CHAPTER I

INTRODUCTION

Tissue engineering originates from reconstructive surgery which the replacement of organs is practiced to repair the function of damaged tissue. (Stamatialis, D.F et al., 2008) A large number of research groups focus on tissue engineering therefore tissue engineering constructs have been significantly improved in recent years. (Tessmar, J.K. and Gopferich, A.M., 2007) One of the major research themes is the scaffold fabrication. Scaffolds represent the space available for the tissue to develop and the physical support for cell growth before there are transplanted back to the host tissue. Compatible to the structure of the tissue, scaffolds should be designed to have a specific three dimensional shape, high porosity, a fully interconnected geometry, high surface area, and structural strength. Scaffold porosity and interconnected geometry are a fundamental characteristic for providing facile cell growth, blood vessel invasion, and effective metabolic nutrient/waste transport. Additionallly, highly specific surface areas and highly pore volume fraction of scaffolds allow for cell attachment and enable cell growth, migration, and effective transportation of fluids and nutrients. Scaffold mechanical properties should allow shape maintenance during tissue regeneration and enable stress transfer and load bearing. (Tessmar, J.K. and Gopferich, A.M., 2007, Stamatialis, D.F et al., 2008, Carletti, E. et al., and Hutmacher D. W., 2001)

A variety of natural and synthetic polymers have been utilized to fabricate tissue engineering scaffolds. These materials must be inherently biocompatible, biodegradable, and highly cell adhesive. Additionally, the polymers must be porous and mechanically stable and exhibit a three-dimensional (3D) structure that can be obtained via a facile manufacturing processes. Aliphatic polyesters, such as polycaprolactone (PCL), polylactic acid (PLA), polyglycolic acid (PGA), and related copolymers, are the most extensively used in biodegradable scaffolds because of these polymers' excellent biocompatibility, biodegradability, bioresorbability, and mechanical strength. These materials have been approved by the Food and Drug

Administration (FDA) and are easily processed into various structures with 3D matrices. (Hyun, J. C et al., 2007 and Woesz, A., 2008)

PCL is a semi-crystalline polyester that is degraded by the hydrolysis of its ester linkages under physiological conditions, such as in the human body. In particular, PCL is useful for the preparation of long-term implants due to its markedly slow degradation rate. This polymer has received FDA approval and is already used in drug delivery devices, sutures, and adhesion barriers. Additionally, due to its excellent biocompatibility, mechanical strength, lack of toxicity, and low cost, PCL is one of the biodegradable polyesters that has attracted the most attention in bone tissue engineering. (Woesz, A., 2008 and Sinha, V.R. et al., 2004)

The potential use of porous biodegradable scaffolds as 3D templates to encourage initial cell attachment and subsequent tissue formation has been studied both in vitro and in vivo. Various tissues, such as cartilage, bone, heart, valve, nerve, muscle, bladder, and liver tissues, have been produced using such scaffolds. In these applications, the scaffolds should be highly porous to allow cell seeding and facilitate invasion by blood vessels, which supply nutrients to and remove waste from the transplanted cells. The scaffold architecture should permit cell intrusion, nutrient and waste product permeation, and new capillary network formation. A scaffold's porosity is another fundamental characteristic that is necessary to provide for cell migration and tissue vascularization. Furthermore, cellular interactions increase as the available surface area is expanded. Generally, the biological activity of a scaffold is determined by the construct's ligand density, which is determined by the scaffold's composition and porous fraction, which is the total surface of the structure that is exposed to the cells. Highly customized surface areas can encourage cell attachment and anchorage, and a high pore-volume fraction enables cell growth and migration and the effective transport of fluids and nutrients. In sum, to best mimic a tissue, scaffolds should be designed to be highly porous and to exhibit a high surface area, fully interconnected geometry, structural strength, and a specific 3D shape. (Nam, Y. S, et al., 1999, Hutmacher D. W., 2001, and Lee, J. H et al., 2003)

Various techniques have been reported for the preparation of porous polymeric scaffolds. A wide range of techniques commonly used in tissue engineering generate constructs with random structures, unpredictable pore sizes, and

reduced pore interconnectivity. Any variation in porosity within the 3D structure cannot be controlled, and the mechanical strength, structural stability, and reproducibility of these scaffolds are generally low. Among these techniques, solvent casting, freeze drying, phase inversion, fiber bonding, melt-based technologies, and high pressure-based methods are the most commonly used. (Carletti, E. et al.) Porous polymers can be prepared by embedding soluble ingredients, known as porogens, into the polymer, which form pores in the polymer matrix upon removal. In modern tissue engineering, solvent casting is often used in combination with other common methods to produce a porous 3D structure. For example, solvent casting combined with particulate leaching has been used to successfully fabricate 3D scaffolds. The porogens typically used in the field are sodium chloride, ammonium bicarbonate, and glucose with different crystal sizes. (Kim, H.J. et al., 2007, Pattison, M.A. et al., 2005, Yoon, J.J et al., 2004, Shin, M. et al., 2008, and Sokolsky-Papkov, M. et al., 2007)

The porogen leaching technique for solvent-cast scaffolds involves pouring a polymer solution (e.g., PLA in chloroform) onto a bed of porogens, such as salt particles, paraffin microspheres or emulsion particles of a defined size. The solvent is evaporated, resulting in the solidification of the polymer around the porogen particles. The entrapped porogen is then leached out of the scaffold using numerous rinses with distilled water (in the case of NaCl) or an organic solvent (in the case of paraffin), thereby creating a defined pore structure. This leaching strategy has been applied to the creation of porous scaffolds for the growth of endothelial cells. (Cao, Y. et al., 2005) Such a construct's biochemical properties and architecture should support cell attachment, migration, growth, and ultimately, tissue maturation. (Ekaputra, A. K et al., 2008) In particular, the achievement of an open, interconnected pore system within a 3D structure is critical for ensuring proper nutrient and waste transport, tissue ingrowth, vascularization, and eventually, the integration of the construct within the host. (Lin,W.J. and Lu, C.K, 2002)

Blended polymers play an important role in the development of microporous controlled-delivery systems. Typically, there are two types of polymers present in blended membranes: one polymer remains for the end use, whereas the other polymer, used only to produce pores in the membranes, is removed. Due to its

specific properties, such as water solubility, polyethylene glycol (PEG) is frequently used as a polymeric additive to generate membranes and is a common coating material in biomaterial applications. The pores can be created in scaffolds by leaching out the water-soluble PEG using the solvent casting and polymer leaching methods. (Lin,W.J. et al., 2002, Lin, W.J. et al., 2003, Kesting, R.E., 1985, and Harris, J.M, 1992)The preparation method proposed here combines solvent casting and salt particulate leaching with polymer leaching, with the purpose of improving the pore interconnectivity of 3D polymeric scaffolds.

Among the numerous bioceramics, bioactive glass, silica, tricalcium phosphate, biphasic calcium phosphate, and hydroxyapatite (HA) have been widely investigated for bone tissue engineering. (Johari, N. and Fathi, M.H. et al., 2012) Hydroxyapatite (HA) becomes one of extensively investigated due to its composition and structure close to natural bone mineral. HA, which has the general formula $Ca_{10}(OH)_2(PO_4)_6$, has been demonstrated to have good biocompatibility, good biological properties (osteoconductivity, and osteoinductivity), and partially good mechanical stability. (Wang, Y. et al., 2010 , Wang, Y. et al., 2010, Wei, G. and Ma, P.X., 2004, Yang, F. et al., 2006, Webster, T.J. et al., 2000, Webster, T.J. et al., 1999, Webster, T.J. and Ejiofor, J.U., 2004, Ren, J. et al., 2008, and Kumar, P.T.S. et al., 2011) However HA is difficult to form into complex shape, and unsuitable for load-bearing application due to its stiffness, and brittleness. (Ren, J. et al., 2008)

Currently, composite materials have been widely studied and recognized for the development of scaffolds for bone tissue engineering. Composite or nano composite materials made by a polymeric matrix and ceramic reinforcers such as hydroxyaptite (HA), tricalcium phosphate (TCP), silica or bioactive glass have been suggested to design porous materials with adequate mechanical properties for bone tissue engineering. (Johari, N. et al., 2012 and Deplaine, H. et al., 2010) The main disadvantage of polymeric scaffolds is their low mechanical strength and the one of ceramic scaffolds is their inherent brittleness. (Wei, G. and Ma, P.X., 2004) A possibility to overcome these problems is to fabricate composite scaffold which combines the toughness of the polymer component with the strength and stiffness of ceramic component such that are expected to have superior mechanical properties. (Raucci, M.G. et al., 2010, Johari, N. et al., 2012, Webster, T.J. et al., 2004, and Ma,

P.X., 2008)Wei et al. showed that the introduction of HA into HA/polymer composite scaffolds greatly increased the mechanical properties and improved the protein adsorption capacity. (Wei, G. and Ma, P.X., 2004) Many research works also verified that the incorporation of ceramic into the polymer scaffolds improved the mechanical properties and protein adsorption of the composite scaffolds. Moreover the combination of polymers with bioceramic component also has improved the osteoconducting properties of scaffolds. (Wang, Y. et al., 2010, Cao, Y. et al., 2005, and Lin,W.J., 2002)

In our previous work, the PCL scaffold with highly interconnected networks has been fabricated by our modified solvent casting, particulate leaching, and polymer leaching techniques using sodium chloride and polyethylene glycol (PEG) as a porogen. Although increased porosity and interconnected network facilitate bone ingrowth, the result is a reduction in mechanical properties of scaffold. Not only scaffold should be highly porous with good pore connectivity to ensure sufficient nutrient transport towards the cells and removal of waste products but also scaffold should have suitable mechanical properties comparable to in vivo tissue at the site of implantation. (Karageorgiou, V. and Kaplan, D, 2005) For these reason, the another aim of the study was to improve the mechanical properties of the dual-leached PCL scaffold by the addition of hydroxyapatite. Moreover due to hydrophobic nature of PCL, the alkaline treatment (NaOH treatment) of PCL/HA scaffold requires for the improvement in the water retention behavior. The morphology, physical and mechanical properties of the PCL and PCL/HA scaffold were investigated. Finally, the indiect cytotoxicity of L929 and MC3T3-E1, MC3T3-E1 cell attachment, proliferation, and differentiation were also evaluated the potential of scaffolds for used in bone-tissue engineering applications.

Tissue engineering applies methods from materials engineering and life science to create artificial constructs for regeneration of new tissue. With tissue engineering, we can create biological substitutes to repair or replace failing organs or tissues. The task of tissue engineering demands a combination of molecular biology and materials engineering, since in many applications a scaffold is needed to provide a temporary artificial matrix for cell seeding. (Amir, N. H. et al., 2012)

Polymeric scaffolds play a pivotal role in tissue engineering through cell seeding, proliferation, and new tissue formation in three dimensions, showing great promise in the research of engineering a variety of tissues. (Masami, O. and Baiju J., 2013, Rezwana, K. et al., 2006, Yung-Chih, K., and Cheng-Ting, W., 2012, Hutmacher, D.W. 2001) Interconnected porous architecture, sufficient porosity, appropriate mechanical properties, suitable degradation rate, biocompatibility and good cell attachment properties are the major requirements of an ideal scaffold for bone tissue engineering applications. (Ilaria, C. et al., 2013, Long, Y. et al., 2006, Perrine, B. et al., 2009)

As a consequence, the scaffold fabrication method should allow for the control of its pore size and shape and should enhance the maintenance of its mechanical properties and biocompatibility. (Joel, R., and Michel, A. H., 2006, Prae-ravee, K. et al., 2011) During the past year, many techniques have been applied for making porous scaffolds. Among the most popular are particulate leaching , temperature-induced phase separation , phase inversion in the presence of a liquid non-solvent , emulsion freeze-drying , electrospinning, and rapid prototyping(Lida, B. H. et al., 2009, Jian, T. et al., 2009) Solvent casting of biocomposite scaffolds involves the dissolution of the polymer in an organic solvent, mixing with ceramic granules, and casting the solution into a predefined 3D mould. The solvent is subsequently allowed to evaporate. (Marrakchi, Z. et al., 2012, Lijun, J. et al., 2015)

For tissue engineering applications, aliphatic polyesters, such as poly(L-lactic acid) , polycaprolactone (PCL), poly(L-lactide/ε-caprolactone) copolymers, poly(lactide-co-glycolide), poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate), have been widely used because of their favorable biocompatibility and degradability. (Rezwana, K. et al., 2006, Xian-Yi, X. et al., 2010, Donghua, G. et al., 2008, Shuai, W. et al., 2008, Sanaz, A. et al., 2012, Shaun, E. and Suman, D., 2010)

PCL is also an important member of the aliphatic polyester family. Among the biocompatible polyesters, PCL is a semicrystalline aliphatic polymer linked with ester bonds, which can be hydrolyzed in mammalian cells, metabolized via tricarboxylic acid cycle, and eliminated by kidney. (MyllymaÈ, K.O. et al., 1998, Ken-Jer, W. et al., 2007, Chern, C. E. et al., 2013) PCL is flexible semicrystalline

polymer with low melting point and exceptional blend compatibility. Therefore, PCL can be blended with other polymers to improve stress crack resistance, dye-ability, and adhesion. (Chern, C. E. et al., 2013)

PHAs have been investigated as a tissue engineering scaffold due to its biocompatibility, biodegradability, and adjustable mechanical properties. (Korakot, S. et al., 2007, Chuan, Y. et al., 2009) PHB is a biodegradable, thermoplastic polyester with high crystallinity and high melting temperature that possesses excellent mechanical strength and modulus. It is also the simplest and most common member of the PHA family. In vitro tests have shown that PHB is biocompatible to various cells including osteoblasts, fibroblasts, chondrocytes, endothelium and epithelium cells. (Zhao, K. et al., 2003) Polyhydroxybutyrate (PHB) and poly(hydroxybutyrate-cohydroxyvalerate) (PHBV) are the most well-known polymers of the polyhydroxyalkanoates family. (Johari, N.et al., 2012) Material properties can be tailored by varying the HV content. An increase of the HV content induces an increase of the impact strength and a decrease of the melting temperature and glass transition, the crystallinity, the water permeability and the tensile strength. (Murali, M. R. et al., 2013, Chunyan, Z. et al., 2013, Christina, W. C. et al., 2014)

The one approach to modify and tailor material properties are by blending two polymer components either in molten or in dissolved state, aimed at superior thermal and physical properties. The fragility of PHB thus restricted its application in cartilage repair. Many research works have been made to improve the mechanical properties of PHB by blending PHB with other polymers. (Kai, Z. et al., 2003)It has been demonstrated that PHBV based blends present improved biological performances. (Korakot, S. et al., 2007, Orawan, S. et al., 2007)

In our previous work (Thadavirul, N.et al., 2013, Thadavirul, N. et al., 2014) the PCL and NaOH treated PCL/HA dual leached scaffolds with high porosity have been prepared by our combining modified solvent casting, particulate leaching, and polymer leaching techniques using sodium chloride and polyethylene glycol (PEG) as porogens. The purpose of this research work was to fabricate and biological evaluations of the PCL blend with PHB (PCL-PHB) and PCL blend with PHBV (PCL-PHBV) dual-leached scaffolds. Moreover, the morphology, physical and mechanical properties, and weight remaining after degradation of the PCL-PHB and

PCL-PHBV dual-leached scaffolds were also investigated. For biological evaluations, the indirect cytotoxicity of L929 and MC3T3-E1 cells, MC3T3-E1 cell attachment, proliferation, and differentiation were evaluated the potential of scaffolds for used these construct as bone scaffolding materials.

Tissue engineering facilitates the creation of biological substitutes to repair or replace the failing organs or tissues. One of the most promising approaches toward this direction is to grow cells on scaffolds that act as temporary support for cells during the regeneration of the target tissues, without losing the three dimensional (3D) stable structure. (Masami, O. and Baiju J., 2013)

Consequently, tissue engineering typically involves the use of porous, bioresorbable scaffolds to serve as temporary, three-dimensional scaffolds to guide cell attachment, differentiation, proliferation, and subsequent tissue regeneration. (Wang ,Y. et al., 2010) Recent research strongly suggests that the choice of scaffold material and its internal porous architecture significantly affect regenerate tissue type, structure, and function. In addition to possessing the appropriate material composition and internal pore architecture for regenerating a specific target tissue, scaffolds must also have mechanical properties appropriate to support the newly formed tissue. (Rezwana, K. et al., 2006) Briefly, a biomaterial scaffold suitable for use in tissue engineering should be biodegradable and have nontoxic degradation products, be highly porous with an interconnected pore structure, have suitable physical and mechanical properties, and be biocompatible and suitable for cell attachment, proliferation, and differentiation. (Yung-Chih, K., and Cheng-Ting, W., 2012)

Conventional methods for scaffold fabrication rely on a variety of techniques involving the use of woven and non-woven fabrics, solvent casting and particulate leaching, solution casting and gel casting with porogens, pressurized gas foaming, forging, injection molding, cold or hot pressing, and electrospinning.(Kai, Z. et al., 2003) The solvent casting/salt leaching method has the advantage of controlling pore size by manipulating the size of the salt particulate. Techniques like freeze-drying often allow the fabrication of porous scaffolds with a high compressive modulus. Researchers have used combinations of these techniques to obtain desirable porosity ratios and pore dimensions. (Ilaria, C. et al., 2013)

Polycaprolactone (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(hydroxy butyrate) (PHB), and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and poly(butylene succinate) (PBS) are examples of synthetic biodegradable polymers. PCL, PHB and PHBV are extensively used in tissue engineering for treating patients suffering from damaged or lost organs or tissues. (Long, Y. et al., 2006), Perrine, B. et al., 2009, Murali, M.S. et al., 2013, Hutmacher, D.W., 2001) They have been demonstrated to be biocompatible, degrading into non-toxic components, and have a long history with FDA (US Food and Drug Administration) approval for clinical use. (Lida, B. H. et al., 2009, Jian, T. et al., 2009, Marrakchi, Z. et al., 2012, Lijun, J. et al., 2015)In general, scaffolds must exhibit high porosity, proper pore size, biocompatibility, biodegradability and proper degradation rate. The scaffold must provide sufficient mechanical support to maintain stresses and loadings generated during in vitro or in vivo regeneration. (Masami, O. and Baiju J., 2013)

PCL is also an important member of the aliphatic polyester family. (Carletti, E. et al., 2011) PCL degrades at a significantly slower rate than PLA, PGA, and PLGA. The slow degradation makes PCL less attractive for biomedical applications, but more attractive for long-term implants and controlled release application. (Xian-Yi, X. et al., 2010) PCL has been used as a candidate polymer for bone tissue engineering, where scaffolds should maintain physical and mechanical properties for at least 6 months. (Masami, O. and Baiju J., 2013) Poly(3-hydroxybutyrate) (PHB) and and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), as the member of polyhydroxyalkanoates (PHA) family, have attracted much attention for a variety of medical applications because of its biodegradation and excellent biocompatibility. (Shuai, W. et al., 2008) However, the brittleness and narrow processing window of these polymers restrict their application. Blending of these polymers with other biocompatible polymers has been widely studied to improve their mechanical and thermal properties. (Shuai, W. et al., 2008)

Other important categories of materials are bioactive ceramics such as calcium phosphates (with hydroxyapatite being the prominent family member), bioactive glasses, and glassceramics which elicit a specific biological response at the interface of the material resulting in the formation of a strong bond between the bone

tissue and the material. (Amir, N. H. et al., 2012, Shaun, E. and Suman, D., 2010, MyllymaÈ, K.O. et al., 1998, Ken-Jer, W. et al., 2007) Currently, polymer/ceramic composite materials are being developed with the aim of enhancing mechanical properties and improving cell and tissue interaction of scaffolds . Nanocomposites based on HA particles and biopolymers have attracted attention for their good osteoconductivity, osteoinductivity, biodegradability and high mechanical strengths PCL/nHA nanocomposites were prepared and they combine the osteoconductivity and biocompatibility shown by HA ceramic with PCL properties. (Amir, N. H. et al., 2012, Chern, C. E. et al., 2013, Jian-Zhong, H. et al., 2013, Sanaz, A. et al., 2012, Azadeh, A. et al., 2011, Steaphane, S.and Monique, L., 2006)

In recent years, many studies have been carried out on composite scaffolds consisting of PHB or PHBV reinforced with bioactive ceramics. Incorporation of HA microparticles into PHB or PHBV resulted in composites with improved mechanical properties and in vitro bioactivity. (Amir, N. H. et al., 2012) The PCL-PHB and PCL-PHBV dual-leached scaffolds in our previous work provided better support for bone cell adhesion and proliferation. (Thadavirul, N. et al., in preparation)Therefore, the objective of this work were to prepare the PCL-PHB/HA and PCL-PHBV/HA dual leached scaffolds, and improve the mechanical properties of the dual-leached PCL-PHB and PCL-PHBV scaffolds by the addition of hydroxyapatite. The water absorption capacities, degradation behavior, morphology, physical and mechanical properties of the PCL-PHB/HA and PCL-PHBV/HA dual-leached scaffolds were investigated. Then, the indiect cytotoxicity of L929 and MC3T3-E1, MC3T3-E1 cell attachment, proliferation, and differentiation were also evaluated the potential of scaffolds for used in bone-tissue engineering applications.

In this study, sodium chloride and polyethylene glycol (PEG) were used as water-soluble porogens for the formation of porous polycaprolactone (PCL) and its blend scaffolds. The purpose of this work was to evaluate the feasibility of the preparation method, which combines the polymer and particulate leaching techniques for the preparation of highly interconnected, three-dimensional (3D), porous polymeric scaffolds. Moreover, the potential use of these scaffolds in bone tissue engineering applications was evaluated in vitro using mouse calvaria-derived pre-osteoblastic cells (MC3T3-E1).