/Mg	Yield Stress (MPa)	Young's modulus (MPa)	Toughness (MJ/m ³)
100/0	11.6 ± 2.9	209.3 ± 72.0	1075 ± 480
90/10	7.2 ± 2.7*	157.6 ± 36.7	103 ± 78*
85/15	5.3 ± 0.9*	94.1 ± 27.3*	79 ± 42*
80/20	6.9 ± 2.2*	80.4 ± 22.5*	110 ± 74*

5.4.6.6 Magnesium release and associated morphological assessment

The Mg release characteristics of PCL/Mg films were evaluated in vitro (Figure 5-9A). Expectedly, more Mg was released with higher incorporation of Mg into PCL. In general, the release characteristic over the 4 hour period was linear. Correspondingly, the SEM images (Figure 5-9B) of PCL/Mg films taken after release showed that microporous pits were formed. There was also a

positive correlation between the size of the pits and the amount of incorporated Mg.

Figure 5-9 (A) Magnesium release profile at stipulated time points of 30 minutes, 1, 2 and 4 hours. (B) SEM images of various PCL/Mg films after release. Magnification: 300x; Scale bar: 50 µm.

5.4.7 Degradation

5.4.7.1 Morphological assessment

SEM of all PCL/Mg films after accelerated degradation was conducted and summarized in Figure 5-10. PCL films served as controls against which other PCL/Mg films were compared. Over the 120h degradation period, PCL films did not exhibit major changes in their morphologies, only adopting a slightly cobblestone appearance at 120h. On the other hand, the incorporation of Mg into PCL resulted in faster degradation, with pits forming as early as 12h into the degradation process (80/20).

Figure 5-10 Comparison of SEM images of PCL/Mg films at 0, 12, 24, 72 and 120 hours (300x). Scale bar represents 50 μm .

5.4.7.2 Mass loss analysis

The mass losses from PCL/Mg films were recorded and presented as a function of time in Figure 5-11. PCL films recorded the lowest mass loss over the 120h degradation period, while 90/10 and 80/20 films degraded the earliest by 72h. The degradation profiles of PCL and PCL/Mg films were distinctly different, with 85/15 and 80/20 films taking on a similar, almost linear degradation profile.

Figure 5-11 Percentage mass loss of PCL/Mg films over 120h.

5.4.7.3 Thermal property changes in PCL/Mg post-degradation

The thermal properties of PCL/Mg films were evaluated post-degradation, at stipulated time-points (Table 5-4). In general, the crystallinities decreased with increasing degradation, suggesting the disruption of the crystalline structure. 80/20 recorded the largest changes in crystallinity, decreasing by approximately 20% over the first 12h.

Table 5-4 Summary of crystallinities of PCL/Mg films post-degradation at various time-points.

PCL/Mg	0	12	24	72	120
100/0	50.4 ± 6.2	35.5 ± 2.4	41.1 ± 0.7	41.4 ± 0.9	41.1 ± 3.8
90/10	51.0 ± 1.6	41.4 ± 1.5	41.7 ± 5.7	NA	NA
85/15	51.9 ± 1.7	41.2 ± 13.2	42.0 ± 7.8	NA	NA
80/20	63.9 ± 13.0	44.0 ± 1.9	45.3 ± 4.6	NA	NA

5.4.8 Biocompatibility

The biocompatibility studies in relation to incorporation of Mg into PCL (PCL/Mg 80/20) films have been evaluated in a porcine model, where the films were inserted into fat pockets in the abdomen of pigs, for a period of 3 months (Figure 5-12). Comparatively, PCL/Mg films resulted in lower inflammation.

Figure 5-12 H&E stains of PCL and PCL/Mg (80/20) films implanted into fat tissue of pigs, for a period of 3 months. Inflammation surrounding the tissue with PCL could be observed (arrows).

5.5 Discussion

5.5.1 Biological effects of Mg – Transient exposure to elevated levels of Mg led to increased mineralization

The intriguing role of Mg in directing osteogenesis has been of recent interest, due to the phenomenological observations of enhanced osseointegration in coated implants.²⁴⁶ As such, many groups became interested to understand if Mg has a direct effect on bone formation. However, Mg plays an important role in many cellular processes, one of which includes being complexed to adenosine triphosphate (ATP), which is ternary complex of the catalytic

subunit of cAMP-dependent protein kinase²⁵⁸. Other possible effects of Mg include that of cell adhesion to substrates²⁵⁹. However, its purported role in osteogenesis remains profound knowledge, and this thesis has attempted to understand its importance by hypothesizing that Mg exhibits its influence on osteogenic activities through a temporal effect.

This thesis first sought to demonstrate that the sources of Mg used here resulted in negligible differences in terms of hfMSC proliferation and differentiation. This was shown in Figure 5-2, where both MgCl₂ and MgSO₄ supported cell proliferation over time. This result corroborated well with other studies^{252, 260} where both were used as media supplements for the study of osteogenesis. In this study, higher levels of Mg (8mM) resulted in higher hfMSC proliferation over time (Figure 5-3) in both culture and osteogenic medium, while the lack of Mg (Mg free) resulted in reduced cell proliferation. This was similarly reported in human osteoblast-like cells (MG-63, SaOS, and U2-OS)²⁶¹, clearly cementing the role of Mg in DNA and protein synthesis through melastatin-like transient receptor potential 6 and 7 (TRPM6 and 7)²⁶². More accurately, studies have shown that mTOR, a protein kinase in the PI3-K pathway, is regulated by MgATP.²⁶³ These studies, together with the results in this thesis, verified that Mg has a positive influence on MSC proliferation.

In the presence of soluble osteogenic factors such as dexamethasone and β -glycerophosphate, hfMSCs already have a strong predisposition towards the osteogenic lineage. When further supplemented with higher Mg (8mM), characteristic hallmarks of osteogenesis such as ALP activity and calcium deposition were further upregulated (Figure 5-6). Over 7 days, temporal expression of ALP in response to varying Mg concentrations was observed,

with ALP expression peaking on day 3 in 8mM of Mg as compared to basal levels (0.8mM), which possibly occurred either on day 7 or beyond. In tandem with ALP expression on day 3 in the 8mM Mg group, calcium deposition was significantly expressed on day 7. From the literature, Leem et al.²⁶⁴ also reported enhanced ALP activity within the first 72 hours (3 days) in the presence of 2.5mM of Mg. While the expression of ALP is understandably transient, it is an important, early indication of osteogenesis.²⁶⁵ ALP may traditionally be known as pyrophosphatase, an enzyme that is responsible for the production of inorganic phosphate²⁴², which is transported through the cell membrane via vesicles for interaction with available, unbound calcium ions (Ca) to form calcium phosphate (CaP) crystals.

Earlier in the introduction, various studies that investigated the effect of degradation products of magnesium alloys on BMSC and MSC differentiation were cited. According to a report by Li et al.²⁵³, MSCs proliferated well in the presence of 0.5 – 0.8mM of Mg, while at high levels of Mg (ca. 5 – 8 mM), they showed poorer proliferation. On the other hand, when MSCs were exposed to higher levels of Mg, their differentiation towards the osteogenic phenotype was increased (14 day culture), which is in agreement with the results here. However, the medium extracts used in their study were diluted with other alloying metals, possibly resulting in the delayed onset of ALP activity. In this study, supplementation of Mg to culture medium was a more direct way of understanding its effects on MSC differentiation, to which an earlier peak in ALP expression on day 3 was observed.

Beyond the expression of ALP and Ca, expression of middle-late osteogenic marker such as osteocalcin (OC) was also evaluated in this thesis. From the

results shown, expression of OC was not seen at the end of a 14 day culture period in osteogenic media (Figure 5-5, H8-bottom row). This observation contradicts with earlier results that suggested osteogenic activity (temporal expression of ALP, late deposition of Ca). In addition, von Kossa stains belonging to the H8 group did not show significant dark staining when compared to hfMSCs cultured in basal level (0.8mM Mg) conditions (data not shown). To understand this better, a hypothesis that the delivery of Mg should be done in a temporal manner that decreases over time was formulated. This effect will be much like that in the in vivo conditions, where a local high concentration of Mg will be diluted over time. To simulate this, hfMSCs were cultured in 8mM of Mg over 4 days, and in one group switched to 0.8mM for another 4 days (H-L-0.8) while a second group was maintained in Mg-free conditions for another 4 days (H-L-Mg-). From the results presented in Figure 5-6, it was shown that the osteogenic potential of hfMSCs increased significantly with decreasing concentrations of Mg, as observed by the upregulation of osteogenic genes such as ON and coll-1, while the converse was true. This is in agreement with a previous study by Leidi et al.²⁶⁶ and Yang et al..²⁶⁷ In the latter study by Yang et al.²⁶⁷, human bone marrow MSCs (bMSCs) maintained in culture extracts taken from Mg alloys (AZ91D, NZ30K) and Mg metals demonstrated upregulation of osteopontin (OPN) at day 6 at the transcription level but not at the protein level. ALP levels were also similar to control (no added Mg) throughout the study period. These results, taken together with the observations in this thesis, suggest that transient exposure to elevated levels of Mg may potentiate early differentiation of MSCs.

5.5.2 Mg-incorporated PCL films: Characterization of physical properties, degradation response, and biocompatibility

As demonstrated earlier, the transient, exogenous supply of Mg appeared to potentiate mineralization events in vitro. To harness the potential of Mg through the use of scaffolds and/or films, this thesis has elected to incorporate soluble Mg into PCL films as a proof-of-concept of this platform. As part of the evaluation of scaffolds and/or films meant for bone tissue engineering, the mechanical, degradation, and release characteristics of Mg in this particular case, are important parameters that need to be evaluated.

From the tensile testing results of PCL/Mg films, the incorporation of Mg into PCL appeared to reduce the yield stress, Young's modulus, and toughness significantly ($p < 0.05$). Among the films that have incorporated Mg, 90/10 appeared to mechanically more robust than the other groups. Thereafter, there did not appear to be significant differences between 80/20 and 85/15 PCL/Mg films. The incorporation of trace elements into scaffolds for directing osteogenic activity has previously been explored, with researchers focusing on improving the host response to magnesium metal-based scaffolds^{268, 269}, and minimizing the undesirable side effects of magnesium metal corrosion in vivo through the use of polymeric²⁷⁰ and/or ceramic coatings²⁷¹. Others have also worked on harnessing the potential of Mg more directly without experiencing the undesired corrosion through substitution of lattice sites in the calcium phosphate crystal lattice.^{272, 273} All of these strategies require specific techniques that increase the complexity of materials processing. Here, cryomilling was employed to homogeneously distribute Mg in PCL, while ensuring mechanical integrity and release properties. To further substantiate

the point that cryomilling resulted in homogeneously distributed PCL/Mg composites, Figure 5-13 demonstrates the extent of inhomogeneity that resulted in the detaching of Mg from PCL (arrow), a phenomenon explored earlier in Chapter 4.

Figure 5-13 Films produced via cryomilling and melt pressing, as opposed to direct melt pressing.

Due to the solubility stability constant of MgCl_2 , its dissolution into an aqueous environment is effective, as demonstrated by its burst release profile (Figure 5-9). Effectively, the concentration of Mg released into the microenvironment was similar to in vitro hfMSC evaluations (8mM). This suggested that this PCL/Mg films fabricated were able to provide fixed, pre-determined amounts of Mg by varying the amount of Mg incorporated initially (by weight). Along with the rapid dissolution of Mg, micron-sized pores were formed (Figure 5-9B). These pores allowed intimate interactions between the films and the surrounding microenvironment, resulting in significant mass losses over time. The rate of degradation was also clearly dependent on the amount of Mg (Figure 5-11).

The biocompatibility of these PCL/Mg composites was evaluated in a porcine model, where the films were placed alongside fat tissue. PCL was used as a

control. PCL/Mg films appeared to induce markedly less inflammation than PCL did (Figure 5-12). Importantly, this suggests that PCL/Mg may in the future be used as tissue engineering films and/or scaffolds without concerns relating to biocompatibility issues.

5.6 Summary

This chapter has demonstrated that trace metal ion Mg may be incorporated into PCL as part of the strategy to develop bioactive composites for stimulating osteogenic responses. Through a myriad of evaluation techniques ranging from in vitro biological evaluation to preliminary biocompatibility testing, the potential of PCL/Mg in directing osteogenic differentiation of hfMSCs was demonstrated. In addition, PCL/Mg films were characterized and shown to be able to release Mg into the surrounding microenvironment. Taken together, these results encourage further development of PCL/Mg composites for bone tissue engineering applications, with a focus on tuning the mechanical properties to enhance mechanical integrity.

6. In vivo brain tissue compatibility of PCL/TCP composites designed for the delivery of therapeutics in cranial repair^{††}

6.1 Introduction

6.1.1 Brain tissue trauma and associated pathoanatomical consequences

Following traumatic brain injury (TBI), complex biochemical and molecular cascades may lead to neurological complications. The blood brain barrier (BBB) helps to maintain neurological function. Shlosberg et al. cited that the high incidence of BBB breakdown is commonly associated with TBI.²⁷⁴ The consequences following BBB dysfunction are severe, with BBB breakdown leading to transcriptional changes in the neurovascular network that cause neuronal dysfunction and degeneration.²⁷⁴ In addition, severe pathoanatomical (hematoma, subarachnoid hemorrhage, diffuse axonal injury²⁷⁵) and mechanical consequences (cerebral edema and ischemia^{276, 277}) may result. Primary BBB damage may be a result of traumatic injury²⁷⁸, which may be inflicted via closed head injuries or penetrating brain injuries (PBI). Despite the severity of PBI, their relative complexity²⁷⁹, compounded with the lack of understanding has led to non-ideal clinical management (typically defaulted to that closed head injuries).²⁸⁰ It was suggested that the paracrine signaling effects among the astrocytes, glial and cerebrovascular endothelium play a role in maintaining the function and phenotype of the BBB.²⁸¹⁻²⁸³

^{††} Published in: J Lim et al., TE(A). 2014; DKS Choy, VDW Nga, J Lim et al., TE(A). 2013.

Cerebral inflammation that arises after injury is also a major concern in the regulation of the BBB. On this note, Schwartz et al. has made significant contributions to the field's understanding of the brain-immunity relationship, stating that the choroid plexus (CP) is an on-alert gate for the recruitment of leukocytes capable of resolving inflammation, going further to suggest that the CP might also be a target for therapeutics.^{284, 285} More importantly, they have also suggested that circulating immune cells are critical to the normal functioning of the central and peripheral nervous system.²⁸⁶

6.1.2 Treatment modalities and their limitations

Direct injections of pharmaceutical agents such as cells^{287, 288} and growth factors²⁸⁹⁻²⁹¹ may diffuse from the site of insult to surrounding tissue, greatly affecting the effectiveness of this technique²⁹². Tissue engineering (TE) may be able to provide an alternative to enhancing current treatment methods, by using a tissue-engineered cranial scaffold to contain pharmaceutical agents, cells and growth factors for efficient delivery.^{3, 293} In order to prevent and/or restore neurological function after PBI, targeted delivery of biomolecules may be necessary. As such, one proposed strategy for targeted delivery could be attained either through the development of cytoprotective genes²⁹⁴ or proteins²⁹⁵. However, these biomolecules require suitable carriers that prolong their bioavailability for effective therapeutic effects.^{296, 297}

6.1.3 Polymeric scaffolds as vehicles for delivery of therapeutic agents

A suitable delivery vehicle for cranial TE should allow for tissue ingrowth from host, immunological compatibility, biocompatibility, suitable degradation characteristics and by-products in order to avoid undesirable inflammatory responses that further worsen injury.²⁹² The combination of suitable materials

and biological agents may provide an alternative route to successful regeneration of the injured parenchyma.

In this light, a variety of materials have been investigated as delivery vehicles for TBI. Tate et al. developed synthetic methylcellulose (MC)-based gel constructs with pore sizes in the range of 30-50 μm , as a biocompatible injectable scaffold.²⁹⁸ Polymer stability in *in vitro* studies over a 2 week period encouraged their use for TE applications, while MC was recently shown to promote peripheral nerve regeneration.²⁹⁹ Tian et al. prepared a freeze-dried hyaluronic acid and polylysine hydrogel, and investigated its biocompatibility in adult rats.³⁰⁰ Hyaluronic was chosen due to its presence in natural extracellular matrix (ECM) of brain, and due to its critical role during brain formation. Infiltration of glial fibrillary acidic protein (GFAP)-positive astrocytes and contiguity between hydrogel and host tissue indicated its biocompatibility. Yasuda et al. used a fibrin scaffold for the transplantation of bone marrow stromal cells in rat cortical injury³⁰¹ due to its biocompatibility, biodegradability, and ability to adhere to brain tissue. Recently, Wong et al. has reported positive effects of using polycaprolactone (PCL) sponges in regeneration following TBI.²⁹² As aforementioned, the desirable properties of scaffolds have, in one way or the other, been considered in the chosen materials.

6.1.4 Bioactive PCL/TCP composites

Bioactive PCL/TCP composites have been continually explored in this thesis for directing osteogenic events *in vitro* and *in vivo*. In fact, PCL/TCP composites have been widely studied for bone regeneration, in particular by Teoh's group.³⁰²⁻³⁰⁴ These composites have also been demonstrated to be useful in the regeneration of the cranium^{305, 306}, which is important for

preventing neurological deterioration and significant depression after PBI.^{307,}

308

Over the years, Teoh's group has developed PCL/TCP as viable biomaterials for tissue engineering applications including bone^{42, 99, 309}, and for drug delivery^{98, 213}. By using a solvent-free approach of fused deposition modeling, PCL/TCP scaffolds have been fabricated with a variety of porosities ranging from 70-90%.^{13, 310} Morphological observations under scanning electron microscope (SEM) revealed interconnected microarchitecture that has been shown to be a conducive environment for the infiltration, retention, proliferation and differentiation of cells.^{11, 311} In treating a large cranial defect, scaffold support with geometrical integrity may hold advantages over hydrogels by providing sturdier architecture for efficient cell, growth factor and drug delivery. The degradation profile of PCL and PCL/TCP scaffolds have already been well-characterized by Teoh's group both *in vitro* and *in vivo*.¹⁰¹ Of particular interest would be the ability of PCL/TCP scaffolds to maintain pore interconnectivity despite having a higher degradation rate as compared to PCL, which is conducive for the promotion of vascular infiltration, subsequently initiating a cascade of molecular events to promote tissue ingrowth.

6.2 Materials and Methods

6.2.1 Surgical procedure

The animal studies reported in this work have been approved by the animal ethics committee Institutional Animal Care and Use Committee (IACUC, protocol: IACUC 096/11) at the National University of Singapore. Female Sprague-Dawley rats weighing approximately 250g were anesthetized with

isofluorane, and their skulls were surgically exposed. The bregma was located, and three positions were marked: 3 mm anterior and 3.5 mm left and right. Holes were drilled at the three locations with a 3 mm outer-diameter (OD) trephine (Figure 6-1). Bone chips were removed, and 3 mm deep holes were drilled into the cerebral cortex using the same trephine. The drill was held in position and maintained perpendicular to the surface of the skull.

PCL and PCL-TCP scaffolds (Osteopore International Pte Ltd, Singapore) were fabricated using a solvent-free approach of fused deposition modeling^{11, 13}, to achieve a lay-down pattern of 0°/60°/120° with a porosity of 70%, as reported earlier²⁸. In order to fit the defects created in the rat cranium, the scaffolds were designed in a plug-like manner, as depicted in (Figure 6-1, inset). PCL plugs with a diameter of 3 mm were fitted snugly into the cavity left of the bregma, while PCL-TCP scaffolds of similar geometry were fitted snugly into the cavity right of the bregma. Control (3 mm anterior) was left without scaffold implantation.

Two time-points were investigated: 1 week and 1 month. At each time-point, 5 rats were sacrificed, their brains harvested and cryoprotected in 30% sucrose, embedded in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Electron Microscopy Sciences, Hatfield, PA) and then serially sectioned in the coronal plane at 14 mm thickness intervals for further analysis.

Figure 6-1 (A) Visualization of the morphology of the implanted PCL-based scaffolds taken by scanning electron microscopy (SEM), at 100x magnification. TCP particles (white arrows) were observed distributed within each PCL filament. (B) Representative image of a harvested Sprague-Dawley rat brain, with the bregma, and locations of the scaffolds and controls clearly shown. The scaffolds used were of 3 mm diameter and 3 mm height (inset)

6.2.2 Histopathology and immunohistochemistry

Following perfusion, the brains were harvested and post-fixed in 10% buffered formalin. The brains were then dehydrated in an ascending series of alcohol, cleared with xylene, and then embedded in paraffin wax. Paraffin sections of 4µm thickness were then cut and microwaved in citrate buffer for antigen retrieval and blocked with peroxidase blocking reagent (S2023, DAKO UK Ltd, UK). For general histopathology, sections were stained in hematoxylin and eosin. For immunohistochemistry, sections were incubated with mouse monoclonal anti-neuronal nuclei (NeuN) (MAB377, Chemicon International, Inc, MA USA) diluted 1:600 in PBS; mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (MAB360, Chemicon International, Inc, MA USA) diluted 1:1800 in PBS; rabbit polyclonal anti-ionized calcium binding adaptor molecule (IBA-1) (019-19741, Wako Pure Chemical Industries, Japan) diluted 1:500 in PBS; mouse monoclonal anti-nestin (MAB353, Chemicon International, Inc, MA USA) diluted 1:500 in PBS; for detection of NeuN, GFAP, IBA-1 and nestin respectively. Subsequent antibody detection was

carried out using either anti-mouse (rat absorbed), or anti-rabbit IgG (ImPRESS Ig reagent kit, Vector Laboratories). All samples were then visualised using 3,3'-diaminobenzidine (DAB) and digitalized using Leica SCN400 Automatic Slide scanner and corresponding Image Viewer Software (Leica Microsystems CMS GmbH, Germany). Japan). All images were taken in one sitting, using the exact same exposure and processing parameters. Baseline readings were taken at regions far from the defect site (>5mm), with the assumption that at those regions, there is minimal elevation of GFAP or IBA-1 positive cells. For immunohistochemistry, sections were incubated with rat blood-brain-barrier (SMI-71) monoclonal antibody (SMI-71R, Covance, CA, USA) diluted 1:500 in PBS; polyclonal rabbit anti-IL-1 β (ab9722, Abcam) diluted 1:500; mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (MAB360, Chemicon International, Inc, MA USA) diluted 1:1800 in PBS; rabbit monoclonal anti-aquaporin (AQP)-4 affinity (5582-1, Epitomics, Inc, CA, USA) diluted 1:200 in PBS for the detection of VEGF, SMI-71, IL-1 β , AQP-4 and GFAP respectively. Subsequent antibody detection was carried out using anti-mouse (rat absorbed), anti-goat or anti-rabbit IgG (ImPRESS Ig reagent kit, Vector Laboratories). For fluorescence staining, FITC-conjugated and Cy3-conjugated secondary antibodies were used instead. For brightfield microscopy, all samples were visualized using 3,3'-diaminobenzidine (DAB). All samples were examined using a brightfield and fluorescence slide scanner (SCN400, Leica, Germany).

6.2.3 Determination of pixel area

Pixel areas for GFAP and IBA-1 were calculated using ImageJ (version 1.46r, National Institute of Health, USA, from <http://imagej.nih.gov/ij>). Thresholding

of the colored images was done, to highlight the regions of interest in black. Following that, images were converted into 8-bit images and then into binary images. Noise was removed by removing outliers, and the binary image was compared to the original image carefully to ensure a good representation of the original. Cell counting and area measurement was then conducted on the processed images. At least three regions were chosen for analysis for each image. A total of 4 images per anatomical location were used in the calculation of the pixel area. In this study, the proximal region is defined as the region within 0.9mm of the defect, while the distal region is defined as the region more than 1mm away from the defect.

Pixel areas and intensity for SMI-71 and AQP-4 respectively, were calculated using the same method described above. At least three regions were chosen for analysis for each image. A total of 3 images per anatomical location were used in the calculation of the pixel area (thus n=9). In this study, the defect (R1) and periphery (R2) region are as defined in Figure 6-2.

Figure 6-2 Illustration of the selected regions used: R1 indicating the defect region; R2 refers to the periphery. (scale bar represents 1 mm).

6.2.4 Statistical analysis

To determine statistical significance, two-tailed student's *t*-test was conducted, and p values less than 0.05 were considered statistically significant.

6.3 Results

6.3.1 Gross observations

Post-operatively, the rats were monitored for any significant weight loss, signs of pain and severe brain injury. Subcutaneous Caprofen and oral Neomycin were given for 3 and 7 days respectively based on the rats' body weight. There were no deaths post-operative, and the rats recovered spontaneous locomotion within 15 minutes post-operatively. The rats were inspected daily for the 1st 7 days and then on a weekly basis. The area of the TBI sites were not measured due to the geometrical and physical properties of PCL and PCL-TCP scaffolds which prevented accurate measurement.

6.3.2 Microglial immunoreactivity

Figure 6-3 Immunohistological staining for (A) microglial (IBA-1), (B) astrocytes (GFAP), (C) neurons (NeuN), and (D) neurogenesis (nestin). (A) and (B) indicate that the levels of inflammation across all groups decreased over time. Observable difference in the intensity of staining for IBA-1 (A) in the PCL-TCP scaffolds as compared to PCL was noted. Neuronal arrangement was disordered across all groups (C). The neurogenic potential was not retained over time, with a down-regulation of nestin at 1 month (D). Images were taken at 10x magnification. Scale bar represents 100 μ m. Reproduced with permission from Choy et al.

IBA-1-positive activated microglia were observed from in all groups at 1 week and declined by 1 month (Figure 6-3A), reflecting a decrease in microglial activation. Notably, there was significantly less activated microglia (Figure 6-3A, $p<0.05$) in the PCL/TCP group at week 1. By 1 month, all groups had approximately the same number of activated microglia. The intensity of IBA-1-positive microglia also decreased with increase in time, reflecting a decline in the level of inflammation.

6.3.3 GFAP immunoreactivity

Figure 6-4 Cellular count and area were measured at regions proximal to the defect site (within 0.9 mm of defect). (A) IBA-1 activated microglial over time. There were significantly fewer inflammatory cells for the PCL-TCP group at 1 week ($p<0.05$). Inflammation decreased to similar levels across all groups by 1 month. (B) Astrocyte activation was found to be similar across all groups at both 1 week and 1 month. (C) Astrocyte area was measured using ImageJ software following image processing. There were no significant differences among all groups at both 1 week and 1 month. The white bar represents astrocyte area measured at distal regions from the defect site. (D) Nestin stain intensity quantified using ImageJ analysis. A higher intensity, as represented by lower brightness, was reported for PCL/TCP scaffolds as opposed to PCL ($p<0.05$) on the first week. Reproduced with permission from Choy et al.

A significant elevation in GFAP immunoreactivity was observed (Figure 6-4B) at week 1 (Figure 6-4B, $p < 0.05$). GFAP immunoreactivity was marked by a distribution of GFAP-positive astrocytes with hypertrophic soma and processes in the hippocampal area. In particular, measurement of astrocyte area proximal to the TBI site confirmed hypertrophy, with a 75% increase in astrocyte area in the control group, and approximately 55% increase in astrocyte area in both PCL and PCL/TCP groups (Figure 6-4C, $p > 0.05$). By 1 month, GFAP immunoreactivity was greatly reduced, with astrocyte area proximal to the TBI site measuring at the most 12.1% larger (PCL-TCP, $p > 0.05$) larger than astrocytes distal to the TBI site. GFAP-positive cells were observed in all groups at week 1, and declined by 1 month. Post-hypertrophy astrocyte area measured from GFAP images revealed a significant decrease ($p < 0.05$) by 1 month among all groups, suggesting that astrocyte activation has declined (Figure 6-4C). No significant differences were found among all groups at 1 month.

6.3.4 Nestin immunoreactivity

In all operated rat brains, nestin-positive cells with morphological characteristics similar to that of reactive astrocytes (hypertrophic soma and processes) were observed in the hippocampal area, proximal to the TBI site (Figure 6-4D). By using ImageJ to calculate the level of brightness of the nestin stains belonging to the respective groups (where a lower level of brightness indicated a higher intensity of nestin staining), it was found that at week 1, PCL/TCP induced a significantly higher intensity of nestin stain (Figure 6-4D, $p < 0.05$). Nestin immunoreactivity, a populated marker of neural

progenitor cells, was intense at week 1 and had reduced in intensity by 1 month across all groups (Figure 6-4D).

6.3.5 Neuronal damage— NeuN and H&E staining

Figure 6-5 H&E staining images, taken at 20x magnification. In all groups, eosinophilic neurons could be observed (darkly stained), indicating irreversible neuronal damage. A representative overview of the histological section has been shown in the inset. Scale bar represents 50 μm.

Table 6-1 Neuronal size differences among the various groups. Significant decreases in neuronal nuclei size were recorded in all groups between proximal and distal regions after 1 week ($p < 0.05$, *). Proximal to the defect site, PCL-TCP implants recorded a significantly smaller neuronal nuclei size as compared to PCL ($p < 0.05$, ^), and which was comparable to that of the control. The results are an average of 800 measurements over 3 images per implant group.

Implant	Proximal (<0.9mm)	Distal (>0.9mm)	Decrease (%, proximal vs. distal)
PCL	114 ± 47	156 ± 75	26.9,*
PCL-TCP	106 ± 45, [^]	156 ± 75	32.2,*
Control	110 ± 47	165 ± 76	33.3,*

NeuN-positive neuronal nuclei could be observed throughout the hippocampal region. Proximal to TBI site (within 0.9 mm), neurons were shrunken and darkly stained (Figure 6-4C). Similarly, H&E stains (Figure 6-5) revealed a portion of neurons that were eosinophilic with pyknotic nuclei. Through observation, neuronal cells did not appear to infiltrate the area of insult regardless of control, PCL, or PCL/TCP groups.

By conducting a measurement of cell nuclei size, it was found that at week 1 in the PCL-TCP scaffolds, the neurons proximal (within 0.9mm) were 32.2% smaller (Table 6-1, $p < 0.05$) than those distal to the defect site (>1mm). In the PCL group, neuronal size was approximately 26.9% smaller (Table 6-1, $p < 0.05$). Between PCL/TCP and PCL scaffolds, the former has the smaller neuronal size (Table 6-1, $p < 0.05$). In the control group, neurons were found to be 33.3% smaller than in non-disturbed neurons (Table 6-1, $p < 0.05$).

6.3.6 Expression of Endothelial Proteins (SMI-71)

Figure 6-6 (A) *SMI-71* immunohistochemistry and (B) ImageJ quantification of total capillary number and average capillary area. *SMI-71* were clearly expressed in the peripheral region of all groups. In the defect region (dark circled), morphological observations that the capillary count and area were superior in the PCL/TCP group as compared to the PCL group was confirmed by ImageJ analysis, with the results presented in (B). Notable superiority in total capillary count and average capillary area in PCL/TCP could be observed. Scale bar represents 500 μ m.

The expression of SMI-71 could be clearly observed in the peripheries of the sham, PCL, and PCL/TCP groups (Figure 6-6A, 1 week and 1 month), with most taking on a circular cross-sectional morphology. At the defect region however, PCL and PCL/TCP groups exhibited lower expression of SMI-71. At endpoint (Figure 6-6A, 1 month), general recovery in the expression of endothelial barrier antigens could be observed at the defect region across all groups.

6.3.7 Capillary Area

The number of capillaries and their individual areas were determined, and by comparing against the peripheries of each individual group (i.e. defect vs. periphery), the average capillary area in the PCL/TCP scaffold group was 30% smaller, whereas the PCL scaffold group was 60% smaller (Figure 6-6B).

Between PCL and PCL/TCP scaffold groups at the defect site, capillary area in the PCL/TCP group was 23% larger (Figure 6-6B, $p>0.05$). Interestingly, the number of capillaries and their area in the PCL/TCP group remained at approximately the same levels between 1 week and 1 month, while an increase was observed in the PCL groups.

6.3.8 AQP-4

Figure 6-7 AQP-4 immunohistochemistry. Expression of AQP-4 in PCL/TCP scaffold group appeared to be lower than that of PCL and sham, and was confirmed by ImageJ analysis for stain intensity. Scale bar represents 1 mm.

AQP-4 expression was observed across all groups (Figure 6-7), as evidenced by the appearance of AQP-positive astrocytes. Compared to sham (no scaffolds), the intensity of AQP-positive astrocytes in the PCL/TCP scaffolds was upregulated to a lower extent (Figure 6-7, $p<0.05$). Conversely, PCL scaffolds attenuated BBB alterations less when compared to PCL/TCP (Figure 6-7, $p>0.05$).

6.4 Discussion

In this work, 70% porous PCL and PCL/TCP scaffolds were implanted into rat brains to primarily investigate brain tissue inflammatory response to PCL/TCP scaffolds. While PCL has been shown to support regeneration in TBI²⁹², no prior work has been conducted on the interaction of PCL/TCP porous scaffolds with brain tissue. Nonetheless, PCL/TCP scaffolds have been investigated for bone tissue engineering applications with reasonable success.^{28, 99, 212}

Inflammatory response at the edges of the scaffold could still be determined with IBA-1 expressing microglial, and GFAP-expressing hypertrophic astrocytes. IBA-1 results indicated that PCL/TCP scaffolds activated fewer microglial cells initially (Figure 6-4B, $p < 0.05$), suggesting that PCL/TCP elicits similar responses from the immune system as that of a defect without an implant. This suggests that PCL/TCP, as compared to PCL, attenuates the immune response. A possible reason for the attenuated response could lie in the degradation characteristics of PCL/TCP scaffolds. According to previous published results on the degradation of PCL³¹² and PCL/TCP scaffolds¹⁰¹, the addition of TCP to the PCL matrix was reported to result in an increase in pH over time¹⁰¹ due to the presence of TCP which are alkaline in nature. The pH reached a level of approximately 8 by 4 weeks in vitro. Importantly, Lardner et al.³¹³ reviewed the effect of pH on immune function, and has suggested that a slightly alkaline pH attenuates immune response, while clinical acidosis is accompanied by impaired immunoefficiency. In view of the above, a locally elevated pH might have led to the lower activation of microglial cells in PCL/TCP scaffolds.

An earlier study by Wong et al.²⁹² reported that OX-42-positive activated microglia decreased over the duration of 4 weeks in control and PCL groups, which was similar to the results here. However, in terms of astrocyte activation, PCL sponges maintained astrocyte activation over time, while the PCL scaffolds used in this work reported a significant decline in GFAP-positive activated astrocytes, as did PCL-TCP scaffolds (Figure 6-4B). This result, taken together with the lowered activation of IBA-1 microglial, suggested that inflammation has decreased in PCL and PCL/TCP scaffolds. Additionally, a drop in microglial levels over time without a corresponding decrease in astrocyte activation suggested microglial activation might have mediated astrocytic activation, which subsequently affected the ability of astrocytes to form glial scars. This phenomenon was labeled as a time-dependent effect of activated microglia-related glial scar formation. In this chapter, a decrease in activated microglia over time (Figure 6-4A) resulted in a corresponding decrease in activated astrocyte count and size (Figure 6-4B, 4C) over time. Therefore, microglial activation did not appear to have a time-dependent effect on glial scar formation here. It was suggested by Streit³¹⁴ that microglial performs neuroprotective roles, but the author believes that their roles have manifested in a different fashion. Initial astrocyte activation in PCL and PCL/TCP scaffolds were comparable to control, while at the same time, microglial activation was significantly lower in the PCL-TCP scaffolds. Thus, it appears that the neuroprotective function that microglial has on astrocyte activation is more significant when a critical number of microglial cells are present.

It is known that following any injury or disease to the CNS, a typical reaction known as astrogliosis occurs. This reaction is characterized by hypertrophy and hyperplasia, in synchronous with the expression of the GFAP gene.³¹⁵ Astrocyte activation following TBI may be beneficial to damaged neurons by regulating neurotransmitter levels, repair of extracellular matrix, control of blood-CNS interface and transport processes, and trophic support to damaged cells. However, gliosis has also been suggested to interfere with residual neuronal circuits, negatively impacting the repair process, or preventing remyelination. Despite this, reactive astrocytes and microglia regulate the migration and function of each other via the release of cytokines like interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- α), which subsequently up-regulates expression of IL-6 and transforming growth factor-beta (TGF- β), of which TGF- β has been shown to limit inflammation.^{315, 316}

The levels of astrocytes and microglia found in the parenchyma can serve as a gauge to the level of inflammation. In general, acute inflammation sets in within the first week, following which chronic inflammation occurs.^{317, 318} As a result, differences in tissue response would have begun in the first week, and become apparent by 1 month. The degradation of PCL/TCP scaffolds, both in vitro and in vivo, has been reported by Teoh's group.¹⁰¹ Of interest to current work, the in vivo degradation of PCL/TCP scaffolds implanted in rabbit abdomens was reported to result in a significant decrease in molecular number (79.6%) and molecular weight (88.7%) over a period of 24 weeks. Despite the long degradation time, the mechanical properties of the PCL/TCP scaffolds could sufficiently match that of the cancellous bone, suggesting good scaffold mechanical integrity. Previous results investigating the

degradation of PCL scaffolds suggested that they have a long degradation life of 2-4 years, depending on the initial molecular weight.^{319, 320} Based on the information above, PCL/TCP has a higher degradation rate than PCL, which could potentially lead to the formation of low molecular weight by-products (8000g/mol).¹⁰¹ This could potentially have resulted in higher macrophage activation, leading to phagocytosis of the degradation by-products. This phenomenon was, however, not observed. In fact, the levels of inflammation of PCL/TCP scaffolds were significantly lower (IBA-1-positive) initially as compared to PCL and control (Figure 6-4A, $p < 0.05$). As mentioned earlier, the locally-elevated pH could have mediated the inflammation response. Considering that acute inflammation has been significantly lowered, it reflects the advantage that PCL/TCP has over PCL scaffolds in terms of brain tissue inflammatory response.

Presently, scaffolds in tissue engineering and regenerative medicine have bi-functionality: as structural supports as well as biomolecule delivery vehicles. This chapter evaluated PCL-based scaffolds that may function as biomolecule delivery vehicles²⁹² and for the regeneration of the cranium.³⁰⁶ PCL is a biomaterial that has traditionally been used as a drug delivery device³²¹, and more recently as a scaffold for promoting bone regeneration³²². Previously, Teoh's group has also demonstrated that PCL and PCL-based scaffolds were able to deliver recombinant human bone morphogenetic protein-2, as well as provide sufficient structural support to promote bone healing.⁹⁸ Given the bi-functionality of PCL-based scaffolds, they may have potential for use in PBI, both as biomolecule delivery vehicles as well as for the regeneration of the cranium. As a preliminary study to evaluate this, PCL-based scaffolds were

evaluated for their compatibility with brain tissue, by investigating their effects on the BBB, and cerebral inflammation, which plays a significant role in disrupting / maintaining the BBB.

Cerebral ischaemia has severe clinical complications that will affect patient mortality rates.³²³ Therefore, it is important to maintain or promote regeneration of the vascular supply to injured brain tissue after PBI. To ascertain this, this thesis chose to evaluate the expression of EBAs, which are proteins located within the plasma membrane of microvascular endothelium, and are selectively expressed in the normal nervous system.³²⁴ Here, PCL and PCL/TCP scaffolds were comparable in the expression of EBAs as compared to the sham group (no scaffolds), suggesting that they do not cause further changes to vascular supply in and around the defect.

The use of scaffolds for the treatment of TBI is not unprecedented, with Mahmood et al.³²⁵ showing that collagen scaffolds may be used vehicles for transplanting human mesenchymal stem cells (hMSCs). In addition, Wong et al.²⁹² illustrated the use of PCL and PLGA sponges as potential biomolecule delivery vehicles. Common to both these reports would be the similarities in terms of stiffness to brain tissue, which is reportedly between $(1.0 - 3.5 \times 10^3 \text{ dyn/cm}^2)$.³²⁶ While the stiffness of both PCL and PCL/TCP scaffolds are considerably higher than that of brain tissue¹⁰⁰, tissue ingrowth into the bioresorbable scaffold is still possible, owing to its highly-porous structure.³²⁷ In fact, a highly porous structure is a necessary requirement to facilitate cellular and vascular infiltration.³²⁸ The results here suggested that both PCL and PCL/TCP did not obtund vascularization, which is a significant finding.

Cerebral vascularization and inflammation are key events that determine the extent of neurological dysfunction. The attenuated expression of IL-1 β was an expected outcome, as it is known from previous studies that its expression does not persist more than a few days.³²⁹ However, due to foreign body reaction (FBR), it was of interest to ensure that inflammation was not prolonged further. Here, the results demonstrated that prolonged inflammation did not occur in both scaffold groups.

AQP-4 is a water channel protein that allows for the transmembrane transport of water.³³⁰ It is strongly expressed in the brain, and has been postulated to be the main membrane protein regulating water flux.³³¹ The need for AQP-4 stems from its involvement in brain edema³³²⁻³³⁵, the swelling of tissue due to increase water and sodium loading³³⁰. Previously, AQP-4 deficient mice have been reported to have poorer neurological deficit scores and poorer survival rate, a direct consequence of increase tissue water content and astrocytic swelling.³³³ Therefore, it is important that the transcellular transport of water is regulated tightly by the aquaporins, specifically AQP-4 in the brain. This has clear, potential clinical implications such as reduction in cerebral edema, which may improve patient prognosis, as suggested by Ding et al. recently²⁷⁷.

6.5 Summary

In this chapter, the effect of PCL and PCL/TCP scaffolds on the BBB and cerebral inflammatory response to PBI was studied. EBA expressions were recovered by 1 month across all groups, suggesting that PCL-based scaffolds did not cause further changes to the vascular supply in and around the defect region. In terms of cerebral inflammation, prolonged inflammatory responses were not present despite the presence of PCL-based scaffolds for 1 month,

demonstrating that these scaffolds do not induce undesired and prolonged inflammation in the long run. Finally, AQP-4 expression was downregulated initially in the PCL/TCP group, suggesting that interleukin induced AQP-4 expression was reduced, potentially leading to better clinical prognosis. Taken together, the results of this study provide evidence that PCL-based scaffolds may be used in direct contact with cerebral tissue safely.

7. Conclusions and future work

7.1 Conclusions

This thesis began by reviewing the literature for processing techniques that are solvent-free, and have the ability to improve the homogeneity of composites. Cryomilling presented itself as a suitable candidate, as processing was conducted in an entirely solvent-free approach. In addition, through Chapters 4 and 5, this thesis has shown that cryomilling is able to generate homogeneous composites that retain this characteristic in downstream post-processing techniques such as melt pressing, both using inorganic TCP and trace metal ion magnesium. To ensure that cryomilling is not restricted to semi-crystalline polymers such as PCL, PLGA (50:50) was also evaluated through cryomilling, in Chapter 3. By taking these three chapters in entirety, Specific Aim 1 was partially addressed. This thesis then demonstrated in Chapter 4 through in vitro cellular differentiation assays, that cryomilled PCL/TCP composites were able to direct osteogenic events in a robust manner. An understanding on the physical properties of PCL/TCP composites, such as mechanical strength and degradation properties was also developed therein. In addition, biocompatibility and regenerative capabilities of PCL-based composites were clearly demonstrated through a 3-month study in a porcine model. These results, taken together with the previous results, fully address both Specific Aims 1 and 2, and in part Specific Aim 3. In vivo studies presented in Chapter 6 completes the biocompatibility studies to fully address Specific Aim 3, demonstrating the biocompatibility of PCL/TCP composites fabricated through a solvent-free approach.

7.2 Future work

7.2.1 Inorganic nanocomposites for tissue engineering and regenerative medicine

Tissue engineering combines principles from biology, medicine, material, and engineering science to create functional replacements.³³⁶ Driven by a shortage of tissue/organs for transplantation along with an aging population, this field has been under intense development in the past decades and since developed three major strategies for engineering tissues, namely: (1) creating implantable pieces of the organism with the use of living cells seeded on a natural or synthetic extracellular substrate; (2) delivering tissue-inducing substances, and (3) injecting therapeutic cells placed on or within matrices.

Although these strategies sound technically different, the goal is the same: to fabricate new and functional living tissues by incorporating living cells within a matrix or scaffold. Scaffolds can be natural, man-made, or a composite of both. Living cells are able to associate with the matrix in culturing before implantation or migrate into the implant after implantation. Successful tissue engineering would happen if the scaffold can guide the organization, growth, and/or differentiation of cells to form a functional tissue or organ.

Because of the key role that scaffolds play here, the development of tissue engineering strategies has been closely related with the advancement of material science.³³⁷ For example, the first generation of scaffolds for bone tissue engineering was bioinert and adopted from common materials (e.g. metals). They have similar mechanical properties to the tissue of interest but do not promote tissue regeneration. Later, the focus on resorbable biomaterials led to the development of resorbable polymers such as

polycaprolactone (PCL), poly(glycolic acid), poly(lactic acid), and poly(lactic-co-glycolic acid) (PLGA). These materials would have significant advantages over their non-resorbable counterparts, as the body is able to process the degradation byproducts through the citric acid cycle, a natural metabolic pathway.¹⁸⁴ Together with the 3D printing technology, a new generation of scaffolds that could induce the migration of host cells into scaffold and the regeneration of natural tissues is ushered in.

Now, after more than 20 years of intensive research, nanomaterials have evoked a great amount of attention from many technological and industrial sectors including biomedical field. The applications of nanomaterials in biomedical field include but are not limited to diagnosis, *in vivo* imaging, device coating, implantable materials, drug delivery, surgery, and tissue engineering. Liposomes, micelles, inorganic and organic nanoparticles (NPs) are extensively used, each of which has its own unique set of properties presenting only in nanoscale, such as the tunable fluorescent emission of semiconductor nanocrystals, switchable magnetic properties of magnetic NPs, and laser-induced hyperthermia mediated by gold or carbon NPs. Given the important role of materials in tissue engineering discussed above and the desired interaction between nanostructures and cells, nanomaterials have inevitably been introduced to this field for seeking better performances in tissue engineering than traditional materials.

Nanomaterials can be broadly grouped into organic and inorganic, depending on their chemical make-up. Organic nanomaterials include phospholipids, dendrimers, or polymers, while inorganic nanomaterials are comprised of metals, ceramics, semiconductors, or oxides. Compared to organic

nanomaterials, the mechanical properties of inorganic materials such as strength and modulus of elasticity tend to be significantly different due to the inherent strength of the chemical bonding. For example, collagen fibrils, a natural extracellular matrix (ECM) structure with a diameter of approximately 200 nm, has a reported elastic strength of 0.5 GPa,³³⁸ while inorganic nanomaterials like tricalcium phosphate has a Young's modulus of between 2-5 GPa, approximately 10 times that of ECM.³³⁹ The ionic bonding and lattice structure of ceramics tend to form stronger primary bonds that require more energy to break them, which is manifested in terms of their physical properties (mechanical and thermal). Furthermore, inorganic nanomaterials are more stable in biological and physiological environments, which is an advantage for the long-term implantation *in vivo*. Finally, from a fabrication perspective, inorganic nanomaterials offer the flexibility of either a top-down or bottom-up approach, depending on the structure required.

It is the interest of this review to uncover the impact of inorganic nanomaterials on tissue engineering, which include: (1) improving structural support and cell-matrix interactions, (2) tracking cells engrafted on scaffolds, and (3) delivering drugs/genes/growth factors.

7.2.1.1 Structural Support and Cell-Matrix Interactions

The crux of tissue engineering lies in employing scaffolds that provide physical support for cells and which also regulate essential cellular functions such as adhesion, morphogenesis, proliferation, migration, and differentiation. Traditionally, most scaffolds are constructed with single-components, using only polymers, metals, or ceramics. However, single component systems are generally unable to provide appropriate strength and stiffness, particularly in

hard tissue applications. Classic examples where structural support is important include biomaterials developed for vascular,³⁴⁰ skeletal muscle,¹⁹⁶ bone,^{50, 341} and cartilage³⁴² tissue engineering. As such, the creation of adequate structural support in tissue-engineered materials has been a long-standing interest of researchers.³⁴³⁻³⁴⁶

To address this issue, composite materials (those that incorporate two or more constituent materials presenting uniquely different chemical properties from the individuals) were proposed. In scaffolds made from composite materials, the structural stability and mechanical reinforcement can be improved either through particle- or fiber-reinforcement. The theoretical basis for the mechanical improvement in particulate and/or fibrillar composites follows the Hall-Petch equation, where material strength is related to particle size, fiber length and fiber orientation,¹⁰⁶ as shown in the equation:

$$\sigma_y = \sigma_0 + \frac{k_y}{\sqrt{d}}$$

for particle reinforced composites, where σ_y is the yield stress, σ_0 is a materials constant (for the initial stress for dislocation movement), k_y is the strengthening coefficient (constant specific to each material), and d is the average particle diameter. Apart from that, filler-matrix cohesion has been suggested to influence the effectiveness of structural strengthening as well.³⁴⁷

Although many different composite systems could be designed to address the need for structural support, the key is to match the mechanical properties of engineered matrices to those of targeted physiological tissue. Taking bone tissue as an example, German anatomist and surgeon Julius Wolff proposed the theory of stiffness-matching in 1892, which laid down strong foundations

for the subsequent development of bone scaffolds and matrices.⁸⁹ It stated that bone growth and remodeling were positively correlated to increasing stiffness and an ideal composite material for bone tissue engineering (BTE) should have similar stiffness to physiological bone and avoid stress shielding that would lead to host bone resorption due to a lack of stress transfer from the implant to host bone. To address this, composite scaffolds have been fabricated by incorporating calcium phosphates (CaP), which contains up to 70% of hydroxyapatite (HA) nanocrystallites,³³⁶ and the results are encouraging: composite scaffolds showed similar stiffness, compressive strength, and toughness to physiological bone.³⁴⁸⁻³⁵³ In a study by Ramay and Zhang³⁴⁸, they developed electrospun nanofibers of β -tricalcium phosphate/HA (β -TCP/HA) as load-bearing scaffolds for BTE, and reported desirable compressive strength and toughness comparable to that of cancellous bone (2 – 10 MPa³⁵⁴), demonstrating the mechanical advantages of composite biomaterials.

Although rarely discussed, the success of this technology relies on the extent and homogeneity of fillers within the matrix. Non-homogenous dispersion of fillers within matrices is detrimental to the structural stability of scaffolds.²⁰⁰ On this note, significant challenges lie in preventing the agglomeration of nanomaterials, which occurs due to their high surface energies.³⁵⁵ Significant efforts have thus been made to disperse inorganic nanomaterials during the fabrication process. For example, Avella et al. presented an *in situ* polymerization methodology to disperse inorganic calcium carbonate (CaCO_3) NPs into a polymeric matrix.³⁵⁶ Homogeneous distribution of CaCO_3 NPs at low concentrations (4%) was confirmed through the mechanical and

morphological analyses. Zhao et al. presented an impactful method of directing NP assembly without the need for surface modification,³⁵⁷ achieved through non-covalent interactions such as hydrogen bonding and electrostatic interactions. The attractiveness of this method is further enhanced by the ability to strengthen/break through external stimulation,^{358, 359} consequentially leading to controlled variations in spatial distribution of these NPs. In this thesis, cryomilling was demonstrated to be capable of homogeneously distributing inorganic tricalcium phosphate (TCP) in a PCL matrix via low-temperature processing. This method is devoid of organic solvents and heat,¹⁰ potentiating its applicability in tissue engineering.

To further explore the importance of filler-matrix interactions, Rong et al. designed a chemical grafting method for the surface modification of nano-alumina, silicon carbide and silicon nitride.³⁶⁰ Before surface modification, these materials did not exhibit strong adhesion properties to the matrix, leading to poor mechanical performance. However, after covalently conjugating polyacrylamide onto their surface, favorable filler/matrix interfacial adhesions were achieved, and mechanical improvement was observed in terms of its tribology (wear rate, friction coefficient).

Apart from providing strong structural support, the incorporation of inorganic nanomaterials also has the potential to alter cell-matrix interactions, influencing downstream cell responses. For example, Webster et al. found that osteoblast adhesion, which was dependent on the desirable surface property of the nanophase-HA, was enhanced on nanophase-HA (~67 nm) as compared to other conventionally sintered HA (>132 nm).³⁶¹ In another study, Wu et al. elucidated the effect of nanostructure titanate on cellular adhesion

and proliferation via the development of 1D nanostructured titanate on 3D microporous Ti-based metal scaffolds.³⁶² Recently, Lock et al. fabricated HA/PLGA nanocomposites and showed that despite the absence of osteogenic factors in normal growth conditions, osteogenic differentiation of mesenchymal stem cells (MSCs) was achieved, suggesting that CaP alone may already support osteogenic differentiation. The use of inorganic nanomaterials is not restricted to eliciting positive responses from cells; suppression of bacterial growth could also be achieved. For instance, nanophase zinc oxide (ZnO) significantly enhanced osteoblast adhesion and suppressed *Staphylococcus epidermidis* colony forming units (CFU).³⁶³

It is interesting to note how far inorganic materials have progressed over the years, to the point where a variety of clinical trials and commercial products have been conceived, often finding their places in the field of dental restoratives.³⁶⁴⁻³⁶⁶ For example, Palaniappan et al. studied Filtek Supreme nanocomposites (Bis-Glycidyl methacrylate (Bis-GMA) impregnated with silica NPs of size circa. 75 nm) for their clinical performance and wear. Results during the sequent 3 years post-implantation met the guidelines for restorative materials for posterior teeth laid out by the American Dental Association (ADA).³⁶⁷ There have been reports of inorganic nanocomposites in the orthopedic field as well.^{364, 368, 369} As an example, Kon et al. nucleated collagen fibrils with HA NPs and implanted them in the medial/lateral femoral condyles, patellas, and trochleas as an osteochondral scaffold. Short-term follow-up results were encouraging, and suggested safety and good implant stability without the need of fixation.³⁶⁹

From the preceding paragraphs, the incorporation of inorganic nanomaterials into polymer matrices either as a form of structural support or for elucidating specific cellular responses is not only of academic value; clinical applications of these inorganic nanocomposites are also highly valued and needed.

7.2.1.2 Enhancing Cellular Imaging

An indispensable and key element of tissue engineering is the cell. The generation of functional tissues or organs depends on the survival, growth, differentiation, and/or organization of cells after transplantation. Traditionally, the effectiveness of a tissue-engineered strategy is evaluated through histological methods which are destructive, time-consuming, and do not provide interactive feedback. The emergence of molecular imaging opens the door for real-time visualizing of cellular function and molecular process in living organisms.^{370, 371} This is particularly valuable in two key areas of tissue engineering (cardiovascular and neurological) where stem cell based therapies are widely studied.

To be visualized by imaging modalities in molecular imaging, cells of interest have to contain specific “signal molecules”. Although small molecules such as gadoliniumtetraazacyclododecanetetraacetic acid (an MRI contrast agent) and Fludeoxyglucose (18F), a positron emission tomography (PET) contrast agent, have been widely used in both research and clinical settings, inorganic nanomaterials or inorganic NPs are receiving increased attention as next-generation contrast agents because of their unique properties. These NPs can be synthesized to possess tunable sizes, shapes, compositions, and physical properties (i.e. electronic, magnetic, optical, and thermal properties).

The basic criteria for the design and selection of inorganic nanomaterials for tracking cells include reproducible procedures for standardization, good ability to maintain physical properties after being internalized by cells, and most importantly have minimal cytotoxicity or biological effect on cells.³⁷²

Several independent groups have explored the idea of labeling cells with inorganic nanomaterials for their localization, proliferation, differentiation, and migratory behavior in tissue engineering.³⁷³ The demonstration of live, *in vivo* tracking of cellular behavior and distribution post-implantation was reported by deVries³⁷⁴ in 2005 and Ko³⁷⁵ in 2007. Briefly, human bone marrow-derived MSCs (bMSCs) were labeled with superparamagnetic iron oxide (SPIO) NPs (i.e. Feridex I.V.) and cultured in gelatin sponges before implanting into the right proximal thigh of nude mice. Using clinical 1.5T MRI imaging modality, bMSC behavior could be detected, and was verified by histological studies. Remarkably, imaging of labeled cells was sustained for 4 weeks, allowing for the *in vivo* visualization of cells and scaffold in real time. Although the signal intensity of labeled cells decreased with cellular division and proliferation, this study has demonstrated that SPIO NPs were capable of facilitating *in vivo* visualization implanted cells and scaffolds in a minimally invasive manner.

In a recently reported clinical trial,³⁷⁶ SPIO NPs labeled bMSCs were injected into patients with multiple sclerosis and amyotrophic lateral sclerosis. Although the study was conducted to investigate the safety and immunological effects of bMSC transplantation, it also demonstrated that SPIO NP-labeled bMSCs were safe to use in patients. From this, the possibility of *in vivo*, real time cellular tracking was demonstrated by observing

that labeled cells disseminated from the site of inoculation (lumbar) to the occipital horns, spinal parenchyma, and other locations.

Another group of inorganic nanomaterials that have specific applications as cell trackers include gold NPs,³⁷⁷⁻³⁸⁰ due to their strong light absorption cross-sections. Sokolov et al.³⁸¹ demonstrated topical delivery of gold NPs throughout the entire epithelium, which has potential applications in the early detection of molecular changes associated with cancer progression. Negesha et al.³⁸² labeled mouse embryonic stem cells with gold NPs and visualized them through multiphoton-absorption-induced luminescence, which generated efficiently by near-infrared laser (800 nm). More recently, Song et al.³⁸³ took a combinatorial approach involving gold NPs with polymeric vesicles for cancer-targeted drug delivery. The visualization of labeled breast cancer cells through strong fingerprint Raman signals, and fluorescence imaging suggested that these newly developed plasmonic vesicles assembled from surface-enhanced Raman scattering (SERS) may be employed as a theranostic tool in cancer treatment. These two examples illustrate how gold NPs may be used and modified for cell labeling purposes.

Fluorescent inorganic NPs (e.g. quantum dots, fluorescent silica NPs and etc.) are other groups of contrast agents for fluorescence based cell tracking. One well-known fluorescent semiconductor nanocrystals, quantum dots (QD), have been extensively used to label stem cells for tracking their biodistribution post transplantation.³⁸⁴⁻³⁸⁶ The tunable emission especially at the near infrared region (>800 nm) avoids the background signal of autofluorescence of the animal tissues (emissions are mainly at the visible region, ~300-550 nm). Good photostability allows QDs for the long-term tracking of stem cells.

Despite promising, the safety issues (risk of heavy metal leakage) hinder the translation of QDs to clinical applications.³⁸⁷ As an alternative, researchers have designed biocompatible silica NPs containing fluorescent dyes.

Fluorescent silica NPs include cyanine dye-doped silica NPs (IRIS dots) synthesized using a reverse microemulsion method, and fluorescent core-shell silica NPs (C dots) made by the sol-gel process.³⁸⁸ The silica shell not only prevents organic dyes from oxidation or decomposition, but also provides strong fluorescence by concentrating the dyes inside. For example, C dots present higher brightness (brightness is 30 fold as that of the precursor fluorescent dye) and stability than free dyes in aqueous solutions.³⁸⁹ Fluorescent silica NPs were demonstrated not to affect the viability, proliferation and differentiation capability of human MSCs. More interestingly, discrimination between liver and early-stage apoptotic stem cells can be achieved by labeling stem cells with IRIS dots.³⁹⁰ IRIS dots were found mainly in the cytoplasm of live cells but on the outer cell surface of early apoptotic cells due to loss of active endocytosis.

Taken together, labeling cells with inorganic nanomaterials is helpful for elucidating cell-level mechanisms involved in the localization and/or biodistribution of cells during the tissue engineering.

7.2.1.3 Delivery of Drugs, Genes, and Growth factors

Drugs, genes, and growth factors are traditionally delivered into the human circulatory system through the bolus injection. However, it suffers from the poor bioavailability, the lack of containment and specificity.²⁹² One idea is to locally deliver drugs, genes, and growth factors through the inorganic

composite platform. These platforms could be constructed by embedding inorganic NPs in thin films, wafers, and other forms of matrices. Depending on (1) the target, (2) tissue structure, and (3) desired therapeutic effects (sustained or burst delivery), the techniques and platforms for delivery are adequately designed.

Inorganic NPs offer viable solutions due to their unique characteristics such as size, biocompatibility, and drug loading capabilities. First, by encapsulating cargoes (drugs/genes/growth factors) of interest into carrier NPs (e.g. silica NPs,³⁹¹⁻³⁹³ CaP NPs³⁹⁴⁻³⁹⁷), the bioavailability of these cargoes is maintained.³⁹⁸ Secondly, by preloading stimuli responsive inorganic NPs (magnetic NPs, gold NPs) and cargoes of interest in scaffold, controlled release of cargoes on demand can be achieved under external stimuli.^{399, 400} In addition, by tagging these inorganic carriers with cell/tissue-specific ligands, targeted delivery of cargoes may also be achieved.⁴⁰¹⁻⁴⁰³

Shen et al. developed CaP-based nanocomposites for the delivery of DNA, based on the premise that high concentration of localized DNA would result in enhanced gene transfer. When MG-63 osteoblasts were treated with CaP NPs encapsulated DNA, enhanced gene transfer leading to biomineralization was reported, suggesting potential applications in BTE and regeneration.⁴⁰⁴ The delivery of proteins and growth factors, as mentioned earlier, are susceptible to enzymatic degradation, and also face difficulties in retention at the site of insult. As such, Krebs et al. designed CaP-based NPs incorporated with BMP-2, for localized and sustained delivery of BMP-2, resulting in efficacious bone regeneration when subcutaneously injected into the back of

mice.⁴⁰⁵ Through this system, they have addressed two main issues, that of protein degradation *in vivo*, as well as protein retention at the site of injury.

The transport of biomolecules such as RNA and siRNA has huge potential as a therapeutic target including intracellular and transcription factors, but face issues such as instability and poor cellular uptake. Due to the potential of NPs as DNA vectors, they have also been investigated as potential delivery vehicles for the intracellular transport of RNA and siRNA. Hom et al.⁴⁰⁶ predicted in 2009 that the usage of this system was a foreseeable future given its potential and true to their word, a flurry of publications based on the delivery of RNA and siRNAs followed in the subsequent years.⁴⁰⁷⁻⁴⁰⁹ The delivery of DNA and siRNA, which are negatively charged, relies on the creation of a positively charged surface of silica nanoparticles. As shown in other inorganic NP systems (iron oxide, gold), surface modification to convert the surface charge of silica was conducted using polyethyleneimine (PEI). Delivery of DNA and siRNA after conjugation with PEI resulted in enhanced cellular uptake (dependent on PEI molecular weight (M_w), lower M_w resulted in better uptake), and thus highlighted the potential of using silica NPs for intracellular drug and biomolecule delivery.⁴⁰⁸

Magnetic NPs (e.g. iron oxide NPs) and gold NPs have been extensively used in controlled release systems in biomedical applications. This is mainly because the nature of these two types of inorganic NPs which presenting unique properties once under specific external stimuli. For example, macroporous ferrogels scaffold were developed by mixing iron oxide NPs with alginate hydrogel solution before gelation followed by frozen at low temperatures (-20, -80, and -180 °C) in order to generate pores.⁴¹⁰ When

placed in magnetic field, this macroporous ferrogels scaffold will undergo large deformation and over 70% volume change caused by the attraction of the interparticle magnetic force. The pre-loaded drugs or biological agents are able to be released due to the generated water flow through the interconnected pores. Significant increase profile of drugs (mitoxantrone), plasmid DNA and chemokine (SDF-1 α) were confirmed by this delivery system. When modifying the hydrogel with arginine-glycine-aspartic acid (RGD) amino acid before gelation, this ferrogel scaffold allowed increased loading efficiency of cells (fibroblasts or mouse MSC) because of the enhanced cell adhesion on the RGD-peptide modified surface. With this technology, the researchers demonstrated the on-demand release of mouse MSCs after being implanted subcutaneously into the back region of nude mice.⁴¹⁰

MNPs can generate heat under an alternating magnetic field (AMF) based on Brown relaxation (friction generated from particle oscillation) and Neél relaxation (rotation of magnetic moment with each field oscillation).⁴¹¹ Magnetic NP aided hyperthermia for cancer therapy is particular promising due to non-invasiveness and no limitation of penetration depth of magnetic field. By combining this phenomenon with the thermosensitive materials (hydrogel or polymer), on-demand drug release has been developed. For example, composite scaffold was synthesized by incorporation of SPIO NPs and temperature sensitive poly(N-isopropylacrylamide) hydrogels (NIPAAm).⁴¹² Under an external trigger of AMF, the swell behaviour of hydrogel due to the generated heat was studied and controlled drug release was demonstrated. However, limited research of AFM based controlled

release system has been reported in tissue engineering field, which indicated comprehensive investigation are highly needed. Similarly, heating gold NPs with laser at the wavelength at their plasmonic resonance could also be used in laser-triggered drug delivery when incorporating gold NPs in thermosensitive hydrogels.⁴¹³

7.2.2 Preliminary data – Cryomilling of silica nanoparticle-polycaprolactone composites for localized drug delivery

7.2.2.1 Morphological and topographical assessments

Figure 7-1 (A) PCL pellets as-received, approximately 2710 μm in length. (B) cPCL powder with an average size of $180 \pm 70 \mu\text{m}$ (Table inset). (C) Molding defects of PCL/Dox films fabricated without cryomilling (black arrows), using PCL pellets presented in (A). (D) Homogeneous appearance of PCL/Si-Dox films fabricated with cryomilling. Surface morphologies of (E) pristine PCL films and (F) PCL/Si-Dox films. (G – H) AFM imaging of PCL/Si-Dox films confirming the presence of pores. The topographical features also clearly displayed a peak-to-peak variation of approximately 200 nm. (I – L) Surface and spatial distribution of Si-Dox on PCL/Si-Dox films conducted with confocal microscopy and 3D reconstruction.

PCL and cPCL showed a significant decrease in particle size (Figure 7-1A, B, table inset; *: $p < 0.05$). When pressed into films together with Dox, control films (non-cryomilled) showed clear agglomeration (Figure 7-1C, black arrows), while Dox appeared to be homogeneously distributed when PCL and Dox were processed via cryomilling, due to the homogenous coloration of the film (Figure 7-1D). Surface morphological assessment of PCL films at high magnifications indicated that they were featureless, while micro-pores were present both on the surface, and throughout the thickness of Dox-incorporated PCL/Si-Dox films (Figure 7-1F, inset: thickness view). AFM-fluorescence imaging indicated that the Dox was well distributed over the entire film, while within the pores, high intensity fluorescent signals were recorded due to light reflectance. These pores had a depth in excess of 200 nm (Figure 7-1G, H) and the average roughness was 49.650 nm. To validate the distribution of Si-Dox within the films, confocal microscopy images showed even distribution over the surface and throughout the bulk of the material (Figure 7-1I – L).

7.2.2.2 Mechanical characterization of PCL and PCL/Dox

Figure 7-2 (A) Stress-strain behavior of pristine PCL (dark bold) and PCL/Si-Dox films (light colored, dotted) with detailed values (Table, inset). Flexural ability of (B) pristine PCL and (C) PCL/Si-Dox films.

The stress-strain behavior of pristine PCL and PCL/Si-Dox are presented in Figure 7-2A. Both films displayed a clear elastic-plastic transformation point with substantial plastic deformation. Their detailed mechanical properties are shown in the inset table, but importantly, tensile strength, modulus, and strain values were all found to be similar ($p > 0.05$). The flexural capability of pristine PCL (Figure 7-2B) and PCL/Dox (Figure 7-2C) were also presented.

7.2.2.3 In vitro release characteristics of PCL/Dox

The in vitro release characteristics of PCL/Si-Dox and PCL/Dox (Figure 7-3A) demonstrated long-term release characteristics over 50 days, attaining approximately 32 $\mu\text{g/ml}$. From the inset, approximately 0.2 $\mu\text{g/ml}$ of Dox was

released when embedded in Si-NPs, while freely encapsulated Dox recorded a release of close to 0.7 $\mu\text{g/ml}$ over the first 48 h.

Figure 7-3 (A) In vitro, sustained release characteristics of PCL/Si-Dox film over 50 days, with the inset showing the initial release of both PCL/Dox (bold) and PCL/Si-Dox (dotted) over 48 h.(B) Cytotoxicity of PCL/Si, PCL/Dox, and PCL/Si-Dox films on HeLa cells over the course of 5 days. (C) Live/Dead imaging of HeLa cells over the first 3 days (Blue: Hoechst 33342, cell nuclei; Green: FDA, live cells; Red: Dox).

7.2.2.4 HeLa toxicity on PCL/Dox release platforms

Toxicity of the released Dox to HeLa cells were evaluated with cell metabolism assays Figure 7-3B. Controls involved seeding directly on tissue culture plates (TCPS) and PCL/Si films. Results were presented in percentage survival normalized to TCPS, indicated by the dotted line. When

cultured directly on PCL/Si, HeLa survival was maintained throughout the 5 days of culture, with approximately 80% survival by day 5. PCL/Dox resulted in a significant decrease in HeLa viability as early as day 2, where a 50% reduction was recorded when compared to day 1. By the end of culture, approximately 10% of HeLa cells remained viable. PCL/Si-Dox showed a slower but steady decrease in HeLa viability, with a 50% reduction recorded between days 3 and 5; 30% survivability was recorded by day 5. Confocal imaging of live cells corroborated with this observation, with an observed decreased in live cells (green).

Figure 7-4 Confocal imaging of HeLa cell on day 1 at 200x magnification. (B) Confocal z-stack slices of HeLa cells on day 3, at 200x magnification.

7.2.2.5 Si-Dox uptake by HeLa

The uptake of Si-Dox by HeLa were evaluated and confirmed by confocal microscopy on days 1 and 3 (Figure 7-4). Day 1 showed uptake of Si-Dox by HeLa cells (Figure 7-4A), while image slices at steps of 0.95 μm were taken through the depth of two isolated HeLa cells at day 3 (Figure 7-4B). From the images, it was evident that Si-Dox was present at every image slice,

consolidating earlier data on the uptake of Si-Dox. In addition, vesicles were also seen, which is characteristic and indicative of apoptosis (Figure 4, slice $z = 0$ and $z = 5.70 \mu\text{m}$).

7.2.2.6 Discussion

Doxorubicin (Dox) is a chemotherapeutic drug that has been used extensively in clinics and in many in vitro drug delivery platforms^{414, 415} to evaluate drug-release characteristics. However, there are problems that severely impede its bioavailability, primarily due to its hydrophobic nature making it prone to premature renal clearance before achieving its therapeutic effect. As part of the combined efforts to increase the bioavailability of Dox, Dox has been incorporated in carriers like nanoparticles.⁴¹⁶ For example, Doxil is an effective carrier for Dox^{417, 418}, and have shown increased tumoral uptake due to the enhanced permeability and retention (EPR) effect.⁴¹⁹ As such, this study has adopted a similar strategy to achieve increased cellular uptake of Dox in mesoporous Si. In conventional breast cancer therapy, surgical removal of tumors is commonly followed by systemic administration of therapeutic agents like Herceptin® or chemotherapeutic drugs like the anthracyclines (e.g. Dox/Adriamycin®) and taxanes (e.g. paclitaxel/Taxol®). However, the efficiency and potential side effects of these therapeutic agents were reported to be a major concern with reported severe fatigue ($p < 0.01$) and a poorer quality-of-life, both associated with its use in patients.⁴²⁰ In some cases, patients with a higher relapse potential may require high doses of these therapeutic agents, administered either intravenously or orally. These patients were found to suffer from long-term cognitive impairment.⁴²¹

These issues necessitate the development of localized drug delivery platforms with good bioavailability of Dox, sustained release characteristics, and minimized side effects. The current clinical approach to localized treatment takes the form of radiotherapy. However, the possible side effects of lymphoedema^{422, 423} and the rare but unpredictable occurrence of continuous coughing and breathlessness arising from radiation fibrosis remain key concerns in this form of adjuvant therapy. In this light, this study evaluated the PCL/Si-Dox platform in terms of its mechanical function and anti-cancer effects.

Firstly from a mechanical perspective, PCL/Si-Dox composite film possesses good handling properties. The mechanical properties of PCL/Si-Dox films were shown to be similar to that of pristine PCL (Figure 7-2A, table inset), which is an important finding. To achieve this, a materials processing technique reported earlier in this thesis was employed. Importantly, the homogeneous distribution of Dox corroborated with the distribution effects reported earlier. Interestingly, the morphological features of PCL films differed significantly from that of PCL/Si-Dox, with the presence of micropores in the latter. Micropores and microdefects have been reported to influence the mechanical properties of films, but the results here have shown that film pliability and strength were not affected. In fact, the presence of the micropores had facilitated the release of Dox from the bulk of the film, as observed from the release characteristics over 50 days, suggesting desirable long-term release characteristics.

To maintain a sustained release in vivo would require the maintenance of its mechanical integrity. To this end, Teoh's group has shown in a variety of in

vitro and in vivo studies that PCL possesses good degradation stability in vivo.^{27, 70, 100} More specifically PCL films fabricated via a similar melt-press method¹⁷² reported significant mass loss after 40 days of exposure to highly degradative environment. More significantly, yield strength and modulus were maintained for 20 days before reflecting a decrease.

The pharmacological effects of this PCL/Si-Dox platform were shown to occur between 3 – 5 days with HeLa cells. By correlating this observation with in vitro release characteristics, the critical IC_{50} value ($1.12 \mu M^{424}$) was achieved as early as day 2 from the control films (PCL/Dox), while PCL/Si-Dox achieved a Dox concentration of $0.42 \mu M$ on day 4 (Figure 7-3A, inset). Expectedly, significant cytotoxicity was observed on day 2 in the PCL/Dox culture. Interestingly, significant cytotoxic effects were observed between days 3 and 5 in the PCL/Si-Dox system, which appeared counter-intuitive when correlated with the in vitro release characteristics. Detailed investigation of Si-Dox uptake via confocal microscopy then suggested that uptake occurred as early as day 1 (Figure 7-4A), and was retained until day 3 (Figure 7-4B). It is well-known that the endocytic and subsequent retention effects are related to the diameters of nanoparticles.^{425, 426} In fact, it had been reported that 200 nm Si nanoparticles were endocytosed earlier, and retained in the body for longer periods of time compared to 100 nm Si nanoparticles.⁴²⁷ On this platform, the forced uptake of Si-Dox was facilitated by intimate cell-matrix interactions. As such, Si-Dox release was complemented by its concurrent uptake, increasing the exposure of encapsulated Dox to HeLa to achieve pharmacological effects.

Safety concerns about the accumulation of Dox within the circulatory system may also be addressed by the efficient excretion of 200 nm Si nanoparticles via renal and biliary clearance.⁴²⁷ Notably, the benefits of this platform lie in the lowered Dox exposure to surrounding tissue yet being able to achieve pharmacological efficiency through the uptake of Dox via cell-matrix interactions.

7.2.2.7 Summary

This study hypothesized that a PCL/Si-Dox platform fabricated via cryomilling provides homogeneous Dox distribution, desired mechanical integrity, and sustained release characteristics for localized drug therapies. The results have confirmed that the PCL/Si-Dox platform was able to provide sufficient mechanical strength, while displaying pharmacological anti-tumor effects when tested with HeLa cells. Taken together, these results open up the possibility of fabricating flexible film composites via a clinically relevant method in cryomilling and provide encouragement for further evaluation in small animal tumor models.

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