Hybrid Biomimetic Scaffold of Silk fibroin/collagen Type I Film for Tissue Engineering: Preparation and Characterization

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บทคัดย่อ

คอลลาเจนเป็นโปรตีนที่เป็นส่วนประกอบหลักสำคัญของโครงสร้างค้ำจุนรอบนอกเซลล์ สิ่งมีชีวิต(Extracellular matrix; ECM) คอลลาเจลเป็นหนึ่งในวัสดุที่ได้รับการพัฒนาสำหรับการใช้งานที่หลากหลายทางด้านการแพทย์ เพื่อเพิ่มคุณสมบัติเชิงชีวภาพและความเข้ากันได้ของเซลล์ อีกทั้งเพื่อตอบสนองความจำเป็นและความต้องการที่เพิ่มมากขึ้นของวิศวกรรมเนื้อเยื่อ แต่คอลลาเจนยังขาดคุณสมบัติทางด้านกายภาพและเชิงกล

ในการศึกษาวัสดุรองรับเนื้อเยื่อผสมระหว่างโปรตีนไหมกับคอลลาเจนโดยวิธีไบโอมิเมติก เป็นการนำคุณสมบัติทางด้านกายภาพและเชิงกลที่ดีของฟิล์มไหมร่วมกับคอลลาเจนที่ละลายในกรดที่ได้จากผิวของปลาฉลามขาวโดยใช้วิธีเคมีคอลครอสลิ้ง(ใช้สารEDC และ NHS เป็นตัวเชื่อมขวาง)เคลือบพื้นผิวฟิล์มไหมด้วยสารละลายคอลลาเจนที่มีอัตราส่วนความเข้มข้นต่างกัน การศึกษามีวัตถุประสงค์เพื่อศึกษาและเปรียบเทียบลักษณะของฟิล์มโปรตีนไหมที่เคลือบพื้นผิวด้วยสารละลายคอลลาเจนที่มีอัตราส่วนต่างกัน ดังนี้ 0.25, 0.5, 1.0 และ 2.0 mg/ml โดยใช้วิธีการเลียนแบบการจัดเรียงตัวของคอลลาเจนตามธรรมชาติ ติดตามคุณสมบัติทางกายภาพโดยใช้กล้องจุลทรรศน์อิเล็กตรอน (SEM) ผลการทดลองพบว่า เมื่อเพิ่มความเข้มข้นของคอลลาเจน การจัดเรียงตัวใหม่ของเส้นใยคอลลาเจนจะสังเกตพบมากขึ้นและเส้นใยจะมีขนาดใหญ่ขึ้นตามความเข้มข้นของสารละลายคอลลาเจนอีกด้วย นอกจากนี้ผลจากเครื่องวิเคราะห์หาโครงสร้างของสารประกอบอินทรีย์ โดยใช้หลักการการดูดกลืนรังสีอินฟราเรด(FT-IR)แสดงให้เห็นถึงโครงสร้างของฟิล์มโปรตีนไหมเกิดการรวมตัวเคลือบพื้นผิวด้วยสารละลายคอลลาเจนได้หลังจากนั้นจึงทดสอบคุณสมบัติเชิงกลพบว่าฟิล์มโปรตีนไหมที่เคลือบพื้นผิวด้วยสารละลายคอลลาเจนความเข้มข้น 1-2 mg/ml แสดงให้เห็นผลการเปลี่ยนแปลงคุณสมบัติเชิงกลของวัสดุได้

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ABSTRACT

Collagen, one of the proteins in human tissue is the major component in extracellular matrix (ECM). It has suitable functionality and properties for use as a scaffold. Because of its biocompatibility, biodegradation and non-toxicity, but also there are still critical problems because of the poor physical and mechanical properties.

In this study, silk fibroin collagen to improve function of collagen. Acid soluble collagen(ASC) from the shark skin (*Carcharodon carcharias*) were used to produce biomimetic reself-assembly of collagen approach. This study reports in vitro reconstitution of the development of silk fibroin film coated with collagen by chemical crosslink;1–ethyl–3-(dimethylaminopropyl) carbodiimide hydrochloride(EDC/HCl) and N -hydroxysuccinimide (NHS).

The study aimed to investigate and compare characteristics of *Bombyx mori* silk film coated with mimicked re-self assembly collagen type I under various conditions concentration [0.25, 0.5, 1.0, 2.0 mg/ml]. The thin films consist of collagen fibrils and result from the self-assembly of collagen. The morphological characteristics observed by scanning electron microscopy (SEM) showed that when increased in collagen concentration, collagen became increase the size of the fibrils. The structure and thermal behavior all kind of silk coated with collagen were determined by Fourier Transform Infrared spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC)

instrument. FT-IR spectra showed both spectra of silk and collagens are assigned to β-sheet conformation of silk fibroin and collagen type I. The stability thermal peak shifted to slightly higher temperature by increasing collagen concentration content. Mechanical properties of silk film coated collagen demonstrated that coating collagen at concentration 1-2 mg/ml resulted in modification of mechanical features, with increased maximum load, stress, strain, and Young's modulus .

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LIST OF ABBREVIATIONS AND SYMBOLS

AFM	Atomic Force Microscope
ASC	Acid Soluble Collagen
BMPs	Bone Morphogenetic Proteins
BSF	Bovine Sponge form Encephalopathy
DSC	Differential Scanning Calorimetry
EDC/HCl	1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride .
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide)
FTIR	Fourier Transform Infrared Spectroscopy
IGF	Insulin-like Growth Factor
GDF9	Growth Differentiation Factor-9
MPa	Megapascal
NHS	N-hydroxysuccinimide
SEM	Scanning Electron Microscopy
Tg	Glass Transition Temperature
Tm	Melting Temperature
WCA	Water Contact Angle

CHAPTER 1

INTRODUCTION

1.1 Background

Tissue and organ loss or failure resulting from an accident and chronic disease is a major health problem. When the body loses tissue, it can repair the damage site by itself, if it is of moderate size. However, when damage is extensive, the generation of new tissue requires much time and in the case of skin defects there is the risk of bacterial or other microbial infection during treatment. Furthermore, undesirable scarring is likely to occur. Such problems have provided the motivation for many researchers and physicians to create tissue engineering strategies. Tissue engineering is an exciting technique to solve the problem that the level of demand from such patients far exceeds the supply of donor organs. Tissue engineering is a new technique which has the potential to create tissues and organs. It is likely to revolutionize the ways to improve the health and quality of life for millions of people worldwide by restoring, maintaining, or enhancing tissue and organ function.

Basic principle of Tissue engineering, Tissue engineering is a new technology which combines of knowledge's cells, technical engineering, materials methods, biochemical and physiochemical factors to restore, maintain or improve biological functions [3]. The tissue engineering's objective is to support the limitations of tissue transplantation and biomaterial implantation [1]. The principle of tissue engineering was that tissues can be separated from a patient, expanded in a tissue culture, and seeding cells into an extracellular matrix or scaffold prepared from a specific building material. The mature of construct can be implanted in the defect organs or tissues. The tissue engineering technology made various tissue structures such as skin, bone, cartilage, tendon and vessel. The object of tissue engineering was the recovery function of tissue by transfer cells grafted with scaffold matrices into the patient.

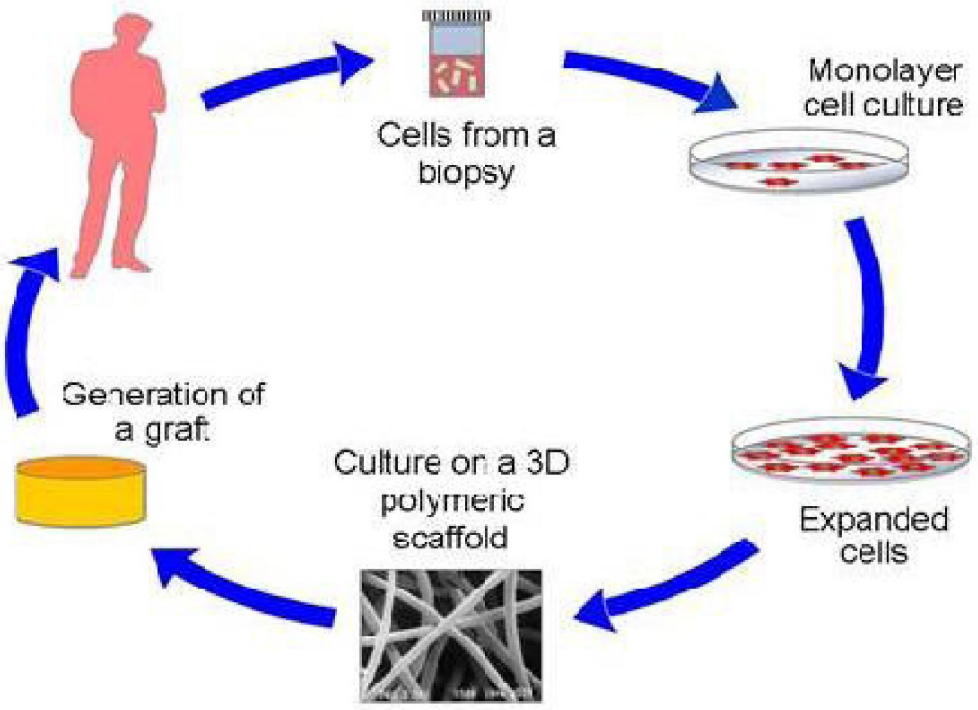


Figure 1.1 Basic principles of Tissue engineering

Many types of material are used in such scaffolds; they may be classified as either natural or synthetic, each with its particular advantages. Natural polymers had been widely for the tissue-engineering scaffolds. It can be considered as the first because of biodegradable properties. It had biocompatibility properties and better interactions with the cells which have enhance the cell's performance in biological system. Silk and collagen type I were natural materials which had good special properties to develop medical application for skin tissue engineering.

Silk from silkworm cocoon is a natural fibrous protein material. *Bombyx mori* (*B. mori*) silk fibroin was the important biomaterial because of environmental stability, high mechanical properties, biocompatibility, and biodegradability [1]. Silk fibroin was studied in the biological function and biomedical application fields have fabricated such as nanofiber, film and 2D or 3D matrix for cell culture [2].

Collagen is a potentially useful biomaterial since it has been a major component of extracellular matrix. Its biomaterial characteristics has many advantages such as bioresorbability, biocompatibility. It can be processed into variety of form such as nano-fiber, gel, and film. The collagen fibrils in animal tissue have properties making them suitable for both biological and mechanical functions [1, 2]. This is the motivation for creating some unique structures and functionalities via processing of collagen self-assembly. Understanding the mechanism of collagen self-assembly in these conditions is an attractive choice to create materials with potential for use as scaffolds. However, collagen has been used for short-term application as it degraded in 6-8 weeks. It had severely limited by lacking of mechanical strength and susceptibility to enzymatic degradation in vivo.

Biomimetic strategy was the attractive approach for biomedical applications. Almost all natural materials have a sophisticated hierarchical structure arising from the self-assembly of biomolecules and producing high performance functionalities. A biomimetic biomaterial for tissue engineering produces scaffolds similar to natural extracellular matrix. These include synthesis to complete properties similar to the extracellular matrix. The innovation processing technologies to achieve structural features was mimicking the extracellular matrix.

The critical problems of collagen and the unique functionalities and properties of silk fibroin noted above provide the motivation for this research, in which we have attempted to create a novel scaffold for use as skin regenerative material from biopolymers based on collagen and silk fibroin. We expect that silk fibroin was used to improve mechanical properties of biomaterials and protected the biological functionality. Re-self assembly of type I collagen are coated for inducing cell recognition and excellent biocompatibility. Furthermore, it is expected that its properties will allow the novel scaffold to be of outstanding value in skin tissue engineering. The aims of this research emphasise four objects based on the concept of material science: the structure, properties, processes and performance of the material under examination. Preparation and characterization are strongly considered from the effects of re-self assembly of collagen on silk fibroin film.

1.2 Hypothesis

The research hypothesis is formulated as follow:

1.2.1 Based on mechanical, biological and manufacturing considerations, only simple shape can be utilized as tissue engineering scaffold.

1.2.2 The information obtained from assessment of tissue engineering scaffold properties can be used to develop process for scaffold design.

1.2.3 The novel biomimetic scaffold is superior to the regular scaffold in terms of mechanical and biological.

1.3 Objectives

The aiming goals of this study are to facilitate the scaffold design with various necessary properties.

1.3.1 To facilitate and modify scaffold by biomimetic approach.

1.3.2 To design a performance scaffold and characterize their structure physical and mechanical properties.

1.3.3 To propose feasibility for using a performance scaffold for skin tissue engineering applications.

1.4 Scope of the thesis

1.4.1 The experiment use type I collagen from shark skin and silk fibroin film from *Bombyx mori*.

1.4.2 The experiment considers to develop methods for coating biomimetic reself-assembly typeI collagen on silk fibroin films as hybrid biomimetic scaffolds.

1.4.3 Hybrid biomimetic scaffolds of silk fibroin/collagen typeI were characterized the physical and mechanical properties.

Chapter2

Basic and Principle

2.1 Tissue engineering overview

Tissue engineering utilized the combination of cells, technical engineering, materials methods, biochemical and physico-chemical factors to restore, maintain or improve biological functions [3]. The goal of tissue engineering is to support the limitations of tissue transplantation and biomaterial implantation [1]. The artificial organ has potential to produce the supply of immunological tolerant transplant tissue. Tissue or organ replacement can grow with the patient and improve physical function of failure. This should lead to a permanent solution to the damaged organ or tissue therapies [4].

The replacement part is transplanted to repair or replace the degenerative organs, which the post-transplantation results are expect to restore organ function. The process of tissue engineering involves by extraction of cell from living body and then increase population of the cell by culturing the cell in vitro under certain controlled environment. During cell culture the period, the cell are seeded onto cell carrier, i.e. scaffold, that serves as a temporary support for cell to proliferate and differentiate after culturing in certain period. The cell carrier was implanted into the body, after the number, function, and morphology of cell are sufficient or met the mechanical and biological requirements of tissue [1].

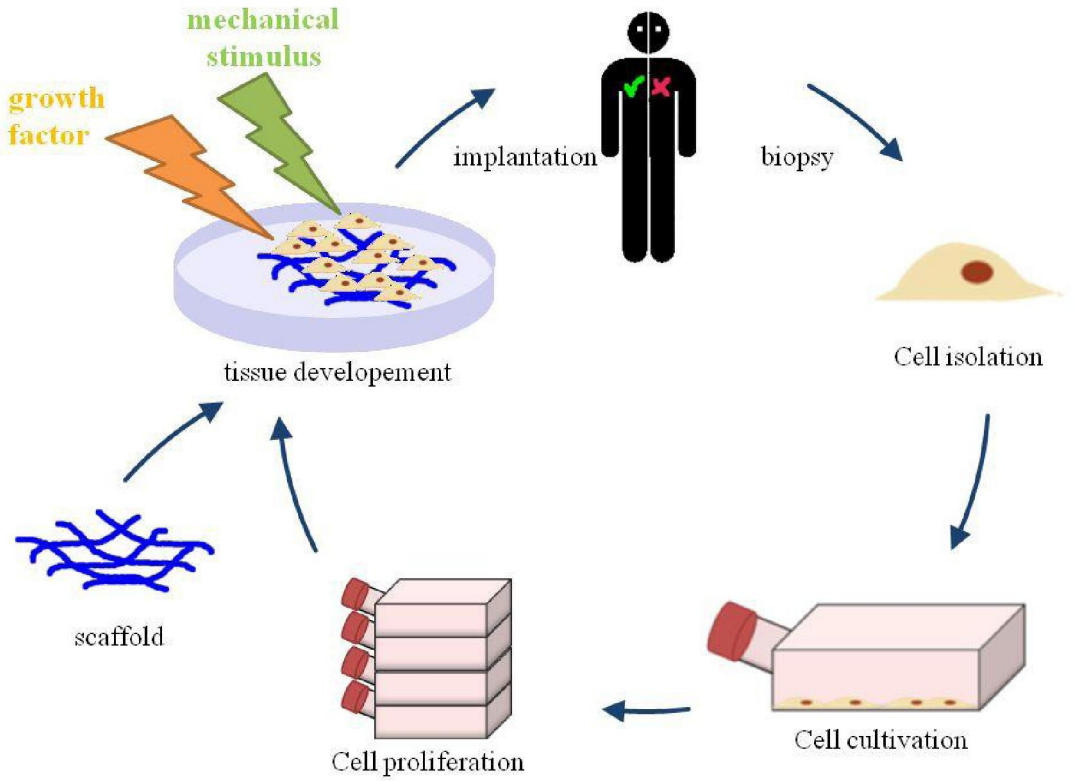


Figure 2.1 Tissue engineering process [10]

2.1.1 Tissue engineering of skin

The skin is an important organ, having the primary function as a barrier to the outside environment. To fulfill this function, the skin must be physically tough, flexible and elastic, in order to protect against toxic substances from the outside and to preserve internal humidity. Beyond these passive roles, skin also has significant active functions, including the important one of providing immunity against foreign substrates and microorganisms. It is also vital in controlling and regulating body temperature.

The anatomy of skin is best described by considering its two main layers, the epidermis and the dermis.

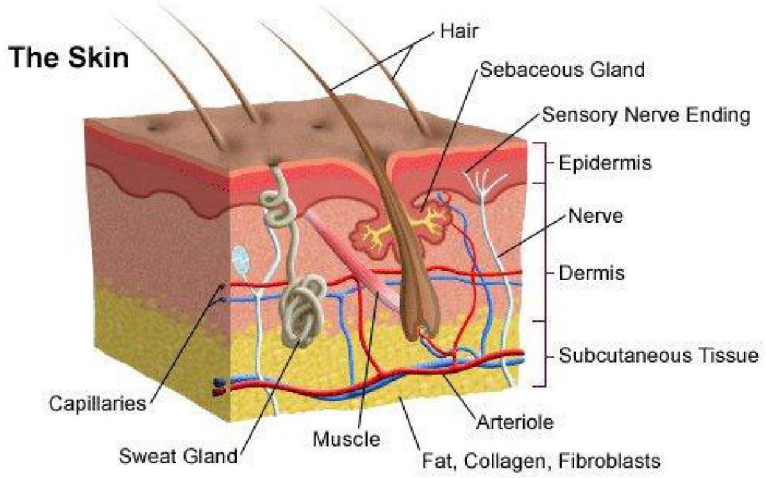


Figure 2.2 The anatomy of skin [1]

Epidermis

The outer epidermal layer contains the primary keratinocytes. These have the important function of producing various proteins, growth factors and cytokines which generate signals between cells to regulate the skin. Basal cells also proliferate in the epidermis, lying between the epidermis and the dermis, anchoring one to the other. The epidermis contains melanocytes, which produce melanin, whose role is to pigment the skin and protect both epidermis and dermis from ultraviolet (UV) radiation. Langerhans' cells are dendritic cells, abundant in the epidermis, which organize themselves into a network of dendrite and interact with keratinocytes and nerves. The role of hairs is to help to preserve the body temperature of mammals. Hair follicles also act as a source to grow keratinocytes after severe wounds and work with sebaceous glands to secrete sebum, an oily substance acting as a moisturizer and lubricant for the hair and epidermis.

Dermis

The dermis is the layer of skin beneath the epidermis. The important physical properties of dermis are the flexibility and strength required to bear the movement of the body. The dermis comprises an extracellular matrix of interwoven collagen fibrils having the properties of elastic fibers. Beside collagen woven fibrils, there are both proteoglycans and glycoproteins in the extracellular matrix (ECM). Collagen, proteoglycans and glycoproteins all have significant biological roles as well as their

physical and mechanical properties. They have biological functional sequences in their structures whose task is to recognize cells, to accommodate them and to provide a substrate for cell-cell communication. The structure, properties and functionality of ECMs provide a model for the design and fabrication of the scaffolds used in tissue engineering. Structurally, the dermis comprises two parts: the papillary dermis lying under the epidermis and the deeper recticular dermis. The important contribution of the latter is to provide the strength, flexibility and elasticity of the skin. This sub-layer has a thicker, dense mesh structure of collagen and elastic fibers than the papillary dermis. In the dermal layer, fibroblasts are the main cells forming the extracellular matrix. There are also endothelial cells having an important role in the skin immune system and hematopoietic cells .Finally, there is a network of nerve fibers extending throughout the dermis serving a sensory role and secreting neuropeptides, which affect inflammation and immunity in the skin.

Generally, when the body receives an injury, it can regenerate new tissue by wound healing process. Nevertheless, if the injury causes a critical wound over a large area in the case of injury, the new tissue is incomplete because of lacks the extracellular matrix that is necessary for the regenerative process. The design and fabrication of the artificial skin were interested to produce scaffolds. Because it contacts and interacts with the wound, the significant properties of artificial skin are as follows: 1) to adhere to the substrate, 2) to be durable and sufficiently elastic to tolerate some deformation, 3) to provide a microbial barrier, , 4) The evaporative water loss was allowed at the typical rate for the stratum corneum, 5) to be able to promote haemostatic, 6) to regenerate the new tissue without foreign-body, inflammatory or non-self-immunologic reactions, 7) to be easy to use and available immediately after injury.

Accordingly, dermal replacement is the choice in such cases, as well as being usual in plastic surgery. The development of dermal scaffold focus on the Tissue engineering field.[7] Tissue engineering can be defined as the application of the healing of skin, stimulate healing, protect the surface while healing, and even replace the skin surface. The scaffolds can be produced specific skin functions: to protect the injury from environment, to reduce fluid loss, to remove exudates, and inhibiting exogenous microorganism invasion. [8]

Reproducibility is important for the performance regenerative matrix. Key to reproducibility is the control of distribution and over size of pores, the inflammatory, immune responses, degradation rate and the removed residual toxic organic solvents. [8]

2.2 Scaffold for tissue engineering

The objectives of tissue engineering seek to restore, maintain or improve the functions of human degenerative tissue. Three main necessary components assisted tissue engineering to achieve the objectives are, harvested cells, and signaling molecule (for example, growth factor-substance encouraging cellular growth which normally is a protein and scaffold[6,7,10].Generally the signaling molecule is often coated onto scaffolds, before the cells are seeded for incubation. During the incubation and later on period, the scaffold and cell signaling together promotes the cell proliferation and differentiation.

The design of tissue engineering scaffold is a challenge. Scaffold is required to satisfy many mechanical and biological requirements. Both requirements need to be carefully optimized in order to effectively produce neo-tissue. These requirements are explained as follows:

(1.) Tissue compatibility: the material used to fabricate the scaffolds and the residual substances after the material degradation must be non-toxic. The material is preferred to provide and encourage cell adhesion, proliferation, and differentiation. In addition, in any case, the scaffold must not present negative immune reaction inflammatory response after implantation.

(2.) Architecture: the scaffold should have appropriate pore size and porosity for specific cell types in order to support tissue growth. For example, Table 2.2 provides preferred pore size of some types of cell. The porous structure of scaffold should also have interconnected structure throughout the scaffold, lack of dead-end pore. The high scaffold porosity is appreciable in order to provide good transportation of nutrient into and waste out from the scaffold.

(3.) Cellular activity: the scaffold should also serve as a carrier for bioactive substances, such as growth factors and cell-adhesive materials, to enhance cell attachment, growth, and differentiation. Some common bioactive cues include BMPs, insulin-like growth factor (IGF), and growth differentiation factor-9 (GDF9). In addition, the surface of scaffold may

also be treated to mimic the autogenously extracellular matrix (ECM) to encourage cell attachment.

(4.) Strength and Stability: the mechanical properties i.e. stiffness of scaffold should be consistent with implanted surrounding tissue. Improper scaffold mechanical properties may result undesired post-operative result. The scaffold should also have sufficient strength to withstand physiological loads. For biodegradable scaffold material, the degradation rate of scaffold is required to be compatible to the tissue regeneration rate. This is to ensure the scaffold stability during throughout the healing period.

(5.) Shape validable: the shape and dimension of scaffold should be anatomically conformed to the defect site. Consequently, it provides the best attachment and uniform stress distribution between surrounding tissue and implant interface.

2.3 Extracellular matrix as scaffold for tissue engineering

The extracellular matrix (ECM) consists of structure and functional molecules. It arranged to 3D structure that is unique to each tissue specific. The proteins of ECM represented collagen, glycosaminoglycans, glycoproteins, fibronectin and elastin.

For a good structure, generally, the scaffold functions as an artificial extracellular matrix, so it is necessary to create the same structure and biological functionality as the native ECM. The requirement for a suitable structure is that it should have the porous mesh or network structure that makes it suitable for cells to reside. As the literature shows, many researchers have attempted to optimize and concentrate on the pore formation and the effect of the porous structure on cell culture [1]. For good performance, one of the important roles of scaffold is in providing strength and flexibility to the tissue. Accordingly, the most important properties of the scaffold materials are strength and flexibility. Because scaffolds have to be used in aqueous solution, must also be stable. Many research studies have focused on improving their stability and mechanical properties by structural modification through cross-linking, blending, the use of copolymers [5, 24]. All requirements can be met by means of these processing techniques.

In the case of good biological functionality, all approaches focus on the strategy of preserving or supplementing the biological functionality of the material, especially for collagen, whose biological functionality always diminishes during fabrication [9]. Collagen was the most

component protein in the mammalian ECM. Collagen represented in tissues and organs more than 90% of the dried weight of ECM. Type I collagen is the major structural protein present in human tissues. It located in connective tissues including tendons, skin, ligaments, cornea, and many interstitial connective tissues. In some cases for synthetic polymers, it is necessary to supplement biological functionality by adding bioactive molecules such as collagen ; the RGD peptide sequence was the recognition group for cells in the ECM for to be good artificial structure. Generally, the scaffold functions as an artificial extracellular matrix, so it is necessary to create the same structure and biological functionality as the native ECM. The requirement for a suitable structure is that it should have the porous mesh or network structure that makes it suitable for cells to reside. The engineering of surfaces to manipulate healing is a rapidly expanding area, with the use of interactive dressing systems with the realization of the impact of surface topography and chemistry on cell expression. [8]

2.4 silk fibroin scaffolds for tissue engineering

Silks are protein-based fibers made by silkworm spun into fibers during their metamorphosis phase. Silkworm's silk is an established textile fiber industry. Silk can apply to use in medical application successful as suture. Silk fibroin is product from de-gummed silks. Silk fibroin has high mechanical properties, biocompatibility, biodegradability and side change amino acid to corporate functional groups. The amino acid composition of silk fibroin containing up to 90% of the amino acids glycine, alanine, and serine .Silk fibroin can form repeating to antiparallel β-sheet formation in the secondary structure [20].

2.4.1 Structure of B. mori silk fibroin

The silk from the cocoon of B. mori composed of two major fibroin proteins, light and heavy chains, 25 and 325 kDa, linked by a single disulfide bond. A third small glycoprotein, known as the P25 protein (30 kDa) is associated via non-covalent hydrophobic interactions. Silk was coated with hydrophilic protein called sericin (20-310 kDa) that was glue protein.The molar-ratios of Heavy-chain: Light chain: P25 are 6:6:1. The heavy-chain is hydrophobic and contains

blocks of Gly-X repeats; with X being Ala, Ser, Threonine (Thr) and Valine (Val) that was crystalline forming domains [21]. The light-chain is more hydrophilic and relatively elastic, the P25 protein is believed to play a role in maintaining the integrity of the complex. These proteins are coated with a family of hydrophilic proteins called sericins, glue-like proteins (20–310 kDa) [22, 23]. The formation of primary structure was disulfide bond between the heavy chain and light chain called silk I. The secondary structure called silk II (β-sheet) which converted from silk I by exposure heat or alcohol immersion. The β-sheet structure was asymmetrical from hydrogen side chains of glycine and methyl side of alanine. Methanol adjusted hydrogen groups and methyl groups of opposing sheets interact to form inter-sheet stacking in the crystals.

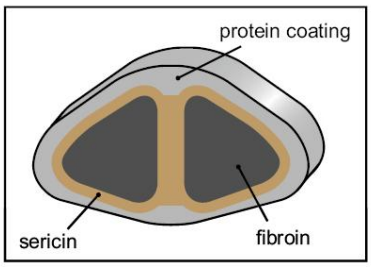


Figure 2.3 Example of silk fibers produced by silkworms [24]

Table2.1 Summary the structure of silk fibers [26, 27]

	Silk fiber	**Molecular Weight**	**Structure**
Silk fibroin (~75%)	heavy chain (H-chain) fibroin	325 kDa	- (Gly-Ala-Gly-Ala-Gly-Ser)n - To form anisotropic β-sheet-rich nanocrystals
	light chain (L-chain) fibroin	25 kDa	- Disulfide bonds linked a second small protein - Hydrophilic and relatively elastic
	P25 protein	30 kDa	- Non-covalent hydrophobic interactions - Maintaining the integrity of the complex
Silk sericin (~25%)	a glue-like glycoproteins	20–310 kDa	Coating protein

2.4.2 Properties of silk fibron

The advantage of *Bombyx mori* (*B. mori*) silk fibroin compared to other biomaterial is high mechanical properties, environmental stability, good biocompatibility, biodegradability and easy obtainable chemical groups for functional modification. Lots of studies have been focused on silk fibroin as scaffold for tissue engineering. Furthermore, it can be processed into variety of form such as nanofiber, gel, and film. Importantly, silk fibroin has predominant mechanical properties over the other biological materials as table2.2. Therefore, it is often selected to develop scaffold for load bearing tissue engineering.

Table2.2 Comparison of mechanical properties of common silks

Material	UTS (MPa)	Modulus (GPa)	% Strain at break
B. mori silk	740	10	20
B. mori silk (with sericin)	500	5–12	19
B. mori silk (without sericin)	610–690	15–17	4–16
Spider silk	875–972	11–13	17–18
Collagen	0.9–7.4	0.0018–0.046	24–68
Cross-linked collagen	47–72	0.4–0.8	12–16
Tendon (comprised of mainly collagen)	150	1.5	12
Bone	160	20	3
Synthetic Rubber	50	0.001	850

The success of silk sutures has made to know biocompatibility of silk. However, native silk contained gum-like protein sericin which was cause Type I allergic immune response in human body. Silk fibroin can purify from sericin by boiling silk cocoons in an alkaline solution. Silk siricin was removed during the de-gumming process [37, 38]. Removal of the silk sericin protein before using dismissed the thrombogenic and inflammatory response of silk fibroin.

The silk had advantages more than other biomaterials aspects biodegradration and bioresorbable. The degradation of silk fibroin depended secondary structure of silk from preparation methods. Silk fibroin had slow degradation rate and retaining strength over along time.

Table 2.4 Advantages and disadvantages of silk as biomaterial.

Advantages	Disadvantages
High mechanical properties, environmental stability to match clinical repair	Side effects from silk sericin induced Type I allergic response
Biodegradable by enzymatic degradation	Lack of biological function
Nontoxic and biocompatibility properties	
Collaborate with bioactive components	
Fabricated to variety of form such as film, fiber and sponge	
Long term to applied in medical application such as sutures	
Available large scale processing in silk textile industries	

Silk fibers can be produced by spinning from cocoons. Silk fibers has been used as biomedical application in varieties of structure. B.mori silk fibroin can be dissolved with neutral salt solutions such as lithium bromide (LiBr), hexafluroisopropryl alcohol (HFIP).The mixtures of silk solution are dialyzed to get pure fibroin solution. Silk fibroin solution can be used to prepare silk fibroin membranes, fiber, hydrogel and scaffolds. Silk fibroin films were produced by casting or spin coating silk fibroin solution to produce thin films.

2.5 Collagen for tissue engineering

Collagen, one of many kinds of protein, is the most abundant in mammals, constituting approximately 25% of total protein in animals. It is a major component in extracellular matrix (ECM), where it functions as a fibrous mesh network embedded in polysaccharide basement gel and has a complex structure. The ECM was cell environment which cells can attach and spread. Cell signal communicated achievement by cell-matrix adhesion and interaction. Collagens fibrils supported tissue's mechanical function, anchorage of macromolecules, storage and release of cell mediators. It maintained the shape and form of tissues. Generally, collagen has the molecular structure of a polypeptide built of amino acid monomers, called alpha chains, each of about 1000 amino acids. Each alpha peptide chain is composed of many types of amino acid monomers. The different types and amounts of amino acid monomer have an influence on the structure, assembly, functionalities and properties of collagen. Accordingly, they are classified into different types, as shown in Table 2.3

Table 2.3 The various collagen types, as they belong to the major collagen families. [14]

Type	Molecular Composition	Tissue Distribution
Fibril-forming collagens		
I	$[\alpha 1\ (I)]_2\alpha 2(I)$	Bone, dermis, tendon, ligaments, cornea
II	$[\alpha 1\ (II)]_3$	Cartilage, vitreous body, nucleus pulpous
III	$[\alpha 1\ (III)]_3$	Skin, vessel wall, reticular fibers of most tissues

Type	Molecular Composition	Tissue Distribution
V	α1 (V), α2 (V), α3 (V)	Lung, cornea, bone, fetal membranes
XI	α1 (XI) α2 (XI) α3 (XI)	Cartilage, vitreous body
Basement membrane collagens		
IV	[α1 (IV)]2 α2(IV); α1–α6	Basement membranes
Microfibrillar collagen		
VI	α1 (VI), α2 (VI), α3 (VI)	Widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils		
VII	$[\alpha 1\ (VII)]_3$	Skin, dermal– epidermal junctions; oral mucosa, cervix
Hexagonal network collagens		

Type	Molecular Composition	Tissue Distribution
VIII	$[\alpha 1\ (VIII)]_2\ \alpha 2(VIII)$	Endothelial cells, Descemet's membrane
X	$[\alpha 3(X)]_3$	Hypertrophic cartilage
FACIT collagens		
IX	$\alpha 1\ (IX)\ \alpha 2\ (IX)\ \alpha 3\ (IX)$	Cartilage, vitreous humor, cornea
XII	$[\alpha 1\ (XII)]_3$	Perichondrium, ligaments, tendon
XIV	$[\alpha 1\ (XIV)]_3$	Dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$[\alpha 1\ (XIX)]_3$	Human rhabdomyosarcoma
XX	$[\alpha 1\ (XX)]_3$	Corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	$[\alpha 1\ (XXI)]_3$	Blood vessel wall
Transmembrane collagens		

Type	Molecular Composition	Tissue Distribution
XIII	$[\alpha 1\ (XIII)]_3$	Epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$[\alpha 1\ (XVII)]_3$	Dermal– epidermal junctions
Multiplexins		
XV	$[\alpha 1\ (XV)]_3$	Fibroblasts, smooth muscle cells, kidney, pancreas
XVI	$[\alpha 1\ (XVI)]_3$	Fibroblasts, amnion, keratinocytes
XVIII	$[\alpha 1\ (XVIII)]_3$	Lungs, liver

Type I collagen provided an important set of materials options and to be in favor of studies collagen. . It was the content ninety percent in tissues of mammalian, mainly located in connective tissues including tendons, skin, ligaments and cornea. The collagen organization reflected the tissue mechanical function. For example, to carry the tensile load tensile strength, and torsion stiffness in skin or tendon, collagen fibrils are arranged into long and parallel fascicles. The structure of collagen type I was the triple helix formed as heterotrimer by two identical α1-chains and α2-chains. Type I collagen is interested using scaffold material for tissue engineering because of its many advantages. Collagen type I monomers can be extracted from bovine, porcine and marine sources. The extracted type I collagen molecules results in the self-assembly into isotropic fibrillar network. The fibrillar collagen can be applied for tissue engineering.

Table 2.4 Advantages and disadvantages of collagen as biomaterial: modified form [16, 29, 39]

Advantages	Disadvantages
Available purified from living organisms such as marine porcine and bovine	High cost to purified type I collagen
Biodegradable and bioreabsorbable	Hydrophilicity properties leads to swelling and more rapid release
Nontoxic and biocompatible	Variability in enzymatic degradation rate
Collaborate with bioactive components	Complex to immobilize methods
Biological plastic due to high tensile strength and minimal expressibility	Side effects, such as bovine sponge form encephalopathy (BSF) and mineralization
Fabricated to multiple form such as film, gel ,fibril and sponge.	Lacked of mechanical strength
Biodegradability can be controlled by cross-linking	
Using its functional groups to produce hybrid material	

2.5.1 Structure of fibrillar collagen

Collagen peptide chains are rich in glycine and proline, both of which are important in the formation of stable triple helices. To date, 28 genetically different types of collagen are known. Fibril forming collagens (i.e. I-III, V, XI) are the main load-bearing components of the ECM which self-assemble into 67 nm cross striated (D-banding) fibrils. Collagen molecules are produced intracellular in the form of soluble procollagen. Procollagen is mostly composed of a linear helical domain (300nm length and 1.5nm diameter) flanked by two globular regions (N- and C- propeptides). The molecular structure of collagen is composed of three left handed polypeptide (α chains) that are wound together to form a right handed helix. The amino acid backbone of the α-chains have a repeating form of Gly-X-Y in which X and Y in most cases are tertiary amides of L-proline and 4(R)-hydroxy-L-proline, respectively. Glycine, the smallest amino acid in nature, is essential for collagen stability. It is small enough to fit in the space between proline and hydroxyproline enabling it to form a hydrogen bond (through its NH- group) with the CO- group of the proline (or any amino acid in the X- position) in the neighboring α-chains. Procollagen are secreted to the ECM, the loose pro alpha chains at the end of the procollagen are removed by procollagen peptidases. In this step, procollagens are converted to new extracellular structures called tropocollagens, which are then converted by self-assembly into large fibril formations at the cell surface. The globular N- and C- propeptides attached to each end of tropo-collagen occupy a large space around the molecules preventing the self-assembly of the molecules. It has been shown that the cleavage of these regions is necessary for the self-assembly of the molecules into the fibrils with morphologies similar to the native collagen fibril. , as shown in figure 2.5

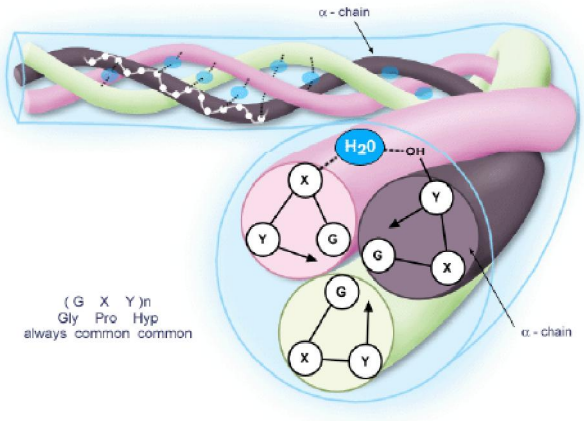


Figure 2.4 Structure of collagen triple helix [1]

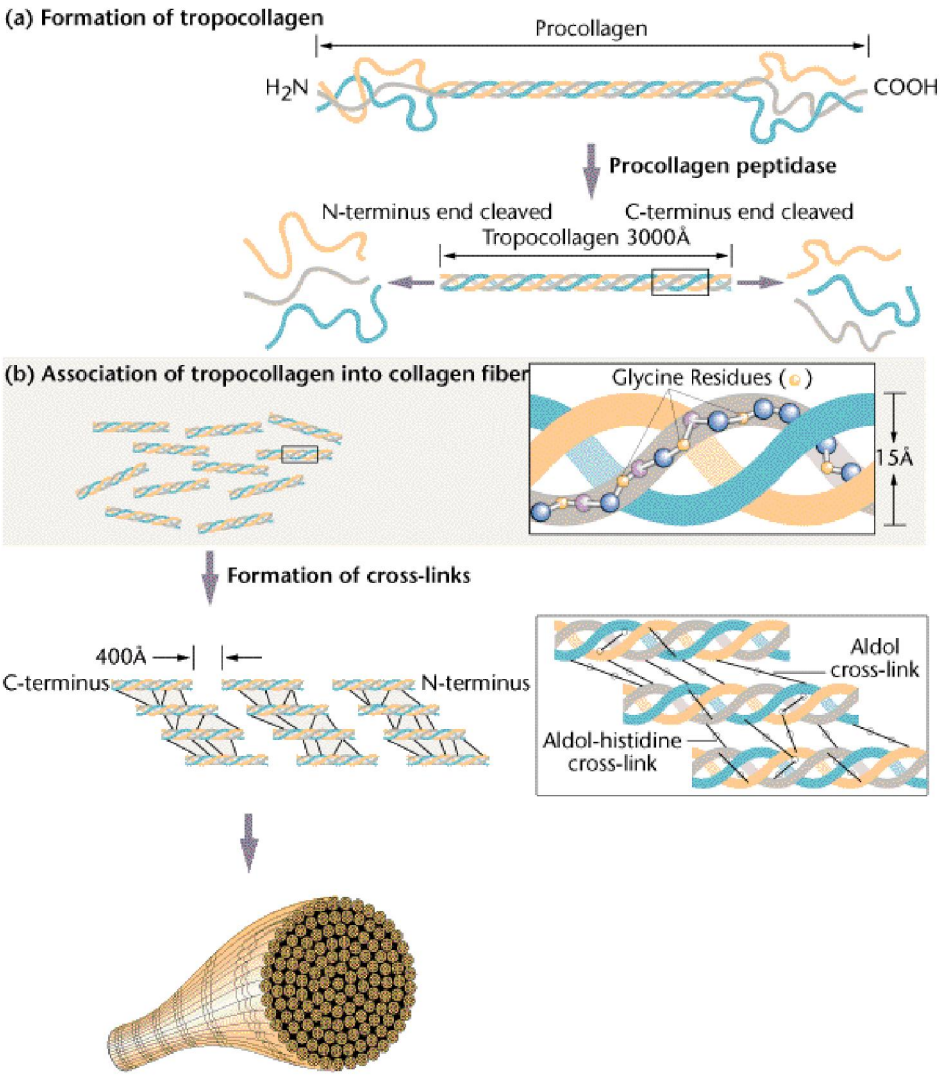


Figure2.5 Collagen molecules secretion and self-assembly into fibrils.

2.5.2 Preparation of fibrillar collagen based biomaterials

The collagen fibril uses for biomedical application. It lack of mechanical properties in aqueous solution, elevated temperature and enzymatic degradation. To improve mechanical properties of collagen fibril, the most commonly used cross-linking method such as physical and chemical crosslink. Various polymeric materials have used for mechanical support in addition to collagen because of its good performance mechanical properties. The chemical was cross-link with glutaraldehyde and formaldehyde. They have cytotoxiccity and increased immune response and stiffness of the tissue. This research has tried chemical cross linking with low toxic chemical substance. The interested chemical cross-linking substance for collagen was the materials 1-Ethyl-3-(3-dimethyl-aminopropyl-1-carbodiimide) (EDC) and N-hydroxysuccinimide (NHS). They were mild reagent for cross-linking and therefore, denatured of proteins is not expected to appear. The EDC/NHS chemical crosslink induced the formation of amide bond by activation of the side chain carboxylic acid group afterwards occurred aminolysis of the O-isoacylurea intermediates by the amino groups, which presents in the formation of interhelical cross links. The EDC/NHS chemical crosslink induced collagen molecules by the formation of iso-peptides without getting itself incorporated into the macromolecule. This process could crosslink collagen and silk film surface treated with EDC/NHS. Moreover, the by-product of the cross-linking reaction is urea which can be easily removed during routine rinsing of the matrices [34].

The random assembly of collagen had control methods to influence organization of self-assembling collagen fibrils have made in controlling collagen organization ex. pH, temperature, electrical gradients, stress mechanic and concentration.

2.5.3 Self-assembly of fibrillar Collagen

In human body, collagen peptide chains are synthesised intracellularly. They generate an assembly in the loose triple helix called procollagen, before being delivered to the ECM by secretion. Because of their loose structure, with few crosslinks in the triple helix, collagens synthesized in the intracellular matrix are able to dissolve. Nevertheless, after they are secreted to the ECM, the loose of alpha chains at the end of the procollagen are removed by procollagen peptidases. In this step, procollagens are converted to new extracellular structures called tropocollagens, which are then converted by self-assembly into large fibril formations at the cell surface.

The classical fibril-forming collagens are characterized by their ability to assemble into highly oriented supramolecular aggregates with a characteristic suprastructure. The fibril formation is depending on ionic strength, concentration, pH, and temperature. Because this unique structure is suitable for utilization in biomedical applications and its fibrous nature has an influence on its potential usefulness, much research has been conducted into the self-assembly and fibrillation of collagen [14]. There are three steps in the self-assembly of collagen. The first is a nucleation step, where the molecules of procollagen begin to form seeding points. The second is a fibrillation step, where other microfibrils attach themselves to the seeding point and organize themselves into fibrils. Finally, fibrillation reaches a steady state and growth of collagen fibrils is more rapid. All three steps are shown in Figure2.6.

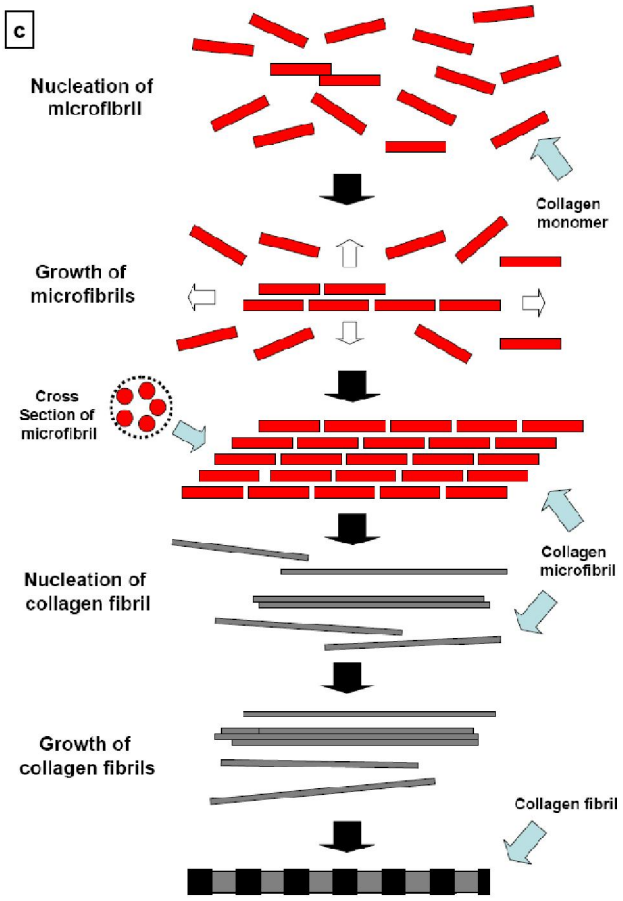


Figure2.6 Model of self-assembly of collagen fibril. [14]

2.5.4 Fibrillar collagen scaffold

The advantages for using of collagen in biomedical application is that collagen can form fibers with stability through it's self-assembly and cross-linking with substrate by physical and chemical crosslink. The extraction and purification collagen source can be produced procollagen. procollagen can be polymerized of collagen functional scaffold for tissue engineering. This step marked the initiation of procollagen self-assembly into 10-300 nm sized fibrils and aggregated of fibril into 0.5-3 μm sized of collagen fiber. The technique can be presented to various crosslink methods and protocols which are applicable to a variety of tissue sources and species of origin.

2.6 Biomimetic Approaches and mimicked reself-assembly of fibrillar collagen

Biomimetic strategy was the attractive approach for biomedical applications. Almost all natural materials have a sophisticated hierarchical structure arising from the self-assembly of biomolecules and producing high performance functionalities. A biomimetic biomaterial for tissue engineering produced scaffolds similar to natural extracellular matrix. The molecular organization of biological structures also supported their mechanical properties. In addition, certain of these structures can self-heal, self-repair, and self-replicate.

The collagen fibrils in animal tissue have properties making them suitable for both biological and mechanical functions. This is the motivation for creating some unique structures and functionalities via self-assembly for use as scaffolds. Understanding the mechanism of collagen self-assembly in these conditions is an attractive choice to create materials with potential for use as scaffolds. This constitutes the biomimetic strategy, which is the utilization of a biological approach to design in engineering and advanced technology. In principle, it is used in tissue engineering to develop artificial extracellular matrices [ECM] as scaffold materials for tissue engineering. The most significant requirement of biomimetic scaffold materials is that their structure and functionality are suitable for cell recognition.

This strategy is a good way to preserve biological functionalities and generate structures closely resembling natural ECM. In tissue engineering, this strategy is used to create high-performance scaffolds, especially for use in skin tissue engineering. The use of a biomimetic strategy in tissue engineering is illustrated in many soft tissue engineering researchers have also studied novel polymers which can be self-assembly to form a structure resembling collagen fibrils ECM.

2.7 Hybridization for scaffolds fabrication

The correct approach to fabrication is important to create an effective scaffold. Generally, the aim of scaffold fabrication is produce good biological functionality, structure and mechanical properties for effective tissue engineering. Many research studies have focused on improving their stability and mechanical properties by structural modification through cross linking, blending, the use of copolymers. In some cases for synthetic polymers, it is necessary to supplement biological functionality by adding bioactive molecules; the RGD peptide sequence is the recognition group for cells in the ECM. For a good structure, generally, the scaffold functions as an artificial extracellular matrix, so it is necessary to create the same structure and biological functionality as the native ECM. The requirement for a suitable structure is that it should have the porous mesh or network structure that makes it suitable for cells to reside. As the literature [1] shows, many researchers have attempted to optimize and concentrate on the pore formation and the effect of the porous structure on cell culture. For good performance, one of the important roles of scaffold is in providing strength and flexibility to the tissue. Accordingly, the most important properties of the scaffold materials are strength and flexibility. Because scaffolds have to be used in aqueous solution, must also be stable. In this research, silk fibroin is used to improve mechanical properties of biomaterials and protected the biological functionality. Re-self assembly of type I collagen are coated for inducing cell recognition and excellent biocompatibility.

Hybrid Materials is a material which included two material mixed on the recognition early on that mixtures of materials can present higher properties compared with their pure materials. The successful samples were the group of composites which are formed by the incorporation of a basic structural material into a second substance. Most of the resulting materials show improved mechanical properties and biocompatibility.

Classification of Hybrid Materials

(1.) Structurally-Hybridized Materials (Composites).

(2.) Materials Hybridized in Chemical-Bond, and Physical bond.

(3.) Functionally-Hybridized Materials.

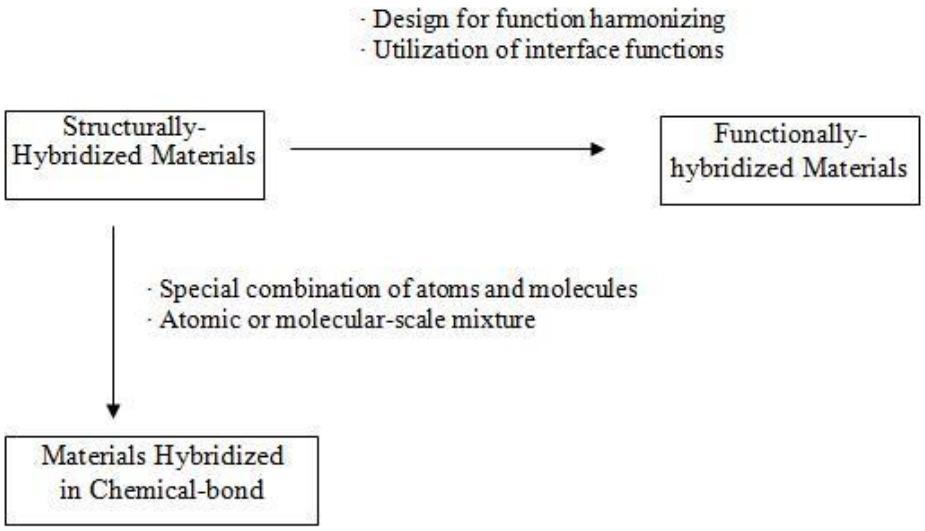


Figure2.7 Relationship between Structurally, Functionally and Material hybridized Materials. [16]

This study used Functionally-hybridized and Materials Hybridized in Chemical-Bond based on the rule of biomimetic (self assembly and immobilization process), our study used advantage of silk fibroin improved mechanical properties of collagen fibril that excellent biological function and cell recognition.

Chapter 3

Preparation and Characterization of Coated Silk Fibroin Films with Mimicked Re-self Assembly Type I collagen

3.1. Introduction

As described in literature reviews, biomimetic use to develop the scaffold for tissue engineering by combine natural polymer to improve functionality and synthetic scaffold to improve mechanical properties. It is necessary for scaffold to provide functional and mechanical properties; it can be used for various clinical complications such as wound healing scaffold and bone scaffold.

Tissue engineering has been investigated for over the past few decade, in this chapter using advantages from *Bombyx mori (B. mori)*. Silk fibroin had the advantages of material options for biomaterial because of high mechanical properties, environmental stability, biocompatibility, and biodegradability. Collagen for hypothesis improved functionality as cell proliferation and cell attachment. To accelerate healing time of Tissue regeneration, a wet environment can enhance the wound healing process. Collagen had potential to use for biomaterial since it has been a major component of extracellular matrix. Its biomaterial characteristics offer several advantages: there are biodegradation, biocompatibility. It can be fabricated the category of forms such as nanofiber, gel, and films. Collagen has been used for short-term application as it degraded in 6-8 weeks. It has severely limited by lacking of mechanical strength and susceptibility to enzymatic degradation in vivo. In this research silk fibroin is used to improve mechanical properties of biomaterials. Re-self assembly of type I collagen are coated for inducing cell recognition [56]. Preparation and characterization are strongly considered. To mimic re-self assembly condition is emphasized for collagen coating development a promised material as tissue engineering scaffolds is aim at this research.

3.2. Materials

3.2.1 Silk fibroin films formation

3.2.1.1. Preparation of silk fibroin films formation

Bombyx mori silkworm cocoons were collected from Silk Commemoration Centre, Naratiwas, South Thailand. *B. mori* silk cocoons cut into small pieces and boiled for 45 min in Sodium carbonate solution 0.02 M and rinsed thoroughly with water to remove sericin proteins. The degummed silk fibroin dried at 60 ˚C in the drying oven. The dried silk was dissolved in 9.3 M LiBr solution at 60°C in the drying oven for 4 hrs. The dissolved silk fibroin in LiBr was dialysed with cellulose dialysis membrane (Spectra/Por molecular weight cut-off 3,500) against distilled water 200 times for 72 hours, changing water every 6 h. to remove LiBr. After being dialyzed, silk fibroin solution was centrifuged at 3000 rpm for 20 min to remove scraps of silk fibroin. Calculated concentration of silk fibroin aqueous solution by Biuret protein assay, used spectrophotometer was adjusted the wavelength to 540 nm. The final concentration of silk fibroin aqueous solution was 8-11 %w/v. The concentration of silk fibroin solution was rechecked by weighing the remaining solid after drying divide by volume of silk solution. The concentrated silk fibroin solution was stored at 4 °C for further study. [23-34]

Figure 3.1 Method of preparation of silk fibroin films formation

A. Silk cocoons boiled for 45 min in Sodium carbonate solution 0.02 M to remove silk sericin protein.
B. Degummed silk fibroin dried in oven at 60 ˚C.
C. The final concentration of silk fibroin solution was 8-11 %w/v after being dialysed.

Dialyzed silk fibroin solutions rinsed to plastic plate concentration 14 mg/cm² and dried at room temperature 24 h. for forming silk fibroin film. Silk film was cut to small pieces 3*4 cm². The pieces of Silk fibroin films were fixed on rectangle plate for anti-deformation of silk film in treatment process which used much solution.

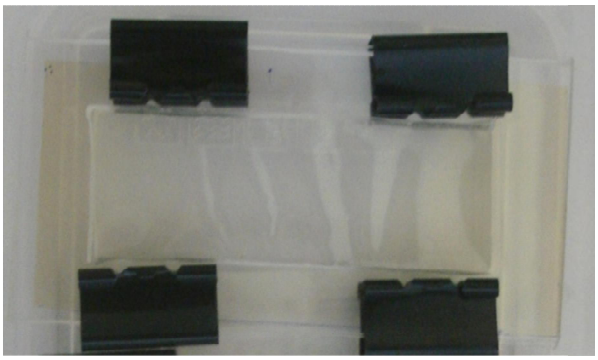


Figure 3.2 The rectangle plate fixed silk film for treatment process .

3.2.2. Preparation of collagen from shark skin

3.2.2.1. Extraction of collagen from shark skin

The collagen from shark skin was produced by acid soluble collagen (ASC) method. It was extracted according to the method of Kittiphattanabawon et al. (2005) and Nalinanon, Benjakul, Visessanguan,and Kishimura (2007) with a slight modification. All procedures were carried out at 4 ˚C [58, 59].

3.2.2.2. Shark (*Carcharodon carcharias*) skin preparation

Skin of shark (*Carcharodon carcharias*) that the average size 20–40 cm in length was supported from Blue Ocean Food Products Co., Ltd. in Samutsakhon Province of Thailand. The skin of shark kept in the freezer at -20 ˚C until use. The pieces of meat was removed from the shark's skin and washed with cool water until smell of ammonia disappeared. Cut the washed shark skin into small pieces (1.0*1.0 cm^2).

Figure 3.3 A. The frozen skin (-20 ˚C) was washed with cool water.
B. Removed remaining meat on shark skin.
C. Cut into small pieces (1.0*1.0 cm^2).

3.2.2.3. Pretreatment of shark skin

The prepared shark skin was mixed with 0.1 M NaOH from solid dissolved to alkali solution ratio of 1:10 (w/v). The blend was stirred continuously for 6 h at control temperature 4 ˚C. The NaOH alkali solution was changed every 2 h to remove non-collagenous proteins.Afterwards, the deproteinised skin was washed with cold water until the pH of wash water was reached neutral pH water.

Figure 3.4 A. Mixed with 0.1 M NaOH solution ratio of 1:10 (w/v).
B. Stirred for 6 h. The alkali solution was changed every 2 h.
C. Washed with cold water until a neutral.

3.2.2.4. Extraction of acid soluble collagen

The 0.5 M acetic acid solution soaked pretreated shark skin with a solid to solvent ratio of 1:15 (w/v) for 48 h at control temperature 4 ˚C with continuous stirring and filtered with two layers of cheesecloth. The filtrate collagen was precipitated by adding NaCl to final concentration of 2.6 M in the presence and adding 0.05 M tris-(hydroxymethyl) aminomethane at pH7.5 in real time measurement. The precipitate collagen was collected by centrifugation at 20,000 g and control temperature at 4 ˚C for 60 min. Afterward the centrifuge pellet was dissolved in 0.5 M acetic acid to recover pure collagen solution, dialysed against 25 volumes of 0.1 M acetic acid for 12 h and control temperature at 4 ˚C. Thereafter, it was dialysed against 25 volumes of distilled water for 48 h control temperature at 4 ˚C. The dialysate collagen was freeze dried to produce mesh collagen [58, 59].

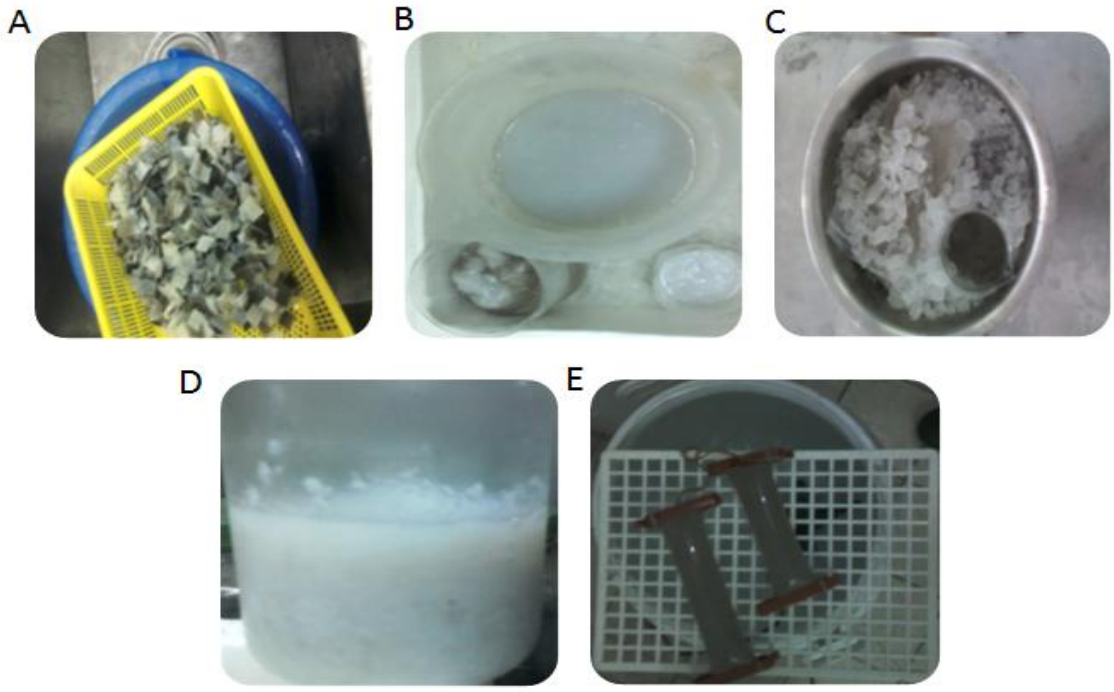


Figure 3.5 A. The 0.5 M acetic acid solution soaked pretreated shark skin.
B. Adding NaCl to a final concentration of 2.6 M.
C. Centrifugation at 20,000 g at 4 °C for 60 mins.
D. The pellet was dissolved 0.5 M acetic acid to recover solution.
E. Dialysed against 25 volumes of 0.1 M acetic acid for 12 h and distilled water for 48 h.

3.3 Methods

3.3.1 Surface modification of Silk fibroin films

The silk fibroin films were fixed on rectangle plate. The silk fibroin films were soaked in 60 % methanol and 80% methanol 20 min to induce beta-sheet in silk films structure. The treated silk fibroin films were hydrated in PBS buffer solution pH 7.4 for 30 min. To activate the hydrated silk fibroin films by reaction with 0.5 mg/ml of 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC/HCl) and 0.7 mg/ml N -hydroxysuccinimide (NHS) solution in PBS buffer solution for 15 min at control temperature 4 ˚C. This process created the stable of amine reactive esters on the water-unsolvable silk surface. The EDC/NHS chemical crosslink induced the formation of amide bond by activation of the side chain carboxylic acid group of glutamic acid residues, afterwards followed the reaction aminolysis of the O-acylosourea intermediates by the amino groups of collagen. The process of treated silk was moist and swelling of silk film. Plastic plate instrumental fixed silk film to recover smooth surface by air cover machine.

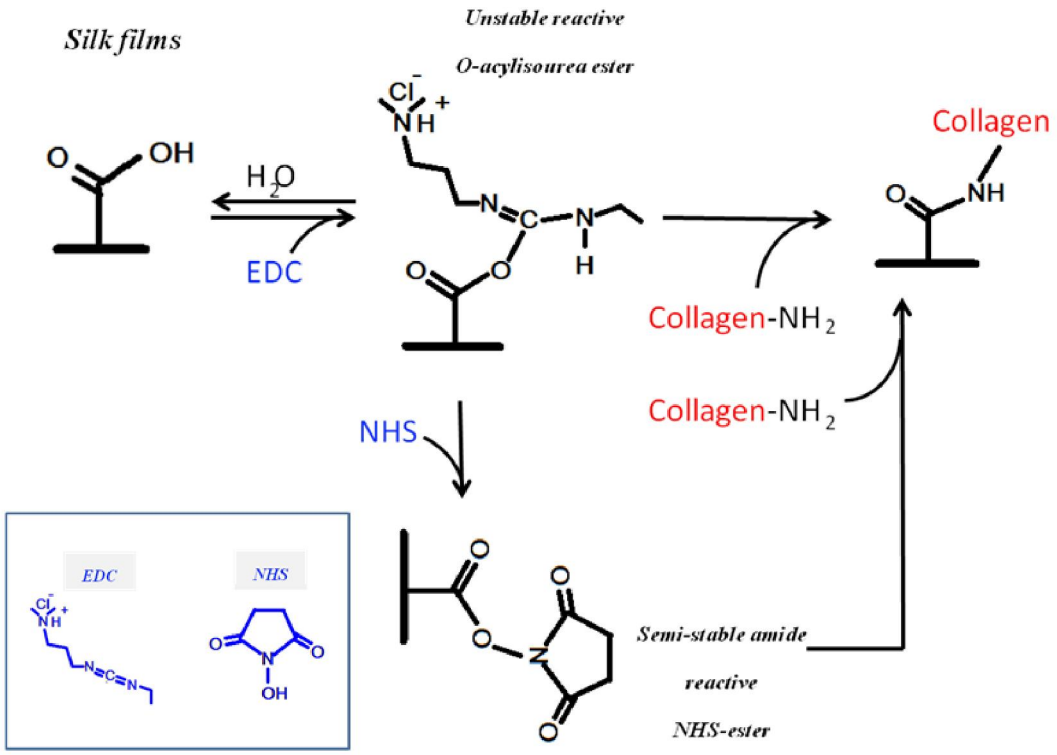


Figure 3.6 Reaction scheme for the surface modification of silk fibroin films by the collagen solution.

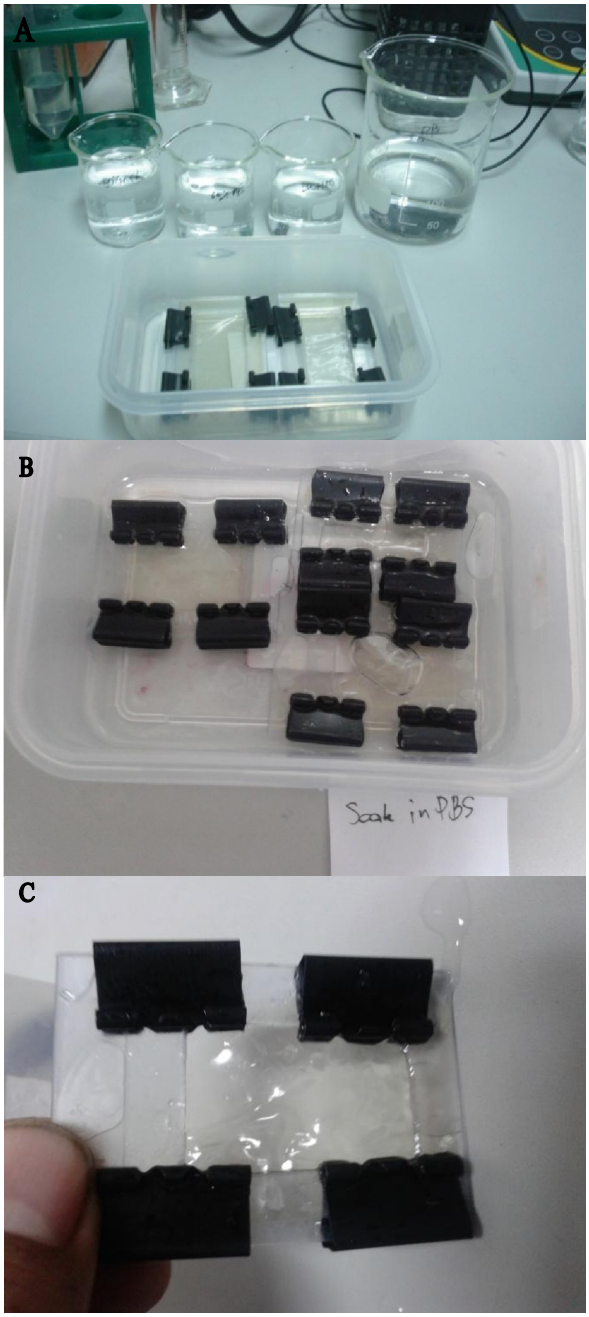


Figure 3.7 A. The surface modification of Silk Fibroin Films used 60% methanol, 80% methanol and EDC/NHS solution.

B. Silk films modified surface by PBS solution.

C. Rectangle plate fixed silk films in process surface modification.

3.3.2 Coating of mimicked re-self assembly collagen on silk films.

The activated of silk fibroin films dipped with PBS buffer pH 7.4 solutions at 4°C temperature to get the surface-modified. The collagen solution 4 mg/ml diluted with 0.01 M acetic to concentration [0.25, 0.5, 1.0, 2.0] mg/ml, followed by dipping the surface-modified silk films in collagen solution for 60 sec. At the completion of peptide coupling, the coated surface silk fibroin films dried in the air cover machine over night. The plastic plate instrumental protected silk film to turn smooth surface.

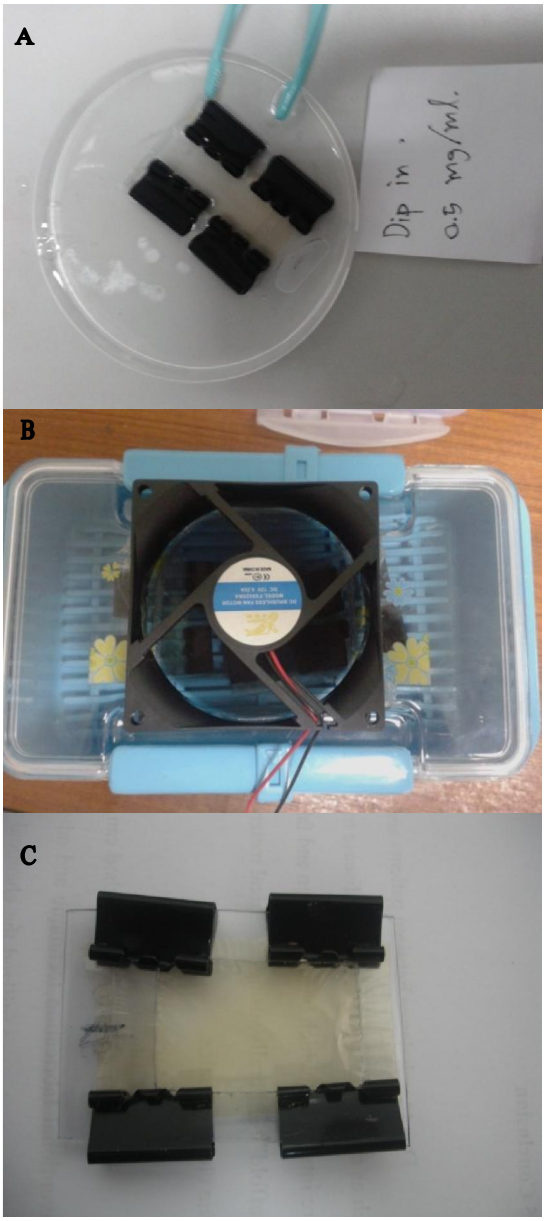


Figure 3.8 A. Dipping the surface-modified silk films in collagen solution.

B. Air cover machine run overnight.

C. The plastic plate instrumental protected silk film to turn smooth surface.

3.3.3 Analysis

(1.) Tensile properties

Tensile properties of silk films without collagen and silk films coated with collagen was performed by a Universal testing machine (Lloyd instruments, LRX-plus, AMETEK Lioyd,instrument Ltd,Hamphshire,UK). The specimen of Silk coated collagen films are average sizes at 5 mm (width) *50mm (length). Specimen was prepared as following standard ASTM D882. Experiment controlled temperature, elongation rate 50 mm/min, Gauge length 100 mm, and testing 5 samples.

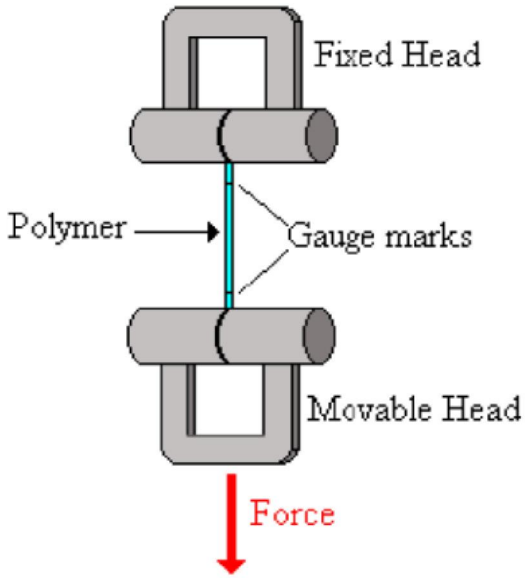


Figure 3.9 An illustration of a set-up of mechanical testing in tensile mode [68]

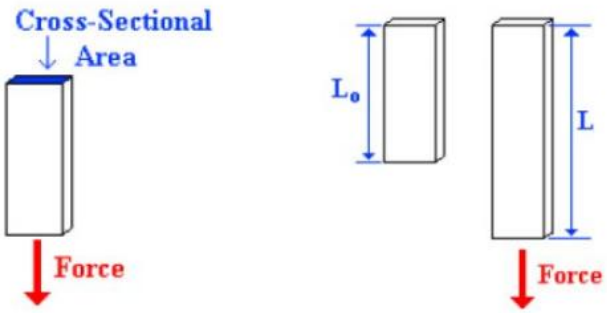


Figure 3.10 An illustration of sample deformation during tension [69].

Stress is calculated as force per unit area:

Stress (σ) = Force (F)/ Cross-sectional area (A0)

Strain is calculated as the change in the length of the sample:

Extensional strain = L/L0

Young's modulus can be calculated by dividing the tensile stress by the extensional strain in the elastic.

(2.) Fourier Transform Infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a popular method for characterizing chemical functional groups in molecular structures, as each group generally has a vibration signature indicating its molecular structure. There are different modes of vibration of chemical functional groups. When IR spectra are added in a sample, some modes of vibration can absorb the IR wave or energy at different wavelengths. From this principle, we can identify the type of functional group and relate this to the structure of the molecule.

For organic materials, there are many different absorption wave-numbers of chemical functional groups, as Fig. 34 shows. In practice, not only the wave-number of the absorbance but also the transmittance is measured, as shown in Figure 3.11

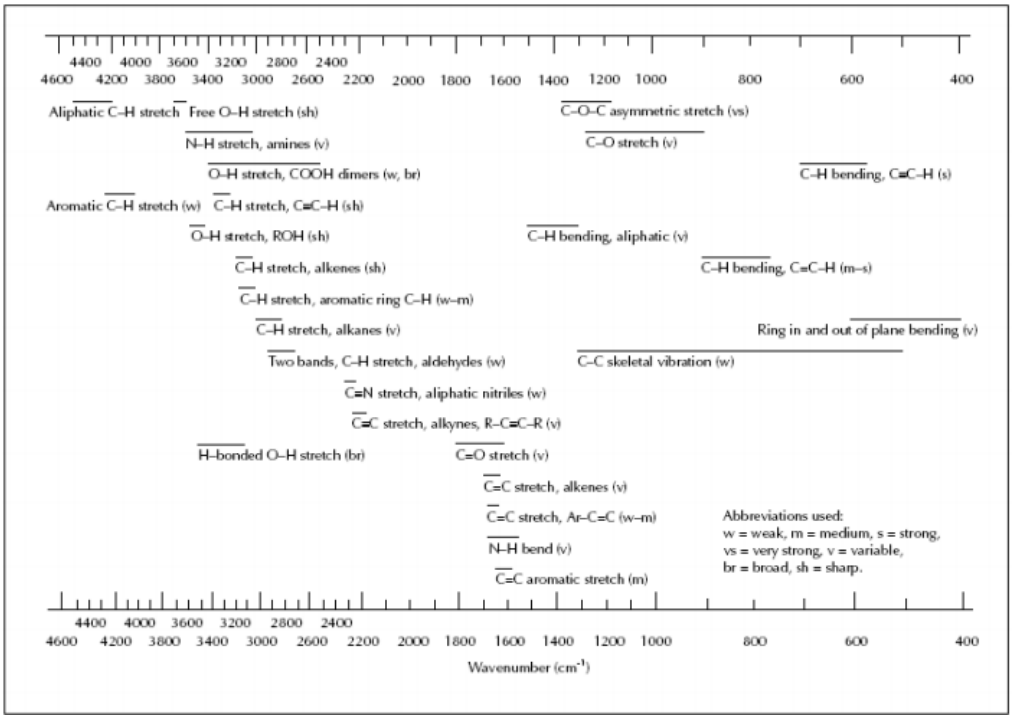


Figure 3.11 Wave's number of absorption bands of chemical functional group vibrations in different modes [70].

FTIR analysis of silk films without collagen and silk films coated with collagen was performed with a EQUI-NOX55 (Bruker, Germany). The wave numbers ranged 400 – 4000 cm^{-1}. The FTIR spectrum of silk films was prepared by powder KBr disc.

(3.) Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) is a popular method used to characterize the thermal behavior of materials, especially polymeric ones, for which DSC can be used to characterize factors including the crystallinity, ordering state, glass transition temperature (Tg) and melting temperature (Tm) of the polymer

The dried silk films without collagen and silk films coated with collagen heated in a DSC7 (Perkin Elmer, USA) with purged dry nitrogen gas flow (50 ml/min). Heated from 20-500 °C Standard mode DSC measurements were performed at a heating rate of 10°C /min.

(4.) Contact angles

The contact angle is the angle, which measured through the liquid interface meets a solid surface. It was the method for measurement outline of water drop and measurement the two dimensional angle contact between solid surface and water drop outline. The hydrophobic surface shown contact angle over 90°. This condition was explained by poor wetting, poor water adhesion the solid surface free energy is low. The hydrophilic surface shown contact angle lower 90°. This condition explained better wetting, better adhesiveness, and higher surface energy.

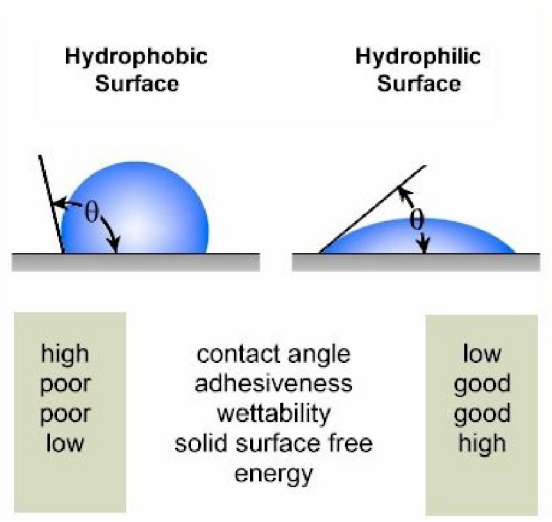


Figure 3.12 Drop technique contact angle measurements [71].

The hydrophobic-hydrophilic properties of silk films without collagen and silk films coated with collagen were determined with sessile drop technique contact angle measurements (Dataphysics, Model OCA15EC, Germany). The water droplet dropped on the sample surface 5 testing.

(5.) Atomic force microscope (AFM)

Atomic force microscope (AFM) is a high-performance instrument used to observe the topology of a sample and some properties at the nano-scale. They are utilized to examine some nano-scale properties, especially for the surface characterization of materials, self-assembly, phase separation. The principle is that the force between the tip and surface of a sample is considered. When an atom at the tip moves towards the surface, the force between them increases, because each generates an attractive force which acts upon the other. To use the AFM, the tip is moved towards the surface of the sample until it reaches this stable state, where it is then held while being scanned across the whole surface of the sample. The changing distance between tip and surface is detected to construct 2D and 3D images, as Figure 3.13 shows.

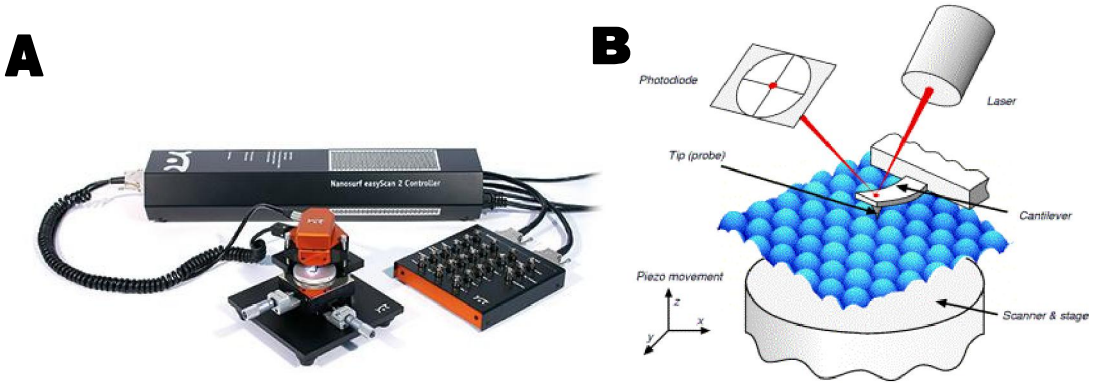


Figure 3.13 A. Nanosurf easyscan 2 was used to image surface of collagen fibril on silk film. [72]

B. The structure operation of AFM [73].

There are two popular modes of operation which are used to characterise the surface of samples: contact and tapping mode. The first, contact mode scan used the end of tip to mechanical contact with the sample and feedback force measured. The second, tapping mode used the cantilever tip to stimulate vibration near the resonant frequency. The vibration amplitude of the cantilever varied from interaction force with the surface shifts the resonant frequency. In our research, tapping mode was used to examine the surface of samples, which were prepared for AFM characterization by Nanosurf easyscan 2.

(6.) Scanning electron microscopy (SEM)

The configuration of a scanning electron microscope (SEM) is shown in Figure 3.14. The electron source is generally a lanthanum boride or tungsten hairpin filament heated with a low voltage. The potential of the filament is negative and the electrons generated are accelerated to an anode. The SEM operates under high vacuum. The electron beam generated by the source approaches a condenser lens before scanning over the surface of the sample under the control of a scanning coil.

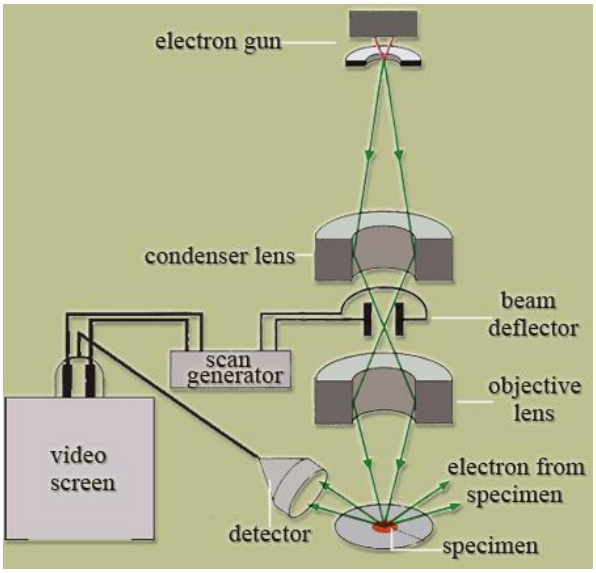


Figure 3.14 The structure of SEM [74].

The principle of the SEM is that an electron beam approaches and strikes the surface of the sample, causing secondary electrons, backscattered electrons, Auger electrons and X-ray radiation to be generated. The different types of electrons or radiation emitted by the sample as the incident beam strikes its surface are shown in Figure3.15.

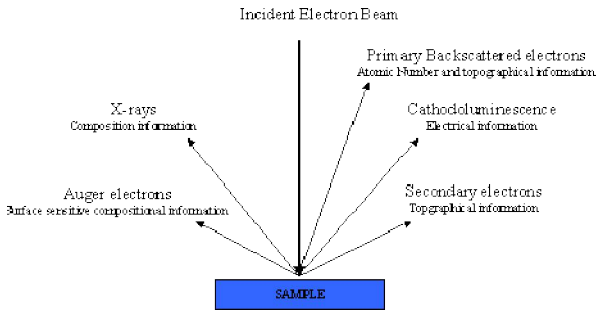


Figure 3.15 Electrons generated by the sample [75].

Both secondary and backscattered electrons are mostly used to form an image. First, a sample is prepared for SEM, and then an image is constructed by secondary or backscattered electrons from the sample surface. When the penetration of incident electrons is considered, the different regions of penetration generate different types of electron. Secondary and backscattered electrons are first generated on the surface. In the present study, SEM was used to observe the topology and morphology of dried membranes and swollen membrane which was dried before being observed. Furthermore, it was used to study the morphology of cells in membranes.

The morphological characteristics of the silk films without collagen and silk films coated with collagen produced by scanning electron microscopy Quanta400, FEI (Czech Republic). The samples size 10×10 mm2 coated with gold sputter coating technique. The study was operated at either 5-20 kV, described scale bars was 5 μm.

3.4 Results

3.4.1. Tensile properties characterization

The specimen of Silk /collagen films cut into sizes 5 mm (width) *50 mm (length). Specimen was prepared as following standard ASTM D882.Experimaent controlled temperature, elongation rate= 50 mm/min , Gauge lenght =100 mm. Mechanical properties of the Silk coated with collagen films are such as maximum load, stiffness and young's modulus.

Figure 3.16 Universal testing machine (Lloyd instruments, LRX-plus, AMETEKLioyd,instrument Ltd,Hamphshire,UK) from Faculty of Dentistry, Prince of Songkla University.

Table 3.1 Mechanical properties of native silk films and silk films coated collagen

Silk film	Maximum Load (N)	Stress (MPa)	% Strain	Stiffness (N/mm)	Young's Modulus (MPa)
Silk/col [0]	14.48±1.92	4.33±0.29	3.68±0.97	29.03±3.64	255.72±20.85
Silk/col [0.25]	13.26±1.29	3.79±0.37	3.39±0.87	21.66±1.29	185.65±11.09
Silk/col [0.5]	13.18±1.31	3.12±0.95	4.10±0.91	17.25±3.81	191.77±15.86
Silk/col [1.0]	17.42±1.33	5.24±0.98	3.78±0.65	24.38±3.00	263.92±29.91
Silk/col [2.0]	19.74±1.15	6.88±1.08	6.70±1.38	32.32±1.59	340.71±30.93

Note: Silk/col[x], x is mean collagen coated concentration on silk films (unit = x mg/ml)

Table 3.1 has shown the mechanical properties of silk without collagen films and silk coated with collagen films. The maximum load of silk without collagen films was 14.48 N. It increased to 19.47 N when coated with collagen 2 mg/ml. As the result, Young's modulus of silk without collagen films is 255.72 MPa. Significantly, Young's modulus of Silk/col[1.0] and Silk/col [2.0] increased to 263.92 and 340.71 MPa, Furthermore, the Silk/col[2.0] has a higher elongation than silk without collagen films about 55%. These results may explain the effect of coated collagen on the tensile properties of silk films which silk coated collagen films can easily alter the silk films structure leading to large changes in the stress–strain and the elongation profiles of silk films. The results are consistent with the perception that experiments to develop coated collagen 1-2 mg/ml in silk films products, their mechanical properties are superior to silk films without collagen.

3.4.2. FTIR spectra characterization

Silk films has three conformations (random coil, α-helix and β-sheet) depend on preparation conditions. Each conformation has its own characteristic absorption bands on FTIR spectrum. The FTIR spectrum of silk films powder KBr disc is presented in Figure 3.9, The spectrum of silk fibroin films showed strongly absorption band at Amide I 1645 cm^{-1} The Amide I region mainly comes from the C=O stretching vibration, that was the characteristic band of β-sheet conformations (1600-1650 cm^{-1}). The amide II 1543 cm^{-1}, resulting from N–H bending vibration coupled with C-N stretching vibration. The amide III 1240 cm^{-1} bands represented N–H bending vibrations and C–H stretching. The absorption band of 700 cm^{-1}(amide IV) represented amide group sericin. In this research, such a peak silk is disappearing because sericin was removed during silk fibroin films preparation.

As FTIR characterization of silk coated with collagen films fig.1, (B, C, D, E) shown FTIR spectra both of silk and collagen. The amide a band was found at a wave number of 3400-3440 cm^{-1}. According to previous literature, a free N-H stretching vibration occurs in the range of 3400–3440 cm^{-1}. The result indicated that the absorption band of N-H groups of collagen shift to low wave number when the concentration increases. As the result, N-H groups at high concentration of collagen were involved in hydrogen bonding, probably with a carbonyl group of the peptide chain. The amide B band positions of ASC of collagens from silk coated with collagen were found wave numbers at 2920-2930 cm^{-1}, respectively representing the asymmetrical stretch of CH_2. The amide I band of collagen from silk coated with collagen films found wave numbers at 1640-1660 cm^{-1}, respectively. This band is associated with C=O stretching vibration or hydrogen bond coupled with COO-. The amide II band of collagen from silk coated with collagen films situated at a wave number at 1540- 1545 cm^{-1}, resulting from N-H bending vibration coupled with CN stretching vibration. The amide III band of collagen from silk coated with collagen films located wave numbers at 1238-1250 cm^{-1}, respectively. The amide III bands represented N-H bending vibrations and C–H stretching. In agreement with reported by Phanat Kittiphattanabawon et.al 2010.Amide I, II, III were found in silk films 100% together. A free N-H stretching of silk 100% vibration 3443 cm^{-1} and when the N-H group of a peptide involved in a hydrogen bond, the position was shifted to lower frequencies in the range of 3400–3440cm^{-1}[58,59]. The result indicated that the N-H groups of this collagen were involved in hydrogen bonding, probably with a carbonyl group of the silk treated with EDC/NHS peptide chain.

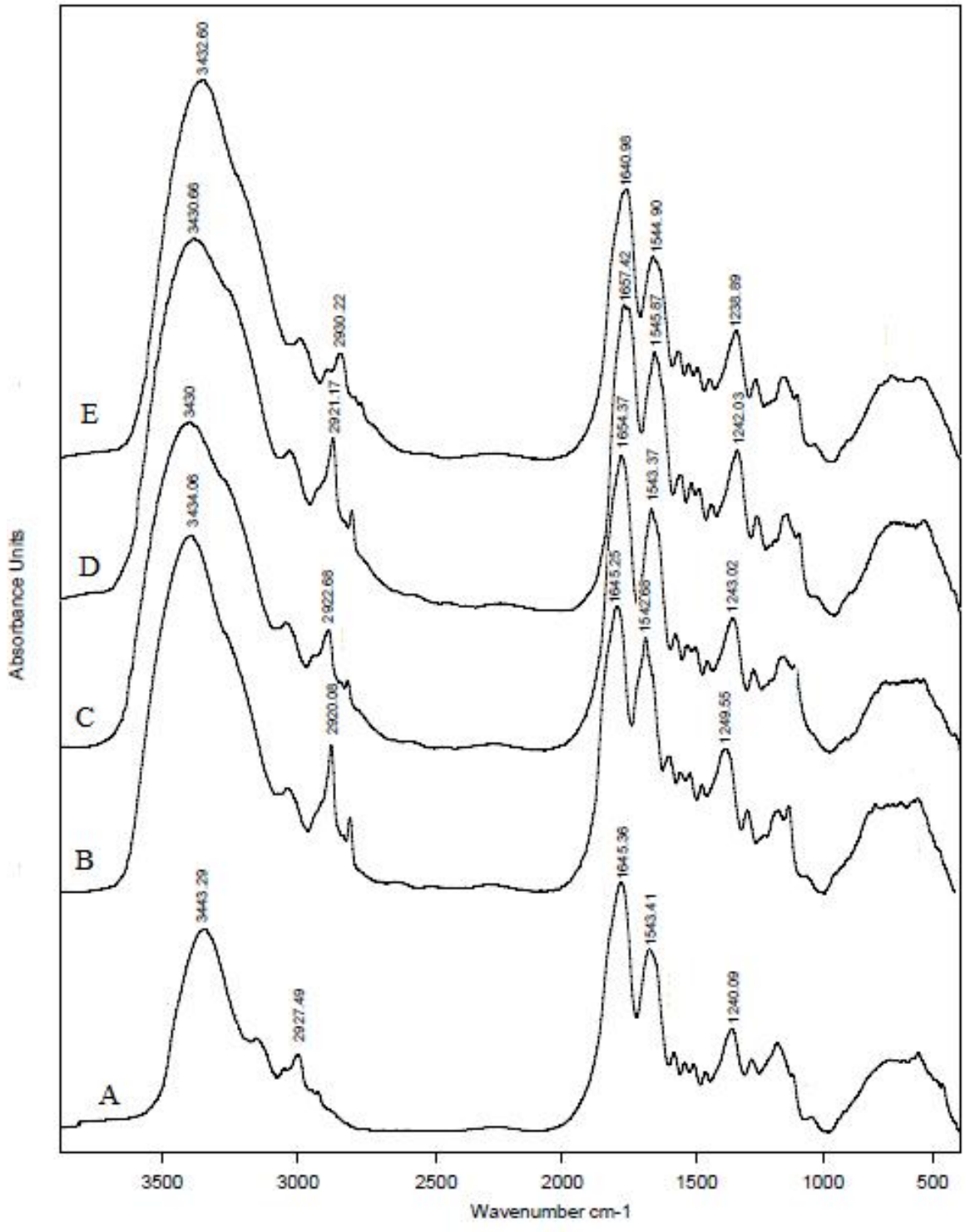


Figure 3.17 Showed FTIR of silk 100% (A), Silk/col[0.25] (B), Silk/col[0.5] (C), Silk/col[1] (D) and Silk/col[2] (E)

3.4.3. DSC Characterization

DSC characterization of silk without collagen films and silk coated with collagen films has shown in Fig.2, The silk without collagen films shown bound water peak at 103°C. All of silk coated with collagen films shown bound water peaks from 80 °C to 88°C. The bound water peak shifted to lower temperature with treatment with coated collagen when silk films treated with collagen increasing concentration content, the bound water peak shifted to slightly higher temperature. After bound water removal, an endothermal peak appeared in 330°C (silk without collagen films), 286-290°C (silk coated with collagen films). The endothermal peak of silk coated with collagen shifted to a lower temperature, can be assigned to the thermal transition/crystallization of the silk-transition decreased when treatment with collagen. The endothermal peak shifted to slightly higher temperature with increasing collagen concentration content (286 to 290°C). All silk coated with collagen films had a lower degradation temperature than silk without collagen films (330°C), which indicated that the collagen decreases the thermal stability of silk protein after coating.

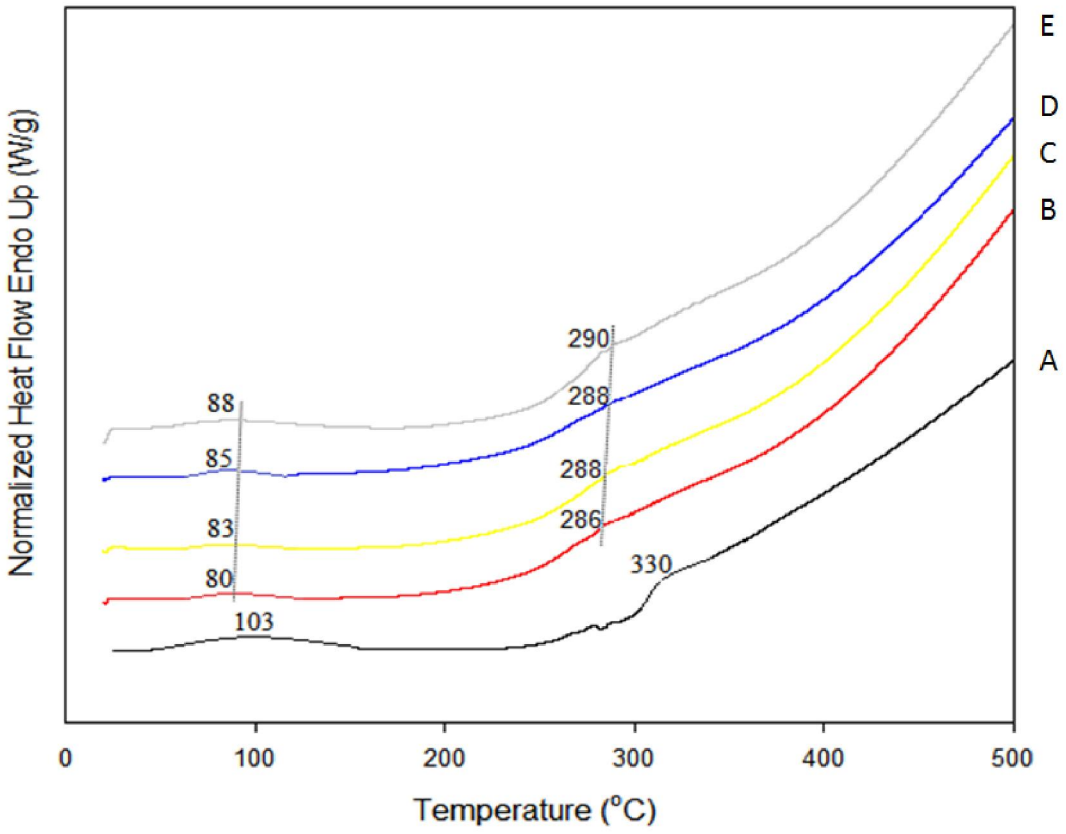


Figure 3.18 Showed DSC of 1000x, 5000x, 10000x of silk 100%(A), Silk/col[0.25] (B), Silk/col[0.5] (C), Silk/col[1] (D) and Silk/col[2] (E)

3.4.4. Contact angles characterization

The hydrophobic-hydrophilic properties of silk and collagen in films were examined by water contact angle (WCA) testing as a function of time. The hydrophobic solid surface shown contact angle over 90° and the hydrophilic surface shown contact angle lower 90° (Table II.). This measurement was reported that the addition of coated collagen causes the initial WCA to increase about 30°.It changed surface property from hydrophilic to hydrophobic. The facts indicated that the hydrogen bonding formation lead the cross-linking of collagen and silk, in turn the hydrophobic of the films. This observation could indicate the N-H group of collagen binding reduced higher polarity of the carboxyl side group in the glutamic acids of silk chains, as silk coated. [36]

Table 3.2 Contact angles of silk without collagen films and silk coated with collagen films

Sample	**CA (M)**
Silk 100%	56.56±3.87
Silk 100% treated Methanol	73.73±5.37
Silk/col[0.25]	103.27±5.91
Silk/col[0.5]	104.27±4.20
Silk/col[1.0]	106.55±6.18
Silk/col[2.0]	103.95±2.03

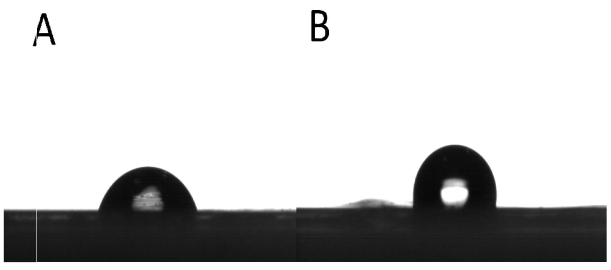


Figure 3.19 A. showed silk 100 % films are hydrophilic properties.
B. showed silk coated with collagen 2 mg/ml is hydrophobic properties.

3.4.5. Atomic Force Microscopy

AFM images of the silk native films and silk coated with collagen. The immobilized collagen fibril has in homogeneities on the surface. It extended up to 10-30 nm above the surface. The silk coated with collagen has a significantly different appearance than the silk native films. By AFM, the thin films of silk native films appear smooth surface and homogeneous. A mesh of collagen fibrils and significant topographical variations can be seen. We can observe appearance fibrils in the silk films coated with collagen.

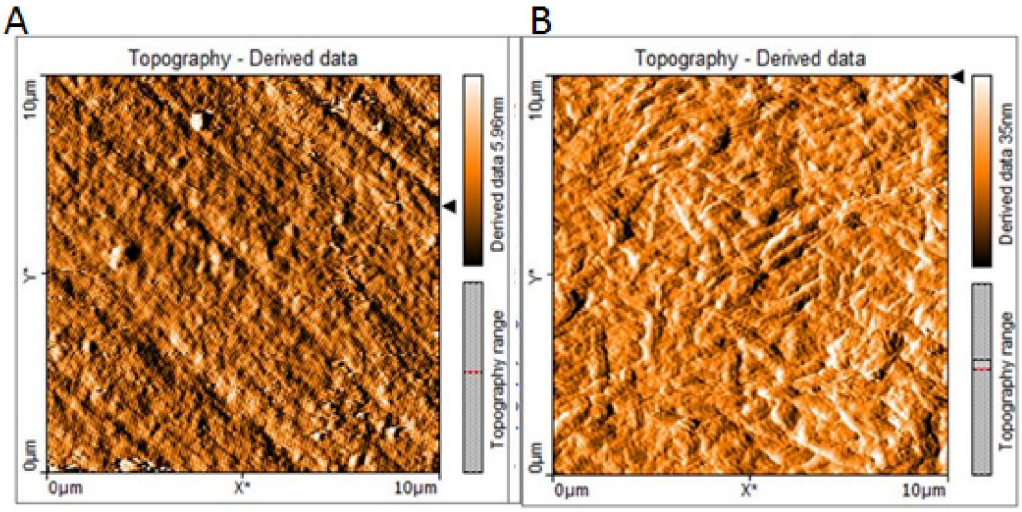


Figure 3.20 AFM images 10*10µm^2 of (A) uncoated silk films, (B) silk coated with collagen 2 mg/ml.

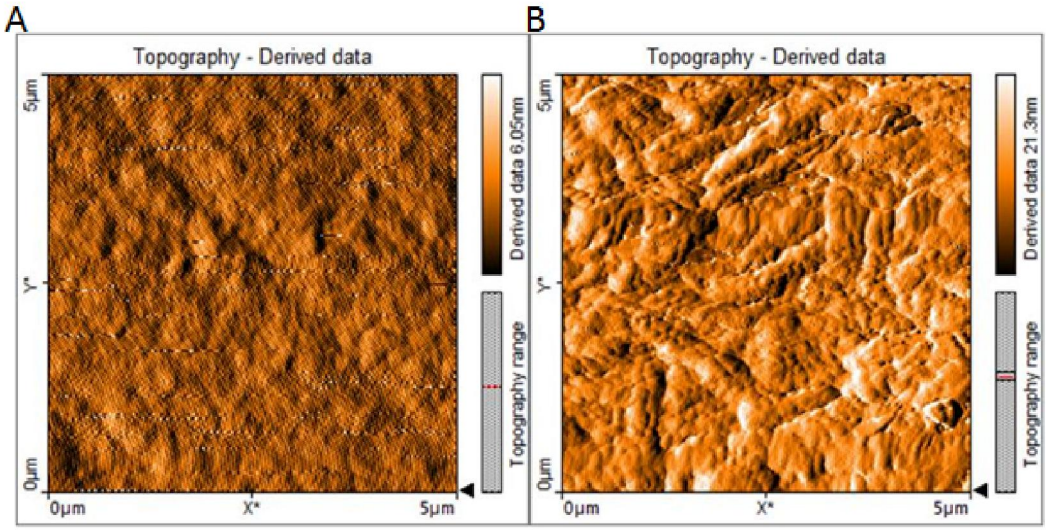
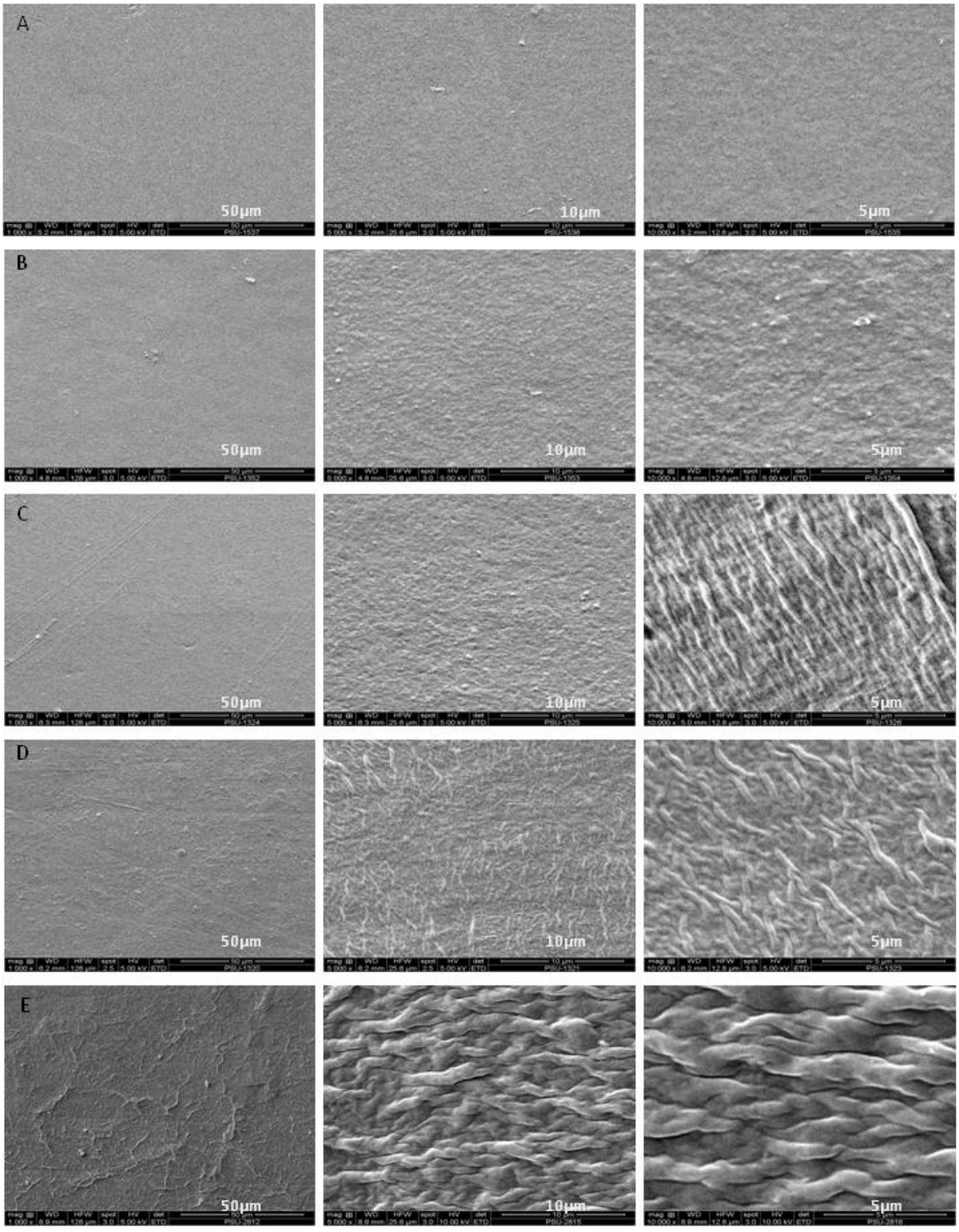


Figure 3.21 AFM images 5*5µm^2 of (A) uncoated silk films, (B) silk coated with collagen 2 mg/ml.

3.4.6. Scanning electron microscopy (SEM)

As can be seen in Fig. 9 that immobilized collagen fibril has in homogeneities on the nanostructure that extend up the surface. The silk coated with collagen has a significantly different appearance than the silk native films the fibrous texture is observed showed the dense nanofibrils films structure. The silk100% sample showed no fibrillar appearance. Fig.9A shows the films showed a homogeneous surface. Therefore, different morphologies observed under SEM for silk coated with collagen obtained under various conditions concentration are expected. Silk/col[0.25] showed a globule structure. It was low collagen concentration to fibril formation. In Silk/col[0.5], the collagen molecules are parallel oriented and loose packed on silk films. In silk/col[1] and silk/col [2],the collagen fibril are highly oriented and packed on silk films. Silk/col[1] is smaller than collagen fibril of Silk/col[2]. With an increase in the collagen concentration, collagen in became the size of the fibrils.



1,000x (scale bar 50µm)	5,000x (scale bar 10µm)	10,000x (scale bar 5µm)

Figure 3.22 SEM images 1000x, 5000x, 10000x of silk 100% (A), Silk/col[0.25] (B), Silk/col[0.5] (C), Silk/col[1] (D) and Silk/col[2] (E) .

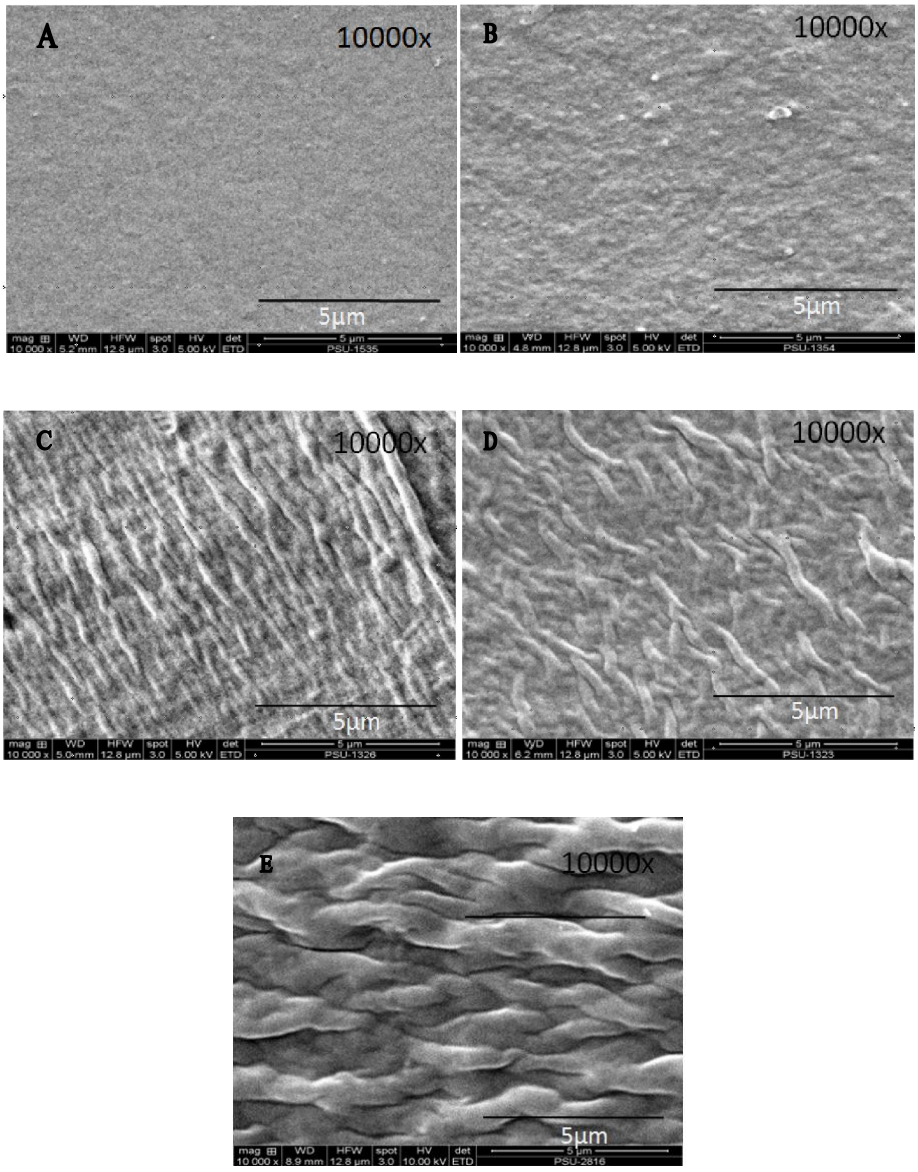


Figure 3.23 SEM 10000x shown silk surface adjusted collagen solution from 0.25-2.0 mg/ml silk 100%(A), Silk/col[0.25] (B), Silk/col[0.5] (C), Silk/col[1] (D) and Silk/col[2] (E) showed surface change when increase in the collagen concentration, collagen in became the size of the fibrils.

3.5 Conclusions

In this study, the fibrils assembly of collagen type I affects on the morphology of silk films. Collagen type I fibrils has been shown to mimetic reself-assembly on silk fibroin films. From the result of SEM, Silk films coated with collagen 1-2 mg/ml shown collagen fibril formation. The collagen fibrils are parallel oriented and packed on silk films. When increased in the collagen concentration content, fibril structure of collagen became increase size and density of the structure. This finding provides the motivation to explain and create a novel extracellular matrix having an attractive structure for cell recognition. Furthermore, it could probably be helpful for explaining the mechanisms of other fibrillation processes in the human body.

Tensile properties may explain the effect of coated collagen on the tensile properties of silk films which silk coated collagen films can easily alter the silk films structure leading to large changes in the stress–strain and the elongation profiles of silk films. The results are consistent with the perception that experiments to develop coated collagen 1-2 mg/ml in silk films products, their mechanical properties are superior to silk films without collagen.

From the result of FTIR spectroscopy analysis, has showed both the spectra of silk and collagen are assigned to β-sheet conformation of silk fibroin and collagen type I. All silk coated with collagen films had a lower degradation temperature than silk without collagen films, which indicated that the collagen decreases the thermal stability of silk protein after coating. It can be assigned to the thermal transition/crystallization of the silk-transition decreased when treatment with collagen.

The hydrophobic-hydrophilic properties of silk and collagen in films indicated the surface changing. Re-self assembly of Type I collagen changed surface property from hydrophilic to hydrophobic. The facts indicated that the hydrogen bonding formation lead the cross-linking of collagen and silk, in turn the hydrophobic of the films.

Chapter 4

Research Conclusions

In this research, we observe that silk coated with collagen were prepared by the fibrils assembly of collagen type I affects on the morphology of silk films. The conditions used in this technique are very mild and would be tailored for a silk films scaffold.

In addition, the developed biomimetic reself-assembly of collagen in this current work also shows the advantages in mechanical properties. At the end of the dissertation, the silk coated with collagen concentration 1-2 mg/ml show higher mechanical properties than native silk films. From the result of SEM, silk fibroin films were coated with collagen concentration 1-2 mg/ml shown collagen fibril formation. The collagen fibrils are parallel oriented and packed on silk films. When increased in the collagen concentration content, fibril structure of collagen became increase size and density of the structure. This finding provides the motivation to explain and create a novel extracellular matrix having an attractive structure for cell recognition. Furthermore, it could probably be helpful for explaining the mechanisms of other fibrillation processes in the human body.

In this present study, the developed technique was use to design scaffold for tissue engineering application. The use of biomimetic re-self assembly with collagen type I should consider based on the mechanical strength, biological compatibility, and manufacturing feasibility considerations.

There are implications for future work in this field: because we can control the substitution of N-H group of collagen. It can fabricate scaffold which has good protein binding, making it desirable for use in tissue engineering applications. The further study of this research may be extended into two steps. Firstly, to use silk coated with collagen for the in vivo biological test to ensure the sufficient biomechanical performance. Secondly, the animal model should be performed after in vitro test to validate the possibility for human use.

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