Development of silk fibroin tissue engineering scaffolds via indirect additive manufacturing for cartilage regeneration

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DEVELOPMENT OF SILK FIBROIN TISSUE ENGINEERING SCAFFOLDS VIA INDIRECT ADDITIVE MANUFACTURING FOR CARTILAGE REGENERATION

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List of Acronyms

3D	3-dimensional
3DP	3-dimensional printing
ABS	Acrylonitrile butadiene styrene
ACI	Autologous chondrocytes implantation
ACM	Acellular matrices
ACT	Autologous chondrocytes transplantation
AVA	Agri-food and Veterinary Authority
AM	Additive manufacturing
AMem	Human amniotic membrane
CAD	Computer aided designs
CaP	Calcium phosphate
COLI	Collagen type I
DBRP	Desktop-based RP
DNA	Deoxyribonucleic acid
DOD	Drop-on-demand
ECM	Extracellular matrix
ESR	Equilibrium swelling ratio
FDA	Food Drug Administration
FDM	Fused deposition modelling
FTIR	Fourier transform infrared spectroscopy
GFs	Growth factors
HA	Hydroxyapatite
HyA	Hyaluronic acid
HLE	Human limbal epithelial
IHC	Immunohistochemical
LDFM	Liquid-frozen deposition manufacturing
MMII	ModelMaker II TM
MMP	Metalloproteinases

MSCs	Mesenchymal stem cells
MRI	Magnetic resonance imaging
NaOH	Sodium hydroxide
OA	Osteoarthritis
OCT	Optical coherence tomography
PBT	Poly(butylenes terephthalate)
PCL	Poly-caprolactone
PDLLA	Poly (D,L-lactide)
PEEK	Poly(etheretherketone)
PEG	Poly(ethylene glycol)
PEGT	Poly(ethylene glycol)-terephthalate
PEMA	Poly (ethyl methacrylate)
PEO	Polyethylene oxide
PGS	Poly(glycerol sebacate)
PGs	Proteoglycans
PHBV	Poly(hydroxybutyrate-co-hydroxyvalerate)
PLGA	Poly (lactic-co-glycolide acid)
PLLA	Polylactic acid
POC	Poly(1,8 Octanediol-co-Citrate)
PU	Polyurethane
PVA	Poly-vinyl alcohol
PVP-I	Povidone-iodine
RP	Rapid prototyping
NaCl	Sodium chloride
SEM	Scanning electron microscopy
SF	Silk fibroin
sGAG	Sulphated glycosaminoglyans
SLS	Selective laser sintering
TCP	Tri-calcium phosphate

TE	Tissue engineering
TMJ	Temporomandibular joint
UV	Ultraviolet

Abstract

The development of cartilage tissue scaffolds in both craniofacial and orthopaedic surgeries still remains a great challenge in tissue engineering (TE). The architecture of scaffold constructs is one of the important criteria required for cartilage TE as it meets aesthetics and functional purposes in craniofacial and orthopaedic surgeries respectively. TE has been explored in recent years to create desirable cartilage substitutes so as to circumvent the limitations faced in the usage of autogeneous implants and allografts. TE approaches provide an opportunity to construct tissue scaffolds with geometrical properties and mechanical integrities that are comparable to that of native tissues. A viable TE scaffold should include the presence of adequate morphologies to allow optimal cell attachment and mass transportation of essential nutrients. Moreover, the scaffold should provide sufficient physical support for tissue ingrowth and degrade at a comparable rate with the regenerated tissue.

Vast varieties of methodologies have since been developed to produce TE scaffolds addressing connective tissues as well as organs. One of the viable approaches includes additive manufacturing (AM) which allows complete user control over the architecture of the scaffold construct. Commercialised AM systems have been widely explored for TE purposes and a main limitation of the direct AM applications include the difficulty to process naturally-derived biomaterials such as proteins and polysaccharides. High temperature and use of toxic solvents are factors that contribute to the disadvantages of direct AM methods. Conversely, indirect AM techniques allow the use of naturally-derived materials and provide user-defined control through the use of a sacrificial mould. The present study proposed a scaffold fabrication technique which involves the application of solvent-casting (a conventional TE approach) on top of the AM-based inkjet printing technique to yield TE scaffolds with both macro- and micro-architectural features. The formation of macro-channels aims to facilitate cell migration towards the interior of the scaffold while the presence of micro-pores serves as anchorage sites for cell attachment, proliferation and extracellular matrix (ECM) secretion.

The main objective of this project is to fabricate silk fibroin (SF) scaffold using an indirect AM approach and investigate the viability of the scaffold for cartilage TE. SF, a poly amino-based biopolymer extracted from the Bombyx Mori cocoons, has been

widely used for biomedical applications, owing to its superior biocompatibility and mechanical properties. In this study, SF protein was used in its regenerated form in aqueous state to produce SF foams of varying concentrations for the assessment of its mechanical characteristics and biodegradability. Subsequently, simple empirical models were formulated based on experimental results to provide a good basis for the identification of appropriate SF concentration for specific TE application. The TE potential of SF protein was further verified using permeability theory and biodegradation model. The factors that affect the permeability of SF foams were elucidated, while the biodegradation model enables the identification of erosion mode and the prediction of biodegradation life-time of a specimen. The theories were further verified by *in vivo* studies on nude mice. From the experimental observations, a suitable SF concentration was chosen and used for the fabrication of TE scaffold.

Two processes of inkjet printing were analytically modelled in this work to develop further understanding on the operational mechanics of the AM technique. The impaction of a single droplet on a solid surface was first studied. The dynamics of the droplet impact was modelled using energy conservation principles and equations to determine the maximum spreading ratio of droplet upon impact on a surface. The mechanics of line formation via printing was next being modelled. A thermodynamics approach was used to study the coalescence between two deposited droplets and predict the critical time at which the droplets could no longer merge as a result of cooling effect. The coalescence of droplet and its subsequent spreading were also evaluated to be influenced by the effects of drawback which would result in the formation of discontinued line.

Finally, SF TE scaffolds, with both macro- and micro- sized morphologies, were produced using the proposed indirect AM technology with the preservation of the protein's intrinsic properties and without inducing cytotoxicity. The quantitative results obtained from *in vitro* cell cultures demonstrated the TE constructs as suitable templates for chondrocyte attachment and proliferation. This observation was verified visually from the histology of the specimens after a prolonged period of cultivation. To further elucidate the TE potential of SF scaffolds, the constructs were implanted into nude mice models. The *in vivo* work revealed that the SF scaffolds promoted cartilage regeneration. Based on the immunohistochemical analysis, the key cartilage components such as the sulphated glycosaminoglycans (GAGs) and collagen type II

were observed and vascularization networks were found to permeate within the TE constructs.

Chapter 1 - Introduction

1.1 Background

Cartilage tissues serve as important musculoskeletal components to meet the aesthetics and functional needs in both craniofacial and orthopaedic surgeries. In the craniofacial or head and neck regions, cartilages are found in the external ear (auricular), temporomandibular joint (TMJ) and nose (nasal septum) respectively [1-3]. The auricular and nasal septum cartilages provide defined shapes for aesthetic purposes while the TMJ cartilages are known to have moderate weight-bearing capabilities for effective masticatory actions. In orthopaedics, articular cartilages provide low-friction gliding surfaces that are essential for smooth functional movements while distributing the bearing load evenly across the relevant joint [4, 5].

Defects of cartilages in the orthopaedic and craniofacial surgeries can occur as a result of trauma, cancer, congenital abnormalities or degeneration. At present, the degeneration diseases of joints, such as osteoarthritis (OA) have emerged as a major health concern in developed countries [6, 7]. The projected increase in head-and-neck malignancies and incidences of microtia, representing cosmetic and functional disorders in craniofacial surgery, has indicated a surge in demand for therapies to regenerate or replace musculoskeletal tissues [8].

Cartilages intrinsically have limited self-repair capacity due to its avascular nature and sparse population of cartilage cells (chondrocytes) within the extracellular matrix (ECM) [9-11]. Unlike the other musculoskeletal tissues such as muscles, tendons and ligaments, cartilages experience a slow matrix turnover and the recovery outcomes were generally reported to possess inferior functional properties compared to the host tissue. Existing treatments for cartilage ailments include the substitution of impaired native tissue with autogeneous tissue, autologous cells or alloplastic implant [12]. The harvesting of autogenous tissues remained the gold standard for reconstruction of craniofacial cartilage [13]. Cartilages from low-weight bearing sites such as rib cartilages are often used to replace the native tissue. Nevertheless, this treatment approach may lead to disadvantages such as donor-site morbidity, surgical complexity, graft rejection and rapid resorption of the graft tissue [14-16]. In cases of congenital microtia, ear reconstruction with autogenous rib cartilage has been widely accepted

and was reported to improve the psychosocial outcomes of microtic patients [17]. However, due to heavy scarring at operative sites, lack of regrowth of healthy normal tissues and undesirable blood circulation, secondary revisions are usually performed to eliminate these unfavourable results [18]. For adult patients, the rib cartilages are often calcified and brittle, thus posing challenges in shaping into a fine cartilaginous framework of the ear [19]. In orthopaedics surgery, autologous chondrocytes implantation (ACI) with a periosteal graft is one of the treatments for cartilage injuries [10]. Despite showing encouraging results, the reliability of cartilage formation from the treatment still remain questionable. Aesthetics alloplastic implants approved by the Food and Drugs Administration (FDA) are composed of silicon or polyethylene [20]. These devices do not induce toxicity, elicit minimal foreign body responses and possess adequate mechanical support for low load-bearing craniofacial tissues. Knee injuries associated with diffused cartilage defects require partial or total knee replacements with metal, polymeric or ceramic-based alloplastic implants while minor osteoarthritis conditions could be treated by synthetic fillers [6]. Although alloplastic implants do not lead to donor site morbidity and are available easily, possible risks of using them include: high rate of complications such as inflammation, erosion, infection, chronic irritation, dislodgement and extrusion after prolonged period of implantation and sometimes even carcinogenicity [12].

Tissue engineering (TE) has provided a revolutionary possibility for the treatment of cartilage ailments faced in craniofacial and orthopaedic surgeries. TE is defined as a multi-disciplinary field which integrates the principles of engineering and life sciences to provide patient-specific biological substitutes that could replace or circumvent the existing clinical solutions [21]. One of the approaches to TE includes the development of a temporary physical support or scaffold for the healing of injured and defective tissues. In contrast to alloplastic implants, the aim of using TE scaffolds is to provide a biomimetic environment for the regeneration of neo-tissues. The scaffolds are biodegradable and their structural features are generally fabricated to (1) serve as an anchorage to cells, (2) provide the relevant biological signals for the secretion of tissue-specific ECM, (3) contribute sufficient mechanical support for tissue growth, and (4) assist in tissue remodelling [22, 23]. Occasionally, the topographies of the scaffold constructs are used to trap and deliver growth factors timely so as to simulate

cellular growth, proliferation and cellular differentiation [24]. The biodegradability of the scaffolds is also required to match the rate of tissue formation [25, 26].

The fabrication process of tissue scaffolds can be grouped into two categories: conventional and advanced TE approaches. Conventional techniques include electro-spinning, thermal induced phase separation, particulate leaching and gas foaming that involve laborious efforts to create a scaffold construct [27]. These methods are known to pose several limitations. Firstly, it is difficult to control the pore size, pore geometry and spatial distribution of pores for the facilitation of cellular processes and the distribution of mechanical load when implanted into the defect site [28]. The pores present within scaffolds are random, thus leading to inconsistencies in the scaffold architecture. The partial pore interconnectivity within the scaffold network could restrain the supply of nutrients and ingrowth of tissues into the deeper regions of the TE construct. Therefore, additive manufacturing (AM) technologies are viable alternatives to the fabrication of TE scaffolds due to its flexibility in controlling pore size, porosity and interconnectivity through computer aided designs (CAD). AM is a manufacturing know-how that is used in the automobile and aerospace industries. The manufacturing technology builds state-of-the-art physical models in a layer-by-layer manner upon the input of a CAD file [29]. Selective laser sintering (SLS), 3D printing, stereolithgraphy, fused deposition modelling (FDM) are the existing AM techniques used for the manufacturing of TE scaffolds [30-33]. Except for the preliminary preparation of raw materials, the entire fabrication is automated thus requiring lesser laborious effort as compared to conventional methods. Moreover, AM is capable of fabricating constructs with regular and reproducible architecture, and design customization could be established through the manipulation of the CAD file. CAD files from imaging modalities such as magnetic resonance imaging (MRI) and optical coherence tomography (OCT) could be incorporated to obtain an exact replicate of patient's native tissue which are often geometrically complex [34, 35]. Recently, data retrieved from these modalities were assessed for the design optimization of scaffolds to its physiological performance when implanted; material choice(s), mechanical properties and geometry of the tissue are taken into consideration to ensure adequate functionality of the biological substitutes.

AM has been equipped with precise control over the geometrical features of the resultant constructs as well as meeting the functional requirements of TE scaffolds.

However, the processing conditions for the fabrication of TE constructs directly on the building platform are often harsh. The application of high temperature during the manufacturing process discouraged the use of thermosensitive materials such as naturally-derived proteins and hydrogels. As a result, the material choices for AM techniques are limited to synthetic polymers that require toxic solvents to convert them to molten or aqueous state before beginning the manufacturing process. Conversely, indirect AM eliminates the use of harsh conditions, thereby offering a wider range of materials choices [36]. The indirect AM technology involved the casting of aqueous biomaterial into a negative mould derived using an AM method. The mould is subsequently dissolved using non-toxic solvents. Although the indirect approach requires an additional step on top of direct AM, the former technique is able to integrate both conventional and advanced TE fabrication knowledge. The developed TE constructs through the combination of techniques acquires an exact replicate of the shape of the native tissue, interconnected macro-channels for the mass transportation of essential nutrients and removal of metabolic waste, and pores in micro scale that are essential for the entrapment of cells and secreted ECM [37]. The mild processing conditions also allow natural biopolymers such as collagen, gelatin and composites consisting of both synthetic and naturally-derived materials to be used [38, 39]. The hydrophilic properties of proteins have been widely known to promote cell adherence and tissue remodelling when swelled to a desirable percentage.

Silk fibroin (SF), extracted from the cocoons of *Bombyx Mori* silkworm, is composed of highly repetitive homogenized amino acid sequences. The protein-based biopolymer is biocompatible and possesses superior mechanical characteristics as compared to existing protein-based TE materials. SF is also thermally stable for sterilisation via autoclave and has been used for biomedical applications in its native form or regenerated as films, foams or electro-spun fibres. Native and regenerated SF fibres have been used as sutures and weaves as meshes for the replacement of ligaments and tendons, while SF films and foams have shown potential in the treatment of ocular surface disorders, bone and cartilage ailments [40-42]. An interesting characteristic of SF is its ability to undergo chemical structure conformation transitions when exposed to elevated temperature and non-toxic organic solvents. Literature reviews have revealed extensive SF studies associated with its secondary structure [43, 44].

1.2 Motivation

Cartilage is a unique tissue that does not contain vascular vessels or nerves and thus possess limited self-healing potential. The replacement of impaired cartilages using autogenous tissues and synthetic alloplastic grafts, as discussed in the previous section, can result in unsatisfactory surgical outcomes. The promise of TE for cartilage repair has been emphasized as it illustrates the possibility to obtain scaffolds that are capable simulating tissue regrowth without inducing toxicity. TE also offers the customization of scaffold constructs according to patient's need. The advantageous option thus leads to reduced complexity during surgery and hasten the recovery process.

ACI is commonly performed on TE scaffold before implantation into the defective site. This is because of the low availability of chondrocytes present within the native cartilage and to minimise the invasion of non-cartilage cells within the construct [45]. ACI is also used to prevent the need for immunosuppressive therapy. In this procedure, cartilage bits are retrieved from the low load bearing area and chondrocytes are obtained via enzymatic digestion of the ECM. The autologous cells are subsequently cultured *in vitro* and the scaffolds are seeded with cells when adequate cell number is achieved. As the secretion of cartilage ECM is observed on the pre-seeded TE construct, the specimen is deemed ready to replace the impaired native tissue. It could be inferred that the architecture of the TE scaffold plays a crucial role during the preliminary steps of ACI.

Conventional and advanced TE techniques are employed to produce scaffolds for chondrocyte seeding. However, they do have their advantages and disadvantages. Meshes, fibre bundles and foams derived from conventional TE methods displayed sufficient topographical features or surface morphology for the cell anchorage and cell motility. However, cell growth and regeneration is only concentrated on the peripheries of the TE constructs [36]. This observation is attributed by the non-uniform distribution of pores and the lack of interconnectivity between the pores. As a result, cell migration and mass transport of nutrients are hindered by the deficiency in architectural features. Thus, the healing process is affected while the regenerated cartilage may possess inferior properties as compared to the native tissue. AM technology provides complete user control over the architecture of TE scaffolds. Using this technology, interconnected networks could be introduced into scaffolds so that

spatial tissue growth is acquired. A major limitation of AM techniques is the inadequacy of surface roughness to promote cell adhesion [30]. TE constructs fabricated by additive-based fibre deposition exhibit smooth surfaces due to the rapid cooling after fibre extrusion. The limitation of particle size in laser sintering and printing AM methods is another attributing factor to lower probability of cell attachment.

Naturally-derived materials such as proteins and hydrogels are preferred choices for TE scaffold fabrication. Their ability to absorb water reflects a similar behaviour of connective tissues and the swelling properties has been revealed to facilitate tissue remodelling and maintaining the phenotypic characteristic of chondrocytes [37, 46]. SF is compatible to native tissues due its composure of protein sequences. Despite broad research being conducted to evaluate SF for TE purposes, limited attention is focused on the SF biodegradability and the incorporation of complex architectures into SF specimens for the exploration of further TE potentials [47-49]

A basis of TE scaffold fabrication has been established based on the earlier discussion. It was highlighted that having substantial control over the design of TE scaffold and use of natural biomaterials would be beneficial to the regeneration of cartilage and other musculoskeletal tissues. Thus, this study aims to tissue engineer scaffolds for cartilage repair, in accordance to the highlighted factors. The present work unifies the conventional fabrication method and AM technology to develop SF scaffolds without altering the intrinsic properties of the protein. A series of pathway was established to identify and relate the processing conditions appropriate for the manufacturing of cartilage scaffolds. Firstly, suitable parameters were analysed for the incorporation of channel networks within SF proteins. A 3D inkjet printer was chosen as the AM technology to yield interconnected channels while conventional foam forming method was employed to introduce pores into the interspaces between the channels. The developed SF constructs were subsequently assessed for their biocompatibility under *in vitro* and *in vivo* conditions. Finally, the study concludes with the implantation of the pre-designed SF tissue scaffolds into animal models to examine their potential for cartilage regeneration. The process flow of the proposed work is presented in Figure 1.1.

Figure 1.1 The entire process flow of the proposed research work. The contributions of each TE fabrication method are broken down into different segments.

1.3 Objectives

The objectives of this study are:

- To understand and interrelate the properties of SF protein and determine the optimal micro-porous design for TE applications
- To further analyse the TE potential of SF protein through permeability theory and biodegradation model

- iii. To describe and model the fabrication process of inkjet printing which focuses on droplet impaction and line formation.
- iv. To create a 3D SF construct with macro- and micro-architectural features to facilitate cell migration, mass transport of essential nutrients and cell entrapment respectively. A negative sacrificial mould is built by a 3D inkjet printer in order to introduce macro-channels into the TE construct.
- v. To assess the 3D SF constructs for cartilage TE potential based on *in vitro* and *in vivo* experiments.

1.4 Scope

This project includes:

- i. A thorough literature review on the scaffold requirements for TE applications. Existing scaffold fabrication techniques were also described and compared to each other.
- ii. Establishing a model relating the geometrical, swelling and mechanical properties of SF protein.
- iii. The formulation of analytical models for the evaluation of permeability of porous matrices and illustration on the erosion modes of the biodegradation process. The degradation behaviour of SF protein foams was also illustrated based on *in vivo* examinations.
- iv. Describing the operating mechanics of 3D inkjet printing and fabrication of negative sacrificial moulds using AM technology.
- v. Developing 3D SF constructs with macro- and micro-architectural features by the employment of conventional foam forming method.
- vi. Analysing the fine structure of SF specimens and evaluating the macro- and micro-architectures of the fabricated scaffolds.
- vii. Carrying out *in vitro* and *in vivo* experiments to assess 3D SF scaffolds' biocompatibility and potential in cartilage TE.

1.5 Organization of Report

This report is organized as follows:

Chapter one provides an overview of cartilage ailments in the orthopaedics and craniofacial surgeries and the prevailing treatments for impaired cartilages. The challenges in cartilage repair were highlighted while TE was offered as a viable option to circumvent existing limitations. The motivation, objectives and scope of this work were proposed based on the background description.

Chapter two presents the literature reviews of TE. The review content includes: the requirements of TE scaffolds, biomaterials, fabrication processes of scaffolds and challenges faced in TE. The importance of architectural features in TE scaffolds was emphasized. Different cartilage TE approaches were briefly discussed and related work presented in this study.

Chapter three presents the concentration effects of SF on its properties which include equilibrium swelling ratio (*ESR*), water binding ability and mechanical properties. The empirical models were obtained based on experimental investigation and the findings formed a good basis to evaluate a suitable SF concentration for TE application.

Chapter four formulates a permeability theory that is expressed as a function of the porosity, effective pore size, sphericity of the pores and the fluid path within a SF porous matrix. A model was also presented to elucidate the possible erosion mechanisms occurring during a biodegradation process. The biodegradability of the SF foams was assessed based on *in vivo* observations and the degradation behaviour of SF was illustrated.

Chapter five elaborates on the operating principles of 3D inkjet printing. Detailed theoretical models associated with the AM fabrication process, particularly on droplet impact and line formation, were described.

Chapter six presents the methodology on scaffold characterization. Firstly, cytotoxicity test was carried out to examine if the 3D SF construct induce any toxicity. Fine structural and thermal analyses were used to ensure that the intrinsic properties of SF protein were maintained. SF constructs were subsequently subjected to *in vitro* cultivation using porcine chondrocytes, where the cell isolation and seeding

procedures were clearly elaborated. Cartilage regeneration was assessed based on results obtained from biochemical and immunohistochemical (IHC) assays. Imaging methods such as scanning electron microscopy (SEM), confocal microscopy and histological staining were conducted to verify the quantitative observations. The *in vivo* implantation of 3D SF constructs into nude mice models was conducted and examined.

Chapter seven focuses on the results and discussions of the experiments highlighted in chapter six.

Finally, chapter eight concludes the report and recommends future work for this work. A list of publication resulting from this study was also presented.

Chapter 2 - Literature Review

2.1 Cartilage

Cartilages are soft biological composites consisting of cells (chondrocytes) surrounded by a multi-component matrix. The three main phases in cartilage extracellular matrix (ECM) include collagen, water and proteoglycans. This connective tissue is mainly composed of collagen type II (10-20 % per wet weight) which organized arrangement within the ECM serves to provide as a mechanical support to the entire cartilage framework. Proteoglycans (PG, 4-7 % per wet weight) are complex macromolecules with central long protein core backbones known as hyaluronic acid (HyA) (Figure 2.1). These un-branched polysaccharides are found within the interstitials of the collagen type II network. The HyA backbone is capable of attaching sulphated glycosaminoglycan (sGAG) chains which are crucial elements to bind with water molecules via Van der Waals forces [50]. Approximately 70-80 % of the weight of cartilage is water and its interaction with GAG is capable of withstanding the mechanical forces applied to the cartilage.

Figure 2.1 An illustration of the extracellular matrix (ECM) organization of cartilage [7].

There are three types of cartilage present in adults: hyaline cartilage, elastic cartilage and fibrocartilage [2, 51] (Table 2.1). The types of cartilage at various locations in the

body, such as the articular surface of bones, mandible, nose, ear, trachea, bronchi and intervertebral discs, have distinct characteristics and serve different functions (Figure 2.2). The function of each cartilage type is dependent upon its ECM composition and architecture. For ear and nose, cartilages are required to provide defined shape and flexibility for aesthetic purposes. These two organs are rarely exposed to load-bearing effects and with the presence of elastin content, the auricular cartilages are less vulnerable to degenerative changes. The TMJ fibro-cartilages are characterized to have moderate weight-bearing capabilities for effective jaw movements. In orthopedics, articular cartilages, which are also known as hyaline cartilages, are located at joint surfaces. The presence of the hyaline cartilages serve to provide smooth functional movements by withstanding high load bearing effects, providing a frictionless support and distributing load evenly across the relevant joint. These superior mechanical properties of the articular cartilages are generally attributed to the alignment of collagen type II fibrils within the different zonal regions. However, the degeneration of articular cartilage can result in diseases such as osteoarthritis [52].

Figure 2.2 Different types of tissue needed for reconstruction in head and neck area [8].

Cartilage Type	Composition	Function(s)	Source(s) of cartilage	References
Hyaline cartilage	Numerous cells embedded in a matrix consisting of collagen type II fibrils and sGAGs	<ul style="list-style-type: none"> - Provide smooth functional movements - Withstanding high load bearing effects - Provides a frictionless support and distributes load evenly across the relevant joint 	<ul style="list-style-type: none"> - Knee (articular) - Nose - Larynx - Bronchi 	[53, 54]
Fibrocartilage	Dense bundle of collagen matrix with scattered chondrocytes	<ul style="list-style-type: none"> - Provide structure and rigidity to organs - Moderate weight bearing capacity - Strongest among all cartilage types 	<ul style="list-style-type: none"> - Temporomandibular joint (TMJ) - Vertebral disk 	[3, 51]
Elastic cartilage	Mainly threadlike elastin fibers with chondrocytes located in the interstitials	<ul style="list-style-type: none"> - Helps to define and maintain shape of organ - Great flexibility to withstand repeating bending 	<ul style="list-style-type: none"> - Ear (auricular) 	[14]

Table 2.1 Tabulation of the three cartilage types and their functions.

The properties of cartilage change with depth from the tissue surface, hence resulting in the zonal variation that is contributed by difference in the composition of ECM and tissue organization. The zonal architecture is divided into four zones: superficial, middle, deep and calcified [50] (Figure 2.3). The concentration of chondrocyte is the highest within the superficial surface. The cells appear flatten and aligned to the surface while the collagen fibrils run parallel to one another on the surface. Below the superficial zone is the mid zone. In this zonal region, the cells are lesser, more spherical in shape and randomly orientated. In the deep zone, cell density is the lowest and cells are larger in size. The chondrocytes are also organized in vertical columns.

The concentration of PGs and collagen fibril diameter are the highest and widest in this zone. In articular cartilages, a layer of calcified cartilage forms that provides a good interface between the deep zone and subchondral bone.

Figure 2.3 Organization of hyaline cartilage, showing the superficial, middle, deep and calcified cartilage zones [50].

Despite having distinctive tissue organization and remarkable functions, cartilages possess limited availability for self-repair as they contain minimal traces of blood supply and nerves [55, 56]. Thus, any disrupted cartilage would require surgical option since the tissue is incapable of self-healing. Typical options of treating cartilage ailments include autogenous and alloplastic grafts. Autogenous graft is a cartilage tissue taken from a part of patient's body and the graft is implanted to replace the damaged or diseased cartilage. The surgical approach involving autogenous grafts are recognized as the gold standard for aesthetics reconstruction. Often, costal (rib) cartilage is used as an autogenous graft for the auricular (ear) and nasal septum (nose) reconstruction. The graft can replace the injuries associated with the superficial zone of articular cartilage. The removal of costal cartilage has been reported to cause pain with larger transplant and there are possible risks of pneumonia and thorax deformities [57]. The correction of impaired aesthetic cartilage requires the shaping and assembly of costal cartilage flaps into the relevant organ, indicating the demand for substantial

surgical skills. For the repair of articular cartilage, the procedure is simpler as it only requires the shaping of the autogenous tissue to the worn out cartilage surface. However, since the knee joint experiences load bearing, full restoration of the cartilage architecture is desirable. The autogenous graft approach can be challenged when the patient is a burn victim where abundance of tissues is required. Thus, donor-site morbidity is a major concern. Other disadvantages of autogenous graft include graft rejection and rapid resorption of graft tissue. Surgical complications such as unsatisfactory tissue growth, experience of inappropriate healing pathways can lead to revision surgery. As a result, a higher expenditure is imposed on the treatment and surgical effort on the patient and surgical team respectively. Alloplastic are devices beneficial for cartilage replacement as they eliminate the limitation of autogenous grafts. Firstly, donor-morbidity is avoided. Secondly, the alloplastic grafts are designed and made readily for implantation but they are not “made to measure”. Nevertheless, a risk involved when using alloplastic devices include medical complications, where long-term prescription of immunosuppressant drugs have to be taken to prevent rejection.

The architecture of aesthetic and orthopedic cartilage tissues, which includes their external shape and organization of ECM, is crucial when replacement of the impaired tissue(s) is/are concerned. The ability to regain the organ’s shape, as well as function, would lead to improvements to the key psychological variables such as body image, self-esteem and mental health [58]. The effect of treatment is also a central motivation of osteoarthritis sufferers whose psychological well-being is significantly associated with disability [59, 60]. It can be inferred that any disruptions to cartilage tissues can have a great impact on their intrinsic anatomical performance and subsequently patients’ quality of life. Since cartilages are avascular inherently, cartilage nutrients and metabolism are facilitated by local diffusion and cell-matrix interaction [61]. In addition, with drawbacks faced when using autogenous and alloplastic grafts for cartilage ailment treatment, scaffold-based tissue engineering (TE) has emerged as a viable solution for musculoskeletal tissue replacements and shall be thoroughly discussed in the next section.

2.2 Tissue Engineering

Tissue engineering (TE) is an interdisciplinary field which integrates the knowledge between biomedical sciences and engineering to alleviate or heal diseased or damaged tissues. Scaffold-based TE is an approach which creates a temporary biological substitute to replace the impaired tissue. Tissue scaffolds are generally shaped according to the impaired tissue using a biomaterial. The scaffold construction process takes several factors into consideration, they are: the material type, architecture feature(s) and mechanical properties of the engineered construct. These factors are crucial in the performance of the resultant tissue scaffold as the regeneration of appropriated tissue is desired. Nonetheless, scaffold-based TE strategies are developed to replace of the existing gold standard treatments such as the use of autogenous and alloplastic grafts as discussed in the earlier section. These surgical options require substantial amount of surgical skills and the results achieved can be unsatisfactory.

The rationale of regenerating cartilages with TE scaffolds is to create artificial ECM which is capable of regulating the important cellular behaviors as well as bio-mimicking the native tissue [62]. Cell survival, migration, proliferation and differentiation are the various biological processes which are necessary for tissue regeneration [23]. Besides using tissue scaffolds, acellular matrices (ACM) from cadaveric skin and animal tissues (xenogeneic implants) have obtained satisfactory results as cartilage substitutes [63]. ACMs have been reported to provide a suitable microenvironment for mesenchymal stem cells (MSCs) to differentiate into chondrocytes [64]. However, the decellularization technique involves various reagents and protocols, which are described to be harsh processing conditions leading to denaturation of collagen, loss of sGAG content and biomechanical properties [65, 66]. The possibility of adverse antibody-mediated reaction in xenogeneic tissues has been raised as well. On the contrary, recellularization of the biological scaffold with autologous chondrocytes might potentially encourage the production of sGAG and restore the tissue's function. Skin samples are also tested for bacterial or fungal infections according to the FDA regulations to ensure that the cartilage substitutes do not cause an allergic reaction or inflammatory response. Although the xenogenic tissues can be easily shaped and manipulated, the disadvantages faced using this substitutes include long-lasting edema, inappropriate resorption rates and challenges when repairing larger defects [67].

Macro-aggregates of chondrocytes are autologous cartilage grafts formed by the *in vitro* culturing of isolated cells on a membrane [68]. This method focuses on the development of cartilage substitutes without the use of biomaterials. The aggregation of cells also promotes the 3D culturing of human chondrocytes, thus maintaining the phenotypic expression of cartilage. The engineered chondrocyte macro-aggregate presents longer survival time and good viability [69]. However, poor mechanical stability of the autologous grafts is experienced. It was further suggested that the combination of cell aggregates and a biodegradable support will be a more beneficial strategy for tissue regeneration [70].

2.3 Clinical Applications of Tissue Engineering Scaffolds

Cartilage repair relies on cell-based therapy approaches to induce the regrowth of tissues since it has limited self-repair ability. Autologous chondrocyte implantation (ACI, also known as autologous chondrocyte transplantation (ACT)) is a common cell-based approach employed. The procedures involved in ACI include harvesting of a cartilage biopsy from the patient, isolation of chondrocytes, expansion of cell population and culturing of cells in a medium that will be subsequently implanted in the defective site (Figure 2.4). To date, ACI is clinically used to treat non-traumatic cartilage defects (arthritis) and to repair complex tissues such as osteochondral site [2]. Currently, there exists three generation of ACI in the clinical setting and they can be described as follows:

1. First-generation ACI

Two-stage procedure: cells are mixed with serum-containing suspension and the cell-suspension was then injected into a sutured native tissue flap or glued collagen membrane [6]. The limitations of this ACI technique include cell leakage, inhomogeneity in cell distribution and delamination of the flap or membrane. The hypertrophy of the flap or membrane may require another surgical procedure to remove the excess tissue.

2. Second-generation ACI

Matrix-induced technique: culture-expanded chondrocytes are seeded on matrices such as collagen membranes and implanted into the defect site and fixed with fibrin glue [71]. The matrix is then implanted using minimally invasive arthroscopy.

Although no complications and better clinical outcomes are achieved using this technique, the cells tend to lose their characteristic morphology or “de-differentiate” after culturing in monolayer thus affecting the functionality of the regenerated tissue.

3. Third-generation ACI

3D matrix scaffold: Chondrocytes cultured on 3D scaffold constructs were reported to attain the relevant phenotype after *in vivo* implantation. The physical support offered by 3D constructs serves as a suitable substrate for the production of stable cartilage matrix [72].

Figure 2.4 Schematic representation of cartilage tissue engineering (TE) using autologous chondrocyte implantation (ACI) [62].

The second- and third-generation ACI is currently being researched widely since they are potential strategies to yield functional cartilages. Before clinical applications, standard experimental and clinical procedures and outcome must be standardized. The aspects to be considered include:

1. Cell seeding density

Cell efficiency, which is defined as the number of cells that survive the implantation process, is one of the factors that identifies the optimal seeding density for ACI. The other contributing components include distribution of cells in the defect and the use of cell carriers/scaffolds [73]. For scaffold-based ACI, chemical and physical properties such as cell-biomaterial interaction, pore size and porosity also influence the cell density used [5].

2. Cell multiplication rate

It is critical to attain a desirable amount of cells rapidly during *in vitro* cultivation so that the cell population is large enough for the repair of a major defect. Cell doubling has been examined to be more efficient with the incorporation of growth factors (GFs) [1]. It was recommended that bovine serum should not be used for cell culture since the supplement was recognized as a source for virus and prion contamination.

3. Cell sources and selection

Chondrocytes arise from pluripotent MSCs through a series of differentiation pathways [71]. The cell selected must be renewable and non-immunogenic. The science of bone marrow-derived MSCs for cartilage TE is advancing more rapidly than other types of stem cells (adipose- and embryonic-derived stem cells) [74]. The chondrogenic potential of the cells are also dependent the patient's age; chondrocytes from older patients are metabolically less active *in vitro* [1, 75].

4. Autologous versus allogeneic cells

Autologous cells are often retrieved in limited quantities from cartilage (chondrocytes), blood or bone marrow (stem cells). In addition, the isolation of cells and expansion of cell population is difficult and time consuming respectively [1]. Thus, allogeneic cells can be offered as a viable option to resolve the problem. This approach is suitable for old patients whom harvested cells are characterised with low metabolism. However, the treatment might be susceptible to host rejection and hence require the patient to be constantly on prescribed immunosuppressive drugs.

5. Biomaterials

The purpose of using biomaterial scaffolds is to provide a biologically comfortable environment for the cells to secrete cartilage matrix and temporarily substitute the native tissue. This includes the incorporation of necessary physical and chemical signals to enhance the tissue repair process. The resultant functions of the scaffold constructs are considerably dependent on its fabrication method and the composition of the biomaterial used [75].

Lastly, it should be noted that these attributing factors would be reviewed to ensure they are optimal for ECM simulation of the required cartilage type. The zonal organization of cartilages should be one of the main considerations as well. This is especially important to articular cartilage repair since the osteochondral tissue acts as an interface or mineralized zone between cartilage and bone tissues [22, 76].

2.4 Requirements of Tissue Engineering Scaffolds

Cartilage repair by the implantation of pre-seeded TE scaffold into defective site has been recommended as a viable solution to circumvent the limitations of existing treatment options. However, the ACI technique requires more investigation in order to validate its applicability for cartilage regrowth. The contributions of TE constructs during the tissue healing process should include [54, 77]:

1. To substitute the defective native tissue using an artificial ECM.
2. To provide a mechanically strong framework to support tissue regrowth.
3. To serve as an anchorage site for cells to adhere and proliferate
4. To allow spatial distribution of cells and thus uniform secretion of ECM.
5. To provide adequate cell-to-cell spaces so that nutrients can be supplemented to cells in the deeper regions as well as enhancing tissue growth.
6. To release the necessary biological signals for maintaining the cell phenotype and subsequently tissue remodelling.
7. To degrade linearly with the rate of tissue formation.

Material, physical and mechanical requirements of TE scaffolds (or artificial ECMs) have to be considered specifically for each TE application as the criteria vary according to the targeted tissue or organ [78]. Hence, TE scaffolds should be developed using these basic guidelines:

1. Biomaterials

Biomaterial selection is the fundamental step to achieve a biomimetic environment that is capable of simulating the required tissue components. The range of scaffold materials includes ceramics, polymers, metals, proteins and polysaccharides [23]. The usage of these materials for TE applications has to comply with the FDA guidelines. In order to employ the material for scaffold construction, the chemical composition of biomaterial has to be understood. It is important to understand the structural organization (crystallinity and functional groups) of the material used and thus deciding if it is suitable for treating the targeted defect. After implantation of the scaffold, the biomaterial should not result in unwanted tissue response and must possess the necessary surface chemistry for cell adherence [79].

Under physiological condition, the scaffold material will degrade in a timely manner unless it is specified to be bio-inert. The degradation mechanism is propagated through hydrolysis or enzymatic interactions and the degraded by-products should not induce any side effects or reduce the pH level (autocatalysis) of the implanted site [80]. Any autocatalysis effect can lead to cell apoptosis and disruptions to the newly secreted ECM. The intrinsic mechanical properties of the biomaterial should be adequately stable for the scaffold fabrication process and then TE application. It is preferred that the material can be processed into different shapes and forms so as to meet the requirements of the targeted tissue [54]. Finally, the material must maintain its inherent properties such as melting point and chemical structure after heat treatment or interaction with solvents.

2. Physical properties

Tissues are biological composites which are composed of cells distributed within the ECM network for specific functions. Similarly, TE scaffold aims to provide a temporary substrate that mimics the function of the native tissue. The architecture

of the scaffold has to be designed for uniform distribution of cells and ECM within implanted site [27]. The pore size of the TE construct is required to be at least the average size of the cell that is within the micro scale level (Table 2.2). The pores can also act as “pockets” to store and release biological modifiers such as drugs, growth factors or other bioactive molecules [81, 82]. It is crucial to have high porosity and interconnectivity so that the regenerated ECM has sufficient space to infiltrate and grow. As the biomaterial degrades, the pore volume would increase and be sufficient for the accommodation of cell masses and allow nutrients to diffuse into the central regions of the scaffold. Gradually, vascularization would occur so that hypoxia (inadequate oxygen level) is avoided and the tissues of greater size can survive [83]. However, the vessel formation process completes in the order of weeks and thus leading to nutrient deficiencies and/or hypoxia deeper in the tissue or scaffold. The effects of autocatalysis would be significant when the cellular waste and degradation by-products are not removed.

The issues faced in the inner regions of the implanted scaffold can be prevented by having macro-channels within the scaffold. The channels should be interconnected so that transportation of the nutrients and removal of the metabolic waste can take place [38]. Any manipulation of the width of channels and/or pore size (surface area and pore volume) could alter the mechanical integrity and degradation rate of the tissue scaffold. Moreover, the input of progressive dimensional changes of the channel sizes (also known as functional gradient) along the scaffold had demonstrated to be a good template that closely mimics the bone structure [84, 85]. In orthopaedics applications, it is crucial for TE implants to restore the shape of their native articular cartilage as a dimensional mismatch of 10 % can lead to detrimental loads across joints [86].

3. Mechanical properties

Biomechanical interactions between cells and biomaterial regulate the pathways leading to tissue growth [87]. Tissue scaffolds are required to possess sufficient mechanical stability when implanted as a tissue replacement. This characteristic is exceptionally important for weight-bearing organs as it is undesirable to achieve inferior properties for the regenerated tissue. When subjected to *in vivo* conditions, the scaffold must be able to withstand the physiological forces so that

cellular process and tissue remodelling process can be attained. The ability to resist mechanical forces would allow the scaffold to maintain its dimensional stability for facilitating the cell migration and nutrient transport.

4. Surface texture

The topography or surface roughness of tissue scaffolds is important to the characteristics of the developed neo-tissue [88]. Firstly, the roughness profile of constructs enables the cells to cling on and adhere to the scaffold. Based on this initial step, cell-biomaterial interaction is promoted [89]. Secondly, the topographical features can be used as guides for tissue growth as well as remodelling. The cell-instructive feature of the artificial ECM is also capable of releasing the necessary biological signals to maintain the cell phenotype.

Cell or cellular pathway	Optimal pore size (μm)	References
Neovascularization	5	[90]
Blood vessel ingrowth	250	[91]
Chondrocyte ingrowth	70 to 120	[92]
Chondrogenic differentiation	200 to 860	[93, 94]
Bone ingrowth	100 to 400	[95]
Osteoconduction	200 to 350	[96, 97]

Table 2.2 Guidelines to the optimal pore size for the respective cell growth or cellular pathway.

2.5 Biomaterials

To date, different types of biomaterials have since been explored as potential scaffold materials for cartilage tissue engineering. The biomaterials can be naturally-derived or synthetic polymers. More recently, both groups of materials are combined to form composites, which are proposed to provide a more favourable environment for the formation of neo-cartilage tissue.

2.5.1 Natural Polymers

Naturally occurring polymers, such as polysaccharides and proteins, have been identified as potential substitutes to autogenous grafts and bone-stimulation techniques for the repair and regeneration of cartilages. These polymers often are available in

abundance and their chemical nature elicits excellent biocompatibilities as well as provides flexibilities for processing. An extensive list of natural materials has been used to the development of physical constructs for the repair of cartilage defects, they include:

1. Collagen type II

Collagen type II is a protein used commonly for articular cartilage repair. The collagen type II matrix forms a large composition of the articular cartilage and its alignment within the ECM is crucial for the tissue remodelling processes. In contrast to the fibrin clot formed in microfracture treatments, the implantation of collagen type II porous matrix within the microfracture site demonstrated the formation of hyaline-like cartilage which offers better load-bearing capabilities [98]. It was observed that only matrices seeded with autologous cells facilitate the re-growth of hyaline-like cartilage. In addition, constructs that are comprised of collagen type I and type III showed lower expressions of proteoglycans as compared to collagen type II matrices [99]. Nevertheless, collagen's tissue engineering capabilities is dependent upon its antigenicity due to the animal origin. The drawback of collagen protein is thus proposed with the substitution of its denatured form, known as gelatin.

2. Gelatin

The denatured collagen exhibits high solubility under normal physiological conditions. Temperature treatments to the gelatin scaffolds have indicated higher compressive strength due to the ability to form cross-links [100]. Likewise, gelatin is inexpensive as opposed to the cost of collagen therefore it will serve as a more economical choice as cartilage scaffolds.

3. Agarose, fibroin and alginate

The gel-like constructs were found to promote the synthesis of sGAG fine structures more effectively as compared to synthetic scaffolds due to their abilities to retain cells within the matrices [101]. As a result, the further proliferation of cells within the matrices will increase the chances of cell-to-cell interactions, hence contributing to the formation of quality cartilages.

4. Macroaggregate culture grafts

Porous cell-derived ECM scaffolds are offered as another promising option to cartilage regeneration due to its tissue-compatible morphological structures and the ability to promote biological pathways more effectively [102]. Despite its diversity in clinical applications, the use of ECM for cartilage TE is still in the exploratory stage. Under *in vitro* conditions, the ECM scaffolds, which are yielded from lyophilized chondrocyte pellets, produced high quality cartilages and the functionalities of the newly formed tissues were evident by the increase of compressive strength with culture time.

5. Chitosan

Chitosan is produced by deacetylation of chitin and is particularly useful for engineering of cartilages as the polysaccharide contains β 1-4 linked D-glucosamine and N-acetyl-D-glucosamine due to its structural similarities to the GAGs found in cartilage tissues. Upon seeded with chondrocytes, chitosan scaffolds demonstrated higher cell viability and better ability to retain the phenotypic nature of the cartilage cells [103].

6. Gellum gum

A polysaccharide named gellum gum was introduced into cartilage TE because of its similar chemical composition to that of sGAGs [104]. Despite its indistinguishable processing versatilities with chitosan, the gellum gum was revealed to undergo gelation under 36 °C hence suggesting its application as an injectable scaffold.

7. Silk fibroin

Degradable silk fibroin (SF) proteins, which are derived from *Bombyx mori* silkworm cocoons, were suggested to promote the formation of specific cartilage ECM from MSCs given its unique mechanical and structural properties. To closely mimic the infrastructure of the native articular cartilage, aqueous SF solutions are lyophilized to form foams and the resultant protein networks were shown to promote cell differentiation, uniform cell growth and cell aggregation in the presence of growth factors [105]. Another advantage of SF proteins includes the ability to undergo phase transition into β -sheets in the presence of organic solvents such as methanol and ethanol for user-control over the mechanical properties and biodegradability of the

cartilage scaffolds. Furthermore, from the later findings by the same research group, it is worth noting that SF scaffolds seeded with human articular chondrocytes displayed differences in cellular behaviours as compared to the MSC-seeded constructs [106]. This observation suggests that the architectural features of the 3D scaffold constructs may play a significant role in the newly synthesized ECM for cell-based TE methods.

8. Altelopeptide collagen

In order to eliminate the adverse effects during post-surgery, collagen type I hydrogels were digested with protease to show lower immunogenicity, hence forming altelopeptide collagen [107]. Together with the encapsulation of auricular chondrocytes, these modified collagen gels were able to express $\beta 1$ integrin molecules that are essential for cell adhesion.

9. Hyaluronic acid (HyA) gel

HyA macromer most resembled the properties of native cartilage. The sGAG-like HyA hydrogels photo-encapsulated with the auricular chondrocytes were implanted into nude mice models. The *in vivo* work demonstrated that the HA gels exhibited comparable mechanical properties and biochemical compositions to that of native auricular cartilages [108].

2.5.2 Synthetic Polymers

For decades, synthetic polymers have been used for medical devices and osseous implants, owing to its high mechanical properties, biocompatibility and biodegradability. Depending on the process methods and parameters, these polymers can form different shapes and internal architectures that are appropriate for cartilage tissue constructs. The more commonly used synthetic biomaterials in TE include:

1. Polylactic acid (PLLA)

Poly-L-lactide (PLLA) human ear models with complex internal architectures can be fabricated via the particulate-leaching method [109]. Concerns over PLLA constructs have been highlighted due to their immunological response to the tissue engineered auricular cartilages. The transplantation of PLLA cartilage scaffolds alone in nude mice models either showed significantly lesser collagen type II expression and elastic content and/or localization of macrophages, while the *in vitro* inclusion of co-culture

chondrocytes into PLLA transplants indicated the formation of immune privilege as well as better mechanical properties [110, 111].

2. Poly-caprolactone (PCL)

For articular cartilage repair, polycaprolactone (PCL) sponges developed were reported as unsuitable substrates for *in situ* chondrogenesis, unless cultured *in vitro* with expanded chondrocytes under serum-free conditions [112]. Conversely, PCL fibre-based scaffold studies achieved chondrogenic differentiation of hMSCs regardless of the growth media used *in vitro* while suggesting that the alignment of fibres within the 3D mesh network may lead to varying cell adhesion behaviours [113].

3. Poly (lactic-co-glycolide acid) (PLGA)

PLGA is characterised as a flexible polymer because surface modification can incorporate chondrogenic stimulatory factors such as steroids and growth factors [114]. Despite its versatility, a concern regarding the acidic degraded products produced by PLGA was highlighted to affect cartilage tissue formation [115].

2.5.3 Composites

The tissue engineering of cartilage scaffolds using composites serve to provide sufficient mechanical stability and biocompatibility for the regeneration of fully viable functional cartilages. The materials' blends can generally be consisted of two or more synthetic polymers, natural-occurring materials, a combination of both polymeric types and occasionally with ceramics, while the compositional ratios of the polymeric materials are particularly dependent on the scaffold requirements. Synthetic copolymers are commonly created to meet the mechanical demands of the cartilage scaffolds in both reconstructive and orthopaedics surgeries. There are limited studies involving composites consisting of natural-occurring polymers. This is because the composites are generally weak mechanically despite having the superior ability to enhance cell process. Lastly, hybrid composites (blend of natural and synthetic biomaterials) serve to retain the advantages, at the same time, minimize the deficiencies of both synthetic and naturally-derived polymers.

1. Poly-caprolactone/ hydroxyapatite (PCL/HA)

PLLA/PCL composite scaffolds were demonstrated to provide long-term structural integrity by sustaining complex ear morphologies up to 40 weeks under *in vivo* conditions [116]. Moreover, with the appropriate polymeric compositions, the copolymers showed rubber-like elasticity which are efficient in the delivery of mechanical signals for tissue remodelling and subsequently regeneration of functional articular cartilage [117].

2. Poly-vinyl alcohol/ polyurethane (PVA/PU)

The copolymerization between PVA hydrogels and PU via cross-linking was reported to reduce the swelling ratio of PVA significantly [118]. The formation of the PVA/PU composite scaffolds minimized water uptake abilities of PVA hydrogels, resulted in increased compressive modulus comparable to native articular cartilages, and yet displayed better cell viability and GAG expression than PVA.

3. Poly (lactic-co-glycolide acid)/ hydroxyapatite (PLGA/HA)

The blended combination of PLGA and HA exhibited better mechanical integrity, more hydrophilicity and showed the ability to promote chondrocyte attachment and proliferation more efficiently than the PLGA controls [119].

4. Hyaluronic acid/ collagen type I (hyA/Col I)

A study conducted for articular cartilage TE involving hyA/Col I composites highlighted two challenging issues [120]. Firstly, although the incorporation of hyA gels contributes to slower degradation rate of Col I, the diffusional transport within the composite scaffold may be limited with increased hyA concentration. Secondly, the composite hydrogels are unlikely to be able to withstand physiological loads, hence additional physical supports are required to provide the hydrogels with a mechanically protective environment.

5. Hybrid (synthetic/natural polymer) scaffolds

PLLA/collagen type I hybrid scaffold models experienced an increase in implantation period and more spatially grown cartilaginous tissues [121]. The inclusion of polyethylene oxide (PEO) to chitosan, thus the formation of PEO/chitosan composites,

also indicated improvements to the mass transport of nutrients, chondrocyte attachments as well as modified elasticity for better load resistance [122]. More recently, the structural effects of PLGA/collagen type I hybrid scaffolds were investigated [123]. This study was achieved by introducing thin PLGA meshes within and on the peripheries of collagen sponges respectively. Based on the findings, it was revealed that hybrid models with collagen sponges surrounding PLGA meshes yielded better mechanical properties as well as higher expression in collagen type II and sGAG production. To maintain the delicate contours of the human ear models, biosynthetic constructs composed of alginate and PVA were created [53]. These hybrid gels demonstrated the flexibility for moulding into different shapes while the non-degradable nature of PVA gels allows prolonged shape retention. Under biochemical examinations, the hybrid gels were observed to be infiltrated with neo-cartilage in the absence of inflammatory response, hence indicating their potentials for auricular and nasal reconstructions.

2.6 Silk Fibroin

SF is a poly amino acid-based biopolymer with unique properties such as high mechanical strength and hydrophilicity which are attributed to its overall chemical structure (Figure 2.5) [124]. SF synthesis begins with mechanical interaction between the amino acid residues to achieve secondary conformational structures. The prominent SF secondary structures include: β -sheets, α -helices and β -turns (as shown in Figure 2.6) [124].

Figure 2.5 Chemical structure of silk fibroin composed of amino repeat units that assembles into β -sheet conformation [124].

SF's amino acid sequence favours the formation of β -sheet structures which are exceptionally stable in nature. β -sheet conformations can be induced via cross-linking by submerging SF specimens into organic solvents or using thermal treatment. The β -sheet conformation is much more resistant to water solubility and mechanical loading than α -helix and β -turn structures [125]. This is because the chemical bonds, particularly the hydrogen bonding between the functional groups of the amino acid residues, within the β -sheet structure are much stronger as opposed to the other two conformations. Moreover, β -sheets formed the highly crystalline regions in protein peptides while α -helices and β -turns contributed the amorphous components. The hydrophobic β -sheet domains prevent the penetration of water and enzymes, therefore SF-based materials degrade more slowly than commonly used materials such as collagen, fibrin and chitosan. The slower degradation rate may be useful to allow more time for tissue regeneration and integration. By varying the proportion of β -sheet structure, the rate of degradation can be controlled.

Figure 2.6 Secondary structures in proteins [125].

A significant advantage of SF, as compared to synthetic materials, reside in the nature of the degradation products which include protein peptides. The degraded by-products are less likely to cause local changes in tissue osmolality and pH that can be expected with degradation of aliphatic polyester such as PCL, PLLA and PLGA [47]. For this reason, and with the flexibility to tune the properties of SF by manipulating the relevant process parameters, SF offers clear advantages to be a useful biomaterial for TE purposes. Besides SF's processability, SF can be autoclaved for sterilization purposes without affecting its intrinsic characteristics.

2.7 Applications of Silk Fibroin in Tissue Engineering

SFs are used in its raw or regenerated versions in TE applications. To date, a wide variety of techniques is available for the development of SF into thin films, fibres and porous matrices.

2.7.1 SF Thin Films

SF thin films have been explored specifically for the engineering of corneal tissues because of its inherent optical clarity, mechanical robustness and controllable biodegradability. Under *in vitro* investigations, human limbal epithelial (HLE) cells cultured on SF films were found to display growth behavior comparable to that observed on tissue culture plastic and human amniotic membrane (AMem) [41]. AMem is the innermost layer of the placenta and is commonly used as a graft for ocular surface reconstruction. The phenotype of the HLE cells attached to SF demonstrated similar observations to the cells cultured on AMem, therefore suggesting SF film as a viable substitute to AMem. Moreover, SF thin films have been verified to possess adequate permeability for the diffusion of nutrients, oxygen, waste products and cell-signalling molecule. More recent work of SF thin films includes topographical patterning where grooved features are introduced into SF films that serve as guides to cell alignment and proliferation [126].

2.7.2 SF Fibres

Raw SF fibres obtained directly from silkworm cocoons has demonstrated as viable substitutes to injured or impaired tendons and ligaments. The bioactivity and mechanical properties of the SF fibre-based scaffolds are improved by knitting the SF fibres and combining SF matrices with micro-porous SF or collagen sponges [127, 128]. The enhancements serve to favour more cellular attachment as well as deposition of collagenous tissue matrix. The woven SF fibre matrices have shown to promote the secretion of ligament specific ECM components and also support adult stem cell differentiation towards ligament lineages [129, 130].

Electrospinning provides the opportunity for the production of nano- scaled SF fibres, and the formation of composite fibrous matrices. SF nanofibres, with diameters ranging from 30 to 120 nm, were characterized by high porosity and high specific area that are favourable conditions for cell adhesion and deposition of ECM [131]. The nanofibres were thus revealed as potential candidates for wound healing and scaffold for TE. SF is often used to blend with synthetic polymers to form electrospun composite-based fibres to enhance their biocompatibility and biodegradability. For instance, SF/PCL nanofibres of 50:50 blend ratio, when compared to PCL nanofibres, presented better cell adhesion and eliminates the bioinert nature of PCL [132].

2.7.3 SF Foams

SF porous foams or sponges have received much attention as 3D scaffolds for cartilage and bone TE in the recent years. SF foams are often prepared using a technique that combines freeze drying and salt-leaching (Figure 2.7). Salt particles, ranging from 250 to 1000 μm , are used as porogens during the fabrication process to create interconnectivities within SF constructs that serves to facilitate the mass transport of essential nutrients.

The applications of porous SF foams for cartilage reconstruction in the existing literature have presented promising results. Cell culture studies performed on SF foams demonstrated uniform cell distribution as well as high proliferation rates when compared to monolayer culture [42]. In addition, the evaluation of chondrocyte differentiation on SF scaffolds revealed that the chondrocytes were able to retain their phenotype and the critical chondrogenic markers such as collagen type II and glycosaminoglycan were up-regulated. Porous SF constructs has shown their ability to support the chondrogenesis of bone marrow-derived stem cells over 3 weeks of cultivation within the presence of inducers such as dexamethasone and growth factors [105, 133]. The mechanical robustness of the SF scaffold was reported to provide sufficient structural support for the stem cells to form dense tissue in cartilaginous ECM. The porous SF constructs were also presented as suitable substrate for the re-differentiation of culture-expanded chondrocytes which are widely known to quickly differentiate during *in vitro* expansion [106, 134].

For bone repair, the scaffold should degrade at a comparable rate with the new bone growth so as to permit full restoration of the native tissue structure and function. SF offers the advantage to the production of porous scaffolds with different degradation rates for the establishment of a correlation between degradation rate and osteogenesis *in vitro* [135]. SF scaffolds, when cultured with human adipose-derived stem cells, demonstrated the ability to produce increased bone protein, enhanced calcium deposition and total bone volume [136].

Figure 2.7 Scanning electron micrographs of cross-sectional morphology of the SF scaffolds obtained by the salt-leaching methodology [134].

2.8 Challenges of Silk Fibroin in Tissue Engineering

SF has been increasingly studied in the recent years due to the biocompatibility, slow degradability and superior mechanical properties. Although the various formats of SF such as thin films, fibers and foams have shown to promote tissue repair, some challenges of SF in TE are still faced, and they include:

1. Meeting tissue-specific mechanical and architectural requirements

Based on existing literature, it is inferred that the biological performance of SF constructs have been emphasized more than any other requirements of TE scaffolds. The mechanical properties and architecture of scaffolds are crucial TE components that provide temporary physical support and facilitate cellular processes respectively. The correlation between these two properties has been qualitatively established with the aim to elicit SF as a useful biomaterial [137, 138]. Nevertheless, limited studies have integrated these properties to SF's specific TE purposes.

2. Reproducibility of SF TE scaffolds

Porous SF constructs are commonly produced by the use of salt-leaching technique. The inclusion of the salt particles, which serve as porogens, does not guarantee uniform distribution of pores within the SF construct [38]. There is a possibility that interconnectivities cannot be established due to the randomly distributed pores.

Furthermore, SF constructs produced under the same processing conditions would tend to possess varying properties, thus defining the salt-leaching technique as being non-reproducible.

3. Incorporation and delivery of cell signalling molecules

SF scaffolds have been evaluated as suitable substrates for the phenotypic differentiation of stem cells into cartilage- and bone-like cells. This promising result is often due to the presence of cell signalling molecules such as tissue growth factors which are included in the culture medium. However, these tissue inducers are likely to work less efficiently due to convection and diffusional effects [139]. Therefore, a more viable option would be the embedment of the cell signalling molecules into the SF matrices which can be subsequently improved to achieve sustained released functions [140].

2.9 Scaffold Fabrication Techniques

2.9.1 Conventional Tissue scaffold Engineering Approaches

Conventional approaches in TE are considered to be the simplest and most economical way to produce promising scaffolds that are structurally similar to the ECM. The tissue constructs made from these techniques usually appeared in the form of foams or meshes and the internal morphological features such as pore geometries and interconnectivities within the scaffolds are dependent on the processing conditions.

2.9.1.1 Porous foam-like tissue scaffolds

Particulate leaching

In the particulate-leaching method, porogens such as salt or sugar are used to fill a mould and the aqueous form of scaffold material is cast into the porogen-filled mould. Once the scaffold material has solidified, the fillers are leached out by water immersion, thus yielding a porous matrix. The technique has been used to develop naturally-derived, synthetic as well as composite scaffolds for cartilage TE.

1. Salt and sugar particles

Regenerated silk fibroin porous matrices, formed using sodium chloride (NaCl) of particulate sizes between 600 - 700um, were reported to serve as suitable substrates

for the chondrogenic differentiation of MSCs and articular chondrocytes [105, 106]. Using identical porogen size range, synthetic PLGA scaffolds were able to substantially increase the GAG and collagen type II content [115]. Similarly, PLLA and PCL porous cartilage constructs formed by the sugar-leaching approaches, were shown to provide adequate microstructures for the secretion of ECMs [109, 111]. The formation of the sponge-like networks, in addition, allowed the penetration of cell-encapsulated hydrogels within the matrices to inhibit foreign body reactions [110]. Given the flexibility for degree of control over pore geometries, and despite the need for skilful hands, the particulate-leaching technique provides the opportunity for the investigation of suitable pore sizes for optimal cartilage tissue growth in silk scaffolds [42]. The morphological features within the porous scaffold were found to introduce mechanical elasticity to PLLA/PCL composites scaffolds for effective tissue remodelling during cartilage growth [117]. Given the hydrophobicity of synthetic composites, which attributes to weaker cell-biomaterial interactions, the salt leaching method was applied to hybridized matrixes composed of PCL and collagen. Besides being mechanically stable as the PLLA controls during the cell culture period, the hybrid PCL/collagen sponges demonstrated the formation of cartilaginous tissue that is quantitatively higher and homogeneously distributed [121].

2. Polymer beads

A study by *Izquierdo et al* [141] presented PCL scaffolds with interconnected spherical pore network using poly (ethyl methacrylate) (PEMA) beads sized at 200 μm . These thermoplastic beads were subsequently removed by soaking in ethanol for 4 days.

3. Ice particles

Another interesting particulate-leaching technique was applied using ice particles [142]. The primary step to scaffold fabrication involves the formation of an ice particle template which is obtained by spraying water onto a polymeric film and freezing subsequently. By controlling the spraying repetitions and freezing temperature, ice crystals of sizes between 180 to 720 μm can be yielded. This method has been examined to be feasible for the fabrication of collagen sponges, which were observed to provide optimal cartilage regrowth with an average pore size of 400 μm .

4. Microspheres

More recently, microspheres made of synthetic and naturally-derived materials were incorporated as porogens to provide uniform structure within the tissue scaffolds [143]. These spherical particulates are produced by means of a micro-fluidic device that uses the stable oil-in-water (O/W) emulsion. The approach, in brief, yields uniformly size microspheres when the porogen solution (discontinuous phase) is injected into the surfactant (continuous phase) at a constant flow rate. The resultant pore diameter can be varied by (1) the flow rate of the continuous phase and (2) the concentration of the discontinuous phase. Based on the versatility of the technique, studies have shown to produce homogeneously distributed pores within chitosan and PLGA tissue scaffolds, using PCL and gelatin microspheres respectively [24, 27].

Thermal-induced phase separation

The phase separation method forms porous structures by subjecting the frozen scaffold matrices to sublimation. Under low temperature and pressure conditions, the solvent ice crystals convert to gaseous state, vaporize and leave porosities within the constructs. The average pore size of the processed scaffold is dependent upon the material concentration as well as the freezing temperature prior to the freeze drying. The technique was applied to process naturally-derived chitosan sponges where the presence of micro pores induced by the phase separation method led to enhancements in the mass transport process. Composites such as PEO/chitosan and PLLA/PCL composites were also achieved to enhance the biocompatibility for cartilage tissue engineering applications [116, 122]. Using the similar method, scaffolds composed of PLGA and nano-sized HA can be produced and the constructs were shown to be capable of inducing osteochondral healing without the use of growth factors [144]. However, in comparison to particulate-leached scaffolds, SF constructs yielded from the phase separation approach demonstrated pore sizes which were only adequate for cell attachments, hence resulting in the suppression of cell migration and proliferation [42].

2.9.1.2 Mesh-based tissue scaffolds

Woven or non-woven meshes assembled using micro/nano fibres were experimented as the next potential constructs to simulate the production of cartilage ECM and

promotion of chondrogenesis. The fibres within the scaffold meshes are often produced using electro-spinning, which has been recognized widely as a simple and economical method. The spinning process, in general, begins with the loading of molten or solvent-based biomaterial into a syringe where liquid fibres are extruded out at a controlled rate from a hypodermic needle. An electric field is concurrently applied to the needle tip to evaporate the solvent, hence resulting in the formation of solidified fibres. The fibres are then consolidated, in an oriented or non-oriented manner, on a collector platform where the scaffold meshes are produced. This tissue scaffold fabrication method has since been applied to both synthetic and natural materials.

A comparison made between PGA and non-absorbable polypropylene (or Prolene) meshes had demonstrated that the PGA fibre-based scaffolds were capable of enhancing the proliferation of chondrocytes while the Prolene meshes did not display good cell attachment [145]. However, when assessing the phenotype of chondrocytes on the PGA scaffolds, it was found that the overall cell morphology flattens with increased culture period, indicating signs of differentiation. These findings were consistent to the observations of PCL fibrous scaffolds which highlighted that the mesh networks only attributes to the alignment of cells due to their ability to mimic the ECM structural organization [113]. In the presence of chondrogenic markers, the use of nano-scaled fibrous networks (500 nm) displayed higher efficiency in the chondrogenic differentiation of MSCs, which further illustrated the structural importance of the mesh-like scaffolds. The macro fibre scaffolds (3000 nm), on the other hand, experienced a suppression of collagen type II expression. The nano fibre meshes were also shown to serve as suitable substrates for the formation of cartilaginous tissues when cultured in a multi-chamber flow perfusion bioreactor [146]. However, the nano-scale mesh networks are mechanically weak and hence may result in a detrimental effect on the neo-cartilage formation.

To address this drawback, *Shim et al* [103] fabricated a double-layered membrane formed by rolling up a matrix consisting of both nano- and micro-sized chitosan fibres. The nano-/micro fibrous 3D matrix were reported to result in a higher secretion level of cartilage-specific ECM in comparison to the micro-fibrous network, thus suggesting the dual-scale scaffold as a preferred physical and biological support for chondrocyte cultivation. An alternative method to obtain fibrous matrices includes replica moulding. This approach developed fibres of pre-designed diameters by casting the

biomaterial into elastomeric moulds which are selectively etched using the photolithography technology [147]. The novel moulding method had shown to produce non-woven scaffolds which consist of chitosan fibres ranging from 4 to 22 μm and matrices containing the smaller fibres were found to be more favourable for chondrogenesis.

2.9.2 Advanced Tissue Scaffold Engineering Approaches

Additive manufacturing (AM) or rapid prototyping (RP) has provided an alternate viable option to tissue engineering applications in recent decades. Despite its relatively higher cost, this technology is preferred to the conventional approaches due to (1) the ease of use, (2) high reproducibility and (3) the ability to generate fine architectural features. AM technology builds tissue constructs using a layer-by-layer, or additive approach based on computer-aided design (CAD) data. The data, in general, contains the architectural information of the physical model which can be simply manipulated using existing commercialized CAD software. Moreover, patients' anatomical geometries from image modalities such as the magnetic resonance imaging (MRI) and coherence tomography (CT) can be regenerated as CAD data via the use of image processing software, thus allowing the design customization of tissue scaffolds to suit individuals' needs. This option is exceptionally useful for both maxillofacial and orthopaedics applications, where the overall shapes of the tissue scaffolds are critical for mental well-being and functionality respectively. Using AM systems, the internal morphologies such as pore size, porosity and interconnectivity can also be altered to achieve tissue constructs of desirable mechanical integrity as well as biocompatibility.

2.9.2.1 Direct additive manufacturing approaches

AM approaches can be applied directly or indirectly. The direct AM methods manufacture tissue scaffolds directly from the input CAD files. These tissue scaffold fabrication techniques have been further classified by *Yeong et al* [89] into melt-dissolution deposition and particle-bonding techniques.

Melt dissolution deposition techniques

1. Fused deposition modelling (FDM)

The melt-dissolution typically involves the extrusion of semi-liquid state or molten material through a nozzle moving along the horizontal x- and y-axes, while the building platform lowers along the z-axis as each layer is fabricated and adjoined to the previous layer as it cools and solidifies. In orthopaedic surgery, the fused deposition modelling (FDM) has been used to fabricate tissue scaffolds which are targeted for the repair of osteochondral defective sites. Poly(ϵ -caprolactone) (PCL) scaffolds of coral-like structures produced by the FDM demonstrated that the interconnected networks (of pore sizes between 300 – 580 μm) supported the co-culturing of osteoblasts and chondrocytes as well as linked and integrated the rich ECM secreted by both cell types [148] (Figure 2.8).

Figure 2.8 Microscopic images of (a) 3D fibre-based interconnected network built by the FDM [149] and (b) a typical lay-down pattern after FDM processing [150].

To match the mechanical properties of tissue scaffolds with that of the native medial femoral condyle, the FDM technique was used to build hybrid scaffolds consisting of PCL and tricalcium phosphate-reinforced PCL, which were respectively used for the regeneration of cartilage and bone [151]. The co-polymeric scaffolds were shown to provide a more robust structural support up to 6 months postoperatively; this allowed sufficient time for neo-tissue formation and tissue re-modelling, thus forming newly synthesized osteochondral tissues of mechanical characteristics which were comparable to that of the native tissues. In reconstructive surgery, the auricular frameworks constructed using autogenous tissues were often lack of the delicate external profiles of a human ear. Nevertheless, auricular scaffolds manufactured via the FDM technology were found to replicate the CT scans of the patient's contralateral ear satisfactorily [152] (Figure 2.9). This observation indicated that the direct AM method exhibited as potential substitutes to the traditional auricular reconstruction techniques, and since the applications of AM in craniofacial surgery is relatively new, further work should be conducted to identify the suitable internal morphologies for tissue regeneration.

Figure 2.9 (a) 3D reconstruction models of the auricular framework derived from CT data, (b) an image of the contralateral ear and (c) a porous auricular scaffold created by FDM [152].

Despite having the ability to form tissue constructs of precise dimensions, FDM also has its limitations. A major drawback of the approach is the need to reshape material

into filaments of specific diameters before the start of the manufacturing process. As the materials are processed in molten or semiliquid state, subsequent rapid solidification of the fabricated layers in room temperature may result in the formation of struts with insignificant topographical features. The lack of surface roughness within the construct may limit cell attachment and neovascularization, which are crucial to the initial stages of tissue re-growth. Moreover, the operation of the FDM involves high temperature processing, thus discouraging the use of naturally-derived materials and the incorporation of hydrophilic components to the resultant scaffold constructs for enhanced biocompatibility.

2. Bioplotting

Bioplotting can create scaffolds with pre-defined architecture, strut thickness and porosity. Firstly, the biomaterial has to be loaded into the reservoir and be melted according to its melting point in the bioplotter's heating unit. Secondly, the molten material is extruded through a nozzle or needle with a preferred diameter. As each parallel strand is deposited, the constructed portion would be air-dried.

This fibre-based approach allows more choices of biomaterial and composite blends since it eliminates the need for shaping the biomaterial into specific diameter for processing. For instance, bioplotting poly(hydroxymethylglycolide-co- ϵ -caprolactone) (pHMGCL) bone TE scaffolds exhibited faster cell growth and increase cell metabolic activity when compared to PCL [153]. 3D PLLA- tri-calcium phosphate (TCP) developed demonstrated lesser ability for bacterial adhesion in comparison to PLGA and PLLA, thus promoting significantly higher cellular proliferation [154].

Geometrical modifications such as pore size gradients and shifted pattern can be achieved in bioplotting techniques. PCL scaffolds with varied distances between fibres improved seeding efficiency and a more homogenous distribution of cells [155]. The presence of shifted pattern, which is formed by changing the position of struts gradually for every deposition layer, has shown to provide a better environment for cell attachment, proliferation and attachment [156].

Hydrogels with low viscosities tend to impose difficulties for the construction of TE scaffolds. *Maier et al* [157] has successfully fabricated a 3D hydrogel matrix by dispensing the viscous material into a plotting medium with similar rheological

properties (Figure 2.10). The process is also capable of changing the lay-down pattern of each deposited layer and produce woven type pattern. As a result, this viable approach has offered more opportunities to create 3D scaffolds from hydrogels such as poly(ethylene glycol) (PEG), gelatin, alginic acid and agarose. More recently, a cell-plotting system performed the fabrication of agarose, alginate and Pluronic® F127 3D scaffolds respectively [158]. In order to achieve sufficient structural integrity for the 3D TE constructs, the hydrogels were derived with moderate viscosity and the plotting pressure was varied to identify the optimal conditions for the plotting process. Under *in vitro* investigations, these 3D hydrogels were produced successfully for soft tissue regeneration.

Figure 2.10 A schematic of a bioplotter setup [99].

3. Custom-built fibre-based AM systems

As existing direct AM techniques do not offer the flexibility in terms of material choices for fabrication of tissue scaffolds, several “in-house” AM systems have been developed for TE purposes. An example for such AM-based TE approaches is the 3D

fibre deposition (3DF) which produces porous articular cartilage scaffolds using biodegradable poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) copolymers [159] (Figure 2.11). In addition to the AM technology, a co-polymeric system was introduced so that tailor-made properties such as swelling, biodegradability and mechanical strength can be achieved by adjusting the composition of the hydrophilic (PEGT) and hydrophobic (PBT) polymer blocks. In contrast to the FDM technology, the 3DF method eliminates the need for pre-formed fibres as material feeds; granules of the copolymers can be directly placed into the heating jacket for melting and subsequent processing. The melting process of the copolymers was also conducted with the presence of nitrogen gas so as to preserve the intrinsic properties of the hydrophilic component. The resultant co-polymeric constructs showed no changes in molecular weight and thermal properties. In addition, under *in vivo* conditions, the PEGT/PBT constructs were found to form significantly more cartilaginous tissues in comparison to compression-moulded/particle-leached scaffolds, even though collagen type II was detected regardless of scaffold type [160]. This observation suggests that the co-polymers (1) can be offered as a potential material for the maintenance of chondrogenic phenotype and (2) should be incorporated with pores and interconnectivities so as to support cell migration, nutrient perfusion and finally rapid growth of the neo-cartilages.

The 3DF methodology was further explored with the combination of the phase separation process to create shell-core biphasic scaffolds [161]. The shell-core articular cartilage scaffolds were obtained by viscous encapsulation, which is a rheological phenomenon to coat a stiff and hydrophobic core polymer with a shell polymer that can provide adequate surface properties for specific cell-material interactions (Figure 2.12). Under *in vitro* investigations, the shell-core structures revealed enhanced mechanical stability and physicochemical properties, which are attributed to the shell and core polymers respectively, for improved cartilage tissue engineering.

Figure 2.11 The 3D fibre deposition device consisted of five components: (1) a thermostatically heating jacket; (2) a molten co-polymer dispensing unit; (3) a plunger to regulate flow of molten co-polymer; (4) motor driven x-y-z table; and (5) a position control unit linked to a computer to generate fibre deposition paths [159].

Viscous Encapsulation during Extrusion

Figure 2.12 A schematic drawing of the viscous encapsulation phenomena [161].

To accommodate a larger variety of synthetic biomaterials for additive manufacturing, a desktop-based RP (DBRP) technique was developed to fabricate tissue scaffolds consisting of one or more polymeric materials [162]. The DBRP method has been used to assess the feasibility in the production of PCL homo-polymeric and PCL-PEG co-polymeric scaffolds. The pathway to achieve desirable tissue constructs using the DBRP technology encompasses several factors. They include: (1) the material properties of the raw materials, such as the molecular weight and crystallinity, (2) processing parameters, which consists of the liquefier temperature, extrusion pressure and deposition speed. The optimization of the process conditions has led to the formation of highly interconnected matrices for both PCL and PCL-PEG tissue scaffolds. In addition, the hydrophilicity was found to be significantly enhanced for the co-polymeric constructs and the observation was subsequently verified by higher yield of DNA content through cell culture studies. The ability to integrate hydrophilic materials into the existing AM technology has indeed led to improvements made on the surface properties of cartilage tissue scaffolds, thus resulting in increased cell attachment and tissue growth.

Nevertheless, it is necessary to have AM systems that are feasible for processing non-thermo-sensitive materials or material blends. This hence had led to the development of liquid-frozen deposition manufacturing (LDFM), which is a modified version of the FDM and involves no heating process [163]. Instead of using molten form of the biomaterial, polymeric solution was used as the feed and each layer built was frozen via the low-temperature platform. Once the building process had ended, the scaffolds will be lyophilized to remove the unwanted solvent. The concentration of the polymeric solution was evaluated to affect the architectural and mechanical properties of the resultant tissue scaffold. Furthermore, the LDFM scaffolds displayed the capability to retain the intrinsic properties of the PLGA biomaterial used; FDM PLGA scaffolds, in contrast, exhibited degrading properties such as increase in swelling ratio and broader polydispersity (significant drop in molecular weight).

Challenges in melt-dissolution deposition techniques

Although the melt-dissolution deposition techniques have elucidated their potential in cartilage TE, due to the manufacturing nature of these methods, the control over the internal morphologies can only be manipulated by varying the lay-down patterns. The

lay-down patterns are generally 2-dimensional fibre-based assemblies and may eliminate the incorporation of complex patterns consisting of polyhedral and cylindrical unit cells which are critical for the anatomical requirements of cartilage tissues [164]. For instance, work to include porosity gradient radially using the fibre deposition technique has shown discontinuities in scaffold structures that subsequently pose challenges such as limitation to the mass transportation of essential nutrients and weakness in mechanical properties.

Particle-bonding techniques

Particle-bonding techniques such as 3-dimensional printing (3DP) and selective laser sintering (SLS) have emerged as the preferred direct AM methods for the fabrication of tissue scaffolds with complex geometries and surface morphological features. Both additive manufacturing methods exhibited the ability to use powder-based materials, which can be available commercially or produced via mixing, milling or spray-drying. At the primary stage of fabrication, the two approaches require entirely-filled and levelled powder beds before the processing of 3D scaffold builds. For both methods, the un-bonded particles were suggested to serve as temporary supports to the model and are usually removed after the building process via compressed air or post-treatment procedures. The particle-bonding processes distinctively differ between the SLS and 3DP.

1. Selective laser sintering (SLS)

SLS operates by selectively sintering the powder materials using a carbon dioxide laser beam to form individual 2D sliced layers on the part-built chamber or part-bed (Figure 2.13). The part-bed is encompassed with a constant heat source which serves to bond the adjacent layer together. The resultant 3D SLS physical model is then progressively produced as the building platform lowers along the z-axis after each sintered layer is constructed. The SLS processing conditions such as the laser powder and scan speed of the laser beam are important controlling factors to the resultant build.

Since the SLS is not specifically designed for TE applications, the proprietary materials used in the commercialized systems are often non-biocompatible. This hence has led to substantial efforts for the identification of suitable material choices and their

processing conditions for the respective particle-bonding procedures. For instance, the material choices for SLS have been widely researched. The material selections include poly(etheretherketone) (PEEK), Poly(vinyl alcohol) (PVA), poly(L-lactic acid) (PLLA), poly(L-lactide-co-glycolide) (PLG) and PCL [32, 165].

Figure 2.13 A schematic diagram of SLS [166].

PEEK is known for its bio-inertness, the bioactivity of the PEEK scaffolds can be increased by the incorporation of hydroxyapatite (HA) particles via physical blending in a roller mixer [167]. The formation of composite blends such as PVA/HA by physical blending was justified to be more feasible in contrast to spray-dried composite powders [31]. The physically blended materials were used to build scaffolds addressing to craniofacial and joint defects via the SLS and its TE potential of the SLS constructs was verified [168]. PCL SLS fabricated constructs were first explored for drug delivery devices [169-171]. These findings were subsequently applied to create laser sintered PCL/HA TE scaffolds which are examined to be feasible for bone TE [172]. The research group further challenged the SLS system with PLG/HA/TCP blend for more options for bone repair and regeneration [173]. More recently, PCL homopolymeric constructs built via the SLS, together with the

incorporation of the *Chua's* in-house Computer-Aided System for Tissue Scaffolds (CASTS) [174-177], were demonstrated to provide the necessary architectural features for the treatment to myocardial infarction (MI) [150].

2. 3-dimensional printing

3DP involves the joining of neighbouring powder particles and layers by the injection of a liquid adhesive or binder (Figure 2.14). To eliminate the use of organic solvent, the starting powders for the fabrication of TE scaffolds consist of a blend of starch, cellulose and a thickener-like substance [178]. 3DP PLGA constructs manufactured were evaluated to provide structural characteristics for the facilitation of bone tissue formation and growth [179]. An attempt to build HA interconnected scaffold networks for bone using custom-made 3DP met both structural and mechanical requirements [180]. The porosity of each individual construct can be increased by the inclusion of particulate-leaching technique [181].

Figure 2.14 A schematic of 3D printing process [182].

Challenges in particle-bonding techniques

A disadvantage of the SLS process includes the harsh processing conditions such as high temperature and laser power. This factor is often the reason for limited material choices for TE scaffold fabrication. Moreover, the surface topography of the scaffold constructs is limited by the particle size of the powder-based feed since only micro granules can be obtained.

The existing issues faced are recently circumvented using biocomposites with nano-features. This strategy requires a pre-processing step to yield microspheres as raw materials for the SLS process [183]. The microspheres can then be combined to form a nanocomposite blend of poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) and calcium phosphate (CaP) which demonstrated improved bioactivity as well as mechanical properties for bone TE.

The spot size of the laser beam of commercialized SLS system may possibly contribute to the inability to achieve nano-scaled features. Thus, customized SLS system with minimum spot size of 50 μm has since been built to manufacture nano-HA models as biomimetic substrates for bone TE [184]. In addition, the SLS working principles were also improvised to include ceramics such as bioactive glass for achieving fully dense cranial implants [185]. The laser scanning concept was also attempted to fabricate human ear model cartilages for auricular reconstruction [35].

Although 3DP does not subject the physical build to harsh processing conditions like SLS, the post-processing step which is used to strengthen the TE construct or remove the binder may result in shrinkage as high as 27 % [186]. The shrinkage was found to be isotropic but the density of the supposed build was significantly increased. The compromise in accuracy was avoided by using an alternate 3DP system, known as the Thermojet, which is capable of producing micro-assay channels for effective cell guidance [187]. Despite the presence of the binding reagent, each resultant 3D construct is very fragile and will dissociate in aqueous medium instantly. The limitation faced is resolved by the post-treatment of the scaffold structure, either by infiltrating the tissue construct with co-polymeric solutions or inducing the formation of stronger chemical bonds through incubation in relevant reagents.

2.9.2.2 Indirect additive manufacturing approaches

The inclusion of thermosensitive materials such as naturally derived proteins and polysaccharides are common challenges for direct AM applications. Indirect AM approaches are thus used to incorporate user-defined 3D designs and micro scale morphologies into these materials. The indirect process can also be defined as a procedure that unifies both direct AM and conventional TE fabrication methodologies. The fabrication method requires the casting of aqueous or molten raw material into a sacrificial mould that is built using an AM system. Struts present within the CAD model is replicated as the interconnected macro channels while the lyophilisation of frozen cast material will provide the micro surface features. The sacrificial moulds are subsequently removed via dissolution in non-toxic solvents, therefore allowing the preservation of the biomaterial's intrinsic properties.

Droplet deposition technique

1. ModelMaker IITM (MMII) and PatternMasterTM

ModelMaker IITM (MMII) and PatternMasterTM are inkjet printing systems where each process uses two ink-jet print-heads, each delivering a different material. The printer head dispenses droplets of materials, overlapping them to form a line and overlapping the lines to form a layer (Figure 2.15). After a layer is completed, a rotary cutter is used to flatten the top surface and control the thickness. The building platform is then lower and the process is repeated to build the next layer. The sacrificial moulds were built using the proprietary materials, InduraCastTM and InduraFillTM, which act as the model and support material respectively.

The inkjet technology was primarily explored to produce collagen scaffolds with pre-defined internal morphology (Figure 2.16). It was found that negligible traces of thermoplastic remained in the scaffold construct and the collagen protein was not denatured despite going through the multiple-step fabrication approach [39, 188]. The mould was resistant to drying techniques such as lyophilisation and critical point drying [189]. The mould can subsequently be removed by using ethanol, which is an organic solvent used for sterilization of biomedical devices [49]. The additional advantages of MMII include lesser raw materials compared to direct AM approaches

and the possibility to incorporate composite blends so that the scaffold requirements are met [37].

Figure 2.15 Schematic representation of droplet-based technique [36].

Figure 2.16 (a) A sacrificial mould fabricated via the inkjet printing technique, (b) the resultant collagen construct with interconnected channels [189].

Biomimetic composites consisting of chitosan and HA have been produced via the inkjet technique for bone TE applications [190]. A highly viscous mixture of chitosan solution and HA microspheres was cast into the pre-designed sacrificial moulds. The resultant constructs were shown to be a good substrate for osteo-conduction and osteo-

induction. In addition, the HA microspheres were established as a potential drug delivery system and provides rough surface area for cell attachment and proliferation.

PatternMaster™ was used to fabricate poly(1,8 Octanediol-co-Citrate) (POC), PCL and Poly(glycerol sebacate) (PGS) scaffolds to investigate the materials effect on cartilage regeneration [191]. The wax moulds were used directly to melt-cast PCL while PGS and POC were cast into HA secondary moulds. Despite integrated with 3D design, the three materials were found to de-differentiate chondrocytes. Nevertheless, this concern was resolved by the injection cell/hyA/Col I suspension (cell carriers) into the 3D scaffolds [192].

Photosensitive-based technique

1. Stereolithography

Stereolithography manufactures 3D constructs by spatially controlling the selective solidification of a liquid polymeric resin via photo-initiated cure reaction using a laser beam [28]. An indirect AM approach was performed by integrating pre-designed moulds and gel casting method to produce β -TCP porous scaffold. *In situ* polymerisation was performed to bond and sinter the ceramic particles together and the resin moulds were subsequently burnt out [193].

Commonly, reactive diluents such as diethyl fumarate are required to provide an appropriate reaction rate and viscosity of the resin for the stereolithography process. In addition, significant shrinkage upon the extraction of the photo-polymerised network poses a challenge in the optimisation of resin. *Melchels et al* [194] developed photo-curable poly (D,L-lactide) (PDLLA) porous scaffolds that are free of reactive diluents and can be potentially used for bone TE. The architecture of these PDLLA constructs, compared to salt-leached scaffolds, demonstrated large cell populations growing in the centre of the scaffolds [195].

2. Polyjet™ droplet-based printing technology

This droplet-based approach encompassed the fabrication of two sacrificial moulds before obtaining the resulting tissue scaffold [38]. A sacrificial mould resembling the architecture of the tissue scaffold was first built using the Polyjet™ technology (Figure 2.17) where the model and support materials are ultraviolet (UV) curable photopolymers that are soluble in sodium hydroxide (NaOH) and water respectively.

The support material was dissolved and the second sacrificial mould (with negative architecture) was produced by casting paraffin wax into the 3D model. The model was subsequently dissolved in NaOH. The resultant tissue scaffold was then obtained by casting the biomaterial and dissolution of the wax mould using hexane. Gelatin scaffolds with uniform pore distribution and channel architectures were produced. Moreover, cytotoxicity tests revealed that the constructs do not induce cell toxicity thus elucidating their potential for TE applications [38].

Figure 2.17 The Object PolyJet inkjet technology-based process [28].

Particle-based manufacturing

1. 3-dimensional printing (3DP)

Zcorp, a 3DP system, is used to fabricate negative resin or plaster moulds as templates for the casting of biomaterials. The negative moulds aims to provide the overall shape of the tissue scaffold while particulate leaching is included into the casting process to control the pore sizes. When the biomaterial is dried, the scaffold detaches from the mould surface and the porogen is removed via immersion into its solvent. This approach has proven to support the growth of intestinal epithelial cells and guiding smooth muscle cell growth due to the presence of villi architectures [46, 196]. Ear

constructs engineered with CAD via 3DP provides a useful strategy the reconstruction of auricular cartilages [35].

Fibre-based deposition

1. Fused deposition modelling

FDM has been used to fabricate a negative mould for the construction of medial meniscus [86]. CAD models were first generated via the visualization of a surface image from raw data obtained from CT scan. The image of the solid meniscus was then embedded into an acrylonitrile butadiene styrene (ABS) plastic replica for the injection of the biomaterial.

2.10 Challenges of Additive Manufacturing-based Tissue Engineering Scaffolds

The pre- and post-processing steps of scaffold fabrication via AM technology are crucial to the clinical application. Despite AM's recognition in the TE field, challenges are still faced as technology advances [197]. One of the advancements in AM technology includes cell-based deposition or dispensing approach. In this study, the challenges described are based on their sequence in the stages of TE progression. The different stages of TE progression include knowledge integration, design and development, *in vitro* tissue regeneration, *in vivo* tissue regeneration and clinical applications (Figure 2.18).

Figure 2.18 Stages of TE progression and their challenges

1. Knowledge integration between the mechanical properties and material science

To date, there exist limited models which precisely compute the material and mechanical properties for effective scaffold functions. In the mechanical aspect,

geometric features such as porosity and different scaffold microstructures should be related to the effective stiffness and permeability of the 3D construct [166]. The relationship can be established with the help of voxel data sheets that are derived from CT scan and MRI to define patient's anatomy based on density distribution. The final scaffold design can be generated by intersecting the anatomical shape defect with the CAD design. Following this model, the material properties can be included to obtain the desirable properties and biological environment for TE applications.

2. Surface topography of TE scaffolds

Surface roughness is a crucial component to cell-matrix interaction. Essentially, the scaffold design should include micro- and nano-scaled features as these architectures synergize optimal cell adhesion and facilitation of vascularization. Pore sizes within the meso-range (2-50 nm) showed great potential in TE as bioactive molecules such as growth factors embedded in these pores and released timely according to the tissue's requirements [198]. The limiting factors to achieve such architectures are commonly attributed to the particle size in powder-based bonding techniques and nozzle diameter of fibre-based deposition approaches [182]. The effect of laser curing and rate of droplet solidification also contributes to the resolution of SLS and inkjet printing systems as well.

Patterning method is an approach that is applied to create scaffolds with architectures as robust cell culture platforms to mimic the *in vivo* cellular micro-environment [199]. Other viable solutions to obtain nano-structures include etching and fibre spinning [200]. The combination of multiple fabrication techniques can also promote the formation of diverse architectural features to improve cellular mechanics.

3. Complex interior architecture

The interior architecture of tissue scaffolds works out as a dynamic cellular environment in scaffold design. This is because a controlled tissue framework would provide the spatial and temporal resolution to improve cell response [201]. Polyhedral-based CAD library system has been developed to optimize the design selection for the targeted tissue [176, 177]. The assemblies of differently-shaped unit cells also impart different mechanical properties and varied biological signals to the resulting scaffold.

The implementation of geometric gradients was suggested to precisely mimic the ECM composition and mechanical properties. Automated CAD programs have been developed to generate functionally graded constructs for bone TE [164, 202].

4. Rapid assembling of tissues and organs

Existing TE approaches do not support the regeneration and growth of tissue rapidly. Nevertheless, understanding the knowledge of developmental biology has revealed tissue fusion as a potential option to accelerate the healing process [203]. Currently, the probable candidates for tissue fusion processes include the remodelling of ECM, cell migration, consistent cell-to-cell interactions. These mechanisms are particularly useful for TE of large complex organs.

5. Developing the desirable environment for *in vitro* cartilage reproduction

The chemical, physical and mechanical parameters should be varied according to the stages of cartilage growth. For instance, it was revealed that compressive loading is not helpful during the early stages of chondrogenesis [204].

Bioreactors are offered as better options to simulate the growth of cartilage under *in vitro* condition [205]. This is because the cell and tissue development can be influenced when the major bioreactor components are altered. They include the delivery of nutrients and metabolites, regulation of oxygen and gas exchange and exertion of mechanical and hydrodynamic forces.

6. Elimination of the pre-seeding procedure

Using direct cell- plotting or writing techniques, which are known as cell-based AM, *in vitro* cultivation of the scaffolds with cells can be eliminated [28]. These approaches aim at the rapid production of TE scaffolds and involve low risk of contamination and increasing the chances of direct clinical applications [206].

7. Prediction of cell damage for cell-based AM techniques

Cell damage varies with different TE applications and is significantly affected by the processing method and scaffold design. Even though cell damage can be quantitatively measured experimentally, the system relationship between cell damage and cell-based techniques has yet to be developed [207].

8. TE scaffold progress towards clinical applications

Besides requiring the engineering basics like design, material synthesis, manufacturing and characterization, the success of scaffold-based research has to demonstrate the ability to enhance tissue regeneration in animal models. The translation from scaffold-based therapies to clinical applications usually involves a substantial amount of time and expense [208]. It remains questionable how long more will TE scaffolds be included in clinical applications. It is believed that the translation process can be accelerated by early surgeon acceptance and medical insights of material or design that can be readily used. Meanwhile, the gold standard in clinical practice remains as experimental control to validate the TE scaffold's ability.

9. Cellular differentiation of stem cells

Cascades of biochemical interactions involving extensive regulatory networks, hundreds of different gene and effector molecules have been shown to influence the cellular differentiation of stem cells. The recruitment of environmental factors was used as a more practical strategy to promote chondrogenesis [204]. The approaches include the manipulation of cell culture conditions (from 2D to 3D culture), surface modification of the scaffold to inhibit the formation of non-essential ECM components and mechano-transduction to regulate the genes transcribed for cell growth and differentiation.

2.11 Summary

Cartilage is a unique musculoskeletal tissue that has a defined firm shape containing negligible blood supply and nerves. However, once the connective tissue is disrupted due to traumatic or degenerative reason, the property change imposed in the impaired cartilage is irreversible. This effect confirms that cartilages have limited self-repair capacity and surgical options remain as the only treatment method to repair the damaged or diseased tissue. Moreover, the quantity of chondrocytes present within the native cartilage is limited and thus the ACI procedure has to be performed to ensure optimal healing of the tissue. The use of autografts remained as the golden standard for reconstructive and orthopaedic surgeries, however, the strategy can be challenged when inadequacy of the donor tissue is faced. Moreover, the use of alloplastic, a foreign body, may lead to adverse immunological effects. The ultimate aim of the ACI

approach is to restore the external shape of the cartilage as well as recovering the functionality of the connective tissue.

The third generation ACI approach encompassed the seeding of expanded autologous chondrocytes to an engineered 3D scaffold before implanting into the defective site. The 3D tissue scaffold serves to provide as a biological substitute or substrate to facilitate cell attachment and subsequently tissue growth. The architecture within the scaffold construct is crucial to the function of the regenerated cartilage tissue. Other influencing factors include the choice of biomaterial and the general properties of the cell type.

Tissue engineering is a technical approach that is relevant to the formation of an engineered 3D scaffold. A list consisting of scaffold requirements are commonly established to ensure the TE construct execute its intended purpose. The general factors considered for engineering TE scaffolds include the biomaterial(s) and its/their physical, mechanical and surface properties. Naturally-derived proteins are preferred biomaterials for scaffold fabrication owing to their superior biocompatibility. In particular, silk fibroin is selected for this work as the protein peptides can be easily extracted from silkworm cocoons, possess high mechanical strength and thermal properties, and its versatile processability. Furthermore, the properties of SF can be varied by altering the crystallinity of the protein via thermal treatment or immersion in non-toxic solvents.

AM technology has been widely applied in the recent decades to manufacture TE constructs. The ability to incorporate pre-designed CAD models and additive building concept has attracted wide attention in the TE community. Despite being more costly, AM techniques are reproducible and allow complete user control over the design of the scaffold. The data extracted from imaging modalities can also be used to customize the TE construct according to the patient's needs.

Based on the discussion on the existing AM approaches in this chapter, it can be inferred that there is a need to elucidate further TE potentials of the indirect AM. The indirect method has been proposed as it can process wide range of materials. However, the extension of the indirect AM techniques to include more biomaterial selections is limited. Thus, the proposed work involves the fabrication of SF constructs with desirable architectural features for cartilage tissue engineering. This experiment

presents the first generation of SF tissue constructs with pre-defined internal features to enhance cell process and promote effective tissue generation. The current work also addresses to some challenges of SF which include the establishment of empirical models of SF properties for specific TE applications and the development of manufacturing method that ensures reproducibility of SF scaffolds. Issues faced with AM-based TE scaffolds such as surface topography of scaffolds and developing an environment that favours cartilage formation are addressed to elicit the potential of SF scaffolds for cartilage repair. The next chapter presents established empirical relationships between the geometrical, hydrophilic and mechanical properties of SF foams of varying concentrations. The biodegradability of the SF foams is also assessed based on *in vivo* observations and the mechanism of the dissolution is described and modelled.

Chapter 3 - Modelling of Silk Fibroin for TE Scaffolding

This chapter describes the effects of SF concentration on porosity, swelling ability and mechanical property. Simplistic empirical linear models are presented so as to provide a good basis for identifying suitable SF concentration for TE scaffold fabrication.

3.1 Experimental Investigation of Silk Fibroin Protein

SF porous foams of different concentrations (5, 7.5, 10, 12.5 and 15 % (wt/wt)) were produced and investigated for the concentration effects on porosity, compressive mechanical properties, swelling ratio and water uptake. The protocols for the preparation and characterisation of SF foams, and the graphical plots to derive the empirical relationships are provided in Appendix A. SF concentration is expressed as % (wt/wt), which is the ratio between the weight of the SF solute (after drying the aqueous SF mixture) to the weight of the aqueous SF mixture (Appendix A1.1.1 and Appendix A1.1.2). Three procedures were used to ensure the accuracy of the SF concentration. The first step was to ensure the homogeneity of the aqueous SF by placing the solution on a shaker before every casting take place. Next, the weight measurements were taken by an analytical micro weighing scale with a readability of 0.1 mg. Finally, the accuracy of the SF concentrations was assessed by porosity measurements using the liquid-displacement method. Each part of the investigation is presented sequentially with the following subsections: background, theory and empirical model. The analysis based on different SF concentrations shall provide a reference model to achieve a TE scaffold that is appropriate for the targeted tissue. The targeted tissues may include hard tissues such as bones, soft tissues such as cartilages and soft-hard interface such osteochondral sites.

The empirical models presented are appropriate for SF foams with concentrations of 15 % (wt/wt) and below. It is not applicable for SF porous matrices of higher concentrations because it was suggested that they tend to induce more structural complexities as their concentration or density increases [134].

3.1.1 Investigation Part One: Concentration Effects on Porosity

Background

Porosity and pore size are crucial factors in TE scaffold design as it formulates permeable pathways for cell migration and transportation of nutrients into the deeper regions of the scaffold constructs. This geometrical property is often compromised as there is a need to establish a balance between the mechanical stability of the TE construct and diffusional mechanisms within the scaffold for a specific tissue regeneration process.

Theory

The concentration of biomaterials such as polymers and naturally-derived materials used corresponds to the architecture of the resulting porous foam. The architectural features that vary with concentration include pore size and porosity. Based on the principles of particle kinetics, lower concentration of SF protein molecules will experience wider distribution within a fixed volume of mixture. In this case, the average molecule-to-molecule distance increases as the molecules are widely spread. This hence leads to the formation of bigger pores as the mixture is lyophilized. As a result, the larger pore sizes will contribute to higher foam porosity.

Observation

The explanation above is validated with the investigations conducted in this study. The SEM images of SF foams of different concentration are shown in Figure 3.1. The micrographs for 7.5 and 12.5 % (wt/wt) were not shown. For the 5 % (wt/wt) SF foams, the pore size distributions were identified to be bimodal whereas the higher SF concentrations exhibited homogeneous pore size distributions. In addition, the pores are generally spherical and elliptical for the 10 % (wt/wt) SF while the leaf-like pores were obtained for the other concentrations. The size of the leaf-like pores became smaller and the pore walls appeared more rigid as the concentration increases. The increase in the rigidity of the pore walls is suggested to influence mechanical properties. The interconnectivity between the pores was not obvious from the SEM images for all concentrations. However, this aspect shall be verified with the porosity measurements.

Figure 3.1 Scanning electron micrographs images of the 5, 10 and 15 % (wt/wt) ((a) – (c)) SF foams respectively.

Empirical Model

Porosity measurements serve as a better gauge to relate the mechanical characteristics and transport-based processes since it takes the effects of architectural features into account. In this study, a linear equation was found to relate the SF concentration to porosity of the foam (Appendix A1.1.3, Equation A1.1). The linear expression with a regression coefficient (R^2) of 0.989 is

$$\phi = -3.068 C + 85.5 \quad (\text{Equation 3.1})$$

where ϕ (%) is the porosity (Equation A1.1) and C (% (wt/wt)) is the concentration of SF.

3.1.2 Investigation Part Two: Concentration (or Porosity) Effects on Equilibrium Swelling Ratio and Water Binding Ability

Background

The swelling behaviour of articular cartilages is attributed to osmotic pressure that arises from the interactions between the negatively charged proteoglycans (PGs) and ionic interstitial ECM fluid [209]. The effects of swelling thus maintain a certain stress level within the collagen type II network so that the cartilage tissue acquire the capability to resist physiological loads. The volume change within the connective tissue is also suggested to result in the transport of solvents through the cell membrane [210]. Thus the swelling properties (or volumetric variation) offers an insight to the mass transportation through the porous material. Equilibrium swelling ratio (*ESR*) is a property which estimates when the osmotic pressure is equivalent to the elastic reaction (straining or swelling) of the porous network. The cartilage matrix was further viewed as a gel that is highly cross-linked that can expand to a limiting thickness which is dependent on its collagen fibril network arrangement.

The water binding ability, also known as water uptake, indicates the hydrophilicity of the biomaterial as well as providing the cartilage tissue with mechanical enhancements. The scaffold's ability to absorb fluid is studied to ensure that the porous construct is capable of promoting nutrients uptake (a crucial pathway) for the cell growth, proliferation and differentiation. Moreover, the interstitial fluid present in the cartilage ECM contributes to the viscoelastic properties of the connective tissue.

Quasi-static testing by imposing constant strain to cartilage was suggested to model the behaviour of tissue deformation and provide implications to the onset of osteoarthritis [211].

Theory

Under physiological conditions, the swelling behaviour of the tissue is often coupled with mass transfer as well as generalized diffusion. This section aims to put the interactions between these concepts in a general context for better theoretical understanding.

The osmotic effect occurs due to the presence of a chemical potential (μ' or potential energy) that is created between the ionic interstitial fluid and porous construct [209]. In this case, it was presumed that the porous construct is pre-seeded with cells before implantation into the defect site. The ion concentration between the interstitial fluid and pre-seeded cartilage substitute (scaffold) creates a chemical potential gradient, owing to the presence of negatively charged PGs (generated within the newly secreted ECM). The potential difference can be expressed in the form of pressure gradient based on the law of conservation of energy principle. The Bernoulli's principle is applied and neglecting gravitational effects, the equation is generalized as [212]

$$\Delta P = \frac{1}{2}\rho(v_1^2 - v_2^2) \quad (\text{Equation 3.2})$$

where ΔP is the pressure difference experienced across the foam, ρ is the density of the fluid, and v_1 and v_2 , the velocities of the fluid before and after penetrating through the porous construct. The pressure difference induces a driving force that encourages the fluid flux to permeate into the porous foam [213] (Figure 3.2).

$$J_{fluid} = \kappa_h (\Delta\pi - \Delta P) \quad (\text{Equation 3.3})$$

where J_{fluid} is the fluid flux, $\Delta\pi$ is the difference in osmotic pressures between the physiological environment and the porous construct; and κ_h is the permeability of the fluid-foam interface.

From the Maxwell relationship [214],

$$\left(\frac{\partial\mu}{\partial P}\right)_{T,n} = \left(\frac{\partial V}{\partial n}\right)_{T,P} \quad (\text{Equation 3.4})$$

where T is the temperature which is a constant, V is the volume of the foam and n is the number of osmotic molecules. This relationship illustrates that under isothermal conditions, increase chemical potential with respect to pressure gradient induces volume expansion (due to increase in n).

Figure 3.2 A schematic illustrating the essential parameters and fluid exchange between the matrix-fluid interface.

The viscoelasticity, a time-dependent mechanical property, of cartilage is mainly attributed to the interactions between the interstitial fluid and solid components (sulphated glycosaminoglycan (GAGs)). The porous constructs can thus be modelled similarly with bonded water and scaffold structure as liquid and solid phase respectively. Overall, the normalized stress relaxation function used to model the biological substitute is given by [215]:

$$\sigma(t) = \sigma_0 \int_0^t G(t - \tau) \frac{\partial \varepsilon(s)}{\partial s} \partial \tau \quad (\text{Equation 3.5})$$

where σ_0 is stress at $t = 0$. $G(t - \tau)$ represents the reduced relaxation function which is obtained experimentally by subjecting completely wetted construct under a constant strain. The change in material properties associated deformation is represented by $\frac{\partial \varepsilon(s)}{\partial s}$. This term is generally influenced when a step change in strain is implemented. The stress relaxation behaviour, as a result of step increase in strain, reveals the variation of PG network, in particular to the change in water fraction within the scaffold (Figure 3.3). This hence affects the interactions among the ECM components leading to degenerative symptoms.

The reduced relaxation function is expressed as an exponential relaxation function [216]

$$G(t) = e^{-\left(\frac{t}{\tau}\right)^{\beta'}} \quad (\text{Equation 3.6})$$

where τ is relaxation time which is the time taken to reach stress equilibrium and β' is a constant that takes into the account of material characteristics that impedes the viscoelastic behaviour. The factors affecting β' include molecular structure of SF (which include concentration effects) and the number of cross-links present within the protein.

Observation

The effect of concentration on the *ESR* of porous SF was investigated so as to determine the swelling limit that is suitable for the relevant TE application. *ESR* also assesses the degree of biopolymer-solvent reaction that would subsequently provide the necessary mechanical rigidity for the relevant TE application.

The water binding ability, in addition, allows the prediction of viscoelastic property of the resultant construct. The percentage of water uptake has a significant effect on the interaction between the interstitial fluid and collagen fibres. It is emphasized that adequate fluid should be present within the porous network matrix during tissue regeneration process so as to maintain the mechanical functionality of the native tissue. The contribution of protein chemistry as reflected in β' shall influence the stress relaxation response of the biological substitute.

Figure 3.3 Schematics showing the stress relaxation behaviour in tendon. (a) is a typical graphical plot of the viscoelastic behaviour of the connective tissue; (b) shows an overall view of a tissue fibre being strained (elongating axially with reduction in cross-sectional area; (c) the releasing of water molecules (circles) which were bonded by sGAGs due to the viscoelastic effects. This phenomenon applies for cartilage tissues where the release of water molecules that bond to the cartilage matrix is experienced upon physiologically loaded, thus leading to degenerative effects [211].

Empirical Model

Based on experimental findings, the effects of concentration (or porosity) on *ESR* were investigated (Appendix A1.1.4, Equation A1.2) and the following equation ($R^2 = 0.9643$) was obtained:

$$ESR (\%) = 17.383\phi - 412.5 \quad (\text{Equation 3.7})$$

where ϕ is expressed in percentage in this equation.

The percentage of water uptake (Equation A1.3) is related to the porosity based on the following equation ($R^2 = 0.9587$):

$$Water\ uptake (\%) = 0.4677\phi + 57.151 \quad (\text{Equation 3.8})$$

3.1.3 Investigation Part three: Concentration Effects on Mechanical Properties

Background

An ideal TE porous scaffold should possess the ability to balance mechanical function with diffusional mechanism. Equilibrating the two properties often compromises a denser and more rigid architecture of the porous foam, is usually a trade-off for better mechanical characteristics so as to withstand weight-bearing loads. However, craniofacial tissues such as the auricular and nasal cartilages, which experience only negligible physiological loadings, should be designed to maximise surface area for effective cellular processes. Nevertheless, the quantitative relationship between the architecture of the porous foam and its mechanical properties should be formulated for the fabrication of a desirable TE construct.

Theory

The protein-based porous foams possessed interconnected pores and hence their mechanical properties can be analysed using models associated with open-cell foams. Mechanical integrity of open-cell foam is sustained by a series of cylindrical struts (cell walls) connected and joined together. The protein-based struts are assumed to be homogeneous, isotropic (geometrically similar) and exhibit linear elastic behaviour. The mechanism of strut deformation and failure were used to estimate the mechanical properties of the open-cell foams.

The modified Euler's buckling equation was used to evaluate the compression load exerted on the cylindrical strut of length, l , and radius, R [217]:

$$F = \frac{\lambda E_0 I}{l^2} \quad (\text{Equation 3.9})$$

where λ is the effective length constant which varies with the type of strut support; E_0 is the biomaterial's bulk modulus; I is the moment of inertia of the cross-section ($I = \frac{\pi R^4}{4}$ for a circular cross-section with radius R); and l is the length of the strut.

This expression can also be presented alternatively as follows:

$$F \propto \frac{E_0 R^4}{l^2} \quad (\text{Equation 3.10})$$

The relative density (the ratio of foam density, ρ_f , to that of the solid biomaterial density, ρ_b) was evaluated by *Gibson et al* [218] to be proportional to $(\frac{R}{l})^2$, thus the relationship becomes

$$F \propto E_0 \left(\frac{\rho_f}{\rho_0}\right)^2 R^2$$

The relative density of the porous protein-based foam is equivalent to the volume fraction of the solids. Since $\sigma = \frac{F}{a}$, where a is the cross-sectional area of the strut, the elastic modulus ($E = \frac{\text{Stress}(\sigma)}{\text{Strain}(\varepsilon)}$) of each cylindrical strut is expressed as

$$E \propto \frac{\sigma}{\varepsilon} \propto E_0 \left(\frac{\rho_f}{\rho_0}\right)^2$$

Following this relationship, the relative density is expressed in terms of porosity:

$$\frac{\rho_f}{\rho_0} = \frac{\text{vol}_{\text{solid}}}{\text{vol}_f} = (1 - \phi_f)$$

where ϕ_f is the porosity of the open-cell foam. Thus, obtaining

$$E \propto E_0 (1 - \phi_f)^2$$

By introducing the logarithmic scale, an equation is formulated and expressed as

$$\log E = \ln E_0 + 2\omega \ln(1 - \phi_f) \quad (\text{Equation 3.11})$$

where ω is introduced into the equation as a correction factor to account for the variability in the interconnectivity of pores (such as non-permeable cell walls). The value of this factor ranges between 0 to 1 ($0 < \omega < 1$).

Observation

All porous foams, regardless of concentration or architecture, exhibited typical mechanical behaviour of cellular materials when subjected to compression. Three regions were identified in the stress-strain plots of the SF foams, they include: a toe region at the beginning (region I), followed by a linear response (region II) and then stiffening behaviour (region III) (Figure 3.4). The compressive modulus of each graph is evaluated from region II (Appendix A1.1.5), where the cell walls are deforming elastically. During the transition from region II to region III, the struts began to deform plastically where the porous networks would no longer return to its original state. As the strain increases, the stress-strain plot stiffens. This behaviour is observed due the densification of the tested SF specimen as the sample is relatively stout.

The graphical plots of different SF concentrations obtained from the experimental investigation are presented in Figure 3.5. It was shown that the compression curves exhibited steeper gradients as the concentration of SF increases. This observation is attributed to the increase in the density of the porous SFs where the thicker cell walls require more load to undergo further deformation. It was deduced that the 10 % (wt/wt) foams possessed compressive moduli quantitatively similar to that of native cartilage tissues [219, 220].

Figure 3.4 A typical plot showing the compressive behaviour of a SF foam. The inflection point, which determines the compressive modulus, was obtained after the implementation of a polynomial fit.

Empirical Model

The compression moduli of foam constructs were related to their concentration (or porosity) based on the following equation ($R^2 = 0.9611$):

$$\log E = 1.4008 + 1.686 \ln(1 - \phi_f) \quad (\text{Equation 3.12})$$

After extrapolating the data plot, the bulk modulus of SF was found to be 25.1 MPa and the factor ω to be 0.843. Using this relationship, the moduli of the porous constructs can be estimated upon the substitution of the porosity value and vice versa. Moreover, the empirical model is useful for the construction of hyaline cartilage scaffolds since the mechanical properties within the zonal organisations are depth-dependent.

Figure 3.5 Typical stress-strain plots of 5, 7.5, 10, 12.5 and 15 % (wt/wt) SF foams.

3.2 Summary

This chapter relates SF concentrations (or porosity) with properties such as equilibrium swelling ratio (*ESR*), water uptake and mechanical properties. Experimental investigations had been conducted to elucidate the relationships so that an appropriate SF concentration can be identified for TE scaffold. Moreover, the established empirical models are applicable for the fabrication of devices or tissue substitutes as long as the SF preparation protocol is followed.

As discussed in chapter 2, water is a large component of cartilage tissues and its purpose is to act as a damping mechanism to resist physiological loadings. This “cushioning” effect helps to reduce chances of cartilage injuries or deterioration symptoms. Thus, the understanding of the water binding ability of the scaffold material is important. This property is often associated with viscoelastic properties which are useful for the assessment of mechanical integrity. The SF porous matrices presented in this chapter demonstrated the ability to retain water, thus indicating their

potential to withstand mechanical forces as well as providing sufficient interstitial spaces to accommodate cell growth and tissue regeneration.

The mechanical properties of SF foams with different concentrations had also been evaluated. Based on establish empirical findings, the compressive moduli of 10 % (wt/wt) SF foams were observed to coincide with that of native cartilage tissues. Nevertheless, further analysis is required to ensure that the SF concentration is suitable for tissue formation and the investigation is described in chapter 4.

Chapter 4 - Biodegradation of Porous Silk Fibroin Matrices

The degradation property of biomaterial is one of the key components to TE scaffold design. It is important for the rate of degradation to correspond proportionally to the rate of tissue growth. Nevertheless, the mechanical integrity may sometimes be compromised and result in the formation of tissue that behaves inferior to that of the native tissue. In this chapter, theoretical models associated with the biodegradation process are presented. The first theory formulates permeability as a function of pore size, sphericity of the pore and tortuosity. The second theory is an analytical model which allows the definition of the erosion mode of the degradation process followed by the prediction of the degradation lifetime of the biopolymer. Finally, the end of the chapter describes the degradation observations of SF foams of three different concentrations (5, 10 and 15 % wt/wt), which lead to the identification of the suitable SF concentration for TE scaffold fabrication.

4.1 Permeability Theory

Permeability is a fluid-based characteristic that assesses the ability of a porous material to allow fluids to pass through it. A typical experiment for permeability measurement involves the application of fluid through the cross-section of the porous construct by applying direct pressure.

To formulate the permeability model that is applicable to a porous construct, the following assumptions were made:

1. The thickness of the porous matrix is small and permeable to fluid flow;
2. The fluid flow is laminar, viscous and incompressible;
3. Gravitational effects are negligible;

U_0 , superficial velocity, is the velocity flow of fluid before entering the porous foam (as shown in Figure 4.1) and is represented as [221]

$$U_0 = \frac{Q}{A} \quad (\text{Equation 4.1})$$

where Q is the fluid flow rate through the porous foam and A is the cross-sectional

area of the porous foam. As the fluid passes through the porous foam, the presence of pores reduces the area available for fluid flow so that the fluid continuity is preserved. The fluid continuity equation follows the following form [221]:

$$\nabla Q = 0 \quad (\text{Equation 4.2})$$

where ∇ is a vector operator. From the above continuity equation, it indicates that the velocity within the porous network (U , interstitial velocity) will be greater as the entering superficial fluid flow enters through a smaller area. Assuming that the porosity is an isotropic property, the interstitial velocity can be simply related to the superficial velocity based on the following expression.

$$U = \frac{U_0}{\phi_f} \quad (\text{Equation 4.3})$$

where ϕ_f is the porosity of the foam.

Figure 4.1 A schematic illustrating the fluid flow through a porous medium.

Two relationships in fluid dynamics describe the flow of a fluid through a porous medium. They include Darcy's law [222];

$$\frac{dP}{dL} = \frac{\mu Q}{kA} \quad (\text{Equation 4.4})$$

and Kozeny-Carman equation (modified) [223]:

$$\frac{dP}{dL} = B \frac{\mu}{d_p^2} U \quad (\text{Equation 4.5})$$

For both equations, $\frac{dP}{dL}$ is the pressure drop across the sample divided by the thickness of the sample, μ is the dynamic viscosity of the fluid, κ is the permeability of the porous sample, B is a dimensionless constant which accounts for the sphericity (spherical measurement) of the pores, and d_p is the effective pore size. Methodologically, d_p can be determined according to the protocol specified in ASTM-F316 [224].

Darcy's law is derived based on the concept of conservation of momentum while the Konzeny-Carman equation is associated with the flow effects within the internal foam architecture. From Equation 4.4 and Equation 4.5, it can be deduced that

$$\kappa \propto d_p^2 \quad (\text{Equation 4.6})$$

In this project, this relationship shall be further expanded based on two factors. The first factor illustrates the variation of effective pore size with change in porosity. The next factor takes the pore shape within the foam network into account so that the fluid flow path can be elucidated.

The porous network is assumed to be made of N identical spherical pores embedded in a solid block. The characteristic of each pore is represented by the specific surface area (S) which is the ratio of the pore surface area to the total volume of the block. Figure 4.2 illustrates a simple model for calculation.

$$S = \frac{N4\pi r^2}{AL} \quad (\text{Equation 4.7})$$

where r is the radius of each spherical pore, A and L are the area and length of the solid block respectively. The foam's interconnectivity is assumed to be uniform throughout the porous matrix to avoid any discrepancy.

Figure 4.2 A 3D schematic of a spherical pore within a solid block.

The effective pore diameter is subsequently expressed as a reciprocal of S :

$$d_p = \frac{\phi_f}{S} \quad (\text{Equation 4.8})$$

The porosity within each foam block, ϕ_f , is given by:

$$\phi_f = \frac{N4\pi r^3}{3AL} \quad (\text{Equation 4.9})$$

where $\phi_f + \phi_m = 1$. Subscripts f and m denotes the porosity and material matrix respectively.

Substituting Equation 4.8 into Equation 4.5 yields:

$$\frac{dP}{dL} = B \frac{\mu S^2}{\phi_f^2} U$$

Alternatively, the above equation can be expressed as:

$$\frac{dP}{dL} = B \frac{\mu S^2}{\phi_f^3} U_0 \quad (\text{Equation 4.10})$$

where B is an average representation of the pore shape and lies within the range from 0 to 1. A sphericity of 1 describes a complete spherical shape.

Comparing Equation 4.10 with Equation 4.4, the permeability of the porous foam is derived to be:

$$\kappa = B \frac{S^2}{\phi_f^3} \quad (\text{Equation 4.11})$$

The expression obtained relates the permeability of the porous foam with its internal architecture. As mentioned, B constitutes to sphericities of pores within the foam and is given by

$$B = \pi^{\frac{1}{3}} \frac{\sum_{i=1}^n \frac{(6V_i)^{\frac{2}{3}}}{A_i}}{n} \quad (\text{Equation 4.12})$$

where V_i is the volume of a sphere (pore) and A_i is the surface area of individual pore.

The path of fluid flow is often tortuous (having many turns) as a result of varying pore arrangements and pore surface roughness. Hence, a tortuosity factor, τ , is inserted into Equation 4.11 to provide a more accurate estimation of κ :

$$\kappa = B \frac{S^2}{\phi_f^3 \tau^2} \quad (\text{Equation 4.13})$$

Tortuosity is defined as the ratio between the actual fluid path and thickness of porous construct, which cannot be measured (Figure 4.3). Thus, an experimental technique known as the Arnold diffusion cell is used where tortuosity is evaluated through the determination of effective diffusivity of a gas within the porous construct. Taking the pore details into account, the relationship is presented as [225]

$$D_{eff} = \frac{\phi_f}{\tau} D_{gas} \quad (\text{Equation 4.14})$$

where D_{eff} is the effective diffusion coefficient which is numerically smaller than the bulk diffusion coefficient of gas (D_{gas}). This is because the bulk diffusion of gas is hindered by the presence of pores.

Based on mass continuity principles, D_{eff} can be calculated from the following equation [225]

$$N_{gas} = \frac{c D_{eff}}{L} \ln\left(\frac{1}{y_{gas}}\right) \quad (\text{Equation 4.15})$$

where N_{gas} is the molar flux, c is the molar concentration of the gas and y_{gas} is the molar fraction of the gas that has diffused through the porous construct.

Figure 4.3 A scanning electron micrograph of an open-pore cellular structure [226]. The path traced by the red arrow indicates a possible diffusion path that can contribute to tortuosity measurement.

Finally, substituting equation 4.13 into 4.4, the Darcy's law is modified to be

$$\frac{dP}{dL} = \frac{\mu Q \tau^2 \phi_f^3}{B S^2 A} \quad (\text{Equation 4.16})$$

4.1.1 Modified Reynolds Number

The Reynolds number, Re , is defined as the ratio of inertial forces to the viscous forces of fluid for a clear flow channel. Re is used to assess the significant contributions by these two forces for the given flow conditions and is presented as

$$Re = \frac{\rho_f U L}{\mu} \quad (\text{Equation 4.17})$$

Where ρ_f is the density of the fluid mixture (medium and other supplementation),

In the presence of porous medium, the modified Reynolds number, Re' , is used for flow assessment:

$$Re' = \frac{U_0 \rho_f}{S \phi_f \mu} \quad (\text{Equation 4.18})$$

Similarly, the determination of Re' indicates if the inertial effects become significant. From existing literature [225], for $Re' < 2$, flow is considered laminar with viscous effects being dominant. While for $2 < Re' < 150$, flow is still laminar and combined with inertia effects. The onset of turbulence occurs when $Re' > 150$ or 300 in porous media.

4.1.2 Non-rigid, Swelling Porous Foam

Based on the context of this work, the equilibrium water uptake of the polymeric biomaterial is reflected by its swelling ratio and can be expressed as a function of the biopolymer concentration. The polymeric-based foams with different concentrations were fully submerged into buffer solution (pH 7.4) individually and upon reaching their swelling equilibrium, the volume fraction ϕ_s of the swollen polymer is calculated based on the following equation:

$$\phi_{swell} = [1 + \frac{\rho_0}{\rho_s} \left(\frac{m_0}{m_{swell}} \right) - \frac{\rho_0}{\rho_s}] \quad (\text{Equation 4.19})$$

where ρ_0 and ρ_s represent the density of the biomaterial and solvent, respectively; m_0 and m_{swell} are the mass of the biomaterial before and after swelling. ϕ_f would be replaced with $(1 - \phi_{swell})$ when biomaterials with swelling abilities are involved in the experiments. Moreover, a network parameter M_c is calculated based on the Flory-Rehner equation to evaluate the molar mass between the cross-links (or bonds between the protein peptides). This parameter term is expressed as [227]:

$$M_c = \frac{-d_0 V_s \phi_{swell}^{\frac{1}{3}}}{[\ln(1 - \phi_{swell}) + \phi_{swell} + \chi \phi_{swell}^2]} \quad (\text{Equation 4.20})$$

where V_s is the molar volume of the solvent and χ is the Flory-Huggins numerical factor associating the polymer-solvent penetrant interaction.

4.2 Biodegradation Model

The degradation process leads to the loss of mass from degradable matrices through diffusion of aqueous solutions and hydrolysis mediated by water and/or enzymes. There are a few known factors that have been identified to limit the rate of biopolymer erosion, they include: surface area, porosity, chemical structure, crystallinity and temperature. Some of the key factors shall be discussed in this section.

4.2.1 Erosion Modes

Bulk and surface erosion are the two mechanisms which control the degradation rate of a biopolymer. The dominating degradation mechanism is generally based on the examination of the hydrolysis kinetics controlled by diffusion.

Diffusion is a transport phenomenon that occurs as a result of concentration gradient. The movement of particles from regions of high concentration to regions of low concentration is a simplistic definition for diffusion. When a process is controlled by diffusion, the Fick's equation is used to evaluate the water concentration in the case of isotropic polymers, which is given by [228]

$$\frac{dW}{dt} = \bar{D} \frac{\partial^2 W}{\partial t^2} \quad (\text{Equation 4.21})$$

$\bar{D}$ is the diffusion coefficient and is defined as the inverse of the swelling ratio:

$$\bar{D} = \frac{W_d}{100(W_s - W_d)} \quad (\text{Equation 4.22})$$

where W_d and W_s are the dry weight and wet weight of the porous matrix respectively. If ℓ is the sample thickness, the characteristic time of diffusion is expressed as

$$t_d = \frac{\tau \ell^2}{\bar{D}} \quad (\text{Equation 4.23})$$

It should be noted that the tortuosity (presented in Equation 4.4) is included as the diffusivity is expected to be hindered by the path of fluid.

Hydrolysis is a step in the degradation of a biomaterial that ruptures a chemical bond by the addition of water. The breaking of a chemical bond is also known as chain scission. As a biopolymer undergoes hydrolysis, there is a high tendency that the plastic flow properties of the polymer cause a transition from ductile to brittle behaviour. The hydrolytic process is described using a first-order kinetic equation [229]:

$$\frac{dW}{dt} = -\frac{dA^*}{dt} = \tilde{k} A^*_0 W = k' W \quad (\text{Equation 4.24})$$

where A^* is the concentration of the amorphous (soluble) regions present within the protein peptide, $\tilde{k}$ is a rate constant depends only on temperature (determined by the

Arrhenius equation, $\tilde{k} = \tilde{k}_0 e^{-\frac{E_h}{RT}}$, A^*_0 is the initial concentration of the soluble regions and k' is the first-order pseudo rate constant for water consumption.

$\tilde{k}$ can be obtained experimentally based on the following relationship [230]

$$M_t = M_0 e^{-\tilde{k}t} \quad (\text{Equation 4.25})$$

where M_0 and M_t denote the initial ($t = 0$) and time-dependent average molecular weights, respectively.

It should be noted that only the amorphous regions, which consist of helical and beta turns, are permeable to water. A^*_0 can simply be calculated as

$$A^*_0 = \frac{C}{1-X_c} \quad (\text{Equation 4.26})$$

where C is the concentration of the protein peptide and X_c is the crystallinity ratio.

The characteristic time of the hydrolysis, which is independent of the fluid path, is then expressed as

$$t_h = \frac{1}{k'} \quad (\text{Equation 4.27})$$

The comparison of the characteristic times for diffusion and hydrolysis defines the dominant erosion mechanism of the overall degradation process. When t_d is lesser than t_h , the water will reach the core of the biopolymer before it reacts and results in homogenous degradation or bulk erosion. However, if t_d is greater than t_h , the water only reacts up to the surface of the polymer, thus resulting in heterogeneity in the degradation or surface erosion. Enzymes such as metalloproteinases (MMP, regulators of wound healing) are macromolecules which are considered to have a diffusion rate of zero. Thus, surface erosion is always the case for enzymatic degradation.

By equating t_d and t_h , the critical thickness of the material is defined as

$$\ell_{crit} = \sqrt{\frac{\bar{D}}{\tau k'}} \quad (\text{Equation 4.28})$$

It should be noted any sample thickness greater or lesser than ℓ_{crit} used in laboratories will be insignificant in the prediction of the dominating degradation mechanism.

4.2.2 Degradation Lifetime

The chain scission number (n_t), at time t , is given by [231]

$$n_t = \frac{1}{M_t} - \frac{1}{M_0} = \tilde{k}A^*_0Wt \quad (\text{Equation 4.29})$$

Rearranging the equation, the degradation lifetime (t_L) is determined by

$$t_L = \frac{1}{\tilde{k}A^*_0W} \left(\frac{1}{M_L} - \frac{1}{M_0} \right) \quad (\text{Equation 4.30})$$

M_t is replaced by M_L , the average molecular mass at which the ductile-brittle transition takes place.

4.3 *In vivo* Degradation Behaviour of Silk Fibroin Foams

In this section, blank SF foam constructs of concentrations 5, 10 and 15 % (wt/wt) were implanted subcutaneously into nude mice models to elucidate their degradation behaviour. A typical staining image of a blank SF foam is presented in Figure 4.4. The foams were then harvested for histological staining after implantation for 2, 4 and 8 weeks. The staining aims to reveal the progress of tissue formation with increased implantation duration.

Since musculoskeletal injuries occur in varying degrees, the work in this section aims to evaluate the appropriate SF concentration for optimal cell infiltration and proliferation. The correlation between the concentration and the architecture of the SF foams has been presented in Chapter 3. The *in vivo* findings obtained aims to describe the theories formulated in the earlier sections in a qualitative approach. For better clarity in analysis of tissue formation, images were taken at the edge and centre of the foams.

Figure 4.4 A staining image of a blank SF foam construct.

4.3.1 5 % (wt/wt) SF Concentration

The staining images of the 5 % (wt/wt) foam are presented in Figure 4.5. Two weeks after *in vivo* implantation, ECM formation was formed on the edge of the 5 % (wt/wt) foam. The staining profile of the edges was consistent up to 4 weeks. It was inferred that process of cell migration and proliferation was more dominant in the central of the construct due to the space availability (higher permeability). To establish sufficient cell-biomaterial interaction, “pools” of ECM fluid were observed to fill up the larger centre pores. The growth of cells in the central regions increased as the 4th implantation week approaches. At this time point, the architecture of the SF foams was maintained and insignificant SF degradation was observed despite being subjected to physiological conditions.

It is interesting to note that the 5 % (wt/wt) SF foams were completely degraded, showing signs of accelerated degradation 8 weeks after implantation. The spontaneous breakdown of the SF specimens was seen in the edge as well as central regions while the protein peptides were replaced with fat tissues. This observation is agreeable to the mechanism of bulk polymer erosion. The mass loss profile of the polymer was suggested to undergo a lag period due to the lack of interaction between the erosion medium and inner pores [232]. As SF degraded to a critical degree, the degraded components in the inner regions stretches to the edges of the protein, thus causing spontaneous erosion.

The acceleration erosion process may be attributed to the swelling ability of the SF foam [233]. As illustrated in chapter 3, lower SF concentration yields a higher swelling ratio. The swelling effect then increases the overall porosity of the foam (refer to Equation 4.19), thus resulting in a decrease in the molar mass of cross-links (crystalline regions) within the protein peptide. The strain effects due to swelling and transportation of physiological fluids would further induce tension on the pore walls and decrease its resistance towards fluid flow, therefore collapsing the SF structure. It is concluded that the degradation of 5 % (wt/wt) SF foams was too fast to sustain tissue growth.

4.3.2 10 % (wt/wt) SF Concentration

The staining images of the 10 % (wt/wt) foam are presented in Figure 4.6. The staining images of the 10 % (wt/wt) SF foam showed cells infiltrating into the narrower pores in both the edge and centre of the specimen after 2 weeks of *in vivo* implantation. Since the pore sizes were smaller compared to lower SF concentration, minimal spaces within the pores were filled with ECM fluid, thus indicating better cell-biomaterial interaction.

As week 4 approached, signs of SF degradation were observed; the pores at the edge and centre appeared wider in dimensions. The degraded areas were substituted with small clusters of cells and/or ECM. This finding suggests that partial SF degradation has taken place in order to accommodate further cell attachment and tissue formation. This deduction is consistent with results after 8 weeks. The content of SF was seen to reduce significantly while the 10 % (wt/wt) foam was almost fully filled with regenerated tissue.

4.3.3. 15 % (wt/wt) SF Concentration

The staining images of the 15 % (wt/wt) foam are presented in Figure 4.7. The central region of the 15 % (wt/wt) SF foam, in contrast, was found to be infiltrated with more cells compared to its edge after two weeks of subcutaneous implantation. Despite having smaller pores, the filling of the interstitials revealed good cell-biomaterial interaction.

The degree of degradation of 15 % (wt/wt) SF foam at week 4 was similar to the 10 % (wt/wt) SF. After 8 weeks, the pore interstitials at edge and central regions of the 15 %

(wt/wt) SF foam were infiltrated but not as much compared to that of 10 % (wt/wt) SF. These findings suggest that the degradation of the 15 % (wt/wt SF) foam was sufficiently slow to allow enough time for cell infiltration and tissue growth.

Figure 4.5 Staining images of the 5 % (wt/wt) SF specimens. Images lined in column 1 presents the edge of SF while images in column 2 show the centre of SF. The row order (from top to bottom) signifies the increase in time point for *in vivo* implantation. The black arrows indicate the “pools” of ECM fluid and the green arrows show the remaining SF fragments. (Scale bar = 500 μ m)

Figure 4.6 Staining images of the 10 % (wt/wt) SF specimens. Images lined in column 1 presents the edge of SF while images in column 2 show the centre of SF. The row order (from top to bottom) signifies the increase in time point for *in vivo* implantation. The black arrows show the degraded SF areas which, in some cases, are replaced by cell clusters. (Scale bar = 500 μ m)

Figure 4.7 Staining images of the 15 % (wt/wt) SF specimens. Images lined in column 1 presents the edge of SF while images in column 2 show the centre of SF. The row order (from top to bottom) signifies the increase in time point for *in vivo* implantation. The black arrows show the degraded SF areas which, in some cases, are replaced by cell clusters. (Scale bar = 500 μ m)

4.4 Summary

This chapter presented the theoretical models that relates to the biodegradation behaviour of porous foams. Firstly, the permeability theory was expressed as a function of the pore geometry such as effective pore diameter and pore sphericity as well as the tortuosity of the fluid path. The formulation of the permeability function allowed a better estimation on the ability of fluid flow within a porous matrix.

Next, a biodegradation model was presented to assist in elucidating the dominant erosion mechanism during degradation. The kinetics of hydrolysis controlled by diffusion of water was shown. Based on the comparison between the characteristic time of the hydrolysis and diffusion steps, the dominant erosion mechanism can be defined. In addition, the degradation lifetime derived from the chain scission number was expressed as a function of the average molecular weights before and after the entire degradation process.

The last section of this chapter described the degradation process of vary SF concentrations. SF foams of three different concentrations (5, 10 and 15 % (wt/wt)) were subcutaneously implanted into nude mice models and observed at three distinct time points (2, 4 and 8 weeks). The permeability of the 5 % (wt/wt) SF foam was high and the wide pore feature indicates the ability for tissue regeneration. However, the mechanical integrity of the foam was compromised as a result of its architecture and possibly swelling effects. As a result, the SF structure collapse before the porous matrix was fully infiltrated with cells and ECM.

Conversely, the 10 % wt/wt revealed a suitable porous network for cell attachment and tissue ingrowth. Even though the first time point did not indicate good cell-biomaterial interaction, the degradation of the SF leaflets at the second time point facilitates cell cluster formation which populated and filled up the majority of the pore interstitials. This work has identified 10 % (wt/wt) SF as a suitable concentration for the manufacturing of TE scaffolds since its degradation rate matched the speed of tissue formation. The highest concentration, 15 % (wt/wt), produced an appropriate mechanical architecture to support physiological load but it was not considered because tissue regeneration was hindered by its slow degradation rate.

Chapter 5 - Droplet Impaction and Line Formation

Model of Inkjet Printing

This chapter presents analytical models on the principal processes of inkjet printing: droplet impaction and line formation. The theoretical approach developed for inkjet printing serves to provide the understanding of the manufacturing process and allow the prediction of maximum spreading ratio and tendency of droplet recoil upon droplet impaction. This study also identifies properties and phenomena that contribute significantly to the resultant shape of the deposited droplet and topography of the printed line respectively. Finally, the manufacturability of the 3D parts using the inkjet printer (Model: T612, Benchtop, Solidscape Inc.) used in this study will be examined.

5.1 Inkjet Printing

Inkjet printing is a highly flexible AM technology that enables the deposition of small amount of material to manufacture 3D parts without the need for costly patterning mask. This AM technique manufactures 3D physical models by the precision placement of liquid droplets. Piezoelectric drop-on-demand (DOD) technology was used in the current work to build the sacrificial moulds.

The DOD printing process begins with the dispensing of droplets from the piezoelectric print head. The amplitude and frequency of the driving pulse applied to the piezoelectric actuator determines the size and the velocity of the dispensed droplet [234]. The study of impaction of a single droplet on a solid surface is required so that the geometrical parameters, such as spread diameters and contact angles of the impact droplets, are consistent. The single droplet impact process involves the establishments of energy models relating to fluid mechanics in order to achieve minimal energy at the solid-liquid interface. Upon impact, the surface energy between liquid and atmosphere defines whether the spread droplet will recoil from the solid surface. Surface roughness, in addition, plays a significant role in the interaction between the droplet and the substrate which is defined by the apparent contact angle.

The interaction and coalescence between two adjacent printed droplets are processes which affects the line formation. For instance, it is crucial to identify the cooling rate of the first droplet so that the two droplets can coalesce to form an equilibrated shape

on the solid surface. The time taken to solidify the first droplet, as obtained from a heat transfer model, is used as a critical parameter in this study for the coalescence between the two droplets. Furthermore, the drawback effects during coalescence are taken into account and are suggested to contribute to the discontinuity of the printed line.

5.2 Droplet Formation

The dynamics at impact occurs based on the interplay between kinetic energy and surface tension of the droplet. The droplet will oscillate upon impact onto the surface unless it possesses negligible inertia, splatters or rapidly freezes. Spreading and splashing are the common behaviours seen upon impact onto a surface (Figure 5.1). The initial mechanisms of the droplet impact are similar in both behaviours; the water droplets flatten in the horizontal direction upon impact and reshape into circular disks. The Weber number of a droplet, We , is used to estimate the possible behaviour of the droplet upon impact [234].

$$We = \frac{\rho u^2 d}{\gamma_{LV}} \quad (\text{Equation 5.1})$$

where ρ is the density of the droplet, u is the initial impact velocity, d is the diameter of the spherical droplet (before impact) and γ_{LV} is the liquid-vapour surface tension.

The surface tension of droplet is strong enough to break the spreading process when We is small ($We < 4$) [235]. For a droplet with larger We ($We > 90$), the role of kinetic energy dominates and is capable of splitting the droplet up into smaller droplets, which indicates the occurrence of splashing [236]. Subsequently, after spreading or splashing the surface tension creates an upward flow of droplet in a jet form, a process known as recoil, before reaching an equilibrium state.

Figure 5.1 Stages of droplet impinging on surface. The top row shows the spreading mechanism while the bottom row presents the splashing process.

5.2.1 Spontaneous Spreading

Spontaneous spreading is a process which occurs at a solid-liquid interface and influences the impact process significantly. As the droplet spontaneously spread on the substrate surface, the area of solid-vapour interface is substituted with a unit area of liquid-solid and liquid-vapour interface. The liquid-surface area increases and achieves a maximum spreading diameter on the substrate, without affecting the volume. The change of energy between the three interfacial components is defined as Gibbs free energy (ΔG) and is given by the following relationship [237]:

$$\Delta G = \xi = \gamma_{SV} - \gamma_{SL} - \gamma_{LV} \quad (\text{Equation 5.2})$$

where ξ is the spreading ratio, γ_{SV} is the solid-vapour surface tension and γ_{SL} is the solid-liquid surface tension. Spontaneous spreading occurs when $\xi > 0$ and if $\xi < 0$, the droplet will flow away from the supposed deposition position.

These three types of surface tensions are related based on the Young's equation [235] (Figure 5.2):

$$0 = \gamma_{SV} - \gamma_{SL} - \gamma_{LV} \cos \theta \quad (\text{Equation 5.3})$$

where θ is the static contact angle.

Figure 5.2 Schematic of a droplet showing surface tensions in Young's equation.

γ_{SV} and γ_{SL} are not easily available. Thus, the spreading ratio can be normalized by dividing by γ_{LV} :

$$\frac{\xi}{\gamma_{LV}} = \frac{\gamma_{SV} - \gamma_{SL}}{\gamma_{LV}} - 1 = \cos \theta - 1 \quad (\text{Equation 5.4})$$

This equation indicates that, for $\frac{\xi}{\gamma_{LV}}$ to be less than zero, the droplet possess a finite contact angle that minimizes the surface energy. This hence results in the occurrence of spontaneous spreading.

5.2.2 Impaction of Droplet on a Solid Surface

Before impact, energy is conserved in a system of a constant volume containing a liquid droplet, a substrate surface and the surrounding atmosphere. The droplet is driven by kinetic energy and surface energy. The total surface energy contains the liquid-vapour surface energy ($SE_{1,LV}$) and solid-vapour energy ($SE_{1,SV}$) which are defined respectively as

$$SE_{1,LV} = \pi d^2 \gamma_{LV} ;$$

$$SE_{1,SV} = \frac{\pi D_m^2}{4} \gamma_{SV}$$

where D_m is the maximum spreading diameter and γ_{SV} is the solid-vapour surface tension respectively. $SE_{1,SV}$ is the surface energy of the substrate at impact where

maximum spreading of the droplet or spontaneous spreading occurs. The total impact energy is hence expressed as

$$E_1 = KE_1 + SE_{1,LV} + SE_{1,SV} \quad (\text{Equation 5.5})$$

where KE_1 is the kinetic energy possessed by the droplet before the impact and is represented as

$$KE_1 = \frac{\pi}{12} \rho u^2 d^3$$

The kinetic energy is consumed to overcome surface energy and viscous forces as the droplet deforms and flattens. The surface energy is the sum of the liquid-surface energy ($SE_{2,LV}$), the energy at the solid-liquid interface ($SE_{2,SL}$), subtracting the solid-vapour surface energy ($SE_{2,SV}$). The deformed droplet at maximum spreading is taken as a thin circular disk. The total surface energy (SE_2) after the impact, is thus presented as

$$SE_2 = SE_{2,LV} + SE_{2,SL} - SE_{2,SV} \quad (\text{Equation 5.6})$$

where

$$SE_{2,LV} = \gamma_{LV} \left(\frac{\pi}{4} D_m^2 + \frac{2}{3} \pi \frac{d^3}{D_m} \right);$$

$$SE_{2,SL} = \frac{\pi}{4} D_m^2 \gamma_{SL};$$

$$SE_{2,SV} = \frac{\pi}{4} D_m^2 \gamma_{SV}$$

Equation 5.6 can be further simplified to

$$SE_2 = \gamma_{LV} \left[\frac{\pi}{4} d_m^2 (1 - \cos\theta) + \frac{2}{3} \pi \frac{d^3}{D_m} \right] \quad (\text{Equation 5.7})$$

Energy is also consumed to deform the droplet against viscosity. The viscous dissipation is expressed as [238]

$$E_{2,v} = \int_0^{t_c} \int_V \phi dV dt \approx \phi V t_c \quad (\text{Equation 5.8})$$

where $V = \frac{1}{4}\pi D_m^2 \delta$ is the volume of the flatten droplet, $\delta = 2 \frac{d}{\sqrt{Re}}$ is the layer thickness of the deformed droplet, Reynolds number is $Re = \frac{\rho u d}{\mu}$ and $\emptyset$, the dissipation function is given by

$$\emptyset = \mu \left(\frac{u}{\delta}\right)^2 \quad (\text{Equation 5.9})$$

where $t_c = \frac{3}{8} \frac{d}{u}$ is the time taken to obtain a maximum spread diameter [238]. Combining equations 5.8 and 5.9,

$$E_{2,v} = \frac{\pi}{3} \frac{\rho u^2 d D_m^2}{\sqrt{Re}} = \frac{\pi}{3} \frac{We}{\sqrt{Re}} \gamma_{LV} D_m^2 \quad (\text{Equation 5.10})$$

It should be noted that the viscous dissipation modelled above is used for liquids with low viscosity where δ is less than the flattened droplet height, h ($\delta < h$).

For viscous liquids, where $\delta > h$, the following viscous dissipation expression is used

$$E_{2,v} = \frac{8}{15} \frac{\pi \mu u D_m^2}{d^2} \quad (\text{Equation 5.11})$$

The energy conservation between before and after the droplet's impact onto the substrate is established as:

$$KE_1 + SE_{1,LV} + SE_{1,SV} - SE_{2,LV} - SE_{2,SL} + SE_{2,SV} - E_{2,v} = 0 \quad (\text{Equation 5.12})$$

The maximum spread diameter of the droplet for liquid of low and high viscosities is determined by the following relationships:

For $\delta < h$,

$$\left(\frac{We}{12} + 1\right) D^* - \left[\frac{1}{4}(1 - \cos\theta) + \frac{\pi}{3} \frac{We}{\sqrt{Re}}\right] D^{*2} + \frac{2}{3} = 0 \quad (\text{Equation 5.13})$$

For $\delta > h$,

$$\left(\frac{We}{12} + 1\right) D^* - \frac{8}{15} \frac{We}{Re} D^{*5} - \frac{1}{4}(1 - \cos\theta) D^{*3} + \frac{2}{3} = 0 \quad (\text{Equation 5.14})$$

where $D^* = \frac{D_m}{d}$ is the spreading ratio of the droplet which can be simply resolved using factor theorem.

5.2.3 Droplet Recoil

It is important to assess the degree of recoil of the droplet after the deformation and flattening upon impact. As the droplet recoils, the droplet is assumed to be at rest and the kinetic energy is zero ($KE_3 = 0$) and the total energy only includes gravitational potential energy (PE_R) and liquid-vapour surface energy ($SE_{R,LV}$) which are defined respectively as

$$PE_R = \frac{\pi}{12} \rho g d^4 ;$$

$$SE_{R,LV} = \pi d^2 \gamma_{LV}$$

where g is the gravitational acceleration.

The gravitational potential energy can be ignored as its change is smaller than surface energy and its value never exceeds 2 % of the total energy as specified by *Kim et al* [239]. Rebounds occur when the total energy during droplet recoil (E_3) exceeds ($SE_{R,LV}$).

E_3 can be related to the difference between the total energy after droplet impact ($E_2 = SE_2 - E_{2v}$) and the viscous dissipation during the droplet recoil ($E_{3,v}$):

$$E_3 = E_2 - E_{3,v} \quad (\text{Equation 5.15})$$

A recoiling limit, E^* , is then used to assess the tendency of droplet recoil.

$$E^* = \frac{E_3 - E_R}{E_R} = \frac{E_2 - E_{3,v} - E_R}{E_R} \quad (\text{Equation 5.16})$$

where $E_R = SE_{R,LV}$, $\frac{E_{3,v}}{E_R} = x(D^*)^y(1 - \cos\theta)^z$; x, y and z are empirical constants determined from experimental impact data using least-square data regression. The recoiling limit is finally expressed as

$$E^* = \frac{1}{4} D^{*2}(1 - \cos\theta) - x(D^*)^y(1 - \cos\theta)^z + \frac{2}{3} D^* - 1 \quad (\text{Equation 5.17})$$

where $E^* \leq 0$ indicates that the droplet remain on the surface (negligible recoil). From the above equation, it is inferred that the contact angle and spreading ratio of the droplet plays a significant role in defining the recoil tendency of the droplet after impact on the substrate surface.

5.2.4 Surface Roughness

For drop impact study, it is suggested that the surface roughness of the substrate, in this case, the building platform or deposited layer, will affect the spread diameter. This is because the deposited area is milled by the cutter to achieve the specific layer thickness. The texture of the surface is geometrically modelled with identical pillars spaced equally on the substrate surface.

It is assumed that the liquid drop wets the rough surface completely; the grooves, which are the spaces between the pillars, are filled with liquid after the droplet impact (Figure 5.3). This means that a capillary effect is created. The capillary pressure, p_{cap} , that has to be overcome to allow liquid penetration within the roughness grooves is given by [237]

$$p_{cap} = \frac{-\cos\theta}{(1+\frac{b}{a})^2-1} \cdot \frac{\pi d^2 \gamma_{LV}}{a} \quad (\text{Equation 5.18})$$

where a is the width of the pillar and b is the pitch between the pillars. The liquid will enter the grooves if p_{cap} is balanced by the Bernoulli pressure ($\frac{\rho u^2}{2}$). Equating these two components will provide critical value $(\frac{b}{a})_{crit}$ which is useful in assessing the effect of substrate roughness on the successive deposited layer.

Figure 5.3 Schematic showing complete penetration of liquid droplet into the grooves of the rough surface.

Moreover, for rough surfaces, an apparent contact angle is defined and is related to the static contact angle (θ) based on Wenzel's model [240]

$$\cos\theta_r = \Psi (1 + \cos\theta) - 1 \quad (\text{Equation 5.19})$$

where $\Psi = \frac{1}{(\frac{b}{a}+1)^2}$.

5.3 Line Formation

The printing process begins by considering the ejection of two droplets with an initial diameter, d_0 , and temperature T_i . The droplets are dispensed at a frequency f on a substrate at temperature, T_s . During the deposition of each droplet, the print head moves with velocity, U .

The first droplet experiences drop impact before reaching an equilibrium state where the maximum spread diameter is achieved. The second droplet is then ejected and lands on the substrate after a time interval, $\Delta t = \frac{1}{f}$.

The droplet centre-to-centre distance, L , is calculated from the speed of the print head and the droplet ejection frequency:

$$L = \frac{U}{f} \quad (\text{Equation 5.20})$$

A schematic illustration is presented in Figure 5.4. The diameter of each deposited droplet, D_s , is formed after impact and solidification. D_s is dependent on the fluid flow during impact and T_s . If T_s is less than the melting temperature of the droplet (T_m), the resulting D_s will be dependent on the solidification rate of the droplet.

5.3.1 Coalescence of Droplets

During the printing process, the droplets coalesced to form an equilibrium shape on a flat surface. The coalescence re-shapes upon combining the two droplets and serves to minimize surface energy. The two dispensed droplets will merge if L is less than the final spread diameter of each droplet (D_s). The degree of overlap between the two deposited droplets is described by the overlap ratio

$$\lambda = 1 - \frac{L}{D_s} \quad (\text{Equation 5.21})$$

where $\lambda = 1$ for a complete overlap between the centres of the two droplets. While for $\lambda < 0$, there is no overlap between the two dispensed droplets. If the overlapping

droplets equilibrate equivalently to the first droplet, the resulting stabilised shape is a spherical cap.

Figure 5.4 Schematic showing the deposition of two droplets and the parameters (Δt , D_s and L) for the description of the overlapping droplets. The numerical figures represent the droplets' sequence.

Drawback is a process whereby the edges of the droplets are drawn back in a direction perpendicular to the flat surface as the centres of the two drops join. As shown in Figure 5.5, the retraction mechanisms between the two edges are not identical due to the printing sequence (as the first droplet cools faster than the other). This asymmetry becomes more obvious if the droplets are rapidly cooled or frozen.

The effects of drawback should be emphasized as the droplet coalescence process may lead to line breaks and/or varying thickness, hence influencing the dimensional requirements of the resultant build.

The equilibrium diameter of the combined droplet should be at least $(D_s + L)$ when both deposited droplets do not interact. The drawback index is then defined as

$$\epsilon = \frac{D_x}{D_s + L} \quad (\text{Equation 5.22})$$

where D_x is the measured combined length from experimental observation. For $\epsilon = 1$, it indicates that there is no interaction between the overlapping droplets; $\epsilon < 1$, denotes the retraction of the liquid-solid contact line; while $\epsilon > 1$ droplets have spread further as they would perform individually.

Figure 5.5 Schematic showing the drawback mechanism occurring between two droplets. The arrows indicate the directions in which the edges of the droplets are moving along. The numerical figures represent the droplets' sequence.

5.3.2 Heat Transfer Modelling

It is crucial to ensure that the second droplet is deposited before the first droplet solidifies. Thus, the time taken for the first droplet to cool is essential in determining if the coalescence between the two droplets is successful. This duration is calculated based on a simple thermal energy model.

At the start of the printing process, a droplet with temperature T_1 (greater than its melting temperature, T_m) at impact lands on a substrate ($T_s < T_m$). The total thermal energy in the droplet is given by [241]

$$H_1 = \frac{\pi d^3}{6} \rho C_p^* (T_1 - T_m) \quad (\text{Equation 5.23})$$

where ρ is the density of the droplet and C_p^* is the total heat capacity which includes the latent heat of fusion (L_f),

$$C_p^* = C_p + \frac{L_f}{T_1 - T_m}$$

Assuming the droplet has a constant surface temperature at the solid-liquid interface, the heat flux boundary is expressed as [242]

$$\dot{q} = \frac{k (T_s - T_1)}{\sqrt{\pi \alpha t}} \quad (\text{Equation 5.24})$$

where k is thermal conductivity and $\alpha = \frac{k}{\rho C_p}$ is the thermal diffusivity of the droplet.

As the second droplet approaches the substrate (at Δt), the remaining thermal energy of the first droplet is given by

$$H_2 = H_1 + \frac{\pi d^2}{4} \int_0^{\Delta t} \dot{q} dt \quad (\text{Equation 5.25})$$

The contributing effect of droplet spreading is neglected since the spreading time is much lesser than the time taken for droplet cooling. As H_2 is set to zero, the time at which the thermal energy of the first droplet is completely lost is represented by [242]:

$$\Delta t_c = \frac{\pi d^6}{9\alpha D_s^4 (1 + \frac{T_m - T_s}{T_1})^2} \quad (\text{Equation 5.26})$$

If Δt is less than Δt_c , then the second droplet lands on a droplet that is cooling down (yet to solidify completely). Furthermore, it should be emphasized that processing conditions and parameters such as change in droplet overlap, substrate temperature and deposition frequency of droplets may attribute to the cooling rate of droplets [234].

5.4 Discontinuity of Line

The maximum possible length of line with n number of droplets overlapping one another is defined as,

$$D_x' = D_s + (n - 1)L \quad (\text{Equation 5.27})$$

where n is the number of deposited droplets.

Due to the effects of drawback, the change in length (ΔL) of the line printed tends to decrease as n increases. The sequential plotting of three droplets is used to illustrate the discontinuation of a printer line due to drawback (Figure 5.6). As the second droplet is dispensed, overlapping the first deposited droplet and equilibrated before the third droplet impacts, the length of the coalesced drops is defined by

$$x = (D_s + L)\epsilon \quad (\text{Equation 5.28})$$

where ϵ is the drawback index between the coalescence of the first deposited droplets.

The discontinuity of the printed line is evaluated by using the following relationship:

$$\Delta x = (D_s + L)\epsilon - 2L \quad (\text{Equation 5.29})$$

If Δx is less than zero, the third droplet will not touch the coalesced droplet.

Figure 5.6 Schematic showing the deposition of the first three droplets sequentially (numerically named respectively). The parameters presented are used to examine if the discontinuity of the line formation is reached.

5.5 Manufacturability Evaluation of the 3D Inkjet Printing Process

To assess the dimensional deviation between the designed and measured feature size, a slab of 1 mm in thickness with circular through-hole profiles of 0.5, 0.75, 1 and 2 mm diameters were printed. The slab consists of five sets of through-hole profiles. The built features were imaged using the scanning electron microscope (SEM) and subsequently measured using a public domain image-processing program (ImageJ, National Institute of Health (NIH), USA) (Figure 5.7). The roundness profiles of the measured feature were also evaluated using the out-of-roundness (OOR) measurement

according to ASTM Y14.5M-1994 [243] and ANSI B89.3.1-1972 [244]. The establishment of the dimensional measurements served to provide a basis to understand the geometric tolerance of the AM built as well as to identify the effects of control parameters on the resulting built models

Figure 5.7 Left: A scanning electron micrograph showing a through-hole profile. Right: The processed image which shows a typical out-of-roundness measurement.

Table 5.1 shows the dimensional deviations between the designed and as-built features. It was observed that the percentage diameter error increased as the designed featured size decreased. Nevertheless, the measured roundness demonstrated better circular profile with decrease in diameter of through-hole samples; the observation suggests that the shape configuration was maintained by the printing system. It was postulated that the dimensional discrepancies were due to the change in properties of the build and support materials, reported previously by *Liu et al* [245].

Designed diameter (μm)	Measured diameter (μm)	Diameter Error (%)	Out-of-roundness
2000	1887.34 ± 19.72	5.63 ± 0.96	118 ± 21.61
1000	889.17 ± 14.3	11.08 ± 1.43	78 ± 16.14
750	610.12 ± 8.88	18.65 ± 1.18	68.5 ± 12.07
500	361.34 ± 7.98	27.73 ± 1.60	51.5 ± 8.22

Table 5.1 Dimensional measurements of the slab template manufactured by the 3-D inkjet printer.

5.6 Summary

This chapter has presented the models for droplet impaction on a substrate and line formation due to droplets coalescence. The inkjet printer discussed operates based on a DOD additive technology that forms 3D built models by depositing small droplets sequentially. Low material wastage remains as one of the main advantages of this AM method.

The dynamics of droplet impact is important in defining the resulting geometry of equilibrated droplet. Using the energy conservation principles, equations to determine maximum spreading ratio of the droplet were developed and the modelled equations were found to be strongly dependent on the viscosity and impact velocity of the liquid. The frequency and amplitude of the driving pulse applied to the piezoelectric actuator of inkjet printer are important control parameters in defining the size and velocity of the droplet. Hence, the spreading effects of droplets can be adjusted by tuning these two factors during the printing process. Under circumstances where energy imbalance is experienced between the kinetic energy and surface energies, the droplet has a tendency to recoil. This theoretical study identifies the contact angle and spreading ratio as significant contributing factors to the tendency of droplet recoil. The surface roughness of the solid surface has also been found to affect the contact angle of the droplet.

The mechanism of line formation has been established based on the modelling of the coalescence between two droplets. A heat transfer model has been proposed for the prediction of the critical time at which the first droplet could no longer merge with the second droplet as a result of cooling. The change in droplet overlap, substrate temperature and deposition frequency of droplets are some attributes that may affect the cooling rate of droplets. Furthermore, the effect of drawback between two droplets was used to predict the conditions where the line formed via printing becomes discontinued.

Finally, the manufacturability of the parts built by the inkjet printer was evaluated. The round configuration of the built parts was maintained regardless of the size of the designed feature. In addition, larger dimensional deviations between the designed and as-built features were observed as the size of the designed feature decreases.

Chapter 6 - Experimental Procedures

This chapter presents the experimental procedures carried out for this research. Firstly, the SF scaffold fabrication process, which includes the building of sacrificial moulds, casting of aqueous SF into the moulds and mould removal, is elaborated. The SF tissue constructs were then subjected to chemical structure and thermal analysis to compare their properties to that of the porous SF foams. The purpose of this comparison is to ensure that the intrinsic properties of SF protein were maintained. The *in vitro* evaluation of the SF scaffold was then conducted with cytotoxicity test to ensure that the SF constructs induced negligible traces of toxicity. The *in vitro* cultivation of SF scaffolds using porcine chondrocytes was carried out to examine the constructs potential for cartilage regeneration. Finally, pre-seeded SF constructs were implanted into nude mice models to elucidate their ability to maintain the phenotypic characteristic of chondrocytes under physiological conditions.

6.1 Fabrication of Silk Fibroin Tissue Constructs Using Indirect Additive Manufacturing Method

6.1.1 Design of Sacrificial Mould

The negative sacrificial mould was designed using commercial computer aided design (CAD) software (ProEngineer, PTC). The overall diameter and channel width of each negative mould were 10 mm and 700 μm respectively. The CAD model was then saved as a StereoLithography (STL) file and subsequently exported to the 3-D inkjet printer (Model: T612, Benchtop, Solidscape Inc) application software, ModelWorksTM. The CAD model was duplicated to maximize fabrication within the building platform and the models were orientated along the building axis. In ModelWorksTM, the manufacturing parameters such as cutter feed rate, cooling profile and jet velocity were selected from a library of configurations. The layer thickness specified was 0.0508 mm for the fabrication of moulds.

6.1.2 Fabrication of Sacrificial Mould

Using the droplet-based inkjet printing approach, each thin layer formed consists of two distinct materials, InduraCast™ (thermoplastic) and InduraFill™ (wax), to produce the mould and temporary supporting features respectively.

Once the printing process has been completed, the building platform was removed and placed on a hotplate. The heating effect of the hotplate softens the substrate layer, hence easing the mould removal process. The support materials of the mould builds were removed by immersing the printed parts into mineral oil at 65 °C, with constant stirring. The unwanted solutes and oil residues within the mould structures were blown off using an air gun. Subsequently, the moulds, as shown in Figure 6.1 (a), were left to dry in the fume hood for 3 days.

6.1.3 Casting of Regenerated Silk Fibroin

Aqueous SF with concentration of 10 % (wt/wt) was cast into the sacrificial thermoplastic moulds. The cast moulds were allowed to stand for 15 minutes under room temperature to ensure full penetration of the regenerated SF within the scaffold template.

6.1.4 Processing of Cast Moulds

The moulds containing the SF solution were subsequently frozen at –80 °C for at least 12 hours and freeze dried at -85 °C for 24 hours to obtain the SF constructs. A low freezing temperature (faster freezing rate) was chosen because constructs frozen at a higher freezing temperature (lower freezing rate) tend to be more mechanically fragile. The low freezing rate resulted in the formation of larger ice crystals, thus leading to wider pore sizes and easier breakage during handling. After the lyophilisation process, the specimens were immersed in ethanol (95% in methanol) for 10 minutes to induce β -sheet structure of silk and insolubility in aqueous solutions. This procedure was followed by another 2 hours of freeze drying to sublimate the solvent before removing the moulds.

6.1.5 Sacrificial Mould Removal

Upon achieving the cross-linked SF foams, the specimens were immersed into 50 % (v/v) ethanol at 80 °C to remove the thermoplastic mould materials. The concentration

of the alcohol solvent and treatment temperature were chosen to reduce the duration for mould removal and minimize further β -sheet phase transformation. Once the mould materials have been completely removed, the SF scaffolds were immersed in deionized water at 80 °C to leach out the solvent. The image of the resulting SF scaffold is presented in Figure 6.1 (b).

Figure 6.1 (a) Printed thermoplastic mould (after removal of support material) and (b) SF scaffold obtained after using the RP-fabricated mould.

6.2 Characterization of Silk fibroin Foams and Scaffolds

6.2.1 Fourier Transform Infrared Spectroscopy (FTIR)

The analysis of the amide bands of the protein-based SF foams and scaffolds was conducted using FTIR spectrometer (Model: Spectrum GX, Perkin Elmer). For each sample set, approximately 1 mg of specimen was pressed into a pellet with 9 mg of potassium bromide (KBr) and each spectrum was recorded in transmittance mode with an accumulation of 10 scans with a resolution of 4 cm^{-1} and a spectral range of 4000 to 400 cm^{-1} .

6.2.2 Differential Scanning Calorimeter (DSC)

The thermal properties of the SF sponges and scaffolds were analysed using a differential scanning calorimeter (Model: Diamond DSC, Perkin Elmer). For each measurement, specimens of approximately 2.5 mg were sealed in aluminium cell and the course of investigation was conducted under a nitrogen atmosphere, at a heating rate of 10 °C/min from -30 to 400 °C.

6.2.3 Morphology and Topographical Analysis

The morphological analysis of the SF scaffolds was conducted using a scanning electron microscope (SEM, Model: JSM-1600, JEOL). The specimens were air-dried and sliced axially to expose the cross-sectional area for the observation of internal morphology. The concentration effects of SF foams on the average pore size were analysed while the SF scaffolds were examined for both macro- and micro-morphological features. Subsequently, the samples placed on an aluminium holder, sputtered with gold-palladium and mounted onto the SEM and observed using an accelerating voltage of 10.0 kV.

6.3 Preliminary Cell Culture Investigation

6.3.1 Cell Culture Process

In generally, cell lines can be purchased from a commercial vendor or isolated from specific tissue components from animal models. The culturing of cells was conducted to provide sufficient viable cells for seeding onto the scaffold constructs.

In this work, data from two cell types will be presented. The first cell type is 3T3 embryonic fibroblasts and the cells are used for the cytotoxicity testing of the SF scaffolds. The test is conducted to examine if the scaffold construct induce toxicity after the multiple-step fabrication process. Moreover, the results drawn from the assessment will serve as a guide to evaluate whether the SF scaffold is feasible for further *in vitro* and *in vivo* examinations.

The second cell type used is porcine cartilage cells. Chondrocytes are generally isolated from fresh tissues due to its high tendency undergo phenotypic differentiation. The SF constructs were seeded with the harvested chondrocytes to reveal its potential for cartilage regeneration.

To avoid cross-contamination, the two cell types were cultured at different time periods. The general procedure to maintain 3T3 cells are briefly elaborated in the following sections.

6.3.2 Essential Cell Culture Equipment and Reagents

This section illustrates the required consumables for the culturing of embryonic fibroblasts (N1H/3T3, Catalogue no.: CRL-1658) purchased from American Type Culture Collection (ATCC). The cell culture works were carried out in a Gelman Class II Biosafety cabinet with vertical laminar flow for the establishment of an environment with work, health and safety. The handling of cell culture required the user to be donned with personal protective equipment (PPE) which includes laboratory coat, covered shoes and gloves. The cells were cultured in tissue culture flasks (75 cm³ and 150 cm³ in capacity) and were stored in an incubator (Their-cycle, Thermo Scientific). The conditions within the incubator were maintained at 37 °C and 5.0 % CO₂ atmosphere.

The standard reagents required for the culturing cells include:

1. Cell culture medium

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) was used as the standard culture medium which contains the essential inorganic salts, amino acids, vitamins and glucose. The inorganic salts are crucial for equilibrating the culturing conditions due to cell metabolism while the other constituents are necessary for cell growth and reproduction. A pH indicator, phenol red, was also included in the DMEM standard culture medium to assess the level of nutrient absorption by the cells. Further supplementation of the culture medium include 10 % v/v Fetal Bovine Serum (FBS) for enhanced cell growth and 1% penicillin-streptomycin mixture to inhibit bacterial growth and microorganism contamination. The change in colour of the cell culture medium from deep red (original colour, pH between 7.2-7.4) to yellow (pH at approximately 6.8) indicates the presence of high amount of acidic metabolic waste. Once the colour change is reflected, the medium is removed and replaced with fresh culture medium.

2. 0.25 wt% trypsin-ethylenediaminetetraaceticacid (Trypsin-EDTA, Invitrogen)

When the cells are cultured within the culture flask, the culture surface serves as an anchorage site for the cells to adhere and reproduce. As the cell culture reaches 80 to 90 % confluency, subculturing is required to prevent the overpopulation of cells which may lead to cell apoptosis. Trypsin-EDTA is a

common reagent used for the detachment of cells from the culture surfaces. The cells can then be collected and passaged or split into other flasks.

3. Phosphate buffered saline (PBS)

The osmolarity and ion concentrations of PBS have been known to match human's isotonicity. The buffered solution has a pH of 7.4 and contains mineral salts that are non-toxic to cells. The saline solution is used to rinse off the metabolic waste and cellular debris that may be secreted or discharged during the culturing period.

6.3.3 Cell Resuscitation

In cell resuscitation, the frozen vial of cells purchased from the vendor was rapidly thawed in a water bath at 37 °C. The vial was placed carefully in the bath to prevent water from contacting the vial cap. Once thawed, the contents inside the vials were transferred into a centrifuge tube containing cell culture medium. The suspension was centrifuged at 2500 rpm for 5 minutes to condense the cells into pellet form. The supernatant was carefully aspirated and fresh culture medium was added to re-suspend the cells. Once homogenised with cells, the suspension was pipetted into the culture flask and incubated.

6.3.4 Cell Banking by Freezing

The purpose of cell banking is to store cells for future usage. For preservation reasons, the cell pellets (after centrifuge) were suspended in cryoprotective mixture containing DMEM, 20 % v/v FBS and 10 % v/v dimethyl sulfoxide (DMSO). The suspension was transferred into a cryo-vial and stored in -80 °C overnight before storing in the liquid nitrogen tank.

6.3.5 Sub-culturing

When confluency was almost reached, the cells had to be sub-cultured. The culture medium from each flask was removed and the culture surface was rinsed with PBS. The PBS was aspirated from the flask and 3 mL trypsin-EDTA was pipetted onto the culture surface. The flask was incubated for 3 minutes to detach the cells. Once the cells have been completely detached, 10 mL cell culture medium was pipetted into the

flask to neutralise the cell-detachment reagent. The sub-cultivation ratio was 1:3; cells from one flask were split equally into three flasks.

6.3.6 Cell Counting

Cell counting is a procedure to obtain the cell density for cell culture purposes and was performed using a haemocytometer (Marienfield, Germany). The haemocytometer is a modified glass slide with nine square grids (each 1×1 mm with 0.1 mm depth) engraved on it. Before counting the cells, the haemocytometer and its cover slip were sterilized using 70 % ethanol and blotted dry. After the cells have been detached from the flask and re-suspended, 50 μ L of the suspension was mixed with Trypan Blue solution (Invitrogen) and diluted to a total of four times. The mixture was then pipetted onto the haemocytometer under the cover slip. Cells that were viable would appear clear and unstained, while the dead cells would appear to be stained by the blue dye. The cells within A, B, C, D and E square grids were counted (see Figure 6.2).

Figure 6.2 Haemocytometer grids and counting chamber [246].

After cell counting, the cell density can be obtained as follows [246]:

$$\text{Cell density} = \frac{\sum N}{5} \times d \times 10^4$$

where $\sum N$ is the total number of viable cells in the 5 square grids (A – E) and d is the dilution factor ($d = 4$ for the present work).

6.3.7 Cytotoxicity Test

In vitro cytotoxicity test, a form of biocompatibility assessment, was used to evaluate whether the SF scaffold will have any toxic effect due to leachable on living cells, which could be induced by the scaffold material or the fabrication process. The

examination was carried out towards the growth of 3T3 fibroblasts with reference to the ISO 10993 (Part 5 and Part 12) [247, 248]. The procedure involves the exposure of near confluence cell monolayers to fluid extracts obtained from the negative (scaffold), positive (para rubber) and control (cell culture medium) sample groups. More details on the protocol can be found in Appendix B.

The quantitative results of the cytotoxic test were obtained by the use of the Celltiter 96® AQueous One Solution cell proliferation assay (MTS, Promega), which contains a tetrazolium compound and an electron coupling reagent. The MTS compound, after being added to each cultured well plate, is bio-reduced by the hydrogenase enzymes in metabolically active cells to form coloured formazan product that is soluble in the tissue culture medium. The quantity of the formazan product, which is directly proportional to the number of living cells, can be measured by the absorbance at 490 nm. Finally, the cell viability of each culture well, in terms of percentage, was evaluated using the following formula [247]:

$$Viability (\%) = \frac{(100 \times OD_{490e})}{OD_{490b}}$$

where OD_{490e} is the mean value of the measured optical density of the 100 % extracts of the test sample and OD_{490b} is the mean value of the measure optical density of the negative control. If the viability of the extract is reduced to less than 70 % of the negative control, it is concluded to have a cytotoxic potential.

6.4 Cartilage Regeneration Using Porcine Articular Cartilage Cells

6.4.1 Reagents

Porcine articular chondrocytes were handled using identical equipment and cultured under the same conditions, as illustrated in section 6.3.2. However, the culture medium used, which is known as chondrocyte growth medium, was prepared using different culture reagents. 1:1 DMEM/Ham's F-12 was the standard culture medium while supplementations of the chondrocyte growth medium include 10 % v/v FBS, 1 % antibiotic-antimycotic solution (100×), 50 mg/l ascorbic acid and 1.2 g/l sodium bicarbonate. This cell culture recipe has been used by past researchers and the combination of these ingredients was shown to promote enhanced cell proliferation of cartilage cells [66, 112]. The addition of Ham's F12 provides extra components such

as biotin, putrescine, lipoic acid, glycine, proline, copper and zinc that are not present in DMEM. The antibiotic solution is a mixture of penicillin, streptomycin and amphotericin B mixture, where amphotericin B was used to prevent fungus infection. Ascorbic acid has been shown to retard the differentiation of chondrocytes while sodium bicarbonate serves as a physiological buffer for *in vitro* studies. The other standard reagents such as trypsin-EDTA and PBS remained the same for chondrocyte culturing.

6.4.2 Chondrocyte Isolation

Chondrocytes used in this study were harvested from the articular cartilages of 5-month-old porcines. The fresh knee joints of the porcines were purchased from the local abattoir with the approval by the Agri-food and Veterinary Authority (AVA), Singapore. For each knee joint, it was requested that the tissues surrounding the articular cartilage be intact so that the cartilage could be maintained under physiological conditions and the risk of contamination would be reduced. The knee joints were dissected as soon as they were obtained from the abattoir. Each dissection procedure could only accommodate one knee joint and the rest were kept in 4 °C until use. The details of the dissection procedures are described as follows:

1. Before dissection, the entire knee joint was soaked in a povidone-iodine (PVP-I) solution bath for 30 minutes.
2. The knee joint was rinsed thoroughly with sterile PBS to remove PVP-I completely, disinfected by spraying with 70 % v/v ethanol and transferred into the laminar flow biosafety cabinet.
3. Dissection was carried out using sterile surgical instruments such as scalpels and scissors. Once the articular cartilage has been exposed, the scalpel blade was changed to harvest cartilage bits by slicing the tissue peripherally. During this procedure, caution was exercised to avoid contaminating the tissue. For instance, non-sterile surfaces/instruments were not allowed to contact the cartilage tissue. To prevent dehydration of the cartilage, the tissue was dripped with PBS containing 3 % v/v antibiotic-antimycotic (ABS) every 5 minutes using a dropper pipette.
4. The cartilage bits were harvested and transferred into a sterilised bowl containing ABS.

5. Once the harvesting has been completed, the cartilage bits were rinsed with ABS thrice and minced into $1\text{ mm} \times 1\text{ mm}$ in sizes.
6. The minced cartilage bits were transferred into to a 50 mL centrifuge tube and incubated until the rest of the knee joints were dissected.
7. The ABS in each centrifuge tube was removed and the minced cartilages were subjected to enzymatic digestion using 0.2 % w/v collagenase in chondrocyte growth medium. The digestion process takes up to 16-18 hours in an incubator.
8. After digestion, the suspension was filtered through a 70 μm cell strainer to obtain the chondrocytes and removed the undigested tissues. The filtrate was then centrifuged at 3500 rpm for 8 minutes.
9. The supernatant was removed and the cell pellet was suspended in ABS solution. The suspension was centrifuged to rinse off any contaminants. This procedure was performed thrice.
10. The cell pellet was re-suspended in chondrocyte growth medium and pipetted into 75 cm^3 or 150 cm^3 tissue culture flasks.

A flowchart of the procedure is illustrated in figure 6.3. Since chondrocytes have a tendency to differentiate causing a change in its phenotype, cell seeding was conducted when the cells have reached confluency in the 2nd passage.

Figure 6.3 A schematic of the chondrocyte isolation process.

6.4.3 Cell Proliferation

The scaffold constructs were cultured in the medium for 1, 3, 7, 10 and 14 days for the assessment of cell proliferation. As evaluated in the previous section, the appropriate cell density was determined to be 10^7 cells/mL. The chondrocyte growth medium was changed every 3 days. The DNA content of the specimens was then extrapolated from the standard curve. The cell number can then be estimated using the conversion factor of 7.7 pg DNA per chondrocyte [120, 249].

The total DNA content measurement was carried out according to the protocol of the DNA Quantification Kit (Sigma-Aldrich) [250], with slight modifications. The kit contains Hoechst 33258 and fluorescent assay buffer which are components to stain the nuclei acid. 20 μ L of the digestion extract was added to 80 μ L of 1 μ g/mL Hoechst 33258 dye in fluorescence assay buffer. For calibration, calf thymus DNA, prepared in aliquots of 100 μ g/mL and 10 μ g/mL in sterile ultra-pure water, was used to obtain a standard curve with DNA ranging from 0 to 1000 ng. The fluorescence of the Hoechst-

DNA complex in the samples, standards and blanks were detected at excitation/emission wavelengths of 360 nm/460 nm.

6.5 Prolonged Cultivation of Silk Fibroin Scaffolds for Cartilage Regeneration

To evaluate the suitability of SF scaffolds for cartilage TE, the constructs were subjected to *in vitro* chondrocyte culture for longer experimental durations. The cell density remained as 10^7 cells/mL. The time points for culturing included 4, 8 and 12 weeks, which were selected so as to allow sufficient duration for the chondrocyte to differentiate (if applicable) or secretion of the relevant sulphated glycosaminoglycan (sGAG) and collagen type II. A series of *in vitro* characterisations were conducted to examine the regeneration of cartilage tissue on SF scaffolds and their ECM components, they included: DNA and GAG quantification, immunohistochemical (IHC) evaluation of collagen type II, fluorescence confocal microscopy and scanning electron microscopy.

6.5.1 Total DNA and sGAG Quantification

Before the quantification of total DNA and sGAG content, the harvested samples were required to be digested to extract the protein segments from the cells. At each time point, the SF specimens were rinsed with sterile PBS once and the digestion process was performed by immersing each harvested sample into an eppendorf tube containing 1 mL of papain solution (50 µg/ mL in PBS with 55 mM sodium citrate, 150 mM sodium chloride, 5 mM cysteine HCL and 5 mM EDTA, Sigma Aldrich). The sample digested lasted for 24 hours in an ambient temperature of 60 °C.

The total sGAG content used 50 µL of the digestion extract. The extract was added to 1 mL of 1, 9 dimethylemethyle blue (DMMB) dye solution. The DMMB dye solution was prepared by dissolving 16 mg of DMMB in 1 L of sterile ultra-pure water containing 3.04 g of glycine, 2.37 g of NaCl and 95 mL of 0.1 M HCL. The mixture was allowed to react for 30 minutes so that the blue dye bound to the sGAG and formed a purple dye-sGAG precipitate which was dissociated with 5 µL of the digestion buffer (papain solution). Once the mixture has been obtained for each sample, the absorbance of the sGAG was measured using a spectrophotometer at 525 nm. The sGAG amount was calculated based on an established calibration curve where shark chondroitin sulphate (Sigma Aldrich) ranging from 0 to 100 µg was measured.

6.5.2 Collagen Type II Quantification

Harvested specimens from the *in vitro* culture were digested in 1 % w/v pepsin in acetic acid on a shaker for 24 hours at 4 °C. The digested solution was further solubilized in pancreatic elastase solution in Tris buffer saline (TBS, pH 8.0) at 4 °C for 24 hours. After the entire digestion procedure, the samples were centrifuged and the supernatant was extracted and diluted to at least 10-fold with the assay buffer from the Collagen Type II Enzyme-linked Immunoabsorbant Assay Kit (ELISA kit, Mdbioprodukt). 100 µL of each sample set was incubated within a well in 96-well plates coated with anti-collagen type II monoclonal antibody for 2 hours. Collagen type II present in the sample solution will be bound to the well plate by the antibody. The unreacted constituents in each well was aspirated and the wells were washed 6 times with 200 µL per well of wash buffer. 100 µL of conjugate reagent was added to each well for the labelling of the antibodies and incubated at room temperature for 2 hours. The washing procedure was repeated and 100 µL of Streptavidin-Horseradish peroxidase (SAv-HRP) was pipetted in each well to reinforce the antibody labelling. Another set of washing was conducted and 100 µL of substrate was added to each well. After incubation for 20 minutes in room temperature, 100 µL of stop solution was added and the absorbance of the collagen type II expression was measured at 450 nm.

6.5.3 Normalization of Quantitative Data

The standard calibration for the biochemical assays are shown in Appendix B. From section 6.4.3, the cell count of chondrocytes can simply be obtained through the standard DNA calibration curve. Moreover, it is likely that the chondrocyte number varies from each harvested SF specimen. Thus, the results obtained from sGAG and collagen type II quantifications are normalized with their individual DNA content to determine the averaged data for comparison between different time points of culture. The normalized sGAG and collagen type II shall be expressed as sGAG/DNA and Col type II/DNA respectively.

6.5.4 Confocal Imaging

The viability of chondrocytes in the SF constructs was evaluated using a live/dead assay kit (Molecular Probes). The harvested specimens were washed thrice in sterile

PBS, transferred into a new 24-well plate and immersed in PBS solution containing 4 mM calcein and 2 mM ethidium. Calcein labels viable cells with green fluorescence while ethidium labels dead cells with red fluorescence. The well plate was incubated for 15 minutes at 37 °C. Subsequently the constructs were observed under a fluorescent confocal microscope using lasers at excitation wavelengths of 488 nm (green fluorescence) and 543 nm (red fluorescence). The emission wavelengths for the green and red fluorescence were 500 nm and 555 nm respectively. The intensities of the viable (green) and dead (red) cells were recorded at a magnification of 40×.

6.5.5 Scanning Electron Microscopy

SF constructs cultured for 2, 4 and 8 weeks were pre-fixed for 2 hours (3 % v/v glutaraldehyde) and the concentration of the fixative was changed to 5 % v/v for overnight fixation. The preserved SF constructs were rinsed with PBS and immersed in 1 % Osmium Tetroxide in 0.1 M sodium cacodylate for 1 hour at 4 °C. Following three rinses with PBS, the constructs were subjected to a series of ethanol treatments and air-dried. After drying, the specimens were placed on an aluminium holder, sputtered with gold, mounted on the SEM for observation.

6.6 *In vivo* Transplantation of Silk Fibroin into Nude Mice Models

For *in vivo* studies, a subdermal implantation was used to evaluate the biocompatibility of SF constructs and their potential to cartilaginous tissue formation. SF scaffolds were seeded with chondrocytes and cultured *in vitro* for 2 weeks, until the cell-protein constructs were implanted within the dorsal subcutaneous area of six-week-old male athymic mice (SLC, Japan) for further verification of their feasibility in cartilage TE applications. A transverse incision was made on the dorsum of each mouse to create a subcutaneous pocket with sterile surgical technique as shown in Figure 6.4. Each nude mice model received two constructs on the back. After 4, 8 and 12 weeks of follow-up, the animals were sacrificed by an overdosed inhalation of anaesthesia. The SF scaffold implants were then harvested for histological analysis.

Figure 6.4 Subcutaneous implantation of SF scaffold constructs into a nude mice model.

6.6.1 Histological and Immunohistochemical Evaluation

After implantation, SF constructs were harvested and washed with PBS. The specimens were fixed with 10% formalin for 24 hours, embedded in paraffin, sectioned (5 μ m thickness) and mounted on microscope slides. The sections were stained with hematoxylin and eosin (H&E). sGAG in the harvested constructs were detected by Safranin O staining.

For the IHC analysis, expression of collagen type II detected by the mouse type II collagen monoclonal antibody (abcam®, Cambridge MA) with rabbit anti-mouse secondary antibody (HRP polydetector, Thermoscientific). All histological sections were observed with a light microscope.

6.7 Statistical Analysis

All data were expressed as mean $\pm$ standard error of the mean (SEM). The data obtained from biochemical analysis were statistically assessed with one-way analysis of variance (ANOVA). A p value of less than 0.05 was considered as statistically significant.

6.8 Summary

The experiments presented in this chapter are summarised using Table 6.1 and Table 6.2. They are divided into two categories: the material-based experiments and cell-based experiments.

Experiment	Purpose of experiment
Fabrication of SF scaffolds	To produce a cartilage TE construct using the proposed indirect AM method
Fourier transform infrared spectroscopy	To analyse the conformational structure (amide bands) of the SF foams and scaffolds
Differential scanning calorimeter	To measure the thermal properties of SF foams and scaffolds
Scanning electron microscopy	To assess the morphology of the SF scaffolds

Table 6.1 Summary of the material-based experiments

Experiment	Sample number (n)	Cell type	Cell concentration	Time point(s)	Characterisation method(s)
Cytotoxicity test	8	3T3 embryonic fibroblasts	10^4	48 hours	Optical density measurement
Cartilage regeneration	6	Porcine articular chondrocytes	10^7	1, 3, 7, 10, 14 days	DNA assay
Prolonged cartilage regeneration	6	Porcine articular chondrocytes	10^7	4, 8, 12 weeks	<u>Assays</u> <ul style="list-style-type: none"> • DNA • sGAG • Collagen type II <u>Optical characterisation</u> <ul style="list-style-type: none"> • Confocal imaging • SEM <u>Staining</u> <ul style="list-style-type: none"> • Hematoxylin & eosin • Alcian blue • Safranin O
<i>In vivo</i> implantation into mice models	5	Porcine articular chondrocytes (pre-seeded)	10^7	4, 8, 12 weeks	<u>Staining</u> <ul style="list-style-type: none"> • Hematoxylin & eosin • Safranin O • Immunohistochemical

Table 6.2 Summary of the cell -based experiments.

Chapter 7 - Results and Discussion

This chapter presents the results obtained according to the experimental details described in Chapter 6. The results include: the morphological details of the SF TE scaffold, chemical structural and thermal characterisation, cytotoxicity assessment, immunohistochemical analysis, *in vivo* implantation and histological staining.

7.1 Silk Fibroin Scaffold Fabrication

Regenerated SF was incorporated with indirect RP technology to develop TE scaffold with both micro- and macro-structural features. The micro-porosity served to promote cell entrapment and adhesion while the macro-scaled interconnected channels were designed for cell migration and mass transportation of supplements respectively. Thermoplastic moulds built by an inkjet printer were used to negatively replicate the design, thus forming the resultant TE construct. The reproducibility of the SF scaffolds can be assured as long as the process steps and conditions are preserved. It should be re-emphasized that this reproducibility is combined with flexibility in user-defined control over CAD models, allowing the customization of TE constructs that address patients' needs. Constructs derived from conventional methods, in contrast, may have an issue with consistency in pore size distributions for the optimization of cellular-based processes.

SEM images were taken to verify the presence of micro-pores and interconnected channels. The leaf-like pores within the SF scaffolds exhibited similar morphologies as SF sponges which are useful for cell entrapment and adhesion (Figure 7.1). The channel walls were measured to be approximately 740 μm in width using OCT. The condensed morphology of the channel walls is attributed by the change in crystallinity of SF. As the scaffold constructs were exposed to boiling deionised water, the elevated temperature ambience and repetitive bombardment by the energetic surrounding water molecules might induce further β -sheet formation or cross-linking. This hence resulted in lesser porosity or densification of SF protein in the exposed regions [251]. The β -sheet crystalline conformation is converted from its random coil structure through ethanol immersion and thermal treatment. The presence of the crystalline secondary structure served to enhance mechanical properties and improve SF's solubility towards

aqueous solution. Moreover, it was suggested that the β -sheet content and topography of the SF constructs could induce better cell adhesion and spreading [43].

Figure 7.1 Scanning electron microscopic images of the (a) top view (30 $\times$) and (b) close-up view (75 $\times$) of the SF TE scaffolds. The arrows in the images indicate the presence of the macro-channels.

7.2 Chemical Structure and Thermal Analysis

The FTIR spectra showed the presence of β -sheets in the amide I (1610 - 1645 cm^{-1}) and amide III (1220 - 1320 cm^{-1}) regions. The characteristic bands from the two spectral regions exhibited formation of distinctive “shoulder” and peaks respectively after the de-convolution process (Figure 7.2 (a) and 7.2 (b)). De-convolution is a normalization procedure which reveals the presence of amide bands distinctively. The amide I region was recognised as a more obvious indication of β -sheet [252]. In contrast, the non-cross-linked SF foams demonstrated the absence of the secondary structure in Figure 7.2 (c).

Figure 7.2 Typical de-convoluted FTIR spectral data of SF specimens processed under different conditions, (a), SF TE construct; (b), 10% (wt/wt) cross-linked SF foams; and (c), non-cross-linked 10 % (wt/wt) SF foams. The data plots of the SF TE constructs were identical to 10 % (wt/wt) cross-linked SF foams. Both set of plots demonstrated β -sheet formation as highlighted by the arrows in FTIR spectra. The result obtained from the non-cross-linked 10 % (wt/wt) SF foams was included as a control for comparison.

In the thermal analysis, the first endotherm observed for the 3D constructs and porous SF foams were found to be broader as compared to the non-cross-linked SF foams. The peak observed between 50 to 100 °C was an indication of the breakage of hydrogen bonds between water molecules and SF protein peptides. The broadness of the peak, shown in Figure 7.3 (a) and 7.3 (b) relates to the amount of water content present in each specimen [253]. The narrower endotherm indicates a better ability to achieve a higher swelling ratio. The second endotherm, which was represented by a sharp peak between 280 to 300 °C in Figure 7.3, was experienced by all SF specimens. This consistent observation among the SF specimens was attributed to the thermal decomposition of the SF protein peptides. These endothermic peaks were also noted to be independent of the degree of cross-linking within each sample. The thermal plot for the 3D scaffolds was evaluated to follow a similar trend as the cross-linked SF foams. The formation of β -sheet was further verified by the absence of the exothermic peak at

approximately 220 °C which was encountered by the non-cross-linked SF foams in Figure 7.3 (c). Several literatures have illustrated the process of heat energy release as an indicator of β -sheet formation when the sample is subjected to dynamic heating at a constant rate [253-257]. Moreover, there was no endotherm seen at 50 °C and 95 °C, which represents the melting point of the support material and thermoplastic mould respectively. This observation suggests that the resultant SF constructs contain negligible traces of the sacrificial mould and its support material.

Figure 7.3 Typical and DSC thermogram of SF specimens processed under different conditions, (a), SF TE construct; (b), 10 % (wt/wt) cross-linked SF foams; and (c), non-cross-linked 10 % (wt/wt) SF foams. The data plots of the SF TE constructs were identical to 10 % (wt/wt) cross-linked SF foams. Both set of plots showed absence of exothermic peak during the thermo-analytical process, which indicates β sheet formation. The results obtained from the non-cross-linked 10 % (wt/wt) SF foams were included as a control for comparison.

7.3 Cytotoxicity Test

The cytotoxicity examination of the 3D SF scaffolds was conducted using the 3T3 fibroblast cells. From the quantitative results displayed in Figure 7.4, it can be inferred that the 3D scaffolds portrayed cell viability effectiveness similar to that of the negative control. Since the cell survival rates of the scaffold extracts exceed 70 %, it can be postulated that the indirect RP method imposed on the SF protein induce

insignificant toxicity. In contrast, cells exposed to the positive control experienced obviously lower cell viability, thus showing indications of toxic potential.

Figure 7.4 The relative cell viability percentage of cells cultured in extracts derived from standard culture medium (negative control), scaffold extracts (100, 50, 25 and 10 % v/v respectively) and Para rubber extracts (positive control). A total of 8 samples ($n = 8$) were tested in each group. * indicates a significant difference was observed when compared to the negative control group ($p < 0.05$).

7.4 Cell Proliferation

Porcine chondrocytes were used in this study to assess the SF scaffolds potential for cartilage regeneration. Cartilage cells were first seeded on the 3D constructs to examine their cell proliferation ability. DNA was used to quantitatively evaluate the metabolic activity of chondrocytes cultured on the SF tissue scaffolds. During the culture period, as shown in Figure 7.5, the cell growth experienced a significant dip ($p < 0.05$) after 7 days of seeding on the constructs. The cell number then increased significantly in the subsequent days of culture. The DNA content measured at days 10 and 14 were significantly higher when compared to day 7 ($p < 0.05$). The observed trend was attributed to the presence of the interconnected channels, which may have

led to the cells dropping to the bottom of the seeding well. This observation was supported by *Ragety et al* [147] who suggested that the architecture of the TE scaffold may influence the cell proliferation trend. Nevertheless, it should be noted that the emphasis of the macro-channels served to provide a pathway for the transport of essential nutrients and metabolic waste within the SF construct. The microstructural characteristics were recognised as important elements for the relevant cell signalling to regulate cell proliferation and differentiation [191, 258, 259]. The pores served as “pockets” to provide space for the cells to infiltrate, adhere and multiply. As the cartilage ECM was being regenerated, the newly formed tissue filled up the pore to preserve structural integrity as the SF protein biodegraded with time. It was believed that the cells required up to 7 days for adaption and adherence to the SF substrates even though the constructs had been conditioned in the growth medium before the cell seeding process. To minimize the significant drop in cell attachment, a higher cell seeding density was recommended [260]. A larger population of cells can lead to enhanced cell-to-cell interaction which subsequently increases the sGAG and collagen type II production. The subjection of TE constructs in dynamic cultures has also shown that cell viability was increased since the cells attached within the constructs received adequate nutrition [146, 261].

Figure 7.5 DNA content quantification of chondrocytes cultured on SF TE constructs cultured for 1, 3, 7, 10 and 14 days under *in vitro* conditions. The results were expressed as mean $\pm$ SEM.

7.5 Prolonged Cultivation of Silk Fibroin Scaffolds for Cartilage Regeneration

The culturing period was maintained for 12 weeks to investigate the seeded-SF construct's capability to secrete cartilage-specific extracellular matrix. The biochemical compositions such as DNA, sGAG and collagen type II contents of the cartilage-ECM expressed were evaluated. For the total DNA content, presented in Figure 7.6, an inconsistent trend was observed. When compared with the seeded constructs at 4 weeks, the 8-week group experienced a significant decrease ($p < 0.05$) in the DNA expression (cell number) while a comparative quantity was evaluated after 12 weeks of culture. This study inferred that the gene expression plateaued, an implication that the cell saturation density has been reached. This observation was consistent with Whittenbreger *et al* [262], with similar experience for 3T3 embryonic fibroblasts. Another possibility of inconsistent result includes the dislodgement of cell clusters attached to the SF construct in the process of changing culture medium; the cell aggregates may have been removed as the old culture medium was aspirated via vacuum. Concurrently, the time points involved in the present study were relatively

distant. The 10-fold increase in DNA expression from 14 days to 4 weeks of culture postulated that a rapid expansion of the chondrocyte population was occurring during this time interval.

The quantitative measurements of the sGAG and collagen type II was used to determine the ability of the seeded cells to produce a cartilage-like ECM. The sGAG expression, as shown in Figure 7.7, was found to increase with culture time although the growth observed was not statistically significant ($p > 0.05$). Collagen type II expression was evaluated and a significant increase ($p < 0.05$) was noted from 4 weeks to 12 weeks (Figure 7.8). Although the number of cells within the TE constructs plateaued after 4 weeks of cultivation, the ECM-specific polysaccharides and protein were produced increasingly. This observation suggested that micro- and macro-morphological features of the SF constructs promoted chondrogenesis, which is a physiological process crucial to restoring the intrinsic properties of the native tissue. Naturally-derived SF protein consists of amino acids that can emit the appropriate biological signals for cell adhesion and specific cell-to-biomaterial interactions on the micro-scaled level, and maintaining the phenotypic expression of chondrocytes. Conversely, synthetic polymers are generally hydrophobic and may hinder smooth cell seeding. On the macro-level, the interconnected channels served as passages for the migration of cells and transportation of culture medium to the cells located in the central regions of the scaffold constructs.

Figure 7.6 DNA content measurements of SF TE constructs cultured for 4, 8 and 12 weeks under *in vitro* conditions. The results were expressed as mean $\pm$ SEM.

Figure 7.7 sGAG content measurements of SF TE constructs cultured for 4, 8 and 12 weeks under *in vitro* conditions. Normalization was conducted on sGAG quantification based on the total DNA amount obtained for each time point. The results were expressed as mean $\pm$ SEM.

Figure 7.8 Collagen type II content measurement of cell-seeded SF TE constructs cultivated for 4, 8 and 12 weeks under *in vitro* conditions. Normalization was conducted on collagen type II quantification based on the total DNA amount obtained for each time point. The results were expressed as mean $\pm$ SEM.

7.6 Microscopic Imaging of Silk Fibroin Tissue Engineering Scaffolds

The stages of neo-cartilage formation were examined qualitatively by SEM and confocal imaging. Majority of chondrocytes were stained fluorescence green, indicating the presence of viable cells. At 2 weeks, the distribution of the cells within the porous network (shown in Figure 7.9 (a)) was even and the adherence of the chondrocytes to the leaf-like morphological features was observed. The SF constructs cultured for 4 weeks showed increase in cell number quantitatively (Figure 7.9 (b)). After 8 weeks of culture, the secreted ECM filled the pores entirely (Figure 7.9 (c)) and the walls of the microstructural features were visually hindered by the extensive growth of tissue-specific ECM.

The results obtained from the SEM images were coherent with the live/dead assay. With the proposed manufacturing method, the scaffold pores exhibited leaf-like morphological details and the macro-channels were distributed at equal distances as shown in Figure 7.10 (a). After 2 weeks of culture, the micrographs showed that the chondrocytes were attached and filling up the micro-pores (Figure 7.10 (b)) while the macro-channels were preserved for mass transport and waste removal. Individual cartilage cells were difficult to detect as the ECM had a tendency to submerge the cells. The deposition of ECM can be seen at leaflet borders at a higher magnification and extended deep into the pores (Figure 7.11 (b)). At 4 weeks, abundant ECM had formed while the larger pores experienced a filled-out phenomenon (Figure 7.11 (c)). By 8 weeks of culture, a majority of the leaf-like pores were filled with ECM as illustrated in Figure 7.10 (c). Confluent layers of ECM were secreted out of the scaffold surface, indicating either the chondrocytes' capability to slowly migrate within the secreted ECM, or the expression of ECM a certain distance away from the cell. The macro-channels were preserved despite 8 weeks of culturing. Thus, it can be inferred that the channel networks could continue to facilitate the mass transport of nutrients and removal of metabolic waste.

Figure 7.9 The viability of cells observed via confocal microscopy using the live/dead assay after (a) 2 weeks, (b) 4 weeks and (c) 8 weeks of culturing. At $40\times$ magnification, the leaf-like structures are seen as the wavy lines dispersed between the round, bright green cells.

Figure 7.10 SEM observation of the SF TE constructs. At $50\times$ magnification, (a): micro-scaled morphology of a blank SF scaffold; (b), (c): 2 and 8 weeks after *in vitro* chondrocyte cultivation. The black arrows indicate the preservation of macro-channels despite undergoing a long culture period.

Figure 7.11 SEM observation of the SF TE constructs. At 150 $\times$ magnification, (a), (b), (c): blank, 2 and 4 weeks cultured SF scaffolds. The black arrow indicates the ECM deposition upon the leaflet borders. The white arrows showed some pores have been filled out.

7.7 Histological Staining of Silk Fibroin Scaffolds Cultured *in vitro* for 8 Weeks

Cartilage constructs were harvested from *in vitro* cultures at week 8 and sent for histological processing. The histological results of the shorter time points (2 weeks and 4 weeks) were eliminated due to the detachment of cells from the microscope slide after the multiple-steps of immersion into staining solvents. Structurally, the general architecture of the SF scaffold has been preserved throughout the culturing process. Most of the ECM has been deposited on the surface of the scaffold, indicating that the cartilage formation preferentially grew outward under *in vitro* conditions, with penetration seen between the leaflets of the SF scaffold. Cells were distributed in a fairly uniform fashion throughout the ECM, although clusters could occasionally be seen. Lacunae-like spaces were noted around cells, and cells possessed a typical dark, round nucleus. From the Haematoxylin and Eosin (H & E) stains presented in Figure 7.12, a bi-layered staining of the secreted ECM could be seen, with the hematoxylin stained area on the outer half of the neocartilage. Classically, native cartilage is stained light blue, similar to the outer layer of the tissue-engineered cartilage. A more basophilic staining was noted around certain clusters of cells, similar to the histological appearance of isogenous groups of chondrocytes in normal cartilage tissue, indicating that mitotic divisions of the cells have taken place.

From both Alcian Blue (AB) and Safranin-O (SO) stains, which are shown in Figures 7.13 and 7.14 respectively, observation of the outer neo-cartilage layer could be further confirmed. The deep blue region in AB staining, indicating mucosubstances, and the orange-red region of the SO staining, indicating sGAG, was mostly noted in the outer half of the secreted ECM as well. Through histological staining, it was apparent that the SF scaffold has successfully supported cartilage tissue neo-generation and also confirmed the observations noted on SEM and biochemical analysis.

Figure 7.12 Microscopy slides of SF TE constructs stained with Haematoxylin and Eosin after being cultured for 8 weeks: (a) at $100\times$ magnification and (b) at $200\times$ magnification. The arrows show the distinct cartilage layer (light blue) formed superficially.

Figure 7.13 Microscopy slides of SF TE constructs stained with Alcian Blue after being cultured for 8 weeks: (a) at $100\times$ magnification and (b) at $200\times$ magnification. . The arrows show the distinct cartilage layer (deep blue) formed superficially.

Figure 7.14 Microscopy slides of SF TE constructs stained with Safranin O after being cultured for 8 weeks: (a) at $100\times$ magnification and (b) at $200\times$ magnification. The arrows show the distinct cartilage layer (orange-red) formed at the superficial region, verifying the H & E and Alcian Blue stain observations.

7.8 *In vivo* Transplantation of Silk Fibroin Scaffolds into Nude Mice Models

A subdermal implantation protocol was used to assess the ability of the seeded scaffolds to produce cartilage in an *in vivo* environment. The histological findings included H & E and SO staining which were used to assess the secretion of ECM and sGAG expression respectively. The H & E staining results of constructs cultured at 12 weeks did not reveal typical obvious lacunae formation within the micro-pores (Figure 7.15). However, ECM-like material was seen throughout the scaffold. Chondrocytes appeared round in shape, were not spindle-shaped, and were situated near the walls of the leaf-like pores. No apparent inflammatory cell infiltrates were noted and adipose tissue infiltration of the scaffold was not seen as well. However, capillaries have begun to invade by week 4 (Figure 7.15 (a)), suggesting an alternative source of nutrition and waste transport. As the culture period reached 8 weeks, a significant increase of ECM formation (Figure 7.15 (b)) was observed and the distribution of chondrocytes within the micro-pores (and ECM) exhibited homogeneity. A distinguishable difference between the 4 weeks and 8 weeks culture was the cell density within the interstitial matrix. More clustering of cells was noted, which might allow the cells to interact more and thus stimulate more ECM production [92]. The biological explanation was further justified at 12 weeks (in Figure 7.15 (b)) where the ECM production was more extensive within the pores.

The presence of sGAG filled within the interstices of the ECM provided the swelling capabilities of cartilages, hence enabling the connective tissue to be resistant against compressive loading. To demonstrate the secretion of collagen-like ECM, histological staining by SO revealed the growth of sGAG production with respect to the culture period (Figure 7.16). The increase in staining intensity within the construct illustrates increased sGAG detection, demonstrating the filling of cartilaginous tissue in the 3D SF constructs seen on SEM. Overall, the distribution of sGAG was uniform for every time point.

Figure 7.15 Hematoxylin and eosin staining of SF TE constructs after (a) 4 weeks, (b) 8 weeks and (c) 12 weeks of subcutaneous implantation. The arrows indicate the formation of capillaries.

Figure 7.16 Safranin O staining of SF TE constructs after (a) 4 weeks, (b) 8 weeks and (c) 12 weeks of subcutaneous implantation.

Collagen type II is one of the cartilaginous components synthesized by chondrocytes to build the ECM of the connective tissue. The secretion of collagen type II also characterises the chondrogenic phenotype of the regenerated connective tissue and plays an important role in the facilitation of chondrogenesis. Positive staining of cartilage-specific marker was evident for the cell-seeded constructs for 4, 8 and 12 weeks (Figure 7.17). The immunohistochemical staining was found to be more widespread as the constructs were cultured longer. In addition, it was noted that the tendency of collagen type II secretion was more prominent in wider pores up to 8 weeks of culture (Figure 7.17 (b)). The expression of the cartilage tissue marker, as seen in Figure 7.17 (c), was then observed to infiltrate into the smaller pores, which indicates the development of a stable infrastructure to support connective tissue growth. Staining intensity was also higher around the scaffold material. This observation requires further investigation to better illustrate the cell-scaffold interactions.

As a general observation from all time points, the distance between of pore walls, which represents a marker for the onset of scaffold biodegradation, did not indicate any observable change. This suggested that degradation of the SF protein requires a longer period of time than the observed 12 weeks under subdermal conditions.

Figure 7.17 Immunohistochemical staining of SF TE constructs after (a) 4 weeks, (b) 8 weeks and (c) 12 weeks of subcutaneous implantation.

Chapter 8 – Conclusions and Future Work

8.1 Conclusions

This study has presented the fabrication of cartilage TE scaffolds using AM technology and SF, a naturally-derived protein, as the biomaterial. An indirect AM approach has been applied to manufacture pre-designed negative sacrificial moulds using a 3D inkjet printer. The design of the sacrificial mould was subsequently transferred to the scaffold material by casting aqueous SF into the moulds. After lyophilisation and mould removal, a 3D TE construct with macro-channels and micro-pores was obtained.

8.1.1 Modelling of Silk Fibroin for Tissue Scaffolding

Through experimental evaluation, the relationships were obtained between the concentration of silk fibroin and its properties, which include equilibrium swelling ratio, water binding ability and mechanical properties. These formulations contribute significantly to the design of SF scaffolds as the geometrical characteristics such as porosity and pore size, which varies with respect to SF concentrations, serve as interstitial sites for cell anchorage and ECM secretion.

From the empirical models developed, it can be deduced that SF protein possesses the ability to retain significant amount of water in its porous matrix. This deduction suggested the viability of SF as cartilage substitutes since the native tissue requires 70 - 80 % of water in order to resist the mechanical forces applied to the cartilage [50]. In addition, the mechanical properties of SF foams were within the range of native cartilages, hence further supporting the cartilage TE potential of SF protein. The models were also suggested to provide a basis for determining the required SF properties for the development scaffolds for other tissue or organs.

8.1.2 Biodegradation of Porous Silk Fibroin Matrices

A permeability theory was first established as a function of the pore geometry such as effective pore diameter and sphericity as well as the tortuosity of the fluid path. The permeability function allows a better estimation on the ability of fluid flow within a porous matrix. The understanding of the permeability provides an appropriate

reference to evaluate the effectiveness of the mass transport of essential nutrients towards the foam's interior.

The permeability concept is also useful for the assessment of the interaction between water and porous matrix since hydrolysis is the main attribute to biodegradation. The kinetics of hydrolysis controlled by diffusion of water was modelled for the identification of the dominant erosion mechanism during degradation. Furthermore, the degradation lifetime derived from the chain scission number was expressed as a function of the average molecular weights before and after the entire degradation process.

The biodegradability of SF porous matrices of three different concentrations was assessed by implanting the constructs into nude mice models up to 8 weeks. Histological observations from this experimental study have determined 10 % (wt/wt) SF foams as suitable matrices for the fabrication of cartilage TE scaffolds as the porous specimens demonstrated the ability to serve as a temporary physical support and provide adequate morphological features for optimal cell infiltration.

8.1.3 Droplet Impaction and Line Formation Model of Inkjet Printing

The dynamics of droplet impact is important in defining the resulting geometry of equilibrated droplet. The concept of droplet impaction involves the interplay of kinetic energy and surface energies. Based on the energy conservation principles, the total energy before and upon impaction should be equivalent in order to achieve a maximum spreading ratio of the droplet and hence the equilibrated diameter. However, if any energy imbalance is encountered, which is likely to affect the contact angle and spreading ratio, the droplet has a tendency to recoil. Furthermore, the presence of surface roughness on the solid substrate has been evaluated to alter the contact angle of the droplet upon impact.

Line formation of the inkjet printing process has been established based on the model involving the coalescence between two droplets. A thermodynamic model was presented for the estimation of the critical time at which the first droplet could no longer merge with the second droplet as a result of cooling. As the effects of drawbacks are known to produce undesirable lines, a theoretical model was formulated

to predict the conditions in which discontinuity of a line formed during the printing process.

8.1.4 *In vitro and in vivo Characterisation of Silk Fibroin Tissue Engineering Scaffolds*

SF scaffolds, which were successfully produced using the proposed indirect AM technique, demonstrated the preservation of the protein's intrinsic properties and without inducing cytotoxicity. The findings from *in vitro* cell cultivation showed that the TE constructs served as suitable templates for chondrocyte adhesion and proliferation. The observation was further verified using histology staining images of the specimens after a prolonged period of cultivation. To address the SF scaffolds' interaction with actual physiological environment, the constructs were implanted into nude mice models. After several weeks of implantation, it was revealed that the SF scaffolds promoted cartilage regeneration based on the qualitative results obtained through immunohistochemical assessments which include the identification of essential cartilage components such as sulphated glycosaminoglycans (GAGs) and collagen type II.

8.2 Key Contributions

The key contributions from this research include:

1. The formulation of novel empirical models relating to SF protein as a basis to evaluate the suitable SF concentration for general TE applications. The models derived were established based on experimental investigations.
2. The presentation of a permeability theory that satisfies the criteria for the design of porous foam in TE scaffold. The study of the biodegradation mechanisms occurring within porous matrices using a model was conducted and validated with *in vivo* experiments.
3. Illustration of the crucial processes in the inkjet printing which include droplet impaction and line formation models. Following the descriptive models, SF TE scaffolds possessing both macro- and micro-architectural features were fabricated using the indirect AM technique.

4. *In vitro* studies to ensure that the intrinsic properties of SF protein were maintained and SF scaffolds induced no cytotoxicity after the application of indirect AM approach. The examination of the cartilage TE potential of SF scaffolds included *in vitro* cultivation with chondrocytes and *in vivo* implantation into nude mice models.

8.3 Suggestions for Future work

8.3.1 Design Optimization of Silk Fibroin Tissue Scaffolds

This research can be further developed to identify the desirable scaffold designs for optimized cartilage growth in craniofacial and orthopaedic surgeries. Parameters which can be manipulated include the concentration of SF, the size of the channels and the distance between the channels. The ability to exactly replicate the impaired tissue is important for aesthetics reconstruction as the recovery results will impact the mental well-being of patients considerably. For orthopaedics, the architectural design of scaffold should mimic that of the damaged or injured cartilage because any dimensional mismatch of the cartilage implant can lead to detrimental loads across joints. Nevertheless, the geometrical properties have to be carefully adjusted so that the mechanical properties are not significantly compromised.

8.3.2 Further Characterisation of Silk Fibroin Tissue Scaffolds

The SF scaffolds can be tested mechanically to ensure that their mechanical properties are comparable to that of the native tissue. However, the scaffold dimensions required for standard mechanical testing will lead to high material usage, especially on the raw building materials of the inkjet printer. Therefore, indentation tests and/or flexural strength assessments can be used as alternative testing methods so that samples of smaller sizes can be tested.

8.3.3 In vivo Implantation of Silk Fibroin Tissue Scaffolds into Cartilage Defect Site

This report has shown the viability of SF TE scaffolds for cartilage regeneration through subcutaneous implantation into nude mice models. The applicability of the 3D SF construct should be further justified by implantation of the scaffold sample into the impaired site. It is important to ensure that the architecture of the TE scaffold is suitable for cartilage-specific ECM secretion. Secondly, for osteochondral sites, the

interactions between the bone and newly generated cartilage have to be observed closely so that the anatomical features are restored and behaved comparatively to the native tissue. For instance, the calcification of cartilage tissues is undesirable as it may contribute to brittleness of the joint surface and subsequently lead to degenerative disease.

8.4 Publications

The publications generated based on this research are listed as follows:

8.4.1 International Journal Paper

1. **Liu, M.J.J.**, Chou, S.M., Goh, K.L & Tan, S.H., 2008. Cross-Sectional Area Measurement of Soft Tissue *In Vitro*: A Non-Contact Laser Scan Method. *Journal of Mechanics in Medicine and Biology*, 8 (3), pp 1-9.
2. **Liu, M.J.J.**, Chou, S.M., Chua, C.K., Tay, C.M.B. & Ng, B.K., 2011. The development of silk fibroin scaffolds using an indirect rapid prototyping approach: Morphological analysis and cell growth monitoring by spectral-domain optical coherence tomography. *Medical Engineering & Physics*, 35 (2), pp 253-262.
3. Chua, C.K., Leong, K.F., Sudarmadji, N., **Liu, M.J.J.** & Chou, S.M., 2011. Selective laser sintering of functionally graded tissue scaffolds. *MRS Bulletin*, 36, pp 1006 – 1014.
4. Tay, B.C.M., Ng, B.K., **Liu, M.J.J.**, Chua, C.K. & Chou, S.M., 2013. Monitoring Cell Proliferation in Silk Fibroin Scaffolds using Spectroscopic Optical Coherence Tomography. Accepted.

8.4.2 Conference Papers

1. **Liu, M.J.J.**, Chou, S.M. & Goh, K. L: Age effects on the tensile and stress relaxation properties of mouse tail tendons. Presented in the 13th International Conference of Biomedical engineering, 6-10th December 2008, Singapore
2. **Liu, M.J.J.**, Chua, C.K. & Chou, S.M.: Development of silk fibroin scaffolds using an indirect rapid prototyping technique. Presented in the 2nd International Conference on Cellular & Molecular Bioengineering, 2-4th August 2010, Singapore.

3. **Liu, M.J.J.**, Chua, C.K. & Chou, S.M.: Protein-based scaffold fabrication using Polyjet technology. Presented in the 4th International PMI conference, 15-17th September 2010, Ghent, Belgium.
4. **Liu, M.J.J.**, Chou, S.M., Chua, C.K., Tay, B.C.M. & Ng, B.K.: The study of micro- and macro-structural features of silk fibroin scaffolds. Presented in the 1st Bilateral Symposium on Bioengineering, 18-19th January 2011, Singapore.
5. **Liu, M.J.J.**, Chou, S.M., Chua, C.K.: A novel protein-based scaffold with macro- and micro-structural features for tissue engineering applications. Presented in the International Conference on Advanced Research in Virtual and Rapid Prototyping, 28th September – 1st October 2011, Leiria, Portugal.
6. Chua, C.K, **Liu, M.J.J.** & Chou, S.M.: Additive manufacturing-assisted scaffold-based tissue engineering. Presented as a keynote in the International Conference on Advanced Research in Virtual and Rapid Prototyping, 28th September – 1st October 2011, Leiria, Portugal.

8.4.3 Journal Papers Submitted

1. Cartilage tissue engineering with 3-dimensional silk fibroin scaffolds fabricated via additive manufacturing technology. *Chen, J.H., **Liu, M, J.J.**, Chua, C.K., Chou, S.M. & Chen, J.P., submitted to Journal of Tissue Engineering and Regenerative Medicine.*

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3. Wang L, Detamore MS. Tissue engineering the mandibular condyle, *Tissue Engineering* 2007 13: 1955-1971
4. Kalson NS, Gikas PD, Briggs TWR. Current strategies for knee cartilage repair, *International Journal of Clinical Practice* 2010 64: 1444-1452
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Appendix A

A1.1 Scaffold Material Preparation

A1.1.1 Regenerated Silk Fibroin

Cocoons of Bombyx Mori silkworm (Treenway Silks, Canada) were heated at 95 ± 5 °C for 30 minutes in an aqueous solution of 0.02M Na₂CO₃ (Sigma Aldrich) [42]. This process was repeated thrice. The cocoons were then thoroughly rinsed with deionised water to ensure complete removal of the glue-like sericin proteins. The degummed silk was left to dry for 24 hours in a fume hood.

SF solution was obtained by the dissolution of the degummed silk cocoons in a solvent mixture of calcium chloride (CaCl₂), ethanol (CH₃CH₂OH) and deionised water (molar ratio = 1:2:8) at 80 ± 5 °C for four hours [42]. This solution was then dialyzed in deionised water using a cellulose dialysis membrane (Molecular weight cut-off (MWCO) = 6000 – 8000 g/mol, Thermo Scientific) for 3 consecutive days. The deionised water was changed twice a day to ensure the complete removal of CaCl₂. The diluted SF solution was subsequently centrifuged at 3500 rpm for 30 minutes and filtered to remove the unwanted solutes. Finally, the SF solution was concentrated by dialyzing against 20 % (w/v) polyethylene-glycol (PEG, molecular weight = 20,000 g/mol, Sigma Aldrich) and the concentration of the silk solution was determined by weighing the remaining dried silk solid after air drying. The aqueous SF was stored at 4 °C until use.

A1.1.2 Casting of Regenerated Silk Fibroin

Aqueous SF with various concentrations was cast into the Teflon beakers and allowed to stand for 15 minutes under room temperature to eliminate air bubbles from forming within the foams. The solutions were subsequently frozen in -80 °C and lyophilized. The dried SF foam matrices were then immersed in boiling ethanol to induce β -sheet formation.

A1.1.3 Porosity Measurement

The porosity measurement of the SF foams were conducted a liquid displacement technique. Hexane was used to infiltrate into the pores of the foams as it permeates

through SF without swelling or shrinking the matrix. Each foam sample was first immersed in a 50 ml centrifuge tube containing a known volume of hexane (V_1). The total volume of the SF foam and hexane was recorded as V_2 . After 5 minutes, the hexane-impregnated foam was removed from the tube and the residual volume of hexane (V_3) was recorded. The volume of SF foam was calculated $V_2 - V_1$, while the volume of the hexane infiltrated into the pores of the foam was represented by $V_1 - V_3$. The total volume of the foam can be expressed as [137]:

$$V = (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3 \quad (\text{Equation A1.1})$$

The porosity of foam, ϕ , was obtained using the following equation:

$$\phi = \frac{V_1 - V_3}{V_2 - V_3} \quad (\text{Equation A1.2})$$

A.1.1.4 Equilibrium Swelling Ratio and Water Uptake

The equilibrium swelling ratio (*ESR*) and water uptake were measured to assess the water-binding ability of SF foams with different concentrations. Each SF sample was immersed in a 50 ml centrifuge tube containing deionised water at room temperature for 24 hours. The excess water was removed and the wet weight of the foam specimen was measured as W_s . Samples were subsequently lyophilized under vacuum conditions and the dry weight of the each foam (W_d) was obtained. The swelling ratio (*ESR*) of the foam constructs and the water uptake were calculated as follows [137]:

$$ESR = \frac{W_s - W_d}{W_d} \times 100\% \quad (\text{Equation A1.3})$$

$$\text{Water uptake} = \frac{W_s - W_d}{W_s} \times 100\% \quad (\text{Equation A1.4})$$

A.1.1.5 Mechanical Characterisation of SF Foams with Different Concentrations

The compressive mechanical properties of SF foams with concentrations 5, 7.5, 10, 12.5 and 15 % (wt/wt) were measured using a 500 N load cell in the mechanical tester machine (Model: 5566 series, Instron). The protocol for testing was conducted with reference to ASTM-D1621 [263]. Briefly, SF sponges, approximately 25 mm in

diameter and 14 mm in thickness, were tested with a crosshead speed of 2.5 mm/min until 40 % strain has been reached. Six samples were used for each SF concentration.

Stress-strain graphs were plotted for the compression tests. The deflection recorded for each test were divided by the specimen thickness to calculate the average compressive strain across the SF foam while the load was divided by the cross-sectional area of the SF sample to yield the compressive stress. The data set was then fitted with a polynomial function using a least-squares curve fit. The compressive moduli of the SF foams were then deduced as the first derivative of the polynomial at the inflection point of the linear region.

A1.2 Graphical plots to obtain empirical models

Figure A1 Graphical plot of porosity against concentration.

Figure A2 Graphical plot of equilibrium against porosity.

Figure A3 Graphical plot of water uptake against porosity.

Figure A4 Graphical plot of log(modulus) against log(porosity).

Appendix B

Cytotoxicity Test Protocol

Calibration curves for biochemical assays

Calibration curve for DNA content

Calibration curve for sGAG content