BIOCOMPATIBILITY OF ELECTROSPUN *Bombyx mori* SILK FIBROIN WITH POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) AS A BIOMATERIAL FOR TISSUE-ENGINEERED SCAFFOLDS

ANG SHAIK LING

UNIVERSITI SAINS MALAYSIA

2019

BIOCOMPATIBILITY OF ELECTROSPUN *Bombyx mori* SILK FIBROIN WITH POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) AS A BIOMATERIAL FOR TISSUE-ENGINEERED SCAFFOLDS

by

ANG SHAIK LING

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

December 2019

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude to my main supervisor Prof. Dr. K. Sudesh Kumar. His guidance, patience and endless support throughout these years, which was an invaluable contribution towards my research work.

Next, I would like to express my sincere thankfulness to my co-supervisor Assoc. Prof. Dr. Bakiah Shaharuddin from Advanced Medical and Dental Institute (AMDI) for her continuous guidance and assistance with various techniques in this work. As I had very little experience in the field of cell culture, I am, therefore, appreciative to the people who worked with me at the beginning of my research. I want to offer my special thanks to Ms Siti Maisura who skilled me and helped to get me started with cell culture work.

I am indebted to USM-RIKEN International Center for Aging Science for providing research grant. Special thanks also go to Kaneka Corporation, Japan for generously providing P(3HB-*co*-3HHx); Dr. Keiji Numata from RIKEN, Japan for providing silkworm cocoons and CryoCord Sdn Bhd, Malaysia for the stem cells.

I would also like to take this opportunity to thank all members and staff of Ecobiomaterial Laboratory, Regenerative Cluster, Electron Microscopy Unit and Earth Material Characterization Laboratory for their help and guidance throughout the whole duration of my project. I wish all of them great luck in their future undertakings.

Lastly, I would like to extend my sincere thanks to all of those who have involved directly or indirectly in this work.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
ALP	Alkaline phosphatase
ATR	Attenuated total reflection
bp	Base pair
BSP	Bone sialoprotein
cDNA	Complementary deoxyribonucleic acid
COL 1	Collagen type 1
Ct	Threshold cycle
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy dispersive X-ray spectrometry
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FT-IR	Fourier transform-infrared
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPC	Gel permeation chromatography
HFIP	1,1,1,3,3,3-hexafluoro-2-isopropanol
HMDS	Hexamethyldisilane
hUC-MSCs	Human umbilical cord-derived mesenchymal stem cells
ISCT	International Society for Cellular Therapy
MCL	Medium-chain-length
MSCs	Human mesenchymal stem cells
MWCO	Molecular weight cut-off
OCN	Osteocalcin
OPN	Osteopontin

P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PDLLA	Poly (D, L-lactic acid)
PDS	Polydioxanone
PE	Polyethylene
PET	Poly(ethylene terephthalate)
PGA	Polyglycolide/ poly(glycolic acid)
PHA	Polyhydroxyalkanoates
PLA	Polylactide/ poly(lactic acid)
PLCL	Poly(lactide-*co*-caprolactone)
PLLA	Poly(L-lactic acid)
PP	Polypropylene
PTFE	Poly(tetrafluoro-ethylene)
qPCR	Quantitative real-time polymerase chain reaction
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCL	Short-chain-length
SEM	Scanning electron microscopy
SF	Silk fibroin
TAE	Tris-acetate-EDTA
TCPS	Tissue culture polystyrene plates
ThS	Thioflavin S
UV	Ultraviolet

LIST OF UNITS AND SYMBOLS

α	Alpha
β	Beta
cm^2	Centimeter square
°C	Degree Celsius
°	Degree
GPa	Giga pascal
g	Gram
h	Hour
kDa	Kilo Dalton
kV	Kilovolt
L	Liter
MPa	Mega pascal
µM	Micro molar
µL	Microliter
µL/min	Microliter per minute
µm	Micrometer
mL	Millimeter
min	Minute
M	Molar
mol%	Mole percentage
ng	Nanogram
nm	Nanometer
cm^{-1}	Number of wavelengths per centimeter
%	Percentage
pH	Potential hydrogen
x g	Relative centrifugal force
s	Second
v/v	Volume per volume
w/v	Weight per volume
M_w	Weight-average molecular weight

BIOSERASI ELEKTRO FIBRION SUTERA *Bombyx mori* DENGAN POLI(3-HIDROKSIBUTIRAT-*ko*-3-HIDROKSIHEKSANOAT) SEBAGAI PERANCAH BIOBAHAN UNTUK KEJURUTERAAN TISU

ABSTRAK

Polihidroksialkanoat (PHA) adalah poliester yang dihasilkan oleh mikroorganisma di bawah keadaan pertumbuhan tidak seimbang. Biodegradasi dan bioserasi PHA telah menjadikan PHA sebagai biobahan yang sesuai bagi applikasi kejuruteraan tisu. Poli(3-hidroksibutirat-*ko*-3-hidroksiheksanoat) adalah salah satu jenis PHA yang mempunyai sifat mekanikal dan pemprosesan yang lebih baik berbanding dengan poli(3-hidroksibutirat) [P(3HB)] dan poli(3-hidroksibutirat-*ko*-3-hidroksivalerat) [P(3HB-*ko*-3HV)]. Bagaimanapun, bioserasi P(3HB-*ko*-3HHx) masih tidak mencukupi kerana sifat hidrofobiknya telah menghalang penggunaannya secara langsung sebagai bahan perancah. Dalam kajian ini, fibroin sutera (SF) yang diperolehi dari kepompong *Bombyx mori*, satu polimer protein yang telah digunakan secara meluas dan dikaji sebagai aplikasi biobahan untuk memperbaiki afiniti sel dan bioserasi P(3HB-*ko*-3HHx). Perancah berserat telah disediakan melalui teknik pemintalan elektro dari campuran P(3HB-*ko*-3HHx) dan SF. Nisbah campuran yang berbeza P(3HB-*ko*-3HHx)/SF telah difabrikasi dan dicirikan. Kajian bioserasi telah dijalankan pada sel tunjang mesenkima dari tali pusat manusia (hUC-MSCs) di atas filem elektro dengan menggunakan 'Presto Blue cell viability assay', potensi pembezaan tiga keturunan dan ekspresi gen. Penggabungan SF dengan P(3HB-*ko*-3HHx) telah meningkatkan hidrofilik dan menurunkan diameter serat purata. Filem elektro P(3HB-*ko*-3HHx)/SF dengan nisbah campuran 1:3 menunjukkan kesan tertinggi terhadap daya tahan sel selepas 28 hari dalam kultur berbanding kesan yang

ditunjukkan oleh P(3HB-*ko*-3HHx) atau SF. Dalam kajian potensi pembezaan tiga keturunan, sel-sel tunjuang mesenkima didapati menunjukkan potensi dalam pembezaan osteogenik, melalui analisis histologi, analisis sinaran penyebaran tenaga (EDX) dan analisis qPCR. Filem elektro P(3HB-*ko*-3HHx)/SF telah memberi kesan dalam meningkatkan ekspresi gen penanda osteogenik untuk fosfat alkali (ALP) dan osteokalsin (OCN) dengan masing-masing 1.6 dan 2.8 kali ganda selepas 21 hari induksi. Kesimpulannya, filem elektro P(3HB-*ko*-3HHx)/SF telah meningkatkan bioserasi dengan membantu perlekatan, pertumbuhan dan pembezaan osteogenik sel-sel tunjang mesenkima tali pusat. Hasil kajian ini menunjukkan bahawa filem elektro P(3HB-*ko*-3HHx)/SF berpotensi menjadi perancah biobahan untuk kejuruteraan tisu tulang.

BIOCOMPATIBILITY OF ELECTROSPUN *Bombyx mori* SILK FIBROIN WITH POLY(3-HYDROXYBUTYRATE-*co*-3-HYDROXYHEXANOATE) AS A BIOMATERIAL FOR TISSUE-ENGINEERED SCAFFOLDS

ABSTRACT

Polyhydroxyalkanoates (PHAs) are polyesters produced by microorganisms under unbalanced growth conditions. They are well documented as a biodegradable and biocompatible polymer, making them attractive as biomaterials for applications in tissue engineering. Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx) is one of the members of the PHA family that has shown superior mechanical properties and processability compared to poly(3-hydroxybutyrate) [P(3HB)] and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [(P(3HB-*co*-3HV)]. However, the biocompatibility of P(3HB-*co*-3HHx) is still inadequate because of its hydrophobic properties that has hampered its direct use as scaffold material. In this study, silk fibroin (SF) derived from *Bombyx mori* cocoons, which is a widely used and studied protein polymer for biomaterial applications have been used to improve the cell affinity and biocompatibility of P(3HB-*co*-3HHx). Non-woven fibrous scaffold was prepared via electrospinning technique from the blends of P(3HB-*co*-3HHx) and SF. Blends of electrospun P(3HB-*co*-3HHx)/SF films with different ratio have been fabricated and characterized. Biocompatibility study was performed on human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) using Presto Blue cell viability assay, trilineage differentiation and gene expression. The incorporation of SF into P(3HB-*co*-3HHx) had increased the hydrophilicity and decreased the average fibers diameter. Electrospun P(3HB-*co*-3HHx)/SF film with blending a ratio of 1:3

showed the highest cell viability after 28 days of cultivation compared to either electrospun P(3HB-*co*-3HHx) film or electrospun SF film. The trilineage differentiation study of hUC-MSCs on electrospun P(3HB-*co*-3HHx)/SF film revealed that hUC-MSCs showed preferential differentiation into the osteogenic lineage, confirmed by histological analysis using alizarin red staining, energy dispersive X-ray (EDX) and quantitative real-time PCR analysis (qPCR). The electrospun P(3HB-*co*-3HHx)/SF film increased the expression of hUC-MSCs osteogenic marker genes for alkaline phosphatase (ALP) and osteocalcin (OCN) by 1.6-fold and 2.8-fold, respectively, relative to control tissue culture plate, after 21 days of osteogenic induction. In conclusion, electrospun P(3HB-*co*-3HHx)/SF film improved the biocompatibility properties in term of cellular adhesion, growth and proliferation and osteogenic differentiation of hUC-MSCs. The results from this study suggest that electrospun P(3HB-*co*-3HHx)/SF film is a promising biomaterial for bone tissue engineering.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Tissue engineering-based treatment is a promising approach that combines cells, growth factors and scaffolds to repair and regenerate damaged tissues from disease, injury and trauma (Shin *et al.*, 2003; O'brien, 2011; Rosa *et al.*, 2012). Among the three components, scaffold plays a vital role as the matrix to accommodate cells and guide their growth by providing an appropriate surface and adequate space for cells to adhere, proliferate and differentiate (Hubbell, 1995). Various biomaterials including ceramics, synthetic and natural polymers have been studied and used to fabricate scaffold for tissue engineering purpose (Vacanti & Langer, 1999; O'brien, 2011). The biomaterials are fabricated using techniques such as solvent casting, electrospinning, phase-separation to shape them into desired scaffold structure like film, fibrous, foam, hydrogel and so forth (Dhandayuthapani *et al.*, 2011). The selection of biomaterial and the technique used to fabricate the scaffold will significantly affect cell–scaffold interaction and ultimately tissue formation and function.

Polymers are widely used in various medical applications such as in the fabrication of tissue engineering scaffolds, drug delivery matrixes and implantable devices (Dhandayuthapani *et al.*, 2011; Wei & Ma, 2014). The most important criterion that must be fulfilled by a polymer for it to be applied as biomaterial is biocompatibility- whereby the polymer must not induce adverse tissue reactions (Vacanti & Langer, 1999; Nair & Laurencin, 2007). Besides, the polymer must be formable into various shapes either by molding, extrusion or 3D printing.

Polyhydroxyalkanoates (PHAs) are a family of microbial polymer that is synthesized and accumulated in the cell cytoplasm as carbon storage compound (Anderson & Dawes, 1990). They are well known for their biodegradability and biocompatibility, making them attractive biomaterial for tissue engineering (Williams *et al.*, 1999; Chen & Wu, 2005). Among them, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] possesses superior mechanical properties and processability compared to other widely studied PHAs such as poly(3-hydroxybutyrate) [P(3HB)], poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] (Doi *et al.*, 1995b; Martin & Williams, 2003). Despite PHA showing some potential for drug delivery and medical uses, PHA's characteristics such as the hydrophobicity properties of PHAs (Mei *et al.*, 2006), lack of recognition domains for cells (Yang *et al.*, 2011) have limited their interactions with cells, which is not suitable to be utilised as scaffold biomaterial for tissue engineering application. Hence, much effort has been channelled towards improving the surface properties of PHA which include, grafting (Grande *et al.*, 2017), coating (Li *et al.*, 2015), blending (Qu *et al.*, 2006c), and treated with sodium hydroxide solution (Li *et al.*, 2005a).

Silk fibroin (SF) derived from *Bombyx mori* cocoons, is a natural polymer and a promising biomaterial because of its excellent biocompatibility, slow rate of degradation *in vivo* and impressive mechanical properties in term of strength and toughness (Altman *et al.*, 2003; Numata & Kaplan, 2010). Previous studies have shown that the surface coating of P(3HB-*co*-3HHx) with SF improves the biocompatibility of P(3HB-*co*-3HHx) (Mei *et al.*, 2006; Xie *et al.*, 2008; Sun *et al.*, 2009; Yang *et al.*, 2010; Yang *et al.*, 2011). Thus, SF has the ability to modify the surface properties of P(3HB-*co*-3HHx).

Electrospinning technique is a simple and effective method to make nano to micro-scale fibers with the help of electrostatic force (Subbiah *et al.*, 2005). The distinctive properties of electrospun films such as high surface area to volume and high porosity with interconnected structure make the electrospun film an ideal scaffold structure. In addition, electrospun films with nanoscale diameters mimic morphological nano features of native extracellular matrix (ECM) and will be able to provide contact guidance for the attached cells (Li *et al.*, 2002).

Recent advances in stem cell research have brought about the possibility to regenerate damaged tissue by tissue engineering approach. Among the variety of stem cells, mesenchymal stem cells (MSCs) are an attractive candidate for tissue engineering due to its multilineage differentiation potential, homing capability and immunoregulatory functions. MSCs are adult stem cells which can isolate from various body tissues such as umbilical cord. The umbilical cord is a new source to isolate MSCs nowadays due to its relatively easy and non-invasive isolation method. Moreover, the usage of MSCs derived from the umbilical cord does not raise ethical issues as it was once treated as biological waste after birth. (Troyer & Weiss, 2008).

Among various PHA surface modification methods, blending is the easiest and straightforward method. Several research groups have been investigated the blending of P(3HB) (Lei *et al.*, 2015), P(3HB-*co*-3HV) (Paşcu *et al.*, 2013; Karahaliloğlu, 2017) with SF; however, there is no report about the biocompatibility and differentiation potential of multipotent MSCs when grown on electrospun P(3HB-*co*-3HHx)/SF films.

In this study, non-woven fibrous scaffolds were prepared via electrospinning technique from blends of these two natural polymers: P(3HB-*co*-3HHx) and SF. The

resulting electrospun films were characterized and the biocompatibility of the electrospun films was evaluated by morphology, growth and proliferation of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs). Besides that, the trilineage differentiation potential of hUC-MSCs on the electrospun P(3HB-*co*-3HHx)/SF films was further investigated. Since hUC-MSCs have huge potential in regenerative medicine application, therefore, this study fulfils the gap of knowledge for the biocompatibility and differentiation potential of hUC-MSCs on electrospun P(3HB-*co*-3HHx)/SF films in order to be applied as tissue-engineered scaffold.

1.2 Research objectives

The objectives of this study were:

1. To fabricate electrospun P(3HB-*co*-3HHx)/SF films by electrospinning technique.
2. To characterize the electrospun P(3HB-*co*-3HHx)/SF films.
3. To study the biocompatibility and differentiation of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) on electrospun P(3HB-*co*-3HHx)/SF films.

CHAPTER 2

LITERATURE REVIEW

2.1 Biomaterials

Biomaterials are defined as a material that is designed to contact with biological systems for the intended method of application and period, which is actively studied for biomedical applications which includes tissue engineering, implant, and medical devices (Piskin, 1995; Dhandayuthapani *et al.*, 2011). One of the main goals in tissue engineering is to develop biocompatible materials, however, until now there is no single biomaterial defined as perfect biomaterial that fully compatible with the living organism (Volova *et al.*, 2017). Therefore, the development of various biomaterials is still going on. In general, biomaterials can be classified into four classes: metals, ceramics, polymers and composites (Piskin, 1995).

Metallic biomaterials have been used as biomaterial for load-bearing application due to their high mechanical strength. Example of metals used in the medical application are stainless steel, cobalt-based alloys, titanium-based alloys that usually used for orthopedic, dental and cardiovascular implants (Ratner *et al.*, 2004; Niinomi, 2008). However, metal implant tends to corrode by chemical or electrochemical action of the body fluid and release the metal ions into surrounding body tissues (Jacobs *et al.*, 1998; Okazaki & Gotoh, 2005; Radenković & Petković, 2018). The released metal ions can cause several consequences, which include, clinical implant failure, cutaneous allergic reactions, osteolysis, and formation of tumor which may need revision surgery (Jacobs *et al.*, 1991).

On the other hand, ceramics cover a wide range of inorganic compositions, mostly is calcium phosphate-based such as hydroxyapatite, tricalcium phosphate and

bioglasses, which is silica-based (Barradas *et al.*, 2011). These class of biomaterial shows inert, anti-corrosive, high to compression and low electrical and thermal conductivity desired properties, making them very suitable as implant material in orthopedics, maxillofacial surgery and dental implant (Sáenz *et al.*, 1999; Thamaraiselvi & Rajeswari, 2004). One of the drawbacks of ceramics biomaterial is poor fracture toughness, which limits their use for load-bearing applications (Davis, 2003).

Considering most metals are vulnerable to corrosion and ceramics are susceptible to mechanical failure, composite that comprise two or more biomaterials are developing to confer more desirable material properties (Salernitano & Migliaresi, 2003). Various types of composite biomaterials have been studied and reviewed for different medical applications, such as stainless-steel screws coated with ceramics to enhance the biocompatibility (Hayashi *et al.*, 1989), ceramics coated with polymer to improve the mechanical properties and bioactivity (Yunos *et al.*, 2008), metal stent coated with drug-containing polymer for drug delivery purpose (Lee *et al.*, 2015). Composite biomaterials are not restricted to coating one biomaterial upon another biomaterial, but solvent-based blending, grafting and melt extrusion procedures also have been used to fabricate composite biomaterials (Azevedo *et al.*, 2003; Mathieu *et al.*, 2006; Tanase & Spiridon, 2014).

Lastly, polymer is the most diverse class of biomaterials and widely applied in the fabrication of tissue engineering scaffold and medical devices (Piskin, 1995; Dhandayuthapani *et al.*, 2011). Polymeric biomaterials can be classified into natural polymer and synthetic polymer; degradable and non-degradable polymer. Figure 2.1 shows the classification of polymeric biomaterials that commonly used in biomedical

applications. Both natural and synthetic polymer has its advantages and disadvantages. In general, natural polymer is non-toxic, biocompatible and biodegradable; its bioactive properties have made them have better interactions with the cells and improve the cells' performance in the biological system. However, natural polymer possesses poor mechanical strength, complex structure and tends for batch to batch variation due to the production depends on the environment and various physical factors (Vats *et al.*, 2003; O'brien, 2011). In contrast, the synthetic polymer is produced under controlled conditions. Its mechanical and physical properties can be reproduced and tailored for specific applications (Dhandayuthapani *et al.*, 2011). Nevertheless, synthetic polymer lacks bioactivity and the degradation products from the synthetic degradable polymers, such as polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL) and their copolymers, have raised the concern about the potential foreign body reactions and implication to the tissues (Bostman *et al.*, 1990; Taylor *et al.*, 1994; Liu *et al.*, 2006). Besides, the additives like stabilizers, emulsifiers and plasticizers which were added during the polymerization process may leach out from the synthetic polymer and may cause severe side-effects to the host (Piskin, 1995)

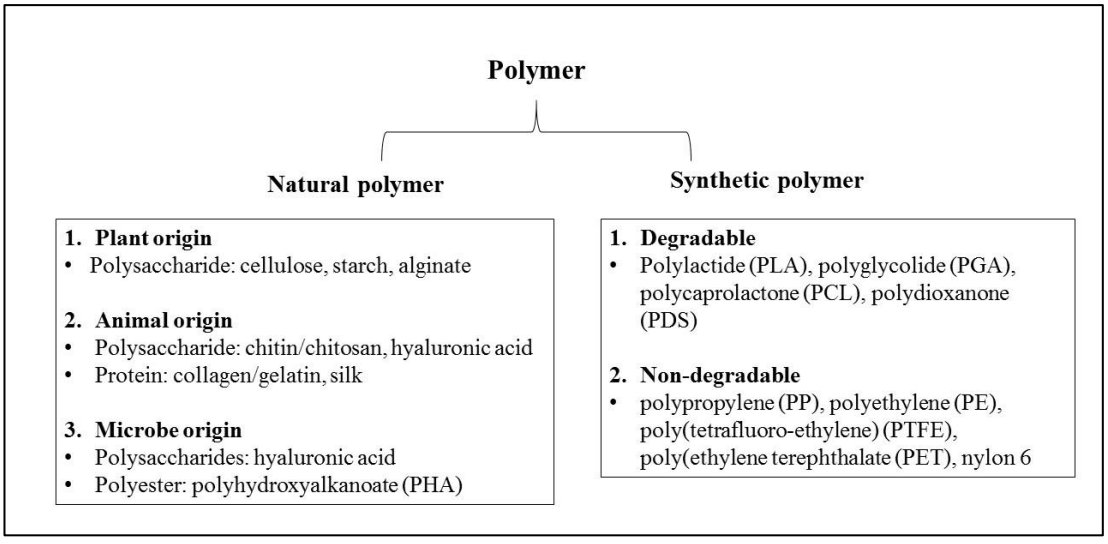

Figure 2.1 Classification of polymeric biomaterials. Adapted from (Ikada & Tsuji, 2000; Cheung *et al.*, 2009)

2.2 Tissue engineering and scaffold

Tissue engineering has emerged as an alternative solution for transplantation and served as a potential therapeutic approach for the damaged or diseased tissue. In the past and present, the damaged tissue is either removed and replaced with synthetic implant or undergoes graft transplantation (Hench, 1998). The source of graft tissue can be either autologous (from one's own body) or allogeneic (from a donor). The challenges associated with autologous grafts involve donor site morbidity, and several incision sites may be required due to the limited amount of tissue available; whereas in the case of allogeneic grafts, it may present with immunological problem, risk of disease transmission (O'brien, 2011) and scarcity of organ donors (Kaihara & Vacanti, 1999).

Tissue engineering is an interdisciplinary field which covers engineering and life sciences to develop biological substitutes to restore tissue function (Langer & Vacanti, 1993). In general, tissue engineering involves isolating the cells, expanding the cells *in vitro*, seeding these cells into the scaffold and inducing them to proliferate and differentiate and lastly implant the scaffold with cells into the injured site. Therefore, scaffold, cells and growth factors are the three key components that usually involved in tissue engineering for successful tissue regeneration (O'brien, 2011).

2.2.1 The general criteria for scaffold

Scaffold is defined as a temporary porous structure that served as artificial extracellular matrix (ECM) to accommodate cells and guide their growth by providing an appropriate surface and adequate space for cells to adhere, proliferate and differentiate (Hubbell, 1995). Scaffold can be fabricated from a variety of biomaterials into various size and structure depending on the purpose and applications area.

However, there are several general criteria should take note when designing scaffold for the use in tissue engineering.

(i) Biocompatibility

Since the ultimate application of scaffold is to be utilized in contact with living tissue, thus, it is crucial that the implanted material must be compatible with living tissue and surrounding physiology environment (Chen *et al.*, 2013). Biocompatibility refers to the ability of the scaffold to support cellular activities like adhesion, proliferation, migration and differentiation. The scaffold also should not induce measurable harm to the host's tissues and organs such as severe inflammatory response that leads to rejection and necrosis (O'brien, 2011).

(ii) Biodegradability

In tissue engineering, scaffold is not intended to function as a permanent implant. It is used as a temporary structure to guide new tissue formation and gradually degrade and replaced by host's regenerated tissue. Therefore, the scaffold should be biodegradable. The degradation products must be non-toxic and able to be removed from the body via metabolic pathways without interference with other organs (Kim *et al.*, 2000).

(iii) Sterilizable

It is vital to keep the scaffold sterile, free of contamination from living microorganisms in order to avoid infections happened associated with *in vivo* use. The scaffold should be able to undergo sterilization process without changing its predefined structure and functionality after sterilization process. Ethylene oxide, gamma-irradiation and electron beams are the common sterilization techniques applied in the current clinical setting (Dai *et al.*, 2016).

(iv) Architecture

The biomaterial intended to design as scaffold should be extrudable and moldable into various shape and structure (Patel & Gohil, 2012). The processability of biomaterial allows the scaffold to have unique features such as three-dimension, large surface area to volume ratio, porous and permeability properties. All of these features are necessary in order to promote cell-in growth, gas permeation, nutrients and metabolite diffusion (Kim *et al.*, 2000).

(v) Mechanical properties

The ability of the scaffold to withstand the *in vivo* forces exerted by the surrounding tissue at the anatomical site is a critical criterion, especially for those scaffolds, is intended to be applied for orthopedic and cardiovascular (O'brien, 2011). Thus, the mechanical properties of the biomaterial should take into account when fabricating scaffold. Besides the mechanical properties, the degradation rate of scaffold and healing rate of the patient also need to optimize and monitor carefully, so that the scaffold can provide structural stability until the regenerated tissues have adequate capability and mechanical integrity to hold itself (Kim & Mooney, 1998).

2.3 Scaffold fabrication techniques

Scaffold architecture design is particularly important in tissue engineering because it can significantly influence the cells' behavior and the properties of the scaffold (Ghalia & Dahman, 2016). Different techniques have been introduced to synthesize the scaffold with different architecture, such as sponges, hydrogel, films and fibers.

Scaffold in the form of sponges are mainly characterized by three-dimension porous structure and can be prepared through gas foaming and emulsion lyophilization

techniques. Gas foaming is the technique that uses gas to create porous structure. The molded polymer is pressurized and saturated with gas, typically carbon dioxide. Following reducing the gas pressure, it caused the gas bubbles to nucleate and create the pores inside the polymer, which can up to 100 μm. Solvent-free is the greatest advantage of this method (Gorth & Webster, 2011). However, the pores created using this technique is close pore and limited interconnectivity (Hutmacher *et al.*, 2014). In contrast, emulsion lyophilization produces porous structure based on the principle of immiscibility between the solvent and non-solvent of the polymer. The homogenous emulsion solution is prepared and poured into a mold, freeze under low temperature and then lyophilize. Both solvent and non-solvent are removed by sublimation process (Whang *et al.*, 1995). The pores produced using this method typically have good interconnectivity (Sultana & Wang, 2012).

Hydrogels are physically or chemically cross-linked three-dimensional polymer network, which can absorb and retain large amounts of water. It is characterized by soft tissue-like properties, flexibility, high swelling potential and high water content (Singh *et al.*, 2016). Therefore, it is widely applied as wound dressing, soft contact lens and drug delivery (Van Vlierberghe *et al.*, 2011). The highly swollen networks which facilitate the permeability for oxygen, nutrients, and water-soluble metabolites also make hydrogel widely studied as scaffold for tissue engineering, for example in the skin (Sun *et al.*, 2011) and cartilage (Holland *et al.*, 2005).

Next, scaffold in film format can be prepared by solvent casting. It is a simple method by dissolving the polymer with a suitable solvent and casting in a mold. If the certain pore size and porosity are required, water-soluble porogen like sodium chloride, sodium citrate, saccharose can be added and leaching out the porogen after

the film was cast. This technique is called particulate leaching (Mikos *et al.*, 1994). The scaffold in film format usually appeared as a thin membrane and lack of interconnectivity (Ghalia & Dahman, 2016).

Fibrous scaffold is characterized with fine fiber structure that gives the properties of large surface area to volume ratio, high porosity and tight pore size. Since the native extracellular matrix (ECM) have the diameter in the range of 50-500 nm, therefore synthesized fiber in nanoscale is important. Self-assembly, phase separation and electrospinning are the three primary techniques available currently to synthesize nanofiber and electrospinning is the most widely study technique (which is discussed in the following section) (Barnes *et al.*, 2007). Self-assembly is an approach to produce fiber based on the spontaneous organization of molecules into an ordered and stable structure mediated by non-covalent bonds, for example, hydrogen bonds, van der Waals forces, hydrophobic and electrostatic interaction (Zhang, 2003). It has been reported the diameter of the fiber produced from this method can obtain around 5-10 nm which is much finer than the diameter of fiber which can be achieved through electrospinning (Ma *et al.*, 2005). Nevertheless, self-assembly involves complex procedures and limited to only a few polymers with specific configurations such as diblock copolymers, triblock copolymers, dendrimers and biomolecules amphiphilic peptides (Barnes *et al.*, 2007).

On the other hand, phase separation is a relatively easy technique compared to self-assembly to produce nanofibers. Phase separation is a process which separates a polymer solution into polymer-rich and a polymer-poor layer which either induced by lowering the temperature or addition of non-solvent to create a gel and subsequently removed the solvent (Nam & Park, 1999). The product which is prepared by this

method usually has sponge-like porous structure or nanofibrous structure. A nanofibrous matrix with fiber diameter of 160 nm, which make up from poly(L-lactic acid) (PLLA) has been reported (Ma & Zhang, 1999).

2.3.1 Electrospinning

Electrospinning is a polymer-processing technology that generates polymer fibers with the aid of electrostatic forces. Electrospinning is a not a novel technology which the history of electrospinning can trace back to more than 100 years ago (Tucker *et al.*, 2012) and the first filed electrospinning patent was in 1902 by J. F. Cooley (Cooley, 1902). However, electrospinning has gained attention only in the 1990s due to the emerge of nanotechnology (Wang & Ryan, 2011).

Figure 2.2 shows the schematic diagram of a typical electrospinning setup which consists of high voltage supplier, a syringe with needle and collector. In order to perform electrospinning process which, to generate fiber from polymer, it is required to dissolve the polymer into solution form. High voltage is then applied to the polymer solution which is loaded into the syringe to provide charge to the solution. The mutual charge in the polymer solution produced the electrostatic repulsive force. Taylor cone is formed when the electrostatic force is equilibrium with the surface tension of the solution, characterized with cone shape of the suspended polymer solution at the tip of the needle (Taylor, 1969). When the applied voltage is increased beyond critical voltage, the electrostatic repulsive force within the polymer solution overcomes its surface tension, and a polymer jet is ejected from the tip of the Taylor cone. While polymer jet travels toward the collector, the charged jet undergoes bending instability which stretches and further elongate the polymer jet and simultaneously evaporates

the solvent. As a result, continuous micro- to nanoscale polymer fibers are formed and deposited on the oppositely charged collector (Doshi & Reneker, 1995).

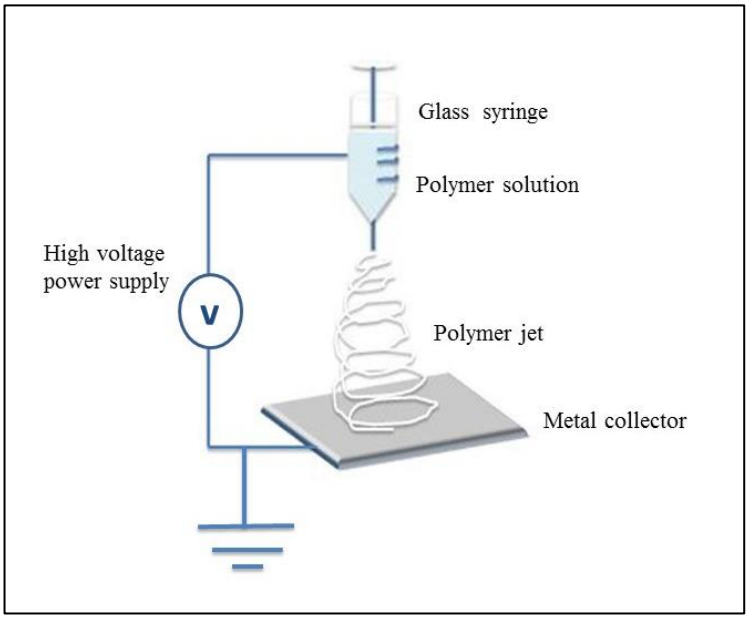

Figure 2.2 Schematic diagram of the electrospinning setup.

Although electrospinning is considered as the most straightforward technique to fabricate the nanofibrous structure, yet there are various parameters that can affect the fiber formation and its morphology. Table 2.1 summarized the parameters which can be divided into three main categories: polymer solution, processing and ambient parameters. In general, the electrospun fibers should have diameter with narrow distribution and absence of beads. Therefore, optimum electrospinning condition must establish in order to obtained desired fibers morphology.

Table 2.1 Electrospinning process parameters (Tan *et al.*, 2005; Ray *et al.*, 2019).

Parameters	**Factor to considered**
Polymer solution parameter	• Viscosity • Concentration • Molecular weight of the polymer • Solvent properties • Surface tension • Conductivity
Processing parameter	• Voltage applied • Flow rate of the solution • Distance of the needle tip to collector • Needle diameter
Ambient parameter	• Temperature • Relative humidity

The main properties of fibrous structure fabricated by electrospinning technique are it can produce ultrafine fibers with high surface area to volume ratio and interconnectivity porous mesh. Electrospinning also is a versatile process, which the fiber diameter and pore size can be modified by adjusting the parameters stated above. The arrangement pattern of the fibers, whether it is randomly oriented or parallel aligned also can be customized by changing the type collector such as static plate, rotating drum (Long *et al.*, 2019). Moreover, different of polymers (synthetic and natural polymer) have been shown able to electrospun to form fibers with diameter ranging from 3 nm to more than 1 μm and the ability of electrospinning to scaled-up for mass production of nanofibers, make it becomes commercial interest in many fields (Huang *et al.*, 2003). Currently, many potential applications of electrospun fibers have been studied such as filtration membranes (Kusumaatmaja *et al.*, 2016), tissue engineering scaffolds (Li *et al.*, 2002), protective clothing (Dhineshbabu *et al.*, 2014), food packaging (Wen *et al.*, 2016) and facial masks in cosmetics application (Fathi-Azarbayjani *et al.*, 2010).

2.4 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are a family of microbial polymer that is synthesized under unbalanced growth conditions. It is well established that many microorganisms can synthesis PHAs and accumulated in the cell cytoplasm to serve as carbon and energy storage compound (Anderson & Dawes, 1990). Figure 2.3 shows the general formula of PHA. PHAs can be made up of various types of monomers. Homopolymer of poly(3-hydroxybutyrate) [P(3HB)] which polymerized form single monomeric unit of 3HB was the first type of PHA being discovered in 1926; later other types of monomer have been reported. To date, approximately 150 different types of monomers have been identified (Steinbüchel & Lütke-Eversloh, 2003). Typically, the monomer can be classified into short-chain-length (SCL, contains 3-5 carbon atoms) and medium-chain-length (MCL, contains 6-14 carbon atoms). Table 2.2 shows some of the PHA monomers that are commonly being studied.

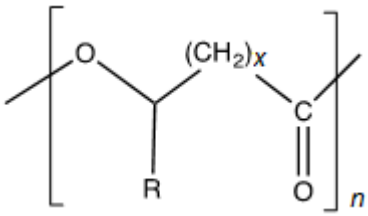

Figure 2.3 The general chemical structure of PHA. R refers to the side-chain (it may contain up to 11 carbons), *x* refers to the number of alkane group on the main-chain, *n* refers to the number of repeating units in the PHA chain. Both R and x determine the type of hydroxyalkanoate monomer unit.

Table 2.2 Various types of hydroxyalkanoate (HA) monomer formed with different R and *x*. (Loo & Sudesh, 2007)

x	R side chain	Type of HA monomer	Monomer size
1	hydrogen	3-hydroxypropionate; 3HP	SCL
	Methyl (CH_3)	3-hydroxybuytrate; 3HB	SCL
	Ethyl (C_2H_5)	3-hydroxyvalerate; 3HV	SCL
	Propyl (C_3H_7)	3-hydroxyhexanoate; 3HHx	MCL
2	hydrogen	4-hydroxybutyrate; 4HB	SCL
3	hydrogen	5-hydroxyvalerate; 5HV	SCL

PHAs are considered as natural polymer and have attracted much interest in the academic and industrial field, due to their physical and mechanical properties resemble commodity thermoplastics such as polypropylene (PP), with an additional plus point of being degradable (Sudesh & Iwata, 2008). Therefore, PHAs are termed as environmentally friendly thermoplastics. However, until today, PHA is still not prevalent in the market due to the high production cost of PHA.

2.4.1 Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)]

The brittleness and high crystallinity properties of P(3HB) which synthesized from a single repeating unit of 3HB, has restricted it to become favourable thermoplastic material. In order to improve the material properties of P(3HB), copolymer P(3HB-*co*-3HHx) is introduced. P(3HB-*co*-3HHx) is a copolymer with the incorporation of 3-hydroxyhexanoate (3HHx) as a second monomer which results in improved physical properties such as lower melting temperature, lower tensile strength, and higher elongation to break than P(3HB) (Doi *et al.*, 1995). The

improvement of the physical properties is primarily due to the longer 3HHx units cannot fit into the crystalline lattice of 3HB and thus avoid isodimorphism, which is co-crystallization of the two monomers as seen in P(3HB-*co*-3HV) (Asrar *et al.*, 2002). As a result, P(3HB-*co*-3HHx) is characterized as a more flexible material with low tensile strength compared to P(3HB) and therefore, P(3HB-*co*-3HHx) have been studied for bulk applications, such as medicine and pharmaceutical (Williams & Martin, 2002), food packaging (Vandewijngaarden *et al.*, 2016), facial oil blotting material (Sudesh *et al.*, 2007). Figure 2.4 shows the chemical structure of P(3HB-co-3HHx), and Table 2.3 shows the properties of PP, P(3HB) and P(3HB-*co*-3HHx).

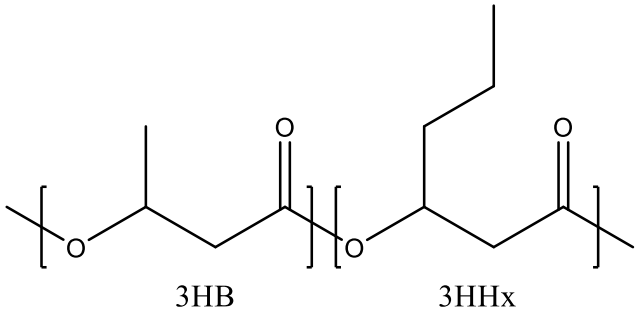

Figure 2.4 Chemical structure of P(3HB-*co*-3HHx).

Table 2.3 Properties of PP, P(3HB) and P(3HB-*co*-3HHx) (Doi *et al.*, 1995; Tsuge, 2002)

	PP	P(3HB)	P(3HB-*co*-10 mol% 3HHx)
Melting temperature (°C)	176	177	127
Glass transition temperature (°C)	-10	4	-1
Crystallinity (%)	50-70	60	34
Tensile strength (MPa)	38	43	21
Elongation to break (%)	400	5	400

As mentioned above, PHA is biodegradable, and thus the same goes to P(3HB-*co*-3HHx). The degradation process of P(3HB-*co*-3HHx) is mainly through enzymatic degradation (either by extracellular PHA depolymerase or lipase). Shimamura *et al.* and Doi *et al.* have reported solution-cast films P(3HB-*co*-3HHx) was able to be degraded by P(3HB) depolymerase from *Alcaligenes faecalis* at 37 °C, pH 7.4. It was shown that the rate of enzymatic degradation of solution cast film of P(3HB-*co*-3HHx) was higher than P(3HB) due to the lower crystallinity of P(3HB-*co*-3HHx) (Shimamura *et al.*, 1994; Doi *et al.*, 1995).

In the case of *in vivo* degradation study, it was reported there was no remarkable degradation of the electrospun P(3HB-*co*-3HHx) film after 12 weeks of subcutaneous implantation in rats in term of physical appearance and molecular weight (Ying *et al.*, 2008). Therefore, the biodegradation of P(3HB-*co*-3HHx) under *in vivo* condition is consider very slow, unlike other members of the PHA which constitutes of 4-hydroxybutyrate (4HB), 5-hydroxyvalerate (5HV) and 6-hydroxyhexanoate (6HHx) monomer units, that were found to be able degrade by lipase in addition to PHA depolymerase due to simple chemical structure without any side chain that facilitate the action of hydrolytic enzyme (Chuah *et al.*, 2013). However, Wang *et al.* (2004b) have reported P(3HB-*co*-3HHx) has experienced 2-6% of weight loss after incubating in phosphate buffer saline containing 0.1 g/L of lipase for 50 days.

2.4.2 Use of Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] in medical application

One of the extensively studied applications of P(3HB-*co*-3HHx) is for medical and therapeutic tools. The reasons which attract it to be applied in this field are due to the excellent biocompatibility, biodegradability and adjustable physical properties, by

varying 3HHx monomer composition. P(3HB-*co*-3HHx) also has hemocompatibility properties. In a study carried out by Qu *et al.*, they revealed P(3HB-*co*-3HHx) showed less haemolytic activity and fewer platelets adhesion to their surface, thus, it is promising for blood-contact materials (Qu *et al.*, 2006a). Moreover, the degradation products of P(3HB-*co*-3HHx): oligo(3-hydroxybutyrate) (OHB) and oligo(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (OHBHHx) have been proved are non-toxic to mouse fibroblast L929 and murine beta cell line NIT-1 (Sun *et al.*, 2007; Yang *et al.*, 2009). Examples of the potential applications of this polymer in the medical field include drug carriers and scaffold in tissue engineering which have been evaluated in both *in vitro* and *in vivo* (Bhubalan *et al.*, 2011; Chang *et al.*, 2014).

Various studies have been carried out to use P(3HB-*co*-3HHx) as drug carrier for controlled drug release. For example, Lu and co-workers have formulated the P(3HB-*co*-16% mol 3HHx) into nanoparticles and microparticles and entrapped with 5-fluorouracil, an anticancer drug into these particles (Lu *et al.*, 2010). Besides, Peng and the team have actively studied the sustained delivery system of insulin to reduce the frequency of insulin injections for diabetic patients. They reported that hydrophilic insulin could be encapsulated into hydrophobic P(3HB-*co*-3HHx) through phospholipid complex (Peng *et al.*, 2012). They further developed thermosensitive hydrogel which loaded with these insulin-phospholipid loaded P(3HB-*co*-3HHx) nanoparticles, which are injectable and slow release of insulin. This system showed promising results which it extended the hypoglycaemic effect for more than five days following single injection into diabetic rats (Peng *et al.*, 2013).

P(3HB-*co*-3HHx) have also demonstrated their potential as tissue engineering scaffold in both soft and hard tissues regeneration, which include bone (Wang *et al.*,

2004a), cartilage (Li *et al.*, 2015), tendon (Lomas *et al.*, 2012), nerve (Bian *et al.*, 2009; Xu *et al.*, 2010) and eyelid reconstruction (Zhou *et al.*, 2010). These show P(3HB-*co*-3HHx) is a biocompatible material.

2.5 Silk

Silk referred to as a fibrous protein that is externally spun into fibers by some living organisms such as silkworms, spiders, caterpillar, mites and flies (Altman *et al.*, 2003). Generally, silk is produced by the specialized glands inside the body and functions differently in nature; for example, spider webs are for predation; silkworm cocoons are for protection. Moreover, the silks produced by different organisms have different amino acid composition and thus exhibits different properties (Holland *et al.*, 2019).

2.5.1 *Bombyx mori* silk fibroin (SF)

Sericulture, which originated in China, has been practice growing the mulberry leaves to feed the silkworms for silk production for textile application over 5000 years (Kaplan *et al.*, 1997). Silkworm is the larva of the *Bombyx mori* moth, due to sericulture, nowadays the *B. mori* are no longer live in wild and flightless and these silkworms referred to as domesticated silkworms. *B. mori* silkworms synthesized the silks in the silk glands and secreted it at the end of the fifth instar of the larvae stage. It secreted and spun the silk fibers around its body and form cocoon, as shown in Figure 2.5 (A) (Hsueh & Tang, 1944). It is worth mentioning the silkworm silk produced by *B. mori* is one of the most well-studied silks, other than spider silks (*Nephila clavipes* and *Araneus diadematus*) (Altman *et al.*, 2003).

There are two proteins can be found from the single strand of silk fiber secreted by *B. mori*, namely, sericin and fibroin. Sericin (20-310 kDa) is a hydrophilic protein which made up 25-30% of the mass of silk cocoon. It acts as surface coater for the fibroin and holds the two fibroin strands together, as shown in Figure 2.5 (B) (Vepari & Kaplan, 2007). On the other hand, fibroin comprises heavy chain (325 kDa) and light chain (26 kDa) which linked by a disulfide bond and responsible for load-bearing capacity of the silk. The heavy chain exhibited as block copolymer, which contains hydrophobic domain alternate with hydrophilic domain. The hydrophobic domains are characterized with repetitive amino acid sequence which comprises mainly by three short side-chain amino acids: glycine (G), alanine (A) and serine (S), that can self-assembly into anti-parallel β-sheet structure. While the hydrophilic domains are characterized by more complex sequence which consists of larger side-chain amino acids and charged amino acids (Wang *et al.*, 2006b; Koh *et al.*, 2015).

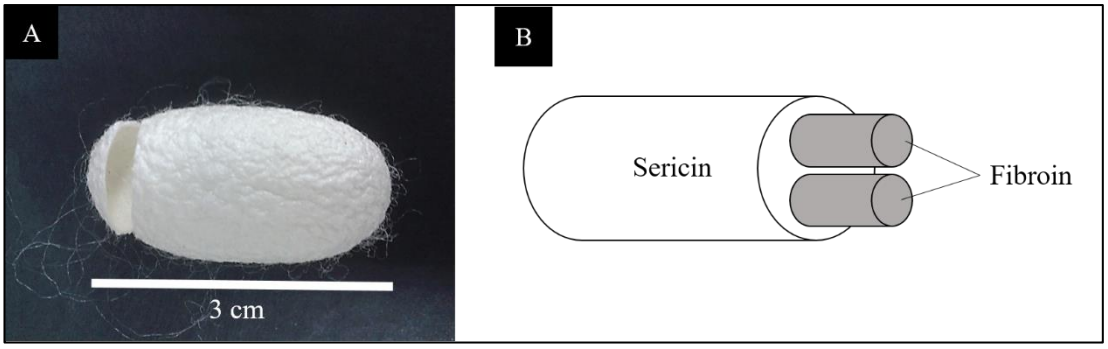

Figure 2.5 (A) Image of *Bombyx mori* silk cocoon. (B) Schematic illustration of silk fibers produced by *B. mori* silkworms. The silk fiber is composed of two fibroin fibers held together by sericin.

Today, silk fibroin (SF) not only used in the textile industry but have been extensively used in biomedical applications since SF is approved by Food and Drug Administration (FDA) as surgical suture biomaterial (Bettinger *et al.*, 2007). The sericin is usually removed by degumming process due to sericin can induce allergic response to the host (Altman *et al.*, 2003). Upon sericin removal, it was reported the

in vivo inflammatory responses of SF were comparable to commonly used biomaterials such as polylactic acid (PLA) and collagen (Meinel *et al.*, 2005).

SF is also attractive for its mechanical properties in term of strength and toughness. Table 2.4 showed the comparison of mechanical properties of *Bombyx mori* SF with collagen and PLA. It has been reported that the tensile strength and Young's modulus of *B. mori* SF (after removing sericin) are higher than collagen and PLA.

Table 2.4 Mechanical properties of *Bombyx mori* SF, collagen and PLA (Altman *et al.*, 2003).

Material	Tensile strength (MPa)	Young's modulus (GPa)	Elongation at break (%)
Bombyx mori SF	610-690	15-17	4-16
Collagen	0.9-7.4	0.0018-0.046	24-68
PLA	28-50	1.2-3.0	2-6

SF is demonstrated as slowly degradable biomaterials, although according to United State Pharmacopeia's definition, silk is classified as non-degradable due to silk suture maintain 50% of its tensile strength after 60 days *in vivo* implantation (Moncada-Saucedo *et al.*, 2019). It is reported that silk fibers commonly will lose their tensile strength within one year and fail to recognize at the site within two years (Altman *et al.*, 2003). This slow degradation property of silk is useful for tissue engineering application because it is able to retain its strength over a long time while gradual transfer load-bearing burden to the developing tissue (Horan *et al.*, 2005; Kundu *et al.*, 2013).